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Blood Flow to Lymphatic Metastases in Conscious Rats*

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Abstract—*The blood flow of lymphatic metastases in conscious rats was estimated with radioactively labelled microspheres 25 μm in diameter. The relative blood flow of the outer 'grossly viable' tissue was twice that of the predominately necrotic central region of the SMT-2A mammary adenocarcinoma. Tumor blood flow to both regions was independent of tumor weight and whether the malignant tissue was transplanted into either the inguinal or axillary mammary gland or had spontaneously metastasized to the axillary lymph nodes. At all tumor weights studied the blood flow of the 'grossly viable' tissue, on a per gram basis, was significantly greater than that of the skin, mammary gland and muscle.*

INTRODUCTION

EXPERIMENTAL tumor treatment modality research is primarily performed in isogeneic animals with transplantable tumors. The site of transplantation has not usually been considered of importance. However, recently Auerbach *et al.* [1] have demonstrated that when malignant cells were inoculated either intradermally or subcutaneously (s.c.) the tumor growth was 3–4 times as rapid anteriorly as posteriorly. The relative blood flow of the MTW-9B mammary adenocarcinoma has similarly been shown to have an anteroposterior dependence when transplanted into mammary tissue [2]. By transplanting the V2 carcinoma into the liver, spleen, kidney, testicle and ear of the rabbit, Young *et al.* [3] have shown that the tumor growth rate, degree of necrosis and relative blood flow is also determined by the normal tissue in which the tumor is proliferating. Thus it has become evident that the effectiveness of tumor treatment modalities may be partially dependent upon the site of transplantation.

Since the treatment of metastatic tumors is of clinical importance and the efficacy of most treatments is partially determined by tumor blood flow and its distribution, it was felt that the blood flow physiology of lymphatic metastases should be investigated. In this study radioactively labelled microspheres were used to estimate the blood flow of lymph node metastases from a spontaneously metastasizing mammary adenocarcinoma.

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MATERIALS AND METHODS

Animal and tumor system

Isogeneic female W/Fu rats weighing approximately 240 g were housed in hanging cages in a temperature-controlled room with 12 hr of light daily. Purina laboratory rat chow (Ralston Purina Co., St. Louis, Missouri) and water were given *ad libitum*.

The spontaneously metastasizing SMT-2A mammary adenocarcinoma was used throughout these studies [4]. It was chemically induced with 3-methylcholanthrene and has been shown to be nonimmunogenic. When transplanted into the inguinal mammary glands it rapidly metastasizes to the ipsilateral axillary lymph nodes. Suspensions of the tumor were prepared for transplantation with a Snell cytosieve as previously described [5], and were adjusted to a 20% volume of centrifugally packed cellular material. The experiment was divided into two groups. In group A, 0.1 ml of the tumor suspension was injected into both axillary mammary glands (2nd nipple) and the right inguinal mammary gland (4th nipple). After approximately 4 weeks the tumors were palpable and blood flows were estimated at various times thereafter. In group B, 0.1 ml of the tumor suspension was injected

into the right and left inguinal mammary glands. Four weeks later, when the inguinal tumors averaged 0.1 g, they were surgically removed under ether anesthesia. Eight weeks after the initial transplantation of the malignant tissue the metastatic tumors in the axillary lymph nodes were palpable, and blood flow estimates were made at various times thereafter.

Blood flow estimation

Full details and the rationale for the use of microspheres for estimating tumor blood flows are provided elsewhere [2], and thus will only be briefly described. When the tumors reached the desired size, the rats were surgically prepared under ether anesthesia. A 4 cm PE10 (i.d. 0.011 in., o.d. 0.024 in.) catheter was heat fused with a 10 cm PE50 (i.d. 0.023 in., o.d. 0.038 in.) catheter. It was subsequently filled with heparinized saline (25 U/ml) and placed via the right common carotid artery into the left ventricle of the heart. The PE10 portion of a similarly prepared catheter was also placed into the left femoral artery. Both catheters were tied off, placed s.c. and exteriorized at the dorsal aspect of the neck. All animals were returned to individual cages and allowed to recover for at least 3 hr before the microspheres were injected.

