ABSTRACT

The role of vascular permeability in the preferential accumulation of photosensitizers in tumor tissue was investigated. Two murine tumors [experimental mammary tumor carcinoma (EMT-6) and methylcholanthrene-induced rhabdomyosarcoma (MIS)] and a human bladder carcinoma (EJ) were grown s.c. on the flank in athymic nude mice and analyzed for in vivo vessel permeability, vascular permeability factor (VPF) secretion, and accumulation of the photosensitizer, chloroaluminum sulfonated phthalocyanine. In vivo tumor vessel permeability and vascular volume were quantitated by measuring Evans blue extravasation and accumulation of a high molecular weight fluoresceinated dextran, respectively. VPF was isolated from serum-free tumor cell conditioned medium using heparin-Sepharose affinity chromatography. Dot and Western blots stained with anti-VPF antiserum positively identified VPF in samples from each tumor. Chloroauminum sulfonated phthalocyanine pharmacokinetics in tumor-bearing mice were measured using a fiber-based spectrofluorometer.

In vivo vessel permeability was found to be greatest in MIS tumors, next in EMT-6 tumors and finally in EJ tumors. Consistent with in vivo data, MIS and EMT-6 tumor cells in culture secrete significantly more VPF than EJ tumor cells. Chloroauminum sulfonated phthalocyanine accumulation was approximately 2 times greater in MIS and EMT-6 tumors compared to EJ tumors. Our data present evidence that photosensitizer accumulation can be correlated to in vivo tumor vessel permeability and VPF secretion of that tumor. Taken together, the data support the hypothesis that vascular permeability differences among tumors play a significant role in the uptake and retention of photodynamic agents.

INTRODUCTION

Selective photosensitizer retention in tumors is the hallmark of photodynamic therapy with many porphyrins, chlorins, and phthalocyanines. Although it is believed that photodynamic destruction of tumors is largely due to effects on tumor vasculature (1, 2), the mechanisms responsible for photosensitizer retention in tumors are less clear. It is well documented that tumor: muscle ratios of a given photosensitizer vary with respect to the tumor type (3); however, the reason for this variability has yet to be elucidated. Tumor properties such as poor lymphatic drainage (4), variability in vessel density (5, 6), incomplete subendothelial barrier (7, 8), unique microvascular endothelium (9), and increased vessel permeability (10, 11) may all play a role in the selective retention of photosensitizers. The relative contribution of each one of these tumor characteristics may determine the variation in photosensitizer retention among different tumor types.

VPF* (also known as vascular endothelial growth factor) is a highly conserved heparin-binding protein (12) which has recently been purified and sequenced (13, 14). This tumor-secreted factor has a molecular weight of 34,000–43,000 (15) and is specific and mitogenic for endothelium in vitro (16) and angiogenic in vivo (17). A variety of tumor cells secrete this growth factor which is responsible for increased vessel permeability characteristic of solid tumors (14, 15, 18, 19). This increased permeability results in greater extravasation of macromolecules, such as low density lipoprotein (20) and albumin (21). Once injected into an animal, most photosensitizers bind low density lipoprotein or albumin, both of which then act as carriers (22, 23). Therefore, any differences in vascular permeability among tumor types may critically influence the ability of a tumor to retain the photosensitizer.

We have recently demonstrated that increased vessel permeability without the presence of neovascular endothelium is not sufficient to provide selective retention of a variety of photosensitizers; however, permeable vessels resulted in increased uptake of all photosensitizers (24). We hypothesized that increased vessel permeability may be an additive factor in photosensitizer retention providing that neovascularization is present. In this article, we test this hypothesis by comparing the amount of photosensitizer uptake and retention among three tumors, the amount of vascular permeability activity secreted by the respective tumor cells in vitro, and the relative permeability of each tumor in vivo. We clearly demonstrate a positive correlation between photosensitizer retention, VPF secretion, and in vivo permeability among EMT-6, MIS, and EJ tumors.

MATERIALS AND METHODS

Sensitizers

CASPc (M, 835) was a gift from Ciba-Geigy (Basel, Switzerland) and was stored in the dark at ~20°C and diluted in phosphate-buffered saline (Gibco, Grand Island, NY) to a concentration of 1 mg/ml before use (25).

