Tumor Necrosis Factor α Mediates Homogeneous Distribution of Liposomes in Murine Melanoma that Contributes to a Better Tumor Response


Abstract

Successful treatment of solid tumors with chemotherapeutics requires that adequate levels reach the tumor cells. Tumor vascular normalization has been proposed to enhance drug delivery and improve tumor response to chemotherapy. Differently, augmenting leakage of the tumor-associated vasculature, and as such enhance vascular abnormality, may improve tumor response as well. In the present study, we show that addition of low-dose tumor necrosis factor α (TNF) to systemic injections with pegylated long circulating liposomes augmented the tumor accumulation of these liposomes 5- to 6-fold, which strongly correlated with enhanced tumor response. Using intravital microscopy, we could study the liposomal distribution inside the tumor in more detail. Especially 100 nm liposomes effectively extravasate in the surrounding tumor tissue in the presence of TNF and this occurred without any effect on tumor vascular density, branching, and diameter. Next to that, we observed in living animals that tumor cells take up the liposomes intact, followed by intracellular degradation. To our knowledge, this is an unprecedented observation. Taken together, TNF renders more tumor vessels permeable, leading to a more homogeneous distribution of the liposomes throughout the tumor, which is crucial for an optimal tumor response. We conclude that delivery of nanoparticulate drug formulations to solid tumor benefits from augmenting the vascular leakage through vascular normalization with vasoactive drugs like TNF. [Cancer Res 2007;67(19):9455–62]

Introduction

Delivery of drugs in solid tumors is still a major problem faced in chemotherapy and frequently responsible for failure of initially promising agents. It is well recognized that the pathophysiology of the tumor vasculature and stromal compartment presents an important obstacle (1). This inadequate drug delivery leads to poor responses and regrowth of tumors. Several studies have shown that encapsulation of anticancer agents in pegylated long circulating liposomes can reduce systemic toxicity while retaining or even improving in vivo efficacy (2–4). Because of their small size, long circulation time, and reduced interaction with serum proteins, these liposomes tend to accumulate in tumors, presumably due to leakage through the often-compromised tumor vasculature (5, 6).

Recently, a combination of tumor vascular therapy with chemotherapy gained interest. Administration of the anti–vascular endothelial growth factor compound bevacizumab improved tumor response and survival in patients with metastatic colorectal cancer when used in combination with 5-fluorouracil/leucovorin (7, 8). Although anti–vascular endothelial growth factor therapy is thought to have its effect through inhibition of angiogenesis, the tumor vascular normalization inflicted by bevacizumab has been proposed as a major contribution to the observed clinical outcome (9, 10). In addition, tumor vascular manipulation, next to destruction or inhibition, may be a useful approach in solid tumor chemotherapy. We propose that apposed to tumor vascular normalization, further abnormalization, in particular vascular leakage, as well may benefit chemotherapy (11). Indeed, administration of tumor necrosis factor α (TNF), histamine, or interleukin-2 improved local drug delivery and tumor response (12–14). An augmented vascular permeability and disruption of the vascular lining was the crucial observation in all these settings. With respect to TNF, we showed in previous studies that coadministration of liposomal doxorubicin (Doxil) and TNF resulted in a drug accumulation accompanied by pronounced tumor response in both rat and murine tumor models (15, 16). In addition, in the isolated limb perfusion, in which TNF is coadministered with a chemotherapeutic agent for the treatment of patients with limb-threatening tumors, addition of TNF results in increased accumulation of drug inside the tumor accompanied by improved response rates (14, 17). TNF is likely to augment the leakiness of the vasculature by increasing the gaps in the endothelial lining, leading to improved extravasation of chemotherapeutic drugs into the tumor interstitium (18).

The goal of this present investigation was to dissect the effect of tumor vascular manipulation by TNF on intratumoral distribution of pegylated long circulating liposomes. For this, we used labeled liposomes from different sizes and did intravitral microscopy in the mouse dorsal skin-fold chamber for a detailed insight in the intratumoral localization of the administered liposomes. Here, we show that TNF not only increases the leakiness of some tumor vessels but also renders more vessels permeable to liposomes while leaving the vascular function (e.g., flow) intact. Consequently, a more homogeneous drug distribution in the tumor is reached explaining an improved tumor response.
Materials and Methods

Agents. Recombinant murine TNF (mTNF), with a specific activity of 3 × 10^7 γ/mg and endotoxin levels below 1 unit per milligram protein (19) was kindly provided by Boehringer Ingelheim (Boehringer Ingelheim Pharma GmbH & Co.). Doxorubicin hydrochloride (Adriablastina) was purchased from Pharmacia and pegylated long circulating liposomal doxorubicin (Doxil/Calex) was purchased from Schering-Plough.

