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Epidermal Growth Factor Receptor (EGFR)-targeted Immunoliposomes Mediate Specific and Efficient Drug Delivery to EGFR- and EGFRvIII-overexpressing Tumor Cells[1]

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ABSTRACT

We hypothesized that immunoliposomes (ILs) that target epidermal growth factor receptor (EGFR) and/or its truncated variant EGFRvIII can be constructed to provide efficient intracellular drug delivery in tumor cells overexpressing these receptors. Monoclonal antibody fragments included Fab′ fragments derived from C225, which binds both EGFR and EGFRvIII, or novel anti-EGFR scFv C10, which binds EGFR only. Monoclonal antibody fragments were covalently linked to liposomes containing various reporters or drugs. ILs were evaluated for specific binding, internalization, and cytotoxicity in EGFR/EGFRvIII-overexpressing cell lines *in vitro*. Flow cytometry and fluorescence microscopy showed that EGFR-targeted ILs, but not nontargeted liposomes or irrelevant ILs, were efficiently bound and internalized by EGFR-overexpressing cells, including glioma cells (U-87), carcinoma cells (A-431 and MDA-MB-468), and EGFRvIII stable transfectants (NR-6M). Furthermore, EGFR-targeted ILs did not bind to non-EGFR-overexpressing cells (MCF-7 and parental NR-6). ILs showed 3 orders of magnitude greater accumulation in NR-6-EGFRvIII stable transfectants *versus* parental NR-6 cells. Quantitative internalization studies indicated binding of EGFR-targeted ILs to target cells within 5 min, followed by intracellular accumulation beginning at 15 min; total uptake reached ~13,000 ILs/cell. ILs were used to deliver cytotoxic drugs doxorubicin, vinorelbine, or methotrexate to EGFR/EGFRvIII-overexpressing target cells *in vitro*. In each case, the IL agent was significantly more cytotoxic than the corresponding nontargeted liposomal drug in target cells, whereas it was equivalent in cells lacking EGFR/EGFRvIII overexpression. We conclude that EGFR-targeted ILs provide efficient and targeted delivery of anticancer drugs in cells overexpressing EGFR or EGFRvIII.

INTRODUCTION

To create agents capable of both drug delivery and molecular targeting, we and others have studied approaches for antibody-targeted liposomes (ILs[3]), in which liposomes are endowed with the specificity of MAbs (1, 2). For example, we have developed ILs directed against HER2 (ErbB2), which bind and internalize in HER2-overexpressing cells but not in normal cells (3, 4).

In principle, this approach can be applied to other antigenic targets, particularly other RTKs capable of mediating endocytosis after ligand binding to enable IL internalization. The EGFR is the prototypic member of the class I (ErbB, HER) family of RTKs, which includes EGFR, HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). As a target antigen, EGFR is a readily accessible cell surface receptor (5), and when overexpressed, provides a basis for selective antibody-based targeting of tumor cells (6, 7). EGFR overexpression occurs in many human solid tumors, and in some cases has been correlated with poor prognosis (reviewed in Ref. 8).

In addition to overexpression of wild-type EGFR, *EGFR* gene rearrangement also occurs in a number of cancer types. Class III mutants (EGFRvIII) contain a deletion of exons 2–7 within the ECD, resulting in an in-frame deletion of 801 bp of the coding sequence and the generation of a novel glycine residue at the fusion junction (9, 10). This mutant form is the most frequently detected genomic variant in brain tumors and other cancers (11). Expression of EGFRvIII is a potentially tumor-specific event in some cases of breast carcinoma, non-small cell lung carcinoma, and high-grade glioma (12–14), and may represent an ideal antigen for immunotherapies.

MAbs against EGFR such as MAb 225 can inhibit the proliferation of EGFR-overexpressing cells *in vitro* and in tumor xenograft models (15). For example, MAb 225 competitively inhibits binding of growth factors such as EGF and transforming growth factor α to EGFR, thus blocking ligand-dependent EGFR signaling. A chimerized version of this MAb, C225 (cetuximab, Erbitux; Ref. 16), has been generated to retain these properties while reducing the potential for immunogenicity. MAb C225 has been studied in clinical trials for the treatment of various cancer types, such as head/neck and colorectal cancer (17, 18). C225 binds to both EGFR and EGFRvIII.

Here we describe the design, preparation, and characterization of EGFR-targeted ILs, including intracellular delivery of multiple drugs in cancer cells that overexpress EGFR and/or EGFRvIII.

MATERIALS AND METHODS

Materials. Reagents for liposome preparation included: $DiIC_{18}$(3)-DS (Molecular Probes, Eugene, OR); 1–2-oleoyl-3-sn-glycerophosphocholine, DSPC, and mPEG-DSPE (Avanti Polar Lipids, Alabaster, AL); Chol (Calbiochem, San Diego, CA); Mal-PEG-DSPE (Shearwater Polymers, Huntsville, AL); organic solvents, high-performance liquid chromatography grade (Fisher, Pittsburgh, PA); and other chemicals of reagent purity (Sigma Chemicals, St. Louis, MO). Vinorelbine (GlaxoSmithKline, Triangle Park, NC), doxorubicin (Bedford Laboratories, Bedford, OH), and pegylated liposomal doxorubicin (Doxil; Alza Pharmaceuticals, Palo Alto, CA) were obtained commercially from the pharmacy. Methotrexate was purchased from Sigma Chemicals. MAb C225 was kindly provided by Dr. Daniel Hicklin (ImClone Systems, New York, NY).

Cell Lines. MDA-MB-468 human breast cancer and U-87 human glioblastoma cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). A-431 epidermoid cancer, and SK-BR-3 and MCF-7 breast cancer cell lines were obtained from the University of California San Francisco Cell Culture Facility (San Francisco, CA). NR-6 and stable EGFR-

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[3] The abbreviations used are: IL, immunoliposome; EGFR, epidermal growth factor receptor; Chol, cholesterol; $DiIC_{18}$(3)-DS, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE, 1,2-distearoyl-3-sn-glycerophosphoethanolamine; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; Mal-PEG-DSPE: β-(*N*-maleimido)propionyl poly(ethylene glycol)-1,2-distearoyl-3-sn-phosphoethanolamine; PEG: poly(ethylene glycol); MAb, monoclonal antibody; RTK, receptor-tyrosine kinase; ECD, extracellular domain; EGF, epidermal growth factor; VRL, vinorelbine.

vIII-transfected NR-6M cells were kindly provided by Dr. Daryl D. Bigner (Duke University, Durham, NC; Ref. 19). MDA-MB-468 cells were maintained in Leibovitz L-15 medium, SK-BR-3 cells in McCoy 5a, and all of the other cell lines in DME H-21 medium supplemented with 10% bovine calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C (except L-15 medium in 100% air).

