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Research Paper

Development of 5-FU and Doxorubicin-Loaded Cationic Liposomes against Human Pancreatic Cancer: Implications for Tumor Vascular Targeting

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Purpose. Human pancreatic adenocarcinoma is a major leading cause of cancer mortality in the United States. Given that current strategies are relatively ineffective against this disease, new treatments are being developed. Liposomes possessing relatively high cationic lipid content preferentially accumulate in tumor angiogenic vessels compared to vessels in normal tissues. We therefore seek to develop cationic liposomes for targeting pancreatic tumor vessels.
Materials and Methods. We report development of 5-fluorouracil (5-FU) and doxorubicin hydrochloride (DOX) loaded in PEGylated cationic liposomes (PCLs). We evaluate cell association, intracellular fate, and cytotoxicity. Human pancreatic cancer cells HPAF-II and Capan-1, and endothelial cells HMEC-1 and HUVEC were used in this study. Intratumoral distribution of PCLs in (HPAF-II) tumors was determined by intravital microscopy.
Results. HUVEC and HMEC-1 were most susceptible to 5-FU after 24 and 48 h, compared to HPAF-II and Capan-1. We observed >90% incorporation of 5-FU and DOX in PCLs for 3–20 mol% preparations, with reduced incorporation for >20 mol% formulations. PCLs showed significantly higher association with human endothelial versus pancreatic cancer cells, and improved growth inhibitory properties of DOX. Intravital microscopy revealed distribution of PCLs along HPAF-II vessels.
Conclusions. Targeting human pancreatic cancer with PCLs may represent a rational alternative to conventional strategies.

KEY WORDS: cationic liposomes; doxorubicin hydrochloride; drug delivery; pancreatic cancer; 5-fluorouracil.

INTRODUCTION

Human pancreatic cancer remains one of the leading causes of mortality in the United States with a 5-year survival of <5%, the poorest of all the gastrointestinal malignancies (1,2). The majority of patients are diagnosed with locally advanced non-resectable disease due to involvement of major vessels (1). To date, approximately 95% of diagnosed patients are in advanced stages of disease, so surgical resection offers no meaningful impact on survival (3,4). In general, when patients present with metastasis in the liver, lung, and brain most treatments are ineffective (3–6).

The infusion of 5-FU concurrently with radiation therapy has been extensively evaluated against human pancreatic cancer, and is one of the standard treatment approaches (5,7,8). Other first line treatments involving the use of chemotherapeutic agents alone (as in the case of gemcitabine (5,9)), or in combination with 5-FU (i.e., mitomycin, cisplatin, epirubicin and doxorubicin) have demonstrated some clinical success (9–11). Unfortunately, reports from clinical trials indicate highly variable treatment responses with no significant improvements in overall survival (4–6). The main priority is therefore to identify non-surgical clinical treatments for effective management of human pancreatic cancer.

It was previously demonstrated that cationic liposomes generally accumulate in the tumor and liver anatomical sites (12); interestingly, the liver is also a favorable site for distant metastasis in pancreatic cancer. Subsequent *in vivo* distribution studies involving human and murine tumor models confirmed that PEGylated cationic liposomes (PCLs) associate with approximately 27 and 5% of vessel areas in tumors

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ABBREVIATIONS: Capan-1 and HPAF-II, human pancreatic cancer cells; Chol, cholesterol; DOPC, 1,2-dioleoyl-*sn*-glycerol-phosphatidylcholine; DOPE-PEG, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-5000; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOX, doxorubicin hydrochloride; EBM-2, endothelial cell basal medium; FITC-dextran, fluorescein isothiocyanate-dextran; HMEC-1, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cell; MEME, eagle's minimum essential medium; PBS, phosphate buffer saline; PCLs, PEGylated cationic liposomes; Rhodamine-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; SCID, severe combined immunodeficient; 5-FU, 5-fluorouracil.

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and normal tissues respectively, with less than 15% of tumor interstitial area involvement (12). Tumor vessels are more accessible to circulating chemotherapeutic agents compared to cancer cells. Tumor endothelial cells also proliferate much faster than endothelial cells in normal tissues (13–15). Several groups have provided evidence demonstrating superior antitumor activity of various chemotherapeutic agents when loaded in cationic liposomes, compared to when the drug was administered alone against different tumor xenografts (16–19). This has created opportunities to both develop and characterize additional cationic liposome therapeutics. It has also provided a rationale to investigate general susceptibility of human microvascular endothelial cells to chemotherapeutic agents, and to those systems that preferentially target the tumor vasculature.

In addition to introducing new formulations the following questions should be addressed: how susceptible are human endothelial cells to the effect of chemotherapeutic agents, compared to human pancreatic cancer cells. Next, if human endothelial cells are more susceptible to the effects of chemotherapeutic agents, do PCLs selectively target human pancreatic tumor vessels (as has been demonstrated with other tumor types)? Finally, is there sufficient evidence to support future development of tumor vascular-specific technologies against human pancreatic cancer as well?

In this study we report the development and pharmaceutical evaluation of 5-FU (inhibitor of thymidylate synthase), and doxorubicin (inhibitor of topoisomerase II) loaded in PCL preparations. We investigated the interaction of PCLs with human endothelial and pancreatic cancer cell lines. In addition, we studied the growth inhibitory properties of free drug (5-FU and doxorubicin hydrochloride) and liposomal formulations against our cellular models of tumor vascular and interstitial tumor compartments. Finally, we used dorsal skin fold window chambers and intravital microscopy to demonstrate the distribution of PCLs in a human pancreatic tumor model in mice.

