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Research Paper

Radiation-Guided Targeting of Combretastatin Encapsulated Immunoliposomes to Mammary Tumors

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Purpose. Radiation upregulates expression of endothelial cell adhesion molecules providing a potential avenue for targeting drugs to irradiated tissue. Induced upregulation of E-selectin can be used to target immunoliposomes to solid tumors. The effects of targeting immunoliposomes containing the antivascular drug combretastatin disodium phosphate (CA4P) to irradiated mammary tumors were investigated in this study.
Methods. Mice bearing transplanted MCa-4 mouse mammary tumors were assigned to one of the factorial treatments permuting the administration of free CA4P, tumor irradiation, CA4P encapsulated liposomes, and CA4P encapsulated immunoliposomes (conjugated with anti-E-selectin). Single and fractionated dosing of radiation and/or CA4P was evaluated.
Results. For single dose treatments the group that received a single dose of radiation plus a single dose of immunoliposomes showed a significant delay in tumor growth compared to all other treatment groups. Fractionated radiation plus fractionated doses of immunoliposomes resulted in further tumor growth delay; however, it was not significantly different from other fractionated dose treatment groups that combined radiation and CA4P.
Conclusions. Targeting of antivascular drugs to irradiated tumors *via* ligand-bearing liposomes results in significant tumor growth delay. This effect can be further potentiated using a fractionated irradiation dosing schedule combined with fractionated immunoliposome treatments.

KEY WORDS: adhesion molecules; combretastatin; immunoliposomes; targeted drug delivery; tumor vasculature targeting.

INTRODUCTION

Administration of antivascular drugs (such as combretastatin) causes a significant reduction in tumor blood flow resulting in hypoxia and metabolic deprivation in tumors (1). However, these drugs also significantly alter blood flow in many normal tissues including the heart, brain, spleen, skin, and kidney (2). In the cases of brain and heart, even relatively small short-lived changes in blood flow may cause serious harm to the patient. In addition, these drugs are often only effective at or above their maximum tolerated doses (3,4).

In the clinic, undesirable side effects of anti-cancer drugs such as doxorubicin have been reduced and blood retention time increased by encapsulating them in liposomes, which can be passively targeted to the leaky vasculature of tumors (5,6). Active targeting of drug-containing immunoliposomes to tumors has the potential to further enhance this normal tissue sparing effect thereby increasing the therapeutic effect of these drugs. A unique opportunity for targeting antivascular drugs to irradiated tumors exists because the maximum effects of several antivascular drugs are only observed when they are combined with radiotherapy (3,7,8) and ionizing radiation is known to upregulate several adhesion molecules (9–12). In this approach antivascular drug (e.g. combretastatin) carrying particles, having appropriate ligands to radiation induced upregulated adhesion molecules on their surfaces, would be preferentially distributed to the irradiated tumor region thereby circumventing the undesirable side effects of antivascular drugs on normal tissue. This targeting could be especially effective in conjunction with modern clinical radiotherapeutic techniques, where radiation exposure is generally limited to a core of diseased tissue and the normal tissue surrounding it.

Previously we have shown that antibody conjugated model drug carriers can be selectively targeted to the vasculature of irradiated tissue bypassing the non-irradiated

CBP and BV made equal contribution to this work.

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(i.e. normal) tissue (9). Furthermore, we have shown the feasibility of targeting immunoliposomes to irradiated tumor vasculature such that administering even a single dose of immunoliposomes encapsulating the lipophilic form of combretastatin (CA4) to irradiated tumors resulted in significant tumor growth delay (10). Here for the first time we show that this proposed therapy can inhibit tumor growth in different models, can target various adhesion molecules at various times after irradiation, is effective under various radiation and targeted drug dosing conditions, and report on the development and kinetics of a more clinically relevant liposomal formulation. We hypothesize that in transplanted mammary tumors, significant tumor volume control can be achieved with a single dose of radiation combined with a single dose of anti-E-selectin conjugated liposomal combretastatin. We also hypothesize that a treatment combining fractionated radiation with fractionated doses of combretastatin disodium phosphate (CA4P) encapsulated immunoliposomes would result in additional tumor growth delay. Our novel findings provide important compelling evidence for the clinical feasibility of this approach.

MATERIALS AND METHODS

Materials and Their Sources

The lipids 1, 2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-*N*-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt) (DSPE-PEG) and 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-*N*-[Maleimide(Polyethylene Glycol)2000] (Ammonium Salt) (DSPE-PEG-Maleimide) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[poly(ethyleneglycol) 2000-*N′*-carboxyfluorescein] (ammonium salt) was purchased from Avanti Polar Lipids. Anti-E-selectin (mAb, clone 10E9.6) was purchased from Pharmingen (San Diego, CA). 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoate (cholesteryl BODIPY® 576/589 C11) was purchased from Invitrogen (Carlsbad, CA). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO). Combretastatin disodium phosphate (CA4P) was custom produced by Biofine International (Vancouver, Canada) according to published methods (13) and determined to be 98% pure by HPLC. Soluble E-selectin was purchased from R & D Systems (Minneapolis, MN).