Liposome Preparation. Unilamellar liposomes were prepared according to the repeated freeze-thawing method (20) using 1–2-oleoyl-3-sn-glycerophosphocholine and Chol, or DSPC and Chol (molar ratio 3:2) with mPEG-DSPE (0.5–5 mol% of phospholipid). Liposomes were subsequently extruded 10 times through polycarbonate filters with defined pore sizes of 0.1 μm, and yielded liposomes of 100–120 nm diameter as determined by dynamic light scattering. Liposome concentration was measured using a standard phosphate assay (21).

For uptake and internalization studies, liposomes were labeled with 0.1–0.3 mol% $DiIC_{18}$(3)-DS, a fluorescent lipid that can be stably incorporated into liposomal membranes (22, 23). For kinetic analysis, the pH-sensitive probe HPTS (pyranine) was encapsulated in liposomes by hydrating the dry lipid in 35 mM HPTS (pH 7.0); the preparation was adjusted to an osmolarity of 280 mOsm/l with NaCl, followed by extrusion as described above. Unentrapped HPTS was removed by gel filtration using a Sephadex G-75 column.

For encapsulation of chemotherapy drugs doxorubicin and vinorelbine, the remote-loading method using ammonium sulfate was performed (24, 25). First, dry lipids were rehydrated in 250 mM ammonium sulfate at pH 5.5, followed by extrusion as described above. Free ammonium sulfate was removed by size-exclusion chromatography using a Sephadex G-75 column/HEPES buffered saline (pH 7.0). Liposomes were then incubated with either doxorubicin or vinorelbine tartrate (Navelbine) for 30 min at 60°C. Notably, in the case of vinorelbine, the pH of the loading solution as well as reduced levels of PEG-DSPE were found to be important for loading. The relatively low pH of vinorelbine tartrate inhibits loading unless adjusted to pH 6.5 before incubation at 60°C. The presence of high concentrations of PEG-DSPE also reduced loading efficiency, and so we used 0.5% PEG-DSPE in this formulation. All of these liposome compositions have been shown to be long circulating, with or without extensive pegylation (26, 27). Under these conditions, loading efficiencies were typically in the range of 95–100% when 150 μg drug/μmol phospholipid was used. In addition, pegylated liposomal doxorubicin (Doxil) was obtained commercially. Methotrexate-loaded liposomes were prepared by passive encapsulation in an aqueous solution containing methotrexate [200 mM and 5 mM HEPES (pH 7.0)]. All of the unencapsulated drugs were removed by size-exclusion chromatography using a Sephadex G-75 column.

MAb Fragment Preparation, Conjugation, and Liposome Incorporation. For C225-Fab′, intact C225 IgG_1 was incubated with pepsin (weight ratio 1:20) in 0.1 M sodium acetate (pH 3.7) at 37°C for 3 h, followed by dialysis against HEPES-buffered saline (pH 6.0). The resulting C225-$F(ab)_2$ was reduced with 2-mercaptoethylamine (15 mM) under argon for 15 min at 37°C, and then recovered by gel filtration using Sephadex G-25. The efficiency of cysteine reduction was assayed using Ellmann's reagent, and was typically 90%. For scFv C10, cysteine reduction was performed according to this procedure; reduction efficiency was typically 80–90%.

Fab′ or scFv were conjugated to Mal-PEG-DSPE as described (4, 27). Conjugation efficiencies were evaluated by SDS-PAGE, allowing comparison of free MAb fragment *versus* conjugate; the resulting bands were scanned and ratios determined using the software package NIH Image (v.1.62). Conjugation efficiencies were typically 30–50% for Fab′ (C225) and 50–80% for scFv (C10). For incorporation into preformed liposomes, including prepared liposomal drugs and probes or commercial pegylated liposomal doxorubicin, MAb fragment conjugates (Fab′/scFv-PEG-DSPE) which form micellar solutions, were incorporated into liposomes by coincubation at 55°C for 30 min at the indicated protein:liposome ratios. As a result, the conjugates become attached to the outer lipid layer of the liposomes via hydrophobic DSPE domains. Unincorporated conjugates and free drug were separated from ILs by Sepharose CL-4B gel filtration. UV absorbance at 280 nm confirmed that ILs were in the excluded volume, whereas unincorporated micelles were retained as a broad peak easily distinguished from the IL peak. When $DiIC_{18}$(3)-DS-labeled liposomes were used, <5% of the fluorescence was coassociated with the micelle fraction, indicating minimal transfer of this marker. Incorporation efficiency of conjugated MAb fragments was estimated by SDS-PAGE using a series of protein standards, and gel scanning and quantitation as described. Typically, 75–85% of added MAb conjugate was incorporated into ILs.

Binding and Internalization Experiments. For flow cytometric assay, cells underwent 2 h incubation with $DiIC_{18}$(3)-DS labeled liposomes or ILs at 37°C. After extensive washing with PBS, cells were detached and stored on ice until subjected to flow cytometry.

For fluorescence microscopy studies, 250,000 cells were coincubated in 12-well plates with liposomes or EGFR-targeted ILs labeled with $DiIC_{18}$(3)-DS for 2 h at 37°C, washed extensively with PBS, incubated for another 2 h without liposomes/ILs, washed with PBS, and subsequently observed using an inverted fluorescence microscope (Nikon Eclipse, TE300) with a 540/25 nm bandpass filter for excitation and a long pass filter at 565 nm for emission.

Cellular Uptake Studies. Quantitative analysis of cell surface bound *versus* endocytosed ILs was performed according to Straubinger *et al.* (28). Various target cells were incubated for 2 h with 0.1 μM (phospholipid concentration) EGFR-targeted ILs, followed by washing three times with ice-cold Hanks solution, and then cell harvesting with PBS/EDTA 0.04%. Fluorescence intensities at excitation wavelengths of 460 nm and 413 nm (isosbestic point) were determined using a Spex Fluorolog 2 spectrofluorometer (Spex Industries, Edison, NJ). On the basis of the pH-sensitivity of the fluorophore HPTS, total uptake, internalized and surface-bound fractions were calculated for various time points (4).

Cellular uptake of doxorubicin was assayed after incubation with EGFR-targeted immunoliposomal doxorubicin or liposomal doxorubicin for 3 h at 37°C. Cells were washed, detached, and subjected to quantitative doxorubicin assay using a FL-600 microplate fluorescence reader (Bio-Tek, Winooski, VT) with a bandpass filter at 485/20 nm for excitation and 590/35 nm for emission.

