Fluctuations in Red Cell Flux in Tumor Microvessels Can Lead to Transient Hypoxia and Reoxygenation in Tumor Parenchyma

Hiroyuki Kimura, Rod D. Braun, Edgardo T. Ong, Richard Hsu, Timothy W. Secomb, Demetrios Papahadjopoulos, Keelung Hong, and Mark W. Dewhirst

ABSTRACT

Hypoxia occurs in two forms in tumors. Chronic or diffusion-limited hypoxia is relatively well characterized. In contrast, intermittent or perfusion-limited hypoxia is not well characterized, and it is not known how common it is in tumors. The purpose of this study was to determine whether spontaneous fluctuations in tumor microvessel flow rate can modify vessel oxygen tension (pO2) sufficiently to cause intermittent hypoxia (IH; tissue pO2 < 3 mmHg) in the tumor parenchyma supplied by such vessels. Microvessel red cell flux (RCF) and perivascular pO2 were measured simultaneously and continuously in dorsal flap window chambers of Fischer-344 rats with implanted R3230Ac tumors. In all vessels, RCF was unstable, with apex/nadir ratios ranging from 1.5 to 10. RCF and pO2 were temporally coordinated, and there were linear relationships between the two parameters. Vascular pO2 was less sensitive to changes in RCF in well-vascularized tumor regions compared with poorly vascularized regions. Simulations of oxygen transport in a well-vascularized region of a tumor demonstrated that two-fold variations in RCF can produce IH in 30% of the tissue in that region. In poorly vascularized regions, such fluctuations would lead to an even greater percentage of tissue involved in transient hypoxia. These results suggest that IH is a relatively common phenomenon. It could affect binding of hypoxic cytotoxins to tumor cells, in addition to being an important source of treatment resistance. Intermittent hypoxia also could contribute to tumor progression by providing repeated exposure of tumor cells to hypoxia-reoxygenation injury.

INTRODUCTION

Hypoxia has classically been thought to be a source of resistance to radiation and drugs. Numerous studies, dating from the early part of this century, have investigated means to ameliorate hypoxia to improve therapeutic response (1, 2). Recently, however, the presence of hypoxia has also been associated with phenotypic changes in tumors that may reflect tumor progression. For example, the expression of a number of cytokines and growth factors is known to be controlled by hypoxia (3, 4). Certain oncogenes and suppressor genes are also known to be up-regulated in the presence of hypoxia (4, 5). Hypoxia may cause selection for cells lacking the wild-type tumor suppressor gene p53. Thus, hypoxia also may play a role in tumor progression by applying negative selection pressure toward mutant forms of the gene (5). There is clinical evidence for a relationship between hypoxia and tumor aggressiveness as well. Recently, we have reported that the presence of tumor hypoxia, before therapy, is associated with a more metastatic phenotype in humans with soft tissue sarcoma (6). Similarly, high lactate levels in human cervix cancer have been associated with a greater likelihood of developing local-regional failure after radiation therapy (7).

Tumor hypoxia can originate in one of two ways. Diffusion-limited hypoxia is classically thought to develop as a result of limitations in the diffusion distance of oxygen from a vessel, and many studies, including our own, have been published that have examined various aspects of this form of hypoxia (8-10). Acute or transient hypoxia typically has been thought to occur as a result of transient stoppages in microvessel flow (11-14), although it has been suggested previously that complete vascular stasis may not be necessary for this form of hypoxia to occur (15). However, this form of hypoxia has not been well characterized, because no direct examinations of the kinetics of blood flow fluctuations in conjunction with measurements of pO2 in tumors have been made. In addition, it has not been previously determined whether fluctuations in microvessel flow rate could lead to changes in tissue oxygenation. We hypothesized that changes in RCF in tumor microvessels could lead to changes in vascular pO2 that would be sufficient in magnitude to affect tumor parenchymal oxygenation.

We used Fischer-344 rats with R3230Ac mammary carcinomas implanted in dorsal flap window chambers to measure the kinetics of microvascular RCF and pO2 in individual tumor microvessels. On the basis of the measurements obtained, oxygen transport to tumor parenchyma was simulated theoretically, leading to the prediction that substantial portions of tumor tissue are exposed to periods of transient hypoxia as a result of such fluctuations in RCF.

