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Original Article

Dog-specific hemorrhagic changes induced by liposomal formulations, in the liver and the gallbladder

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Abstract: Although several liposomal drugs, including liposomal doxorubicin, have been approved, the etiology of the pathological responses caused by their physicochemical properties remains unknown. Herein, we investigated the pathological changes in the liver and the gallbladder of dogs following a single injection of liposomal doxorubicin (1 or 2.5 mg/kg) or an empty liposomal formulation (i.e., liposomal formulation without doxorubicin, ca. 21 mg/kg as lipid content). Injection of liposomal doxorubicin or the empty liposomal formulation induced hemorrhagic changes in the liver and the gallbladder. These changes were accompanied by minimal cellular infiltration with no obvious changes in the blood vessels. As there were no differences in the incidence and severity of hemorrhage between the groups administered comparable amounts of total lipid, the physicochemical properties of the liposomal formulation rather than an active pharmacological ingredient, doxorubicin, were associated with the hemorrhagic changes. Furthermore, decreased cytoplasmic granules with low electron density in mast cells beneath the endothelium of the hepatic vein were observed in the liver of dogs treated with liposomal doxorubicin or empty liposomal formulation. Injection of compound 48/80, a histamine releaser induced comparable hemorrhage in dogs, implying that hemorrhage caused by injection of liposomal doxorubicin or the empty liposomal formulation could be attributed to the histamine released from mast cells. The absence of similar hemorrhagic lesions in other species commonly used in toxicology studies (i.e., rats and monkeys), as well as humans, is due to the lack of mast cells beneath the endothelium of the hepatic vein in these species. (DOI: 10.1293/tox.2019-0029; J Toxicol Pathol 2020; 33: 1–9)

Key words: hemorrhagic changes, Doxil, dog, liposomal formulation, compound 48/80

Introduction

Since the first liposomal drug, Doxil (liposomal doxorubicin), was approved in 1995[1], several liposomal drugs that attenuate the adverse effects and/or enhance the efficacy of active pharmaceutical ingredients (APIs) included in the formulation have been approved[2–4]. More than 10 liposomal drugs are currently used clinically, and other liposomal formulations are in various stages of clinical trials[5, 6]. Liposomal formulations, which are composed of lipid- and/or polymer-based systems that have API, generally have higher solubility, longer circulation time, more favorable bio-distribution, and lower toxicity as benefits, than the API itself[7, 8]. Nevertheless, liposomal formulations sometimes induce unfavorable effects such as acute hypersensitivity reactions (HSRs) and accelerated blood clearance[9, 10], which could be attributed not only the API, but also the physicochemical properties of the formulation. This study focused on newly identified changes in dogs administered a liposomal drug, whereby the changes may be associated with the physicochemical properties of liposomes.

Clinically, HSRs to liposomal formulations, known as infusion reactions were first reported in 1986[10]. HSRs, which affect the cardiovascular, the bronchopulmonary, the mucocutaneous, and the neuropsychosomatic systems causing flushing, shortness of breath, facial swelling, chest/back pain, and hypotension or hypertension, are considered reactions to the physicochemical properties of liposomes such as their particle size, surface charges, polyethylene glycol (PEG), and cholesterol ratio, and hence, are not induced by APIs[11, 12]. In animal models of rats, monkeys, dogs, and pigs, liposomal or lipid-based formulations were found to induce acute reactions including hypertension or hypotension as found in humans[11, 13–17]. As a mechanism of these reactions,

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the compliment system is activated by liposome injections and it leads to compliment C3a production. C3a acts as an anaphylatoxin and causes histamine release from stimulated mast cells. This phenomenon, known as C activation-related pseudoallergy (CARPA), has been well investigated[18–22], although details of the histopathology following the reaction remain unknown.

We investigated the pathological changes induced by a liposomal formulation in standard toxicological animals, including rats, monkeys, and dogs, to understand the additional adverse effects induced by formulating APIs with liposomes. Herein, we reported the hemorrhagic changes in the liver and the gallbladder of dogs following an injection of Doxil or an empty liposome formulation (i.e., liposomal formulation without API: empty liposome). Furthermore, we investigated the mechanisms of the hemorrhagic changes to determine the risks of liposomal formulations for clinical use.

