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Hyperthermia Enables Tumor-specific Nanoparticle Delivery: Effect of Particle Size[1]

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ABSTRACT

The efficacy of novel cancer therapeutics has been hampered by the ability to deliver these agents to the tumor at effective concentrations. Liposomes have been used as a method to overcome some delivery issues and, in combination with hyperthermia, have been shown to increase drug delivery to tumors. Particle size has been shown to affect the delivery of liposomes, but it is not known how hyperthermia affects size dependence. This study investigates the effect of hyperthermia (42°C) on the extravasation of different sized nanoparticles (albumin; 100-, 200-, and 400-nm liposomes) from tumor microvasculature in a human tumor (SKOV-3 ovarian carcinoma) xenograft grown in mouse window chambers. In this model (at 34°C), no liposomes were able to extravasate into the tumor interstitium. Hyperthermia enabled liposome extravasation of all sizes. The magnitude of hyperthermia-induced extravasation was inversely proportional to particle size. Thus, at normothermia (34°C), the pore cutoff size for this model was between 7 and 100 nm (*e.g.*, liposomes did not extravasate). At 42°C, the pore cutoff size was increased to >400 nm, allowing all nanoparticles tested to be delivered to the tumor interstitium to some degree. With hyperthermia, the 100-nm liposome experienced the largest relative increase in extravasation from tumor vasculature. Hyperthermia did not enable extravasation of 100-nm liposomes from normal vasculature, potentially allowing for tumor-specific delivery. These experiments indicate that hyperthermia can enable and augment liposomal drug delivery to tumors and potentially help target liposomes specifically to tumors.

INTRODUCTION

The therapeutic efficacy of most anticancer agents is predicated on achieving adequate local delivery to the tumor site. Many cancer chemotherapeutic agents have been shown to be highly effective *in vitro* but not as effective *in vivo*. This disparity is believed to be attributable to, in part, the difficulty in delivering drug to the tumor site at therapeutic levels and the need for almost 100% cell kill to affect a cure (1, 2). Therapeutic molecules, cytokines, antibodies, and viral vectors are often limited in their ability to affect the tumor because of difficulty crossing the vascular wall (3). Inadequate specific delivery can lead to the frequently low therapeutic index seen with current cancer chemotherapeutics. This translates into significant systemic toxicities attributable to the wide dissemination and nonspecific action of many of these compounds.

To overcome some of the delivery issues seen with *in vivo* use of many cancer therapeutics, liposomes have been identified as promising carriers for therapeutic agents in the treatment of cancer (4, 5). Since their introduction, liposomes have been altered to improve delivery in various ways, including the addition of PEG[3], targeting antibodies, and pH-sensitive polymers (6). Modification of liposome size has been another method to alter the pharmacokinetics of drug delivery. Size can determine circulation half-life (7–11) and accumulation site in the body (12). In the case of drug carriers, size can also influence the delivery volume, release characteristics for drug vehicles (13), and the accumulation site in the body (liver, tumor, or bone marrow; Ref. 14). Ultimately, these factors make particle size an important factor in determining the efficacy of a cancer therapy (15, 16).

HT has been used to modify the local tumor environment to increase liposomal drug delivery to tumors (17). Although classically viewed as a form of adjuvant therapy to increase the efficacy of radiation and chemotherapy, HT can be applied to augment liposomal drug delivery by increasing tumor blood flow and tumor microvascular permeability. At temperatures of 41–43°C, HT has been shown to increase blood flow (18) and oxygenation (19). HT has also been shown to increase permeability of tumor vessels to antibodies (20–23), ferritin (24), and Evans blue dye (25). More specifically, HT has been shown to increase liposome permeability from tumor vessels (26). Extravasation of liposomes and albumin from tumor microvasculature has been explored in the literature (27), but the effect of HT on enabling and augmenting extravasation in a model where extravasation was initially not possible, especially as a function of size, has not been well defined. We now report experiments in which we investigate the ability of HT to enable and augment extravasation of different size particles, from tumor and normal vasculature. We hypothesized that: (*a*) HT will augment nanoparticle extravasation from tumor vessels; (*b*) HT can enable extravasation of nanoparticles into tumor where initially no extravasation occurred; (*c*) this extravasation will be a function of particle size; and (*d*) this increased extravasation will be preferentially seen in tumor vessels compared with normal vessels.

