ABSTRACT

Tumor blood flow is an important determinant of the efficacy of presently available antineoplastic treatment modalities. Using 113 Sn-labeled microspheres, 25 μm in diameter, we measured blood flow to primary tumors and regional lymph node metastases in conscious SMT-2A mammary adenocarcinoma-bearing syngeneic rats following a single i.v. bolus injection of the calcium entry blocker, flunarizine. Tumor blood flow increased in a biphasic dose-dependent fashion; at a dose of 1 mg/kg, primary tumor blood flow increased approximately 50% (p < 0.001) without a significant change in heart rate or blood pressure. The increase in flow was distributed to both the peripheral viable and central necrotic regions of the tumor and was still detectable 45 min following administration of the drug. A similar increase in blood flow was demonstrated for lymph node metastases (p < 0.001).

INTRODUCTION

Since the classic paper by Thomlinson and Gray (31), it has been recognized that tumor cells, for which the distance to the nearest capillary is between 100 and 150 μm, are sufficiently hypoxic to confer a radioprotective effect. It has been postulated that those tumors which are most resistant to ionizing radiation are those which do not undergo reoxygenation following the use of fractionated therapy in an attempt to eradicate this population of chronically hypoxic cells (9). Recently, it has also been suggested that, within the peripheral, grossly viable region of solid tumors, there exist acutely hypoxic cells due to transient interruptions in blood flow (14, 30). Yamaura and Matsuzama (39), studying tumors grown in transparent chambers, found that postirradiation regrowth came from the well-vascularized periphery rather than from the chronically hypoxic cells in the necrotic center, thus providing indirect evidence for the importance of acutely hypoxic cells. In addition to influencing radioirradiability, the hypoperfused regions of solid tumors may also be more resistant to chemotherapeutic drug treatment (6, 37). For example, West et al. (37) found that avascular tumor spheroids greater than 250 μm, exposed to [3H]methotrexate, contained viable cells for which there was no detectable methotrexate incorporation due to limited drug diffusion. Thus, it is apparent that the efficacy of the presently available antineoplastic therapies is directly dependent, in part, upon tumor vasculature and blood flow.

We have reported recently that the calcium entry blocker, verapamil, increases tumor blood flow by approximately 50% in SMT-2A mammary adenocarcinoma-bearing rats (17). However, the use of verapamil as a vasodilator may be limited by its negative chronotropic and ionotropic effects (11, 29). Flunarizine is also a potent calcium entry blocker, but it has greater specificity for peripheral arteriolar smooth muscle with little myocardial depressant activity (7, 32, 33). In several clinical studies (26, 28, 36), this compound has exhibited little or no cardiac toxicity following p.o. administration for peripheral vascular disease. Flunarizine demonstrates a slower onset and longer duration of action than does verapamil in inhibiting agonist-mediated vasorestriction in isolated vascular smooth muscle preparations (23, 32, 33). Additionally, flunarizine has a plasma half-life in vivo of approximately 7 hr with a prolonged elimination phase due principally to accumulation in fatty tissue (35 to 45 hr) (7), whereas the plasma half-life of verapamil is approximately 3 to 4 hr (11).

Because of these pharmacological advantages, we initiated studies to compare the efficacy of flunarizine to that of verapamil in augmenting tumor blood flow. Larger tumors than those in the verapamil study were used to permit a comparison of tumor blood flow changes in the grossly viable and grossly necrotic regions of the tumors. Using a technique described previously by our laboratory (13), blood flow to lymphatic metastases was measured in addition to blood flow to tumors arising in situ following transplantation.

MATERIALS AND METHODS

Animal and Tumor System. Female isogeneic Wistar/Furth rats weighing approximately 250 g were housed in groups of 3 to 4 in filter-top plastic cages with a bedding of wood shavings. They were placed in a temperature- and humidity-controlled room with 12 hr of light daily. Food and water were given ad libitum.

Tumor suspensions were prepared for transplantation with a Snell cytoseive, as described previously (4), and were adjusted to a 10% volume of centrifugally packed cellular material from SMT-2A mammary adenocarcinoma (18). Inocula of 0.10 ml were injected into both the right hind limb musculature and right inguinal mammary gland, and blood flow determinations were performed when the tumors reached approximately 2 g in size. For measurement of blood flow to lymph node metastases, 0.1 ml of tumor suspension was injected into the right and left inguinal mammary glands. Approximately 3 weeks later, the transplanted tumors were removed surgically under ether anesthesia so that the animals would not die from the primary tumors before their metastatic lesions grew to approximately 1.5 g in weight (3 to 4 weeks after surgery).

