Targeting Tumor-Associated Endothelial Cells: Anti-VEGFR2 Immunoliposomes Mediate Tumor Vessel Disruption and Inhibit Tumor Growth

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Abstract

**Purpose:** Angiogenesis is a key process in tumor progression. By binding VEGF, VEGF receptor-2 (VEGFR2) is a main signaling transducer in tumor-associated angiogenesis. Accordingly, therapeutic approaches against the VEGF/VEGFR2 signaling axis have been designed. However, an efficient and specific chemotherapeutic targeting of tumor-associated endothelial cells has not yet been achieved.

**Experimental Design:** We have employed anti-VEGFR2 antibodies covalently linked to pegylated liposomal doxorubicin (PLD) to specifically ablate tumor-associated endothelial cells in the Rip1Tag2 mouse model of insulinoma, in the MMTV-PyMT mouse model of breast cancer, and in the HT-29 human colon cancer xenograft transplantation model.

**Results:** In each model, anti-VEGFR2–targeted immunoliposomes (ILs) loaded with doxorubicin (anti-VEGFR2-ILs-dox) were superior in therapeutic efficacy to empty liposomes, empty anti-VEGFR2-ILs, antibodies alone, and PLD. Efficacy was similar to that of the oral VEGFR1, -2, and -3 inhibitor PTK787. Detailed histopathologic and molecular analysis revealed a strong antiangiogenic effect of anti-VEGFR2-ILs-dox, and the observed antiangiogenic therapy was significantly more efficient in reducing tumor burden in well-vascularized transgenic mouse models as compared with the less-vascularized xenograft model.

**Conclusions:** Anti-VEGFR2 ILs provide a highly efficient approach to selectively deplete VEGFR2-expressing tumor vasculature. They offer a novel and promising anticancer strategy.

Introduction

The distribution of most current drugs in the body is determined by their hydrophilic or lipophilic nature. This constitutes a major obstacle for successful and safe therapies, because drug delivery is not limited to the diseased organ but encompasses the whole body. Nanoparticles, such as liposomes, have been generated for prolonged circulation and redirection of therapeutic drugs. They yield superior accumulation in tumors via a process referred to as the "enhanced permeability and retention" effect (1).

Huge efforts were made to identify specific cell surface markers on tumor cells and to direct anticancer agents selectively to malignant cells. Immunoliposomes (IL), in which monoclonal antibody (mAb) fragments are conjugated to the lipid membrane, represent a next generation of molecularly targeted drug delivery systems. By combining the targeting properties of mAbs with the pharmacokinetic and drug delivery advantages of liposomes, ILs offer the promise of selective drug delivery to tumor cells (2). Using this modular approach, we have developed ILs that successfully target epidermal growth factor receptor (EGFR) on cancer cells (3, 4).

Endothelial cells lining newly formed vessels in a growing tumor express a different set of genes in comparison with those lining physiologic vessels, which translates into a differential expression of surface markers (5). One of the antigens which are selectively upregulated on activated tumor-associated endothelial cells is VEGFR2 [reviewed by Shibuya and colleagues (6)]. This upregulation probably is the consequence of a paracrine loop between tumor cells producing VEGF-A and endothelial cells (7). Not surprisingly, the activation of the VEGF-A/VEGFR2 axis and the differential expression of VEGFR2 on tumor-associated endothelial cells make it a prime target for antiangiogenic treatments in malignant tumors [reviewed by Folkman and colleagues (8)]. The first antiangiogenic drug introduced into the clinic was bevacizumab, a mAb that sequesters VEGF-A and thereby blocks VEGF signaling via VEGFR2. However, the clinical benefit of bevacizumab in combination with chemotherapy for various types of solid tumors has been disappointing. Targeting the tumor vasculature rather than the tumor cells might be a more promising approach for inactivating tumor blood vessels, since antiangiogenic drugs are less toxic and are potentially able to prevent tumor spread by blocking the vasculature of micrometastatic disease (9).

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VEGF-A, the major VEGFR2 ligand. Clinical studies have proven the efficacy of bevacizumab in human colorectal, breast, kidney, and central nervous system cancers (9–12). Other experimental approaches designed to repress tumor-driven angiogenesis include small molecule inhibitors or mAbs suppressing the activity of all or specific VEGF receptors, thus blocking various aspects of VEGF/VEGFR signaling.