Materials and Methods

Animal studies

The study protocols were approved by the Institutional Animal Care and Use Committee of FUJIFILM Corporation and were conducted in compliance with the Act on Welfare and Management of Animals and Code of Welfare of Laboratory Animals of FUJIFILM Corporation.

Animals were maintained under controlled temperature (20–26°C), humidity (30–70%), air changes (10 times or more/h for rats, dogs and monkeys), and lighting (12-h light/dark cycle). Animals had free access to tap water. As diets, 250 g/animal/day PS-A (Oriental Yeast Co., Ltd., Tokyo, Japan) or ca. 300 g/animal/day NVE-10 (Nippon Pet Food Co., Ltd., Tokyo, Japan), CE-2 (CLEA Japan, Inc., Tokyo, Japan) *ad libitum*, and 100 g/animal/day PS-A were provided to dogs, rats, and monkeys, respectively.

Doxil and liposomal formulations

Commercially available Doxil® (Johnson & Johnson, New Brunswick, NJ, USA) was purchased and used as the standard material. For animal studies,doxorubicin liposomal formulation that mimicked the standard material (mentioned as Doxil hereafter) and doxorubicin-free liposomal formulation (mentioned as empty liposome hereafter) were manufactured in-house by FUJIFILM Corp. (Tokyo, Japan), as previously described[23], with minor modifications. For manufacture, hydrogenated soybean phosphatidylcholine (HSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(PEG)-2000] (DSPE-mPEG2k) were purchased from NOF Corp. (Tokyo, Japan). Cholesterol was purchased from Nippon Fine Chemical Co., Ltd. (Osaka, Japan), and doxorubicin hydrochloride was purchased from MicroBiopharm Japan Co., Ltd. (Tokyo, Japan). The molecular ratio of the lipid composition was 57:38:5 (HSPC:cholesterol:PEG-DSPE) in these formulations, and was comparable to that of the standard material. Total lipid concentrations of Doxil and empty liposome were 23.4 mM and 23.1 mM, respectively. The concentrations of doxorubicin in Doxil and empty liposome were determined to be 1.98 and 0 mg/mL, respectively.

One of the manufactured formulations (1 mg/kg and 8 mg/kg) or standard Doxil (1 mg/kg) was intravenously administered via the tail vein to 8-week-old male Sprague Dawley rats (Charles River Laboratories Japan, Kanagawa, Japan). Blood samples at 0.25 h, 1 h, 4 h, 24 h, and 48 h post-administration (N=3 per time point) were collected from the jugular vein under anesthesia with isoflurane (Mylan Inc., Canonsburg, PA, USA). Plasma samples were prepared with heparin sodium as the anticoagulant followed by centrifugation at 800 *g* for 10 min (4°C) and within 1 h after blood collection. Doxorubicin concentrations in these plasma samples were determined using a validated LC/MS/MS method. C_{max}, which was determined as $C_{0.25}$, and $AUC_{0–48}$ were comparable for rats treated with the standard material or the manufactured formulation at doxorubicin dose of 1 mg/kg (Supplementary Table 1 and 2: online only). The$AUC_{0–48}$ of the manufactured doxorubicin formulation at doses of 1 mg/kg and 8 mg/kg increased in a dose-dependent manner (Supplementary Table 1: online only) and was comparable to the data reported previously[24]. Based on these results, bio-equivalency of the manufactured formulation was demonstrated.

Studies with the Doxil and empty liposome formulations

Dog studies: The study designs of Experiment 1, 2, and 3 are summarized in Table 1.

Experiment 1: Doxil at a dose level of 1 mg/kg (20 mg/

Table 1. Test Designs in Dogs

Experiment No.	1	2		3		
Formulation	Doxil	Doxil	Doxil	Vehicle	Doxil	Empty liposome
Number of animals	3	2	2	2	4	4
Number of dosing	Single	Twice	Twice	Single	Single	Single
Dose as doxorubicin (mg/kg)	1	1	1	0	2.5	0
Dose as total lipid (mM/kg)	11.8	11.8	11.8	0	29.5	29.5
Dosing volume (mL/kg)	10	10	10	5	5	5
Dosing rate as doxorubicin (mg/min)	ca. 0.17	ca. 0.17	1	0	1	0
Dosing rate as total lipid (mM/min)	ca. 2	ca. 2	11.8	0	29.5	29.5