Preparation of long circulating liposomes. For the preparation of liposomes, the following chemicals were used: partially hydrogenated egg phosphatidyl choline (PHEPC; Lipoid GMBH), cholesterol (Sigma), and distearoyl phosphatidylethanolamine poly(ethylene glycol) (PEG-DSPE; ALZA Corp.) in a molar ratio of 1.85:1:0.15. Liposomes of 100 nm were prepared by sonication as described before (20). Liposomes of 400 and 800 nm were obtained by multiple extrusions through polycarbonate membranes (Nucleopore) with corresponding pore size. Liposomes were labeled with Gallium^{67} citrate (Nordian) according to Gabizon et al. (21). Pegylated long circulating rhodamine liposomes (Rho-PEG-L) were composed of PHEPC, cholesterol, and PEG-DSPE in a molar ratio of 1.85:1:0.15. The exact size was measured by dynamic light scattering (Malvern 4700 System) and the amount of lipid was determined according to Bartlett (22).

Animals and tumor model. Specific pathogen-free female C57BL/6 mice were purchased from Harlan-CPB, weighing 20–22 g, and fed a standard laboratory diet ad libitum (Hope Farms). The B16BL6 melanoma tumor model was used for this study. All animal studies were done in accordance with protocols approved by the committee on Animal Research of the Erasmus MC (Rotterdam, the Netherlands).

Treatment protocol. Experiments were started when the subcutaneous transplanted B16BL6 tumors reached a diameter of 8 to 10 mm. For tumor response, mice were randomized into the following four groups: saline, mTNF, Doxil, and Doxil immediately followed by injection of mTNF. Mice were injected five times in the tail vein with an interval of 4 days between the injections, first dose of 4.5 mg/kg Doxil and 1.0 mg/kg mTNF. Tumor growth was recorded by caliper measurement and volumes were calculated using the formula 0.4 × (d^2 × L), where d represents the largest diameter and L the diameter perpendicular to B. The tumor size is expressed as tumor size index (TSI), the tumor volume in relation to initial tumor volume.

Biodistribution using the dorsal skin-fold chamber. Preparation of the dorsal skin-fold chamber is an adaptation from previously described procedures (24–26). Briefly, mice were anesthetized 100 µl of a 2:1 (v/v/v) mixture of saline, ketamine, and xylazine and hair was removed from the back of the animal. After dissecting the skin, leaving the fascia, and opposing the skin, the skin-fold of the mouse was sandwiched between two frames, fixed with two light metal bolts and sutures. A small piece of tumor (0.1 mm^3) was transplanted in the fascia, and on both sides, the window was closed with a 12-mm diameter microscopic cover glass of 0.13 to 0.16 mm thick. The mice were housed in an incubation room with an ambient temperature of 32°C and a humidity of 70%. Experiments started 10 to 14 days after implantation of the dorsal skin-fold chamber. Mice were injected via the tail vein with 4.5 mg/kg doxorubicin, 4.5 mg/kg Doxil-Dio, or 48 µmol/kg Rho-PEG-L immediately followed by injection with saline or mTNF (1 µg/mouse).

Fluorescent imaging. At given time points, mice were anesthetized and fixed to the heated microscope stage of a Zeiss LSM 510 META confocal microscope. Injection of 50 µl FITC-bovine serum albumin (FITC-BSA; 1 mg/mL; Sigma) allowed visualization of functional vessels. Randomly selected tumor regions were examined with 20× (NA 0.5) long working distance objective lens and detailed examination was done using 40× water immersion objective lens (NA 0.8). Scans were made with a 488 argon laser for Doxil and FITC-BSA (505–550 nm band pass filter) and a 543 nm helium-neon laser for doxorubicin/Doxil and rhodamine (560–615 nm band pass filter). Vessel morphology, such as vessel density (the area of vessels as percentage of the total field of interest), vessel segments (the number of vessels connected to each other at the branching point), and diameters, was analyzed using the Zeiss LSM Image Browser 4.0 software. Images were analyzed using Image Tool (Don Wilcox, University of Texas Health Science Center, San Antonio, TX). The red green blue color images of 512 × 512 pixels were converted to grayscale in Image Tool. The fluorescence intensity ranged from 0 to 255 and we distinguished specific staining from background using a threshold of 45. The proportion of pixels with fluorescence intensity above the threshold was calculated. To determine extravasation of liposomes, a gridline overlay was applied of 64 × 64 pixels using Adobe Photoshop 6.0. Extravasation of liposomes was scored by counting the number of times the gridlines intersected with intravascular or extravascular presence of liposomes. Second, using a look-up table, the