Transplanted Tumor Models and Treatment

MCa-4 mammary tumor cells (obtained from the laboratory of Dr. Lukas Milas, The University of Texas MD Anderson Cancer Center, Houston, TX) were injected into the mammary fat pads of 6–8 week old C3H female mice (Harlan, Indianapolis, IN). Briefly, cells were centrifuged and resuspended with 200 μL of PBS then approximately 1×10^7 cells were injected into each site using a 1 mL syringe with a 26 gauge needle. Treatments were initiated when tumors reached a volume of approximately 1.0 cm^3. The animals were anesthetized using isoflurane and received either a single dose of radiation (IR, 5 Gy) or fractionated radiation (Frac IR, 2 Gy per day for a total of 20 Gy over 10 days) localized to the tumor from an x-ray machine (X-RAD 320; Precision X-ray Inc., East Haven, CT). Within 5 min post-irradiation, animals received either a single dose or fractionated doses of combretastatin *via* retro-orbital injection.

In the single dose treatment arm the following five different mouse treatment groups were studied: (1) Injection of free CA4P at a concentration of 81 mg/kg (Free CA4P, $n=6$), (2) Single 5 Gy dose of irradiation (IR) only ($n=6$), (3) Single dose of IR in combination with Free CA4P (IR + Free CA4P, $n=6$), (4) Injection of immunoliposomes (IL) at a CA4P concentration of 15 mg/kg ($n=6$), (5) Single dose of IR together with a single dose of IL (IR + IL, $n=6$). Animals treated with a single dose of liposomes (Lip, non-antibody conjugated) at a CA4P concentration of 15 mg/kg ($n=5$) and Lip in combination with IR (IR + Lip, $n=6$) served as controls (Table I).

In the fractionated treatment arm the following five different mouse treatment groups were studied: (1) A dose of free CA4P (81 mg/kg) every other day for a total of four doses over 7 days (Frac CA4P, $n=6$), (2) A dose of IR at 2 Gy a day for a total of 20 Gy over 10 days (Frac IR, $n=6$), (3) A dose of Frac IR and Frac CA4P (Frac IR + Frac CA4P, $n=5$), (4) A dose of IL (15 mg of CA4P/kg) every other day for a total of four doses over 7 days (Frac IL, $n=5$), and (5) A dose of Frac IR and Frac IL (Frac IR + Frac IL, $n=6$). Animals treated with fractionated doses of liposomes (15 mg of CA4P/kg) every other day for a total of four doses over 7 days (Frac Lip, $n=5$) and Frac Lip in combination with Frac IR ($n=5$) served as control (Table II). Untreated tumor bearing animals served as negative control for both single and fractionated dose treatments.

Table I. Single Dose Treatment Groups

Treatment group	Radiation dose	Drug dose	Drug delivery method
Untreated	None	None	None
Free CA4P	None	81 mg/kg	Systemic
Lip	None	15 mg/kg	Liposomes
IL	None	15 mg/kg	Immunoliposomes (anti-E-selectin conjugated)
IR	5 Gy	None	None
Free CA4P + IR	5 Gy	81 mg/kg	Systemic
Lip + IR	5 Gy	15 mg/kg	Liposomes
IL + IR	5 Gy	15 mg/kg	Immunoliposomes (anti-E-selectin conjugated)

Table II. Fractionated Dose Treatments

Treatment group	Radiation dose	Drug dose	Drug delivery method
Untreated	None	None	None
Frac Free CA4P	None	324 mg/kg (81 mg/kg every other day for a total of four doses over 7 days)	Systemic
Frac Lip	None	60 mg/kg (15 mg/kg every other day for a total of four doses over 7 days)	Liposomes
Frac IL	None	60 mg/kg (15 mg/kg every other day for a total of four doses over 7 days)	Immunoliposomes (anti-E-selectin conjugated)
Frac IR	20 Gy (2 Gy per day over 10 days)	none	None
Frac Free CA4P + Frac IR	20 Gy (2 Gy per day over 10 days)	324 mg/kg (81 mg/kg every other day for a total of four doses over 7 days)	Systemic
Frac Lip + Frac IR	20 Gy (2 Gy per day over 10 days)	60 mg/kg (15 mg/kg every other day for a total of four doses over 7 days)	Liposomes
Frac IL + Frac IR	20 Gy (2 Gy per day over 10 days)	60 mg/kg (15 mg/kg every other day for a total of four doses over 7 days)	Immunoliposomes (anti-E-selectin conjugated)

Tumor growth was measured daily with the aid of a metric caliper and tumor volume was calculated by using the equation for a cylinder ($\pi \times r^2 \times h$ where r is half the width of the tumor and h is the tumor length). At the end of the experiment tumors were excised and calculated volumes verified following euthanasia of the animal. The mice bearing MCa-4 tumors were euthanized when tumor volumes reached approximately 10–15% of the animal's total body weight (tumor volume of 3–4 cm^3). The average animal body weight was 23.8±1.7 g and did not change significantly over time. All experiments adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985).

