The Effect of Tumor Size on Necrosis and Polarographically Measured pO_2

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Tumor necrosis and oxygen status were investigated as a function of tumor size in three syngeneic murine carcinomas, MCa-4, OCa-I, and SCC-VII, in C3Hf/Kam mice. Tumor necrosis was estimated histologically, and tumor oxygenation determined by direct polarographic histography. As tumor volume increased necrosis increased significantly in all three tumor types ($p < 0.001$). Similarly, as tumor volume increased from 200 to 1 400 mm^3, hypoxia, defined as the percentage of measured pO_2 values ≤ 5.0 mm Hg, increased from 55.1% to 95.9%, 70.3% to 81.4%, and 56.8% to 98.5% in MCa-4, OCa-I, and SCC-VII tumors respectively ($p < 0.001$). Correcting pO_2 for necrosis reduced the tumor size dependence of measured tumor hypoxia in all three tumor types but in no case was the reduction significant. The main effect of correction was to shift the fitted curves of percent pO_2 values ≤ 5.0 mm Hg down toward lower percentages for all tumors. This change was significant for MCa-4 and OCa-I tumors ($p < 0.001$), but not for SCC-VII ($p = 0.054$). Defining the influence of variables such as necrosis that affect polarographic assessment of tumor oxygenation is important to enhance the technique's reliability and prospect as an investigative and predictive tool.

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In the 1930s it was discovered that the cytotoxic effect of ionizing radiation was retarded by hypoxia (1, 2). Subsequently, radioresistant hypoxic clonogens have been identified in the majority of solid transplanted rodent tumors (3, 4). Together these observations led to the belief that hypoxia was an important factor limiting the success of clinical radiotherapy and motivated a considerable research effort aimed at overcoming tumor hypoxia (5–7). These same studies provided conclusive evidence that hypoxic cells do exist in human tumors and influence the outcome of radiotherapy (8). Furthermore, it has been suggested that hypoxia protects cells in tumors from the cytotoxic effects of some chemotherapeutic agents (9–11).

There has likewise been a considerable research effort aimed at developing methods to detect the presence and magnitude of hypoxia in tumors. Such information may serve as a prognostic indicator of treatment outcome and be useful for stratification of patients to treatments aimed at overcoming tumor hypoxia. Direct polarographic histography is one method used to estimate tumor oxygenation status, both in the laboratory and clinical settings.

Tumor hypoxia is closely correlated with tumor size. As tumors enlarge the fraction of radiobiologically hypoxic cells is increased (12, 13), and measured tumor pO_2 values are decreased (14). It is also well established that tumor necrosis increases with increasing tumor volume (15, 16), but the impact of necrosis on tumor oxygen measurements had not been investigated until recently. Khalil et al., (17) however, reported results of an important study in which they corrected the pO_2 profiles measured in a murine mammary adenocarcinoma by eliminating measurements estimated to have been taken from necrotic regions. Rationally this was appropriate since necrotic cells are clearly nonclonogenic and do not contribute to any radiobiological estimate of hypoxic fraction. These authors found that correction for necrosis abrogated the volume dependence of polarographically measured hypoxia (17).

To determine whether this finding has general applicability to other tumor systems we conducted a similar study using murine tumors of three different histological types. We examined the volume dependence of both tumor necrosis and of polarographically measured tumor hy-

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poxia. Using the method developed by Khalil et al., we also applied a correction for necrosis to measured hypoxia in order to determine the nature and magnitude of the effect of that correction.

MATERIALS AND METHODS

Mice and tumors. Four-month-old male C3Hf/Kam mice bred in our specific pathogen-free facility were used in these experiments. The mammary carcinoma designated MCa-IV, the squamous cell carcinoma designated SCC-VII, and the ovarian carcinoma designated OCa-I, all syngeneic to C3Hf/Kam mice, were grown in the right leg. Tumors were in their 3rd to 6th isotransplant generations and were formed by intramuscular injection of 5×10^5 tumor cells in single-cell suspension prepared by enzymatic and mechanical digestion of parent tumors. Tumor volume (V) was determined by measuring three orthogonal diameters (d_1, d_2, and d_3) with Vernier calipers and substituting them in the formula:

$$V = (\pi/6) \cdot d_1 \cdot d_2 \cdot d_3 \quad [1]$$

When tumors achieved volumes ranging from approximately 150 to 1 500 mm^3, mice were allocated to either histological analysis or tumor pO_2 measurement groups.