MATERIALS AND METHODS

Albumin and Liposomes. Rho-labeled BSA was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in PBS to a concentration of 2 mg/ml. Rho-labeled, sterically stabilized, long-circulating PEG liposomes were prepared by the lipid film hydration and extrusion method (28). The formulation for the liposomes was egg phosphatidylcholine:cholesterol:1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-polyethylene glycol 2000: Rho-PE in the molar ratio of 10:5:0.8:0.1 (29). The final lipid concentration after hydration was 20 mg/ml. Liposomes were sized by multiple passes through polycarbonate filters (Poretics, Livermore, CA) with pore diameters of 0.4, 0.2, and 0.1 μm, depending on the target size. Liposome size was determined by dynamic light scattering using a Coulter N4 MD submicron particle size analyzer (Coulter Electronics, Hialeah, FL). All liposome preparations had a narrow size distribution (95% of the liposomes were within $\pm$10 nm for 100-nm liposomes, $\pm$15 nm for 200-nm liposomes, and $\pm$25 nm for 400-nm liposomes). Liposomes <100 nm in diameter were not made because of instability. Liposomes >400 nm in diameter were also not made because of size nonuniformity.

Animal and Tumor Model. Homozygous NCr athymic nude mice (20 $\pm$ 3 g) were purchased from Taconic (Germantown, NY). Animals were housed in appropriate isolated caging with sterile rodent laboratory chow and acidified water *ad libitum* and a 12-h light/dark cycle. All protocols were approved by the Duke Institutional Animal Care and Use Committee. The athymic nude mouse dorsal skin flap window chamber model was used (30).

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[3] The abbreviations used are: PEG, polyethylene glycol; HT, hyperthermia; Rho-PE, rhodamine labeled phosphoethanolamine; RTIA, relative tumor interstitial amount; $RTIA_{60}$, relative tumor interstitial amount at 60 min.

Briefly, titanium window chambers were surgically placed on the dorsal skin flap of athymic nude mice, and a small volume (~0.1 mm^3) of tumor tissue (human ovarian carcinoma; SKOV-3) was implanted in the window chamber. After 10–14 days, the tumor was visibly well vascularized and ~1–2 mm in diameter. This preparation was then used for experiments. For experiments requiring normal vessels, the same window chamber model was prepared without tumors implanted and allowed to mature for 10–14 days before experimentation.

The permeability of tumor vasculature to liposomes is dependent on the tumor type (31). Some tumors have pore cutoff sizes between 400 and 600 nm (29). After initially screening several tumor types, SKOV-3, a human ovarian carcinoma was found to be highly impermeable to 100-nm liposomes under normothermic conditions. Non-tumor-bearing window chamber vessels were similarly impermeable to 100-nm liposomes under normothermic conditions. Because this tumor represents a limiting case for permeability, it was chosen as the model for this study. It provides a strict model for assaying the ability of HT to enable and increase permeability as well as pore cutoff size in tumor relative to normal vessels.

Experimental Groups. Each experimental group had six mice. Albumin, 100-nm liposomes, 200-nm liposomes, and 400-nm liposomes were each tested in tumor-bearing window chambers at 34°C, the normal s.c. skin temperature of mice (26), and 42°C, a temperature that has been shown previously to have some effect on liposome delivery (17). Liposomes (100 nm) were also tested for extravasation from normal vessels in non-tumor-bearing window chambers at 42°C.