MATERIALS AND METHODS

Materials

DOPC, DOTAP, cholesterol, DOPE-PEG were obtained from Avanti Polar Lipids (Alabaster, AL). The chemotherapeutic agents 5-fluorouracil and doxorubicin hydrochloride, sulforhodamine B, and fluorescein isothiocyanate (FITC)-dextran were purchased from Sigma-Aldrich (St. Louis, MO). Trichloroacetic acid, 1% acetic acid, and ethanol were purchased from Fisher Scientific Company (Fair Lawn, NJ). Cell culture media Eagle's minimum essential medium (EMEM), and Iscove's modified Dulbecco's medium and trypsin-EDTA were purchased from ATCC (Manassas, VA). Endothelial cell basal medium (EBM-2) was purchased from Cambrex (Walkersville, MD).

Cell Culture

The human pancreatic cancer cell lines HPAF-II (CRL-1997) and Capan-1 (HTB-79) were maintained in EMEM and Iscove's modified Dulbecco's medium (ATCC), respectively; required growth media was supplemented with 10% FBS. HUVEC was purchased from Cambrex. HMEC-1 was a gift from Centers for Disease Control and Prevention (Atlanta, GA). Human endothelial cell lines HUVEC and HMEC-1 were maintained in EBM-2 growth medium supplemented by FBS and essential growth factors. All cell lines were grown in a humidified CO_2 atmosphere at 37°C.

Growth Inhibition Studies

Cells were seeded at 1×10^4 cells/ml in 48 well plates. Following a 24 h incubation period at 37°C, cells were exposed to various concentrations of free drug (5-fluorouracil or doxorubicin) solution prepared in media or drug-loaded PCLs in 1X PBS (Cambrex, NJ). Percent of cell viability was determined using sulforhodamine B assay following 1, 6, 24 and 48 h of cell exposure to drug (20,21), and percent of viable cells was calculated as follows:

$$\text{Percent of cell viability} = \frac{\text{Flourescence int. of treated cells}}{\text{Flourescence int. of non}-\text{treated cells (control)}} \times 100$$

Preparation of Liposomes

PEGylated cationic liposomes (PCLs) were prepared using the thin film evaporation method as previously described (22). DOPC, DOTAP, cholesterol and DOPE-PEG lipid stocks obtained from Avanti Polar Lipids were stored at −80°C under an inert atmosphere. When preparing liposomes (DOTAP/DOPC/chol/DOPE-PEG/rhodamine-DPPE label), the cationic charge was contributed by 50 mol% of the cationic lipid DOTAP and the other lipids were added in the following ratio 50:35:10:5 (lipid concentration was typically between 10 and 20 μmol/ml). The concentration of fluorescent label rhodamine-DPPE was normally between 1–2 mol% of the total liposome preparation. For studies involving drug-loaded liposomes we prepared 3, 5, 10, 20 and 50 mol% of 5-fluorouracil or doxorubicin. The respective mixtures were evaporated to dryness at 42°C in a round bottom flask using a rotary evaporator to form a thin film. Additional trace amounts of organic solvent were removed from film by drying for 2 h in a vacuum environment using a Labconco freeze dryer (Labconco Corporation—Kansas City, MO). The film was then hydrated with 1 ml of 1X PBS to form multilamellar liposomes. Liposomes were vortexed intermittently and put in a water bath set at 42°C and in refrigerator at 4°C for 30 – 60 min increments before use. Liposome size and zeta potential was measured at 25°C in distilled water using 90PLUS particle size and zeta potential analyzer (Brookhaven Instruments, New York).

UV Spectroscopic Analysis

The percent of drug (5-fluorouracil or doxorubicin) incorporated into PCLs was measured using a UV spectrophotometer (Bio-Tek® Instruments Inc., VT) and confirmed with use of HPLC-Waters (Milford, MA) and DIC optical analysis (Olympus BX61 WI—Melville, NY). The liposome stock was centrifuged at 5000 rpm to separate free drug from incorporated drug, and UV absorbance from incorporated 5-fluorouracil and doxorubicin were measured at 263 and 232

nm wavelengths, respectively. Percent of drug incorporated was calculated using the following formula:

$$\text{Percent of drug incorporated} = \frac{\text{UV abs. of drug} - \text{liposomes } \textit{after} \text{ centrifugation}}{\text{UV abs. of drug} - \text{liposomes } \textit{before} \text{ centrifugation}} \times 100$$

Cellular Association of PCLs

Cells were seeded in 48 well plates at 1×10^4 cells/ml followed by 24 h of incubation in a humidified environment set at 37°C. The cells were next exposed to between 50 and 1,000 nmoles of rhodamine labeled liposomes and were placed in incubator for an additional 24 h. To determine association of cells with PCLs as function of time, cells were incubated with 1,000 nmoles of liposomes for 1, 6 and 24 h. Cells were next washed with 1X PBS to remove unwanted cellular debris and unbound liposomes from culture media; total fluorescence due to fluorescent liposomes bound to cells was measured at excitation and emission wavelengths of 550 and 590 nm by a fluorescence microplate analyzer (Bio-Tek® Instruments Inc.).

Intracellular Uptake of PCLs

Sterile cover slips were placed in six well plates (Corning, NY). Cells were next seeded at 5×10^5 per ml in the same six well plates. Following an incubation period of 24 h at 37°C, 100 nmol rhodamine labeled PCLs were added to each well. Cells were incubated for an additional 24 h with PCLs and washed with 1X PBS to remove unassociated liposomes. The cover slip from each well was mounted onto a glass microslide (Corning, NY) with fluor mounting media (Trevigen Inc, Maryland). Finally the interaction of PCLs with cells and intracellular uptake was determined using a combination of fluorescence and DIC microscopic applications at 20× magnification (Olympus BX61WI).

Intravital Fluorescence Microscopy

Dorsal skin fold chambers (DSC) were prepared as discussed elsewhere (12). To prepare a tumor source for our intravital microscopy studies 2×10^6 of HPAF-II cells were injected subcutaneously into 8–10 week old male SCID mice. Tumors were allowed to grow for 2 weeks and then surgically removed with the use of aseptic techniques. Upon removal of an established tumor source all necrotic areas were subsequently removed, and the remaining tumor mass was sectioned into 1-mm-size pieces and immediately placed in corresponding culture media. Mice bearing DSCs were anesthetized with a mixture of ketamine and xylazine followed by implantation of a single viable tumor piece into the center of the window chamber. When tumor reached a suitable size experiments were carried out. Mice were anesthetized and placed onto a custom-designed, mouse restraining holder for image acquisition purposes. The mouse holder was put onto the stage of a fluorescence microscope.