Necrotic fraction estimation. Tumor-bearing mice were killed by cervical dislocation, and tumors were immediately excised and placed in neutral buffered formalin. After fixation, tumors were bisected along the midplane and embedded in paraffin blocks from which 4 μm sections were cut and stained with hematoxylin and eosin. A single histological section was prepared for each tumor. The percentage necrosis was determined using a Chalkey point counter with 25 random points, using a method similar to that described by Camplejohn & Penhaligon (18). At 200× the number of points that fell on necrosis were counted in 20 fields distributed evenly across the area of the tumor section. Thus the percentage necrosis was based on scoring 500 points per section as either necrotic or nonnecrotic. The light-microscopic features used to identify necrosis included increased cell size, indistinct cell border, eosinophilic cytoplasm, loss or condensation of the nucleus, and associated inflammation (19).

Tumor oxygenation measurements. Tumor oxygenation was determined by polarographic measurement using the Eppendorf pO_2 histograph (Eppendorf, Hamburg Germany). The abdomen and tumor-bearing hind limb were shaved, and a saline-moistened Ag/AgCl reference electrode attached to the mouse's abdomen. Measurements were performed in awake mice, breathing room air, immobilized in a custom made Perspex jig. The oxygen probe was introduced into the tumor under direct vision after cutting a small fenestration in the overlying skin. pO_2 was measured every 0.4 mm along 4 parallel tracks in each tumor. Track length ranged from 4 mm in 6–7 mm tumors to 11 mm in 13–14 mm diameter tumors, and so 44 to 112 pO_2 values were collected for each tumor. Results for each tumor were stored as a single relative frequency histogram from which the percentage of pO_2 values $\leqslant 5.0$ mm Hg were calculated.

Oxygen probes were calibrated before and after each series of measurements in 0.9% saline saturated alternately with room air and nitrogen for 2 and 3 min respectively. Probes characterized during precalibration by an O_2 current >15 nA, an N_2 current <3 nA, an $N_2/O2$ ratio $<10\%$, and an O_2 drift of $<0.20\%$ per min were acceptable for use. After pO_2 measurement tumor temperature was recorded by a thermocouple microprobe (Bailey Instruments, Saddlebrook NJ), and pO_2 values postcalibrated accordingly. As an internal control, pO_2 was also measured in the contralateral leg muscle of each mouse.

Correction of pO_2 measurements for necrosis. A plot of percentage necrosis versus tumor volume was prepared for each histological tumor type, and data were fitted with the logistic model using maximum-likelihood analysis. Similarly the percentage of pO_2 percentage values $\leqslant 5.0$ mm Hg was plotted against tumor volume, and the data were also fitted using the logistic model.

To correct the percent of pO_2 measurements $\leqslant 5.0$ mm Hg for necrosis in a tumor of volume x, the average proportion of necrosis y, was firstly determined from the logistic model with constants A and B, according to the equation:

$$y = \frac{e^{(A + B \cdot x)}}{1 + e^{(A + B \cdot x)}} \quad [2]$$

The expected number of readings (n) from necrotic areas was then calculated:

$$n = y \cdot N$$

where N is the total number of pO_2 values measured. The number of pO_2 values $\leqslant 5.0$ mm Hg (h) is given by:

$$h = \frac{\%pO2 < 5.0 \text{ mm Hg}}{100} \cdot N$$

The estimated number of readings from necrotic areas was subtracted from both the number of hypoxic values and the total number of measured pO_2 values, and the corrected percentage of pO_2 values $\leqslant 5.0$ mm Hg (corrected % $pO_2 < 5.0$ mm Hg) was calculated as:

$$\text{Corrected \% } pO_2 < 5.0 \text{ mm Hg} = \frac{N - h}{N - n} \cdot 100 \quad [3]$$

The corrected percentage of pO_2 values <5.0 mm Hg was plotted against tumor volume and a new logistic fit to the data determined. Equation [3] is the same as the method used by Khalil et al. to correct of necrosis, but they assumed a linear relationship between tumor size and necrotic fraction in lieu of the logistic model of equation [2].

Statistical methods. To determine if the correction for necrosis significantly altered the estimated relationship

between tumor diameter and measured hypoxia, the increase in the average percentage of $pO_2 < 5.0$ mm Hg as tumors grew from 150 to 1 500 mm^3 tumors, estimated from the corrected fit, was compared to the increase estimated from the uncorrected fit using the likelihood ratio test. The likelihood ratio test was also used to determine if correction for necrosis significantly altered the relative position of the fitted curves of percentage of pO_2 values <5.0 mm Hg versus tumor volume.

