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Ammonium ion level in serum affects doxorubicin release from liposomes

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In this study, we measured the release of drug from liposome-encapsulated doxorubicin (DXR) in human and mouse serum. While human serum did not induce DXR-release, mouse serum significantly induced DXR-release in a temperature- and time-dependent manner. Release of DXR was clearly observed in ultrafiltrated mouse serum, indicating that low-molecular substances affect DXR-release. Therefore, the level of Na^+, Cl^-, NH_4^+, and urea nitrogen in each type of serum was measured. Only the concentration of NH_4^+ in mouse serum was significantly higher than that in human serum. Furthermore, addition of ammonium acetate to human serum induced DXR release at the same level observed in mouse serum. These results indicate that the NH_4^+ concentration in serum might greatly affect the release of DXR from liposomes.

1. Introduction

Recently, various liposomal products have been developed and applied to clinical treatment (Coukell and Brogden 1998; Maurer et al. 2001). It is a global requirement that evaluation standards for liposomal products are established to ensure their quality (Burgess et al. 2002). The main purpose of using liposomalization is to stabilize drugs *in vivo* and to control release. For example, the serum half-life of DOXIL®, which is the antitumor agent doxorubicin (DXR) encapsulated in a PEGylated or so-called 'stealth' liposome, is about 90 h (Fujisaka et al. 2006), while that of injected DXR is less than 1 h (Mross et al. 1988). Therefore, drug release (or leakage) is one of the most important formulation properties of liposomal products for quality assessment. *In vitro* drug-release tests for appropriately measuring drug release from liposomes would be very useful for assessing lot-to-lot variability or the release characteristics of liposome products. At present, however, few studies have examined how we should assess *in vitro* drug-release appropriately. From this standpoint, we have studied whether or not an *in vitro* release test, which is related to *in vivo* stability, can be established. It's preferable that such an in vitro drug-release test is based on the *in vivo* release mechanism and correlates with the *in vivo* release profiles. In order to achieve *in vivo* relevance, drug release should be measured under conditions that are as near as possible to the physiological condition. Thus, as a first step, we have investigated the utility of human or mouse serum in the assessment of DXR release from stealth liposome-encapsulated DXR (DXR-SL).

2. Investigations, results and discussion

DXR-SL were incubated with mouse or human serum at various temperatures (37, 45, or 52 °C), and the ratio of DXR release was measured. As a result, mouse serum induced significant DXR release from DXR-SL in a temperature- and time-dependent manner (Fig. 1). In the case of human serum, however, the DXR-release rate was extremely low, even at 52 °C. To our knowledge, it has not been reported that drug release from liposomes differs greatly between human serum and mouse serum.

To elucidate this difference, DXR release from DXR-SL was measured in the filtrate of each serum after ultrafiltration (3 kDa or 10 kDa cut-off). Ultrafiltrated mouse serum induced significant DXR release, although it was slightly lower than that in unfiltered serum (Fig. 2A). In human serum, the DXR-release rate in the filtrate was also slightly lower than that in unfiltered serum. This result indicates that low molecular substances largely affect the release of DXR induced in mouse serum. When DXR-SL was incubated with rat or bovine serum, in addition to human and mouse serum, only mouse serum induced DXR release from DXR-SL (Fig. 2B). Next, we compared the DXR-release rate in four kinds of serum: two kinds of fresh serum collected from CD-1 mice and BALB/c mice (prepared in our laboratory), commercial mouse serum that had been used in the above tests, and human serum. As a result, significant DXR release was observed in only the commercial mouse serum, while the DXR-release rate in fresh mouse serum was equivalent to that in human serum (Fig. 2C). These results indicate the possibility that the low molecular substances affecting drug release are specific to the commercial mouse serum.

Therefore, we measured the concentration of typical low molecular substances in blood, such as Na^+, Cl^-, NH_4^+ and urea nitrogen, in each type of serum. Surprisingly, the NH_4^+ level of the commercial mouse serum was 100-fold higher than that of human serum (Fig. 3A). Likewise, the NH_4^+ level was significantly high in another commercially available mouse serum. On the other hand, the concentration of urea nitrogen in the commercial mouse serum was one-twentieth of that in human serum. The concentration of sodium or chloride was normal in all serum. Next, we examined the effect of NH_4^+ level on DXR release from DXR-SL. It was expected that the pH of the commercial mouse serum would be higher than that of human serum. However, there were no differences in pH between mouse and human serum (data not shown). Thus, we added ammonium

