ABSENCE OF HOST-SITE INFLUENCE ON ANGIOGENESIS, BLOOD FLOW, AND PERMEABILITY IN TRANSPLANTED RG-2 GLIOMAS

PETER MOLNAR,1,2 ISTVAN FEKETE,1,3 KURT E. SCHLAGETER,1,4 GREGORY D. LAPIN,1,5,6 AND DENNIS R. GROOTHUIS1,6,7

Department of Neurology, Northwestern University Medical School, Evanston Northwestern Healthcare, Evanston, Illinois

(Received November 6, 1998; accepted June 14, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

The host site is believed to regulate tumor angiogenesis, which could result in site-dependent drug delivery parameters, greatly affecting experimental tumor research. In RG-2 rat gliomas we measured cellular proliferation; cell cycle time was the same for RG-2 cells in brain and s.c. tumors (25 h), and was the same for endothelial cells in these tumors (46 h). We measured the transcapillary transfer constant (K) of α-aminoisobutyric acid and blood flow (F) with iodoantipyrine in RG-2 gliomas transplanted into brain, liver, kidney, muscle, s.c. tissue, and into the abdominal cavity. Data was evaluated by quantitative autoradiography and direct tissue sampling. The variation of F (12.6–84.0 ml/g/min) and K (26.1–49.2 μl/g/min) in RG-2 tumors in the different host sites was less than in surrounding tumor-free tissue (F = 20–1500 ml/g/min and K = 1.6–700 μl/g/min). In contrast to other models, RG-2 does not result in tumors with host site-dependent behavior. The RG-2 tumor cells appear to participate in, if not dominate, the angiogenesis process regardless of the host site. Values of F and K were more dependent on tumor topography (center versus periphery) and local histological features (necrosis versus viable tumor) than host site. We believe that the methods used for data acquisition may introduce as much variability in Results as the tumors themselves and that to better understand how tumor angiogenesis affects the vascular phenotype, comparative studies are needed to validate the results obtained with newer methodologies.

Experimental and clinical studies of brain tumors are often hindered because of limited access; when tumors grow in the brain it is difficult to obtain tissue for purposes other than histological examination, and repeated sampling over long periods of time is all but impossible. To circumvent this problem, many investigators use models in which the brain tumors are implanted in more accessible locations, most often s.c.. It has been stated that the barrier properties of the neovasculature induced by brain tumor cells are determined by the host tissue (Arosarena et al., 1994; Yuan et al., 1994; Hobbs et al., 1998) and that “differences in endothelial properties such as permeability . . . may result in s.c. tumors that differ from their intracerebral counterparts” (Yuan et al., 1994). It would not be surprising that the host implantation site may affect the vascular phenotype of an experimental tumor, because cultured endothelial cells from different organs display characteristics unique to the organ of origin (Craig et al., 1998). The potential interdependence of site of tumor growth and tumor vascular phenotype is critical because it raises issues about the validity of experiments in which tumors are grown outside the nervous system.

To understand how the host implantation site may affect drug delivery parameters, we chose to study RG-2 gliomas growing in different host sites. The RG-2 tumor cell line was originally derived from an ethylnitrosourea-induced rat glioma and has been well defined in terms of its growth characteristics, histology, and reproducibility (Aas et al., 1995; Barth, 1998). In the brain, RG-2 gliomas grow as solid tumors with little necrosis (Groothuis et al., 1982; Aas et al., 1995), although s.c. tumors may develop central necrosis (Groothuis et al., 1982). Blood flow (F) and transcapillary transport vary region-
ally within RG-2 tumors (Groothuis et al., 1983a,b), although the magnitude of the regional variation is small compared with many other brain tumor models (Groothuis et al., 1984). The physiology of the RG-2 tumor model has been very stable in many studies of experimental brain tumor physiology (Nakagawa et al., 1984, 1987, 1988; Tjuvajev et al., 1995; Molnar et al., 1995).