RESULTS

Necrotic fraction. In all three tumor types tested there was a clear increase in the amount of necrosis as tumors increased in size. The fitted percentage and 95% C.I. of necrosis, obtained from the logistic fit to the data, increased from 20.0% (11.6, 32.4) to 48.1% (30.4, 66.4) in MCa-4 (Fig. 1A), from 24.5% (14.2, 39.0) to 44.9% (27.1, 64.3) in OCa-I (Fig. 2A), and from 9.7% (4.6, 19.1) to 48.67% (27.4, 70.4) in SCC-VII tumors as volume increased from 200 to 1 400 mm^3 (Fig. 3A). In each case the best fitting logistic model had a significantly positive slope ($p < 0.001$).

Tumor oxygenation. Just as tumor necrosis increased with increasing tumor size, tumor hypoxia, defined as the percentage of polarographically measured pO_2 values $\leqslant 5.0$ mm Hg, obtained from the logistic fit to the data, increased as tumor size increased. The fitted percentage and 95% C.I. of pO_2 values $\leqslant 5.0$ mm Hg increased from 55.1% (11.0, 92.4) to 95.9% (61.6, 99.7) for MCa-4 tumors (Fig. 1B), from 70.3% (24.2, 94.6) to 81.4% (29.7, 97.8) for OCa-I tumors (Fig. 2B), and from 56.8% (5.5, 96.7) to 98.5% (63.2, 100) in SCC-VII tumors as volume increased from 200 to 1 400 mm^3 (Fig. 3B). For all three tumor types, the best fitting logistic models had significant positive slope ($p < 0.002$). The data clearly demonstrate that these tumors became progressively and markedly hypoxic as they enlarged. For MCa-4 and SCC-VII tumors, the curves had an initial steep slope but tended to plateau at tumor volumes greater than 500 mm^3, at which point the percentage of pO_2 values $\leqslant 5.0$ mm Hg was generally greater than 90%. By comparison, OCa-I tumors exhibited greater variability in the percentage pO_2 values $\leqslant 5.0$ mm Hg, and although the logistic model fitted to the data had a significantly positive slope ($p = 0.002$) it was shallower than for the other two tumor types, and smaller tumors were more hypoxic.

Correction for necrosis. To determine whether and to what extent tumor necrosis influenced the measured percentage of pO_2 values $\leqslant 5.0$ mm Hg, corrections were made by eliminating measurements estimated to have been obtained in necrotic areas. In two tumor types, MCa-4 and OCa-I, this correction for necrosis slightly but not significantly ($p = 0.167$ and $p = 0.626$ respectively) reduced the slope of the logistic curve fitted to the corrected percentages of $pO_2 < 5.0$ mm Hg. In both cases, however, the fitted curve was significantly displaced inferiorally toward lower

