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Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro

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Abstract

Receptors for the vitamin folic acid are frequently overexpressed on epithelial cancer cells. To examine whether this overexpression might be exploited to specifically deliver liposome-encapsulated drug molecules in vitro, folate-targeted liposomes were prepared by incorporating 0.1 mol% of a folate-polyethyleneglycol-distearoylphosphatidylethanolamine (folate-PEG-DSPE) construct into the lipid bilayer, and were loaded with doxorubicin (DOX), an anti-cancer drug. Uptake of folate-PEG-liposomal DOX by KB cells was 45-fold higher than that of non-targeted liposomal DOX, and 1.6-times higher than that of free DOX, while the cytotoxicity was 86 and 2.7-times higher, respectively. Folate-targeting is fully compatible with PEG-coating of the liposomes, since incorporation of 4 mol% PEG2000-DSPE does not reduce the uptake or cytotoxicity of folate-PEG-liposomal DOX. Uptake of folate-PEG-liposomes was inhibited by 1 mM free folic acid but was unaffected by physiological concentrations of folate. In HeLa/WI38 co-cultures, folate-PEG-liposomes encapsulating calcein, a fluorescent dye, were found to be almost exclusively internalized by the HeLa cells which overexpress the folate receptors. We suggest that folate targeting constitutes a possible mechanism for improving the specificity of PEG-coated liposomes for cancer cells.

Keywords: Doxorubicin; PEG-coated liposome; Tumor cell targeting; Folate-binding protein; Liposome targeting

1. Introduction

Although doxorubicin (DOX) is one of the most widely used anticancer agents, its clinical application is still limited by its deleterious side effects, including myelosuppression, gastrointestinal toxicity, and especially cardiotoxicity [1]. To avoid these complications, the use of liposomes as carriers for DOX has been recently explored in both animal and human trials [2–9]. In general, liposomal DOX exhibits enhanced antitumor activity as well as improved therapeutic index. The advantages of liposomal DOX over free DOX derive largely from the former's prolonged systemic circulation, reduced peak levels, and sustained release of the drug into the blood stream [5]. Liposomes also have a tendency to extravasate and preferentially accumulate in tumor tissues with leaky endothelia and reduced lymphatic drainage [6].

The attractiveness of liposomes as drug carriers was further enhanced by the recent introduction of long-circulating liposomes coated with polyethyleneglycol (PEG). By protecting the liposome surface from non-specific opsonization by certain plasma components, the PEG coating inhibits the recognition of the liposomes by phagocytes of the reticuloendothelial system [2,10–18]. Unfortunately, recent studies also indicate that steric hindrance introduced by the PEG-coating on the liposome surface can inhibit ligand-mediated targeting of the liposome when the targeting ligand, e.g., an antibody, is directly conjugated to the lipid bilayer [19–21]. We found that liposomes can be efficiently targeted to receptor-bearing tumor cells when conjugated to folate via a long PEG-spacer [22]. Recent studies in other laboratories also indicate that PEG-coated liposomes attached to an antibody via a long PEG spacer show both effective target binding and prolonged circula-

Abbreviations: PEG, polyethyleneglycol; PEG-*bis*-amine, polyoxyethylene-*bis*-amine; PBS, phosphate-buffered saline (136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4); DOX, doxorubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}, drug concentration giving 50% cell killing; PEG2000-DSPE, polyethyleneglycol (M.W. ≈ 2000) derivatized distearoylphosphatidylethanolamine.

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tion times [23,24]. Therefore, it is likely that similar advantages will be shared by PEG-coated liposomes attached to folate via a PEG spacer [22].

Folate binding protein, a glycosylphosphatidylinositol-anchored cell surface receptor for the vitamin folic acid, has recently been shown to be vastly overexpressed in a wide variety of human tumors, especially ovarian carcinomas [25–31]. This observation has raised the possibility of using folate-PEG-liposomes to specifically deliver anticancer drugs to these tumors, since similar studies using antibody as the targeting ligand gave promising results in vitro as well as in vivo [32–36]. As an initial investigation of this potential application, we have examined the uptake and cytotoxicity of folate-targeted liposomes in two lines of cultured tumor cells overexpressing the folate receptor, KB and HeLa, as well as co-cultured HeLa and WI38 cells (a non-transformed line). We report here that encapsulation within folate-PEG-liposomes increases both the potency and specificity of DOX. The intracellular fate of folate-PEG-liposomal DOX and the potential for folate-PEG-liposomes as in vivo drug carriers are discussed.

2. Materials and methods

2.1. Materials

DOX was purchased from Calbiochem. PEG2000-DSPE was acquired from Avanti Polar Lipids. Distearoylphosphatidylethanolamine (DSPE), distearoylphosphatidylcholine (DSPC), folic acid, polyoxyethylene-*bis*-amine (PEG-*bis*-amine, M.W. ≈ 3350), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and non-enzymatic cell-dislodging solution were purchased from Sigma.

2.2. Methods

Cell Culture. KB cells, a human nasopharyngeal epidermal carcinoma cell line, HeLa cells, a human cervical carcinoma cell line, and WI38 cells, a human lung fibroblast primary cell line were kindly provided by the Purdue Cancer Center. All cells were maintained in a medium containing physiological concentrations of folate, i.e., minimum essential medium minus the folic acid additives and supplemented with 10% heat-inactivated fetal calf serum [37]. The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The folate content of the fetal calf serum supplement brings the folate concentration of the medium to a near physiological value for human serum.

Synthesis of folate-PEG-DSPE. The synthesis of the folate-PEG-DSPE construct is illustrated in Fig. 1.

