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Effect of transferrin receptor-targeted liposomal doxorubicin in P-glycoprotein-mediated drug resistant tumor cells

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Abstract

The over-expression of P-glycoprotein (P-gp) has been associated with the development of multidrug resistance (MDR) in cancer cells. In this study, we examined whether transferrin receptor (Tf-R) targeted liposomes can efficiently deliver encapsulated doxorubicin (DXR) into MDR cells (SBC-3/ADM) via Tf-R-mediated endocytosis thus overcoming MDR by by-passing P-gp-mediated drug efflux. We prepared four types of liposome, i.e. untargeted and Tf-R-targeted, made of either egg-PC/cholesterol or hydrogenated egg PC/cholesterol. Only with the targeted EPC-liposome we achieved significant delivery of encapsulated DXR and increased cytotoxicity of encapsulated DXR on the MDR cells (3.5-fold higher than free DXR). Confocal microscopy and an intracellular drug-accumulation assay indicated that the targeted liposomes efficiently delivered DXR into cells where it readily accumulated in the nucleus, in both drug-sensitive and MDR cells. These findings suggest that the targeted liposomes are rapidly internalized via Tf-R-mediated endocytosis followed by release of their contents into the cytoplasm. The rapid internalization and content release, most likely facilitated by the higher fluidity of the EPC-based liposomes, may explain why only targeted EPC-liposomes were able to prevent drug efflux by P-gp and to consequently circumvent MDR. Our results indicate that in order to achieve MDR circumvention by means of liposomal encapsulation of DXR the liposomes not only need to be targeted, but also to have the proper physicochemical properties for adequate release of the drug. Furthermore, these *in vitro* results suggest that Tf-R targeted EPC-liposomes are a potentially useful drug delivery system to circumvent P-gp-mediated MDR of tumors.

Keywords: Liposome; Targeting; Multidrug resistance (MDR); P-glycoprotein (P-gp); Transferrin

1. Introduction

Multiple drug resistance (MDR) is a serious problem in cancer chemotherapy. It has been demonstrated that P-glycoprotein (P-gp), encoded by the human *MDR1* gene (Ueda et al., 1987), plays a key role in tumor drug resistance (Riordan et al., 1985; Gottesman and Pastan, 1993). P-gp serves as an ATP-dependent drug efflux pump which recognizes a broad range of substrates including anthracyclines, *vinca* alkaloids and others, resulting in reduction of intracellular accumulation of the drug in MDR cells (Spoelstra et al., 1991). Many studies have shown that the overexpression of P-gp in tumor cells is associated with a poor response to conventional chemotherapy, especially with anthracyclins (Marie et al., 1993; Goasguen et al., 1996; Del Poeta et al., 1997). Other factors that can affect cell resistance are overexpression of the MDR-associated protein, altered glutathione metabolism, topoisomerase activity, and, possibly, overexpression of the lung resistance related protein (Slovak et al., 1993; Schadendorf et al., 1995; Dalton, 1997; Dalton and Jove, 1999).

Liposomal encapsulation has been demonstrated to allow tumor specific delivery of anticancer agents *in vivo* and to substantially reduce toxicity associated with these agents by altering the distribution of the drug (Allen, 1994; Gabizon, 1992; Mayer et al., 1995; Forssen et al., 1996). However, alterations in the biodistribution of the drug because of its entrapment of in

Abbreviations: CHOL, cholesterol; DPH, diphenylhexatriene; DXR, doxorubicin; EPC, egg phosphatidylcholine; HEPC, hydrogenated egg phosphatidylcholine; MDR, multidrug resistance; PEG, polyethylene glycol; P-gp, P-glycoprotein; Tf, transferrin; Tf-R, Tf receptor

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liposomes can lead to the appearance of new toxicities. Long-circulating, polyethylene glycol (PEG)-coated liposomes have an increased ability to localize in skin, particularly at pressure points such as hands and feet. At higher drug doses, this can cause palmar-plantar erythrodysaesthesia (hand-foot syndrome) (Goebel et al., 1996). While such toxicities has been observed, following accumulation in the tumor, the drug-loaded liposomes are internalized by the tumor cells in one way or another, thus delivering the drug by another route and to another intracellular compartment than the free drug. This, in turn, may lead to improvement of the therapy of P-gp-related MDR tumors, since the entry of the liposomal formulation into the cells may avoid the active drug efflux, e.g. by P-gp. Several researchers have tested this hypothesis *in vitro* and *in vivo* (Oudard et al., 1991; Rahman et al., 1992; Warren et al., 1992; Thierry et al., 1992, 1993; Goren et al., 2000; Matsuo et al., 2001; Rivera et al., 2002; Mamot et al., 2003). It has been shown that liposomal formulations were more toxic than the free drug against the MDR tumors, whereas, liposomal formulations and free drug were equally toxic against non-MDR tumors. However, it has by no means been firmly established that MDR reversal by liposomal formulation is necessarily the result of internalization of the liposomal formulation by the MDR cells. It should also be kept in mind that the reversal of drug resistance obtained by liposomal formulation of the drug may not (only) be attributable to the encapsulation as such, but may also be affected by other factors such as lipid composition and drug/lipid ratio.