Cytotoxicity Studies. Specific cytotoxicity of EGFR-targeted ILs containing various anticancer drugs was evaluated in target cells plated at a density of 9000 cells/well in 96-well plates and allowed to grow overnight. ILs or control treatments were applied for 2 h at 37°C, followed by washing with PBS and readding growth medium. Cells were additionally incubated at 37°C for 3 days and analyzed for cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining (29).

***In Vitro* Stability of ILs in Human Plasma.** EGFR-targeted ILs were prepared by incorporation of C225-conjugated micelles into preformed Doxil as described. Doxil and ILs were mixed with 50% human plasma and transferred into a 10-well microdialyzer (Spectra/Por; Spectrum Med. Ind., Rancho Dominguez, CA). The stability of these ILs and native Doxil was determined after 4 h and 8 h of continuous dialysis at 37°C. Briefly, Sepharose 4B columns (Sigma) were used for each sample to remove the released doxorubicin and unassociated plasma components before calculating the relative stability by determination of the ratio of measured doxorubicin:phospholipid. The amount of entrapped doxorubicin was measured fluorimetrically using the suitable excitation (λ = 470 nm) and emission (λ = 592 nm) wavelengths. Phospholipid concentration was quantified using a standard phosphate assay (21).

RESULTS

Construction of ILs Targeted to EGFR. EGFR-targeted ILs were constructed using the modular design and methodologies initially developed in conjunction with HER2-targeted ILs (1, 3, 4, 27). This includes liposome compositions optimized previously for systemic delivery, including *in vivo* stability and long circulation times. Briefly, ILs were prepared by conjugation of 100-nm liposomes (DSPC/Chol/PEG-PE) with MAb fragments directed against EGFR and/or EGFR-vIII. Conjugation was performed by direct linkage of MAb fragments to modified termini of PEG chains on liposomes (4), or by initial coupling to Mal-PEG-DSPE linker in solution, followed by incorporation of the resulting micellar conjugates into liposomes (30, 31). The latter method allows conjugation of MAb fragments to existing drug-loaded liposomes, such as pegylated liposomal doxorubicin (Doxil), obviating the need for specially prepared liposomes engineered to contain appropriate functional sites.

EGFR-targeted ILs contained either Fab′ derived from C225 (cetuximab; ImClone Systems, Inc.) or scFv C10, a novel anti-EGFR

scFv generated from screening of a phage antibody library. C225, a chimeric IgG_1, binds the ECD of EGFR and thereby blocks activation by EGFR ligands such as EGF and transforming growth factor α (32). Although EGFRvIII involves deletion of exons 2–7 in the ECD, which abrogates binding of EGF and other cognate growth factors, C225 still binds EGFRvIII. Hence, the ability of this MAb to bind EGFRvIII is irrelevant to its therapeutic mechanism of action of ligand blockade, because EGFRvIII is constitutively active; yet this MAb represents a potentially useful reagent for targeted delivery strategies in EGFRvIII-expressing tumors. Fab′ fragments derived by pepsin cleavage of C225 were attached via their hinge cysteine to Mal-PEG-DSPE, either on liposomes or as an initial coupling reaction in solution.

In addition to Fab′ fragments from MAb C225, novel fully human single-chain antibody fragments (scFv) were isolated for IL targeting. scFv, which consist of variable domains only in a single polypeptide sequence, are attractive targeting ligands, and can be generated from recombinant phage antibody libraries (33). For anti-EGFR scFv, two phage antibody libraries containing human antibody genes (34–36) were screened for binding to recombinant EGFR ECD. Of the multiple novel and independent clones showing specific binding, scFv C10 was selected for IL conjugation based on its expression level and stability. For this purpose, scFv C10 was subcloned into an alternative bacterial expression cassette that affixes a COOH-terminal cysteine residue for covalent conjugation to the Mal-PEG-DSPE linker construct.

Uptake of EGFR-targeted ILs in EGFR- and EGFRvIII-overexpressing Cell Lines. Binding and uptake of EGFR-targeted ILs in EGFR-overexpressing cell lines were evaluated by flow cytometry. In this assay, liposomes were fluorescently labeled with $DiIC_{18}(3)$-DS with/without conjugated MAb fragments, and incubated with MDA-MB-468 breast cancer cells for 2 h at 37°C. ILs containing C225-Fab′ showed >200-fold greater accumulation in MDA-MB-468 cells than did control liposomes, which produced only background levels of fluorescence in these cells (Fig. 1*A*). Similarly, ILs containing scFv C10 showed >100-fold greater uptake in MDA-MB-468 cells than did nontargeted liposomes (Fig. 1*B*).

The uptake of C225-Fab′-containing ILs was also evaluated in EGFRvIII-expressing stable transfectants NR-6M *versus* nontransfected parental NR-6 cells. ILs showed extensive uptake in EGFRvIII-expressing NR6-M cells (Fig. 1*C*) but minimal uptake in parental NR-6 cells, which was indistinguishable from that of nontargeted liposomes (Fig. 1*D*). This result indicated a selectivity of up to 3 orders of magnitude for IL uptake between the two isogenic cell lines differing in EGFRvIII expression.

The observation of minimal fluorescence uptake in target cells after incubation with control liposomes is consistent with the nonreactive properties of pegylated liposomes (3, 4) and also confirms that $DiIC_{18}(3)$-DS can be used as a stable liposome-based marker without significant exchange into cell membranes. Indeed, target cells did not accumulate additional fluorescence even after 24-h incubation with $DiIC_{18}(3)$-DS-labeled control liposomes (data not shown).

Internalization of EGFR-targeted ILs. ILs were designed to exploit both binding and internalization of anti-EGFR MAb fragments, thus enabling endocytosis of ILs in target cells for intracellular drug delivery (1). The internalization of EGFR-targeted ILs was evaluated by confocal fluorescence microscopy in a variety of cell lines. First, fluorescence-labeled ILs and matched control (nontargeted) liposomes were incubated with MDA-MB-468 cells for 2 h at 37°C. ILs were observed to have accumulated profusely throughout the cytoplasm in a pattern consistent with endosomal uptake (Fig. 2, *A* and *B*). In contrast, control liposomes failed to show any detectable binding or internalization (Fig. 2, *C* and *D*), consistent with the flow cytometric assay results.