To observe the interaction of liposomes with tumor blood vessels, rhodamine labeled liposomes (0.2 cc) were injected via tail vein 24 h prior to intravital microscopic analysis. FITC-Dextan (20 mg/ml, 0.1 cc) was injected via tail vein to observe

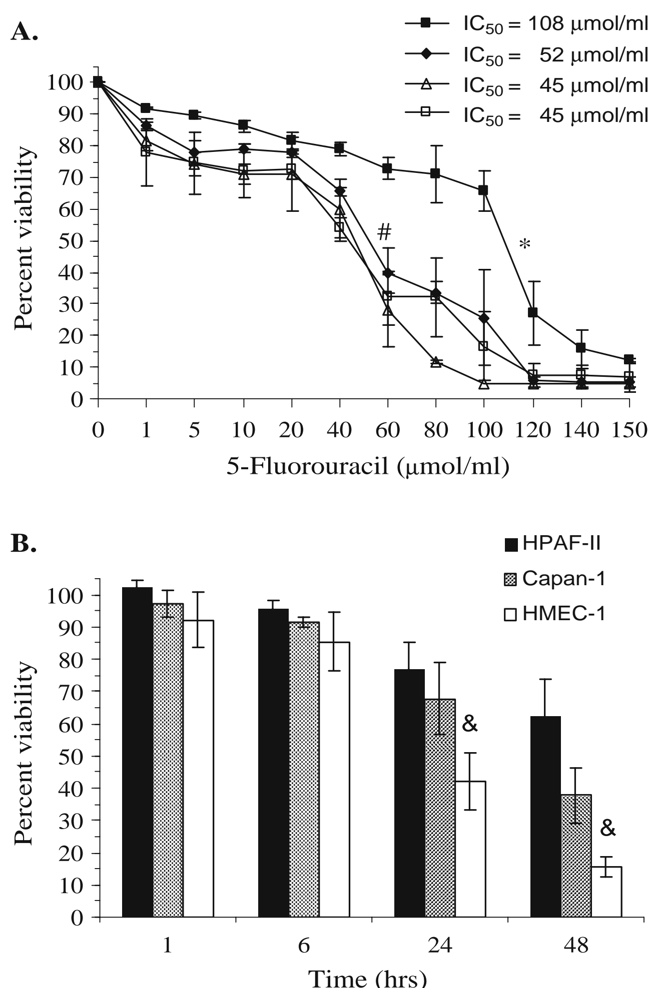

Fig. 1. Evaluation of growth inhibitory properties of 5-FU against human endothelial and pancreatic cancer cells. HPAF-II (*filled square*), Capan-1 (*diamond*), HUVEC (*triangle*) and HMEC-1 (*open square*) were seeded at 1×10^4 cells/ml in a 48 well plate and incubated for 24 h at 37°C. (A) Sulforhodamine B assay was used to determine the percent of viable cells 24 h following exposure of cells to various concentrations of 5-FU ($^*P\leq0.05$; $\#\leq0.05$). (B) Sulforhodamine B assay was used to evaluate time-dependent cytotoxic effect following cellular exposure to 5-FU at 1, 6, 24 and 48 h ($^{\&}P \leq 0.05$). Each point on graph represents the mean of three separate experimental determinations.

location of PCLs with respect to tumor vasculature. We first viewed images under the RGB filter and next, without disturbing the stage, we switched to a FITC filter to acquire images of blood vessels only. Finally we switched to rhodamine filter to observe and capture images of rhodamine-labeled PCLs. Images were captured at 4× and 10× magnifications and superimposed to observe localization of liposomes with respect to tumor vessels in the dorsal skin fold chamber.

Statistical Analysis

To determine the significant difference between different experimental groups nonparametric Mann–Whitney *U*-test was used. Statistical significance was established at *P* value ≤0.05. Analysis was performed using the statistical package SPSS 12.0 (SPSS Inc., Chicago, IL).

RESULTS

Effect of 5-FU *In Vitro* against Cellular Models of the Vascular and Interstitial Tumor Environment

Tumor capillary networks are avid targets of cationic drug carrier molecules (12,23,24). For this reason, endothelial cells lining these vessels have much greater access to circulating cationic liposomes compared to cancer cells that normally invade the interstitial tumor compartment. In this experiment we eliminated the general accessibility factor, and asked whether human pancreatic cancer cells are any more susceptible to the effects of standard chemotherapeutic drugs compared to human endothelial cells. We compared the cytotoxic effect of 5-FU against human pancreatic cells HPAF-II & Capan-1, and endothelial cells HUVEC & HMEC-1.

The growth inhibitory properties of 5-FU against all four cell lines were determined 24 h following exposure of each cell type to 5-FU (Fig. 1A). In general, the percent of viable cells decreased with increase in concentration of drug. The degree of sensitivity was cell line dependent. The IC_{50} values for 5-FU against HPAF-II and Capan-1 cell lines were 108 and 52 μmol/ml respectively; whereas the IC_{50} values observed for 5-FU against HMEC-1 and HUVEC were significantly lower, reported at approximately 45 μmol/ml ($P \leq 0.05$: against *HPAF-II and #Capan-1).