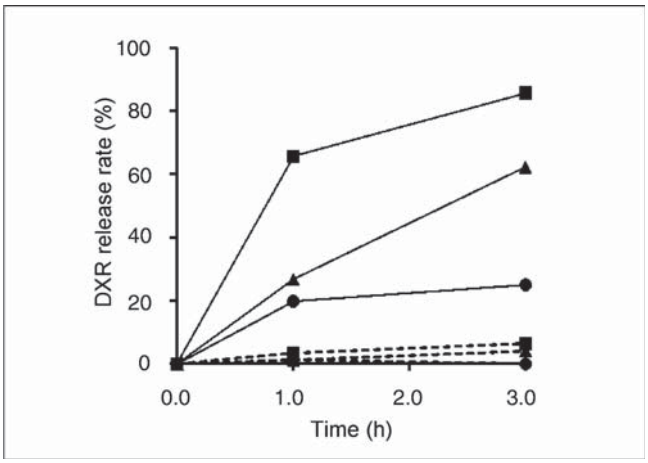

Fig. 1: DXR-release rate in mouse or human serum. DXR-SL (DXR 200 μg/ml) was incubated in human (dashed line) or mouse (solid line) serum (final 90% (v/v)) at 37 °C (circle), 45 °C (triangle), or 52 °C (square) for indicated time

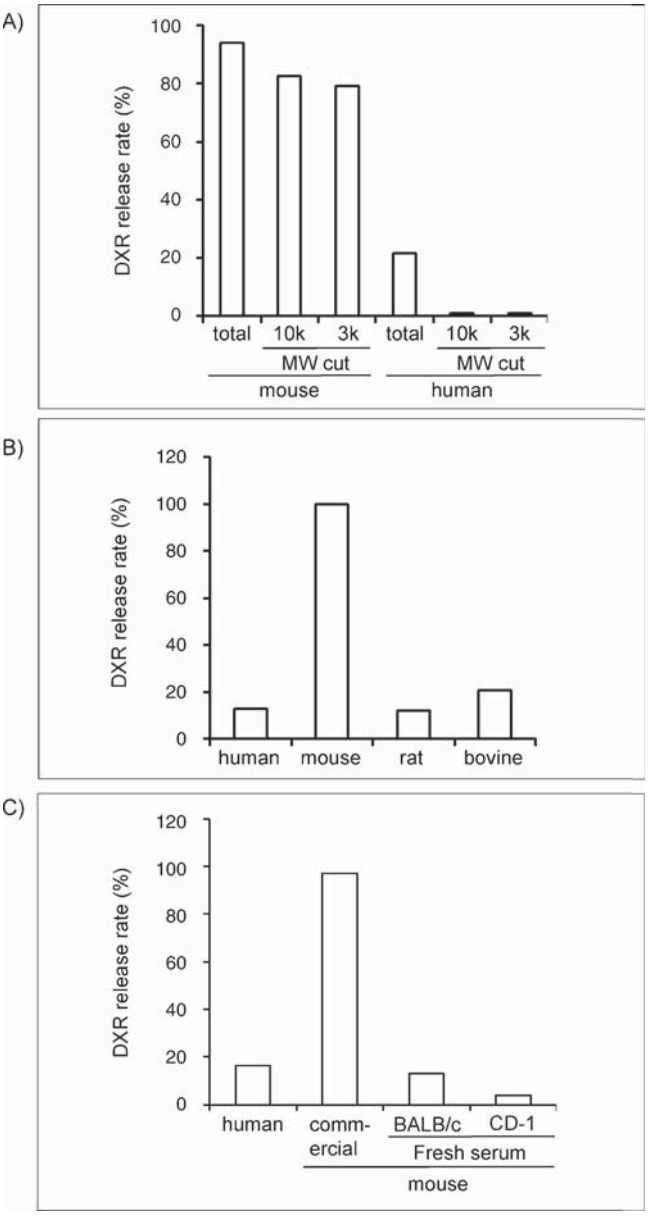

Fig. 2: Effect of difference in serum on DXR-release. A) Effect of ultrafiltrated serum on the DXR-release rate. DXR-SL (DXR 200 μg/ml) was incubated in the filtrate (final 90% (v/v)) for 3 h at 52 °C. DXR-release rate in rat and bovine serum B), and fresh mouse serum collected from BALB/c and CD-1 mice C), in addition to human and mouse serum, were measured after incubation for 3h at 52 °C