MATERIALS AND METHODS

Animal Model. The dorsal skinfold window chamber implanted in Fischer-344 rats was used for these experiments (16). Briefly, sections of epidermis 1 cm in diameter were removed from opposing surfaces of the dorsal skin flap, leaving one to two fascial layers. The two halves of the chamber were sutured on the outside of the window flap. R3230Ac mammary adenocarcinoma cells were transplanted onto the upper fascial plane at the time of the surgery. Cover glasses were placed over the surgically exposed tissue to protect against infection and dehydration. Tumors were allowed to grow to 2–3 mm in diameter, which typically required 8–9 days. These protocols were approved by the Duke Animal Care and Use Committee.

Oxygen Microelectrodes. Recessed tip microelectrodes were manufactured and calibrated in our laboratory, using methods described by Linsenmeier and Yancey (17). The electrodes have a tip diameter of 3–6 μm with a recessed, gold cathode. All electrodes were calibrated before and after each experiment in saline bubbled with certified gas mixtures of 0, 5, 15, and 21% O2 in N2. Electrodes with sensitivities of >0.2 pA/mmHg were used, and experiments in which the calibration slope drifted >20% from pre- to postexperiment were eliminated from analysis.

Fluorescent Labeling of RBCs. Erythrocytes were labeled with Dil using the method of Unthank et al. (18). Briefly, 5 mg of Dil was dissolved in 10 ml of ethanol. Blood from an anesthetized donor rat was withdrawn via cardiac puncture, and the erythrocytes were isolated and washed in PBS. One hundred μl of packed RBCs were suspended in 10 ml of PBS with 100 μl of Dil stock solution and incubated at room temperature for 1 h. The labeled RBCs were separated from excess Dil solution by centrifugation and washing in PBS.

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3 The abbreviations used are: pO2, oxygen tension; RCF, red cell flux; Dil, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine; IH, intermittent hypoxia.
Intravital Video Microscopy. Videomicroscopy of the window chamber, as anesthetized animals were restrained in lateral recumbency, was used to observe microvascular parameters (Zeiss Photomicroscope III, Carl Zeiss, New York, NY). Visualization of window chamber vasculature was accomplished using either transillumination (40-W tungsten source) or epifluorescence (xenon arc source and rhodamine filter sets). Data were acquired using a charge-coupled device camera for transillumination (MTI CCD-72, Dage-MTI, Michigan City, MI) or a silicon-intensified tube camera for fluorescence microscopy (model C2400-08, Hamamatsu Photonics, Hamamatsu City, Japan) and recorded to SVHS videotape (SVO-9500MD, Sony, San Jose, CA). A videotimer image was superimposed on all videotapes for recordkeeping (CTG-55 Video Timer, For.A. Co., Los Angeles, CA).

Flow Cytometry. Samples of peripheral blood (0.1 ml) were obtained in heparinized tubes and after each experiment to determine the fraction of labeled red cells. Determination was made using flow cytometry, as described previously (19).

Liposome Preparation. Liposomes were prepared according to a repeated freezing-thawing and extrusion method (20). Liposome composition consisted of hydrogenated soy phosphatidylcholine/cholesterol/polyethylene glycol (M, 2000)-derivative phosphatidylethanolamine/rhodamine-labeled phosphatidylethanolamine with molar ratio 60:40:2.4:0.48. Phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipids were mixed in chloroform solution, and solvent was removed under reduced pressure at 50°C. Preheated buffer (20 mm HEPES and 145 mm NaCl (pH 7.2)) at 55°C was added to the dry lipid to yield a final phospholipid concentration of 10 mm. Multilamellar vesicles were formed by vortexing. After the lipids were fully hydrated, the sample was put through freezing (~80°C) and thawing (55°C) cycles for five repetitions to reduce the number of bilayers. The sample was then extruded three times at 55°C under pressure (200-300 p.s.i.) through polycarbonate filter membranes with 0.2-μm pore diameters. The resultant liposome diameters had diameters of 200-300 μm.