It has been demonstrated that both the rate of cell proliferation and the specific phase of the cell cycle influence the cytotoxic action of fluoropyrimidines (25). We therefore investigated time-dependent cytotoxic effects of 5-FU (Fig. 1B). The percent of viable cells decreased with increase in drug exposure time, and a significant decrease in viability was observed following 24 h of incubation when compared to earlier exposure time points. The most significant cytotoxic effect was observed against HMEC-1 when compared to HPAF-II and Capan-1 cells at both 24 and 48 h ($^{\&}P \leq 0.05$). The experimental findings support the hypothesis that human microvascular endothelial cells are more susceptible to the effect of chemotherapeutic agents compared to human pancreatic cancer cells.

Physicochemical Characterization: Determination of Size, Zeta Potential, and Efficiency of Drug Loading

It was previously demonstrated that when the synthetic cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) was included as part of several different cationic liposome preparations, DOTAP was shown to improve the incorporation efficiency of paclitaxel (26). Incorporation of paclitaxel varied as a function of acyl chain length and drug to total lipid ratio (26,27).

Our PCLs used in this study contain some additional liposome components (i.e., cholesterol and PEG), so we appropriately evaluated the incorporation of 5-FU and doxorubicn in PCL preparations.

We evaluated 3 to 50 mol% drug-loaded preparations. Percent of chemotherapeutic agent retained by PCLs was determined by UV spec. analysis (see Table I), and this approach was further supported by optical DIC and HPLC analysis (data not shown).

The inclusion of 5-FU in PCLs resulted in the incorporation of more than 95% of drug for all formulations, except for 50 mol% preparations averaging around 85% (see Table I). Doxorubicin-loaded (3 to 20 mol%) formulations showed incorporation efficiency values above 90%; however 50 mol% formulations were less stable reporting values around 46% (see Table I).

We determined liposome size and zeta potential for all 5-FU and doxorubicin formulations in relation to PCLs alone (244 ± 30 nm). Incorporation of 5-FU in PCLs resulted in the following liposome sizes: 3 mol% (248 ± 37 nm), 5 mol% (256 ± 27 nm), 10 mol% (244 ± 43 nm), 20 mol% (221 ± 8 nm) and 50 mol% (216 ± 11 nm). There were no significant changes in size of 5-FU-loaded prreparations when compared to PCLs alone.

The incorporation of doxorubicin in PCLs at drug to lipid ratios between 3 and 50 mol% resulted in the following particle sizes: 3 mol% (247 ± 15 nm), 5 mol% (227 ± 6 nm), 10 mol% (196 ± 24 nm), 20 mol% (233 ± 12 nm) and for 50 mol% preparations average size estimated around 167±19 nm.

In the absence of 5-FU and doxorubicin the zeta potential for PCLs was 32 ± 1.9 mv. Zeta potential for 5-FU (3 to 50 mol%) preparations was as follows: 3 mol% (36 ± 7 mv), 5 mol% (33 ± 6.6 mv), 10 mol% (37 ± 10.6 mv), 20 mol% (46 ± 3.2 mv) and for 50 mol% it was 52 ± 4.6 mv. The zeta potential for PCLs loaded with 3 to 50 mol% doxorubicin was as follows: 3 mol% (36±1.2 mv), 5 mol% (31 ± 5.9 mv), 10 mol% (34 ± 2.3 mv), 20 mol% (32 ± 2.7 mv) and for 50 mol% it was 19±4.8 mv.

The incorporation of 5-FU and doxorubicin in PCLs, and associated influence on bilayer physical properties (such as size and cationic charge potential) were not significant, except for 50 mol% formulations. The percent of 5-FU and doxorubicin incorporated in PCLs significantly reduced with increase in drug to lipid ratios from 20 to 50 mol% (Table I). A more significant decrease in the loading efficiency was

Table I. Characterization of Drug Loaded PEGylated Cationic Liposomes

Mol%	5-Fluorouracil—PCLs			Doxorubicin—PCLs		
	Particle Size (nm)	Zeta Potential (mv)	Percent Incorporation (%)	Particle Size (nm)	Zeta Potential (mv)	Percent Incorporation (%)
No drug	244 ± 30	32 ± 1.9	–	244 ± 30	32 ± 1.9	–
3 mol%	248 ± 37	36 ± 7.0	96 ± 9	247 ± 15	36 ± 1.2	92 ± 4
5 mol%	256 ± 27	33 ± 6.6	94 ± 3	227 ± 6	31 ± 5.9	95 ± 6
10 mol%	244 ± 43	37 ± 10.6	98 ± 2	196 ± 24	34 ± 2.3	95 ± 6
20 mol%	221 ± 8	46 ± 3.2	95 ± 8	233 ± 12	32 ± 2.7	93 ± 6
50 mol%	216 ± 11	52 ± 4.6	85 ± 24	167 ± 19	19 ± 4.8	46 ± 8

observed with doxorubicin at 50 mol%, suggesting that at this ratio we exceeded the critical loading potential. Moreover, the reduced loading efficiency of doxorubicin may well correlate with decrease in liposome size and significant reduction in membrane surface charge potential (Table I). Finally, 5-FU exerted no additional effects on size or cationic charge potential in any of the preparations suggesting that the overwhelming majority of the (low molecular weight) highly water soluble drug is mainly incorporated in the liposome aqueous core. This is also supported by DPH polarization studies showing no change in fluorescence polarization values upon inclusion of 5-FU in liposome preparation (data not shown).

Association of Human Pancreatic Cancer and Endothelial Cells with PCLs

Depending on the experimental conditions, cytotoxicity may result from the use of synthetic cationic lipids, but may be avoided by manipulating liposome size, charge, composition and concentrations (28–30). Some of our *in vitro* studies required that we eliminate all cytotoxicity issues related to the use of PCLs alone. For this reason we evaluated toxicity of PCLs against our experimental cell lines in a concentration-dependent manner. We observed no cell death ≤1,000 nmoles of liposomes against HPAF-II, Capan-1, HUVEC and HMEC-1. Experiments involving various amounts of cationic liposomes did not exceed this critical concentration.