m^2) was intravenously administered once to 8- or 10-month-old male beagle dogs (N=3) (Kitayama Labes Co., Ltd., Nagano, Japan). Dose volume was 10 mL/kg, and dose rate was approximately 0.17 mg/min of doxorubicin. Clinical pathology examinations of hematology, clinical chemistry, and coagulation were conducted. Animals were anesthetized through an intravenous administration of sodium pentobarbital (Kyoritsu Seiyaku Corp., Tokyo, Japan) and were euthanized 24 h after administration. Gross pathology examination was conducted, and the liver with the gallbladder was fixed in 10% neutral buffered formalin solution. The fixed tissue samples were embedded in paraffin, and the sections were stained with hematoxylin and eosin (HE) for light microscopic examination.

Experiment 2: Doxil at a dose level of 1 mg/kg (20 mg/m^2) was intravenously administered twice at an interval of one week to 8- or 9-month-old male beagle dogs (N=2/group) (Kitayama Labes Co., Ltd.). Dose volume was 10 mL/kg, while dose rate was 0.17 or 1 mg/min of doxorubicin. Other procedures, such as euthanasia, and clinical pathology, gross pathology, and histopathology examination were the same as those of Experiment 1.

Experiment 3: Doxil at a dose level of 2.5 mg/kg (50 mg/m^2), an equivalent lipid amount of empty liposome formulation (ca. 21 mg/kg as lipid content), or 5% w/v glucose solution as a vehicle was intravenously administered once to 8-month-old male beagle dogs (N=2 or 4 per group) (Kitayama Labes Co., Ltd.). Dose volume was 5 mL/kg, and dose rate was 1 mg/min of doxorubicin. Subsequent procedures consisting of euthanasia, and clinical pathology, gross pathology, and histopathology examination were the same as those in Experiment 1. The sections were stained with toluidine blue (pH 4.0) for light microscopic examination. In addition, small liver tissues were fixed in 2% glutaraldehyde solution followed by 1% OsO_4 fixation and embedded in epoxy resin. Electron microscopic examination (JEM-1200EX, JOEL, Tokyo, Japan) was conducted for samples with double staining using uranyl acetate and lead citrate.

Rat and monkey studies: Doxil at dose levels of 2.5 mg/kg and 4 mg/kg (15 mg/m^2 and 24 mg/m^2) or vehicle (9.4% w/v sucrose solution containing 10 mM histidine) was intravenously administered once weekly forfour weeks to 7-week-old male Sprague Dawley rats (N=3 per group) (Charles River Laboratories Japan). Doxil at a dose level of 1.6 mg/kg (20 mg/m^2) was intravenously administered twice at an interval of three weeks to 3-year-old cynomolgus monkeys (N=2) (Ina Research Philippines, Muntinlupa, Philippines). Dose volume and rate were the same as those in the dog study. At 24 h post-injection, rats were euthanized under isoflurane inhalation anesthesia, while monkeys were euthanized with an intravenous pentobarbital injection. Gross pathology and histopathology examinations, using light microscopy, were conducted in the same manner as Experiment 1.

Studies with Compound 48/80

Dog study: Compound 48/80 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline was intravenously injected via the hepatic artery to 13-month-old male beagle dogs (N=2) (Marshall BioResources Japan, Tsukuba, Japan). Dose level and volume were 2 mg/kg and 5 mL/kg, respectively. Animals were anesthetized with an intravenous pentobarbital injection (ca. 30 mg/kg) followed by a subcutaneous injection (ca. 4 mg/kg) of carprofen (Rimadyl®, Pfizer Inc., New York, NY, USA) and an intramuscular injection (ca. 0.05 mg/kg) of atropine sulfate (Mitsubishi Tanabe Pharma Corp., Osaka, Japan). After median laparotomy, the left gastroepiploic artery was ligated. Compound 48/80 solution was infused for 20 min through a flexible catheter inserted into the hepatic artery. Animals were euthanized immediately after the infusion, and gross pathology examination was conducted following necropsy. The liver and the gallbladder were collected and reserved in 10% neutral formalin. Pathology slides with HE staining, prepared in the same manner as the studies with liposomal formulations, were subjected to light microscopic examination.