Experimental Protocol. Animals were anesthetized with 40 mg/kg Nembutal, given i.p. A femoral artery was cannulated for blood pressure measurement (CODAS, Micro Software, Akron, OH), and the tail vein was cannulated to provide venous access. Animals were placed on the microscope stage in lateral recumbency on a thermostatically controlled heating blanket (Harvard homeothermic blanket; Harvard Apparatus, Ltd., South Natick, MA), which maintained rectal temperature at 37°C. Aliquots of femoral arterial blood (0.1 ml) were obtained before and at the end of each experiment for determination of blood gases.

The upper window glass was removed from the window chamber, and the tissue was suffused with Earl's balanced salt solution and bubbled with 5% CO2 and 95% N2. To further minimize the diffusion of oxygen from air, a tent was formed around the window chamber out of plastic wrap (Saran Wrap, Dow Brands, L.P., Indianapolis, IN), and 5% O2/95% N2 was blown into the tent. The suffusion medium was kept at 33°C (pH 7.3) and suffused at a rate of 1.0 ml/min. These conditions created an average pO2 of 4 mmHg on the tissue surface, as described previously (10). Vessels had to be at least 50 μm beneath the tissue surface to minimize artifacts that could be created by the suffusion medium. Vessels had diameters of 0.2-300 μm. The configuration of vessels used for the simulations, consisting of 22 vascular segments in a region 0.25 X 0.37 X 0.2-mm, has been described previously (21). Length densities were measured within circles of 100- and 150-μm radius, around the point of pO2 measurement. The results are reported as mm/mm3.

The statistical distribution of pO2 in the tissue was estimated from computed pO2s from the video recording onto transparency film, and the vessels to be analyzed were identified. RCF in each vessel was determined at each time point in the current experimental series. The oxygen content of blood was assumed to be governed by the Hill equation, with 0.1 cm3 O2/cm3 at saturation, half saturation at 38 mmHg, and exponent n = 3. The product of oxygen diffusivity and solubility in the tissue is 4.2 x 10^-10 cm2 O2/cm3/min/mmHg, and the oxygen consumption rate averages 1.52 cm3 O2/100 g/min, based on previous studies (23).

The statistical distribution of pO2 was generated using a Green's function method (9, 22). Briefly, the network of vessels within a cuboidal tissue region was represented as a set of finite cylindrical segments, which were subdivided into many (100-200) short pieces. Reduction of consumption in hypoxic regions was modeled by a Michaelis-Menten relationship, with half-maximal consumption at 1 mmHg. The oxygen fluxes from each vessel piece were computed so as to satisfy the condition that the vessel pO2 matched tissue pO2 at the blood-tissue boundary of each vessel piece. The distribution of pO2 in the tissue was estimated from computed pO2s at 1000 randomly selected points.

The configuration of vessels used for the simulations, consisting of 22 vascular segments in a region 0.25 X 0.37 X 0.2-mm, has been described previously (9) and is similar to the more densely vascularized regions among those observed in the current experimental series. The oxygen content of blood was assumed to be governed by the Hill equation, with 0.1 cm3 O2/cm3 at saturation, half saturation at 38 mmHg, and exponent n = 3. The product of oxygen diffusivity and solubility in the tissue is 4.2 x 10^-10 cm2 O2/cm3/min/mmHg, and the oxygen consumption rate averages 1.52 cm3 O2/100 g/min, based on previous studies (23).

Theoretical simulations were designed to predict the effects of observed fluctuations in RCF and peripheral pO2 on levels of parenchymal pO2 and on hypoxic fraction. Two states were defined: a control state in which the total blood flow entering the region was 8 x 10^-3 cm3/s, as observed experimentally (24), and a reduced-flow state, in which flow in all segments was reduced by 50%. The pO2 of inflowing blood was assumed to be 30 mmHg in the control state and 19 mmHg in the reduced-flow state, based on experimental observations described below.

RESULTS

Thirteen experiments were conducted. Two were excluded from analysis. In one case, blood pressure was well below the normal range for our anesthetized subjects (normal range = 90-110 mmHg; this individual’s mean arterial pressure averaged 70 mmHg). In the second case, the postexperimental calibration of the electrode showed drift in the calibration that exceeded our stated limit (>20% drift in slope). Thus, 11 experiments were completed and available for analysis.