Cells

EMT-6 carcinoma cells were grown in tissue culture flasks with RPMI 1640. MIS cells were grown in Dulbecco’s modified Eagle’s medium. EJ cells were grown in McCoy’s 5A medium. All media were purchased from Gibco (Grand Island, NY) and contained 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum. Cells were incubated at 37°C under 5% CO₂. For tumor inoculation, cells were trypsinized and resuspended in medium at a concentration of 1 × 10⁶ cells/ml.

Anti-VPF Antiserum

The antiserum was raised in rabbits against a synthetic amino-terminal guinea pig VPF sequence (13). The antiserum was kindly provided by Dr. Don Senger.

Animals

Mice. For all murine experiments, male athymic nude mice (4-5 weeks old) were used. All protocols were approved by an independent animal use committee.

Guinea Pigs. Male Hartley albino guinea pigs (300–450 g) were used for the Miles assay to quantitate the vascular permeability activity secreted from each tumor cell type (26).

Received 11/2/91; accepted 10/20/92.

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1 This work was supported in part by ONR Contracts N00014-86-K0117 and R29 AR38918. W. G. R. received support from the Ford Foundation Postdoctoral Fellowship and NIH Postdoctoral Fellowship (GM13763).

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4 The abbreviations used are: VPF, vascular permeability factor; CASPc, chloroauminum sulfonated phthalocyanine; EMT-6, experimental mammary tumor; MIS, methylcholanthrene-induced rhabdomyosarcoma; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; i.d., intradermally.
Tumor Inoculation

Mice received a s.c. inoculation of 1 × 10⁶ cells on the right flank. Within 10–14 days (20–30 days for EJ tumors), the tumors were well vascularized and 5–8 mm in diameter. Tumors averaged 1.15 cm² at the time of measurements.

**In Vivo Spectroscopy**

Animals received an i.p. injection of CASPc at a concentration of 10 mg/kg body weight. CASPc distribution at 30 min and 1, 2, 3, 6, 9, and 24 h was determined spectrophotometrically in the tumor and contralateral flank (normal) using a SPEX Fluorolog 2 spectrofluorometer (SPEX, Edison, NJ) coupled to a bifurcated quartz fiber optic assembly (24). The Y-shaped fiber optic assembly consists of numerous quartz fibers, one half of which transmits light from the excitation source to the sample (e.g., tumor), while the other half transmits emitted fluorescence from the sample to an emission monochromator and photomultiplier tube. All measurements were completed in the dark with the fiber in contact with the tissue. CASPc was excited at 610 nm and emission was read from 630 to 800 nm. The fluorescent dextran was measured by exciting at 492 nm and monitoring emission from 500 to 550 nm. Data represent the average ± SD of four animals. Statistical significance was determined using Student’s t test.

**Tumor Vessel Permeability**

Tumor-bearing mice received a 0.1-ml i.v. injection of Evans blue dye (M, 961; 0.1% in saline; Baker Chemical Co., Phillipsburg, NJ). Twenty min later the animals were sacrificed and Evans blue was extracted from tumors and normal muscle as described under Mice vascular permeability assay (13, 27). To determine tumor vascular volume, a high molecular weight fluoresceinated dextran (M, 2,000,000) (Sigma Chemical Co., St. Louis, MO) was continuously monitored after i.v. injection (10 mg/kg) for 20 min. The peak fluorescence was used to calculate relative vascular volume among the tumor types. Data listed in Table 2 have been corrected for the vascular volume of each tumor. Data represent the average ± SD of 10–15 animals. Statistical significance was determined using Student’s t test.