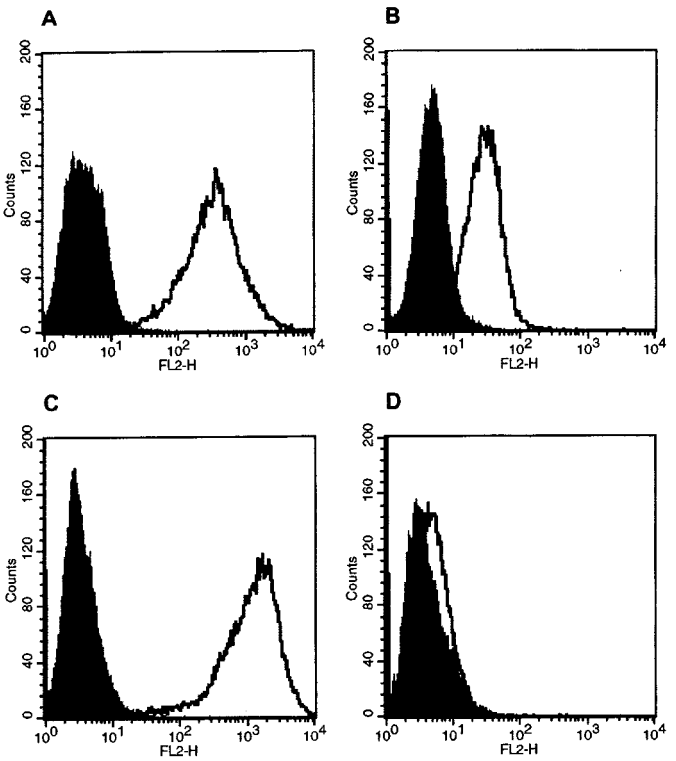

Fig. 1. Uptake of EGFR-targeted ILs in various cell lines. The uptake of EGFR/EGFRvIII-targeted ILs was evaluated in target or control cell lines by flow cytometric assay. The indicated cells were incubated with $DiIC_{18}(3)$-DS-labeled ILs containing either C225-Fab′ or scFv C10 for 2 h at 37°C, extensively washed, detached, and stored on ice until subjected to flow cytometry. *Open histograms*, EGFR/EGFRvIII-targeted ILs. *Filled histograms*, control liposomes prepared identically as ILs except for omission of MAb fragments. *A*, uptake of C225-Fab′-containing ILs in EGFR-overexpressing MDA-MB-468 breast cancer cells. *B*, uptake of scFv C10-containing ILs in MDA-MB-468 cells. *C*, uptake of C225-Fab′-containing ILs in NR-6M, stable transfectants of EGFRvIII. *D*, uptake of C225-Fab′-containing ILs in control cell line NR-6 lacking EGFR or EGFRvIII expression.

IL internalization was further evaluated in additional EGFR-overexpressing or nonoverexpressing cells lines. In EGFR-overexpressing U-87 malignant glioma cells (Fig. 3*A*) and A-431 epidermoid cancer cells (Fig. 3*B*), incubation with EGFR-targeted ILs resulted in extensive internalization and cytoplasmic accumulation. In contrast, irrelevant (HER2-targeted) ILs showed no detectable binding or internalization in either cell type (Fig. 3, *C* and *D*), which was also the case with nontargeted control liposomes (data not shown). Finally, EGFR-targeted ILs were not observed to bind or accumulate in non-EGFR-overexpressing MCF-7 breast cancer cells (Fig. 3*E*). Because MCF-7 cells have low levels of EGFR expression ($\sim 10^4$ receptors/cell), this result indicates a threshold effect in which higher levels of EGFR density than this basal level are required for detectable IL uptake; this appears analogous to the threshold observed for HER2-targeted ILs in HER2-overexpressing target cells (3, 4).

Internalization of C225-Fab′-containing ILs was also studied in cells with EGFRvIII expression. EGFR-targeted ILs demonstrated a high degree of intracellular accumulation in NR-6M cells stably transfected with EGFRvIII (Fig. 3*F*). However, in parental NR-6 cells lacking EGFR or EGFRvIII, no detectable binding or internalization were observed (Fig. 3*G*).

Kinetic Analysis of IL Internalization. Quantitative studies of IL uptake and internalization were performed using EGFR-targeted ILs loaded with the pH-sensitive fluorophore pyranine (HPTS). This method allows quantitative analysis of IL uptake in both neutral pH (surface-bound) and acidic pH (endocytosis-associated) compartments (4). Binding to MDA-MB-468 cells was observed within 5 min,

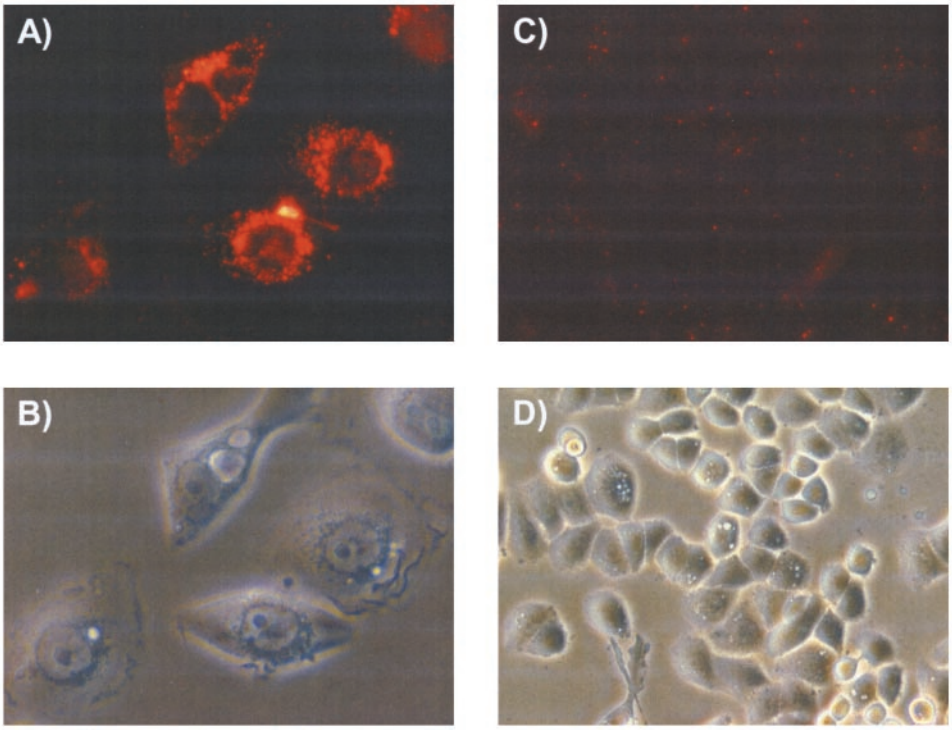

Fig. 2. Internalization of EGFR/EGFRvIII-targeted ILs *versus* nontargeted liposomes in EGFR-overexpressing cells. MDA-MB-468 breast cancer cells were treated with $DiIC_{18}$(3)-DS-labeled liposomes containing or lacking C225-Fab′ for 2 h at 37°C. *A*, internalization of EGFR-targeted ILs in MDA-MB-468 cells as visualized by confocal microscopy. *B*, same field as in *A*, visualized by phase contrast microscopy. *C*, lack of uptake of control (no MAb fragments) liposomes in MDA-MB-468 cells as visualized by confocal microscopy. *D*, same field as in *C*, visualized by phase contrast microscopy.

