Abstract. Background: Folate receptor (FR) is selectively amplified among human tumors, including in 70% of myeloid leukemias. FR-targeted liposomal delivery is an attractive strategy for enhancing the therapeutic efficacy of anticancer agents against FR(+) tumors. In this study, FR-targeted liposomal daunorubicin was evaluated in an FR+ L1210JF murine ascites tumor model for therapeutic efficacy in vivo. Materials and Methods: FR-targeted liposomal daunorubicin (F-L-DNR) and non-targeted liposomal daunorubicin (L-DNR) were prepared by polycarbonate membrane extrusion followed by remote loading of DNR. FR-targeted liposomal uptake by L1210JF cells was characterized in vitro using fluorescent liposomes entrapping calcein. For in vivo therapeutic study, B6D2F1 mice on a folate-free diet were intraperitoneally implanted with FR (+) L1210JF cells and treated with 4 intraperitoneal injections of 10 mg/kg liposomal DNR at 1, 5, 9 and 13 days following tumor cell inoculation. Animal survival was then monitored daily. Results: L1210JF cells showed ~10^3 times greater uptake for FR-targeted liposomal calcein compared to the non-targeted control. Uptake of the targeted liposomes could be blocked by 1 mM folic acid. In the therapeutic study, mice treated with F-L-DNR showed significantly greater tumor inhibition and 40.7% greater increase in life-span compared to those that received identical doses of L-DNR. Meanwhile, free DNR given at the same dose failed to prolong the survival of the treated mice. Conclusion: F-L-DNR can effectively target FR(+) leukemia cells in vivo. Further preclinical evaluation is warranted to determine its potential application in leukemia therapy.

Folate receptor (FR) is a high affinity folate binding protein that has two glycosylphosphatidylinositol (GPI)-anchored isoforms, α and β. Normal tissues generally lack FR expression. In contrast, FR-α is frequently overexpressed in epithelial cancers including over 90% of ovarian carcinomas (1-3). On the other hand, FR-β is expressed in a non-functional form in placenta and mature neutrophils (4, 5), and in a functional form in activated macrophages, chronic myelogenous leukemias (CML), and ~70% of acute myelogenous leukemias (AML) (5, 6). A number of FR-targeted therapeutic and imaging agents have been evaluated in preclinical studies, including liposomal agents, with promising results (7, 8).

Liposomal anthracyclines, including doxorubicin and daunorubicin, have prolonged circulation time and reduced cardiotoxicity compared to the parent drugs (9-11). In this study, the therapeutic efficacy of FR-targeted liposomal DNR was evaluated in a murine L1210JF ascites tumor model.

Materials and Methods

Reagents. Distearoylphosphatidylcholine (DSPC) and methoxy-polyethyleneglycol (M.W. 2,000) distearoyl phosphatidyl-ethanolamine (PEG2000-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Folic acid, cholesterol, Sepharose CL-4B resin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Daunorubicin (DNR)
hydrochloride and folate (g)-PEG3,400-DSPE were obtained from the National Cancer Institute Developmental Therapeutics Program through a RAID project. Polycarbonate membranes and the Lipex™ lipid extruder were obtained from Northern Lipid Inc. (Vancouver, Canada). Tissue culture media, additives and fetal bovine serum (FBS) were purchased from Gibco-BRL (Rockville, MD, USA).

Cell culture. L1210JF, an FR (+) subline of the L1210 murine lymphocytic leukemia cell line generated by culturing in low folate media, was kindly provided by Dr. J. Fan. All cells were cultured at 37°C in 5% CO₂ in folate-free RPMI 1640 media, supplemented with 100 U/ml penicillin, 100 U/mL streptomycin and 10% fetal bovine serum.

Liposome preparation. FR-targeted liposomes, composed of DSPC/Cholesterol/PEG2000-DSPE/Folate-PEG3400-DSPE (60:35:4:1, mole/mole), and non-targeted control liposomes, composed of DSPC/Cholesterol/PEG2000-DSPE (60:35:5, mole/mole), were prepared by polycarbonate membrane extrusion, as described previously (19). Briefly, the mixture of lipids dissolved in chloroform was dried under a stream of nitrogen and desiccated under vacuum for 2 hours. The lipids were then re-hydrated in 300 mM sodium citrate (pH 4.0) and subjected to 6 cycles of freeze and thaw. The liposomes were extruded through a 100 nm pore size polycarbonate membrane using a Lipex extruder at 60°C under 400 psi provided by compressed nitrogen gas. Next, the liposomes were passed through a Sepharose CL-4B gel-filtration column equilibrated in phosphate-buffered saline (130 mM NaCl, 20 mM Na₂HPO₄, pH 7.4) and incubated at daunorubicin at a drug-to-lipid ratio of 1:15, which results in near quantitative loading of the drug into the liposomes. The drug-loaded liposomes were then purified on a Sepharose CL-4B column. The mean diameter of the liposomes was determined by dynamic light scattering on a NICOMP Particle Sizer Model 370. The lipid concentration of the liposomes was determined by an ammonium ferrothiocyanate partitioning colorimetric assay, as previously described (20).

FR-targeted and non-targeted liposomes entrapping a fluorescent dye calcein were also prepared by the extrusion method, as described above, by hydrating the lipids in 20 mM of calcein.

Cellular uptake of liposomal calcein. The uptake of FR-targeted and non-targeted liposomal calcein by L1210JF cells was determined by flow cytometry. L1210 JF cells were briefly washed with acid saline (pH 3.5) to remove folate from FRs on the cell surface. The cells were then incubated for 1 hour with 20 mM liposomal calcein. For FR blocking, 1 mM folic acid was added to the incubation media. After incubation with the liposomes, the cells were washed three times with PBS (pH 7.4), re-suspended in 0.3 ml saline and analyzed on a Beckman Coulter Elite Flow Cytometer. Cellular uptake was determined by mean fluorescence intensity.