Given the apparent stability of the RG-2 glioma model, we reasoned that changes induced by the host implantation site should be easily detected with the RG-2 system. We first studied the effect of host site on tumor cell and endothelial cell proliferation kinetics in RG-2 tumors growing s.c. and in the brain. We then compared F and permeability of RG-2 tumors grown in six different locations. We compared two different methods for obtaining tissue measurements: quantitative autoradiography (QAR) and direct tissue sampling (DTS). QAR allows precise anatomic localization of the studied parameters, because autoradiographic measurements can be based on the histology of the same tissue section. Although DTS is widely used in physiological experiments because it is less labor intensive than QAR, the tissue is destroyed in the process of obtaining measurements and the histologic substrate from which the measurement was made can never be known precisely. This may introduce variation in tissues with regional heterogeneity, which is the hallmark of malignant tumors. In addition to exploring these variables, we also evaluated how the roles of experimental methodology (used to obtain data), the pharmacokinetic model (used to evaluate the data) and the physiological units (used to report the data) can affect the experimental outcome.

Materials and Methods

Tumor Transplantation. All animal experimentation was approved by the Institutional Animal Care and Use Committee of Evanston Northwestern Healthcare. Fisher-344 rats (Harlan Industries, Indianapolis, IN) were anesthetized with halothane/nitrous oxide/oxygen anesthesia (1.5:30:70 v/v/v). For labeled mitoses experiments, 102 rats were injected with RG-2 glioma cells (10⁶ cells/ml); 2 μl was injected intraocularly and 10 μl was injected s.c. into the flank. For the physiological experiments, five groups of rats were injected with RG-2 glioma cells (10⁶ cells/ml): 1) 15 were injected with 10 μl s.c. in the flank; 2) 15 were injected with 10 μl into the gluteal muscles; 3) after a laparotomy, 15 were injected with 5 μl of tumor cells into the liver under direct visualization; 4) after a laparotomy, 15 were injected with 5 μl of tumor cells into the left kidney under direct visualization; and 5) 10 animals injected intracerebrally with 2 μl, as described previously (Groothuis et al., 1983a).

Labeled Mitosis Experiments. Three weeks after tumor cell injections, rats were injected i.v. with 3H-thymidine (3 Ci/kg, 40–60 mCi/mmol, NEN, Boston, MA). At times from 15 min to 56 h, three rats per time point were perfused for 60 s through the left ventricle of the heart with heparinized phosphate buffer (0.1 M, pH 7.38; Karlsson and Schultz, 1965), followed by 0.8% (Sakurada et al., 1978; Groothuis et al., 1983b). The mean value of AIB, 50 μCi of [14C]AIB was injected as a bolus, and timed plasma samples, which were stopped at 10 min, were obtained until the end of the experiment (Blasberg et al., 1983b). Plasma and tissue samples were processed as described previously (Blasberg et al., 1981). At the end of the experiment, the systemic tumors were removed, including a margin of the tissue into which the tumor was growing. A small tumor sample (~10 mg) was surgically dissected from a solid part of each tumor and used for liquid scintillation counting (LSC); the remaining tumor was frozen in liquid freon (~80°C) within 1 min of the conclusion of the experiment. On opening the peritoneal cavities of the rats in which the liver and kidney had been injected, we found that some tumor cells had seeded the peritoneal cavity and were growing on the peritoneum; these tumors were included as a separate group. Brains were removed whole and frozen.

Analysis. Preparation of tissue sections and autoradiographs has been described (Blasberg et al., 1981; Groothuis et al., 1983a,b). Briefly, tumors were sectioned at 20 μm thickness on a cryomicrotome, collected on 1–3-inch slides, and placed on X-ray film along with calibrated 14C-methylmethacrylate standards. The autoradiographs were digitized at a resolution of 50 μm/pixel with a video-based digitizing system. After the autoradiographs had been prepared, the same tissue sections were stained with hematoxylin-eosin, digitized, and aligned with the autoradiographic images in computer memory (Blasberg et al., 1983a, 1984). Measurements of tissue radioactivity (nCi/g) were obtained from the autoradiographs, using the histological images to define the region of interest (ROI). Measurements were made in the following tumor regions: whole tumor (through maximum tumor cross-sectional area); tumor center (consisting of 20% of the tumor located geographically in the center), tumor periphery (consisting of 60% of the tumor geographically at the tumor edge), solid viable tumor tissue (as determined by histological appearance), and in regions of necrosis. In neuro-oncology, the brain immediately adjacent to the tumor mass (called brain adjacent to tumor or BAT) has received special attention because it represents an area with infiltrating tumor cells and generally decreased permeability. In these studies, we analyzed a comparable area of tissue adjacent to tumor (TAT), which represented a 250-μm zone immediately outside the tumor border. Measurements were also made in tissue distant from the tumor, which was free of tumor cells (called “normal”).