In this study, we specifically targeted tumor-associated vasculature by anti-VEGFR2 ILs loaded with doxorubicine, a chemotherapeutic drug. The aim of this study was to assess the feasibility and efficacy of selective drug delivery to activated, VEGFR2-expressing endothelial cells in tumor-associated vessels. Here, we describe the therapeutic potential of anti-VEGFR2 ILs in a series of transgenic and xenograft tumor models.

Materials and Methods

Materials

Reagents for liposome preparation included the following: DiIC18(3)-DS (Molecular Probes); DSPC, cholesterol, and mPEG-DSPPE (Avanti Polar Lipids); Mal-PEG(2000/3400)-DSPPE (Nektar); organic solvents, and other chemicals of reagent purity (Sigma-Aldrich AG). Doxorubicin (C14, C22) and mPEG-DSPE (Avanti Polar Lipids); Mal-PEG-DSPPE (Nektar); organic solvents, and other chemicals of reagent purity (Sigma-Aldrich AG). Doxorubicin (dox; Pfizer AG) and pegylated liposomal doxorubicin (PLD, Caelyx; Essex Chemie AG) were obtained commercially. mAb DC101 was obtained from ImClone Systems Inc.

Liposome preparation

Liposomes were prepared by a lipid film hydration–extrusion method using repeated freeze-thawing to hydrate the lipid films (13, 14). Liposomes were composed of DSPC and cholesterol (molar ratio 3:2) with mPEG-DSPPE (5 mol% of phospholipid). The phospholipid concentration in the liposome solution was measured utilizing a standard phosphate assay (15).

For uptake studies, liposomes were labeled with 0.1 to 0.3 mol% DiIC18(3)-DS, a fluorescent lipid that can be stably incorporated into liposomal membranes (16, 17).

For biodistribution studies, Rip1Tag2 mice were treated with liposomes and anti-VEGFR2-ILs, which were labeled with [3H]-cholesteryl hexadecyl ether, injected via the tail vein at a dose of 31.25 μmol phospholipid/kg mouse (cohorts of 4–6 mice per time point and treatment). At 24 or 48 hours, mice were euthanized and tissues were collected after perfusion with PBS. Tissue samples were homogenized and [3H]-cholesteryl hexadecyl concentration were determined as described (4). The final distribution was expressed as % of injected dose (ID)/g tissue and as % ID for the concentration in the blood circulation. For encapsulation of doxorubicin, the remote loading method with ammonium sulfate was used (18, 19).

Preparation of mAb fragments and ILs

DC101 is a rat mAb against the extracellular domain of mouse VEGFR2 (20). For preparation of DC101-Fab’, intact DC101 mAb was cleaved and reduced as previously described (3).

For ILs, Fab’ were covalently conjugated to maleimide groups at the termini of PEG-DSPPE chains (Mal-PEG-DSPPE; ref. 21). mAb fragment conjugates (Fab’-Mal-PEG-DSPPE) were incorporated into liposomes by coincubation at 55°C for 30 minutes at a protein/liposome ratio of 60 μg Fab’/μmol PL (22, 23). Unincorporated conjugates and free drug were separated from ILs by Sepharose CL-4B gel filtration. The incorporation efficiency of conjugated mAb fragments was estimated by SDS-PAGE (3).

Cell lines

MS-1 mouse endothelial, HT-29 human colon cancer and MDA-MB-468 human breast cancer cell lines were obtained from the American Type Culture Collection. MS-1 and MDA-MB-468 cells were maintained in “Improved MEM Zinc Option” medium (Invitrogen AG) and HT-29 in RPMI-1640 (Sigma-Aldrich AG) supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Specific binding of anti-VEGFR2-ILs in vitro

For flow cytometry studies, 150,000 MS-1 or MDA-MB-468 cells were coincubated in 12-well plates with saline (control), untargeted liposomes, Dil-ILs, or VEGFR2-targeted ILs (anti-VEGFR2-Dil-ILs or anti-EGFR-Dil-ILs as a control), labeled with DiIC18(3)-DS for 2 hours at 37°C, washed extensively with PBS, followed by detaching and storing on ice until flow cytometry. DC101 anti-VEGFR2 or C225 anti-EGFR antibodies (ImClone Systems, Inc.) were inserted into the liposomes as described above.