Immunohistochemistry of Transplanted Tumor Models

MCa-4 tumors used for immunohistochemistry received no radiation, or a single 5 Gy dose of radiation. Irradiated tumors were excised and paraffin embedded at 6, 24, and 48 h following irradiation and untreated tumors were also excised and paraffin embedded (t=0 h). The tissue was sectioned at four microns in thickness, placed on electromagnetically charged slides and stained with hematoxylin and eosin for routine histological analysis. Immunohistochemistry was then used to determine the expression levels of E-selectin, using the Avidin-Biotin-Peroxidase methodology according to the manufacturer's instructions (Vectastain, Vector Laboratories, Burlingame, CA). Briefly, sections were deparaffinized and re-hydrated through alcohol up to water. Non-enzymatic antigen retrieval was performed by placing the slides in citrate buffer pH 6.0, at 96°C for 30 min. After a cooling period of 20 min, endogenous peroxidase was quenched with 30% H_2O_2 in methanol for 20 min. After rinsing with PBS, sections were blocked with 5% normal horse serum for 2 h at room temperature. Then primary antibodies in 0.1% PBS/BSA were added and incubated overnight at room temperature in a humidified chamber. The antibody utilized for this study was a mouse monoclonal anti-E-selectin (Clone 2Q780, 1:100 dilution, Santa Cruz Biotechnology). After rinsing with PBS, biotinylated secondary antibodies were added and incubated for 1 h at room temperature followed by avidin-horseradish peroxidase complexes (Vectastain ABC peroxidase kit, Vector Laboratories), also for 1 h. Finally, the peroxidase on the sections was developed with diaminobenzidine (DAB, Roche Laboratories), for 3 min and sections were lightly counterstained with Hematoxylin (Fisher Scientific), and mounted with permount. Adjacent tissue sections were similarly processed with isotype-matched nonspecific primary antibody (normal mouse IgG from Santa Cruz).

Liposome Formulation

Long circulating liposomes were composed of HSPC, cholesterol, and DSPE-PEG in a molar ratio 50:45:5. For preparation of long circulating liposomes with attached anti-E-selectin, a fraction of DSPE-PEG (2 mol%) was replaced by DSPE-PEG-maleimide functional lipid. Liposomes were prepared by extrusion. Briefly, lipids dissolved in chloroform were mixed in appropriate amounts, the solvent was evaporated by a stream of nitrogen and then the sample was placed in a lyophilizer overnight to thoroughly evaporate excess chloroform. Afterwards, the thin dry film of lipid was suspended in a buffer containing CA4P (20 mM Tris–HCl, 135 mM NaCl, 200 mM CA4P, pH=7.2 if no antibody was to be attached, or pH 6.0 when antibody was to be coupled) preheated at 50°C and vortexed for 3 min. Then the suspension was extruded (Lipidex, Vancouver, Canada) 10 times at 50°C through a membrane of 200 nm pore size.

Long circulating liposomes with no antibody had a mean size of 173.2±3.7 nm; the addition of antibody to the surface of liposomes slightly increased their size to 190.6±3.7 nm. Both liposomes and immunoliposomes remained stable for at least 2 weeks.

Coupling of Anti-E-selectin to the Liposome Surface

Liposomes having 2 mol% of DSPE-PEG-maleimide were used to couple thiolated anti-E-selectin onto the distal end of functionalized PEG polymer thereby forming immunoliposomes. Briefly, anti-E-selectin (0.5 mg/mL) in 60 mM triethanolamine-HCL, 100 mM NaCl, 1 mM EDTA, pH 8 was combined with fivefold excess of 2-iminothiolane and incubated for 1 h at room temperature. A 30,000 MW cutoff Centricon (Millipore) filter was used to remove 2-iminothiolane

by dialysis. The antibody in 20 mM Tris–HCl, 135 mM NaCl at pH 6.0 was added to freshly made liposomes in a molar ratio of 1 antibody per 180 maleimide groups and incubated overnight at 4°C. The unincorporated antibody and CA4P were removed by ultracentrifugation at 30,000 rpm for 1 h at 4°C. The pellet containing IL (or Lip) was then resuspended in 20 mM Tris–HCl, 135 mM NaCl at pH 7.2 and used immediately. The number of antibody molecules attached to the surface of the liposomes were calculated from the size of the liposome and the assumption that, on average a lipid molecule occupies a cross sectional area of 0.7 nm^2 (14). Based on these assumptions each liposome had 16.3±4.1 antibody molecules on its surface.