Microspheres, 25 μm in diameter, labelled with either 57-Co or 113-Sn (New England Nuclear, Boston, Mass.), were suspended in 10% dextran, and to prevent aggregation the solution contained 0.01% Tween-80. To estimate tissue blood flows approximately 70,000 microspheres were infused over a period of 5–10 sec into the left ventricle of the heart with 0.5 ml of isotonic saline. An integral arterial blood sample was withdrawn at a rate of 0.51 ml/min during and for 1 min after the spheres were injected. The blood sample was washed into a wide-mouth gamma ray counting vial for radioactivity determination. After the completion of the experiment the animals were sacrificed by an intracardiac injection of 60 mg of sodium pentabarbital (Nembutal, Abbott Laboratories, North Chicago, IL).

Skin and mammary gland tissue from the inguinal region, soleus and adjacent muscles and tumors were removed, dissected free of surrounding extraneous tissue, weighed and placed in gamma ray counting vials. For each sample, isotope activity and the corresponding number of spheres were determined by appropriate data reduction of the output from a 3-channel NaI well counter equipped with pulse-height analyzers [6].

The blood flow to various tissues was calculated by using the formula: $FT = (WR/NB) \times (NT)$ where FT is the tissue blood flow (ml/min/g), WR is the rate of the arterial blood sample withdrawal (ml/min), NB is the number of microspheres in the withdrawn blood sample, and NT is the average number of microspheres per gram of tissue. The cardiac output was calculated by using the formula: $CO = (WR/NB) \times (NI)$ where CO is the cardiac output (ml/min), and NI is the total number of microspheres injected into the animal. The value of NI was determined as previously described [2]. The systemic arterial blood pressure was measured with a Millar pressure transducer (Millar Instruments, Houston, TX) and was continuously recorded except when an arterial blood sample was being withdrawn. Linear interpolation of the recorded pressure data was performed to estimate the pressure during the time of microsphere injection.

Statistical analyses

The blood flow data have previously been shown to be adequately normalized by a log transformation [2]. Thus all parametric statistical tests were performed after log transformation. The linear correlation coefficient was used to determine the extent of correlation between two variables. The two-sample t-test assuming unequal variances was used to compare sample means [7].

RESULTS

A tumor bearing animal from group A was injected, via the left common carotid artery, with a 40% (w/v) micropaque–gelatin suspension and fixed in 10% buffered neutral formalin. Figure 1 shows a radiograph of an approximately 3 mm thick central section from a SMT-2A tumor growing in the axillary mammary gland. Paraffin sections 6 μm in thickness were also cut from this tissue and were stained with hematoxylin–eosin (Fig. 2). Both figures demonstrate that the SMT-2A mammary adenocarcinoma is comprised of two easily discernible areas. The outer annulus of tissue is well vascularized and devoid of gross necrosis. The central portion of the tumor is comprised of both cords of viable tissue surrounding patent vessels and large areas of necrosis. Due to the clear demarcation between these two regions, they were

Fig. 1. *Radiograph of the vasculature network of a* 3 mm *thick central section from a SMT-2A mammary adenocarcinoma* (10×). *The avascular demarcation between the outer and inner regions is clearly defined* (*arrows*). *Extravasation of the contrast medium into the central region occurred.*