Rat study: Compound 48/80 (Sigma-Aldrich) was dissolved in saline and intravenously injected via the hepatic vein to 7-week-old female rats (N=4) (Charles River Laboratories Japan) under isoflurane inhalation anesthesia at a dose level of 2 mg/kg. Dose volume and rate were the same as those used in the dog study. Animals were sacrificed under isoflurane inhalation anesthesia immediately after the infusion, and gross pathology and histopathology examinations by light microscopy were conducted in the same manner as the dog study.

Results

Liver and gall bladder changes induced by Doxil and empty liposome formulations

Dog study: In Experiment 1, there were no significant changes in clinical signs, body weights, food consumption, or clinical pathology consisting of clinical chemistry, hematology, and coagulation.

Table 2 displays gross pathology at necropsy and the histopathology results. In one out of three animals, reddish foci in the liver were observed. In addition, histopathology revealed mild perivascular hemorrhage in the liver and mild hemorrhage in the sub-adventitial layer of the gallbladder.

There were no notable changes in body weights, food consumption, clinical signs, or clinical pathology consisting of clinical chemistry, hematology, and coagulation, in Experiment 2. In one of two animals in each group, reddish foci in the liver and gallbladder serosa were observed. Histopathology revealed mild to moderate perivascular hemorrhage in the liver and mild to moderate hemorrhage in the sub-adventitial layer of the gallbladder in one or two animals in each group. These results suggest that the gross pathology and histopathology findings in Experiments 1 and 2 were comparable (Table 2).

Based on the results of Experiment 3, there were no

Table 2. Pathological Changes in the Liver and Gallbladder of Dogs

Experiment No.		1	2		3		
Formulation		Doxil	Doxil	Doxil	Vehicle	Doxil	Empty liposome
Dose as doxorubicin (mg/kg)		1	1	1	0	2.5	0
Dose as total lipid (mM/kg)		11.8	11.8	11.8	0	29.5	29.5
Dosing rate as total lipid (mM/min)		2	2	11.8	0	11.8	11.8
Number of animals		3	2	2	2	4	4
Gross findings							
Liver							
Reddish foci, sub-adventitia of gallbladder		1	1	1	0	2	2
Gall bladder							
Reddish foci in serosa		0	1	1	0	1	2
Histological findings	Grade						
Liver							
Hemorrhage, perivascular	1	1	1	1	0	1	0
	2	0	0	1	0	2	2
Dilation of lymphatic vessel, perivascular, focal	1	0	0	0	0	3	0
	2	0	0	0	0	0	2
Gall bladder							
Hemorrhage of adventitial layer	1	1	1	1	0	1	0
	2	0	0	1	0	1	1
	3	0	0	0	0	1	1

Grade of changes; 1: mild, 2: moderate, 3: severe.

significant body weight changes in any group. Moreover, no clinical signs were observed in animals treated with Doxil, other than incontinence and pale oral mucosa, suggesting hypotension during Doxil administration.

For necropsy, reddish foci in the liver and gallbladder serosa were observed in one or two of four animals in Doxil and empty liposome groups respectively (Table 2 and Fig. 1). Histopathology findings in the liver were mild to moderate perivascular hemorrhage and mild to moderate dilated lymphatic vessels around the central vein; these findings were observed in two or three of four animals in each treatment group (Table 2). Mild to severe hemorrhage in the sub-adventitial layer was a histopathology finding of two or three offour animals in each treated group, some of which extended to the serosa and corresponded to the gross finding during necropsy (Table 2). Therefore, there were no obvious differences in the incidence and severity in the gross pathology and histopathology findings between both treatment groups in this experiment. However, these findings are likely to be more severe and observed in more animals compared to those of treated animals in Experiments 1 and 2. These findings suggest that the incidence and severity of the gross pathology and histopathology changes are associated with total lipid contents in liposomal formulations.