Specific binding of anti-VEGFR2-ILs in vivo

For uptake studies in vivo, 12 weeks old Rip1Tag2 mice were injected intravenously with Dil-ILs or anti-VEGFR2-Dil-ILs. Thirty-six hours after injection, mice were sacrificed and a single-cell suspension of pancreatic tumors was prepared by Dispase digestion. For subsequent fluorescence-activated cell sorting (FACS) analysis cells were
stained with PerCp-CD45 (Biolegend) and APC-CD31 (Biolegend) antibodies.

Reverse transcriptase PCR
A single-cell suspension of pancreatic tumors of 12-week-old Rip1Tag2 mice was prepared by Dispase digestion. For isolation of GLP-1Rβ-tumor cells and tumor-derived CD31+ blood endothelial cells by FACS, cells were stained with fluorescein isothiocyanate (FITC) labeled exendin-4 (Phoenix Pharmaceuticals, Inc.; ref. 24), and with APC-CD31 (Biolegend). Total RNA was extracted from isolated cells, cDNA prepared, and the expression of the mRNA evaluated by PCR.

Primers semiquantitative PCR
mActin: ACACGTGCCCATCTAGGAGG and CATGATGGCACAAGTCTCC
mCD31: GGAGTCAGAACCCATCAGGA and CCGCTGCCATTGAGTGAT
mVEGFR1: CCGCAGGAAATACACACAGC and CGGCTGCCTTATAGATGC
mVEGFR2: GCGGTGGTGACAGTATCTT and CAAAGCCAGTCCAGGTCCC
mVEGFR3: GCTGTTGGTTGGAGAGAAGC and CGACGAGATGGAAATCACA
mCD31: GGAGTCAGAACCCATCAGGA and CCGCTGCCATTGAGTGAT
mVEGFR1: CCGCAGGAAATACACACAGC and CGGCTGCCTTATAGATGC
mVEGFR2: GCGGTGGTGACAGTATCTT and CAAAGCCAGTCCAGGTCCC
mVEGFR3: GCTGTTGGTTGGAGAGAAGC and CGACGAGATGGAAATCACA

Primers quantitative reverse transcriptase PCR:
mCD31: CGGTGTTCAGCGAGATCC and CCGCTGCCATTGAGTGAT
mVEGFR1: CCGCAGGAAATACACACAGC and CGGCTGCCTTATAGATGC
mVEGFR2: GCGGTGGTGACAGTATCTT and CAAAGCCAGTCCAGGTCCC
mVEGFR3: GCTGTTGGTTGGAGAGAAGC and CGACGAGATGGAAATCACA

Mice
Phenotypic and genotypic analyses of Rip1Tag2 and MMTV-PyMT transgenic mice in a C57Bl/6J and FVB/N background have been described previously (25, 26). Wild-type C57Bl/6J mice were used for organ toxicity studies. Athymic nude mice were hosts for the HT-29 xenograft experiments. Animals were maintained and treated in compliance with the guidelines of the Swiss Federal and the Cantonal Veterinary Office Basel-Stadt (permissions No. 1878, 1907, 1908, and 2030).

For therapeutic studies, mice were randomized according to their gender. Rip1Tag2 mice were 10 and PyMT mice 8 weeks old at the onset of therapy. Mice were injected into the tail vein with 5 mg dox/kg anti-VEGFR2-ILs-dox, 5 mg dox/kg PLD (Caelyx), or the same quantity of empty liposomes or empty ILs, respectively, intravenously at days 1, 4, 8, and 11. Additional cohorts of Rip1Tag2 mice were injected with free DC101 alone (50 μg/mouse) or with a combination of PLD and free DC101 at the same concentration as present in the anti-VEGFR2-ILs-dox cohort (membrane-bound DC101) or with 100 mg/kg bodyweight PTK787 dissolved in polyethylene glycol 300 (PEG300; Sigma) by daily oral administration for 2 weeks. Tumor size was assessed after sacrifice (day 15) using a caliper or a grid (for small tumors). The mass was calculated assuming an ellipsoid shape of the tumor.