### Table 1. Tumor response after systemic treatment of B16BL6-bearing mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response of B16BL6 melanoma (TSI)</th>
<th>% Mice with TSI &lt;4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 8</td>
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<tr>
<td>Saline</td>
<td>2.4 ± 0.3</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>mTNF</td>
<td>2.3 ± 0.8</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>Doxil</td>
<td>2.1 ± 0.8</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>Doxil + mTNF</td>
<td>1.3 ± 0.4*</td>
<td>1.4 ± 0.3&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*P < 0.05 versus injection with mTNF alone at the same time point.
†P < 0.05 versus injection with Doxil alone at the same time point.

NOTE: Mice were injected five times via tail vein with an interval of 4 d with saline, mTNF, Doxil, and Doxil in combination with mTNF, and tumor size index, the tumor volume in relation to the tumor volume at the start of treatment, was calculated. (n = 4–6, SD).

Abbreviation: TSI, tumor size index.
Addition of TNF increases uptake of long circulating liposomes in tumor tissue. A, the effect of mTNF on $^{68}$Ga-labeled liposome distribution of different sizes was analyzed 12 or 24 h after systemic injection. Addition of mTNF increased tumor localization of liposomes significantly. Columns, liposomal distribution per gram tumor ($n$ = 3–5); bars, SE. *, $P < 0.01$; †, $P < 0.05$ versus no mTNF at the same time point. B, analysis of Doxil in the blood circulation after systemic injection in combination with saline or mTNF. Twelve hours after injection, significantly less liposomes were found in the blood circulation. Points, liposomal blood circulation ($n$ = 3–5); bars, SD. *, $P < 0.05$ versus no TNF at the same time point.

Results

Addition of TNF improves response to Doxil. To evaluate the antitumor activity of Doxil in combination with low-dose mTNF, B16BL6-bearing mice were treated with saline, mTNF, Doxil, and Doxil in combination with mTNF (Table 1). Combination of Doxil with mTNF resulted in a tumor growth arrest and all mice were still in the treatment at the end of the experiment. Control and mTNF-treated mice showed progressive disease and had to be sacrificed between day 8 and day 12. Treatment with Doxil alone showed some tumor response and, at the end of treatment, 33% of the mice had a tumor size index <4.

Addition of TNF increases accumulation of liposomes in the tumor. We speculated that the observed improved tumor response was not due to a direct effect of mTNF on the tumor cells. First, mTNF by itself has no effect on tumor progression. Second, the concentration of mTNF, which can theoretically be received in the tumor, is too low to have a direct effect on these tumor cells (data not shown). Therefore, we studied whether mTNF augmented liposomal delivery in the tumor. The results show a 6.3-fold increased accumulation of 100 nm liposomes in the tumor 12 h after coadministration with mTNF compared with liposomes alone ($P < 0.05$) and a 5.5-fold increase after 24 h ($P < 0.05$; Fig. 1A). Coadministration of mTNF and 400 nm liposomes resulted in a 5.1-fold enhanced accumulation at 12 h ($P < 0.05$) and 9.2-fold at 24 h ($P < 0.01$) compared with liposomes alone. In case of 800 nm liposomes, addition of mTNF did not cause an increased accumulation of these liposomes ($4.1 \pm 3.7\%$ without mTNF; $3.8 \pm 0.9\%$ with mTNF; data not shown). Accumulation of 400 and 800 nm liposomes in tumor was significantly lower compared with 100 nm liposomes, even in the presence of mTNF, indicating the beneficial properties of small liposomes. Besides localization in tumor, liposomal distribution to other organs was investigated. In the spleen, mTNF did not increase uptake of all three liposome sizes. Only in heart and liver we observed a slight increase of liposomal uptake with the addition of mTNF, which could potentially give an increase in cardiotoxicity (data not shown).