The mean (± S.D.) value of F (ml/g/min) was calculated from all pixels within an ROI using Kety-Schmidt equations as described previously, with λ = 0.8 (Sakurada et al., 1978; Groothuis et al., 1983b). The mean ± S.D. value of the unidirectional blood-to-tissue transfer constant (K) value of AIB, 50 μCi of [14C]AIB was injected as a bolus, and plasma samples were used to perform Kety-Schmidt measurements. The mean ± S.E.M. of values from individual tumors. The lowest values of F and K measurable in these experiments were represented by film background: F = 0.01 ml/g/min, K = 0.1 μl/min.

Tissue radioactivity (nCi/g) of tissue samples collected by DTS was measured by LSC. The F and K values of these samples were calculated for the entire tissue sample, using the same methods as described for the SL-QAR procedures.

Data were compared with parametric statistics. Student’s t test was used for...
comparison of means of $F$ or $K$ from two locations (e.g., tumor center versus tumor periphery, etc.). Groups were compared using ANOVA.

**Results**

**Percent Labeled Mitosis (PLM).** The cell cycle time for the RG-2 tumor cells was approximately 28 h for intracerebral tumors and 27 h for s.c. tumors (Fig. 1). The length of the S phase for the RG-2 tumor cells was approximately 7 h for tumors in both locations. The cell cycle time for the endothelial cells was 47 h in brain tumors and 46 h for s.c. tumors. The length of the S phase was 12 h for the brain tumors and 13 h for the s.c. tumors. There were insufficient numbers of labeled mitoses in tumor-free brain to estimate the cell cycle time. In Fig. 1, each point represents the mean ± S.D. from three animals.

**Physiological Status.** The status of animals used for $F$ experiments was: temperature = 36.6 ± 0.2°C; blood pressure = 120.2 ± 3.4 mm Hg; hematocrit = 44.1 ± 1.9; arterial blood values: $pO_2 = 112.7 ± 0.3$ torr; $pCO_2 = 30.1 ± 3.3$ torr; and pH = 7.45 ± 0.02. The status of animals used for transcapillary transport experiments was: temperature = 36.7 ± 0.1°C; blood pressure = 127.6 ± 3.3 mm Hg; hematocrit = 41.5 ± 1.0; arterial blood values: $pO_2 = 104.3 ± 3.1$ torr; $pCO_2 = 31.0 ± 0.9$ torr; pH = 7.46 ± 0.01. Values are mean ± S.E.M.

**Histopathological Findings.** The microscopic appearance of RG-2 tumors in all locations was generally like RG-2 tumors implanted intracerebrally (Groothuis et al., 1982; Aas et al., 1995). Central macroscopic necrosis occurred in tumors in which the long axis exceeded 15 mm and was not observed in any of the brain tumors. There was no consistent relationship between the implantation site and the frequency or location of microscopic necrosis. Tumor shape in the brain was spherical and in other organs was most often an oblate ellipsoid. Inflammatory cells, with either leukocytes and/or lymphocytes, were variably present but were not a prominent feature. Focal hemorrhages were seen in some smaller tumors. In a few tumors (liver, kidney, s.c.) there was conspicuous morphological heterogeneity with areas of sarcomatous appearance that was not seen in intracerebral RG-2 gliomas. Muscle tumors were infiltrative and sometimes destroyed the femoral bone. Kidney and liver tumors were usually well demarcated and, like s.c. tumors, were surrounded by a pseudocapsule.

**Tumor Size.** Tumor size, expressed as the maximum cross-sectional area (mm$^2$) measured on the histological image, is presented in Fig. 2. Tumors from all systemic locations were significantly larger than the brain tumors ($p < .05$, ANOVA). However, except for tumors growing in muscle, there was no difference between the size of tumors used for $F$ and those used for transport experiments within the same organ ($p > .5$, Student’s t test.)