Blood Flow Estimation. When the tumors had reached the desired size, the animals were anesthetized with ether, and catheters were surgically placed into the left ventricle of the heart, the left femoral artery, and the right external jugular vein. The animals were returned to cages and allowed to recover for approximately 3 hr before the experiments were performed. Each conscious experimental animal received, via the external jugular vein, a bolus injection of flunarizine (Ortho Pharmaceutical Co., Raritan, N.J.) varying from 0.125 to 5.0 mg/kg body weight diluted in deionized water, such that the injection volume including 0.9% NaCl solution flush (saline) was 0.5 ml. Control animals were given a bolus of 0.5 ml of saline by the same route. At either 15 or 45 min following the initial bolus, an Edco withdrawal pump (Edco Scientific,
Inc., Chapel Hill, NC) was used to withdraw a femoral arterial reference blood sample for 1 min at a rate of 0.51 ml/min. Simultaneously, approximately 85,000 113Sn-labeled 25-μm microspheres (New England Nuclear, Boston, MA) were flushed slowly into the left ventricle of the heart with 0.5 ml of saline. Following this experimental procedure, the animals were killed with an intraarterial injection of Nembutal, and the radioactivity in the tissues was determined with a Nal well counter. Tissue blood flow, vascular resistance, and cardiac output were estimated as described previously (15).

Statistical Analysis. The standard unpaired t test assuming equal variances was used to compare blood pressure, heart rate, cardiac output, total peripheral resistance, blood flow, and vascular resistance data for the control group to the corresponding values for animals treated with a given dose of flunarizine. A paired t test was used to compare differences in tumor blood flow for the 2 implantation sites within a given animal. All parametric statistical tests involving blood flow and resistance data were performed after natural logarithmic transformation, since this has been shown to adequately normalize the data (15).

RESULTS

Tumor blood flow data for the 2 transplantation sites (mammary gland and hind leg musculature) were found to be highly correlated with a mean percentage difference of less than 5%, which was not significantly different from zero (p > 0.10). For this reason, blood flow and resistance data for the 2 sites were combined in Charts 1 and 2. Chart 1 depicts the effect of flunarizine on tumor blood flow and blood flow resistance as a function of dose 15 min following drug administration. All results are based on total wet tumor weights. A maximal increase in flow was obtained at 1 mg/kg associated with a significant fall in tumor vascular resistance (p < 0.001). Thereafter, a submaximal increase in flow was observed at doses up to 5 mg/kg without significant hypotension (blood pressure < 60 mm Hg) or bradycardia (data not shown).

In Table 1, malignant and normal tissue blood flow data 15 min following flunarizine (1 mg/kg) are compared to corresponding control values. Among the normal tissues, only the kidneys and lung exhibited statistically significant increases in blood flow (p < 0.05). Both the muscle- and mammary gland-implanted tumors exhibited statistically significant (p < 0.001) increases in flow of approximately 50%. The mean tumor weight for mammary gland-implanted tumors was 2.1 g for controls and 1.8 g for treatment animals (p > 0.10). The mean weight for the muscle-implanted tumors was 1.8 g for controls and 2.0 g for treatment animals (p > 0.10). For both the control group and the flunarizine treatment group, the mean number of microspheres contained in the reference arterial blood samples and in the tumors exceeded 400 so as to minimize random error (3).

Blood flow to lymphatic metastases was 0.67 ml/min/g (n = 15; 95% confidence interval, 0.59 to 0.76) 15 min following flunarizine (0.25 mg/kg) compared to a control value of 0.48 ml/min/g (n = 36; 95% confidence interval, 0.43 to 0.54) (p < 0.001). The mean tumor weight was 1.5 g for control animals and 1.3 g for animals treated with flunarizine (p > 0.10).

Chart 2 depicts blood flow to the grossly viable and grossly necrotic tumor tissue at 15 and 45 min following flunarizine administration compared to control values. Bars, S.E. Numbers in parentheses, number of tumors in each group.