**Vascular Permeability Factor Isolation**

Tumor cells were incubated as above with the following modifications. Cells were plated in T750 flasks and allowed to reach 90% confluency. Cells were then washed three times with PBS, and fresh serum-free medium was added. The conditioned medium was harvested 24 h later. Conditioned medium (500 ml) was filtered and passed over a heparin-Sepharose column, washed with PBS, and eluted with 2 bed volumes of 2 × NaCl. This procedure recovers the maximum tumor-secreted permeability-increasing activity. It is an abbreviated method described previously for the purification of VPF (13). NaCl eluants were concentrated using a Centricon 10 (Amicon, Danvers, MA) microconcentrator prior to electrophoresis, immunoblotting, and the Miles assay (described below). SDS-polyacrylamide (12.5%) gel electrophoresis was performed to identify the presence of vascular permeability factor in silver-stained with a commercially available kit (Bio-Rad Laboratories, Richmond, CA) in accordance with the manufacturer’s instructions. Bands were identified visually and using a densitometer. The gel was also transferred to nitrocellulose and stained with antiserum as described below. Concentrated VPF isolates (20 μl) were blotted onto a nitrocellulose strip and blocked with 3% BSA-PBS for 3 h. Anti-VPF antiserum was diluted 200-fold with 1% BSA-PBS and incubated with the nitrocellulose strip at room temperature for 1 h. The strip was rinsed briefly with double-distilled water (ddH₂O) and washed twice with PBS for 10 min. It was then covered with goat anti-rabbit IgG horseradish peroxidase, diluted 1:3000 with 1% BSA-PBS, and allowed to incubate at room temperature for 1 h. After a washing with PBS, diaminobenzidine (Sigma) was added and allowed to develop for 5 min. Western blots were stained as above.

**Miles Vascular Permeability Assay**

In order to quantitate the extent of permeability caused by each VPF sample, shaved and depilated guinea pigs were anesthetized prior to receiving a 0.2-ml i.v. injection of Evans blue dye. After 2 h, 0.05 ml of concentrated VPF was injected i.d. Twenty min later, the animals were euthanized and the injection spot (1 cm in diameter) was surgically removed. Tissues were dissolved in 1 ml of tissue solubilizer (Soluene 350; Packard Industries, Downers Grove, IL) at 37°C overnight and then at 57°C for 1 h. The solution was allowed to cool before the addition of 2 ml of ethyl acetate, the mixture was vortexed, and 2 ml of 1 N HCl were added, vortexed again, and centrifuged at 2500 rpm for 15 min. Absorption of the upper phase was read at 626 nm on a diode array spectrophotometer (Model 8451A; Hewlett Packard, Palo Alto, CA). Concentrations were determined from a standard curve of Evans blue dye. Data represent the average ± SD of eight injections and are corrected for the amount of total protein injected. Statistical significance was determined using Student’s t test for all experiments.

**RESULTS**

CASPc distribution in EMT-6, MIS, and EJ tumors is shown in Table 1. CASPc concentration is significantly greater at 2, 3, 6, and 24 h postinjection in MIS tumors compared to EJ tumors (P < 0.08). Photosensitizer amount in EMT-6 tumors is greater at 3, 9, and 24 h compared to the EJ tumors (P < 0.07). There is also a slight increase in CASPc concentration in MIS tumors versus EMT-6 tumors at 2 and 3 h but this increase is not statistically different. Differential retention of CASPc in MIS, EMT-6, and EJ tumors, grown in the same mouse strain, led to experiments measuring tumor vascular volume and permeability.

Evans blue was extracted from tumors as a measure of tumor vessel leakiness. Differences in vascular volume among the tumor types were accounted for when determining tumor vascular permeability. To measure vascular volume, tumor-bearing animals were monitored spectrofluorometrically after injection with a large Mᵣ fluoresceinated dextran. Peak fluorescence always occurred between 5 and 10 min postinjection, regardless of tumor type. The peak fluorescence signal was used to determine relative vascular volumes among tumor types.