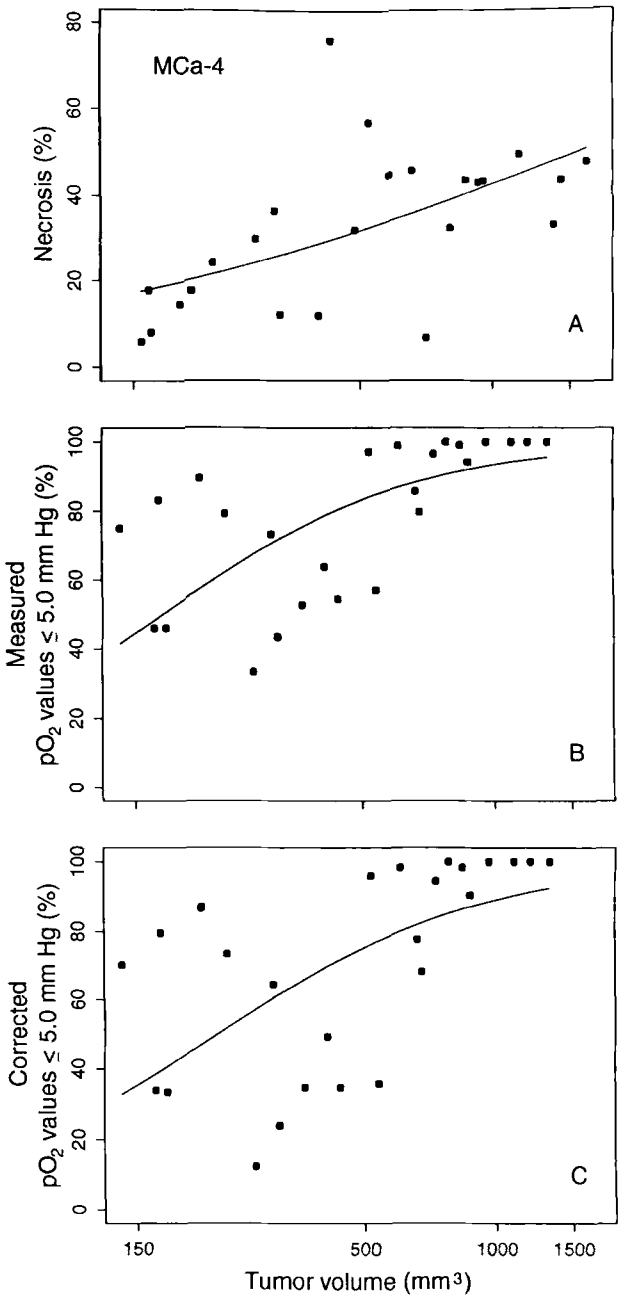

Fig. 1. Tumor necrosis (A) and polarographically measured tumor hypoxia, defined as the percentage of pO_2 readings $\leqslant 5.0$ mm Hg (B), plotted as a function of MCa-4 tumor volume. Each point represents a single tumor. Data were best fit by the logistic model; resultant curves had significant positive slope ($p < 0.001$). Measured tumor hypoxia was corrected by eliminating pO_2 readings estimated to have been taken in necrotic areas. The corrected percentage of pO_2 values <5.0 mm Hg and the logistic fit to the data are shown as a function of tumor volume (C). Maximum-likelihood analysis confirmed significant inferior displacement of the fitted curve ($p < 0.001$) without significant reduction in slope.

percentages of pO_2 values <5.0 mm Hg for corresponding tumor volumes ($p < 0.001$) (Fig. 1C and Fig. 2C respectively). The pO_2 values from the SCC-VII tumor were similarly affected by correction for necrosis: the slope