An alternative approach to achieve MDR circumvention may be to use a liposomal formulation specifically targeted to a tumor-related internalizing antigen that is over-expressed on the tumor cell membrane allowing the liposome to be actively taken up via a receptor-mediated endocytic pathway. This mechanism of uptake presumably will allow the drug to by-pass the membrane-associated P-gp, resulting in an increased therapeutic index.

Human Tf is a serum glycoprotein (80 kDa) that transports ferric ions. It is known to be internalized by cells by means of Tf receptor (Tf-R) mediated endocytosis (Huebers and Finch, 1987). Tf-Rs are over-expressed and feature a high turnover rate on tumor cells (Singh, 1999), their number correlating with the aggressive proliferation potential of the tumor (Elliott et al., 1993). Therefore, Tf-R is considered an effective potential target for specific drug delivery into tumor cells (Xu et al., 1999; Ishida et al., 2001a; Gijsens et al., 2002; Iinuma et al., 2002; Qian et al., 2002; Derycke et al., 2004).

In this study, we encapsulated DXR in liposomes carrying covalently surface-conjugated Tf and examined the *in vitro* cytotoxicity of these liposomes towards a DXR-sensitive cell line (SBC-3) and a DXR-resistant sub-line, SBC-3/ADM (Shrivastava et al., 1998).

2. Materials and methods

2.1. Materials

Hydrogenated egg phosphatidylcholine (HEPC) and egg phosphatidylcholine (EPC) were generously donated by Nippon Oil and Fat (Tokyo, Japan). 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylethanolamine-*N*-[3-(2-pyridyldithio) propionate] (PDP-PE) was purchased from Avanti Polar Lipids (AL, USA). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). Doxorubicin (DXR), transferrin (Tf), dithiothreitol and 3-(2-pyridylthio) propionic acid *N*-hydroxysuccinimide ester (SPDP), verapamil (VER) and cyclosporin A (CsA) were purchased from Sigma (MO, USA). Sephadex G-25, Sephadex G-50 and Sepharose CL-4B were purchased from Amersham-Pharmacia Biotech (Upsala, Sweden). ^{3}H-Cholesterylhexadecyl ether ([^{3}H]-CHE) was purchased from NEN Research Products (MA, USA). The anti-P glycoprotein (P-gp) monoclonal antibody, MRK16 (IgG2a) (Hamada and Tsuruo, 1988), was kindly provided Dr. Saburo Sone (The University of Tokushima). All other reagents were commercial products of reagent grade.

2.2. Cells

Human small cell lung cancer cell, SBC-3, and DXR resistant subline of SBC-3, SBC-3/ADM, were kindly provided by Dr. Saburo Sone (The University of Tokushima). Cells were maintained in EAGLE's MEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Trace Scientific, Melbourne, Australia) at 37 °C in a humidified atmosphere of 5% CO_2 in air. For maintaining the drug resistance, SBC-3/ADM cells were cultured at 100 ng DXR/ml.

2.3. Preparation of liposomes

Liposomes were composed of EPC or HEPC plus CHOL (2:1, molar ratio). For covalent coupling of Tf, 1% of PDP-PE (mol/mol EPC or HEPC) was incorporated. Liposomes were prepared as previously described (Ishida et al., 2004). The dried lipid film was hydrated with 250 mM ammonium sulfate and the hydrated liposomes were sequentially extruded through polycarbonate membrane filters (Nuclepore, CA, USA) with pore sizes of 400, 200 and 100 nm, respectively. The mean diameter of the liposomes was approximately 120 nm, as determined by a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). Phospholipid concentration was determined by colorimetric assay (Bartlett, 1959). DXR was encapsulated into the liposomes by remote loading using an ammonium sulfate gradient, as previously described (Haran et al., 1993). Following extrusion, the external buffer was exchanged by eluting through a Sephadex G-50 column equilibrated with 10% sucrose. DXR was added to the liposomes at a DXR/phospholipid ratio of 0.2:1 (w/w). HEPC-based liposomes were incubated for 1 h at 65 °C, and EPC-based liposomes for 1 h at 37 °C. The liposome-encapsulated DXR was separated from free DXR over a Sephadex G-50 column eluted with HEPES buffered saline (25 mM HEPES, 140 mM NaCl, pH 7.4).