Table 1 illustrates the effect of flunarizine (1 mg/kg) on malignant and normal tissue blood flow.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control (n = 13)</th>
<th>Flunarizine (1 mg/kg)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneyb</td>
<td>4.94 (4.44-5.49)</td>
<td>5.96 (5.45-6.49)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Heart</td>
<td>4.18 (3.55-4.92)</td>
<td>4.39 (4.19-5.54)</td>
<td>NS</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.20 (0.14-0.31)</td>
<td>0.37 (0.22-0.60)</td>
<td>NS</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.23 (0.19-0.29)</td>
<td>0.24 (0.19-0.31)</td>
<td>NS</td>
</tr>
<tr>
<td>Brain</td>
<td>0.96 (0.83-1.11)</td>
<td>0.92 (0.81-1.05)</td>
<td>NS</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>0.18 (0.15-0.22)</td>
<td>0.15 (0.09-0.24)</td>
<td>NS</td>
</tr>
<tr>
<td>Skin</td>
<td>0.20 (0.17-0.25)</td>
<td>0.26 (0.21-0.32)</td>
<td>NS</td>
</tr>
<tr>
<td>Liver</td>
<td>0.08 (0.06-0.12)</td>
<td>0.08 (0.04-0.16)</td>
<td>NS</td>
</tr>
<tr>
<td>SMT-2A (mammary site)</td>
<td>0.41 (0.38-0.48)</td>
<td>0.61 (0.55-0.68)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SMT-2A (muscle site)</td>
<td>0.40 (0.36-0.44)</td>
<td>0.57 (0.52-0.63)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SMT-2A (lymph node metastasis)</td>
<td>0.48 (0.43-0.54)</td>
<td>0.67 (0.59-0.76)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

n, number of observations; NS, not significant (p > 0.10).
Number of observations = 2 n.
Numbers in parentheses, 95% confidence interval for predicted mean.
\( p = 38 \).
Flunarizine dose, 0.25 mg/kg.
\( n = 15 \).

Table 2 illustrates the effect of flunarizine (1 mg/kg) on systolic and diastolic blood pressure, heart rate, cardiac output, and total peripheral resistance after natural logarithmic transformation (15).
temic hemodynamic variables at 15 min. There was a significant fall in the total peripheral resistance ($p < 0.005$) associated with an increase in cardiac output ($p < 0.005$). Blood pressure and heart rate were unchanged ($p > 0.10$).

**DISCUSSION**

The purpose of this investigation was to determine the effect that flunarizine has on tumor blood flow and to compare the results to those obtained with verapamil (17). In this investigation, the tumors were approximately twice the weight used in the verapamil study. However, valid comparisons between these 2 studies can be made, since Jirtle (13) has demonstrated previously that SMT-2A tumor blood flow expressed on a per-g basis is independent of tumor weight over the range of tumor weights we used. We utilized 25-μm microspheres to estimate tumor blood flow because 15-μm spheres readily pass through the large sinusoidal-type vessels in solid tumors, but despite the use of 25-μm spheres, there was a significant increase in pulmonary blood flow following treatment with flunarizine, suggesting an increased passage of spheres through arteriovenous shunts in both normal and malignant tissues.

Also worthy of comment is that all blood flow measurements were performed using conscious animals. We chose to utilize unanesthetized animals because virtually all anesthetics exert some effect on vascular smooth muscle (1, 21, 40). In addition, anesthetics may compete with vasoactive compounds for protein binding and may interfere with their metabolic degradation. Anesthetics, most notably the barbiturates, may also interfere with ganglionic nerve transmission (10). In studies designed to examine tumor blood flow in animals anesthetized with α-chloralose, a compound said to have minimal cardiovascular activity (2), we noted an exaggerated hypotensive response following administration of verapamil associated with marked attenuation of the compensatory increase in cardiac output seen in conscious animals. We have obtained similar results with the use of chorial hydrate anesthesia. Thus, anesthetics were found to greatly alter the hemodynamic effects of calcium entry blockers from those seen in conscious animals. Consequently, unanesthetized animals were used throughout this investigation, and we suggest that conscious, minimally disturbed animals must also be utilized in studies designed to investigate the efficacy of calcium entry blockers as adjuvants to conventional antineoplastic treatments.

Vasodilation by calcium entry blockers appears to be multifactorial in origin, although inhibition of calcium influx across the vascular smooth muscle membrane is postulated to be their principle mechanism of action (27). The approximate 50% increase in primary tumor blood flow observed for at least 45 min following i.v. flunarizine is essentially identical to our results obtained previously with verapamil. There is recent evidence suggesting that there may be a specific drug recognition site to which these agents bind (22), and the observed maximal increase in flow may reflect saturation of the putative calcium entry blocker binding sites. Alternatively, it may reflect, in the absence of marked changes in systemic blood pressure, a maximal obtainable increase in flow imposed by the architecture of the tumor vascular bed and increased tumor interstitial pressure secondary to neoplastic cell replication.