For xenograft experiments, 1 × 10⁶ HT-29 colon cancer cells in 100-μL cell culture medium were injected subcutaneously in the flanks of athymic mice. Once tumor size reached a volume of 150 to 250 mm³, treatment was started following the protocol described for the transgenic mouse models. Tumor size was assessed twice weekly for 6 weeks.

Histologic and toxicity analysis
Tumor tissue analysis was done as described before (24, 27). Blood and lymphatic vessels were visualized using anti-CD31 or anti-Lyve1 primary antibodies, respectively (Sigma). For the analysis of metastasis in Rip1Tag2 mice lung, liver, kidney, and spleen were isolated and fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Histologic analysis was done on hematoxylin and eosin (H&E)-stained paraffin sections. The entire organs were cut into 5-μm sections and every fifth section was stained with H&E for microscopic examination.

Frozen sections were incubated with rat anti-CD31, anti-NG2, or anti-smo (Sigma) as primary antibody. For VEGFR2 staining, goat anti-VEGFR2 mAb (R&D Systems Europe Ltd.) was used. Fab' fragments of DC101 were visualized with monoclonal anti-rat Fab'-FITC (Sigma).

Anti-cleaved caspase 3 (Ab-2; Calbiochem Merck), a rabbit polyclonal antibody, was utilized for apoptotic cell labeling.

Proliferation and apoptosis assay
For bromodeoxyuridine (BrdU) staining (proliferation assay), BrdU was injected at 100 μg BrdU per gram body weight intraperitoneally 2 hours before sacrifice (24). For terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL; apoptosis) assays, slides were incubated for 1 hour in TUNEL reagent (Roche) and apoptotic cells were visualized with the AEC-kit (Vector Laboratories).

Microscopy
Immunohistochemical stainings were analyzed on an AxioVert microscope (Zeiss). Immunofluorescence stainings were analyzed on a LSM510 META confocal microscope (Zeiss). Histologic grading of islet and mammary tumors follows the WHO International Classification of Rodent Tumors, Part II, The Mouse.

Statistical analysis
Statistical analysis was done using the GraphPad Prism software (GraphPad Software, Inc.). Tumor volume and mass were compared using nonparametric statistical analysis (Kruskal–Wallis test) with Dunn’s posttest. Proliferation, apoptosis, and blood and lymph vessel density were...
analyzed by parametric testing (1-way ANOVA and Newman–Keuls posttest).

Results

Target expression, cellular uptake, and biodistribution of VEGFR2-targeted ILs

ILs against activated endothelial cells were coated with Fab’ derived from DC101, a monoclonal rat antibody binding the extracellular domain of mouse VEGFR2 (28). Binding and uptake of VEGFR2-targeted ILs were evaluated by flow cytometry in MS-1 cells, an endothelial cell line expressing high levels of VEGFR2 (29) and in MDA-MB-231 human breast cancer cells, which lack VEGFR2 expression but express high levels of EGFR instead. In this assay, liposomes were fluorescently labeled with DiIC18(3)-DS, with and without conjugated mAb fragments, and incubated for 2 hours with MS-1 or MDA-MB-468 cells. ILs containing DC101-Fab’ showed a one order-of-magnitude higher accumulation in MS-1 cells than did control liposomes (Fig. 1A). In contrast, MDA-MB-468 cells had a 2-log higher uptake of anti-EGFR-ILs compared with anti-VEGFR2-ILs (Supplementary Fig. S1A).

We next asked whether this specific uptake in VEGFR2-expressing cells could also be observed in vivo. Staining of tissue sections of Rip1Tag2 mice treated with anti-
VEGFR2-ILs using a FITC-labeled anti-rat-Fab’ antibody showed specific binding of the targeted ILs to VEGFR2-positive cells (Supplementary Fig. S1B). Further confirmation of in vivo binding and uptake resulted from FACS sorting of explanted tumor-associated endothelial cells from Rip1Tag2 mice (Supplementary Fig. S1C). CD31-expressing vascular cells treated with anti-VEGFR2-ILs loaded with DiI had a 3-fold increase in DiI positivity compared with treatment with nontargeted DiI-positive liposomes.