For the preparation of targeted liposomes, 10 mg of Tf was incubated with 16 μl of 20 mM SPDP in MeOH for 30 min at room temperature followed by gel filtration on a Sephadex G-25 column in 0.1 M acetate buffer (pH 4.5) to remove excess SPDP. Then, dithiothreitol was added at a final concentration of

50 mM and the mixture was incubated for a further 30 min at room temperature to reduce the SPDP. The product was purified over a Sephadex G-25 column in MES buffered saline (25 mM MES, 140 mM NaCl, pH 6.5). The reduced Tf was added to preformed DXR-loaded liposomes at a ratio of Tf to phospholipid of 0.2:1 (w/w) and then incubated overnight at 4 °C. Free Tf was separated from the Tf-liposomes by chromatography on a Sepharose CL-4B column in HEPES buffered saline. The phospholipids concentration in the resulting liposome was determined as described above. The efficiency of coupling was determined by estimating the amount of liposome-associated TF using the CBQCA Protein Quantification kit (Molecular Probes, OR, USA). The density of TF on the resulting liposome was approximately 20–40 Tf molecules per liposome irrespective with the lipid composition. The concentration of liposome-encapsulated DXR was determined by spectrophotometry ($\lambda = 480$ nm) (Hitachi F-4500, Tokyo, Japan) following methanol extraction. For the cellular association assay, to determine the amount of Tf-liposome associated with the cells, the liposomes were labeled with a trace amount of [^{3}H]-CHE (40 μCi/μmol phospholipids) as a non-exchangeable lipid phase marker.

2.4. Cytotoxicity assay

IC_{50} of various DXR-formulations was determined using the MTT cell proliferation assay (Mosmann, 1983). Cells were seeded into 96-well plate at 1×10^4 cells/well, and incubated for 48 h. After addition of various DXR-formulations the cells were incubated for 1 or 24 h at 37 °C in a CO_2 incubator. After washing three times with cold PBS(−), the plate was further incubated in fresh media containing 10% FBS for another 47 h or 24 h (the total culture time prior to the assay was 48 h). After washing three times with cold PBS(−), 50 μl of an MTT solution (5 mg/ml in PBS(−)) was added. Following a 4-h incubation, 150 μl of 40 mM HCl in isopropanol was added to dissolve any crystals formed in the well. Absorption of the dissolved crystals was determined on a microplate reader at 590 nm (Wallac1420 ARVO$_{SX}$, Perkin-Elmer Life Science, MA, USA). IC_{50} was defined as the DXR concentration, which was encapsulated in the liposomes or free form that inhibits cell growth by 50% as compared to the control wells without any drug.

2.5. Cellular DXR accumulation

Cells (5×10^5) were seeded in a 12-well plate. The plate was incubated for the times indicated in the presence of the liposomal DXR at a concentration of 10 μM DXR. After the incubation, the cells were washed three times with cold PBS(−) and collected by treating with 0.25% trypsin/1 mM EDTA followed by centrifugation (1000 rpm, 5 min, 4 °C). The cell pellet was resuspended in 500 μl of cold PBS(−), and a 5 μl aliquot was subjected to protein assay (DC protein assay (Bio-Rad, CA, USA)). The cells were disrupted by addition of 500 μl of 10% Triton X-100. Then, 1.5 ml of 0.15 M HCl/50% EtOH was added to extract the DXR. After centrifugation to remove cellular debris, the extracted DXR was determined by fluorescence spectrophotometry (Hitachi F-4500) at an excitation wavelength of 470 nm and an emission wavelength of 585 nm, respectively.

2.6. Cellular liposome association

Cells (5×10^5) were seeded in a 12-well plate. The plate was incubated for 1 h in the presence of the Tf-coupled liposomes (40 μM phospholipid, corresponding to approximately 10 μM DXR). After the incubation, the cells were washed three times with cold PBS(−) and collected by treating with 1 ml of 0.25% trypsin/1 mM EDTA followed by centrifugation (1000 rpm, 5 min, 4 °C). The cell pellet was resuspended in 500 μl of cold PBS(−), and a 5 μl aliquot was subjected to protein assay (DC protein assay (Bio-Rad, CA, USA)) and residual sample was subjected to radioactivity assay. The amount of [^{3}H]-CHE-liposome associated with the cells was determined by scintillation couting (Aqueous Counting Scintillant scintillation fluid (Clear-sol I, Nacalai Tesque, Kyoto, Japan)) in a LSC-3500 (Aloka, Tokyo, Japan).

2.7. Intracellular distribution of liposomal DXR

Cells (5×10^5) were seeded in a glass-based dish. Liposomal DXR (10 μM DXR) was added, and then the dish was incubated for 1 h. After replacement of media, the cells were immediately examined by confocal laser microscopy (LSM5 Pascal, Carl Zeiss, Germany) with an LP560 filter at an excitation wavelength of 488 nm.