**$F$ Values.** Regional $F$ values from RG-2 tumors in different locations are presented in Fig. 2. Between 5 and 18 tumors were studied in each location (Fig. 2). $F$ in TAT and in tumor-free tissue from the same organ are presented in Fig. 3. $F$ values in Fig. 2 are presented from several different perspectives: 1) as a function of host organ site (e.g., liver, kidney, etc.), 2) topographical location within the tumor (tumor center versus periphery), 3) the effect of sampling from viable versus necrotic tumor, and 4) a comparison of DTS versus QAR analysis.

Except for the brain tumors, which had whole tumor $F$ values ($84.0 ± 8.0$ ml/g/min) that were nearly twice those of the next highest location (s.c. = 49.3 ± 10.7 ml/g/min), whole tumor $F$ values were not significantly different from one another ($p > .05$, ANOVA). However, the range of $F$ within the tumors in different organs was narrow (12.5–84 ml/g/min) in comparison with the large variation in $F$ values in normal tissue, i.e., from $<20$ ml/g/min in muscle to $>1500$ ml/g/min in kidney (Fig. 3). It must be emphasized that the $F$ values in Fig. 3 are for “normal-appearing” tissue in the same organ in which the tumor of interest was growing and may not reflect $F$ values in a truly normal organ. The area of TAT (Fig. 3) represented a buffer zone: the $F$ value in TAT was intermediate between normal tissue and tumor. $F$ was dependent on the topographic tumor region from which the sample came and the rank order was the same for all tissues: $F$ in tumor center was lower than in tumor periphery. Similarly, $F$ in viable tumor exceeded that in necrotic tumor. $F$ to viable tumor tissue was close to 80 ml/g/min in all host locations except liver, where it was 40 ml/g/min. Despite the narrow range of the averaged values, the absolute range of $F$ values in individual tumors varied from a low of 1.9 in a liver tumor to a high of 197 ml/g/min in a brain tumor.

One objective of these experiments was to compare values obtained by DTS with those obtained by QAR analysis. When the whole tumor $F$ values measured by QAR were compared to those measured by DTS (Fig. 2), the values of each group were not significantly different ($p > .05$, ANOVA). In most instances, the value of $F$ determined from DTS was intermediate between $F$ in tumor periphery and tumor center. The DTS values were consistently and significantly less than $F$ values from viable tumor in the same organ as determined by QAR ($p < .001$, ANOVA), suggesting that the DTS samples contained small areas of necrosis. The DTS values consistently exceeded the $F$ values in necrotic tissue determined by QAR.
**K Values.** Regional values of the unidirectional $K$ values of AIB are presented in Fig. 2, and for TAT and normal tissue in Fig. 3. Between 5 and 23 tumors were studied in each location (Fig. 2). The range of whole tumor $K$ values was narrow regardless of the host site (from a low of $26.1 \pm 4.5 \mu l/g/min$ in muscle tumors to a high of $49.2 \pm 5.2 \mu l/g/min$ in liver tumors). The whole tumor $K$ values were not significantly different in any of the systemic implantation sites, nor were the whole tumor $K$ values of the systemic tumors different from the brain tumors ($p > .05$, ANOVA). $K$ values were lowest in tumor center and highest in tumor periphery, although the differences were not significant within an individual organ. Similar to $F$, $K$ values in viable tumor were significantly higher than those in necrotic tumor ($p < .001$, ANOVA). Considerable variation was present in the $K$ values of AIB from normal tissue (Fig. 3) and as in $F$, the TAT behaved as a buffering zone between the normal tissue and the tumor tissue.
Discussion

Our primary objective was to examine the effect of host site on tumor and endothelial cell proliferation and on two drug delivery parameters (F and K) in the transplantable RG-2 rat glioma model. Although RG-2 tumors growing in different locations varied in size and microscopic histological features, the following observations in different host sites indicate that RG-2 tumor cells played a major role in determining the vascular phenotype: 1) Endothelial cells had similar proliferation kinetics in brain and s.c. locations (Fig. 1). The cell cycle times and labeling indices of the endothelial cells were about half those of the RG-2 tumor cells. In contrast, the labeling index of the endothelial cells in the RG-2 tumors was 20 times higher than in contralateral tumor-free brain, indicative of the angiogenesis occurring in the RG-2 tumors. 2) The mean value of whole tumor blood-to-tissue transport, as measured by the K of AIB, varied over a narrow range (26.1–49.2 μl/g/min) and there was no statistical difference among the K values for tumors growing in different host locations (Fig. 2). 3) Although there was more variation in whole tumor F values, from a low of 12.5 (liver) to 84.0 ml/g/min (brain), only whole brain tumor F differed significantly from that in other locations. 4) In contrast with the moderate variation in F and transcapillary transport within the RG-2 tumors, there was a very large range of F and K values in tumor-free tissue in the organs into which the tumors were transplanted (Fig. 3), which suggested that the RG-2 angiogenic phenotype dominated over that of the host site.