Experimental Procedure. In each experiment, animals were anesthetized with sodium pentobarbital (80mg/kg; i.p.). The tail vein was cannulated for i.v. injection of albumin or liposomes during the experiment. The animal was placed on a temperature-controlled microscopic stage to maintain normal body temperature throughout the experiment. The window chamber was fixed in a specially designed HT chamber (32) that allowed for visualization of the tumor, whereas the chamber was being heated (to 34°C or 42°C, depending on the experimental group). The window chamber and tumor were allowed to reach steady-state temperature (~2 min as determined by thermographic imaging) in the HT chamber before injection of liposomes or albumin. The preparation in the window chamber was observed with a ×20 objective lens. A region in the preparation with steady blood flow and few obvious underlying vessels was selected. The images of the selected region were recorded with a SIT camera (Hamamatsu; C2400-08) connected to an S-VHS video tape recorder (Mitsubishi; BV-1000). This region was first recorded under transillumination. Then, under epi-illumination with a filter set for rhodamine (H546; Zeiss), a background image of the selected region was recorded before injection of liposomes or albumin. Next, 0.2 ml of rhodamine-labeled liposomes or albumin was administered i.v. The region was recorded under epi-illumination intermittently (for 10 s every 2 min) for 60 min after the injection of nanoparticles.

The videotape of the experiment was analyzed with image processing software (NIH Scion Image). The fluorescent light intensities of the entire selected region and representative vascular regions were measured at serial time points. The relative fluorescent light intensities of the vascular and interstitial components were determined as described by Wu *et al.* (33). All light intensities were normalized to the initial vascular light intensity in the region after injection of liposomes or albumin. Vascular volumes for all experimental groups were not statistically different. Because the light intensity is proportional to the number of particles present (33), the data are presented as RTIA or accumulation in this report. RTIA values taken at 60 min are defined as $RTIA_{60}$.

Statistics. Changes in RTIA were summarized by averaging data from individual animals in each group (albumin, 100-nm liposomes, 200-nm liposomes, and 400-nm liposomes at 34°C or 42°C). Statistical significance between experimental groups (treatment temperatures for each size or different sizes for same treatment temperature) was determined using the Mann Whitney *U* test. Two quantities were regarded as different if $P < 0.05$.

RESULTS

General Description. After identifying an appropriate region within the tumor by transillumination, epi-illumination was used to measure the extravasation of albumin or liposomes under normothermic and hyperthermic conditions. The typical preparation, seen with epi-illumination, is shown in Fig. 1 using a filter set for the rhodamine label. In general, the fluorescent signal indicating labeled particles appeared in preparations ~10 s after injection and became stable within ~30 s. Throughout the 60-min observation, the tumor blood vessels retained fluorescent signal (Fig. 1, *right top* and *bottom panels*), consistent with previous sterically stabilized liposome pharmacokinetic data (34, 35). Perivascular fluorescent spots usually indicated initial liposome extravasation that later became more diffuse and spread throughout the tumor interstitium (Fig. 1, *bottom row*). These initial fluorescent spots were heterogeneously distributed along