We evaluated association of PCLs with HPAF-II, Capan-1, HUVEC, and HMEC-1 cells by exposing each cell type to various amounts of rhodamine labeled PCLs. Regardless of the cell line employed we observed a significant increase in association of cells with PCLs with increase in concentration of PCLs following 24 h of incubation (Fig. 2A). We observed significantly higher association of PCLs with HUVECs compared to HPAF-II and Capan-1 cell lines ($^*P \leq 0.05$); whereas association of PCLs with HMEC-1 was significantly higher compared to only HPAF-II at 500 and 1,000 nmoles ($^\#P \leq 0.05$).

Polyethylene-glycol (PEG) has been shown to extend the circulation time of liposomes *in vivo*; with PEG the half life of liposomes in blood normally exceeds 24 h (31–33). Due to the ability to avoid rapid elimination, PEG was included as part of our cationic liposome preparations. At the appropriate ratio of PEG to other liposome components extended circulation time can readily be achieved, while also permitting tumor vascular access to circulating liposomes on the basis of molecular charge (12).

Although PEG does not eliminate all cationic surface charge characteristics of PCLs, it does reduce the positive zeta potential to some extent (12,34). We performed time-dependent association studies to determine whether extent of association varies as a function of time. Studies were performed following 1, 6 and 24 h of cell incubation with 1,000 nmoles of PCLs (Fig. 2B). We observed no significant change in cell association with increasing exposure time for each of the three cell lines evaluated; however, there was a significantly higher association of PCLs with HMEC-1 compared to HPAF-II and Capan-1 after 6 h of incubation ($^*P \leq 0.05$). We also note that the interaction of PCLs with HMEC-1 was significantly higher compared to interactions with HPAF-II after 24 h ($^\#P \leq 0.05$).

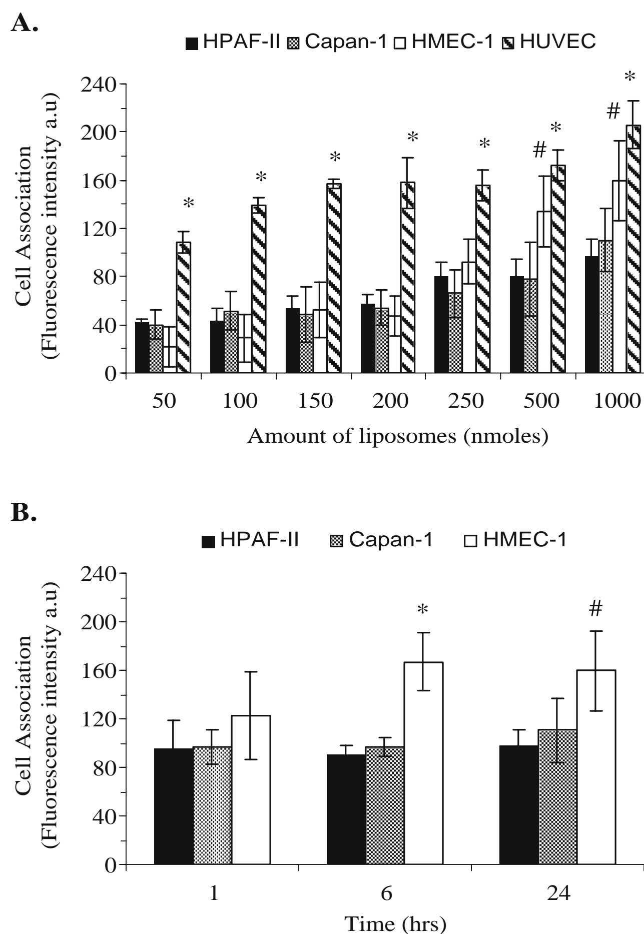

Fig. 2. Association of PCLs with human endothelial and pancreatic cancer cells *in vitro*. Cells were seeded at 1×10^4 cells/ml in a 48 well plate and were incubated for 24 h at 37°C. Following 1, 6 or 24 h of cell exposure to rhodamine labeled PCLs, fluorescence was measured at excitation and emission wavelengths of 550 and 590 nm respectively. The data show (A) fluorescence intensity (used as direct measure of PCL association with cells) observed as a function of liposome amount, and (B) time-dependent association of PCLs (1,000 nmol/ml) with cells ($^*P \leq 0.05$; mean ± SD; $^\#P \leq 0.05$; mean ± SD). Each bar on graph represents the mean of three separate experimental determinations.

We next converted values for cell association to percent of liposomes associated. This is an estimation of the amount of liposomes associated with a given number of cells under similar experimental conditions. Following 6 h of incubation the association was greater for Capan-1 compared to HPAF-II, but not when Capan-1 was compared to HMEC-1 ($P > 0.05$). The values for percent of PCLs associated with Capan-1, HPAF-II, and HMEC-1 were 1.6 ± 0.2, 0.9 ± 0.2, and 1.3 ± 0.5 respectively.

Following 24 h of incubation the values for percent of PCLs associated with each of the three cell lines was significantly higher for HMEC-1 at 1.9 ± 0.3 ($P < 0.05$), but was not significantly higher for Capan-1 or HPAF-II ($P > 0.05$). In general, reducing the amount of PCLs (<1000 nmol) or increasing the cell seed number (>1×10^4 per ml of growth medium) resulted in higher percent association however, the trend was similar (data not reported).