Folate-PEG-NH_2 was synthesized by reacting 500 mg polyoxyethylene-*bis*-amine with an equimolar quantity of folic acid in 5 ml dimethylsulfoxide containing one molar equivalent of dicyclohexylcarbodiimide and 10 μl pyridine. The reaction mixture was stirred overnight in the dark at room temperature. At this point, 10 ml water was added and the insoluble by-product, dicyclohexylurea, was removed by centrifugation. The supernatant was then dialyzed against 5 mM $NaHCO_3$ buffer (pH 9.0) and then against deionized water to remove the dimethylsulfoxide and unreacted folic acid in the mixture. The trace amount of unreacted polyoxyethylene-*bis*-amine was then removed by batch-adsorption with 5 g of cellulose phosphate cation-exchange resin pre-washed with excess 5 mM phosphate buffer (pH 7.0). Although not necessary, the trace amount of PEG-*bis*-folate may be removed by anion-exchange chromatography on a DEAE-trisacryl Sepharose column. Folate-PEG-amine can be easily eluted with 10

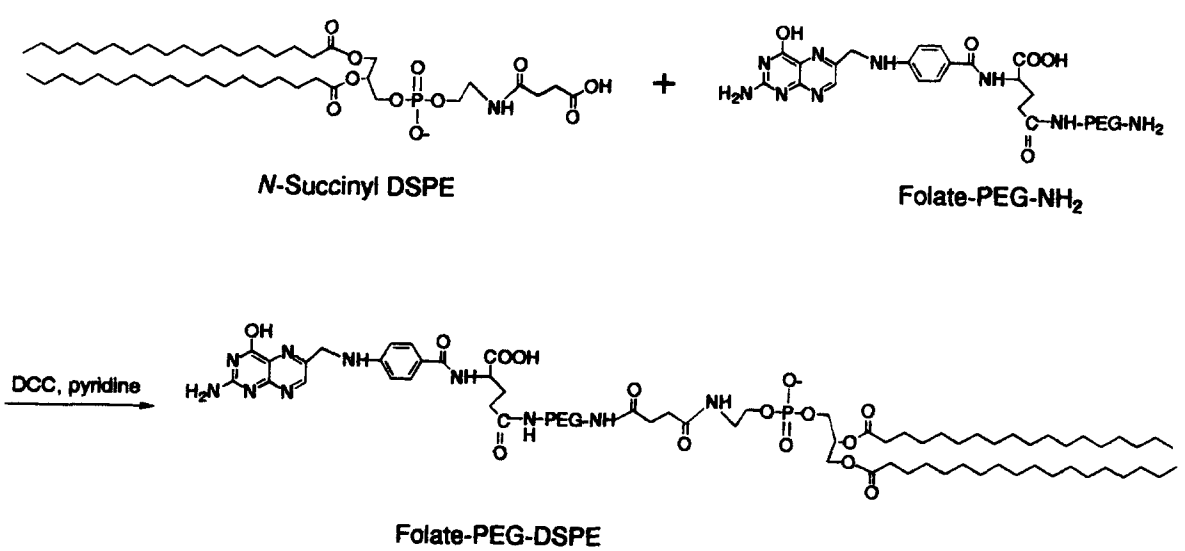

Fig. 1. Synthesis of folate-PEG-DSPE. See text for details.

mM NH_4HCO_3 (pH 8.0). The product folate-PEG-NH_2 was then lyophilized and analyzed for folate content by absorbance at 363 nm and -NH_2 content by the ninhydrin assay. The ratio of folate to free -NH_2 groups in this product was ≈ 1.

N-Succinyl-DSPE was synthesized by reacting overnight 1.1 molar equivalent of succinic anhydride with 100 mg DSPE in 5 ml chloroform containing 10 μl pyridine. The product was precipitated with cold acetone and verified by thin-layer chromatography. *N*-Succinyl-DSPE was re-dissolved in chloroform and its carboxyl group was activated by reacting with one molar equivalent of dicyclohexylcarbodiimide for 4 h at room temperature. An equimolar amount of the above synthesized folate-PEG-NH_2 dissolved in chloroform was then added. After overnight stirring at room temperature, the solvent was removed from the reaction mixture, and the lipid pellet containing the folate-PEG-DSPE conjugate was washed twice with cold acetone, redissolved in chloroform, and stored at -20°C. The formation of folate-PEG-DSPE was confirmed by reverse-phase high-pressure liquid chromatography.

Liposome preparation. Liposomes of the following compositions were used in this study: (a) folate-PEG-liposomes composed of DSPC/cholesterol/folate-PEG-DSPE (56:40:0.1), (b) folate-PEG-liposomes with 4% PEG (M.W. $\approx$ 2000) coating composed of DSPC/cholesterol/PEG2000-DSPE/folate-PEG-DSPE (56:40:4:0.1), (c) control liposomes not targeted with folate composed of DSPC/cholesterol (56:40), and d) control liposomes coated with 4% PEG composed of DSPC/cholesterol/PEG2000-DSPE (56:40:4).

Liposomes were prepared by a polycarbonate membrane extrusion method and were loaded with DOX using a transmembrane pH gradient based on a method described previously [9,35]. Briefly, 100 mg lipid mixture was dissolved in chloroform and dried to a thin film in a round-bottom flask on a rotary evaporator under reduced pressure. The dried lipid mixture was then rehydrated in 2 ml of the low-pH 'trapping' buffer (400 mM citrate, 5 mM phosphate, pH 4.0) by vortexing. The resulting suspension of multilamellar vesicles was then subjected to five cycles of freezing and thawing, briefly sonicated, extruded five times through a 100 nm pore size polycarbonate membrane, and passed through a Sepharose CL-4B (Pharmacia) gel-filtration column (10 cm $\times$ 1.5 cm) pre-equilibrated in PBS. The opaque liposome fractions eluted in the void volume. The liposome suspension was then warmed to 60°C in a water bath, and added to 10 mg solid DOX. The mixture was incubated at 60°C for another 15 min with intermittent vortexing and then passed through a Sepharose CL-4B column to separate folate-PEG-liposomal DOX from unencapsulated free DOX. The DOX concentration in the fractions collected was monitored by absorbance at 475 nm [9]. Approx. 95% of the DOX eluted with the liposome fractions. The DOX-loaded liposomes, with a drug/lipid ratio of 0.1:1 (wt/wt), were then sterilized by filtering through a 0.2 μm cellulose acetate filter (Corning) and stored at 4°C. All liposome samples were used within two weeks of preparation during which period no significant leakage of doxorubicin from the liposomes was detected by gel filtration. The size distribution of the various liposome preparations was determined by light scattering, and the medium size of all liposome preparations was approx. 130 nm in diameter. Since 0.1 mol% folate-PEG-DSPE was incorporated in the targeted liposomes, assuming equal distribution of this lipid in the two leaflets of the liposomal membrane, the number of folate ligands on the outer liposome surface for a 130 nm liposome should be ≈ 75.