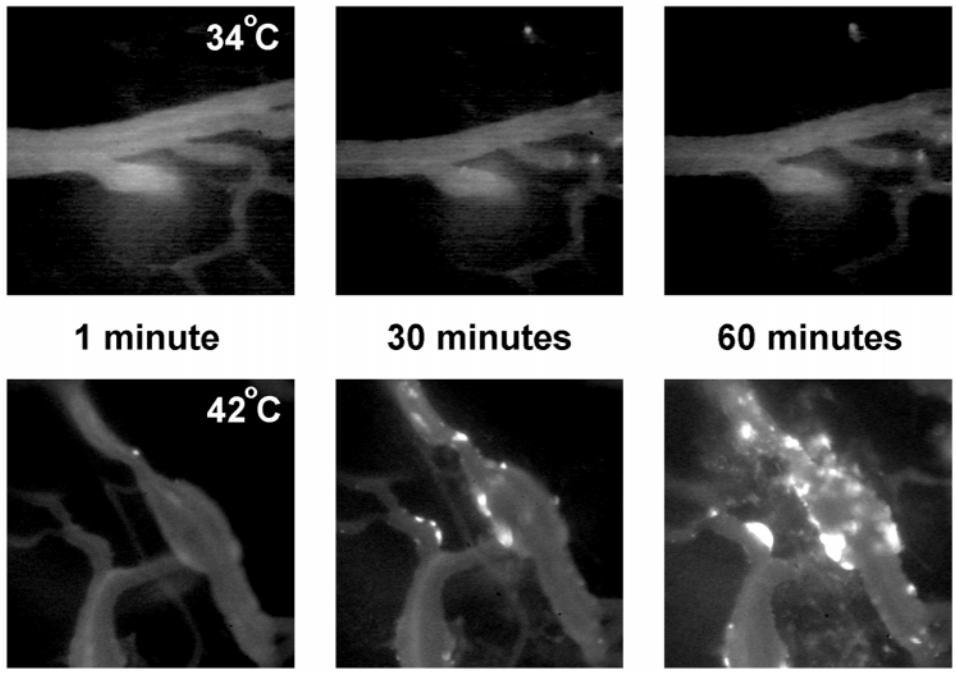

Fig. 1. Extravasation of 100-nm liposomes from tumor vessels at 34°C and 42°C at 1, 30, and 60 min after injection. Minimal extravasation of liposomes was seen at 34°C throughout the 60-min experiment (*top panel*). At 42°C, focal perivascular fluorescent spots developed that increased in size and became more diffuse over time (*bottom panel*).

the vessel. This suggests that the leakage pattern of liposomes from the tumor vessels is dependent on heterogeneously spaced and/or sized pores along the tumor vessel wall (27).

Effect of HT as a Function of Particle Size. Albumin (size ≈7 nm; Ref. 29) extravasation was generally diffuse throughout the time course of the experiment and the tumor interstitium. It freely accumulated in the tumor interstitium at 34°C (Fig. 2*a*) with a $RTIA_{60}$ of ~0.95. At 42°C (Fig. 2*a*), the $RTIA_{60}$ for albumin tended to be greater, ~1.45, but was not statistically different from the $RTIA_{60}$ seen at 34°C. This suggests that albumin extravasation is largely independent of the effect of HT. The accumulation of albumin at 34°C and 42°C did not plateau but rather appeared to increase linearly throughout the hour experiment.

Under normothermic conditions (34°C), 100-nm liposomes showed no accumulation, $RTIA_{60}$ of ~0 (Fig. 1, *top panel*, and Fig. 2*b*). This was significantly lower than the $RTIA_{60}$ for albumin at 34°C ($P < 0.005$). HT (42°C) significantly increased the accumulation of 100-nm liposomes in the tumor interstitium ($P < 0.02$) with a $RTIA_{60}$ of ~1.5. Overall at 42°C, the $RTIA_{60}$ of albumin and 100-nm liposomes was similar. The remaining two liposomes sizes (200 and 400 nm) also did not extravasate significantly at 34°C ($RTIA_{60}$ ~0; Fig. 2, *c* and *d*). At 42°C, all liposome sizes showed increased extravasation ($P < 0.05$) compared with 34°C. The 200-nm liposome at 42°C had a $RTIA_{60}$ of ~0.96. The 400-nm liposomes at 42°C accumulated to a plateau within 25 min and remained there for the rest of the experiment, resulting in a $RTIA_{60}$ of ~0.43 (Fig. 2*d*).

Effect of Particle Size on Extravasation. Although HT enabled extravasation for all sizes ≥100 nm, the degree of enhancement was a function of particle size (Fig. 3). At all sizes, the addition of HT resulted in more accumulation in the tumor interstitium. At 34°C, extravasation quickly dropped off as size increased from albumin (~7nm) to the 100-nm liposome and stayed negligible as liposome size increased. The $RTIA_{60}$ of albumin was significantly higher than all larger particles at 34°C ($P < 0.005$). At 42°C, significant accumulation was maintained from albumin to 100-nm liposomes and then decreased as liposome size increased to 200 and 400 nm. Even at 400 nm, HT still facilitated some significant degree of accumulation over normothermia. At 42°C, the degree of extravasation between the 100-nm liposome compared with the 400-nm liposome was significantly higher ($P < 0.02$), whereas the differences between the 100- and 200-nm liposome and the 200- and 400-nm liposome were not significant.