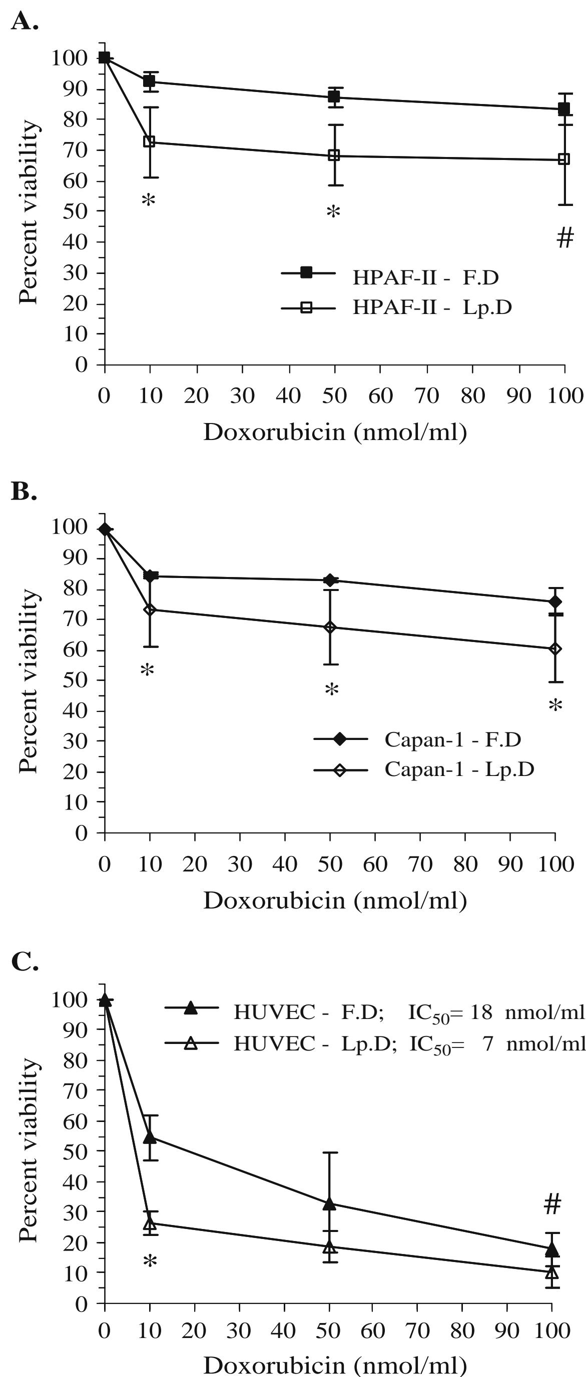

Fig. 3. Evaluation of cytotoxic effects of free doxorubicin and doxorubicin-PCLs against human pancreatic cancer cells (A) HPAF-II (*filled square*), (B) Capan-1 (*diamond*) and (C) HUVEC (*triangle*). The cells were seeded at 1×10^4 cells/ ml in a 48 well plate and incubated for 24 h at 37°C. Sulforhodamine B assay was used to determine the percent of viable cells after 24 h of exposure to different concentrations of free doxorubicin (FD) and doxorubicin-PCLs (LpD). Growth inhibitory properties of doxorubicin-loaded PCLs was significantly lower compared to doxorubicin alone for all three cell lines ($^*P<0.001$ and $^\#P<0.05$). IC_{50} value of doxorubicin-PCLs against HUVEC was significantly lower compared to doxorubicin alone ($^*P < 0.001$, $^\#P<0.05$; mean ± SD). Capan-1 was significantly more susceptible to doxorubicin treatment compared to HPAF-II ($P<0.05$). IC_{50} value for HUVEC was also lower than HPAF-II and Capan-1 cells ($P<0.05$).

The interaction of cationic liposomes with human microvascular endothelial cells (HMEC-1) was significantly greater than with pancreatic cancer cells. Experimental findings suggest that pancreatic tumor vessels may represent a suitable target of cationic drug carrier molecules.

Growth Inhibitory Properties of Doxorubicin-Loaded PCLs

We evaluated the cytotoxic effects of doxorubicin alone against human pancreatic and endothelial cell lines (Fig. 3). The percent of viable cells were significantly lower for HUVEC ($IC_{50}=18$ nmol/ml) compared to HPAF-II and Capan-1 ($P<0.001$). We next compared the effects of DOX-loaded PCLs to doxorubicin alone against HPAF-II, Capan-1, and HUVECs. The percent of viable HPAF-II (Fig. 3A), Capan-1 (Fig. 3B) and HUVEC (Fig. 3C) cells post treatment with doxorubicin-loaded PCLs was significantly lower compared to cells treated with doxorubicin alone ($^*P<0.001$, $^\#P<0.05$). Furthermore, the IC_{50} value (7 nmol/ml) observed for doxorubicin-loaded PCLs against HUVEC (Fig. 3C) was significantly lower compared to effect of doxorubicin alone (18 nmol/ml; $^*P<0.001$). Due to the high concentrations of 5-FU required to induce cell death (Fig. 1A) we were unable to perform *in vitro* cytotoxicity studies with 5-FU-loaded PCLs. Our experimental findings suggest that PCLs enhanced the inhibitory drug action of doxorubicin against both human cancer and endothelial cells, but endothelial cells were more susceptible to treatment.

Intracellular Uptake Studies and Distribution of PCLs in Pancreatic Tumors

DIC and fluorescence microscopy were used to evaluate cell morphology and localization of PCLs in HPAF-II and Capan-1 cells, respectively (Fig. 4). The majority of DIC images confirmed other reports stating that pancreatic cancer cells tend to grow as a multilayer cluster of cells in culture (Fig. 4A and D). We observed avid association of PCLs with cell membrane surfaces (Fig. 4B). Extensive uptake was frequently observed at the periphery of cell clusters (Fig. 4E and F); accumulation at peripheral regions of clusters was more prominent with Capan-1 cell cultures.

We next performed intravital microscopy experiments to observe the distribution of PCLs in tumors, using a dorsal skin fold chamber in tumor (HPAF-II) bearing mice. We systemically administered rhodamine labeled PCLs, and 24 hrs following injection we determined whether HPAF-II vessels were preferential targets of PCLs. To determine the intratumoral fate of PCLs we first acquired images of tumor vessels. The images revealed extensive vascularization, and highly tortuous vascular networks accompanied by extensive capillary branching (Fig. 4G and H). We next observed distribution of PCLs along tumor vessels; in many areas liposomes accumulated at branching points of vessel networks (Fig. 4I). We note heterogeneous localization of PCLs given that some areas showed extensive liposome accumulation compared to other vessels located within the same microenvironment. The data further suggest that PCLs may well be used to develop site-specific delivery systems that can preferentially deliver chemotherapeutic agents (such as 5-FU and doxorubicin) to tumor vascular networks.