Typical experiments lasted 40-60 min once the suffusion medium was established, and the gradient of pO2 from the medium surface to the tissue surface was measured. The mean baseline values for mean arterial blood pressure and blood gas pO2 were well within the range for previous experiments in this laboratory (25) and were also within normal limits for unanesthetized rats (Table 1; Ref. 26). Mean arterial pressure did not change over the period of observation. Blood gas pO2

Table 1 Mean arterial blood pressure and arterial pO2

<table>
<thead>
<tr>
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<th>Blood pressure</th>
<th>Blood gas pO2</th>
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<tr>
<td>Beginning</td>
<td>103 ± 6</td>
<td>89 ± 3</td>
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<tr>
<td>Middle</td>
<td>104 ± 7</td>
<td>95 ± 7</td>
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<td>End</td>
<td>98 ± 8</td>
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TRANSIENT HYPOXIA IN MURINE BREAST CANCER

showed a trend toward an increase, but this was not statistically significant.

Results from two example cases are presented in detail. In the first example, the vascular length density was 41.9 mm/mm³ (100-μm radius), which is relatively high in this series of experiments (Fig. 1A). The point of pO2 measurement was near the side of the network. Temporal changes in RCF were coordinated in vessels in the region (Fig. 1B) and temporally coordinated with changes in vascular pO2 (Fig. 3, experiment 6). In the second example, vascular length density was relatively low (18.9 mm/mm³; Fig. 2A). In this example, the point of measurement was downstream from a point of divergence, and the RCF in the neighboring vessel was considerably larger than that at the point of pO2 measurement (Fig. 2B), but RCF in the two vessels was still coordinated. Again, changes in RCF were coordinated with changes in vascular pO2 (Fig. 3, experiment 8).

A summary of the temporal changes in RCF and perivascular pO2 in all experiments is shown in Fig. 3. Several important observations can be made. First, baseline RCFs were highly variable, ranging from the low thousands to maxima of >100,000 cells/min. Baseline pO2s were also variable, ranging from <10 to >50 mmHg. There was no obvious relationship between baseline pO2 and baseline RCF, but perivascular pO2 generally tracked changes in RCF. The most obvious exception to this trend was experiment 9, in which a reduction in pO2 after 40 min of observation was concomitant with an increase in RCF. RCF was unstable in all cases, although the relative magnitude of fluctuation varied from one experiment to the next. In some cases, variations in RCF followed a cyclical pattern (experiments 2, 3, and 4).

To examine the relationship between changes in RCF and perivascular pO2, plots of pO2 versus RCF were generated (Fig. 4). In all cases except one (experiment 9), there was a positive correlation between RCF and pO2. R²'s ranged from 0.3 to >0.8 for the remaining 10 experiments. The slope of the relationship was variable from one experiment to the next, however, ranging from 0.6 to >3 mmHg/10⁶ cells/min.

We hypothesized that the vascular density in the region of the pO2 measurement could have an influence on the sensitivity of vascular pO2 to changes in RCF. For this reason, we examined the relationship between the slopes obtained from Fig. 4 and vascular density. Two experiments were excluded from the analysis. Experiment 9 was excluded because the relationship between flux and pO2 in that experiment was not significant. Experiment 5 was excluded because the average pO2 in that case was very low compared with the other nine experiments. The average pO2 of all experiments, excluding experiment 5, was 20 ± 5 (range 13–27) mmHg. The average pO2 for experiment 5 was 6 mmHg, which was <50% of the lowest of the

Fig. 1. A, diagram of vascular network surrounding point of pO2 measurement for experiment 6. Directions of vascular flow are indicated by arrows, and the point of pO2 measurement is pictorially indicated by the schematic electrode tip. B, RCF versus time for the vessel segments depicted in A.
remaining experiments. It is interesting that the two experiments for which this analysis did not work were the extremes, in terms of vascular pO2. Experiment 9 had the highest baseline pO2, and experiment 5 had the lowest.