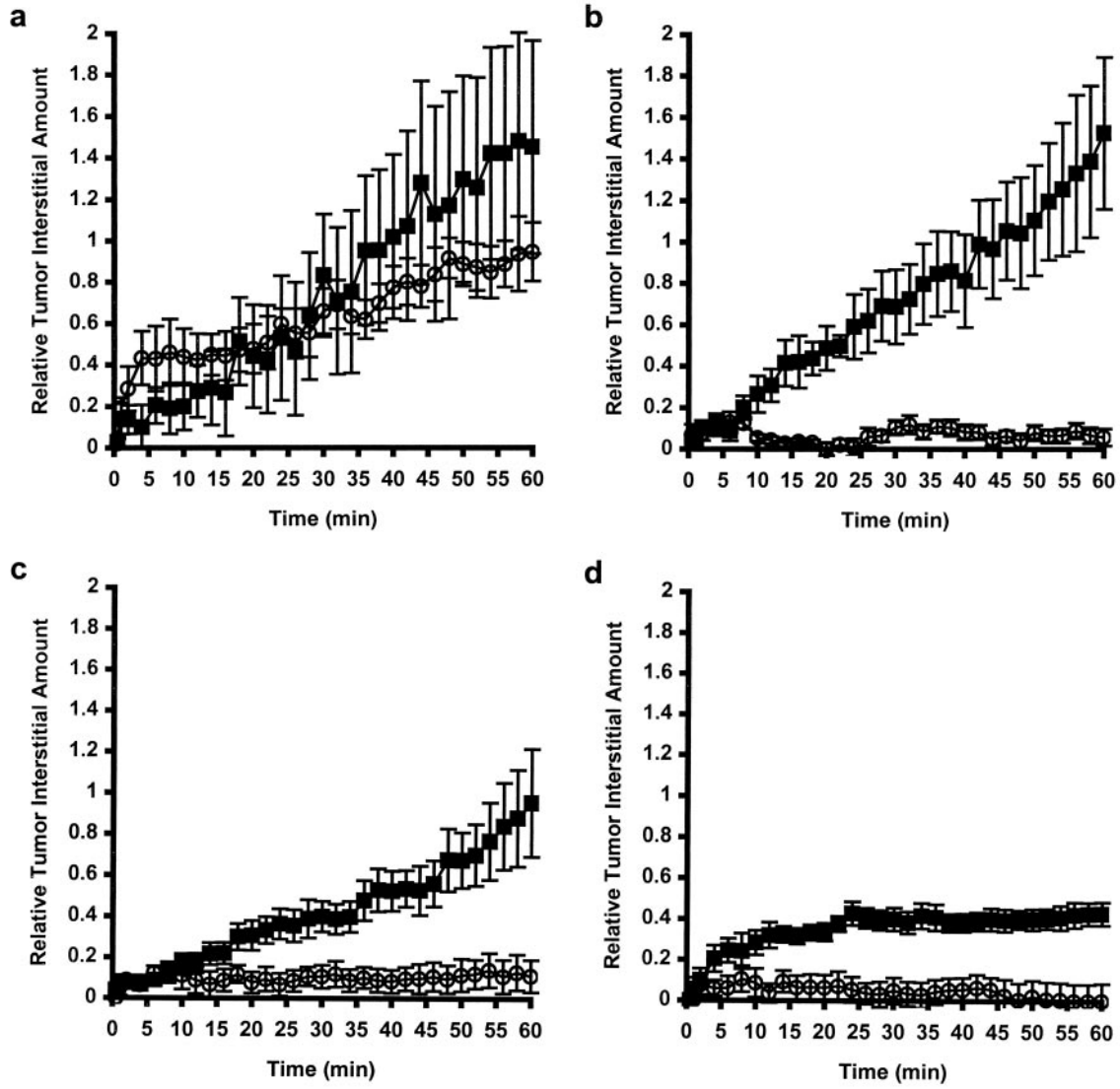

Fig. 2. HT-enhanced extravasation of different sized particles. The RTIA at 34°C (○) and 42°C (■) of albumin (*a*), 100-nm liposomes (*b*), 200-nm liposomes (*c*), and 400-nm liposomes (*d*). The 100-nm liposomes (*b*) showed the largest HT enhancement of extravasation. The means are shown; *bars*, SE.