followed by detectable intracellular accumulation within 15 min, which subsequently increased to a plateau at 240 min (Fig. 4*A*). Total uptake of C225-Fab′ containing ILs at 240 min was 1.70 fmol phospholipid/cell (~13,000 ILs/cell), with virtually the entire uptake in the endocytosed fraction. The uptake of ILs was at least 43-fold greater than that of nontargeted liposomes (<300 liposomes/cell). Similarly, in non-EGFR-overexpressing MCF-7 cells, the uptake of ILs was only 450 ILs/cell. EGFR-targeted ILs containing scFv C10 showed comparable internalization kinetics in MDA-MB-468 cells (Fig. 4*B*). Total uptake for scFv C10-containing ILs reached 1.32 fmol phospholipid/cell (~10,000 ILs/cell) in MDA-MB-468 cells.

The kinetics of internalization of C225-Fab′-containing ILs was similar in cells with EGFRvIII expression. Again, binding to NR-6M (EGFRvIII) stable transfectants started within 5 min, followed by detectable intracellular accumulation after only 15 min and increasing to a plateau after 240 min, reaching 1.45 fmol phospholipid/cell (~11,000 ILs/cell; Fig. 4*C*).

Targeted Drug Delivery Studies. In addition to quantitative analysis of IL internalization, studies of IL-mediated drug delivery were performed using doxorubicin-loaded EGFR-targeted ILs in MDA-MB-468 cells. ILs were prepared by insertion of C225-Fab′ into commercial pegylated liposomal doxorubicin (Doxil) at increasing ratios of Fab′ per liposome and under two reaction conditions. Doxorubicin delivery reached a maximum of 600–700 ng/10^6 cells. Estimating a volume of approximately 0.8–1.2 fl for MDA-MB-468 cells, this number corresponds to an intracellular doxorubicin concentration of 1–2 μM (approximately 0.58–1.16 μg/ml). This is substantially greater than the IC_{50} for doxorubicin in this cell line (~0.3 μg/ml; Table 1*A*). In addition, this uptake corresponds to ~31,500 liposomes/cell, which is somewhat greater than the uptake calculated by the HPTS method. Potential explanations for this 2–3-fold difference in calculated uptake include greater stability and/or less leakiness of commercial liposomal doxorubicin, in which drug is encapsulated in gelated form, than laboratory grade liposomal HPTS, and the possibility of efflux of HPTS via organic anion transporters (37). Doxorubicin delivery was proportional to the amount of Fab′ incorporated in the IL, up to a plateau ratio of 30 μg of Fab′/μmol phospholipid (approximately 30–40 MAb fragments/liposome; Fig. 5). These results are consistent with those observed with HER2-targeted ILs in HER2-overexpressing cells, which similarly show increased uptake with higher MAb fragment density on ILs, up to a plateau of 20–30 MAb fragments/IL (4). Doxil alone without MAb fragments showed negligible delivery of doxorubicin under these *in vitro* conditions.

Targeted Cytotoxicity Studies. For cytotoxicity studies, anti-EGFR ILs containing C225-Fab′ were stably loaded with various chemotherapeutic drugs for targeted delivery to EGFR- or EGFRvIII-overexpressing cells. Drug-loaded ILs were either prepared by active ("remote")-loading or passive loading as described in "Materials and Methods." ILs were incubated with various cell lines, and compared with the respective free and nontargeted liposomal drugs to evaluate efficiency and specificity of delivery.

In EGFR-overexpressing MDA-MB-468 breast cancer cells, EGFR-targeted immunoliposomal doxorubicin showed substantial *in vitro* cytotoxicity after treatment for 2 h (IC_{50} = 1.1 μg/ml), which approached that of free doxorubicin (IC_{50} = 0.8 μg/ml; Fig. 6*A*). Thus, EGFR-targeted IL delivery of doxorubicin was almost as efficient as the rapid diffusion of free doxorubicin, a small, amphipathic molecule that readily transverses cell membranes *in vitro*. On the other hand, EGFR-targeted immunoliposomal doxorubicin, derived by conjugation of C225-Fab′ to Doxil, showed 29-fold greater cytotoxicity than Doxil itself (IC_{50} = 32 μg/ml) in MDA-MB-468 cells, indicating that delivery was antibody-dependent (Fig. 6*A*). Notably, similar treatment with C225 for 2 h showed no cytoxicity in this assay, confirming that IL activity was due to targeted drug delivery and not related to potential antiproliferative effects of C225 during this brief incubation time. Furthermore, ILs containing C225-Fab′ but lacking encapsulated drug ("empty ILs") similarly showed no cytotoxicity under these assay conditions.

The specificity of EGFR-targeted IL delivery was additionally evaluated in MCF-7 breast cancer cells lacking EGFR-overexpression. Free doxorubicin again showed extremely efficient cytotoxicity (IC_{50} = 0.4 μg/ml) because of its high *in vitro* potency. However, treatment with