Determination of Entrapped Combretastatin

The free CA4P was separated from liposomes by column chromatography using Sephadex G-50 (10×150 mm), eluted with a solution of 20 mM Tris, 135 mM NaCl (of pH 7.2 for liposomes and pH 6.0 for immunoliposomes). The fraction containing the CA4P entrapped in liposomes was determined by colocalization of the peak of drug fluorescence (excitation=340 nm and emission=435 nm) and scattering of light (excitation=600 nm, emission=615 nm) using an ISS K2 fluorometer (Champaign, IL). The fractions containing CA4P entrapped in liposomes were combined; 500 μL of this solution was then added to 2 mL of a mixture of chloroform–methanol (2:1), vortexed and centrifuged at 2,000×*g*. After centrifugation two phases were visible. The bottom layer, non-polar phase, contained the phospholipids dissolved in chloroform and methanol. The top layer, polar phase, contained CA4P dissolved in water and methanol. The volume of both phases was estimated and a phosphorus assay (15) was performed for each of the phases to obtain the ratio of CA4P to phospholipid. The encapsulation of CA4P was 0.126±0.04 mg drug/μmol of phospholipid, representing a concentration of drug inside the liposome of about 40 mM. An *in vitro* 96 well plate assay was used to confirm the selective adhesion of the immunoliposomes to E-selectin coated surfaces (16).

Circulation Time of Liposomes and Immunoliposomes

The blood residence time of liposomes and immunoliposomes was determined *in vivo* to ensure that immunoliposomes circulated in blood long enough to attach to E-selectin and deliver encapsulated drug. Long circulating liposomes and immunoliposomes were prepared with 44 mol% of cholesterol with 1 mol% of cholesteryl-BODIPY added as a non-exchangeable fluorescent probe. Liposomes or immunoliposomes in an amount equivalent to 1 μmol of lipid (10 nmol of cholesteryl-BODIPY, 200 μL of liposomal solution maximum) was administered to normal healthy mice by retro-orbital injection. To determine the circulation time of liposomes and immunoliposomes, blood was taken from mice *via* heart puncture at 45 min, 2, 4, 8, 12, 24 and 48 h post injection. Three animals were used for each time point, and each animal was used once for blood drawing. The plasma was recovered by centrifugation of blood samples at 2,000 rpm. The emission spectrum of cholesteryl-BODIPY in the plasma was read at an excitation wavelength of 550 nm and an emission wavelength ranging from 580 to 650 nm using an ISS K2 fluorometer (ISS Inc., Champaign, IL). The fluorescence intensity at 600 nm was normalized by dividing by the weight of the animal and plotting to determine the clearance of circulating liposomes or immunoliposomes with time. The fluorescence of plasma with no liposomes was measured and subtracted from plasma with liposomes. The single exponential $y = A_1 \times e^{-x/t_1} + y_0$ was fitted (Origin 7.5, Origin Lab Corporation, Northhampton, MA) to determine the lifetime (t_1) of circulating liposomes and immunoliposomes in blood. The fluorescence intensity was normalized by weight and by the estimated initial fluorescence intensity (A_1+y_0).

Vascular Perfusion in Treated and Untreated Tumors

DiOC7 (3,3′-diheptyloxacarbocyanine iodide, (Invitrogen, Eugene, OR) 1.0 mg/kg in 75% DMSO) was injected i.v. 1 min before the tissue was removed and rapidly frozen. This dose provides optimal fluorescent visualization of perfused vasculature by preferentially staining cells immediately adjacent to perfused blood vessels (17). Tissue was then sectioned (10 μm thick) at −20°C using a cryostat and mounted on poly-L-lysine-coated glass slides for later imaging to map the location of perfused vessels. Using a digital camera (Qimaging Retiga 1300, Qimaging, Surrey, BC Canada) mounted on a Nikon Eclipse TE200 inverted microscope (Nikon, Melville, NY) and a motorized stage (Ludl Electronic Products, Hawthorne, NY) controlled by Scope Pro Plus (MediaCybernetics, Silver Spring, MD), representative 4×4 montages at ×10 magnification were taken of each tumor section (approximately 1 mm^2). Distance from the nearest perfused vessel for each point in the tumor tissue was calculated, using image calculation software from Image-Pro Plus, for each treatment group.

Statistical Analysis

Unless otherwise noted, data are presented as Mean ± SEM. Analysis of Variance (SigmaStat 3.1, Systat Software Inc., San Jose, CA) with planned contrast (Student–Newman–Keuls) was used to determine significant differences in tumor volume among experimental groups. The Kolmogorov–Smirnov Test (Statgraphics Centurion XV, StatPoint Inc., Herndon, VA) was used to determine significant differences in the distribution of distances from the nearest perfused vessel data.