This relationship for the remaining nine experiments appeared to be linear for both 100- and 150-μm radii, and in both cases the relationship was significant (P = 0.0055 and 0.0027, respectively; Fig. 5; data not shown for 100-μm radius). When vascular density was relatively high, vascular pO2 was relatively insensitive to changes in RCF and vice versa. This dependence occurred over approximately 5-fold ranges of both vascular density and slope.

**Modeling Results.** These experimental results were used to define the conditions for the theoretical simulations. In the reduced-flow state, flow in all vessels was reduced by the same amount, consistent with the observed parallel flow changes in nearby vessels (Figs. 1B and 2B). The assumed flow reduction of 50% was conservative, based on the large observed amplitude of flow fluctuations (Fig. 3). The inflow pO2 in the control state was set at 30 mmHg, in the upper range of observed perivascular values (Fig. 3). The inflow pO2 in the reduced-flow state was estimated according to the following procedure. (a) The vascular density of the network used in the simulations was estimated using the method already described, yielding 40 mm³/mm³ in a circle with a radius of 150 μm. (b) The corresponding pO2 versus RCF slope was estimated from the regression line in Fig. 5, yielding 1.45 mmHg/(10⁴ RBC/min). The decrease in RCF in the major feeding vessel to the region (which carried 64% of total flow) associated with a 50% decrease in flow was 7.6 × 10⁴ RBC/min, based on a hematocrit of 25% (19) and a red cell volume of 55 fl. This implies a decrease in perivascular pO2 of 10 mmHg, and so an inflowing pO2 of 20 mmHg was assumed in the reduced-flow state. This value was in the mid-to-lower range of observed values (Fig. 3).

The results of the simulations are shown in Fig. 6, in the form of cumulative frequency distributions of pO2 in control and reduced-flow states. To interpret this graph, assume that the flow rate fluctuates with time between these two states. At a given pO2 value, the vertical distance between the lower curve and the X-axis represents the percentage of tissue that is always below that pO2 (chronically hypoxic cells), and the vertical distance between the curves represents the percentage of tissue whose pO2 fluctuates above and below the specified level. In particular, if hypoxia is defined as pO2 < 3 mmHg (Fig. 6, vertical line), then it can be seen that approximately 25% of the tissue is chronically hypoxic, 35% is transiently hypoxic, and 40% remains above 3 mmHg. Corresponding predictions for other defined levels of hypoxia may be determined similarly.

**DISCUSSION**

The results of this study indicate that there are fluctuations in RCF in tumors and that these fluctuations lead to measureable changes in vascular pO2. Casual observation of vessel flow would not reveal this transient behavior, which requires repeated measures of RCF over periods of 1 h or more. In some experiments, the RCF fluctuations appeared to show cyclic behavior, with periods of 20–30 min. However, the 60-min observation period was too short to show whether the fluctuations were genuinely periodic. In other experiments, no periodicity in RCF was found, although it is possible that the cycle times were greater than 60 min. In a previous report in which suffusion media were not used, we found cycle times ranging as high as 60 min (27). The observation that the variations seem to be coordinated in groups of vessels would indicate that it is most likely caused by upstream effects. These variations in RCF are similar to variations in feeding arteriolar diameter, which suggests a cause-and-effect relationship (27), although direct manipulation of arteriolar vasomotion with resultant changes in RCF dynamics would be needed to prove a causative relationship. In a therapeutic sense, if it were possible to pharmacologically minimize arteriolar vasomotion, then the impact of this form of hypoxia on treatment outcome also could be minimized. An alternative hypothesis to explain the fluctuations in flow is the recently reported vascular architectural remodeling caused by intussusceptive microvascular growth in tumors (28).

The large fluctuations in RCF reported here are in agreement with findings from several other studies. Thermocouple measurements within unheated tumors showed temporally similar temperature fluctuations, and these temperature changes were attributed to alterations in blood flow (29). More recently, laser Doppler flow studies in both experimental (30) and human tumors (31) have directly demonstrated that microregional changes in RCF occur within tumors. The authors suggested that these RCF fluctuations were consistent with transient perfusion-related changes in tumor oxygenation (31). The time course of erythrocyte flux changes in those studies and the present work were also similar.