The histopathological findings observed in animals treated with Doxil or empty liposome include perivascular hemorrhage around the central vein, the sub-lobular vein, and the Glisson's sheath, dilated lymphatic vessels around the central vein in the liver, and hemorrhage in the sub-adventitial layer of the gallbladder (Fig. 2a–f). Perivascular hemorrhage in the liver was more prominent around the central vein and sub-lobular vein than the Glisson's sheath,

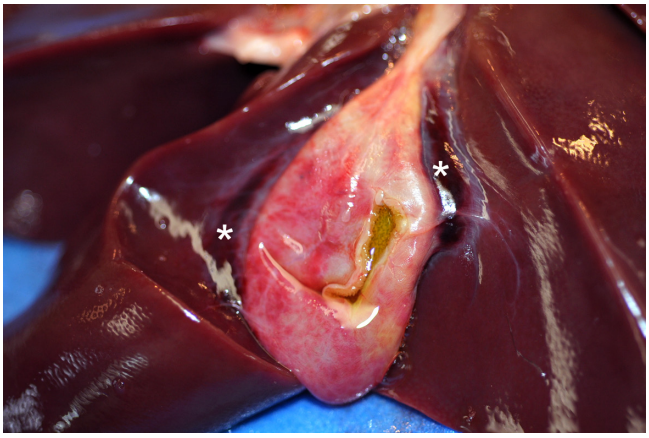

Fig. 1. Macroscopic findings in dogs (Liposomal formulations). Reddish foci (asterisks) in the liver (sub-adventitia of gall bladder) and/or gallbladder serosa (body to bottom) were observed in both Doxil and empty liposome groups.

and was accompanied by dilated lymphatic vessels around the central vein, with little cellular infiltration and no obvious changes in the blood vessels. Hemorrhagic changes were also observed along the sub-adventitial layer of the gall bladder to serosa.

In the liver of animals treated with the vehicle in Experiment 3, numerous mast cells were observed in the hepatic centrilobular sinusoids, the central veins, and the sub-lobular vein in sections stained with toluidine blue (Fig. 2i) as reported previously[25, 26]. Furthermore, mast cells were densely distributed around the central veins compared to the perilobular sinusoids. Among the animals treated with Dox-