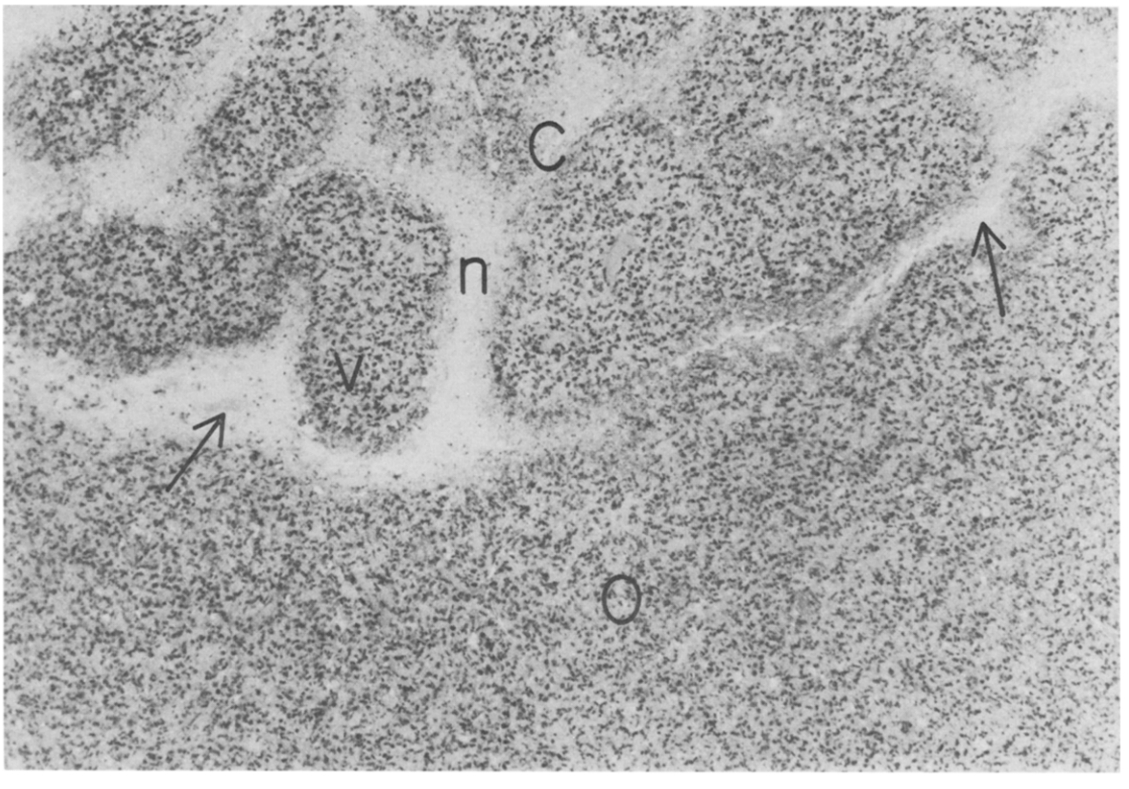

Fig. 2. A hematoxylin–eosin stained 6 μm *section of the tissue radiographed in Fig.* 1 (180×). *The demarcation between the outer* (*O*) *and central* (*C*) *regions is demonstrated with arrows. The central region is comprised of both necrotic* (*n*) *tissue and viable* (*v*) *cells surrounding patent vessels. In contrast, the tissue in the outer region is predominantly viable in appearance.*

easily separated, and the relative blood flow of each area was estimated.

At the time of blood flow estimation the total tumor burden in groups A and B was similar since the inguinal tumors in group B were surgically removed 4 weeks after transplantation. At the time of blood flow estimation the average arterial pressures for groups A and B were 102.6 ± 2.8 mmHg ($n = 19$) and 95.2 ± 3.5 mmHg ($n = 10$), respectively. The average cardiac outputs were 95.7 ± 6.2 ml/min ($n = 19$) and 104.4 ± 6.9 ml/min ($n = 10$), respectively. The differences in both the arterial pressures and cardiac outputs were insignificant ($P > 0.1$).

The blood flow per gram of tissue in the outer region of the tumor is graphed in Fig. 3 vs the 'grossly viable' tumor weight. The correlation between these two variables was insignificant ($P > 0.1$) both for metastatic tumors and tumors growing in either the inguinal or axillary mammary glands. The relative blood flow of the inner tumor region, over the weight range investigated (i.e., 0.07–0.86 g), was similarly independent of the tissue weight ($P > 0.1$). The relative tumor blood flow estimates for the inner and outer regions of this tumor were therefore averaged. The results are shown in Table 1. Analyses of these data show that the relative blood flow of the 'grossly viable' portion of the SMT-2A tumor while growing in the inguinal mammary gland is not significantly different ($P > 0.1$) from that when growing in the axillary mammary gland. Additionally, the relative blood flow to the outer region of the lymphatic metastases did not significantly differ ($P > 0.1$) from that of tumors growing in the mammary glands. The relative blood flow of the central portion of the tumor was similarly independent of whether the tumor was growing in either the inguinal or axillary mammary glands or had metastasized to the axillary lymph nodes. The relative blood flow of the outer viable appearing region was, however, approximately twice that of the central region ($P < 0.01$).