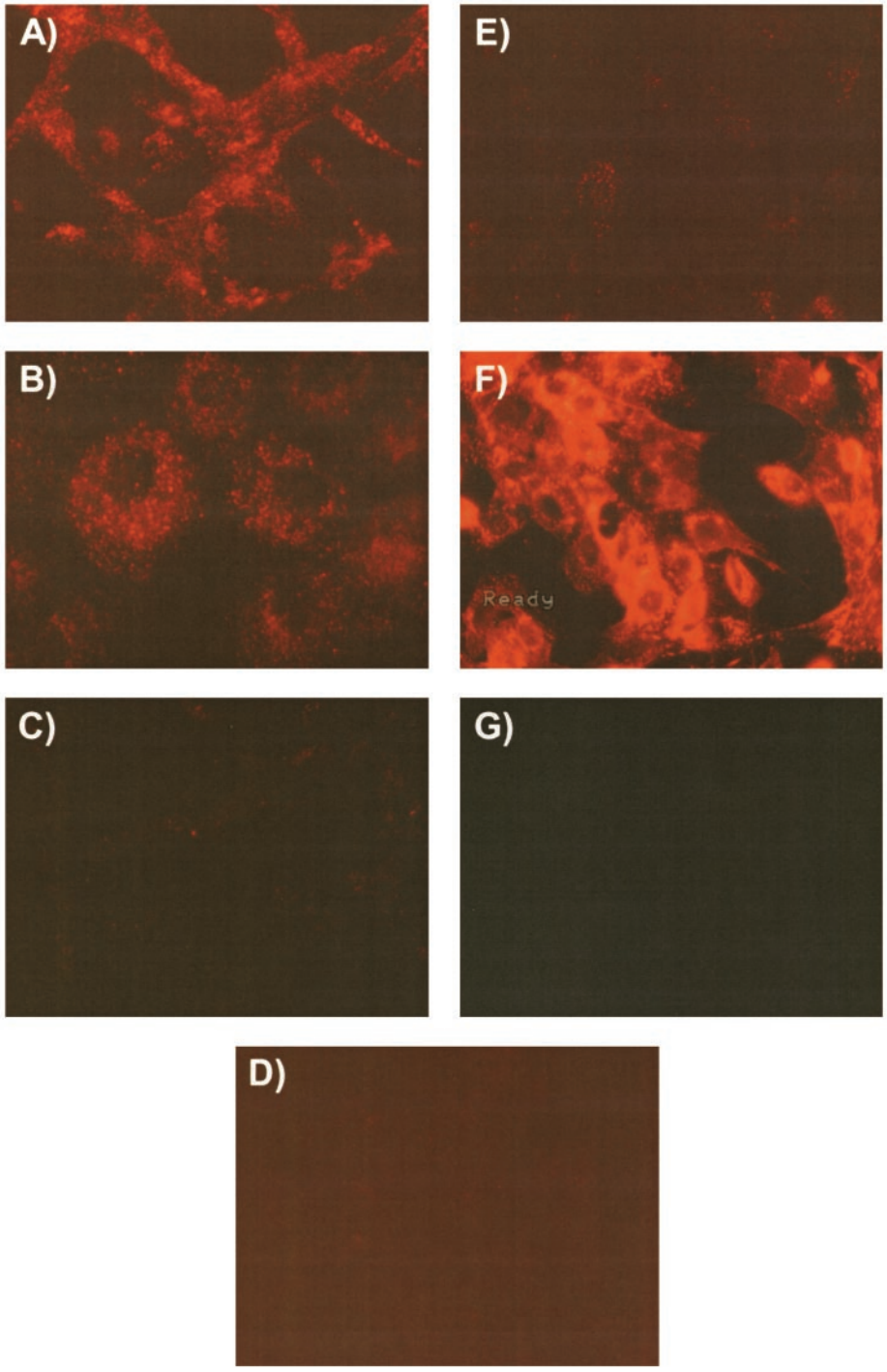

Fig. 3. Internalization of EGFR/EGFRvIII-targeted *versus* irrelevant ILs in various cell lines. Various cell lines were treated with $DiIC_{18}(3)$-DS labeled liposomes containing either anti-EGFR C225-Fab′ or anti-HER2 scFv F5 for 2 h at 37°C. *A*, EGFR-targeted ILs in EGFR-overexpressing U-87 glioma cells. *B*, EGFR-targeted ILs in EGFR-overexpressing A-431 epidermoid cancer cells. *C*, irrelevant (HER2-targeted) ILs in EGFR-overexpressing U-87 glioma cells. *D*, irrelevant (HER2-targeted) ILs in EGFR-overexpressing A-431 epidermoid cancer cells. *E*, EGFR-targeted ILs in non-EGFR-overexpressing MCF-7 breast cancer cells. *F*, EGFR-targeted ILs in NR-6M cells stably transfected with EGFRvIII. *G*, EGFR-targeted ILs in parental NR-6 cells lacking EGFR or EGFRvIII expression.

EGFR-targeted immunoliposomal doxorubicin was >100-fold less efficient than free doxorubicin in these cells and equivalent to Doxil (Fig. 6*B*). Taking together the cytotoxicity results in target (MDA-MB-468) *versus* nontarget (MCF-7) cells, the specificity index (defined as IC_{50} in nontarget cells/IC_{50} in target cells) was 45.5 for EGFR-targeted immunoliposomal doxorubicin, 0.5 for free doxorubicin, and 1.1 for Doxil.

EGFR-targeted ILs were also loaded with vinorelbine (anti-EGFR ILs-VRL) for treatment of MDA-MB-468 and MCF-7 cells. In MDA-MB-468 cells, these constructs were as efficient in delivering vinorelbine as free vinorelbine itself, which has high *in vitro* potency (IC_{50} = 0.3 μg/ml; Fig. 6*C*). EGFR-targeted immunoliposomal vinorelbine was 40-fold more cytotoxic (IC_{50} = 0.3 μg/ml) than nontargeted liposomal vinorelbine (IC_{50} = 12 μg/ml), prepared identically except for omission of MAb fragments. In contrast, in non-EGFR-overexpressing MCF-7 cells, EGFR-targeted ILs showed much reduced cytotoxicity *versus* free vinorelbine, and were comparable with nontargeted liposomal vinorelbine (Fig. 6*D*). For vinorelbine, the specificity index was >90 for EGFR-targeted immunoliposomal vinorelbine, 0.7 for free vinorelbine, and 2.3 for nontargeted liposomal vinorelbine.

EGFR-targeted ILs were also used to deliver methotrexate (anti-EGFR ILs-MTX). Unlike doxorubicin and vinorelbine, methotrexate does not diffuse across the cell membrane, and requires active trans-

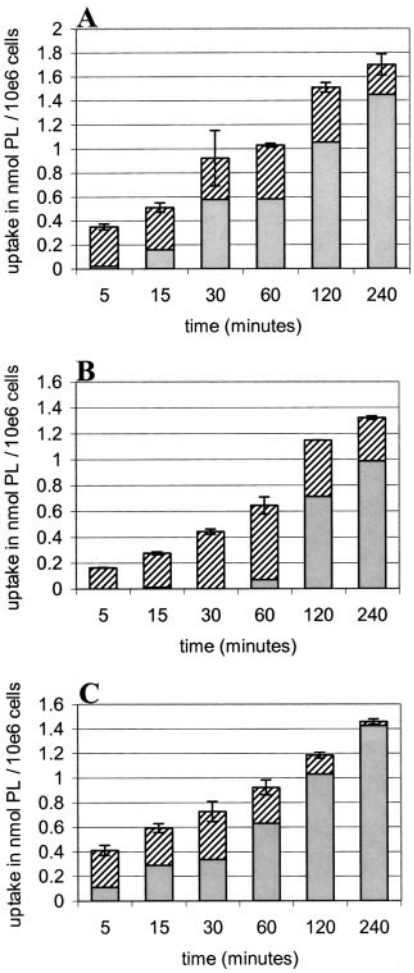

Fig. 4. Kinetics of uptake of EGFR-targeted ILs in cell lines overexpressing EGFR or EGFRvIII. ILs containing the indicated MAb fragments were loaded with the pH-sensitive fluorophore HPTS and incubated with either EGFR-overexpressing MDA-MB-468 cells or EGFRvIII-expressing NR-6M cells for various time points at 37°C. Fluorimetry was then performed to quantify surface-bound ILs at neutral pH (not internalized; ▨) *versus* endocytosed ILs at acidic pH (internalized; ▧). *A*, ILs containing C225-Fab′; MDA-MB-468 cell line. *B*, ILs containing scFv-C10; MDA-MB-468 cell line. *C*, ILs containing C225-Fab′; NR6-M cell line. Data indicate mean; *bars*, ±SD.