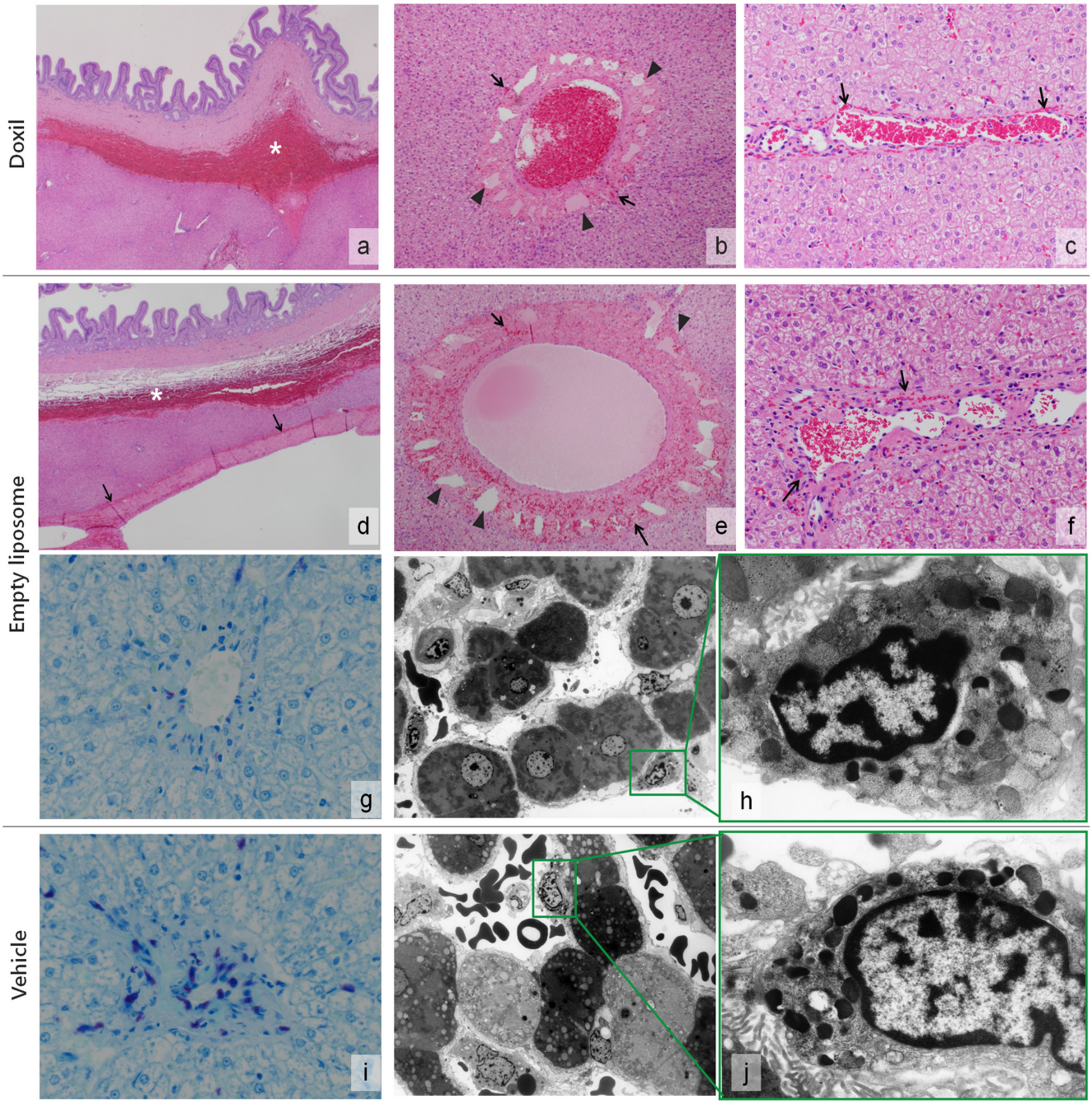

Fig. 2. Histopathological findings in dogs (Liposomal formulations). In the liver and the gallbladder of animals treated with Doxil (a–c) or empty liposome (d–f), hemorrhage in the sub-adventitial layer of the gallbladder (asterisks) (a, d), perivascular hemorrhage around the central and sub-lobular veins (arrows) (b, c, e, f), and dilated lymphatic vessels around the central vein (arrowheads) (b, e) were observed in HE-stained sections. Positive cytoplasmic granules in mast cells decreased in animals treated with both Doxil and empty liposome in TB-stained sections (g). In electron microscopy, granules with lower electron density were decreased in mast cells along the hepatic veins in animals treated with both Doxil and empty liposome (h) compared to the vehicle control-treated animals (j). Magnification, a, d: 12.5×, b, e: 40×, c, f: 100×, g, i: 200×, h, j: 10,000×.

il or empty liposome, a decrease in the cytoplasmic granules of mast cells was observed in the liver with moderate hemorrhage (Fig. 2g), while no change in the cytoplasmic granule was observed in the liver with mild or no hemorrhage. Regardless of the severity of hemorrhage, no changes in the number or distribution of mast cell were observed. Compared to animals treated with the vehicle (Fig. 2j), electron microscopic examination showed a portion of granules with lower electron density in mast cells along the hepatic veins in animals treated with the Doxil or empty liposome formulation (Fig. 2h).

Rats or Monkey Studies: There were no obvious chang-

es in the histopathology of either species. Unlike dogs, when Doxil was administered at equivalent or higher dose levels, hemorrhagic changes in the liver and/or the gallbladder were not observed in rats and monkeys during necropsy.

Liver and gall bladder changes induced by Compound 48/80

Dog study: Compound 48/80, a histamine releaser, was intravenously administered to dogs. Gross pathology findings during necropsy were reddish foci in the liver (the sub-adventitial layer of the gallbladder). Typical histopathological images of the liver or the gallbladder, which were observed in dogs treated with compound 48/80, showed perivascular hemorrhage around the central vein, the sublobular vein, and the Glisson's sheath, dilated lymphatic vessels around the central vein, and hemorrhage in the gallbladder sub-adventitial layer (Fig. 3a and b). These changes were compatible with those induced by liposomal formulations.

Rat study: There were no significant histopathology changes observed in rats. Unlike dogs, hemorrhagic changes in the liver of rat were not observed during necropsy, when compound 48/80 at an equivalent dose was administered.