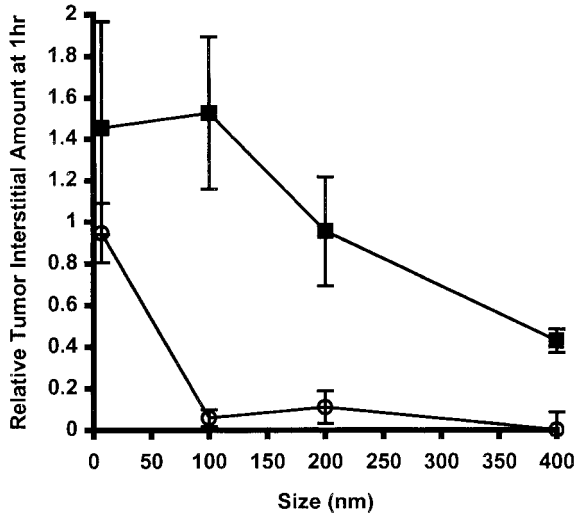

Fig. 3. The effect of particle size on $RTIA_{60}$ at 34°C (○) and 42°C (■). Accumulation decreased as size increased, but HT increased accumulation at all sizes. The means are shown; *bars*, SE.

Effect of HT on Liposome Extravasation from Normal Vessels. HT has already been shown to increase the permeability of albumin from normal vasculature (36). To determine whether the enabling effect of HT on liposome extravasation was unique to tumor vessels, the extravasation of the 100-nm liposomes at 42°C was also studied in normal vessels in the window chamber model. HT did not enable 100-nm liposome delivery to normal s.c. tissue. There was minimal extravasation of 100-nm liposomes at 42°C from normal vessels in the window chamber ($RTIA_{60} \sim 0$; Fig. 4). The accumulation profile was similar to that seen with liposomes at 34°C in tumor vessels (Fig. 2*b*). For comparison, 100-nm liposomes at 42°C in tumor vessels are also shown in Fig. 4. The $RTIA_{60}$ of 100-nm liposomes at 42°C in tumor vessels was significantly higher than in normal vessels ($P < 0.01$).

DISCUSSION

Effect of HT on Pore Size and Nanoparticle Extravasation. In this study, the experiments indicated that albumin, a molecule of ~7 nm in diameter, extravasated easily from tumor vessels under normothermic conditions (Fig. 2*a*). This is likely attributable to the fact that the inherent pore size of the tumor vessel wall is larger than the diameter of albumin. At 42°C, albumin extravasation appeared to be similar and was not statistically different from that at 34°C. This finding is reasonable because an increase in pore size, when the pore is already much larger than the size of the molecule, is unlikely to result in any striking changes. Another study has also found that albumin permeability in tumor vessels is independent of pore cutoff size (37). This same concept of pore cutoff size can explain the extravasation characteristics of the 100-nm liposome species. Under normothermic conditions, the pores in the SKOV-3 tumor vessels are <100 nm in diameter. The presence of HT causes these pores to increase in size and permits the extravasation of 100-nm liposomes.

HT was able to overcome limitations in extravasation of particles and enabled delivery to the tumor interstitium. At 42°C, the 100-nm liposomes and albumin had similar $RTIA_{60}$s, which were not different from the $RTIA_{60}$ of albumin at 34°C. From the particle accumulation viewpoint, HT made the two species functionally equivalent. As the particles got larger in diameter, the $RTIA_{60}$s with HT did decrease; however, the presence of HT always yielded a higher $RTIA_{60}$ than normothermia (which was ~0 for all liposome sizes tested). This decrease in $RTIA_{60}$ for the 200- and 400-nm liposome species may be attributable to heterogeneity in pore size distribution of the tumor vessels created by HT. In other words, as the particle species increases in size, a smaller proportion of these pores in the tumor vessel are large enough to allow for extravasation, thereby resulting in a smaller $RTIA_{60}$.