We further evaluated the specificity of VEGFR2 expression on tumor-associated endothelial cells in the Rip1Tag2 mouse model. There, tumor cells can be sorted on the basis of their selective expression of the glucagon-like 1 receptor (GLP-1R; ref. 24). Quantitative PCR of GLP-1R or CD31 sorted primary cells from tumors of untreated Rip1Tag2 mice showed no expression of VEGFR2 in tumor cells, whereas VEGFR2 was detectable in endothelial cells (Fig. 1B and Supplementary Fig. S2A). Containing of sections of the pancreas of Rip1Tag2 mice confirmed the presence of VEGFR2 in intratumoral endothelial cells (Supplementary Fig. S2B).

Biodistribution studies of nontargeted liposomes versus anti-VEGFR2 ILs were conducted in the Rip1Tag2 mouse model. Tissues were assayed as described before (4) at 24 or 48 hours. Comparable with previously published preclinical models (2, 4), analysis of tissue samples showed a nonsignificant trend to a higher sequestration of ILs in parenchymal organs (lung, liver, and spleen) but not in the tumors. Therefore, a difference in tumor targeting efficiency is rather due to an enhanced and specific uptake of ILs than to distribution (Supplementary Fig. S3).

**Effect of anti-VEGFR2 ILs on tumor burden**

To investigate the antitumor activity of anti-VEGFR2 ILs, we treated 3 different mouse models of cancer: (i) Rip1Tag2 mice express SV40 large T antigen under the control of the insulin promoter and develop pancreatic β-cell tumors; (ii) MMTV-PyMT mice express polyoma middle T antigen under the control of the MMTV promoter and develop breast cancer similar to human ductal carcinoma; and (iii) athymic nude mice subcutaneously xenografted with HT-29 human colon cancer cells. Cohorts of Rip1Tag2 (n = 6 each), MMTV-PyMT (n = 9 each), and nude mice xenografted with HT-29 colon cancer cells (n = 8 each, 1 × 10⁶ cells per flank) were injected with 5 mg doxorubicin/kg anti-VEGFR2-ILs intravenously at days 1, 4, 8, and 11. Control groups were treated by the same time schedule as the interventional group with nontargeted empty liposomes, targeted empty ILs, and nontargeted, doxorubicine-loaded liposomes (PLD, 5 mg doxorubicin/kg). In the experiment with Rip1Tag2 mice, we also analyzed one group treated with DC101 antibody alone, one treated with a combination of PLD and the free DC101 antibody (using the same concentration of the antibody as in the immunoliposomal group), and one treated with the oral VEGFR1, 2, and 3 inhibitors.

![Figure 2. Effect of doxorubicin-loaded anti-VEGFR2 ILs on tumor growth. Treatment of Rip1Tag2 and MMTV-PyMT transgenic mice and HT-29 xenografted mice with anti-VEGFR2-ILs-dox effectively inhibits tumor growth. A, tumor volumes in the Rip1Tag2 tumor mouse model; B, the MMTV-PyMT breast cancer mouse model; C, the HT-29 xenograft model. The tumor volumes in panels A to C were assessed after biweekly injections for a period of 2 weeks. The P value was calculated using the Kruskal–Wallis test. D, kinetics of tumor growth in the HT-29 xenograft model. Tumor growth arrest is observed first in the anti-VEGFR2-ILs-dox group and later in the PLD group. ns, not significant.](image-url)
inhibitor PTK787. Rip1Tag2 and MMTV-PyMT mice were sacrificed at day 15 and the tumor volume was determined. The size of the xenografted tumors in nude mice was determined twice weekly for 6 weeks.

In the Rip1Tag2 model, tumor burden in the experimental group was significantly reduced compared with all control groups, except the treatment with PTK787 (Dunn’s test, \(P < 0.05\), Fig. 2A). The median tumor volumes in the experimental group were 3.6 (0.9–4.6) mm\(^3\), as compared with 21.8 (14.4–37.5) mm\(^3\) in the PLD group, 36.8 (23.5–50.4) mm\(^3\) in the nontargeted empty liposome group, 39.8 (31.9–84.6) mm\(^3\) in the targeted empty IL group, 28.0 (21.7–45.4) mm\(^3\) in the group with the antibody alone, 11.3 (11.3–37.5) in the PLD plus DC101 group, and 6.3 (6.2–7.8) mm\(^3\) in the group treated with PTK787.