port via the folate carrier system for internalization (38). Consistent with this, EGFR-targeted immunoliposomal methotrexate yielded 3-fold more efficient cytotoxicity than free methotrexate in EGFR-overexpressing MDA-MB-468 cells, indicating that IL delivery was superior to cellular active transport mechanisms under these conditions. Anti-EGFR ILs-MTX was also >20-fold more cytotoxic than nontargeted liposomal methotrexate in these cells (Fig. 6*E*). In contrast, in MCF-7 cells, anti-EGFR ILs-MTX and nontargeted liposomal methotrexate showed equivalent minimal cytotoxicity (Fig. 6*F*). For methotrexate, the specificity index was 16.3 for EGFR-targeted immunoliposomal methotrexate, 5 for free methotrexate, and 0.8 for nontargeted liposomal methotrexate.

IL-mediated cytotoxicity with various drugs was also evaluated in EGFRvIII-expressing NR-6M transfectants and compared with results with NR-6 parental cells. These studies permitted an assessment of IL targeting of EGFRvIII alone, because these cells lack wild-type EGFR, and also provided isogenically matched target (NR-6M) *versus* control (NR-6) cells designed to differ only in EGFRvIII expression. ILs containing C225-Fab′ were used to deliver encapsulated doxorubicin, vinorelbine, and methotrexate as described. In each case, the IL drug was markedly more cytotoxic than the corresponding nontargeted liposomal drug in target cells (NR-6M), while equivalent against non-EGFRvIII-expressing cells (NR-6). The specificity indices for doxorubicin were 29.3/1.6/1.1 (EGFR-targeted ILs-drug/free drug/nontargeted liposomes-drug), for vinorelbine 11/2.4/0.9 and for methotrexate 18.7/0.9/1, respectively. A summary of the cytotoxicity studies is shown in Table 1.

Plasma Stability of Anti-EGFR ILs. The EGFR-targeted ILs in this report were derived from an overall design first implemented with HER2-targeted ILs, which were determined previously to be highly stable and with long circulation times *in vivo* (27). To confirm stability and resistance to drug leakage, EGFR-targeted immunoliposomal doxorubicin was prepared by conjugation of C225-Fab′ fragments (approximately 30–40/liposome) to pegylated liposomal doxorubicin (Doxil), and then subjected to prolonged incubation with 50% human plasma. In this model, ILs were stable at temperatures of 37°C for a minimum of 8 h when continuously dialyzed against 50% human plasma. The release of 6% encapsulated doxorubicin after 8 h was comparable with the results observed with commercial pegylated liposomal doxorubicin alone.

DISCUSSION

We have described previously the development of ILs targeting HER2 (3), the relationship of design features to binding and internal-

Table 1 *Immunoliposome delivery of various drugs in NR-6M and NR-6 cells*

Cytotoxic effects (IC_{50}, μg/ml) of various compounds in EGFRvIII-expressing NR-6M cell line compared with NR-6 cell line lacking the mutant EGFvIII receptor (control): free drug, liposomal encapsulated drug (nt, nontargeted), and EGFR-targeted immunoliposomes (ILs drugs).

	NR-6M cells (IC_{50})	Anti-EGFR ILs *vs.* liposome	NR-6 cells (IC_{50})	Anti-EGFR ILs *vs.* liposome
Doxorubicin (Dox)				
-Free Dox	0.22		0.35	
-nt Doxil	40	27-fold	42	None
-ILs Doxil	1.5		44	
Vinorelbine (VRL)				
-Free VRL	0.23		0.55	
-nt VRL	4.2	13-fold	3.9	None
-ILs VRL	0.31		3.4	
Methotrexate (MTX)				
-Free MTX	32		30	
-nt MTX	>54	>23-fold	54	None
-ILs MTX	2.3		43	

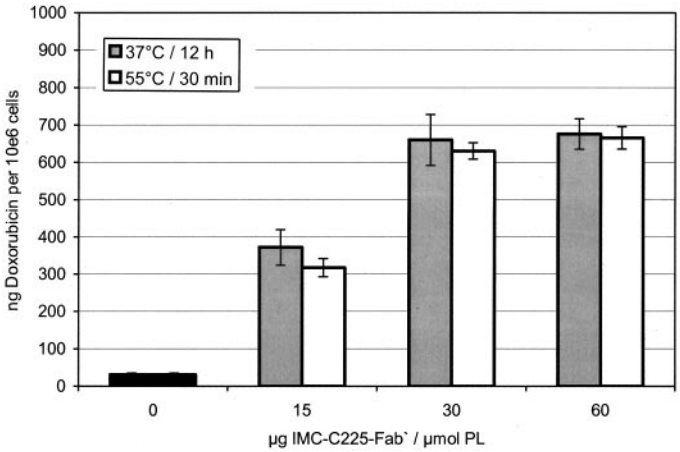

Fig. 5. IL-mediated delivery of doxorubicin *in vitro*. Various amounts of C225-Fab′ (15, 30, and 60 μg/μmol phospholipid) were inserted into pegylated liposomal doxorubicin (Doxil) for conversion of liposomes into ILs. Doxil alone was included as a negative control (liposomal dox). MAb fragment insertion was performed under two different conditions: 37°C for 12 h (▤) or 55°C for 30 min (□). Liposomes or ILs were incubated with MDA-MB-468 breast cancer cells for 3 h at 37°C, washed, detached, and fluorimetry analyzed for quantitation of cellular doxorubicin uptake. Data indicate mean; *bars*, ±SD.