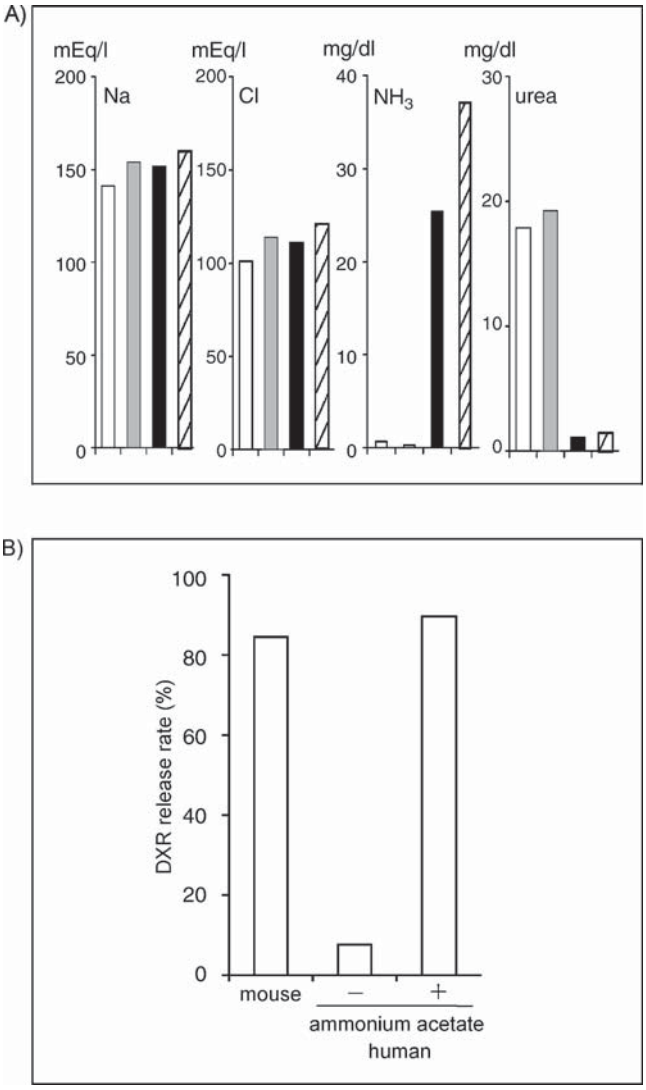

Fig. 3: NH_4^+ level affects DXR release. A) Na^+, Cl^-, NH_4^+ and urea nitrogen in human (white column), fresh mouse serum (gray column), commercial mouse serum (black and shaded column) were measured. B) Ammonium acetate was added to human serum at a final concentration of 1.34 mg/ml which is almost same as the NH_4^+ level in mouse serum. The DXR-release rate in this modified human serum was measured as described in Fig. 1

acetate solution to human serum to the same NH_4^+ level in commercial mouse serum without changing the pH, and measured the DXR-release rate in the adjusted human serum. As in mouse serum, significant DXR release was observed in human serum with ammonium acetate (Fig. 3B). These results suggest that the high NH_4^+ level is one of the causes of the high DXR-release rate in commercial mouse serum.

It is unclear why the NH_4^+ level is markedly increased in commercial mouse serum. Commonly, the blood NH_4^+ level should be measured immediately after blood drawing and centrifugation. Hemolysis and leaving the samples as whole blood at room temperature are causes for elevated test values (Howanitz et al. 1984; Lindner and Bauer 1993). AMP deaminase in red blood cells catalyzes the production of ammonia from protein and amino acids (Nathans et al. 1978). Although we did not investigate the effects in full, we found that, even in human serum, repeating freeze-thaw cycles and long storage tended to increase the DXR-release rate (data not shown). Thus, the high NH_4^+ may be due to a delay in collecting serum after blood drawing, hemolysis, repeating freeze-thaw cycles, or long storage. It is important to stress, however, that the commercial mouse serum used in our examinations is fully compatible with immune assays, such as ELISA or immunostaining, for which it is generally used.

DXR is encapsulated in liposomes by a remote loading method based on the gradient of ammonium sulfate. The mechanism of accumulation is believed to be as follows (Haran et al. 1993). Removal of ammonium sulfate from the extraliposomal medium of liposomes creates an ammonium sulfate gradient $[(NH_4)_2SO_4]_{lip.} > [(NH_4)_2SO_4]_{med.}$ The very high permeability coefficient of neutral NH_3 leads to fast diffusion of NH_3 into the extraliposomal medium. For every NH_3 molecule that leaves the liposome, one proton is left behind, forming a pH gradient across the liposomal membrane. Because DXR is a weakly basic compound (pKa = 8.25), nonionic DXR in the extraliposomal medium diffuses through the lipid bilayer, is protonated and trapped as an ionic form, and accumulates in the intraliposomal aqueous phase by forming a precipitate with sulfate ions. The process can be summarized as an exchange between NH_3 efflux and DXR influx. Therefore, the addition of high concentration ammonium salt to the extraliposomal phase of DXR-SL may induce NH_3 influx into intraliposomes. As a result, the intraliposomal pH may be elevated, and nonionic DXR may diffuse out through the lipid bilayer of liposomes. While the details of the mechanism remain to be elucidated, we speculate that the significant DXR release from DXR-SL in the commercial mouse serum could be caused by high NH_4^+ levels in this way.