Twelve hours after injection, 51 ± 25% of the liposomes were still present in the blood circulation, whereas free doxorubicin was hardly measurable (2.7 ± 0.9%; data not shown). Even after 72 h, still 15 ± 2% of the liposomes were measured in the circulation. With the addition of mTNF, a lower liposomal percentage ($P < 0.05$) was found in the blood circulation (25 ± 18% for 12 h and 6 ± 4% for 72 h; Fig. 1B). Liposome accumulation is selectively augmented in the tumor by the addition of mTNF and is the main cause of the increased clearance from the circulation.

Addition of TNF improves extravasation and homogeneous distribution of 100 nm long circulating liposomes. Ten days after tumor implantation in the dorsal skin-fold chamber, a tumor size of 4 to 5 mm was reached. Few supplying vessels could be observed. Unlike the normal blood vessels around the tumor, the newly formed vessels are not well organized. They display tortuous structure, frequent branching, and irregular diameters (Supplementary Fig. S1). Clearly, addition of a tolerable dose of mTNF resulted in augmented liposome accumulation in tumor. However, it is still unclear how TNF affects the tumor vascular lining. Moreover, insight in the intratumoral localization of the liposomes is lacking. Therefore, we studied the effect of mTNF on intratumoral distribution of liposomes with the use of intravital microscopy. We first investigated extravasation of Rho-PEG-L with a size of 100 nm. We observed hardly any extravasation when only Rho-PEG-L was administered. Interestingly, when liposomes were found, these seemed accumulated in tumor vessels. Addition of mTNF resulted in abundant extravasation of liposomes into the tumor interstitium (Supplementary Fig. S2). Only in a few areas liposomes were still present inside blood vessels. Twenty-four hours after injection of liposomes and mTNF, 50 μL FITC-BSA was administered i.v. to visualize functionality and permeability of blood vessels. With the addition of mTNF, FITC-BSA extravasated at the same spot as the liposomes, indicating that these vessels were still leaky. At higher magnification, we observed that FITC-BSA-positive vessels were quite permeable for 100 nm liposomes. This indicates that liposomes extravasated from blood vessels that remained functional in spite of their increased leakiness (Fig. 2A). Surface plots of the liposomal fluorescence showed fluorescence intensity of the representative grayscale images was converted to a pseudo-color spectrum from 0 to 255 and transformed to a surface plot. The heights of the peaks correspond to the fluorescence intensity.

In vitro uptake. B16BL6 melanoma cells were maintained in DMEM supplemented with 10% heat-inactivated FCS. Tumor cells were cultured on a cover glass and grown till confluence. The cells were washed and Doxil or doxorubicin was added at a concentration of 5 μg/mL. The cells were placed under a Zeiss LSM 510 META confocal microscope equipped with an incubation chamber, which is maintained at 37°C with controlled CO$_2$ flow. Every 5 min, scans were made using the 543 nm laser and 560 to 615 nm band pass filter.

Statistical analysis. Results were evaluated for statistical significance with the Kruskal-Wallis and Mann-Whitney U test. All statistical tests were two sided and $P < 0.05$ was considered statistically significant. Calculations were done on a personal computer using GraphPad Prism version 3.0 and SPSS version 11.0 for Windows 2000.
peaks representing intravascular liposomes. Addition of mTNF resulted in broad areas with high-fluorescence intensity representative of massive liposome extravasation (Fig. 2B). Without mTNF, the incidence that a liposome was found intravascular was 70 ± 16% and 74 ± 12% after 12 and 24 h, respectively. With the addition of mTNF, hardly any liposomes could be found inside the vessels anymore (2 ± 2% after 12 h and 1 ± 1% after 24 h; Fig. 2C).

Microvascular variables were determined by intravital microscopy when the tumors were treated. Addition of mTNF had no effect on vessel density, number of segments, and diameter, indicating that accumulation of liposomes was not the result of a change in vessel number or size (Supplementary Table S3). In summary, the addition of mTNF caused an increased extravasation of 100 nm liposomes into the tumor interstitium in a more homogeneous way. Less liposomes were trapped inside blood vessels when mTNF was coadministered. These results indicate that administration of mTNF results in an increase in the number of leaky vessels.