Angiogenesis is a complex process that represents a balance between stimulatory and inhibitory factors and consists of a multistep cascade including proteolytic degradation of the basement membrane surrounding the endothelial cells, endothelial cell proliferation, migration, and the eventual formation of a new vascular network. Several authors have suggested that the host site environment may dominate the angiogenesis process (Arosarena et al., 1994; Yuan et al., 1994; Risau, 1995; Hobbs et al., 1998). Arosarena et al. (1994) found that immunohistochemical expression of glucose transporter type-1 and endothelial barrier antigen were highly expressed in intracerebral (IC) tumors but not in s.c. tumors; the authors attributed this to host site effects. However, tumor cells may also influence expression of the angiogenic phenotype, regardless of whether the tumors are primary or transplanted. For example, levels of several growth factors, including basic fibroblast growth factor and vascular endothelial growth factor can be directly related to increasing malignancy in brain tumors (Weindel et al., 1994; Yamaguchi et al., 1994; Takahashi et al., 1995), as well as in systemic tumors (Guidi et al., 1995; Takahashi et al., 1995; Toi et al., 1995; Anan et al., 1996).

In addition to the relationship between host site and whole tumor drug delivery parameters, we examined two aspects about the possible impact of methodology on experimental outcome of tumor physiology experiments. First, because ROI's were based on histology in the QAR experiments, we could precisely examine the effect of sample location and histology on the measured result. From a topographical perspective, the value of the measured parameter was always highest in tumor periphery and lowest in tumor center. From a histological perspective, F and K values were consistently highest in viable tumor and lowest in necrotic tumor, regardless of host site. Second, DTS yielded results that were comparable to the whole tumor values obtained from QAR (Fig. 2), although there were instances in which DTS over- or underestimated the parameter compared with the results from QAR. Unless careful attention is paid to the sampling site within an experiment.

![Figure 3](fig3.png)

**Fig. 3.** Drug delivery parameters in tissue around RG-2 gliomas and in tumor-free tissue.

The six locations in which RG-2 gliomas were grown are shown on the left, and are from the same tumors shown in Fig. 2. For each location, F (in ml/g/min) and the K of AIB (in μl/g/min) are shown for TAT and for tumor-free tissue away from the tumor (NOR). Note that the axes used for F and for K have different values in each location. Note the marked variation in values of F and K in TAT and tumor-free tissue, in contrast with those of the RG-2 tumors (Fig. 2).

When the grouped whole tumor values obtained from QAR measurements were compared to those performed by DTS, there was no significant difference (p < .05; ANOVA). In peritoneal tumors, the K measured by DTS exceeded the QAR-determined value in viable tumor tissue, whereas in the muscle and s.c. tumors the K measured by DTS was similar to, or less than, that measured in necrotic tumor tissue. In kidney and liver tumors, the DTS-determined values were similar to those measured by QAR.
tumor, the results may vary over a large range, based simply on which part of a tumor is selected for examination.

If host site interactions are not always responsible for the variability in published results, can other causes be identified? There are major methodological differences in published studies, the effects of which have not been studied. In our experiments, the animals were awake and restrained. Others have also used unanesthetized animals (Lyng et al., 1992), but more commonly the animals are anesthetized (Lyng et al., 1992; Yuan et al., 1994; Vajkoczy et al., 1998; Pardridge, 1998). Some studies have used artificial environments, such as transparent windows (Fukamura et al., 1997; Blasberg et al., 1998; Vajkoczy et al., 1998). In some studies, the tumor may be physically manipulated during the study (Yuan et al., 1995; Fukamura et al., 1997; Blasberg et al., 1998). The effects of variables such as anesthesia, artificial environments, and manipulation on tumor physiology are not known for the most part and methodological comparisons are needed to determine whether these variables can contribute to some of the site-dependent effects that have been reported.