<table>
<thead>
<tr>
<th>Time (postinjection)</th>
<th>MIS</th>
<th>EMT-6</th>
<th>EJ</th>
<th>(MIS, EMT-6 vs. EJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>0.46 ± 0.16</td>
<td>0.42 ± 0.22</td>
<td>0.34 ± 0.12</td>
<td>NS², NS</td>
</tr>
<tr>
<td>1 h</td>
<td>0.79 ± 0.19</td>
<td>0.68 ± 0.20</td>
<td>0.51 ± 0.17</td>
<td>NS, NS</td>
</tr>
<tr>
<td>2 h</td>
<td>1.0 ± 0.21²</td>
<td>0.78 ± 0.21</td>
<td>0.63 ± 0.23</td>
<td>0.02², NS</td>
</tr>
<tr>
<td>3 h</td>
<td>1.0 ± 0.17²</td>
<td>0.84 ± 0.15²</td>
<td>0.63 ± 0.17</td>
<td>0.004², 0.07²</td>
</tr>
<tr>
<td>6 h</td>
<td>0.94 ± 0.18²</td>
<td>0.85 ± 0.13</td>
<td>0.72 ± 0.20</td>
<td>0.08², NS</td>
</tr>
<tr>
<td>9 h</td>
<td>0.91 ± 0.23</td>
<td>0.89 ± 0.07²</td>
<td>0.71 ± 0.19</td>
<td>0.004², 0.003²</td>
</tr>
<tr>
<td>24 h</td>
<td>0.75 ± 0.08²</td>
<td>0.76 ± 0.14²</td>
<td>0.47 ± 0.12</td>
<td>0.005², 0.003²</td>
</tr>
</tbody>
</table>

¹ P values compare CASPc amount in MIS or EMT-6 tumors to amount in EJ tumors.
² NS, not significant.
³ CASPc pharmacokinetics in MIS tumors.
⁴ CASPc pharmacokinetics in EMT-6 tumors.
Vascular leak is greatest in M1S and EMT-6 tumors and least in EJ tumors (Table 2). Since tumor vessel permeability is due, in part, to the tumor-secreted protein, VPF, we decided to analyze tumors for the production of this factor.

The heparin-binding fraction of serum-free conditioned medium from each tumor cell was analyzed for the presence of VPF. Silver-stained SDS-polyacrylamide gels revealed a broad band corresponding to Mr 35,000-50,000 in unreduced samples and Mr 14,000-25,000 with reduced samples common to all isolates. Dow blots stained with antisera to a VPF-synthetic peptide identify the presence of VPF from each tumor cell isolate (Fig. 1A). Western blots stained with antisera to VPF demonstrate the presence of 3 bands (Mr 43,000, 37,000, and 24,000) common to all samples (Fig. 1B). A Miles assay was performed to measure the amount of vascular permeability activity secreted by each tumor (Table 3). M1S and EMT-6 tumor cells secrete more vascular permeability activity than EJ cells.

DISCUSSION

Presently in phase I-III clinical trials, photodynamic therapy has been used to treat a variety of malignancies over the past 20 years (28). Therapeutic response and photosensitizer retention can vary drastically depending on tumor type and size (29, 30). The varied response has been hypothesized to be due to many factors, such as vessel density (5), oxygen perfusion (31), vessel integrity (32), and therapeutic resistance (33, 34). However, the molecular mechanisms involved in differential photosensitizer retention and therefore therapeutic response have not been described. Recently, we have demonstrated that tumor-selective photosensitizer retention necessitates the presence of proliferating neovascular endothelium (24). While increased vascular permeability alone was not sufficient for photosensitizer retention, it did increase photosensitizer uptake. These results prompted our hypothesis that variations in vascular permeability among tumors could account for differences in photosensitizer retention and therefore therapeutic response of the tumors to photodynamic therapy.

CASPc is a well characterized photosensitizer (35) which has proved to be one of the safest (25, 36) and most effective photosensitizers available (37). The M1S and EMT-6 tumors are syngeneic for DBA/2 and BALB/c mice, respectively, whereas the EJ tumor was originally derived from a human bladder carcinoma. Our interest focused on the inherent characteristics of each tumor responsible for photosensitizer retention differences noticed among these tumors, thus necessitating the use of the same animal model for the growth of all three tumors. The growth characteristics are similar for all tumors, with the EJ tumor growing slightly more slowly initially. This slight lag in initial growth was taken into account by injecting the CASPc once the tumors were in identical growth phases, which ranged from 10 to 30 days postinoculation.

We examined the distribution of CASPc in M1S, EMT-6, and EJ tumors and contralateral flank (skin and muscle) spectrofluorometri- cally (Table 1). The in vivo fluorescence spectroscopy method has proven to be an accurate and reliable measure of photosensitizer concentration in superficial tissues, such as a s.c. tumor (38). Our data clearly demonstrate significant differences in CASPc uptake and retention among a variety of tumor types. In preliminary experiments, similar results were obtained with Photofrin and the mono-t-aspartyl-chlorin compound, NP6-MACE.