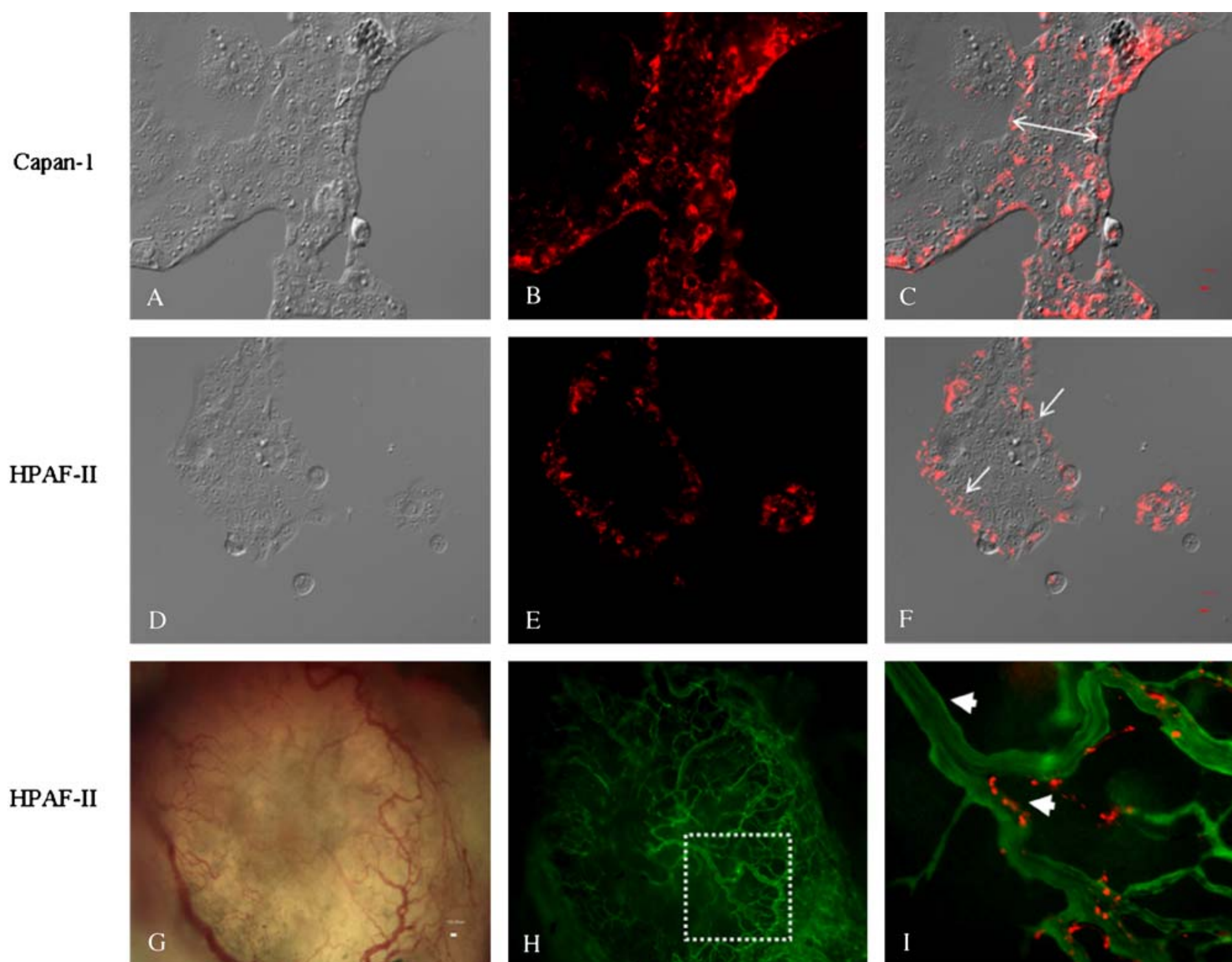

Fig. 4. Intracellular uptake and tumor distribution of PCLs in human pancreatic tumors. (A), (D) HPAF-II and Capan-1 cells were seeded at 5×10^5 cells per ml of required culture media. Approximately 100 nmol of PCLs was added to each well for experimental purposes. DIC microscopy images show multilayers of cells; (B), (E) Rhodamine channel was used to acquire fluorescence images; (C), (F) Merged image show specific region of PCL localization with respect to cell clusters and organelles, and PCLs associated at periphery (*arrow*) of cell clusters (20× magnification); (G) RGB was used to capture image of HPAF-II vessels in dorsal skin fold chamber at 4× magnification; (H) DSC tumor (HPAF-II) bearing mouse was injected with FITC-dextran (2.5 million MW) on day of analysis to visualize tumor vessels (*green*), and PCLs (*red*). Figure 4G and H reveal tortuous capillary networks of tumor. (I) Image is a magnified view of small boxed area located in Fig. G & H. Image was captured in FITC and rhodamine channels and subsequently merged to show PCL accumulation along HPAF-II vessels; we note heterogeneous localization of PCLs with accumulation at some branch points of vessel networks (*arrow*). Near vessels targeted with PCLs are regions with no detectable red fluorescence (*arrow*), suggesting reduced PCL accumulation. Intravital microscopy images were acquired with both 4× and 10× magnifications.

DISCUSSION

The tumor vasculature is the general route of entry by which chemotherapeutic agents gain access to pancreatic tumor cells. The vasculature also represents the life support of these target cells, and so interrupting flow of oxygen and nutrients to them may create an opportunity to effectively manage pancreatic tumor growth and progression of disease. Given that PCLs preferentially accumulate along tumor vessels (12,23,24), and the relatively rapid proliferation rates of endothelial cells in tumors compared to in normal tissues (14,15), delivering chemotherapeutic agents to tumor endothelia may represent a suitable alternative to interstitial drug targeting.