Addition of TNF did not alter the intratumoral distribution of 400 nm long circulating liposomes. It has been shown previously that tumor vessels are permeable for transport of macromolecules with a maximum size of 400 nm, suggesting a cutoff size of the pores somewhere between 400 to 600 nm (27). Strikingly, as shown above, most tumor vessels of the B16BL6 melanoma did not allow extravasation of the 100 nm liposomes, whereas addition of mTNF clearly affected the gap size. To investigate if mTNF has a comparable effect on intratumoral distribution of larger liposomes, 400 nm Rho-PEG-L was injected. Without mTNF, liposomes became mostly trapped inside blood vessels, rendering these vessels nonfunctional as no intravascular presence of FITC-BSA could be observed. Addition of mTNF enhanced the extravasation of liposomes only slightly, mostly at the periphery of the tumor (Fig. 3A; Supplementary Fig. S4). Analysis of the fluorescence intensity showed a 2-fold increase of intratumoral localization of liposomes due to the addition of mTNF (Fig. 3C). However, <10% of the liposomes were present in the surrounding tumor tissue even when coadministered with mTNF (Fig. 3D and E). These results indicate a diminished usefulness of the 400 nm liposomes compared with liposomes of 100 nm for the treatment of solid tumors, also in combination with mTNF.

Addition of TNF improves uptake of liposomal doxorubicin but not of free doxorubicin. It is known from previous studies that total drug accumulation of free doxorubicin in tumor tissue is rather low compared with Doxil (15). In the present study, we

![Figure 2. Addition of TNF promotes liposomal extravasation through hyperpermeable vessels. The effect of mTNF on tumor distribution of systemically injected long circulating liposomes (Rho-PEG-L) was studied by intravital microscopy. Twelve or 24 h later, mice were injected with FITC-BSA to visualize functional vessels. A, in mice injected with Rho-PEG-L alone, some liposomes were seen in the surrounding tumor tissue (*) but predominantly in the blood vessels. In blood vessels filled with liposomes, no green FITC-BSA fluorescence could be seen (arrow), indicating that these intravascular liposomes were obstructing the blood flow. When mTNF was added, liposomal extravasation was observed at sites where also FITC-BSA leaked out (*), indicating that mTNF rendered the vessels hyperpermeable for liposomes. FITC-BSA was seen in the tumor vessels (arrowhead), indicating intact functionality. However, these blood vessels seemed deprived of liposomes, suggesting that all liposomes had extravasated into the surrounding tumor tissue. Bar, 200 μm. B, surface plots show high intensity peaks in the tumor vessels when liposomes alone were given. Consistent with the confocal images, surface plot of images from mice injected with liposomes combined with mTNF show high fluorescence intensity at hyperpermeable sites in the tumor. C, after calculation of the incidence that a liposome was found inside a vessel, a strong reduction in intravascular presence was found when mice were injected in combination with mTNF. Columns, intravascular liposomal incidence (n = 5–6); bars, SE.](cancerres.aacrjournals.org)
examined the intratumoral distribution of free doxorubicin and Doxil. Doxil is labeled with DiO to distinguish between localization of the liposome and doxorubicin, which is red fluorescent. When free doxorubicin was administered, either with or without mTNF, hardly any drug was detectable in the tumor and if any extravasation could be observed, it occurred at the highly vascularized rim of the tumor (Fig. 4A). Limited, focal, and heterogeneous extravasation of Doxil was observed especially at the tumor periphery when injected alone. Addition of mTNF dramatically enhanced extravasation of Doxil (Fig. 4B). Significantly more liposomes (7.1-fold increase after 12 h, \( P < 0.01 \); 3.1-fold increase 24 h after injection, \( P < 0.05 \)) were present extravascular in the tumor tissue when mTNF was coadministered (Fig. 4C). Administration of mTNF augmented total liposomal accumulation 4.7-fold only at 12 h after injection (\( P < 0.01 \); Supplementary Fig. S5). Although liposomes did not penetrate far into the tumor tissue, abundant extravasation could be observed already at 12 and 24 h after injection (Fig. 4D).