Differential Effect of HT on Normal Vessel Extravasation. The SKOV-3 tumor line was used as a limiting case of tumor vessel impermeability for liposomes because 100-nm liposomes were unable to extravasate under normothermic conditions (Fig. 2*b*). Because HT (42°C) was shown to overcome this limitation in this tumor, the next logical step was to study normal vessels and determine whether there was a difference between tumor and normal vessels at this temperature. At 42°C, the 100-nm liposomes did not extravasate from the normal vessels (Fig. 4). Normal vessels were thus a limiting case for the effect of HT-induced extravasation for 100-nm liposomes. These results from the normal vessel experiments show that combination HT and liposomal therapy could potentially be clinically useful because of the size specificity of HT-induced liposome extravasation in this model. In the case of the SKOV-3 tumor, 100-nm liposomes would not significantly accumulate in the tumor or normal tissue (aside from the reticuloendothelial system) at normal body temperature. By administering local HT to the tumor, the 100-nm liposomes would only be able to extravasate in the heated region. The results from the experiments using normal vessels and HT show that the normal vessels in the heated region would not yield significant liposome extravasation either. This allows for regions of normal tissue that may be heated during a HT treatment (*e.g.*, deep seated tumors) to experience minimal toxicity from the liposomal drug. The effect of HT on the tissue immediately surrounding the tumor is still unknown, because this tissue may be influenced by cytokines secreted from the tumor. Overall, the results support the idea that HT can be an important factor in successful liposomal drug delivery to tumors and that liposome size can be used as a mechanism for targeting and modifying the amount of drug delivered when combined with HT.

Possible Mechanisms for HT-induced Nanoparticle Extravasation. The mechanism behind HT-induced extravasation has thus far been described as the pore cutoff size. This theoretical concept schematically facilitates the understanding of the extravasation of different sized particles from tumor vessels. Mechanistically, the exact pathways that lead to increased extravasation have not been determined. There have been many studies and competing theories that attempt to

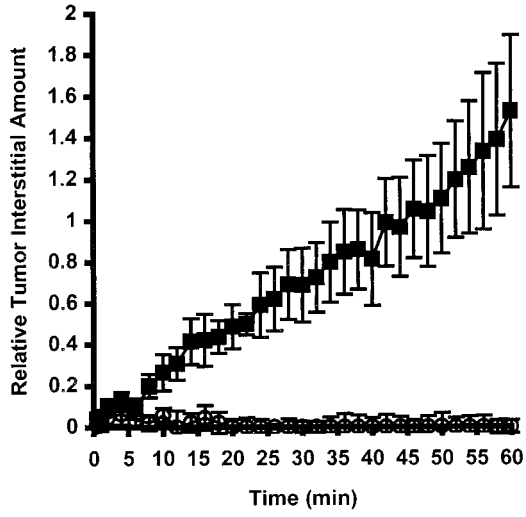

Fig. 4. Tissue specificity of HT-induced extravasation. Comparison of 100-nm liposome extravasation at 42°C in normal vessels (○) and tumor vessels (■). Only tumor vessels showed HT-enhanced extravasation. The means are shown; *bars*, SE.

explain the dominant pathway for particle extravasation. Functional and structural studies have shown that large pores exist in tumor vessels that allow nanoparticles to extravasate (3). HT has been shown previously to increase the permeability of the already hyperpermeable tumor vessels (20–25), but the mechanism is not well established.

One of the more widely accepted explanations for HT-induced extravasation is the "shrinking" of the endothelial cells as a stress response to heat and subsequent widening of the endothelial cell gaps, allowing for larger particles to extravasate (25, 26). The "shrinking" or morphological changes in endothelial cell structure are likely attributable to disaggregation of the cytoskeleton after HT (38–40). This phenomenon has been supported in electron microscopic studies (41).

Modification of tumor blood flow has also been proposed as a possible mechanism for increased RTIA seen with HT. Mild HT (40–42°C) has been recognized to increase tumor blood flow (18, 42). This overall increase in blood flow could translate into more nanoparticles perfusing through tumor vessels, eventually leading to increased extravasation. Although blood flow was not measured in this study, no change in vessel diameter or vessel density was noted over the course of the 1-h experiments. This is most likely because the tumor was allowed to reach steady-state temperature before injection of the nanoparticles. Furthermore, an increase in blood flow alone cannot account for the large change seen in 100-nm liposome $RTIA_{60}$ at 34°C and 42°C because most increases in tumor blood flow attributable to HT have been ~5–20% (26, 43), not orders of magnitude as seen in Fig. 2*b*. Finally, a significant change in blood flow should have also increased the albumin $RTIA_{60}$ at 42°C, but this was not seen.