Animal model. Male DBA/2, B6D2F1 mice were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA). The mice were maintained on a folate-free rodent diet (Cat#117772, Dyets Inc., Bethlehem, PA, USA) for 2 weeks prior to the study and for the duration of the study. To generate ascites tumor, 10⁶ L1210JF cells in 200 ìL were injected i.p. into DBA/2 mice. On day 7, ascites fluid was collected from the DBA/2 and transferred into B6D2F1 male mice i.p. injection in 200 ìL. On day 1 (24 hours post L1210JF cell ascites transplantation), the B6D2F1 mice were divided randomly into 8 groups of 8 mice each, and treated in the following groups: 1) Unloaded liposomes (with the same lipid composition as F-L-DNR, but without DNR); 2) F-L-DNR (10 mg/kg in DNR), 3) L-DNR (10 mg/kg in DNR), and 4) free DNR in PBS (10 mg/kg). The treatments were given via 4 i.p. injections on every fourth day (days 1, 5, 9 and 13). The mice were monitored daily for survival, signs of distress and weight changes. The significance of differences among treatment groups was determined using the log rank test with assistance from the Ohio State University Center for Biostatistics.

Results

Uptake of FR-targeted fluorescent liposomes. L1210JF cells, incubated with FR-targeted and non-targeted control liposomes entrapping calcein, were assessed by flow cytometry to determine the differential in liposomal uptake. Uptake of F-L-calcein was ~ 10³ times higher compared to that of non-targeted L-calcein (Figure 1).
which, in addition, can be blocked by 1 mM free folate. This indicated FR-dependent uptake of the FR-targeted liposomes.

Therapeutic efficacy of F-L-DNR in B6D2F1 mice bearing L1210JF ascites tumor. The mice were treated with 4 i.p. injections in groups of 8 mice each, as described in Materials and Methods. Mice survival is presented in a Kaplan Meier plot (Figure 2). The mean survival time and the percentile treatment/control ratio (T/C) for each treatment group are presented in Table I. The mice treated with F-L-DNR showed a significantly greater increase in life-span (T/C = 179) than mice treated with the non-targeted L-DNR (T/C = 145) and free DNR (T/C = 116). These data suggest that FR-targeted delivery resulted in significant therapeutic advantage in the FR(+) ascites tumor model.

Discussion

In this study, FR-targeted F-L-DNR was compared with non-targeted L-DNR for antitumor activity in vivo and was shown to be more effective in prolonging the survival of ascites tumor-bearing mice.

Compared to doxorubicin, DNR is substantially less cardiotoxic and is the preferred agent for the treatment of leukemia and certain solid tumors (17, 21). There have been a number of reports on FR-targeted delivery of liposomal doxorubicin, both in vitro and in vivo (6, 14-16, 22-24).

Table I. The effect of treatment on the survival time of mice with L1210 JF cell ascites tumor.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Survival after tumor cell inoculation (days) (n=8)</th>
<th>Treatment/Control (%)</th>
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</thead>
<tbody>
<tr>
<td>F-L-DNR</td>
<td>27.3±4.7</td>
<td>184</td>
</tr>
<tr>
<td>L-DNR</td>
<td>19.4±3.8</td>
<td>131</td>
</tr>
<tr>
<td>DNR (free drug)</td>
<td>13.6±0.9</td>
<td>92</td>
</tr>
<tr>
<td>Unloaded Liposomes</td>
<td>14.8±2.4</td>
<td>100</td>
</tr>
</tbody>
</table>

Although a liposomal formulation of DNR (DaunoXome) is currently in clinical use (25, 26), there have been relatively few reports on targeted delivery of liposomal DNR. Previously, our laboratory reported that F-L-DNR could effectively target FR(+) cells in vitro by exhibiting FR-dependent cellular uptake and cytotoxicity (19). In the current study, the in vivo therapeutic efficacy of F-L-DNR was evaluated in an ascites murine tumor model that is FR(+).

Although targeted liposomes generally produced favorable cytotoxicity against tumor cells in vitro, the biodistribution of liposomes in vivo is mostly determined by non-specific factors such as size and surface properties. However, recent studies suggest that, despite the lack of greatly increased solid tumor uptake, FR-targeted liposomes entrapping doxorubicin were more effective against solid tumors than the non-targeted control liposomes in mice carrying FR(+) MA109 subcutaneous tumors (16). This was thought to be due to increased tumor cell uptake and more effective intratumoral distribution of the liposomal drug in the targeted formulation (16).

Leukemias are inherently much more accessible to the blood stream compared to solid tumors, which are separated from the blood compartment by endothelial lining. Targeted liposomes, therefore, should be highly effective in targeting leukemia in vivo. The results of this study confirmed that targeting FR is feasible in vivo against an FR(+) ascites tumor. This finding is consistent with data from another recent report from this laboratory on FR-targeted liposomal doxorubicin (6). Since FR is overexpressed in 70% of AMLs and most CMLs, F-L-DNR might provide a new strategy for the treatment of these diseases. Interestingly, recent studies in Dr. Manohar Ratnam’s laboratory have shown that FR expression in AML cells can be selectively up-regulated by all-trans-retinoic acid (ATRA), either alone or, even more effectively, in combination with histone deacetylase (HDAC) inhibitors such as tricostatin A, dexamethasone and valproic acid (27). FR up-regulation was found to be
independent of cellular differentiation induction and to occur in ATRA refractive AMLs (27). A combination of FR up-regulation using ATRA plus an HDAC inhibitor and F-L-DNR might provide an exciting new strategy for the treatment of AML.

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