**Doxil is taken up intact by the tumor cell and degraded intracellularly.** Insight in the intratumoral fate of liposomal doxorubicin is limited. Doxorubicin-containing liposomes, such as Doxil, are relatively rigid and only slowly release their content resulting in a prolonged exposure of the tumor cells to the drug. However, this also means that low or moderate peak levels of free drug are reached. Elucidation of the liposomal fate and release of contents is useful for drug delivery strategies, certainly if controlled degradation of these carriers is applied (28). Here, we observed in vivo that after extravasation of Doxil-Dio into the interstitial space, the liposomes were taken up as such by the tumor cells. Thirty-six hours after injection of Doxil-Dio in combination with mTNF, a separation of the two dyes (DiO and doxorubicin) was observed and transport of doxorubicin to the nucleus of the tumor cells could be detected, whereas the lipid marker remained in the cytoplasm (Fig. 5A). These results indicate that, presumably, most liposomes are taken up intact by the cells and broken down in the cytoplasm.

In addition, in vitro uptake of doxorubicin or Doxil by tumor cells was studied. Free doxorubicin was immediately taken up by the cells and directly transported to the nucleus. Twenty minutes after incubation, all cells stained positive for nuclear doxorubicin. When B16BL6 cells were exposed to Doxil, accumulation of this drug in the cytoplasm was observed after 15 min. A marginal
intracellular transport of doxorubicin to the nucleus was observed in a period of 2 h (Fig. 5B and C). Uptake by cells as well as intracellular transport of doxorubicin was not affected by mTNF (data not shown). These results confirm our observations in vivo and indicate that Doxil is taken up intact by the tumor cells and remains predominantly in the cytoplasm, whereas intracellular breakdown is slow.

Discussion

Here, we show that addition of low-dose TNF to systemic administration of pegylated long circulating liposomes facilitates extravasation of these liposomes in solid tumor. Strikingly, this is in particular observed when liposomes of 100 nm are used, whereas larger liposomes (e.g., 400 and 800 nm) hardly extravasate, even in the presence of TNF. Our data show that the permeability of the tumor vasculature is not only very heterogeneous but also does not allow passage of liposomes with a diameter of 400 nm or larger. Intratumoral distribution studies reveal that administration of TNF results in a strongly enhanced and more homogeneous distribution of the coinjected liposomes. These results indicate that more vessels become permeable with the administration of TNF. Next to this, we show, and to our knowledge for the first time, by intravital microscopy that liposomes are taken up completely by tumor cells, followed by intracellular degradation of the liposomes on which released doxorubicin enters the nucleus. These findings are confirmed by in vitro experiments. These results are in contradiction to the belief that the liposomes are degraded in the interstitial space followed by uptake of the released chemotherapeutic compound by the tumor cells (29).

In spite of promising results in vitro or even in animal studies, chemotherapeutic agents often disappointed in the treatment of patients. The major prerequisite for a good tumor response is an effective delivery of the systemically injected drug to its target, the tumor cell. However, when injected in the bloodstream, many obstacles have to be overcome (30). Some of these impediments can be obviated with the use of pegylated long circulating liposomes. Unlike free administered drug, their liposomal encapsulated counterpart has limited toxicity and gives the drug prolonged circulation half-life, thereby enhancing the efficacy of targeted drug delivery (31, 32). Second, blood flow in a tumor is very heterogeneous due to the abnormal vascular organization. This irregular blood flow leads to well vascularized parts and necrotic/hypoxic parts obstructing the homogeneous delivery of a drug (1). Third, the drug has to leave the circulation and cross the endothelial lining to reach its target, the tumor cell. In general, the endothelial lining of blood vessels forms a tight, uniform, and continuous monolayer of cells. However, the endothelial cells in tumor blood vessels do not comply with this phenotype and can be best described as disorganized (33, 34). They seem to be permeable to macromolecules and particles, with a determined cutoff of 400 nm (27, 35). The tumor-associated vasculature has become an important target for tumor treatment and several strategies have been reported. (a) The antiangiogenesis

![Figure 4](https://cancerres.aacrjournals.org/content/67/19/9460/F1.large.jpg)
approach interferes with the formation of new tumor blood vessels and normalizes the existing blood vessels to improve a more effective delivery (36, 37). (b) Vascular disrupting agents inflict direct damage to the endothelial cells that will initially lead to vascular shutdown (38, 39). (c) Manipulation of the tumor vasculature with vasoactive drugs, like TNF or histamine, causes a better delivery of the coadministered chemotherapeutic drug that act on the tumor cells (14, 40).