In the introduction, we asked whether the physiological units used to report a study could affect interpretation of the results. This issue was raised by Yuan et al. (1994), who stated that the determination of a transfer constant was “inadequate for understanding mechanisms of the brain-tumor barrier”. Yuan et al. (1994) stated that there has been “no measurement of brain tumor vascular permeability”, by which they meant measurements of the permeability constant, $P$. As we and others have discussed (Fenstermacher et al., 1981; Groothuis and Blasberg, 1985; Blasberg and Groothuis, 1986; Blasberg et al., 1986; Pardridge, 1998), many of the quantitative parameters used to study tumor capillary physiology are interrelated. No single parameter uniquely describes the tumor vascular properties. For example, one relationship between $K$, $P$ (the permeability constant), $S$ (capillary surface area available for exchange), and $F$ is given by the following equation (Fenstermacher et al., 1981):

$$K = FV[1 - e^{-PS/FV}]$$

Other arrangements can be used to interrelate $P$, $S$, $K$, and $F$ and the extraction fraction $E$ (Fenstermacher et al., 1981; Groothuis and Blasberg, 1985; Blasberg and Groothuis, 1986; Blasberg et al., 1986; Pardridge, 1998). An important consequence of eq. 1 is that values of $K$ and permeability-surface area product ($PS$) are equivalent for most water soluble compounds, except at very low $F$ rates. The interrelationship between $F$, the $PS$ and $K$ is shown graphically in Fig. 4. In Fig. 4, the illustrated $F$ values were chosen to be at the low end of the $F$ range, where the divergence between $PS$ and $K$ is greatest. Except when $F \leq 0.01 \text{ ml/g/min}$, $K$ value and $PS$ are equivalent for all $PS$ values less than 0.001 ml/g/min. When $F > 0.1 \text{ ml/g/min}$, $K$ approximates $PS$ for values of $PS < 0.01 \text{ ml/g/min}$. Many chemotherapeutic drugs, as well as oligonucleotides and proteins have $K$ or $PS$ values far less than 0.001 ml/g/min. In other words, in spite of the differences in physiological units, Yuan is measuring the same physiological property as we. As long as quantitatively determined drug delivery parameters can be mathematically related, the studies are likely to have equal value.

In the introduction, we also questioned whether the pharmacokinetic model used in a study could influence the interpretation of the results. In this study we focused on two parameters that influence the initial delivery of blood-borne drugs to a tumor; $F$ and $K$. These two parameters have also been the ones most frequently studied by others. However, the entire drug delivery process must also consider issues such as local metabolism, tissue binding, efflux from tumor back into blood, local movement within the tumor (e.g., convection and diffusion), and for systemic tumors, drug clearance by lymphatics. Aspects of drug delivery that are relevant to the treatment of brain tumors (Blasberg and Groothuis, 1986) and systemic tumors (Jain, 1987, 1988, 1994; Jain and Gerlowski, 1986) have been reviewed. Although the precise calculation of the amount of drug delivered to tumor tissue, as a function of time, cannot generally be accomplished because of the complexity of the models and inadequate knowledge about specific tissue parameters, it is now possible to make general statements about the initial stages of the delivery process. We agree with Jain that additional studies are needed to understand the impact of drug delivery parameters on newer therapeutic agents (Jain, 1996, 1998), but we also need to increase our understanding about the later stages of the delivery and elimination process, as well as to account for the effects of the often large regional variability present in many tumors, especially those growing in brain.

We conclude that the ultimate expression of the angiogenic phenotype for a particular combination of tumor cells and host tissue is a manifestation of interactions between the two and that, depending upon the particular combination, one or the other may dominate. In RG-2 gliomas grown in different host tissues, the angiogenic effects of the tumor cells dominates the process. The similarity of vascular phenotype in the RG-2 tumors, regardless of host location, appears to
be unique among tumor models reported to data and suggest that the RG-2 tumor model may be more suitable for physiological, pharmacokinetic, and drug distribution studies than other tumor models when the tumor is grown in different host locations. However, in addition to the tumor model, more attention needs to be given to the potential variation caused by the experimental methodology itself.

References


Growth Kinetics of Tumors (Walker MD and Thomas DGT eds) pp 211–220, Martinus Nijhoff, Boston.