In the MMTV-PyMT mouse, the median tumor volumes in the experimental group were 162 (99–233.5) mm\(^3\), as compared with 588 (479–769) mm\(^3\) in the PLD group, 3,624 (2,852–4,435) mm\(^3\) in the empty liposome group, 3,624 (2,852–4,435) mm\(^3\) in the targeted empty IL group, 28.0 (21.7–45.4) mm\(^3\) in the group with the antibody alone, 11.3 (11.3–37.5) in the PLD plus DC101 group, and 6.3 (6.2–7.8) mm\(^3\) in the group treated with PTK787.

Figure 3. Changes in tumor cell proliferation and apoptosis and in blood microvessel density in the Rip1Tag2 (top row), the MMTV-PyMT (middle row), and the HT-29 cancer model (bottom row). There is a trend for reduced proliferation in the anti-VEGFR2-ILs-dox group compared with the PLD group. Apoptosis is significantly increased in the anti-VEGFR2-ILs-dox group compared with all other treatment protocols. Blood microvessel density is significantly and specifically reduced in the anti-VEGFR2-ILs-dox cohorts in all 3 models. Bars = mean ± SD, \(P\) values were calculated using 1-way ANOVA testing with Newman-Keuls posttest. ns, not significant.
and 3,945 (3,539–4,677) mm³ in the targeted empty IL group. Tumor volumes in the anti-VEGFR2-ILs-dox group were significantly smaller than in all included control groups (P < 0.05, Dunn’s test, Fig. 2B; values ¼ median and interquartile range).

In nude mice bearing xenografted HT-29 colon carcinoma cells, tumor growth was effectively inhibited in both the groups injected with PLD and with anti-VEGFR2-ILs-dox (Fig. 2C). However, the onset of the growth inhibition occurred earlier in the mice treated with targeted doxorubicin than in mice injected with PLD. In a multivariate rank analysis, the liposomal and anti-VEGFR2 immunoliposomal groups were not significantly different from one another (P > 0.05; Fig. 2C). The two groups with empty (immuno-) liposomes had a significantly higher tumor load than the groups with doxorubicin-containing (immuno-) liposomes (P < 0.001).

Analysis of the tumors by H&E staining of histologic sections revealed smaller tumors and occasional hemorrhagic necrosis in tumors treated with anti-VEGFR2-ILs (Supplementary Fig. S4). Quantification of nuclear atypia in tumors indicated that atypia (anisokaryosis, hyperchromasia, or nuclear enlargement) was significantly more frequent in the group treated with PLD (median: 3% of the tumor cells) than in the anti-VEGFR2-ILs (median 1.5%, P < 0.05 ANOVA testing) or in the empty (immuno-) liposomes groups (median 0.5% of the cells, P < 0.05, ANOVA testing, data not shown). Whole organ examination of lung, liver, kidney, and spleen in Rip1Tag2, PyMT, and xenografted athymic nude mice of all cohorts did not reveal metastases.

**Tumor cell proliferation, apoptosis, and blood microvessel density**

In each experimental system, we analyzed tumor cell proliferation and apoptosis by BrdU and TUNEL assay, respectively (Fig. 3).

In both the Rip1Tag2 and the PyMT model, tumor cell proliferation was significantly reduced if compared with targeted or untargeted empty ILS, but there was no significant reduction in the anti-VEGFR2-ILs as compared with all control groups (P > 0.05, Newman–Keuls test). In contrast, apoptosis was significantly increased in mice treated with anti-VEGFR2-ILs as compared with all control groups (P < 0.05, Newman–Keuls test). In the HT-29 xenograft model, at the time of analysis (day 42, 4 weeks after...
termination of the therapy), there was no difference in proliferation or apoptosis when comparing the anti-VEGFR2-ILs-dox group with the PLD group (Fig. 3).

We next determined blood microvessel density for all groups in all models by staining histologic sections with antibodies against CD31 (Fig. 3). In Rip1Tag2 mice, blood microvessel density was reduced by 25% to 31% in the anti-VEGFR2-ILs-dox group compared with the other groups \((P < 0.05, \text{Newman–Keuls posttest})\). In MMTV-PyMT breast cancer mice, blood vessel density in the anti-VEGFR2-ILs-dox group was reduced by 31% to 33% and in the HT-29 xeno-graft model by 24% to 28% \((P < 0.05, \text{Newman–Keuls test})\).