In this study we report the initial development and characterization of PCLs for selective delivery of chemotherapeutic agents to pancreatic tumor vessels. Our drug-loading efficiency studies showed that PCLs can be used to prepare liposomal formulations with high loading efficiency. The association of PCLs with human endothelial cells (i.e., HUVEC and HMEC-1) was significantly higher compared to human pancreatic cancer cells, further supporting the use of PCLs for targeted delivery of cytotoxic agents to the tumor endothelium.

During our cytotoxicity studies we evaluated the effect of 5-FU and DOX against the growth of two human pancreatic (Capan-1 and HPAF-II), umbilical vein endothelial (HUVEC), and dermal microvascular endothelial (HMEC-1) cells. The *in vitro* experiments involving 5-FU showed significantly higher IC_{50} values for human pancreatic cancer compared to endothelial cell lines. We observed that

doxorubicin was also more effective against our rapidly dividing endothelial cells compared to both Capan-1 and HPAF-II. Our experimental observations to date suggest that the tumor endothelium responds significantly better to the effect of chemotherapeutic agents, and might represent a useful therapeutic approach (35).

Due to relatively high IC_{50} values reported here for 5-FU against our cell lines, it was not possible to perform *in vitro* cytotoxicity experiments using 5-FU-loaded PCL formulations. The lipid concentration required to prepare 5-FU formulations at the appropriate drug to lipid ratio was too toxic. Fortunately, under similar experimental conditions doxorubicin was considerably more cytotoxic compared to 5-FU thus requiring lower drug and lipid concentrations to demonstrate proof-of-principle. In these studies we evaluated the effects of DOX-loaded PCLs on growth inhibition of cells between 10 and 100 nmol/ml of drug. Similar to effects of free drug on cell viability, we observed that HUVECs were more sensitive to the effects of treatment compared to HPAF-II and Capan-1. When doxorubicin was loaded in PCLs the drug was significantly more effective compared to doxorubicin alone supporting subsequent *in vivo* studies.

Although the majority of PCLs taken up by solid tumors accumulate along tumor vessels, a significantly reduced fraction of the dose injected has been observed in the tumor interstitial matrix (12). Anticipating a potentially similar intratumoral fate for 5-FU or DOX-loaded PCL formulations, we investigated intracellular localization of PCLs in HPAF-II and Capan-1 *in vitro* by DIC and fluorescence microscopy. We observed enhanced interaction of PCLs with cells; this might be due to membrane-associated proteoglycans shown to mediate DNA transfer, another cation-mediated event (36,37). Images acquired by DIC microscopy revealed that human pancreatic cells tend to grow as clusters which is consistent with previously published reports (38,39). Furthermore, the images show that PCLs associate with cellular membranes, and accumulate mainly at the periphery of cell clusters with Capan-1 cells (Fig. 4A–C).

PCLs can remain in circulation long enough to selectively deliver doxorubicin to tumor vessels in Ls174T-bearing mice (12). It was noted that after 24 h post systemic injection of DOX-loaded PCLs, extensive accumulation of drug was observed along tumor vessels compared to when doxorubicin was injected alone. Our *in vitro* investigations in this report show enhanced growth inhibitory properties of doxorubicin and 5-FU against human pancreatic and endothelial cells. This was probably due to more significant DNA damage and apoptosis-related cell death due to relatively high intracellular drug uptake; interestingly, our human endothelial cell lines proliferated much faster than Capan-1 and HPAF-II cells (data not shown). This may have contributed to the enhanced sensitivity of immortalized HMEC-1, and primary HUVEC cells compared to Capan-1 and HPAF-II.

Liposomes have been shown to inhibit chemotherapeutic drug binding to P-glycoprotein-enriched membranes and to modulate mutidrug resistance (40,41). This might explain the enhanced cytotoxic effect of DOX-loaded PCLs compared to when HPAF-II and Capan-1 cells were exposed to doxorubicin alone. An additional possibility is that doxorubicin is directly cytotoxic to cellular membranes and at appropriate concentrations the drug need not enter target cells to be considered effective (42). Following this reason one may conclude that the natural electrostatic interactions between PCLs and the net negative membrane surface charge potential of target cells enhanced the cytotoxic activity of DOX when loaded in PCLs.

The tendency for PCLs to localize along the length of vessels in human pancreatic tumors is consistent with previously published reports demonstrating preferential vascular uptake in B16-F10 (murine melanoma), Ls174T (human colon adenocarcinoma) and MCA-IV (murine breast carcinoma) in male SCID mice (12). As with these tumor models PCLs could be used to target chemotherapeutic agents to pancreatic tumors where they can exert their action against the vasculature due to preferential accumulation at this site. Pharmacology and efficacy studies are currently underway.

CONCLUSIONS

In conclusion, we provide evidence to support the use of PCLs to deliver chemotherapeutic agents to pancreatic tumor vessels. On the basis of our experimental findings, human endothelial cells are more sensitive to the effects of 5-FU and doxorubicin hydrochloride (DOX) compared to human pancreatic cancer cells (the traditional target of many chemotherapeutic agents). PCLs can incorporate the water soluble drugs 5-FU and DOX. We provide evidence to support favorable uptake of PCLs by human microvascular endothelial over pancreatic cancer cells, and improved growth inhibitory properties of DOX when loaded in PCLs. Finally, we demonstrated that PCLs accumulate to a greater extent along vessels in HPAF-II (tumors) of dorsal skin fold chambers, compared to the tumor interstitium. PCLs may be useful in the development of effective clinical products against human pancreatic cancer, and hence, subsequent *in vivo* investigations involving the use of other synthetic, chemically unique cationic lipids are warranted.

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