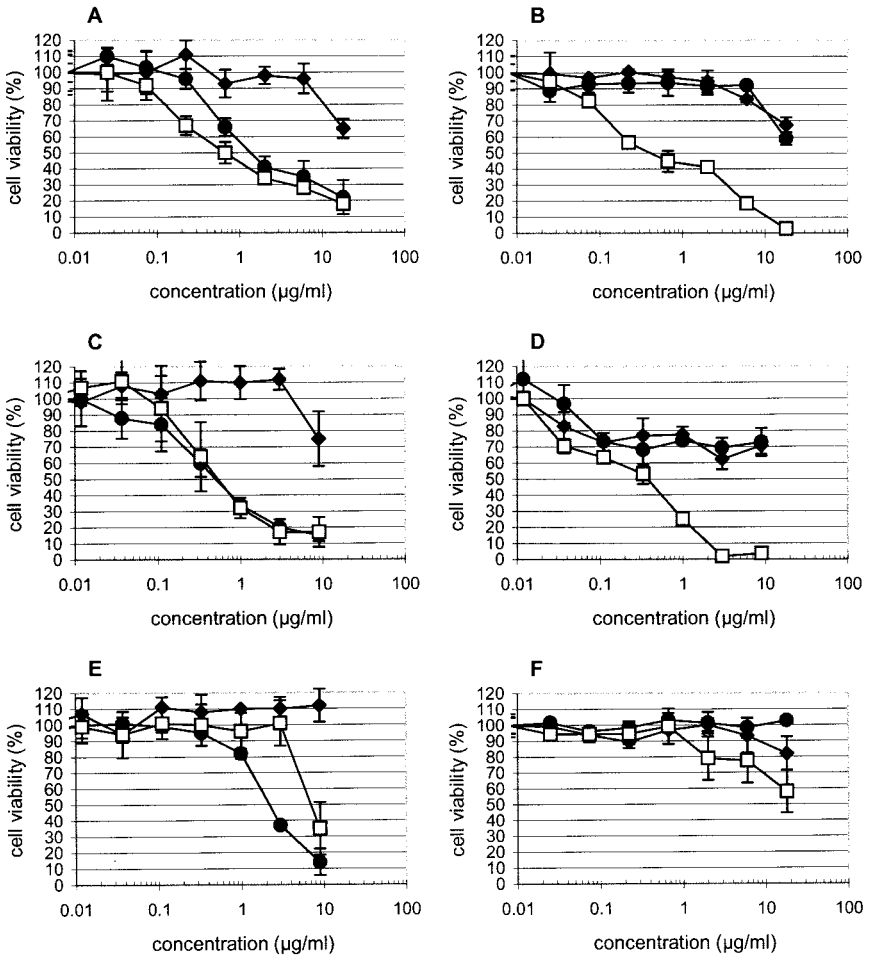

Fig. 6. *In vitro* cytotoxicity of EGFR-targeted ILs containing various drugs. C225-Fab′ were covalently conjugated to stabilized liposomes by incorporation of MAb-linker micelles into preformed liposomes containing various cytotoxic drugs. Cells in culture were treated for 2 h with EGFR-targeted ILs (●), nontargeted liposomes (♦), or free drug alone (□). Cells were incubated for another 3 days and counted using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Doxorubicin: *A*, MDA-MB-468; *B*, MCF-7. Vinorelbine: *C*, MDA-MB-468; *D*, MCF-7. Methotrexate: *E*, MDA-MB-468; *F*, MCF-7. Data indicate mean; *bars*, ±SD.

ization properties (4), and their *in vivo* optimization and therapeutic efficacy in HER2-overexpressing tumor models (27). Because of the modular design of these IL constructs, which allows conjugation of various MAb fragments to stabilized and long circulating liposomal carriers, this approach can in principle be extended to other target antigens. In particular, ILs targeting RTKs can potentially trigger endocytosis after binding, resulting in IL internalization and intracellular drug release.

In this report, ILs were constructed with anti-EGFR MAb fragments to deliver drugs selectively to tumor cells with overexpression of EGFR or the truncated mutant EGFRvIII. MAb fragments included C225-Fab′, which provides targeting of both EGFR and EGFRvIII, as well as scFv C10, a novel anti-EGFR scFv derived from a phage antibody library. EGFR-targeted ILs showed specific binding and internalization in EGFR-overexpressing cell lines MDA-MB-468 (breast cancer), U-87 (glioblastoma), and A-431 (epidermoid cancer). Total cellular uptake of EGFR-targeted ILs in these cells was up to 3 orders of magnitude higher than that detected in non-EGFR-overexpressing cells, indicating substantial specificity for target *versus* nontarget cells. ILs containing C225-Fab′ could also target EGFRvIII-expressing cells, as shown by binding and internalization in EGFRvIII-transfected NR-6M cells but not in the nontransfected isogenic control cell line NR-6. Binding and internalization resulted in highly efficient intracellular drug delivery, because of the combined effects of an accumulation of 10^4 ILs/cell with a carrying capacity of 10^4 drugs/IL. The delivery system was used in conjunction with three chemotherapy drugs representing different classes and mechanisms of action. In each case, the IL drug (doxorubicin, vinorelbine, or methotrexate) was markedly more cytotoxic than the corresponding nontargeted liposomal drug in target cells, while equivalent against non-EGFR-overexpressing cell lines.

An important feature of the IL approach is a threshold effect for target receptor expression. EGFR-targeted ILs internalized in EGFR-overexpressing cells, including those with 10^5-10^7 EGFR/cell, but did not detectably accumulate in low EGFR-expressing cells such as MCF-7 ($\sim 10^4$ EGFR/cell). This suggests that significant drug delivery is unlikely to occur in normal tissues, including epithelial cells expressing up to 10^3-10^4 receptors/cell. These properties of EGFR-targeted ILs are consistent with previous results obtained with HER2-targeted ILs, which indicated a similar threshold for HER2-overexpression (3, 4, 27).

In addition to targeting tumors that overexpress wild-type EGFR, IL agents may be useful against tumors that express truncated mutant receptor EGFRvIII. As a mutant allele, EGFRvIII appears to be a true tumor-specific antigen that is not expressed in normal tissues. Although C225 does not have therapeutic value as a ligand blocker against the ligand-independent EGFRvIII, ILs containing C225-Fab′ can target tumors with overexpression of either EGFR or EGFRvIII. MAbs that specifically recognize the new epitope created by the truncation event in EGFRvIII have also been derived (39). In principle, ILs can be constructed from such MAbs for tumor-specific drug delivery. In addition to existing anti-EGFRvIII MAbs, human scFv can be rapidly selected from phage antibody libraries, which are ideally suited for generation of targeting ligands for IL agents (40). The use of scFv C10 in these studies exemplifies this strategy; this relatively low affinity scFv lacking intrinsic biological activity,

whereas not a therapeutic candidate as a naked MAb, can be very powerful when targeting ILs. Furthermore, the development of methods to select for scFv that internalize in target cells can be rapidly exploited in conjunction with IL technology (36).

In conclusion, these studies describe a molecularly targeted drug delivery system for highly efficient and selective delivery of anticancer drugs in tumor cells overexpressing EGFR or EGFRvIII. This approach may be useful for the delivery of various drugs for enhanced therapeutic index against cancer.

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