Immunohistochemical stainings with antibodies against the lymphatic marker LYVE-1 did not reveal significant differences in lymphatic vessel density between the treatment protocols in all 3 cancer models (data not shown). However, these models exhibit a low frequency of lymphangiogenesis and, hence, may not be suitable to assess the effect of ILs on tumor lymphangiogenesis.

**Tumor-associated endothelial apoptosis**

Next we asked whether anti-VEGFR2-ILs-dox could induce apoptosis specifically in tumor-associated endothelial cells (Fig. 4). Pancreatic sections of Rip1Tag2 mice were costained with anti-CD31 and anti-cleaved caspase 3 (Fig. 4A). In anti-VEGFR2-ILs-dox treated mice, the analysis of endothelial cells within the tumor perimeter indicated a median of 20 apoptotic endothelial cells per visual field. In the exocrine pancreas, anti-VEGFR2-ILs-dox did not induce endothelial cell death. In untreated controls and PLD-treated mice, no significant number of apoptotic endothelial cells was observed, be it in the tumor or in the exocrine pancreas (Fig. 4B, Dunn’s test, \(P < 0.001\)). The results indicated that anti-VEGFR2-ILs-dox exhibit a selective toxicity on activated endothelial cells, whereas quiescent endothelial cells of the nearby exocrine pancreas are not targeted by the compound.

**Disruption of small blood vessels**

The injection of anti-VEGFR2-ILs-dox induced a decline of tumor-associated microvessels. However, when staining for CD31-positive vessels, remnants of the tumor vasculature were still present after treatment with anti-VEGFR2-ILs-dox. To investigate the mechanisms of tumor vessel disruption, we analyzed vessel morphology by immunofluorescence stainings using antibodies directed against endothelial cells (anti-CD31), pericytes (anti-NG2, not shown), or smooth muscle cells (anti-smooth muscle actin; Fig. 5).
We found that major vessels and small capillaries in the exocrine pancreas were unaffected by the treatment with anti-VEGFR2-ILs-dox (Fig. 5A). Although morphologically normal capillaries were present in tumors of mice treated with PLD (Fig. 5B), the number of intact capillaries was reduced by one third in the tumors treated with anti-VEGFR2-ILs-dox (Fig. 5D). Notably, 4’,6-diamidino-2-phenylindole (DAPI) staining showed pyknotic nuclei in the center of interspersed cells that still expressed CD31 (Fig. 5D). However, in the tumors of mice injected with anti-VEGFR2-ILs-dox, we could still detect small vessels lined by a layer of smooth muscle cells (Fig. 5C), suggesting that mature microvessels, such as arterioles, are not targeted by anti-VEGFR2-ILs-dox. However, no increased pericyte coverage of microvessels was detected in tumors of mice treated with anti-VEGFR2-ILs-dox, suggesting that vessel normalization did not occur upon this antiangiogenic treatment regimen. This is in contrast with results found with other antiangiogenic modalities (30).

Toxicity of anti-VEGFR2-ILs
To evaluate long-term toxicity of doxorubicin-loaded anti-VEGFR2-ILs, we injected cohorts of C57Bl/6 mice (n = 3) either with anti-VEGFR2-ILs-dox, PLD, or empty ILs. The injection dose and schedule was the same as for the efficacy experiments.

H&E staining of all internal organs, CAB staining of the liver, and PAS staining of the kidneys 3 days after termination of the therapy did not reveal signs of acute toxicity. Analysis of peripheral blood in Rip1Tag2 mice of all cohorts showed a normal blood count throughout and after treatment (data not shown). H&E staining of histologic sections of hearts 1 month after therapy revealed no signs of fibrosis (data not shown).

All animals survived for 18 months without apparent signs of distress. The body weight corresponded to that of age-matched untreated controls. Histologic analysis of internal organs, including pancreas, liver, and kidneys after this prolonged time period showed no morphologic differences between treated mice and healthy controls (Fig. 6).