The main focus of our study is the use of tumor vascular manipulation as an effective way to improve solid tumor treatment. Previously, we showed that addition of TNF to an isolated perfusion with melphalan or doxorubicin dramatically improves tumor response (14, 41). Moreover, also in a systemic setting, addition of TNF to liposomal chemotherapy improves tumor response (15, 16). This synergy is not, or at least not solely, due to a direct activity of TNF on the tumor cells and does not result from a direct destruction of the tumor-associated vasculature (17). More importantly, addition of TNF, both in the perfusion setting and in the systemic setting, augmented the accumulation of the coadministered drug in the tumors. Other investigators showed that TNF also enhanced tumor accumulation of liposome-encapsulated Adriamycin (42) and radiolabeled antibodies (43). These findings led to our search for alternative agents with known vascular permeability increasing activity, such as histamine and interleukin-2. We have shown that these agents also enhance drug accumulation in the tumor resulting in synergistic antitumor activity in an isolated setting (13, 44). Apparently, this dual approach consisting of a combination of tumor– and tumor vasculature–directed therapy is an important concept for a good tumor response. Although it is known that TNF is capable of increasing the permeability of an endothelial lining, the effect of TNF on tumor vessels in the tumor is largely unknown. Ruegg et al. (45) showed that TNF in conjunction with IFN-γ induced functional down-modulation of the αvβ3 integrin, resulting in detachment and anokis of the endothelial cells. This possibly explains why tumor vessels, in which αvβ3 exposure is increased, are more responsive to TNF than endothelial cells from healthy vessels, which are quiescent. It has also been shown that TNF in combination with IFN-γ and peripheral blood mononuclear cells stimulates elongation and gap formation between endothelial cells, increasing permeability of the endothelial lining (46).

In the present study, we show that low-dose TNF does indeed augment extravasation of liposomes, whereas the endothelial lining is not destroyed and the vessels remain functional. Further investigation is required to determine which type of tumor vessels are affected by TNF and if other components of the vasculature, like pericytes and basal membrane, play a role in this dual regimen. In conclusion, we show that systemically administered low-dose TNF improves drug accumulation in solid tumor by making more vessels permeable to 100 nm liposomes. These results in a more homogeneous drug distribution within the tumor exposing more tumor cells to the drug and causing increased antitumor efficacy. Additionally, we show that the liposomes are taken up intact by tumor cells and are degraded intracellularly, releasing the cytotoxic drug. Our results indicate that the dual approach, tumor vascular manipulation, with TNF combined with chemotherapy, has strong potential for solid tumor therapy and necessitates the further development of clinical applicable formulations.

Figure 5. The complete liposome is taken up by the tumor cell and broken down in the cytoplasm. Using intravital microscopy, the cellular uptake of Doxil was observed. A, 36 h after systemic injection of Doxil-DiO, the liposomes (yellow) were seen in the cytoplasm of the tumor cell, whereas only doxorubicin (red) could be observed in the nucleus (arrow). At higher magnification, doxorubicin (red, arrow) was seen in the nucleus, whereas the liposome (green, arrow) was only found in the cytoplasm. In addition, 72 h after systemic injection, the red fluorescence of doxorubicin in the nucleus of the tumor cell and the yellow fluorescence of the complete liposome inside the cytoplasm could still be observed. B, for better understanding of the uptake of doxorubicin in the B16BL6 melanoma cell, cells were incubated with doxorubicin or Doxil and time lapse microscopy was done. Doxorubicin was immediately taken up by the cell and transported to the nucleus. On the other hand, Doxil took much longer to occur in the cytoplasm. Bar, 200 μm. C, the percentage B16BL6 cells positive for doxorubicin in the cytoplasm (■) and nucleus (▲) after incubation with free doxorubicin or in the cytoplasm (□) and nucleus (△) after incubation with Doxil was calculated. Very rapidly, all cells had taken up the doxorubicin and stained positive for nuclear doxorubicin. In case of cell incubation with Doxil, doxorubicin was observed in the cytoplasm much later compared with the administration of free drug. Transport from the cytoplasm to the nucleus also took much longer.
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