Folate-PEG-liposomes encapsulating calcein were prepared by the method described above except that the lipids were hydrated in 25 mM calcein PBS solution.

Uptake of folate-PEG-liposomal DOX by receptor-bearing tumor cells. KB or HeLa cells were transferred to 33 mm culture dishes at $5 \cdot 10^5$ cells per dish 24 h prior to the assay. The cells were then incubated for 2 h at 37°C with 100 μg/ml free or liposomal DOX diluted in culture medium. In free folate competition studies, 1 mM folic acid was added to the incubation medium. After washing four times with PBS, the cells were scraped and suspended in 2 ml of PBS, and solubilized in 1% Triton-X 100. The cell-associated DOX was then determined by DOX fluorescence at 580 nm (slit = 20 nm) with excitation at 475 nm (slit = 20 nm) on a Perkin Elmer MPF-44A fluorescence spectrophotometer. The amount of DOX was calculated using a pre-determined standard curve and calibrated with the number of cells determined by a bicinchoninic acid (BCA) protein assay (Pierce).

The fraction of cells participating in receptor-mediated uptake of folate-PEG-liposomal DOX was evaluated by flow cytometry. KB cells grown to 50% confluency in 75 cm^2 culture flasks were incubated for 2 h at 37°C with 100 μg/ml folate-PEG-liposomal DOX diluted in 5 ml medium. The cells were then washed with PBS, removed from the bottom of the culture flask with 3 ml non-enzymatic cell-dislodging solution, and analyzed for DOX fluorescence on a Coulter Epics Elite Flow Cytometer using the 488 nm line of an air-cooled argon laser as the excitation source. Fluorescence from cell-associated DOX was detected using a 550 nm long-pass emission filter.

To visualize the intracellular distribution of cell-associated DOX, cells treated with DOX were examined under a MRC600 confocal laser-scanning fluorescence microscope (Bio-Rad). Images were collected in the fast-photon counting mode using the 488 nm line of a krypton-argon mixed gas ion laser as the excitation source. A 60 $\times$ objective and an iris setting of 2 mm were used. Images were analyzed on a Macintosh IIsi computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Spring-

field, VA 22161, part number PB93-504868), and printed on a CODONICS NP-1600 Photographic Network Printer.

Cytotoxicity assay. The cytotoxicity of liposomal and free DOX was determined by MTT assay (a cell viability assay measuring the activity of a mitochondrial enzyme that converts MTT, a tetrazolium salt, into a formazan crystal that absorbs at 570 nm) as described previously [36]. Briefly, KB or HeLa cells were transferred to flat-bottomed 96-well tissue culture plates (Corning) at a density of 5×10^4 cells per well 24 h prior to the assay. The culture medium in each well was carefully replaced with 150 μl of medium containing serial dilutions of liposomal or free DOX samples (in triplicates). For free ligand competition studies, either 1 mM folic acid or 20 nM 5-methyltetrahydrofolate was added to the medium used in the serial dilution. After 2 h incubation at 37°C, the cells were washed twice with sterile PBS and incubated in fresh culture medium for a further 48 h. At this point, 20 μl of 5 mg/ml MTT dissolved in PBS was added to each well and the cells were incubated for another 2 h at 37°C. The medium was then removed and the cells solubilized in 100 μl isopropanol containing 0.1 M HCl. The number of viable cells in each well was then determined by absorbance at 570 nm measured on an automated plate reader.

In experiments evaluating the effect of co-administered empty liposomes on the cytotoxicity of free DOX, free DOX was mixed with empty liposomes at a drug to lipid ratio of 1:10 (identical to the drug to lipid ratio used in liposomal DOX).

Selective uptake and cytotoxicity of folate-PEG-liposomes in HeLa / WI38 co-cultures. HeLa/WI38 co-cultures were prepared by plating simultaneously a mixture of HeLa and WI38 cells in 33 mm culture dishes 24 h prior to each experiment. To demonstrate the selective uptake of folate-PEG-liposomes, HeLa/WI38 co-cultures were incubated with 20 μM folate-PEG-liposomal calcein, 50 μM free DOX, or 50 μM DOX encapsulated in folate-PEG-liposomes diluted in PBS. After 2 h incubation at 37°C, the dishes were washed 4 × with 2 ml PBS to remove unbound fluorophores, and then examined on an Olympus BH-2 phase contrast/fluorescence microscope. The cells were photographed in both the fluorescence (dark field) and the phase-contrast (bright field) modes using a 40 × objective.

To evaluate the selective cytotoxicity of folate-liposomal DOX, HeLa/WI38 co-cultures were treated with 10 μM of folate-PEG-liposomal or non-targeted liposomal DOX. After 2 h incubation at 37°C, the cells were carefully washed four times with 2 ml sterile PBS and incubated in fresh culture medium for another 48 h. The co-cultures were then examined under the microscope and photographed.

3. Results

Cellular uptake of free and liposomal DOX

Uptake of folate-PEG-liposomal DOX by cultured KB and HeLa cells was compared with non-targeted liposomal DOX and free DOX. Folate-PEG-liposomes were prepared by including a newly synthesized folate-PEG-DSPE construct in the lipid bilayer and were loaded with DOX by a 'remote-loading' procedure described in Materials and methods. KB and HeLa cells were then incubated for 2 h at 37°C with liposomal or free DOX, and cell-associated DOX was quantitated by DOX fluorescence. DOX release from the various liposome formulations during the 2 h incubation period was less than 2% of the total encapsulated amount.