Our data revealed that 1) there was almost no DXR release from DXR-SL in human serum, while mouse serum induced significant DXR release; 2) the high NH_4^+ level in mouse serum, especially in commercial mouse serum, is one of the factors leading to the markedly high DXR-release rate; and 3) the concentration of NH_4^+ in the test solution can greatly affect the release of DXR from DXR-SL. Thus, if serum or plasma is used for an *in vitro* drug-release test of liposomal products that are prepared by ammonium sulfate gradient, it will be necessary to control both the lot and the storage period.

3. Experimental

3.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) and (N-(carbonyl-methoxy polyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG2000) were purchased from Nippon Oil and Fat (Tokyo, Japan). Cholesterol (Chol) was of analytical grade (Wako Pure Chemical, Osaka, Japan). Adriacin® injection 10 (Kyowa Hakko Kirin Co., Ltd.), which is doxorubicin (DXR) injection, was purchased from a general sales agency for drugs in Japan. Mouse, rat serum (Valley Biomedical, Inc., VA), and human serum (Biopredic International, Rennes, France) were obtained from KAC Co., Ltd. (Kyoto, Japan). Another mouse serum was obtained from Cedarlane Laboratories Limited (Ontario, Canada). Bovine serum was purchased from Invitrogen (Carlsbad, CA). Fresh mouse serum collected from CD-1 mice was supplied by Charles River (Kanagawa, Japan). Sepharose CL-4B and Sephadex G-25 prepacked columns, PD-10 Desalting Columns, were purchased from GE Healthcare Japan (Tokyo, Japan).

3.2. Liposome preparation

DXR-SL composed of HSPC/Chol/DSPE-PEG2000 (56.5/38/5.4 molar ratio) was prepared by a modified ethanol injection method (Maitani et al. 2001). DXR was encapsulated into liposomes by remote loading using an ammonium sulfate gradient (Lasic et al. 1992). Briefly, all lipids were dissolved in about 5 ml of ethanol, and the ethanol was removed with a rotary evaporator leaving behind about 1 ml of the ethanol solution. Next, 4 ml of 300 mM ammonium sulfate was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual ethanol. After five freeze-thaw cycles, liposomes were extruded through a series of polycarbonate filters (Nucleopore, CA) with pore sizes ranging from 0.4 to 0.1 μm. The mean diameter of resulting liposomes was determined by dynamic light scattering using a DLS-7000 (Otsuka Electronics Co. Ltd., Osaka, Japan). The diameter of extruded liposomes was in the range of 110 ± 30 nm. Following extrusion, liposomes were ultracentrifuged at 80,000 rpm for 45 min at 4 °C, and suspended in normal saline. The concentration of phospholipid was determined by colorimetric assay using Phopholipids C (Wako Pure Chemical Industries, Ltd., Osaka, Japan). DXR was added to the liposomes at a DXR/liposome ratio of 0.2:1 (w/w), and liposomes were incubated for 1 h at 55 °C. The liposome-encapsulated DXR, DXR-SL, was exchanged by eluting through a PD-10 Desalting Column equilibrated with normal saline.

3.3. Release of doxorubicin

DXR-SL (DXR 200 μg/ml) was incubated in each serum (final 90% (v/v)) for indicated time at 37, 45 or 52 °C. After incubation, samples were passed through a Sepharose CL-4B column equilibrated with normal saline to separate the liposomal DXR from serum protein and free drug. The fraction of liposomal DXR was mixed with an equal volume of hydrochloric acid/isopropanol, and the fluorescent intensity was read at 590 nm (excitation 470 nm). The release rate was calculated from the amount of liposomal-DXR. For ultrafiltration, 4 ml of each serum was ultrafiltrated on centrifugal filter units (NMWL 10k or 3k, AmiconUltra, Millipore Corporate Headquarters, Billerica, MA), and 2 ml filtrate was used for release assay. Ammonium acetate was dissolved in water (134 mg/mL) and added to human serum at a final concentration of 1.34 mg/mL which is almost same as the NH_4^+ level in mouse serum.

3.4. Ion levels in serum

Measurement of Na^+, Cl^-, NH_4^+ and urea nitrogen in each serum was outsourced to the Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Na^+ and Cl^- were measured by electrode method. NH_4^+ and urea nitrogen were measured by indophenol colorimetric method (Fujii-Okuda method) and urease-LEDH method, respectively.

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