2.8. Determination of liposome membrane fluidity

The degree of fluorescence polarization of diphenylhexatriene (DPH) in liposomes was used as a parameter to estimate membrane fluidity (Nagayasu et al., 1994). Eight milliliters of 0.5 mM liposome suspension was incubated with 2.5 μl of 5 mM DPH in *N*,*N*-dimethylformamide at 37 °C for 75 min. The fluorescence intensity of DPH was then determined at excitation and emission wavelengths of 360 and 425 nm, respectively (Hitachi F-4500). The degree of fluorescence polarization (r) was calculated by the following equation.

$$r = \frac{(I_{vv} - G I_{vh})}{(I_{vv} + G I_{vh})}, \qquad G = \frac{I_{hv}}{I_{hh}}$$

where I_{vv} and I_{vh} are the vertical and horizontal components of fluorescence intensity of DPH excited by vertically polarized light, respectively. G is the correction factor, and I_{hv} and I_{hh} are the vertical and horizontal components of fluorescence intensity obtained using horizontally polarized light, respectively.

2.9. DXR leakage assay

The leakage assay was carried out according to the method described previously (Ishida et al., 2001b) with minor modification. Briefly, DXR-loaded liposomes were passed over a Sephadex G-50 column immediately prior to use to remove any residual or leaked DXR during storage. One volume of DXR-loaded liposomes (1 mM phospholipid concentration) was

incubated in nine volumes of Eagle's MEM containing 10% FBS at 37 °C. To separate DXR retained inside the liposomes from leaked DXR, the reaction mixture was passed through a Sepharose CL-4B column. Aliquots of liposome fractions were added to 0.15 M HCl/50% EtOH to extract DXR. Fluorescence of DXR was determined by fluorescence spectrophotometry (Hitachi F-4500) at an excitation wavelength of 470 nm and an emission wavelength of 585 nm. The DXR retained following the incubation was calculated by dividing the DXR/phospholipid ratio after the incubation by the initial DXR/phospholipid ratio.

2.10. Analysis of P-gp expression by flow cytometry

Tumor cells were harvested and resuspended in PBS(−) supplemented with 10% human pooled AB serum to prevent non-specific antibody binding. After incubation for 30 min at 4 °C in PBS(−) containing MRK16 antibody or mouse mismatched antibody (10 μg/ml), the cells were washed with PBS(−), and subsequently fluorescein-conjugated rabbit anti-mouse IgG Fc (Organon Teknica, PA, USA) was added as a second antibody. After 30-min incubation at 4 °C, they were washed again and the fluorescence intensity was measured with a FACScan (Beckton Dickinson, CA, USA). Data are expressed as mean fluorescence intensity (MFI).

2.11. Statistics

All values are expressed as the mean ± S.D. Statistical analysis was performed using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p < 0.05$.

3. Results

Before testing the cytotoxicity of the Tf-R targeted liposomes against the resistant cells, we confirmed the P-gp expression in SBC-3 and its drug-resistant variant, SBC-3/ADM, by using flow cytometry. As expected, SBC-3/ADM showed high expression of P-gp compared to the parent cell (38 MFI for SBC-3/ADM cells versus 3 MFI for SBC-3 cells). This is consistent with observations by Parajuli et al. (1999). In addition, the effects of the P-gp-mediated MDR modulators, VER or CyA, on the reversal of DXR resistance of SBC-3/ADM cells were investigated. Treatment for 1 h with 5 μM VER or 1 μM CyA enhanced the sensitivity of SBC-3/ADM cells to DXR 3-fold or 8.5-fold, respectively (59.08 ± 12.12 μM for control versus 19.77 ± 7.67 μM for VER or 6.98 ± 2.53 μM for CyA). This is also consistent with observations reported by Shrivastava et al. (1998). These results clearly demonstrate that SBC-3/ADM cells over-express P-gp in comparison to the parent cells, SBC-3, and that the DXR-resistance of SBC-3/ADM cells is mainly mediated by P-gp.

In addition, the number of Tf-R was determined. Similar densities of Tf-R on the surface of SBC-3 and SBC-3/ADM cell lines ($(2.6–2.7) \times 10^5$/cell) were detected.

3.1. Cytotoxicity of DXR formulations on sensitive or MDR tumor cells

In vitro cytotoxity of DXR formulations is summarized in Table 1. After a 1-h exposure of the drug-sensitive cells (SBC-3) to the DXR formulations, the rank order of cytotoxicity was: Tf-R targeted EPC-liposomes ≈ free DXR > EPC-liposomes ≈ Tf-R targeted HEPC-liposomes > HEPC-liposome. The EPC-based liposomes, either targeted or non-targeted, showed significantly lower IC_{50}s than the HEPC-based liposomes. The IC_{50} of targeted EPC-liposomes was significantly lower than that of non-targeted EPC liposomes. The targeted EPC-liposomes have an IC_{50} comparable to that for free DXR. All cytotoxicities increased 10–40-fold upon increasing the time of incubation with the DXR formulations to 24 h. Under these conditions, the IC_{50} of the EPC liposomes became comparable to that of targeted EPC-liposomes. The IC_{50}s of the EPC-based liposomes, either targeted or non-targeted, were comparable to the IC_{50} of free DXR. The cytotoxicities of HEPC-based liposomes also increased with the time of exposure to the DXR formulations, but remained still much lower than those of EPC-based liposomes, irrespective of Tf-modification.