Uptake of folate-PEG-liposomal DOX by KB cells was found to be 45- and 1.6-times higher than non-targeted liposomal DOX and free DOX, respectively (Table 1). In this experiment, $\approx 1.8 \cdot 10^{10}$ DOX molecules per cell were taken up by the KB cells treated with folate-PEG-liposomal DOX. The level of uptake by these cells was

Table 1
Comparison of cellular uptake and cytotoxicity of various DOX formulations in cultured tumor cells

DOX formulations	Cellular DOX uptake ($\times 10^9$ molecules/cell) ($n = 3$)		IC_{50} (μM)	
	KB	HeLa	KB	HeLa
Folate-PEG-liposomal DOX	18.4 ±0.7	9.04 ± 0.20	0.31	0.41
Liposomal DOX	0.41 ± 0.01	0.31 ± 0.01	26.7	25.3
Folate-PEG-liposomal DOX with 4% PEG-coating	18.7 ±1.0	9.16 ± 0.10	0.29	0.43
Liposomal DOX with 4% PEG-coating	0.42 ± 0.01	0.31 ± 0.01	26.5	25.1
Folate-PEG-liposomal DOX + 1 mM free folic acid	5.64 ± 0.06	2.82 ± 0.03	1.91	1.89
Folate-PEG-liposomal DOX + 20 nM free 5-methyltetrahydrofolate	18.2 ±0.8	9.14 ± 0.17	0.30	0.43
Free DOX	11.3 ±0.4	8.23 ± 0.40	0.83	0.75
Free DOX + empty folate-PEG-liposomes	11.7 ±0.3	8.27 ± 0.38	0.81	0.76
Free DOX + empty control liposomes	11.5 ±0.4	8.24 ± 0.31	0.85	0.77

To determine the cellular uptake, KB or HeLa cells were incubated with 100 μM liposomal or free DOX, after which cell-associated DOX was determined by fluorescence spectroscopy. Cytotoxicity was determined using the MTT assay, as described in Materials and methods. For lipid compositions, see Materials and methods. IC_{50}, DOX concentration giving 50% cell killing

reduced by ≈ 70% by 1 mM free folic acid, but was virtually unaffected by addition of physiological concentrations (20 nM) of 5-methyltetrahydrofolate [37]. Similar results were obtained in HeLa cells (Table 1) except that the levels of folate-PEG-liposomal DOX uptake was ≈ 50% lower, presumably due to the smaller size and lower folate receptor content of these cells compared to KB cells.

Comparison of the uptake efficiencies of folate-PEG-liposomal DOX with and without the additional incorporation of 4 mol% PEG2000-DSPE in the lipid bilayer revealed that folate-mediated targeting was virtually unaffected by addition of PEG-conjugated lipids to the liposome composition. Thus, the PEG segment (M.W. ≈ 3350) between the folate and the DSPE membrane anchor serves as an effective spacer sufficient to completely overcome the steric hindrance introduced by the PEG-coating of liposomes required for prolonged in vivo circulation.

Histograms of KB cell-associated DOX fluorescence generated by flow cytometry (Fig. 2) showed only a single peak. This result indicates that essentially all cells in the flask internalized a similar concentration of the folate-PEG-liposomal DOX.

The intracellular distribution of cell-associated DOX was examined by confocal microscopy. As shown in Fig. 3, DOX fluorescence in cells treated with folate-PEG-liposomes was mainly distributed in endocytic vesicles, in the nucleus, and throughout the cytosol. It is important to recognize that the DOX fluorescence inside these liposomes was quenched ≈ 85% compared to free DOX (data not shown) due to its highly aggregated state inside the liposomes. Therefore, the endosomal compartments of these cells may contain significantly more folate-PEG-liposomal DOX than indicated by their relative fluorescence intensities. Cells treated with control liposomes, however, showed little fluorescence above the background. Cells treated with free DOX displayed a similar DOX distribution to cells treated with the folate-PEG-liposomal preparation, except the endosomal fluorescence was more intense, probably due to lower self-quenching. Since liposomes with the composition we used rarely fuse with the plasma or endosomal membranes, DOX found in non-endosomal compartments must be drug released from the liposomes following endocytosis. This discharge of DOX from folate-PEG-liposomes is probably facilitated by the outward-directed pH

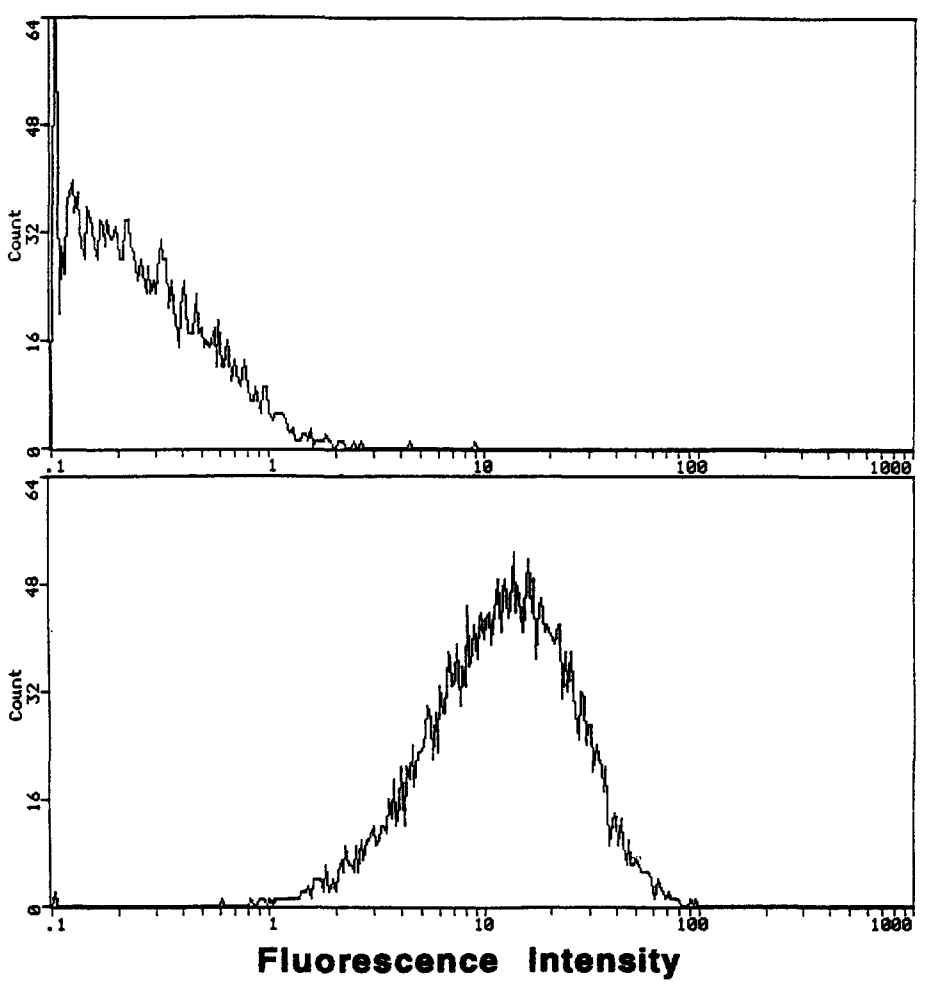

Fig. 2. Flow cytometry profiles of fluorescence from cell-associated DOX. KB cells were incubated with folate-PEG-liposomal DOX as described in Materials and methods. Upper panel, autofluorescence of untreated cells. Lower panel, KB cells treated with folate-PEG-liposomal DOX.