HT has also been shown to increase intravascular pressure in animal models (44, 45) and patients (46). This increased pressure could translate into a larger driving force across the vessel wall and result in more extravasation of nanoparticles. However, increased intravascular pressure cannot account for the increased extravasation seen with HT in this study because no increased extravasation was seen with albumin and HT (Fig. 2*a*).

Ultimately, all or most of these mechanisms for HT-induced extravasation may be important. The lack of HT-induced change in albumin extravasation makes an increase in pore size the most likely explanation.

Consistency of HT-induced Nanoparticle Extravasation. The variation in the data among 100-nm liposome experiments at 42°C (Fig. 2*b*) is likely a function of tumor heterogeneity, vessel heterogeneity, pore size distribution, and liposome size distribution. As the liposome sizes get larger and/or the pore sizes get smaller (*e.g.,* normothermia), there is less extravasation and less variation in the data. This is most clearly seen in the extravasation of all of the liposomes at 34°C, where the pores are too small for any extravasation and thus the data are very consistent. This concept is similarly true in both the 200-nm (Fig. 2*c*) and 400-nm (Fig. 2*d*) liposome data at 42°C. As the liposome sizes get larger, only certain pores will facilitate extravasation and allow for less variation in the data. Thus, for the smallest particle studied, albumin (Fig. 2*a*), the largest variation in results is expected and seen.

Another consideration in the case of the 400-nm liposomes is the circulation half-life. Despite the incorporation of PEG in the membrane, the size of these liposomes results in easy detection by the reticuloendothelial system and rapid removal from circulation (7). At 42°C, whereas all other liposomes continue to extravasate over the course of 1 h, the 400-nm liposomes plateau within ~25 min. After 25 min, only ~30% of the initial vascular concentration of 400-nm liposomes remained in circulation (data not shown). This drop in liposome concentration in circulation reduces the number of liposomes available for extravasation.

Implications for Drug Delivery to Tumors. The $RTIA_{60}$ is a function of both circulation half-life and tumor vessel permeability. Circulation half-life, in this set of experiments, is largely modified by particle size. Tumor vessel permeability is determined by the distribution of pore sizes in the tumor vessel and the distribution of particle sizes in circulation; the former is also potentially modified by HT. It has been shown that particles, such as liposomes, that are too small (<70 nm) or too large (>300 nm) have a reduced circulation half-life (10, 47). Optimization of circulation half-life based on size must be tempered by the optimization for permeability (*i.e.,* the smaller the particle, the easier it exits the vessel). The maximal $RTIA_{60}$ is achieved when the combination of circulation half-life and permeability is optimized.

Although it would initially appear to be ideal to maximize the $RTIA_{60}$, the absolute amount of drug delivered to the tumor is the most important factor in determining efficacy. The amount of actual drug delivered is indeed a function of $RTIA_{60}$, but it is also a function of the internal volume of the drug carrier, a liposome in this case. In the study presented, the 100-nm liposomes at 42°C had the highest $RTIA_{60}$. Although the 100-nm liposomes had a higher $RTIA_{60}$ than the 200-nm at 42°C, the 100-nm liposomes may not actually deliver more drugs to the tumor because of the smaller internal volume. It must be remembered that a 200-nm liposome could have an internal volume that is an order of magnitude higher (depending on the thickness of the membrane) than that of a 100-nm liposome. Thus, the final efficacy for a liposomal treatment is likely to be a function of the product of accumulation and internal volume.

Using HT as the modality for targeting delivery to tumors has several other advantages. Comparable temperatures (~42–43°C) have been shown to trigger drug release from specially designed thermosensitive liposomes (48–51), making it possible to release liposome contents as desired. With a known tumor location, using HT to target and trigger drug release may be advantageous over other methods that require some inherent property of the tissue (pH or certain specific surface antigens), because it is controlled by external means and thus more easily modified to the desired conditions. In addition, preclinical data have indicated that several cancer chemotherapeutic agents in combination with HT have supra-additive cytotoxic effects (52, 53). Lastly, besides targeting and triggering release in liposomes, HT itself has been shown to be directly cytotoxic (54). The therapeutic benefits from liposomes and HT individually, coupled with the potential advantages seen by their combination, make the use of the two modalities together an attractive method for drug delivery to tumors.

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