The reason for the photosensitizer uptake/retention differences among these tumors was further explored by characterizing the vessel leakiness in each tumor. Evans blue has been used extensively to quantitate vascular permeability due to its strong affinity for serum albumin (27, 39). Capillary permeability can be measured based on the extravasation of this dye, which is easily extracted from tissues. Evans blue extravasation can be due to increased tumor vessel density, permeability, or both. This method is appropriate if the tumor vascular volumes are comparable. Vascular volume of each tumor was measured by modifying a standard protocol which uses a high molecular weight dextran to measure vascular volume (40). Our spectroscopic

<table>
<thead>
<tr>
<th>Table 2 Relative tumor vascular permeability*</th>
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<tbody>
<tr>
<td>Tumor**</td>
</tr>
<tr>
<td>M1S</td>
</tr>
<tr>
<td>EMT-6</td>
</tr>
<tr>
<td>EJ</td>
</tr>
</tbody>
</table>

* Data represent mean ± SD of 10-15 animals.
** Normal tissue values have been subtracted.
† Relative vascular volume of each tumor has been factored into the amount of vascular permeability as measured by Evans blue extravasation (M1S = 0.41 ± 0.22; EMT-6 = 0.62 ± 0.22; EJ = 0.89 ± 0.21).
# M1S versus EJ (P < 0.01).

Fig. 1. In A, dot blots of VPF isolated from EJ, EMT-6, and M1S tumor cells stained with anti-VPF antisera confirm the presence of VPF. Concentrated isolates were blotted onto a nitrocellulose strip and stained as described in "Materials and Methods." In B, SDS-polyacrylamide gel electrophoresis of concentrated VPF secreted from tumor cells stained with anti-VPF antibodies demonstrates the presence of three bands (Mr 43,000, 37,000, and 24,000) common to all tumors corresponding to tumor-secreted VPF, kD, molecular weight in the thousands.

Table 3 Tumor-secreted vascular permeability activity

<table>
<thead>
<tr>
<th>Tumor cell</th>
<th>ng Evans blueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1S</td>
<td>1.40 ± 0.33b</td>
</tr>
<tr>
<td>EMT-6</td>
<td>1.36 ± 0.18c</td>
</tr>
<tr>
<td>EJ</td>
<td>0.90 ± 0.24</td>
</tr>
</tbody>
</table>

* Values listed as ng above control (PBS-injected site typically resulted in <0.1 ng Evans blue).
** M1S versus EJ (P < 0.004).
† EMT-6 versus EJ (P < 0.001).
method allows the continuous measurement of circulating dextran which accounts for slight variations in blood flow among animals and tumors. Additionally, fluorescence monitoring of the dextran and Evans blue extraction can be performed on the same animal; thus vascular leak and volume can be measured in the same animal. Vascular permeability is greatest in M15 and EMT-6 tumors. However, only M15 tumors demonstrated a statistically significant increase compared to EJ tumors. Although the difference between vascular permeability of M15 and EMT-6 tumors is not statistically different, it is consistent with the increased CASPc uptake in M15 tumors noticed at 2 and 3 h.

To discriminate whether differences in tumor vessel permeability correlated with tumor cell production of VPF, we isolated and analyzed the amount of VPF secreted by each tumor using an abbreviated method for the purification of VPF. Extensive purification greatly enhances specific activity, but the total activity is decreased (13). Our objective was to isolate the total vascular permeability activity of each tumor; therefore, further purification was undesirable. Undoubtedly, our method isolated more than just VPF. However, it has been demonstrated that heparin binds all the vascular permeability activity in tumor cell-conditioned medium (13). To ensure that our isolates contained VPF, the samples were run on SDS-polyacrylamide gel electrophoresis. Silverstaining revealed many bands as determined by densitometric readings. Common to all samples was a group of bands corresponding to M, 35,000-50,000 in nonreduced samples and M, 14,000-25,000 when reduced, consistent with previously published reports for VPF (13, 18). Dot and Western blots positively identified VPF in each sample. This rabbit antibody generated against a purified guinea pig VPF fragment cross-reacts with mouse and human VPF and binds all the tumor-secreted vascular permeability activity (12). VPF is secreted by all three tumor cell types and is present in all three isolates.