gradient across the liposomal membrane following uptake into the endosomes. Unpublished data in our laboratory show that liposomal DOX release is greatly accelerated when transferred from pH 7.4 to pH 5.0, a pH value found in endosomal compartments of the folate endocytosis pathway. Similarly, when free DOX enters the cell, pH gradient across the endosomal membrane may drive its accumulation into the acidic endosomal compartments via a similar mechanism. However, we recognize that quantitative information of intracellular DOX distribution cannot be extrapolated merely from these fluorescence images, since we do not know the degree of DOX fluorescence quenching in every subcellular compartment.

Cytotoxicity of folate-PEG-liposomal DOX

The cytotoxicities of various DOX formulations to KB and HeLa cells were also compared. KB or HeLa cells grown in 96-well plates were exposed to serial dilutions of liposomal or free DOX for 2 h, and cell viability was determined by the MTT assay following 48 h further incubation, as described in Materials and methods. DOX concentrations leading to 50% cell-killing (IC_{50}) were determined from concentration-dependent cell viability curves. As shown in Table 1, the IC_{50} of folate-PEG-liposomal DOX was 0.31 μM and 0.41 μM for KB and HeLa cells, respectively, which was 86- and 62-times lower than DOX in non-targeted liposomes, but only 2.7- and 1.8-times lower than free DOX. Empty folate-PEG-liposomes or non-targeted liposomes at lipid concentrations 100-times that of the IC_{50} of the corresponding DOX-containing liposomes showed no cytotoxicity against cultured KB or HeLa cells. These empty liposomes also show no effects on the cytotoxicity of co-administered free DOX (Table 1).

Selective uptake of folate-PEG-liposomes by HeLa cells in HeLa / WI38 co-cultures

WI38 cells, a non-transformed human lung cell line, exhibit a morphology easily distinguishable from that of HeLa cells, a human cervical carcinoma cell line. WI38 cells are relatively large and have a fibroblastic appearance, while HeLa cells grown in the same culture dish are smaller with a round or polygonal morphology (Fig. 4). Furthermore, like most non-transformed cells, WI38 cells lack folate receptors, whereas HeLa cells, typical of many cancer cells, greatly overexpress the folate receptors [26–28].

To determine whether folate might mediate the selective targeting of liposomes to HeLa cells in the presence of receptor negative cells, HeLa/WI38 co-cultures were incubated with folate-PEG-liposomes encapsulating calcein, a membrane-impermeant and pH-insensitive fluorescent dye and were examined under a fluorescence microscope. As shown in Fig. 4, a large amount of calcein-containing liposomes were taken up by the HeLa cells. However, WI38 cells in the same field did not take up any significant amount of fluorescence. It was also evident that the calcein fluorescence associated with the HeLa cells was present on the cell surface as well as in internalized endocytic vesicles.

When an analogous study was conducted with folate-PEG-liposomal DOX, essentially the same result was observed. While free DOX was taken up by both HeLa and

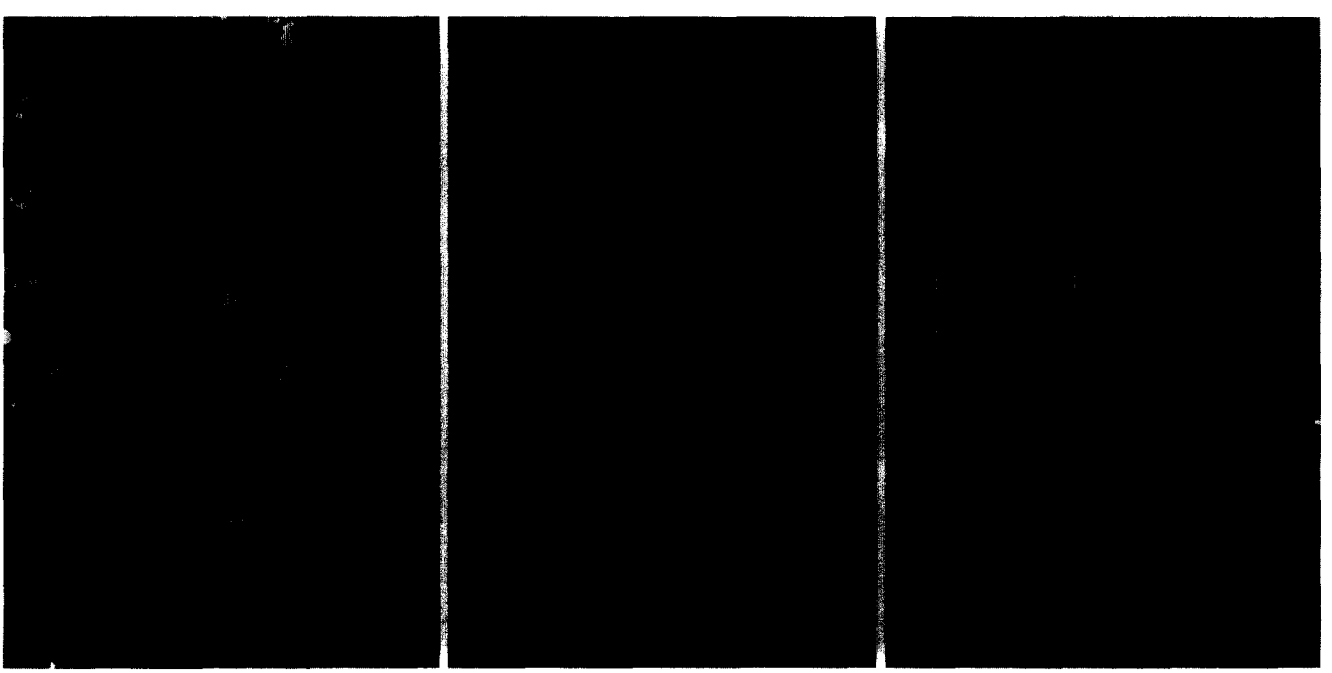

Fig. 3. Confocal images of KB cells treated for 1 h with free or liposomal DOX. Left panel, KB cells treated with folate-PEG-liposomal DOX; middle panel, cells treated with control liposomal DOX; right panel, cells treated with free DOX. DOX is visualized by its fluorescence using the rhodamine dichroic mirror set. Scale bar = 20 μm.