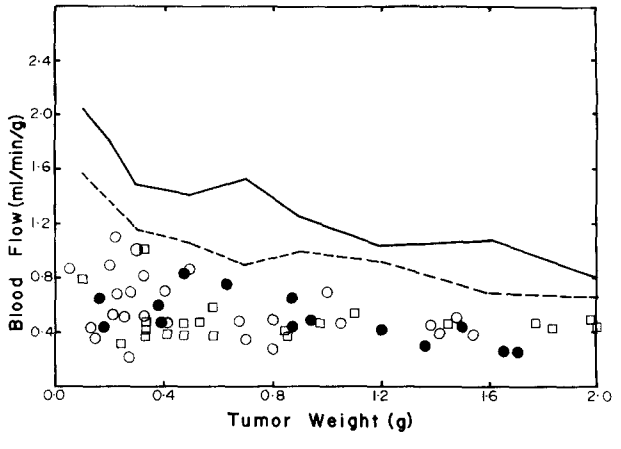

Fig. 3. The relative blood flow (ml/min/g) *of the MTW-9B and SMT-2A mammary adenocarcinomas as a function of tumor weight. The solid* (—) *and dashed* (----) *lines represent the average MTW-9B tumor blood flow when transplanted into either the axillary or inguinal mammary glands, respectively* [2]. *Each symbol represents the blood flow to a single SMT-2A tumor when proliferating in either the axillary* (○) *of inguinal* (●) *mammary glands or the axillary lymph nodes* (□).

For comparison Fig. 3 also displays the average blood flows for the nonmetastasizing MTW-9B mammary adenocarcinoma while growing in either the axillary or inguinal mammary glands [2]. At all tumor weights the relative blood flow of the SMT-2A is significantly lower than that of the MTW-9B tumor ($P < 0.01$). The blood flow of the 'grossly viable' SMT-2A tumor tissue is, however, greater than that to the surrounding mammary gland tissue (0.232 ml/min/g; 95% confidence interval 0.133–0.403; $n = 48$) skin (0.186 ml/min/g; 95% confidence interval 0.118–0.293; $n = 48$) and muscle (0.161 ml/min/g; 95% confidence interval 0.094–0.277; $n = 48$).

Table 1. Blood flow to the outer and central regions of the SMT-2A mammary adenocarcinoma

Site of tumor growth	Outer region (ml/min/g)	n*	Central region (ml/min/g)	n
Inguinal mammary gland	0.495 (0.374, 0.563)†	14	0.262 (0.188, 0.367)	12
Axillary mammary gland	0.463 (0.388, 0.552)	26	0.266 (0.214, 0.331)	16
Axillary lymph nodes	0.467 (0.403, 0.541)	21	0.302 (0.249, 0.366)	12

*Number of tumors.
†95% Confidence interval.

DISCUSSION

Breast cancer is the most frequent malignancy in women, and approximately 70% of all patients with breast carcinoma will eventually present with metastatic disease [8]. Metastases eradication is thus essential for the successful treatment of most mammary carcinomas. Since the efficacy of tumor treatment modalities depends in part upon tumor vasculature and blood flow, this study was undertaken to investigate the tumor vascular physiology of metastatic tumors. The results demonstrate that the relative blood flow of the metastasizing SMT-2A mammary adenocarcinoma is independent of whether the malignant tissue is inoculated into either the inguinal or axillary mammary gland or has spontaneously metastasized to the axillary lymph nodes. The relative blood flow is also shown to remain constant over the range of tumor weights investigated.

The SMT-2A mammary adenocarcinoma was chosen for this investigation since it arose in an inbred strain of rats, and following transplantation into the inguinal mammary glands it readily metastasizes via the lymphatics, a route very common with human mammary tumors. The tumor grows in a well defined spherical mass which can be easily removed from the surrounding normal mammary tissue. Furthermore, the 'grossly viable' tissue can be readily separated from the predominantly necrotic central region, thereby allowing blood flow estimates to be made for each region.

The rationale for the use of microspheres to measure tumor blood flow is as follows. The microsphere method readily permits multiple simultaneous measurements of blood flows to many regions in the same animal [9, 10]. McDevitt and Nies [11] have reported that microspheres can be used to estimate the cardiac output and distribution in rats, provided that the blood samples contain more than 400 spheres. They reported that the injection caused no hemodynamic changes and that results agree with published estimates calculated by other techniques. Tsuchiya *et al.* [12] have used microspheres in unanesthetized rats and also reported that there were no hemodynamic alterations and that the reproducibility of 3 separate injections to each rat was excellent.