As regards the MDR cells (SBC-3/ADM), only targeted EPC-liposomes showed significant cytotoxicity, regardless of the time

Table 1
Cytotoxicity of free doxorubicin (DXR) and liposomal formulations of DXR (with or without Tf-modification) to either drug-sensitive cells (SBC-3) or drug-resistant cells (SBC-3/ADM)

	IC_{50} (μM) (mean ± S.D.)			
	1 h		24 h	
	SBC-3	SBC-3/ADM	SBC-3	SBC-3/ADM
Tf-R targeted EPC-liposome	0.715 ± 0.207	17.69 ± 2.77	0.056 ± 0.001	3.331 ± 2.33
EPC-liposome	11.80 ± 5.59	≫100	0.052 ± 0.004	≫100
Tf-R targeted HEPC-liposome	53.48 ± 5.10	≫100	4.475 ± 1.980	≫100
HEPC-liposome	58.45 ± 18.1	≫100	4.044 ± 2.261	≫100
Free DXR	0.971 ± 0.533	59.08 ± 12.12	0.028 ± 0.013	1.53 ± 0.24

Either SBC-3 cells or SBC-3/ADM (1×10^4) were incubated with free DXR or various formulations of liposome-encapsulated DXR with or without Tf-modification. Tf coupling efficiency via the PDP-PE method was approximately 20–40 molecules per liposome; DXR loading was 0.24–0.28 μmol DXR/μmol phospholipids. After 1 h or 24 h, cells were washed free of drug and then incubated for a further 47 h or 24 h. Cytotoxicities were determined by the MTT assay and are expressed as mean IC_{50} ± S.D. ($n = 3$).

of exposure. The IC_{50} of the targeted EPC-liposomes was significantly lower than that for free DXR following a 1-h exposure. The IC_{50} of free DXR became comparable to that of the targeted EPC-liposomes, when the cells were exposed to DXR for 24 h. None of the liposomal formulations without encapsulated DXR were toxic to either SBC-3 cells or SBC-3/ADM cells after a 24-h exposition at a concentration below 100 μM phospholipid, which would correspond to a DXR concentration of 30 μM, if the formulations contained DXR (not shown). In addition, the cytotoxicity of drug-free targeted EPC-liposomes (100 μM phospholipids) with various concentrations of free DXR was determined. Similar cytotoxicities of free DXR alone and of free DXR added to drug-free targeted EPC-liposomes were observed. Also an excess of free Tf did not show any toxicity to the cells (not shown). It is noteworthy that of all the formulations tested only the targeted EPC-liposomes showed the potential to circumvent MDR.

3.2. Intracellular DXR accumulation

Intracellular accumulation of DXR was determined following incubation of SBC-3 or SBC-3/ADM cells with the various DXR formulations (Fig. 1). As for SBC-3 cells, the rank order of the potential of the liposomes to deliver encapsulated DXR into the cells was Tf-R targeted EPC-liposomes > EPC-liposomes > Tf-R targeted HEPC-liposomes ≈ HEPC-liposomes (Fig. 1A). EPC-based liposomes, either targeted or non-targeted, produced significantly higher intracellular DXR levels, while HEPC-based liposomes delivered relatively little DXR into the cells. The DXR delivered by targeted EPC-liposomes accumulated within the cells at a faster rate and to larger extents than that delivered by non-targeted EPC-liposomes. The DXR level in the cells increased with time of incubation and reached a plateau level at 80 min.

Also for the SBC-3/ADM cells, the EPC-based liposomes, either targeted or non-targeted, delivered the encapsulated DXR substantially better than the HEPC-based liposomes (Fig. 1B). Of the EPC-based liposomes, the targeted formulation delivered the encapsulated DXR more efficiently than the non-targeted one. Yet, the DXR level in the drug-resistant cells, delivered by targeted EPC-liposomes, was much lower (1/4-fold) than that observed in the drug-sensitive SBC-3 cells.

After a 1-h exposure to free DXR, the amount of intracellular DXR was 3.19 ± 0.07 (nmol/mg protein) for SBC-3 cells and 0.75 ± 0.22 (nmol/mg protein) for SBC-3/ADM cells, respectively. Of all DXR formulations used free DXR resulted in the highest drug accumulation: 2.2-fold higher than targeted EPC liposomes in SBC-3 cells and 2.1-fold higher than in SBC-3/ADM cells.

In addition, the association of targeted EPC- or HEPC-liposome was determined following 1 h incubation with SBC-3 or SBC-3/ADM. The amount of associated liposome was quantified from the specific activity of [^{3}H]-CHE counts associated with the cells. There was no significant difference between the targeted EPC- and HEPC-liposome on the association to SBC-3 or SBC-3/ADM cells (data not shown).