WI38 cells in the HeLa/WI38 co-culture with similar efficiencies, folate-liposomal DOX was clearly preferentially taken up by the HeLa cells (Fig. 4). However, the difference in the levels of DOX uptake between HeLa and adjacent WI38 cells treated with folate-PEG-liposomal DOX was less dramatic than in the case with folate-liposomal calcein. This is probably due to the fact that some DOX may release from the folate-PEG-liposomes upon endocytosis and subsequently transfer to adjacent WI38 cells by diffusion. This phenomenon may actually be advantageous for folate-receptor-mediated in vivo targeting of anti-cancer drugs to tumors where cells over-expressing the folate receptor can lie adjacent to receptor-negative cancer cells. Thus the free DOX released from the receptor-positive cells may facilitate the killing of the adjacent receptor-negative tumor cells due to the 'bystander' effect. This effect is likely to be less important when the drug molecules being targeted are very membrane-impermeable.

Selective cytotoxicity of liposomal doxorubicin to HeLa cells in HeLa / WI38 co-cultures

To further demonstrate the specificity of folate-PEG-liposomes for the HeLa cell population, HeLa/WI38 co-

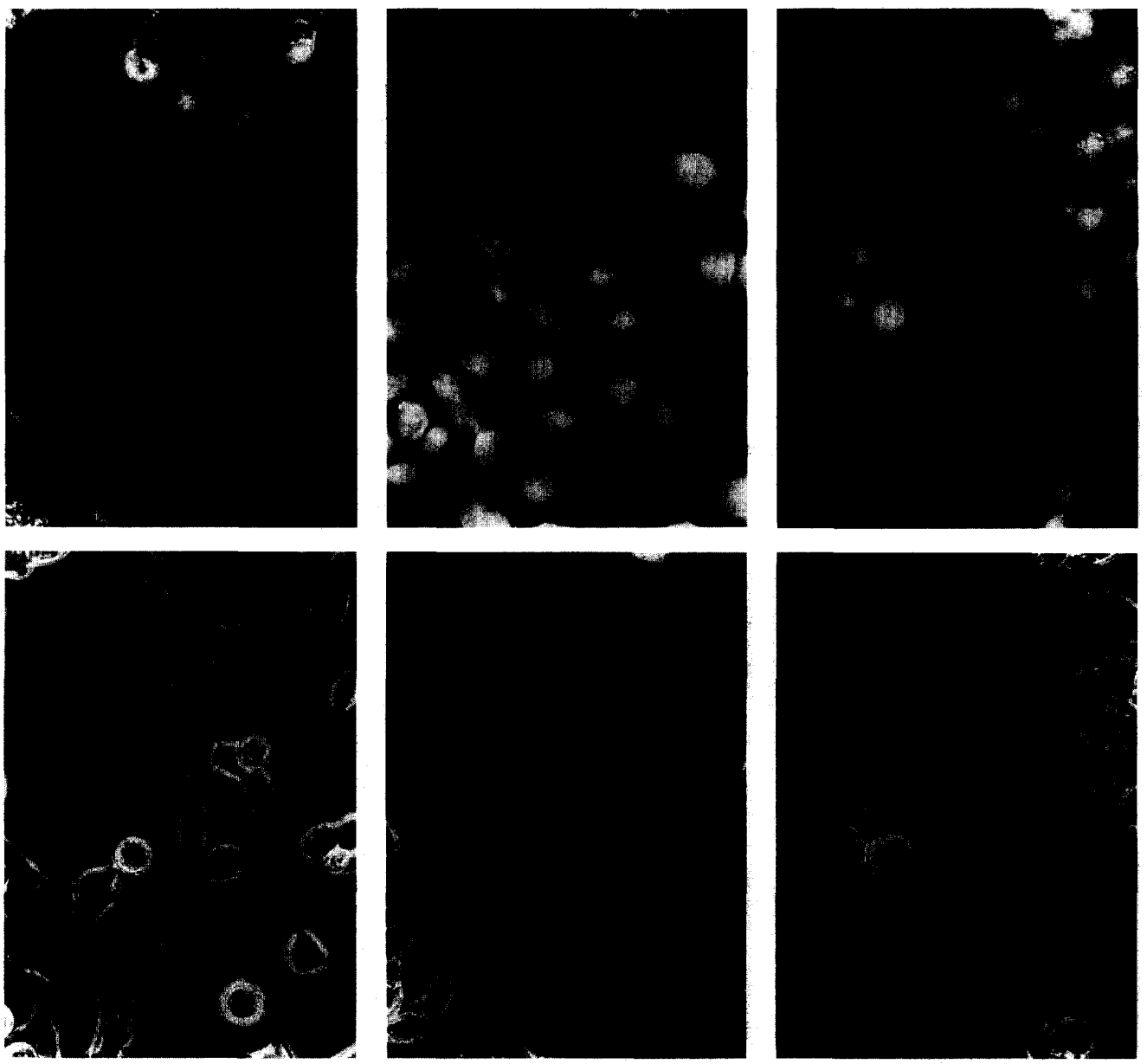

Fig. 4. Selective uptake of folate-PEG-liposomal calcein or DOX by HeLa cells in HeLa/WI38 cell co-cultures. HeLa/WI38 co-cultures were treated with folate-PEG-liposomal calcein, free DOX or folate-PEG-liposomal DOX and photographed in both the fluorescence (dark fields) and phase-contrast mode (bright fields) on a microscope as described in Materials and methods. Left, cells treated with folate-PEG-liposomal calcein; middle, cells treated with free DOX; right, cells treated with folate-PEG-liposomal DOX. Upper panels: micrographs taken in the fluorescence mode; lower panels: the same field viewed in the phase contrast mode. HeLa cells appear as small round or polygonal cells. WI38 cells are considerably larger with a dendritic or fibroblast-like morphology.