Previous reports of blood flow fluctuations in tumors, including our own, suggested that intermittent hypoxia is a relatively rare phenomenon (11, 14, 21), because the assumption was that total vascular stasis was necessary for hypoxia to occur. Results of this work suggest that total vascular stasis is not a requirement for
tumor tissue hypoxia. We observed unstable flow in all of the experiments and, with one exception (experiment 9), this was reflected by correlated changes in vascular pO₂. The simulations suggest that 2-fold changes in RCF, even in a relatively well-vascularized tumor region, could lead to substantial volumes of tumor being subjected to transient periods of hypoxia. In poorly vascularized tumors, the situation would only be more exaggerated, because the sensitivity of vascular pO₂ to changes in RCF is inversely correlated with vascular density. Consequently, transient hypoxia may be more common than was previously believed.

Direct observation of microvessel flow in window chamber tumors has indicated that total vascular stasis is an infrequent event, occurring less than 10% of the time (12, 14, 21). These observations were confirmed in larger three-dimensional tumors by Chaplin et al. (11). The fluorescent dye Hoechst-33342 was administered either before or concomitantly with microparticles that fluoresced at a different wavelength. When the two agents were administered concomitantly, there was good agreement in histological sections between vessels that were stained with Hoechst and the particles. However, when there was a time delay between the two agents, then vessels were identified that contained the particles, but not the Hoechst dye. The frequency of mismatch was dependent on tumor size and type, but was usually less than 10%. In a later study, Trotter et al. (15) used matched pairs of fluorescent dyes instead of the dye-particle combination. In these studies, they found not only dye mismatch, but also differences in staining intensity. They speculated that the latter phenomenon might be due to fluctuations in blood flow, as opposed to total vascular stasis. These results are consistent with our observations.

These results have important implications regarding the use of bioreductive drugs that are being tested to detect and treat hypoxia in tumors (32, 33). Our results suggest that the intensity of binding will be dependent on both the degree and duration of hypoxia once the drug accumulates intracellularly. Consequently, the intensity of binding in immunohistochemistry may not form a sufficient basis for distinguishing this form of hypoxia from true steady-state diffusion limitation of oxygen. In a therapeutic sense, the effectiveness of bioreductive cytotoxins may be quite dependent on the time average
of transiently hypoxic regions, in addition to the proportion of tumor that is at true steady-state chronically hypoxic conditions.

The instability in flow and oxygenation also could be partly responsible for reported discrepancies that have been seen between radiobiological hypoxic fraction and electrode measurements of tumor hypoxia (34). Another factor that could contribute to the lack of correlation is the fact that there may be a discrepancy between the clonogenic hypoxic fraction and that which is measured by an electrode system (35). However, in spite of these issues in rodent models, there have been reports that electrode measurements obtained from patients correlate with treatment outcome in tumors of the head and neck, cervix, and soft tissue sarcomas (6, 36, 37).

In a recent article, it was reported that hypoxia might create negative selection pressure on cells with wild-type p53 suppressor gene, because activation of p53 leads to apoptosis (5). Thus, cells with mutant forms of p53 would gain a survival advantage over cells with wild-type p53. Our results suggest perhaps a more sinister effect of hypoxia. If the process of oxygen transport is as unstable as our results would indicate, then it is possible that once a tumor becomes vascularized, it is subjected to repeated instances of hypoxia-reoxygenation injury. Because the primary bioactive product of this process is superoxide (38), it is possible that mutagenic events leading to tumor progression could result. One would not expect that this process would be responsible for initiation, because tumor angiogenesis, with its associated pathophysiology, would be a prerequisite to this effect.

In summary, by studying tumor vascular oxygenation concomitantly with changes in RCF, we have found that blood flow and oxygenation are unstable in the rat R3230Ac mammary adenocarcinoma. Theoretical simulations suggest that this instability may lead to relatively large fluctuations in the proportion of tumor that is hypoxic at any given time.

Fig. 4. Relationship between RCF and perivascular pO2, ignoring the time factor, for all 11 experiments. Equations for each line are indicated at the top of each figure. The slopes are in units of mmHg/10,000 cells/min.
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