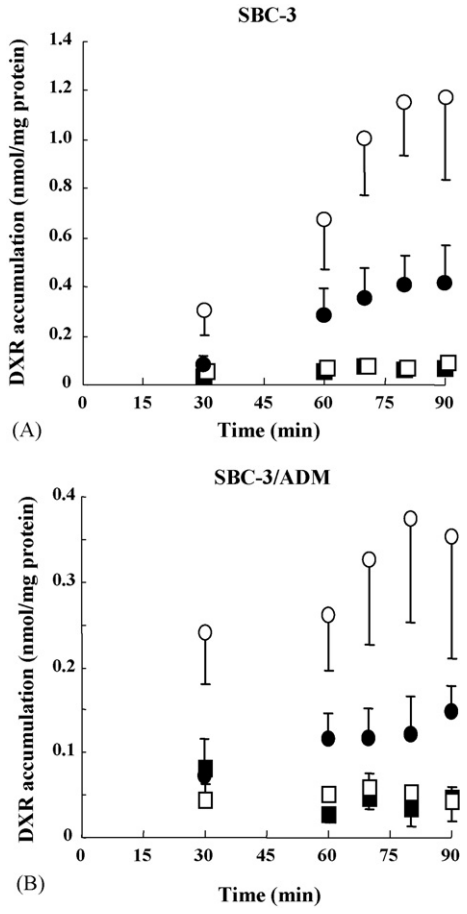

Fig. 1. *In vitro* cellular accumulation of DXR in SBC-3 cells and SBC-3/ADM cells. The DXR cellular accumulation assay is as described in Section 2. Results are from a representative experiment, and are means of triplicate analyses ± S.D. (A) SBC-3 cells, (B) SBC-3/ADM. (◯) Tf-R targeted EPC-liposomes; (●) non-targeted EPC-liposomes; (□) Tf-R targeted HEPC-liposomes; (■) non-targeted HEPC-liposomes.

3.3. Confocal microscopy of interactions of DXR formulations with the cells

The cellular association of the various liposomal DXR formulations was further investigated by confocal microscopy (Fig. 2). After a 1-h exposure, DXR delivered by the targeted EPC-liposomes accumulated to significant extents in the nucleus in both SBC-3 and SBC-3/ADM cells (Fig. 2A and E). For the non-targeted EPC-liposomes, a relatively small amount of liposome-delivered DXR appeared in the nucleus (Fig. 2B and F). On the other hand, for the HEPC-based liposomes, either targeted or non-targeted, only cell surface-bound fluorescence was observed, indicating that no internalization of liposomes occurred (Fig. 2C, D, G, and H).

3.4. Bilayer fluidity and serum stability of targeted and non-targeted HEPC and EPC-based liposomes

The effects on bilayer fluidity of modification of EPC or HEPC-based liposomes with Tf were determined by measuring changes in fluorescence polarization (*r*) of bilayer-