Discussion

The present study identified specific hemorrhagic changes in the liver and the gallbladder of dogs at 24 h after an injection of Doxil at dose levels of 1 mg/kg and 2.5 mg/kg (20 mg/m^2 and 50 mg/m^2) or empty liposome (ca. 21 mg/kg as lipid content). Although up to 1 mg/kg Doxil was tested in dogs in previous study, similar hemorrhagic changes were not observed[24]. The dose levels of Doxil are equivalent to clinical doses and hence, they were selected for further examinations. The hemorrhage observed was a local change and was not considered secondary to systemic deterioration. This is because there were no significant changes in body weights or any clinical pathology parameters. Clinical signs during Doxil administration were considered HSRs, as reported previously[24]. No significant changes were observed in empty liposome treated animals. The severity of the hemorrhagic changes was likely correlated to the doses of Doxil and its dose rates. However, there were no differences in the histopathological features, incidence, and severity of the hemorrhagic changes between the groups treated with Doxil and empty liposome. Moreover, the total lipid content was comparable. These results suggested that the changes were attributed to the common contents between Doxil and empty liposome, which are liposomes including particle size, surface charge, etc. The hemorrhagic changes accompanied by little cellular infiltration were assumed acute. This is because they were observed within 24 h post injection in this study, and not during necropsy, which is seven or eight days post injection, as in a previous study[24]. This assumption is consistent with the lack of difference in the severity of hemorrhagic changes that were induced after a single dose or weekly repeated doses. In addition, hemorrhagic changes were not observed in rats or monkeys at equivalent or higher dose levels even when the dose levels were converted to mg/m^2 from mg/kg[27].

To clarify the pathogenesis of the hemorrhagic changes, species differences in liver histology were investigated. Dogs are the only known species that have mast cells in the space of Disse under the endothelium of hepatic centrilobular sinusoids, central veins, and sub-lobular vein[25, 26]. In addition, mast cells are particularly dense around the central veins compared to perilobular sinusoids in dogs[25, 28]. Mast cells are not generally observed in these areas and are occasionally found only in the Glisson's sheath in other animal species including humans[26, 29]. Liposomal formulations are known to activate mast cells following C3a induction during the process of CARPA [18–21], and the severity depends on the physicochemical properties of liposomes[11, 12]. The light microscopic examination of Doxil- or empty liposome-treated animals revealed a decrease in cytoplasmic granules in mast cells in the liver. In electron microscopic examination, a portion of the granules in mast cells displayed low electron density in Doxil- and empty liposome-treated animals, respectively (Fig. 2h), compared to the vehicle-treated animals (Fig. 2j); this is consistent with typical images of mast cell degranulation[30]. The prominent perivascular hemorrhage coincided with the dense distribution of mast cells, which are typical in dogs. A single injection of compound 48/80 that is known to activate mast cells and release histamine caused identical hemorrhagic changes to that induced by the liposomal formulations in the present study. In fact, the histopathological features of the liver and the gallbladder induced by Doxil or empty liposome were not different from those induced by compound 48/80.

The hemorrhagic changes in dogs treated with liposomal injections were likely to be caused by mast cells located beneath the endothelium of the hepatic veins. Figure 4 displays the postulated etiology of the hemorrhagic changes. The decrease in TB positive cytoplasmic granules and the low density of cytoplasmic granules by histopathology and electron microscopy, respectively, revealed that mast cells stimulated by liposomes in the formulation released physiologically active substances including histamine in the liver of dogs. Histamine is known as a mediator that causes smooth muscle contraction[31] in addition to increasing microvascular permeability, by loosening the endothelium[32]. In dog liver, the sphincter muscles are periodically located along the hepatic veins[25, 33, 34], and they elevate portal vein pressure by contraction following histamine release[35]. Therefore, the release of histamine by liposomes caused hypertension of the sub-lobular vessel and increased the permeability of the blood vessels. It is known that hemorrhage of the sub-adventitial layers in the gallbladder is most common in dogs[36]. Portal hypertension leading to swelling of gallbladder wall is associated with the communication between the cystic vein (portal circulation) and the abdominal wall blood vessels (systemic circulation)[37]; this likely causes hemorrhage of the sub-adventitial layers. Increased blood pressure and permeability of the hepatic vein led to fluid ac-