RESULTS

Upregulation of E-selectin in Irradiated Tumors

Paraffin embedded sections from MCa-4 tumors showed that endothelial cells expressed a basal level of E-selectin (arrows, Fig. 1, Panel A). However, expression of E-selectin significantly increased in irradiated MCa-4 tumors 6 and 24 h post-irradiation (arrows, Fig. 1, Panels B and C respectively), before returning to a near basal level 48 h post-irradiation (arrows, Fig. 1, Panel D). In Fig. 1, arrows point to positively stained endothelial cells in large vessels while other areas expressing E-selectin are smaller capillaries. These experiments verified that the time course for the upregulation of E-

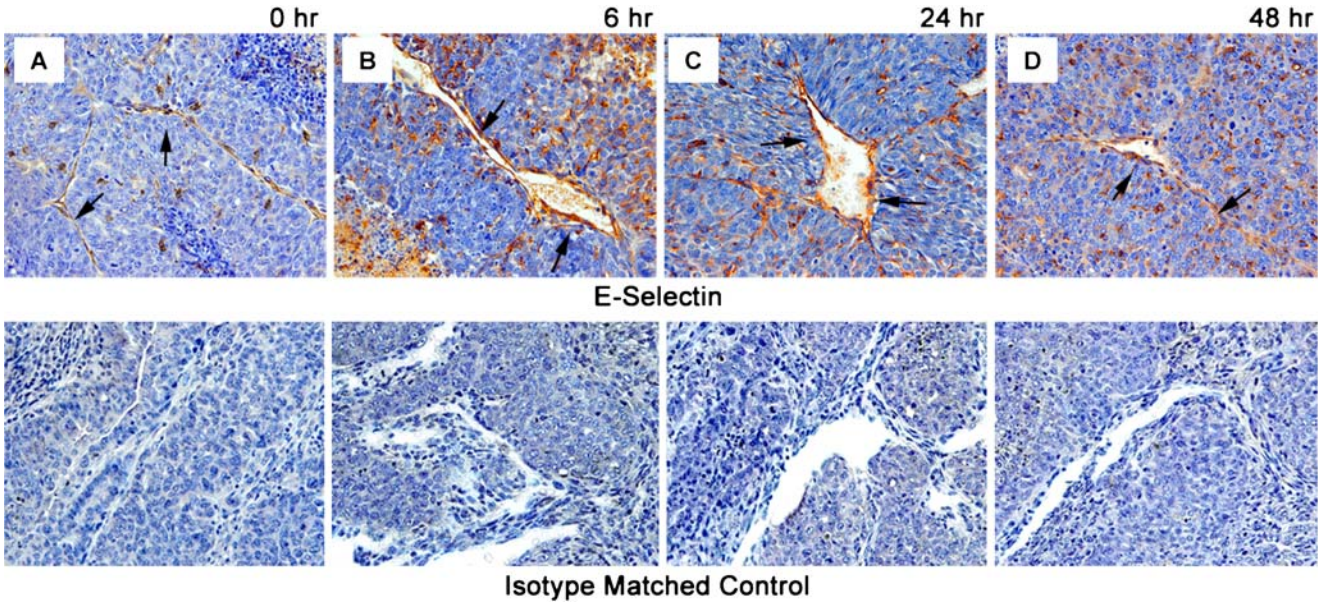

Fig. 1. Immunohistochemistry was used to detect E-selectin in non-irradiated control MCa-4 tumors 0 h (Panel **A**) and irradiated MCa-4 tumors (6, 24, and 48 h) after irradiation. Expression of E-selectin significantly increased in irradiated MCa-4 tumors 6 and 24 h post-irradiation (Panels **B** and **C** respectively), before returning to a near basal level at 48 h post-irradiation (Panel **D**, *arrows*). *Brown color* staining indicates upregulation of E-selectin in capillaries with *arrows* pointing to positive endothelial cells in large vessels. Non-specific staining of necrotic areas is observed in the *lower left corner* of Panel **B**. Isotype matched controls are shown for each time point to demonstrate the specificity of the immunolabeling. All panels original magnification ×400.

selectin within tumor vasculature matched that previously reported in irradiated normal tissue (12). The information gathered during these experiments provided time points following irradiation that were optimal for targeted drug delivery to tumor vasculature.

Circulation Time of Liposomes and Immunoliposomes

The blood residence times of circulating liposomes and immunoliposomes were measured *in vivo* in healthy mice. A single exponential was fitted to the data, with $R^2>0.9$. The half-life in blood was determined to be 10.8±1.8 h for liposomes (Lip) and 9.9±0.9 h for immunoliposomes (IL, Fig. 2).

Targeting of Combretastatin to Tumors Treated with a Single Dose of Radiation

MCa-4 mammary carcinomas were treated with a single 5 Gy dose of radiation (IR) and anti-E-selectin conjugated immunoliposomes (ILs). Tumor volume was measured on a daily basis. As shown in Fig. 3, the increase over time in tumor volume in the IL + IR group was significantly ($p<$ 0.001) different from every other group tested. It is important to note that the treatment with the typical clinical dose of 81 mg/kg free (non-encapsulated) CA4P (i.e. 5.4 times greater than that administered by immunoliposomes) did not result in significant control of tumor volume, indicating that successful targeting of combretastatin to the irradiated tumors can result in effective tumor control at a much lower drug dose.