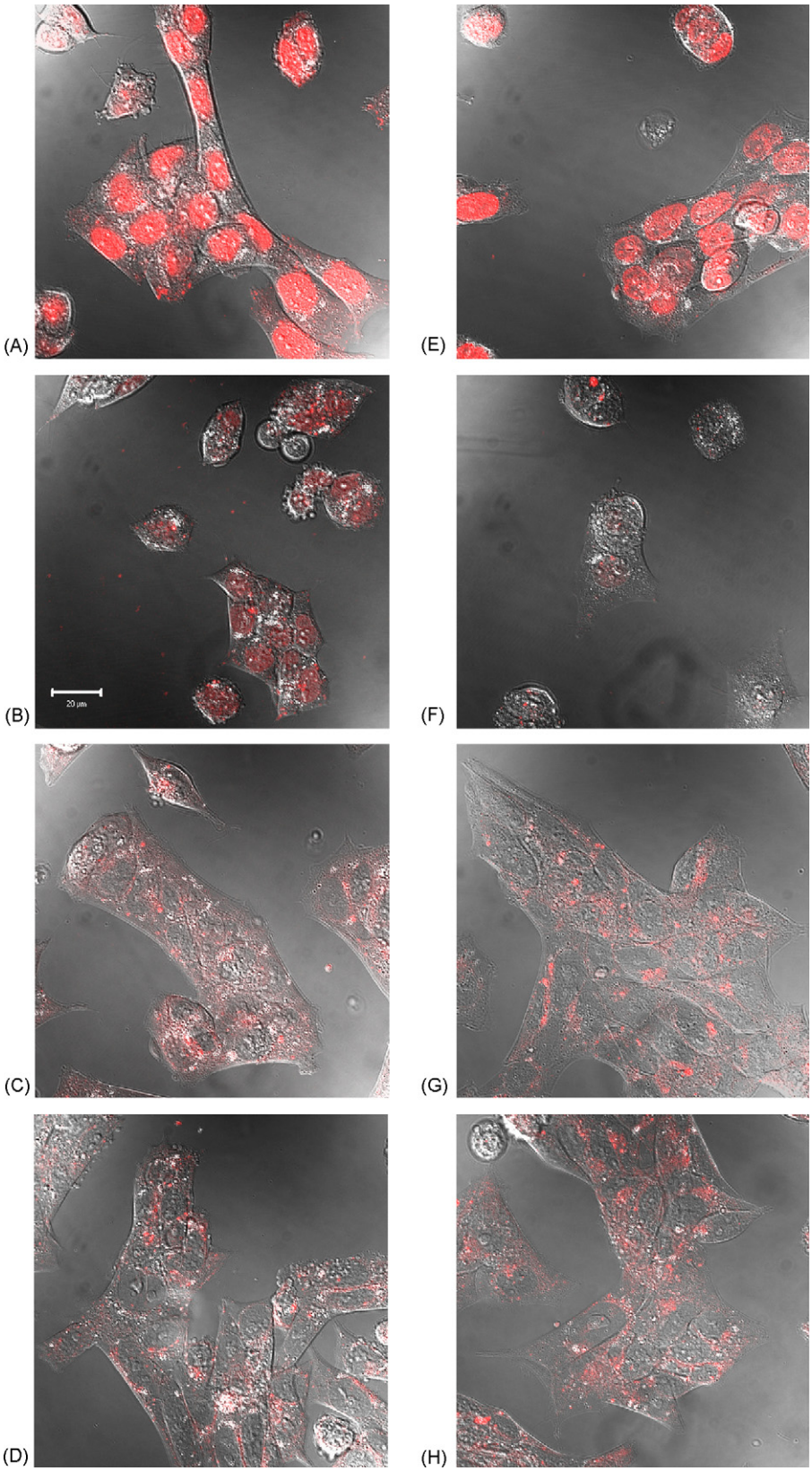

Fig. 2. Confocal microscopy after 1 h of incubation of various liposomal DXR formulations with SBC-3 cells (A–D) and SBC-3/ADM cells (E–H) at 37 °C. (A and E) Tf-R targeted EPC-liposomes, (B and F) non-targeted EPC-liposomes, (C and G) Tf-R targeted HEPC-liposomes, and (D and H) non-targeted HEPC-liposomes.

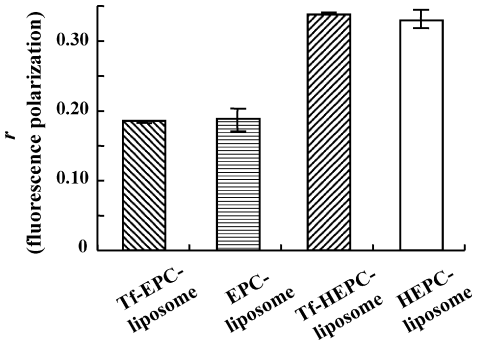

Fig. 3. Membrane fluidity of drug-free EPC-based or HEPC-based liposomes with or without Tf-modification. The degree of fluorescence polarization of diphenylhexatriene (DPH) in liposomes was measured as described in Section 2. Each value represents the mean ± S.D. of three separate experiments.

incorporated diphenylhexatriene (Fig. 3). The *r* value of the non-modified EPC-liposomes was substantially smaller than that of the HEPC liposomes. Modification with Tf did not alter the original *r* values of either liposome type, indicating that, irrespective of modification with Tf, EPC-based liposomes have a much higher membrane fluidity than the HEPC-based liposomes.

The stability of DXR-loaded liposomes in Eagle's MEM containing 10% FBS was examined. In all formulations we used in this study, over 90% of the encapsulated DXR remained liposome-associated following a 24-h incubation in culture medium supplemented with 10% FBS (not shown). Modification with Tf did not affect the stability of EPC- or HEPC-liposomes in the media.

4. Discussion

In this study, we examined the potential of Tf-R targeted liposomes to deliver encapsulated DXR efficiently into target cells and, consequently, to effectuate significant cytotoxicity not only towards drug-sensitive cells (SBC-3) but also to MDR cells (SBC-3/ADM) by by-passing P-gp. The results described in this paper lead a possibility that Tf-R targeted EPC-liposomes can be used as an *in vivo* drug delivery system to bypass P-gp-mediated efflux of DXR from the cells, thus circumventing MDR of tumor cells, in contrast to free DXR, which showed similar cytotoxicity *in vitro*. Because in the case of free DXR the large distribution volume lowers the drug plasma levels following intravenous administration.

The crucial finding of this study was that *only* the Tf-R targeted *fluid*-type EPC-liposomes, but *not* the Tf-R targeted more *rigid* HEPC-liposomes, led to substantially enhanced uptake and nuclear accumulation of encapsulated DXR in both SBC-3 and SBC-3/ADM cells. Although many researchers have studied the importance of liposome size, membrane fluidity and density of ligands/antibodies attached on the liposomal surface for efficiency and rate of internalization via receptor-mediated endocytosis on anticancer drug-sensitive cell lines (Maruyama et al., 1990; Lopes de Menezes et al., 1998; Pagnan et al., 1999; Rensen et al., 2001; Ishida et al., 2001b; Park et al., 2002; Sapra and Allen, 2002; Allen et al., 2005), the influence of membrane fluidity of liposomes has not been studied on MDR cell lines. The results described in Fig. 1 suggest that liposomes with highly fluid membranes are internalized by cells more efficiently than liposomes with rigid bilayers. This was supported by our confocal microscopic study (Fig. 2) showing that the DXR encapsulated in Tf-modified EPC-liposomes was extensively accumulating in the cell interior especially in the nucleus. This clearly indicates that the membrane fluidity of liposomes affects the process of their internalization via receptor-mediated endocytosis.