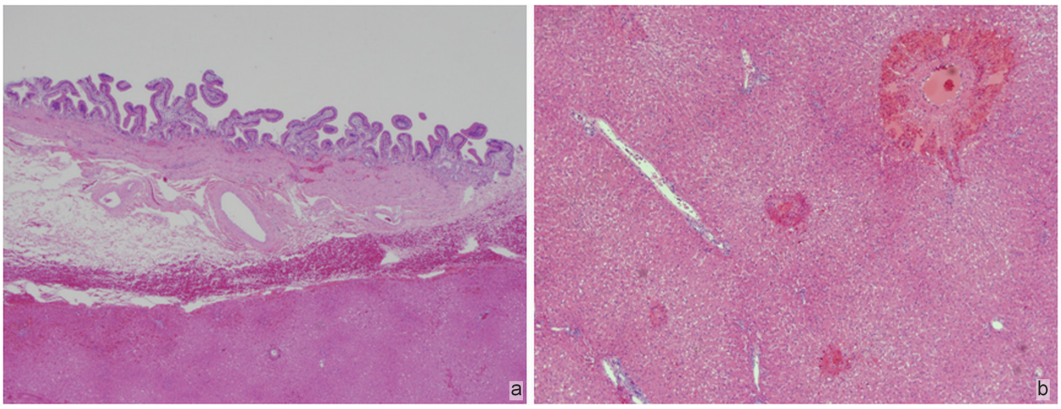

Fig. 3. Histopathological findings in dogs (Compound 48/80). Hemorrhage in the sub-adventitial layer of the gallbladder (a), perivascular hemorrhage around the central vein, the sub-lobular vein, and the Glisson's sheath, and dilated lymphatic vessels around the central vein in the liver (b) were observed in animals treated with compound 48/80. HE stain, Magnification, a: 12.5×, b: 40×.

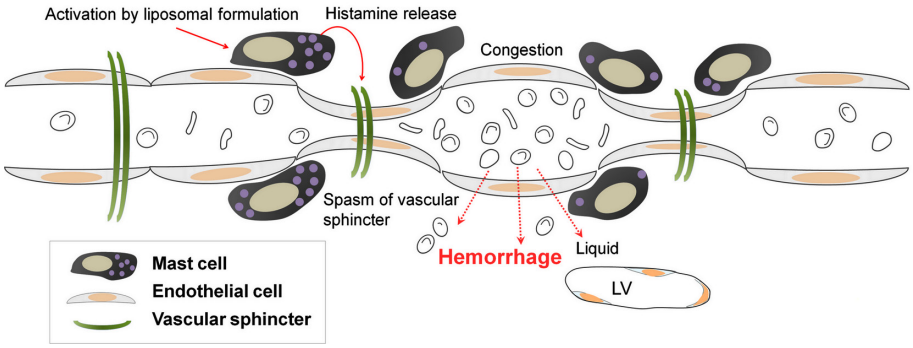

Fig. 4. Postulated etiology. 1. Complement activation by liposome. 2. Histamine release from mast cells by C3a. 3. Congestion caused by spasm of the vascular sphincter. 4. Increased permeability of the blood vessel by histamine. 5. Hemorrhage accompanied by dilation of the lymphatic vessel. LV: lymphatic vessel.

cumulation in the interstitial spaces. Consequently, the lymphatic vessels were filled to maintain pressures in tissues leading to dilation. The dilated lymphatic vessels observed through histopathology were likely to result from the exudation of liquid blood components absorbed into the hepatic lymphatic vessels. Portal hypertension is accompanied by an increase in lymphatic flow in the liver of dogs[38, 39]. Compound 48/80 induces intensive hepatic sub-lobular sphincter contraction[40], leading to swelling of numerous lymphatic vessels around the hepatic vein[26, 40]. Considering the pathogenesis, similar hemorrhagic changes are unlikely to occur in rats, monkeys, and humans based on lack of subendothelial mast cells in their hepatic veins.

Although the severity of the hemorrhagic changes correlated with Doxil doses, the incidence of hemorrhage per group did not clearly indicate any dose relation, which was possibly due to individual differences. Further studies with more dogs are needed to clarify further the correlation of hemorrhage with other factors such as number and distribution of mast cells and contents of granules in mast cells of the liver. Although plasma histamine and C3 concentrations were examined as potential biomarkers, there was no clear correlation with hemorrhage (data not shown).

We revealed that hemorrhagic changes in the liver and the gallbladder of dogs were induced after an injection of Doxil and empty liposome respectively. These changes are attributed to the physicochemical properties of liposomal formulations, and not the API. Furthermore, the hemorrhagic changes are likely to result from the release of histamine from mast cells that are uniquely located beneath the endothelium of the hepatic veins in dogs but are unlikely to occur in other toxicological species, or in humans.

Disclosure of Potential Conflicts of Interest: The authors have no financial conflicts of interest to disclose concerning the study.

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