Vascular Perfusion in Treated and Untreated Tumors

Sections of excised MCa-4 tumors were imaged for perfused vessels (using $DiOC_7$) to determine if various treatments with CA4P result in vascular shutdown and increase in average distance from the nearest perfused vessel. As shown in Fig. 4, the cumulative frequencies of distances from the nearest perfused vessel for all CA4P treatment

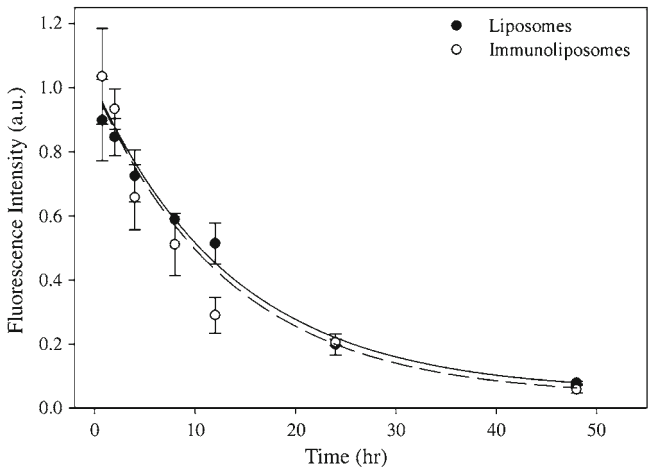

Fig. 2. Cholesteryl-BODIPY fluorescence intensity (F.I.) was used to estimate the blood half-life ($t_{1/2}$) of liposomes (Lip) and immunoliposomes (IL) in healthy mice. The fluorescence intensity was normalized by weight and initial F.I. administrated. For Lip $t_{1/2}$= 10.8±1.8 h and for IL $t_{1/2}$=9.9±0.9 h. The *solid line* is the exponential curve fit for liposomes and the *dashed line* is the exponential curve fit for immunoliposomes.

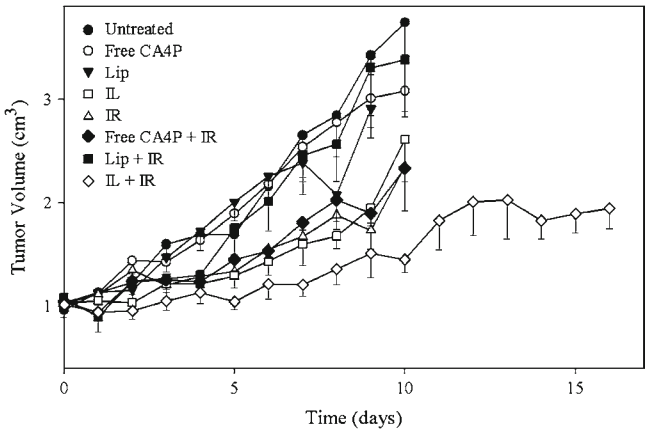

Fig. 3. Targeting anti-E-selectin conjugated immunoliposomes (*IL*) loaded with the antivascular drug combretastatin (*CA4P*) to MCa-4 mammary tumors that were treated with a single dose of irradiation (*IR*) resulted in a significant delay in tumor growth when compared with other treatment groups (see Table I for a description of the various treatment groups).

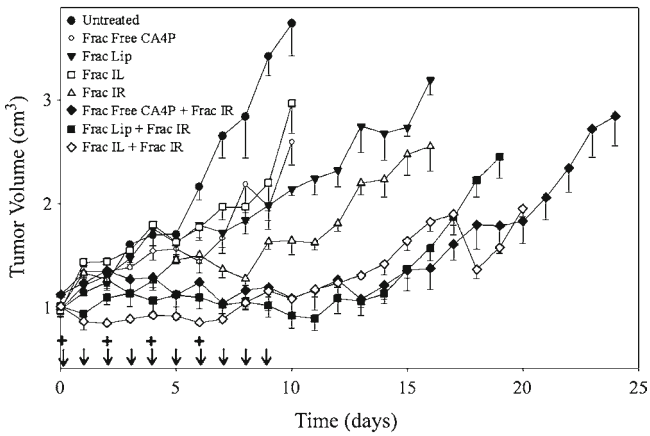

Fig. 5. The antivascular drug combretastatin (*CA4P*) was targeted to MCa-4 mammary carcinomas treated with fractionated irradiation (*Frac IR*) using fractionated doses of anti-E-selectin conjugated immunoliposomes (*Frac IL*). Frac IR plus multiple doses of immunoliposomes resulted in significant tumor growth delay. However, other treatment groups that combined radiation and CA4P also resulted in tumor growth delays that were significantly different from control. *Arrows* indicate days when radiation was administered and *plus signs* indicate days when systemic CA4P, liposomes, or immunoliposomes were administered, depending on treatment group (see Table II for a description of the various treatment groups).

groups were significantly different from untreated or IR treated tumors ($p<0.001$, using the Kolmogorov–Smirnov test) with the IL + IR group exhibiting the largest increase in the distance from the nearest perfused vessel.