The extent of intracellular accumulation of DXR was determined as a function of time (Fig. 1). When EPC-liposomes were targeted with Tf, accumulation of DXR in both cell lines strongly increased, most likely because the receptor-mediated internalization of the Tf-liposomal drug packages occurs far more efficiently than that of the non-targeted EPC-liposomes (Fig. 2). However, even with the Tf-targeted liposomes the extents of DXR accumulation were much lower than those of free DXR. On the other hand, as can be seen in Table 1, free DXR and DXR-containing Tf-R targeted EPC-liposomes produced similar levels of cytotoxicity. This may mean that the *amount* of DXR delivered in the cells is less important in determining the cytotoxic potential of the drug than the *mechanism* by which it is delivered.

We tentatively propose that DXR entering the cell by means of Tf-targeted liposomes is delivered deeper into the cell interior than free DXR, conceivably closer to the nucleus. As a consequence, a major fraction of the drug escapes expulsion by the P-gp pump and is sequestered by the nearby nucleus, where it can exert its cytostatic action. The difference in results between Tf-targeted EPC and HEPC liposomes may be explained by different rates of DXR release from these two liposome types. The more fluid type EPC liposomes (Fig. 3) may be more prone to drug release than the more solid HEPC liposomes. Also intra-lysosomal enzymatic degradation is likely to proceed more rapidly for EPC than for HEPC liposomes (Hertz and Barenholz, 1975; Allen and Everest, 1983).

Nevertheless, we cannot exclude that at least some drug release occurs already during the binding stage at the cell surface. Also that release will be faster from the EPC than from the HEPC liposomes, likewise leading to higher cellular drug uptake through the former liposome type. Thus, the physicochemical properties of the liposomes, in addition to the presence of the targeting device, may be a key factor in determining the extent of intra-nuclear DXR accumulation, and hence the cytotoxic efficiency of the drug. An additional factor that may favor drug release from the fluid type liposomes is the higher rate of flip–flop movement between the two halves of a high-fluidity membrane (Regev and Eytan, 1997). Especially in the MDR cells the combination of a high rate of internalization and the subsequent rapid release of DXR might overload the capacity of drug efflux by P-gp, thus contributing to the relief from drug resistance. The cytotoxicity of non-targeted EPC-liposomes, on the other hand, may have been mainly produced by release of drug from liposomes bound at the cell surface followed by uptake of the released drug into the cells (Ishida et al., 2001b; Sapra and Allen, 2002).

An alternative mechanism possibly accounting for increased cytotoxicity with the targeted EPC-liposome on the MDR cell may involve direct interference of the liposomes with the function of P-gp as described by others (Rahman et al., 1992; Warren et al., 1992). In the present study, however, we observed similar cytotoxicities of free DXR alone and of free DXR added to drug-free targeted EPC-liposomes on SBC-3/ADM (results not shown). This would suggest that the liposomes do not directly interfere with P-gp function. In addition, neither drug-free Tf-coupled liposomes nor free Tf displayed any cytotoxicity against SBC-3/ADM at the concentrations tested, indicating that these Tf formulations were unable to induce cell growth arrest or cell death.

For *in vivo* application with the Tf-R targeted EPC-liposome, it would be necessary to add it long-circulating property since conventional liposome and immunoliposome were usually cleared rapidly from the circulation after intravenous administration. The most popular way to produce such long-circulating liposomes is to sterically stabilize the liposome surface by the incorporation of lipid-conjugated PEG (PEGylated liposome or sterically stabilized liposome) (Allen et al., 1991). The Tf-R targeted liposomes with extended circulation times would efficiently accumulate in sites of tumor progression, and bind to and subsequently being internalized by the cells, resulting in circumvention of MDR *in vivo* by by-passing DXR efflux derived by P-gp. But, such potential of the liposome may be hampered by PEG modification. Although inclusion of PEG-derivative (PEGylated lipid) into the targeted liposome would increase the stability and the circulation half-lives of the liposome *in vivo*, the liposome hardly releases the encapsulated DXR in the cell interior following internalization (Lopes de Menezes et al., 1998). These problems may be overcome by use of cleavable PEGylated lipid instead of PEGylated lipid (Kirpotin et al., 1996; Ishida et al., 2001b). Cleavage of the grafted PEG from the surface of targeted liposome may allow rapid content release and increased cytotoxicity *in vivo*. This possibility is now progress in our laboratory.

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