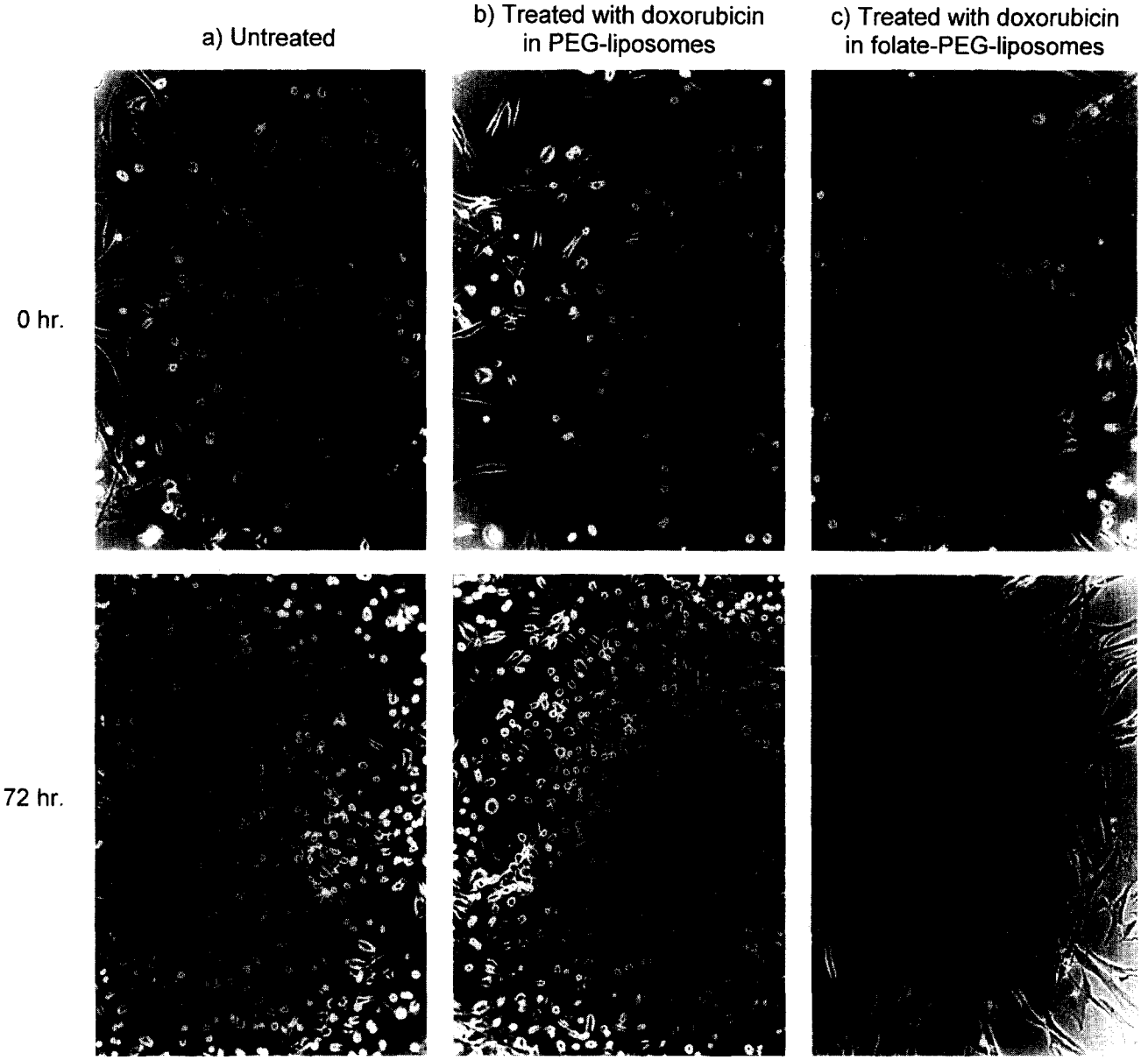

Fig. 5. Selective killing of HeLa cells in a HeLa/WI38 cell co-cultures. HeLa/WI38 cell co-cultures were treated for 1 h with 10 μM doxorubicin encapsulated in either folate-PEG-liposomes or non-targeted liposomes and examined under phase contrast microscope after 72 h further incubation. An untreated control culture is displayed in the left set of panels for comparison.

cultures were treated with 10 μM doxorubicin encapsulated in either folate-PEG-liposomes or non-targeted liposomes. After 1 h incubation at 37°C and washing with PBS, the cells were further incubated for 72 h in 2 ml fresh medium and examined under the microscope. As shown in Fig. 5, co-cultures left untreated or treated with PEG-liposome-entrapped doxorubicin were dominated by HeLa cells due to their faster growth. The population of WI38 cells in the same dishes actually diminished, probably due to unsuccessful competition for limiting growth factors and nutrients. However, in co-cultures treated with the same concentration of folate-PEG-liposome-entrapped doxorubicin, HeLa cells were completely killed, leaving only WI38 cells in the culture dish. The selective cytotoxicity of folate-PEG-liposomal DOX is likely the result of the receptor-mediated enhanced uptake of folate-PEG-liposomes by the HeLa cells, as well as the HeLa cells' higher sensitivity to DOX.

4. Discussion

In this paper, we have demonstrated that the anti-neoplastic drug, DOX, can be specifically targeted to a cancer cell line in the midst of non-transformed cells by entrapment in folate-tethered liposomes. Because such constructs are capable of multivalent attachments to folate receptors on tumor cell surfaces, competition with endogenous fo-

lates at physiological concentrations is not observed. Although DOX administered in liposomal formulations can already boast several advantages over free doxorubicin [2–9], the incorporation of folate-PEG-DSPE molecules into the lipid bilayer could conceivably improve the therapeutic index of the drug still further for the treatment of folate-receptor-positive tumors.