Targeting of Combretastatin to Tumors Treated with Fractionated Doses of Radiation

Combretastatin was targeted to MCa-4 mammary carcinomas treated with fractionated radiation (2 Gy a day for a total of 20 Gy over 10 days) using fractionated doses of anti-E-selectin conjugated immunoliposomes. As shown in Fig. 5, fractionated radiation plus fractionated doses of immunoliposomes resulted in significant tumor growth delay. Additionally, treatment with Frac IL + Frac IR significantly ($p<0.001$) enhanced tumor growth delay compared to a single dose of immunoliposomes and irradiation (Fig. 6). However, the tumor growth delay of the Frac IL + Frac IR treatment group was not significantly different from the two other fractionated treatment groups that combined radiation and CA4P.

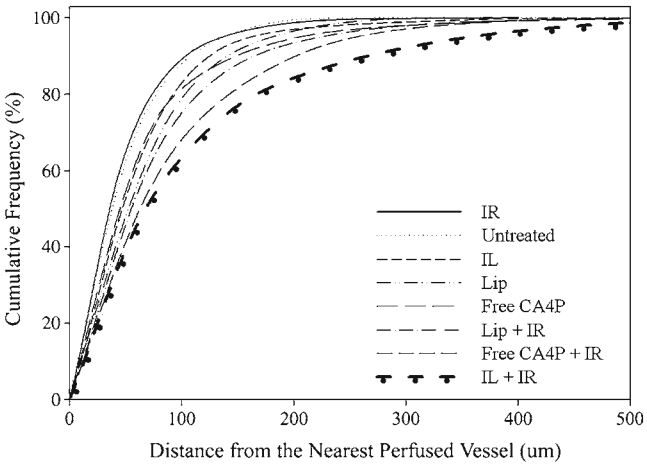

Fig. 4. Tissue distances from the nearest perfused vessel 2 days after treatment. The cumulative frequencies of distances from the nearest perfused vessel for all combretastatin (*CA4P*) treatment groups were significantly different from untreated or irradiated (*IR*) treated tumors with the anti-E-selectin immunoliposomes (*IL*) + IR group exhibiting the largest right shift in the distribution of distance from the nearest perfused vessel (see Table I for a description of the various treatment groups).

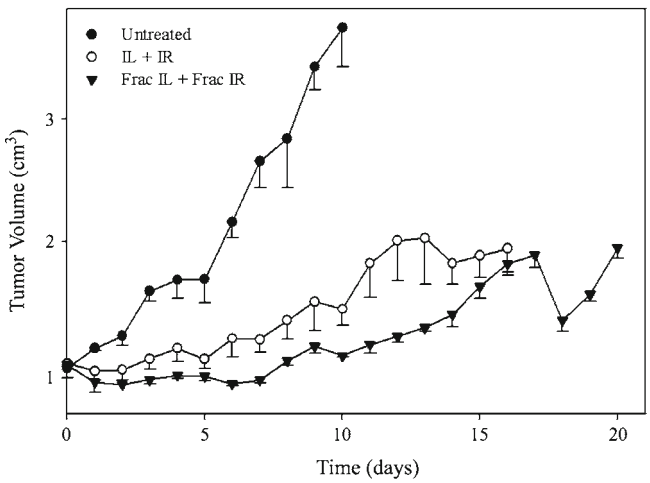

Fig. 6. Treatment with fractionated doses of anti-E-selectin conjugated immunoliposomes combined with fractionated irradiation significantly ($p<0.001$) enhanced tumor growth delay compared to a single dose of anti-E-selectin conjugated immunoliposomes combined with a single dose of irradiation (see Tables I and II for a description of the two treatment groups).

DISCUSSION

During the past 10–15 years several promising antivascular therapies have been developed that attempt to damage the existing tumor microvasculature and cause a rapid and extensive shutdown of tumor blood flow. The antivascular agents used in these therapies have been successful enough to warrant clinical trials (18) but most of these antivascular drugs have undesirable side effects in many normal tissues and are only effective at or above maximally tolerated doses. The goal of this study was to determine if preferential targeting of antivascular drugs to irradiated tumors could cause a significant tumor growth delay compared to treatment with systemic administration of antivascular drugs or with radiation alone.

In our previous study (10) we showed the feasibility of targeting drugs to irradiated tumors. However, to show the clinical relevance of the targeted therapy proposed in this study for treating solid tumors, it must be shown that: (1) the proposed therapy can inhibit tumor growth in different models; (2) there are various adhesion molecules that can be targeted and that these molecules may be upregulated at various times after irradiation; (3) determine the response of this treatment under various radiation and targeted drug dosing conditions; and (4) to make this therapy more clinically relevant, the drug formulation that is used is similar to that currently used in clinical trials.