Malignant tumors derive their vascular bed from newly formed vessels and by incorporation of existing vessels in the host tissue (8). Host arterioles exhibit striking immunity from neoplastic invasion (38) and thus are one site at which calcium entry blockers may act to alter tumor blood flow. As transplantable tumors grow, however, one typically sees recognizable arterioles and venules only in the periphery of the tumor with many of the newly formed vessels transversing the central region appearing as thin-walled sinusoids lacking in smooth muscle (20, 24). As we observed an increase in tumor blood flow to both the central necrotic and peripheral viable regions of the tumor, it is necessary to postulate that the peripheral arterioles are the principle resistance elements influencing blood flow to the central necrotic region and/or that mechanisms independent of vascular reactivity are operative in the improvement of blood flow to the central region. For example, hypoxic conditions within the necrotic region may lead to increases in RBC intracellular calcium secondary to decreased glycolysis and resultant depletion of ATP necessary for active calcium removal (25). Increased erythrocyte intracellular calcium results in decreased deformability of the membrane, giving rise to an apparent increase in blood viscosity (25). The low shear rates associated with sluggish blood flow coupled to a decrease in membrane deformability favor rouleaux formation, which further contributes to an increase in resistance to flow (5, 34). Flunarizine and the calcium entry blocker cinnarizine have been shown to counteract hypoxia-induced decreases in RBC deformability (5). The regurgitant and intermittent nature of tumor blood may potentially result in areas of localized stasis and thrombosis (8). Calcium entry blockers have also been shown to inhibit platelet aggregation both in vitro and in vivo (12, 16). Calcium entry blocker-mediated alterations in the rheological properties of poorly oxygenated blood exposed to low shear rates might also explain the observation that the increase in blood flow we measured was independent of tumor location, given the heterogeneity of vascular smooth muscle response to these agents (35).

The biphasic dose-response curve relating tumor blood flow to flunarizine is qualitatively similar to our results reported previously with verapamil (17). However, flunarizine appears to have a broader therapeutic window with a higher therapeutic index with respect to cardiac toxicity, as we did not observe systemic hypotension (blood pressure, <60 mm Hg) or bradycardia at
doses up to 5 mg/kg. This is consistent with the preferential action of the drug on peripheral vascular smooth muscle. We achieved maximal increases in tumor blood flow with dosages which produced little alteration in normal tissue blood flows. We have postulated previously (17) that this selectivity may be the result of calcium entry blocker inhibition of hypoxia-induced decreases in erythrocyte deformability within the tumor, altered sensitivity of incorporated host vessels to calcium blockade due to the tumor microenvironment, and/or an inherent sensitivity of neovessels to calcium entry blockers. However, it is likely that, if the dose of a vasodilator exceeds the autoregulatory threshold of the normal host vessels resulting in hypotension, one will observe a decrease in tumor blood flow due to the primitive nature of tumor blood flow autoregulation (19) in addition to local normal tissue blood flow "steal" effects.

It is important to note that blood flow to lymph node metastatic lesions was increased to the same maximum extent as that to the tumors transplanted into either the hind leg musculature or the normal mammary gland tissue. This demonstrates that the effectiveness of flunarizine as an adjuvant to conventional treatment modalities would not be dependent upon whether the tumor is metastatic. Finally, for flunarizine to be of clinical importance, it must improve the treatment of tumors more than it enhances the killing of cells in normal tissues. Of the tissues studied, flunarizine increased blood flow only to the kidney and lung. As stated previously, the apparent increase in lung blood flow most likely is an experimental artifact due to increased shunting of the microspheres through the tissue vascular beds after drug infusion. However, it must be kept in mind that the increased blood flow to the kidney may also increase kidney damage from chemotherapeutic agents.

In summary, we have demonstrated that flunarizine was capable of increasing tumor blood flow by approximately 50% regardless of whether the tumor was transplanted into either the hind leg musculature or normal mammary gland tissue. The increase in flow was distributed to both the peripheral grossly viable and central necrotic tumor regions. A similar increase was observed for lymph node metastatic lesions. The maximal increase in tumor blood flow was obtained without a significant decrease in blood pressure or heart rate and was still detectable 45 min following flunarizine administration. We conclude that calcium entry-blocking agents warrant further investigation as possible adjuvants to antineoplastic treatment modalities presently available.

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Blood Flow to Primary Tumors and Lymph Node Metastases in SMT-2A Tumor-bearing Rats following Intravenous Flunarizine

William G. Kaelin, Jr., Shashi Shrivastav and Randy L. Jirtle


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