The microvasculature of tumors is composed of both capillary-like vessels and sinusoidal vessels which are greater than 12 μm in diameter. As tumors increase in size the ratio of sinusoidal to capillary-like vessels has been shown to increase by as much as a factor of 10 [13, 14]. Thus to estimate the 'nutritive' blood flow of tumors, larger diameter microspheres are required than would be necessary to determine the 'nutritive' blood flow of normal tissues. Previous studies demonstrated that microspheres 25 μm in diameter most effectively estimated the blood flow of the MTW-9B mammary adenocarcinoma [2], and similar results were obtained for the metastasizing tumor employed in this study. Following microsphere injection histological examination revealed that the spheres were located randomly throughout the two regions of the tumor rather than primarily at the tumor surface. It is therefore concluded that the microsphere technique as employed in this study can be used to accurately estimate malignant tissue 'nutritive' blood flow in unanesthetized rats.

Previous experimental investigations have shown that pulmonary metastases are more sensitive to chemicals [15] and X-irradiation [16, 17] treatment than s.c. tumors. In these studies, however, the s.c. tumors were much larger than those in the lung. Recently Guichard *et al.* [18] have reported that lymphatic metastases have a larger hypoxic fraction than s.c. transplanted tumors of similar size, and thus would be more resistant to X-irradiation treatment. However, in this study precautions were not taken to insure that the total tumor mass in the experimental animals was always constant. Anemic conditions do occur in experimental animals with large tumor burdens, and the tumors in anemic animals have been shown to have an increased hypoxic fraction [19]. Thus the existing experimental data do not adequately clarify whether metastatic and primary tumors of similar size will respond similarly to various treatment modalities.

It is known that conditions which alter the systemic arterial pressure markedly affect tumor circulation [20] and malignant tissue oxygenation [21]. To minimize these potential problems the transplanted inguinal tumors in group B were surgically removed 4 weeks later. Thus, the total tumor mass in the two experimental groups was similar when blood flow estimates were performed. The cardiac outputs and systemic arterial pressures for both groups of animals were also insignificantly different. Under these experimental conditions the relative blood flow for metastatic tumors is identical to those for tumors proliferating in either the inguinal or axillary

mammary gland tissue. This was true not only for the outer 'grossly viable' tissue but also for the more necrotic appearing tissue in the inner core.

In addition to the relative blood flow of the SMT-2A tumor being independent of whether it is proliferating in either mammary or lymphatic tissue, it is also independent of tumor size. This contrasts to what was observed for the nonmetastasizing MTW-9B mammary adenocarcinoma where the relative blood flow is greater for the axillary mammary gland tumors and it is inversely proportional to the tumor weight (Fig. 3) [2]. The blood flow of the SMT-2A tumor, at all tumor weights investigated, is also lower than that of the MTW-9B neoplasms. This difference is even greater than is apparent in Fig. 3 when it is taken into account that the relative blood flows of the MTW-9B tumor were based upon whole tumor weights (i.e., necrotic tissue included), whereas those for the SMT-2A tumor are based only upon the 'grossly viable' tissue. Thus, the magnitude of tumor blood flow is dependent upon the tumor investigated and may additionally vary with the degree of necrosis, the normal tissue in which it is proliferating, and the antero-posterior location of the tumor. That these variables are important in determining malignant tissue blood flow helps explain some of the variability in the blood flow estimates reported in the literature [2, 3, 22].

The available data concerning whether tumor blood flow is greater or less than that of normal tissue are also conflicting [2]. The present study shows that the MTW-9B tumor and the 'grossly viable' tissue of the SMT-2A tumor have blood flows which though different from each other are both significantly greater than that of the surrounding mammary gland tissue, skin and muscle. Furthermore, small MTW-9B mammary adenocarcinomas [2] and hamster amelanotic melanomas [22] have been shown to actually have blood flows, on a per gram basis, which are similar to that of the brain (Fig. 3) [23]. As a consequence, the viable portions of these tumors would, for example, be more rather than less difficult to heat than the surrounding normal tissue if there is not a redistribution of blood flow during hyperthermic treatment.

The reason for the dissimilarity in the relative blood flows of these two mammary adenocarcinomas is presently unclear. Similar blood flow studies with a variety of tumors with varying metastatic and growth rate characteristics would have to be performed in order for meaningful generalizations to be made. These studies do show, however, that SMT-2A tumors which have derived their vasculature from either mammary or lymphatic tissue have a similar histological appearance and blood flow. Therefore, insofar as successful therapy depends upon tumor blood flow, these results predict that the response of lymph node metastases to various treatment modalities should be similar to that of comparably sized primary mammary carcinomas.

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