The concentrated VPF samples were then used in a Miles vessel permeability assay to measure the permeability activity of each tumor. The data demonstrate that M15 and EMT-6 tumors secrete more vascular permeability activity than EJ tumors, consistent with in vitro tumor permeability. Moreover, the in vitro tumor leakiness, VPF secretion, and CASPc uptake and retention are positively correlated for all three tumors.

In reviewing the data, one may notice the seemingly contradictory correlation among tumor growth rate, VPF secretion, and tumor vascular volume. While the EMT-6 and M15 tumors secrete more VPF and grow more rapidly, they have the smallest vascular volume. These results may provide some insight into the tumor developmental process. It may be expected that tumor cells which divide more rapidly and secrete more angiogenic factors would necessarily have greater vascular density. However, one could hypothesize that a slower growing tumor provides sufficient time for the surrounding host vessels to invade the tumor and form an angiogenic plexus, while rapidly dividing tumor cells outgrow the infiltrating neovascular endothelium which would explain the differences observed among EMT-6, M15, and EJ tumors.

It is interesting to note that photosensitizer retention is relatively unaffected by increased permeability of the tumors. Although the overall amount of CASPc is increased at 24 h in M15 and EMT-6 tumors compared to EJ tumors, the rates of retention are identical. Notice that the slope of retention from 9 to 24 h is very similar regardless of the tumor. The factor accounting for differences in CASPc amount in these tumors at 24 h is the maximum amount of photosensitizer uptake, not differences in retention. Moreover, the maximum amount of photosensitizer uptake is positively correlated to the amount of vascular permeability. These data are consistent with our earlier hypothesis that tumor neovascularature is necessary for photosensitizer retention and increased permeability is responsible for increased uptake, not retention (24).

The potential for tumor vessel leakiness, measured by tumor secretion of VPF, does not exactly predict the in vivo tumor leakiness, as measured by Evans blue extravasation. A direct correlation among CASPc uptake/retention, in vivo vascular permeability, and tumor cell VPF production is not expected. Vascular permeability is only one biological property of tumors among many which are undoubtedly important for photosensitizer uptake and retention. Furthermore, tumor vessels are extremely heterogeneous within a tumor (10) and other variables besides tumor-secreted factors (4) can affect the in vivo vessel permeability. However, the amount of VPF secreted by the tumors reported here does correlate with in vivo leakiness and photosensitizer uptake and retention. It may, therefore, provide a simple method to determine which tumors will have the greatest uptake and retention of an antitumor agent, such as a photosensitizer.

In this article, we have shown that different tumors growing in identical conditions have inherent and different uptake and therefore retention characteristics of the same photosensitizer. These tumors differ in vessel leakiness in vivo which is positively correlated to the amount of VPF secreted in vitro. Most importantly, the relative uptake and retention of a photosensitizer can be correlated to the inherent in vivo vessel permeability and VPF secretion of a given tumor. Measuring tumor vessel permeability may provide a simple method to screen tumors which would retain a given photosensitizer. It is expected that CASPc is representative of other photosensitizers, since pharmacokinetics and tumor retention mechanisms among most compounds are similar (24). We have provided data which support the hypothesis that increased vessel permeability in a tumor positively influences photosensitizer retention by increasing initial uptake. However, other factors including variable expression of proteoglycans or cell adhesion molecules on the tumor microvascular endothelium and the resulting affinity of naturally occurring photosensitizer-carrier molecules, such as albumin or low density lipoprotein, to tumor endothelium have yet to be investigated.

ACKNOWLEDGMENTS

The authors wish to thank Norah Chen for expert technical assistance, Dr. Anthony Cincotta for providing the EMT-6 cells, Quadra Logic Technologies (Vancouver, British Columbia, Canada) for providing the M15 cells, and Dr. Donald Senger (Beth Israel Hospital, Boston, MA) for providing the anti-VPF antiserum and helpful comments.

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