To address the first issue, we show that this proposed therapy inhibits tumor growth in a mammary tumor model which has characteristics that are very different from the melanoma tumor model used in the previous study (10). The fact that our proposed therapy leads to tumor control in two different models is a strong indication that this treatment may be successful in various human tumors.

Secondly, a number of adhesion molecules (e.g., $\alpha_v\beta_3$, E-selectin) are upregulated in irradiated normal tissue and/or tumors (9–12). This therapy can be administered at various times after irradiation by targeting different molecules (e.g. 6–24 h post-IR for E-selectin *vs.* 1–4 h post-IR for $\alpha_v\beta_3$). Furthermore, $\alpha_v\beta_3$ (from the integrin family of adhesion molecules) and E-selectin (from the selectin family of adhesion molecules) are upregulated under different conditions. In clinical applications, it may be desirable to target one or both of these molecules depending on the tumor type.

Thirdly, in the current study we systematically compared the effects of single doses of radiation + targeted therapy *vs.* multiple doses of radiation + targeted therapy. Our findings indicate that treatment with a single 5 Gy dose of radiation followed by a single dose of anti-E-selectin conjugated immunoliposomes encapsulating CA4P results in a significant delay in tumor growth compared to all other treatment groups (Fig. 3). Following a single dose of this dosage form, the tumors grew only gradually and did not double in size even after 17 days while untreated tumors doubled their size in less than 6 days. Based on the results from this study and our previous findings (10), we then tested the hypothesis that a treatment combining fractionated radiation with fractionated doses of CA4P encapsulated immunoliposomes results in additional tumor growth delay. The findings from this part of the study support this hypothesis (Fig. 5). The growth of tumors was completely suppressed during the period of treatment (10 days) and for approximately 4 days thereafter. This growth delay however is not significantly different from other treatment groups that combined fractionated radiation and fractionated doses of systemic (free) or encapsulated CA4P. These findings indicate that combined fractionated radiotherapy and fractionated antivascular therapy, regardless of dosage form, results in significant tumor growth delay.

Finally, in the previous study we used the highly hydrophobic form of combretastatin (CA4) in our targeted formulation (10). However, clinically it is difficult to deliver this highly hydrophobic formulation and in the current study we have used the prodrug form of combretastatin (CA4P) which is currently in clinical trials (19–22). Also, the fact that we have been able to achieve significant tumor control with both CA4 and CA4P provides further support for the versatility of this treatment. Furthermore, we have improved our formulation by significantly increasing entrapment compared to the previous formulation. Our current entrapment is 0.126 mg drug/μmol lipid while the previous formulation entrapment was 0.0077 mg drug/μmol lipid (23). This will enable delivery of more drug with less liposomes. The use of the prodrug CA4P and our optimized formulation makes this therapy more clinically relevant and should facilitate the regulatory approval.

The targeted antivascular therapy developed in this study represents a novel methodology for treating hypoxic tumors that tend to be more resistant to various treatments including radiotherapy (24). Solid tumors are highly dependent on oxygen for survival and average distance in tumor parenchyma to the nearest perfused vessel, i.e. effective oxygen diffusion distance, is an indicator of tumor oxygenation. Antivascular therapies, as well as others, that result in an increase in average distance to the nearest perfused vessel could result in tumor control by reducing tumor oxygenation. Our findings indicate that in the treatment group combining radiation with immunoliposomal targeting of combretastatin, there was an observed increase in distance to the nearest perfused vessel. This treatment was also accompanied by significant tumor growth delay, supporting the idea that effective delivery of CA4P to the tumor inhibited growth of blood vessels and this was responsible for the suppression of tumor growth.

We have developed a targeted liposome system to preferentially and selectively target combretastatin A4, a novel antivascular agent, to irradiated tumors. This liposomal formulation has high drug loading and results in stable drug encapsulation (25). In the *in vitro* cell culture studies, targeted liposomes showed significantly higher binding to their target cells than non-targeted liposomes through specific interaction with upregulated receptors on the cell surface (23). Our *in vivo* studies indicate that selective targeting properties of this delivery system improve the therapeutic benefits of combretastatin A4 to tumors that have been irradiated with a single dose of ionizing radiation. However, further studies are needed to determine why targeted delivery of CA4P to tumors that have been treated with fractionated doses of ionizing radiation was not significantly different from the other fractionated treatment groups that combined radiation and CA4P. Further elucidation of the biodistribution of targeted liposomes and the time course of drug release from liposomes may provide additional insight into this process.

CONCLUSION

Our results strongly suggest that liposomes carrying CA4P and coated with anti-E-selectin are being targeted to irradiated tumors, resulting in a significant growth delay. This effect can be further potentiated using a fractionated irradiation dosing schedule combined with fractionated immunoliposome treatments. The novel findings of the current study combined with those of the previous study provide important compelling evidence for the feasibility of this approach.

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