Results in this study showed a strong correlation between the cytotoxicity of DOX formulations and their cellular uptake efficiency. However, the fold increase in cytotoxicity due to folate-targeting seems to exceed the fold increase in cellular uptake. This is probably due to the enhanced release of DOX from folate-PEG-liposomes following their receptor-mediated internalization into the acidic endosomal compartments. Another factor that may affect the interpretation of the discrepancy in uptake enhancement and cytotoxicity enhancement is that the uptake assays had to be performed at a DOX concentration much higher than the IC_{50} values. At high liposome concentrations, factors such as trace amount of DOX released from the liposomes, and non-specific binding and endocytosis of the liposomes by the cells may affect the observed levels of cellular DOX uptake. Our results also show that while folate-PEG-liposomal DOX was 86-times more cytotoxic to KB cells than non-targeted liposomes, it is only 2.7-times more potent than free DOX. The reason for the much higher cytotoxicity of free DOX compared with non-targeted liposomal DOX is probably that when the cells were exposed to free DOX and liposomal DOX for the same lengths of time, the free DOX is taken up much more rapidly. The situation for the in vivo delivery of DOX, however, is likely to be entirely different, since free DOX is cleared much faster from systemic circulation than is liposomal DOX, especially when a PEG-coating is added. In fact, a recent study has shown that free DOX is cleared 450-times faster than DOX encapsulated in PEG-coated liposomes [6].

Incorporation of PEG-derivatized lipids in antibody-conjugated liposomes has been shown to obstruct target-specific cell recognition [19–21]. In contrast, folate-PEG-DSPE mediated delivery of liposomes to receptor-bearing cells was fully compatible with the inclusion of 4 mole % PEG-conjugated lipid. Although ligation of an antibody or peptide hormone ligand to the distal ends of liposome-anchored PEG molecules may correct the aforementioned targeting defect [23,24], antibody-derivatized liposomes suffer from additional disadvantages not shared by folate-targeted liposomes. Thus, antibodies can be antigenic and trigger complement deposition, leading to rapid liposome removal in vivo, precluding repeated administrations. Also, antibody-liposome conjugates may not be stable during lyophilization or conditions required for remote-loading and prolonged storage. Because antibodies and many peptide hormones are physically large, attachment of multiple copies of the polypeptides to a liposome can significantly increase its size and alter its properties. Since liposome size correlates inversely with its probability of extravasation at the site of a tumor, and since particle size also correlates positively with frequency of capture by macrophages [41–45]), unnecessary enlargement of the liposome is undesirable. Thus, even though antibodies may offer a wider range of cell targeting specificities, folate may still be the preferred ligand for targeting tumors that overexpress the folate receptor [28–34].

Another disadvantage of exploiting polypeptide ligands for targeting liposomes to tumor cells lies in the difficulty of assembling drug, lipid, and lipid-conjugated antibodies efficiently into stable liposomes. Proteinaceous ligands are often not compatible with organic solvents, and therefore, have been commonly conjugated to preassembled liposomes containing lipids with activated head groups [35,37,46–51]. Such protocols, however, may lead to unwanted side reactions such as liposome crosslinking. The unused reactive head groups may also be cytotoxic or they may alter the properties of the liposomes. For example, the incorporation of maleiimidyl lipid head groups has been observed to interfere with the remote-loading of DOX. Furthermore, the site of attachment to the antibody can not be precisely controlled, and consequently, the orientation of many proteins can be incompatible with receptor recognition. As an alternative, antibodies have been conjugated to activated lipids such as *N*-glutaryl-phosphatidylethanolamine in aqueous dispersions and then they have been reassembled with drug and bilayer lipids into targeting liposomes by detergent dialysis [50,51]. This procedure, however, usually results in poor encapsulation efficiency for hydrophilic drug molecules, leading to a significant loss of chemotherapeutic agent. In contrast, folate-PEG-DSPE is synthesized before liposome assembly and can be employed with any liposome assembly method. Thus, reverse phase evaporation, dehydration-rehydration, detergent dialysis, thin film hydration and sonication, etc., all represent drug encapsulation protocol that are compatible with the use of folate as the targeting ligand.

Folate conjugation has also been exploited in the targeting of protein toxins. While folate-toxin conjugates display approximately the same selectivity for killing cancer cells as folate-PEG-liposomal DOX [38–40], the latter formulation may be more effective as an anti-neoplastic agent for several reasons. First, unlike bacterial toxins, folate-PEG-liposomes do not carry foreign antigens and are, therefore, not immunogenic. Further, liposome-encapsulated organic molecules, like doxorubicin, may be more stable during storage and circulation than proteinaceous toxins that rely on enzyme catalysis for toxicity. Whereas proteinaceous drugs must be directly or indirectly conjugated to folate, liposome-encapsulated drugs can be left unmodified, i.e., since the targeting ligand is attached to the packaging liposome. Further, liposome encapsulation protects its bioactive cargo from modification and delivers multiple copies of the drug at each folate receptor. In our study, $\approx 1.8 \cdot 10^{10}$ molecules of DOX were delivered to each KB

cell when folate-PEG-liposomes were used as a carrier. Finally, where competition with endogenous folates is a concern, folate-linked liposomal formulations may be superior due to their higher affinities for the target cell resulting from multivalent binding, which can effectively outcompete any monovalent ligands in vivo. For the same reason, folate-PEG-liposomes are also likely to have higher affinity for the target cells than for soluble folate-receptors released from tumor tissues into circulation.

Besides being a prominent tumor marker, folate receptors are also found in some normal human tissues such as the choroid plexus, the bone marrow, placenta, and the kidney [31]. The effects of these receptors on folate-mediated drug targeting in vivo remain to be elucidated. However, in preliminary live animal studies, uptake of radiolabeled folate conjugates by these normal tissues was small compared to xenographic tumor implants.

In conclusion, we have identified a possible strategy for targeting liposomal doxorubicin to tumor cells. Where a tumor expresses the folate receptor and is accessible to circulating liposomes, the methodology should allow selective delivery of the drug to the cancer tissue. Although the focus of the current studies has been on doxorubicin targeting, the strategy should also apply to other liposome encapsulated therapeutics, such as other cytotoxic drugs, antisense nucleic acids, ribozymes, and imaging agents. A future step in the development of this technology will obviously require testing and optimization of the system in vivo.

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