Discussion

Immunoliposomal strategies combine the advantages of an encapsulated drug with the targeting modalities of a mAb on its surface. In this study, we have evaluated the feasibility and the efficacy of targeting the tumor-associated endothelium by anti-VEGFR2-ILs loaded with doxorubicin.

The treatment with anti-VEGFR2-ILs loaded with doxorubicin induced a reduction of blood vessel density within 2 weeks. The response of the targeted blood vessels was dependent on both localization and morphology of the vessel. First, vessels localized in the tumor were most vulnerable, whereas vessels in healthy tissue were not affected. This probably reflects the differential expression of VEGFR2 between quiescent and tumor-associated vasculature. Second, capillaries consisting of a single layer of endothelial cells strongly responded to anti-VEGFR2-ILs-dox, whereas endothelial cells within vessels covered by smooth muscle cells were protected from the effect of anti-VEGFR2-ILs-dox.

Anti-VEGFR2-ILs-dox tumor therapy was more potent than treatment with PLD or PLD combined with free DC101. In our study, we used free DC101 at a concentration that matches the amount of DC101 found in the anti-VEGFR2-ILs-dox group. This corresponds to about 50 μg of DC101 per animal and is about 20 times less than the concentration used in trials of DC101 monotherapy (31). However, our results clearly show that anti-VEGFR2-ILs-dox are superior to PLD and anti-VEGFR2 antibody at the same dose level. The efficacy of anti-VEGFR2-ILs-dox is similar to that of a strong oral angiogenesis inhibitor, such as PTK787. Of the many treatment regimens tested in Rip1Tag2 mice, including genetic ablation or sequestration of tumor- and angiogenesis-promoting factors (VEGF, insulin-like growth factor II, fibroblast growth factors), and classical chemotherapy, the treatment with anti-VEGFR2-ILs-dox is one of the most efficient, with up to 91% smaller tumor volume in the interventional group, compared with 67% to 89% in
previous studies (24). This also compares favorably with the treatment of Rip1Tag2 mice with another antiangiogenic compound, the multi-kinase inhibitor sunitinib, which resulted in a tumor burden of 1/5 compared with control mice (31).

One limitation of the transgenic mouse models employed is the continuous transgenic expression of oncogenes, such as SV40 large T-antigen in Rip1Tag2 and the polyoma middle T antigen in MMTV-PyMT mice. Tumors regrow after termination of therapy, thus precluding a long-term observation of mice. Experimental results from our and other laboratories (32, 33) indicate that tumor-associated vessels can become independent of VEGFR2 signaling through adopting FGFR signaling, through vascular co-option, or through loss of PTEN expression in vascular endothelial cells. These mechanisms may account for failure of antiangiogenic therapy in preclinical models and in patients, yet it should be investigated whether this also applies to anti-VEGFR2 immunoliposomal strategies.

Recent reports have raised a fundamental concern about the safety of antiangiogenic treatment (31). Therefore, we carefully evaluated side effects of the therapy in our preclinical tumor models. With regard to tumor-bearing mice models, the analysis of nuclear atypia after therapy indicates that treatment with PLD, but not anti-VEGFR2-ILs-dox, elicits pronounced changes in morphology. No increase in tumor progression and in metastasis formation was apparent after treatment with anti-VEGFR2-ILs-dox and no notable long-term toxicity occurred.

A particular concern with antiangiogenic therapy is the possibility of promoting tissue hypoxia and upregulated hypoxia-inducible factors (HIF)-1α/2α signaling. This might counteract the beneficial effect of antiangiogenic therapy (34). First experimental approaches against HIF-1α/2α signaling have been developed, and it will be interesting to test these compounds in conjunction with antiangiogenic therapy.

In conclusion, we have shown that the selective targeting of tumor-associated vascular cells with ILs is feasible and highly effective in repressing tumor growth. These results set the stage for the design of strategies that specifically target other components of tumor-associated vasculature, such as vascular tip cells, pericytes, and smooth muscle cells. In addition, it is conceivable to direct ILs against other cells of the tumor microenvironment, such as lymphatic endothelial cells or tumor-associated fibroblasts, which play important roles in tumor genesis and propagation. Thus, ILs offer a versatile and efficient technological platform to test therapeutic strategies against different cellular compartments of malignant tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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