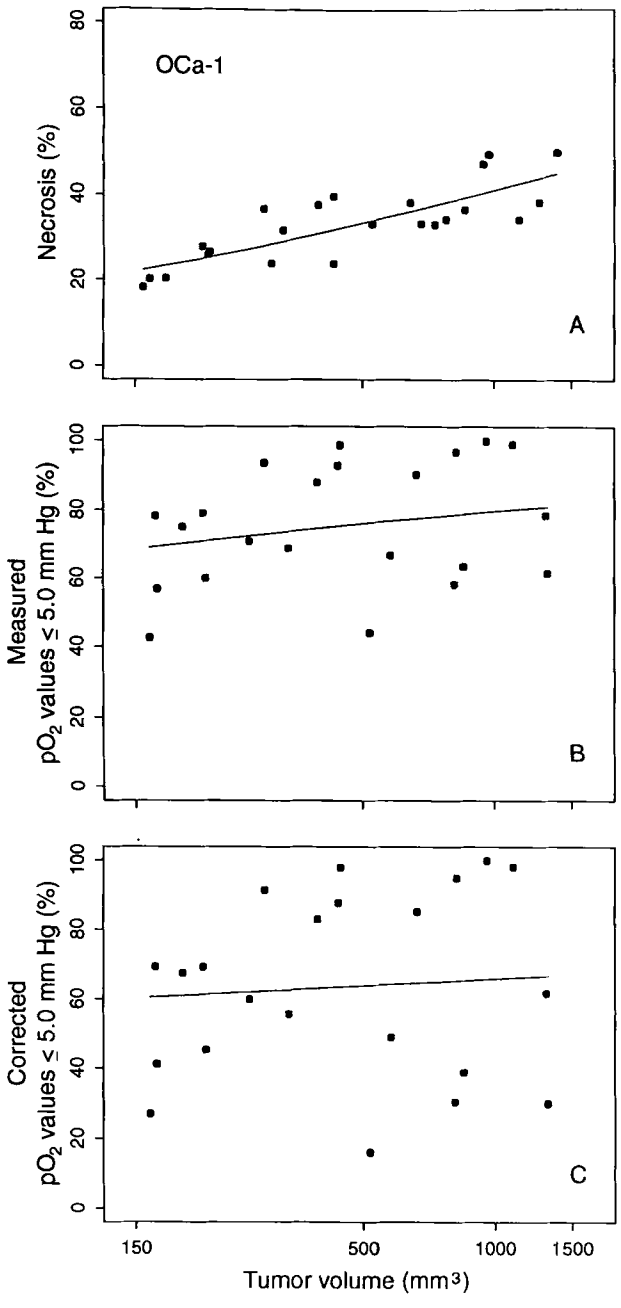

Fig. 2. Tumor necrosis (A) and polarographically measured tumor hypoxia, defined as the percentage of pO_2 readings ≤ 5.0 mm Hg (B), plotted as a function of OCa-I tumor volume. Each point represents a single tumor. Data were best fit by the logistic model; resultant curves had significant positive slope ($p < 0.001$). Measured tumor hypoxia was corrected by eliminating pO_2 readings estimated to have been taken in necrotic areas. The corrected percentage of pO_2 values ≤ 5.0 mm Hg and the logistic fit to the data are shown as a function of tumor volume (C). Maximum-likelihood analysis confirmed significant inferior displacement of the fitted curve ($p < 0.001$) without significant reduction in slope.

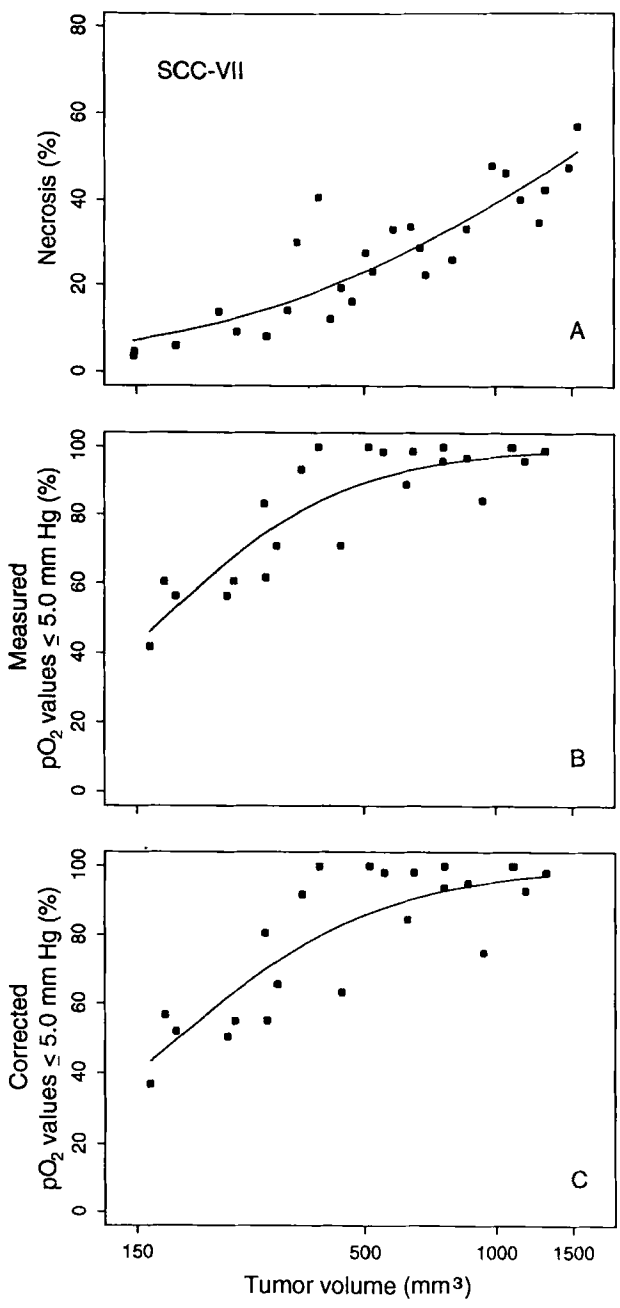

Fig. 3. Tumor necrosis (A) and polarographically measured tumor hypoxia, defined as the percentage of pO_2 readings ≤ 5.0 mm Hg (B), plotted as a function of SCC-VII tumor volume. Each point represents a single tumor. Data were best fit by the logistic model; resultant curves had significant positive slope ($p < 0.001$). Measured tumor hypoxia was corrected by eliminating pO_2 readings estimated to have been taken in necrotic areas. The corrected percentage of pO_2 values ≤ 5.0 mm Hg and the logistic fit to the data are shown as a function of tumor volume (C). Maximum-likelihood analysis confirmed no significant inferior displacement of the fitted curve ($p = 0.054$) or reduction in slope.

of the fitted curve was slighty but not significantly less than that of the uncorrected fit ($p = 0.294$), and the inferior displacement of the curve approached but did not achieve statistical significance ($p = 0.054$) (Fig. 3C).

DISCUSSION

We have quantified necrosis in three transplantable murine tumors with volumes that broadly span the range used in

experimental therapeutics. The percentage of necrosis increased as tumor volume increased. Similarly, as tumors increased in size they generally became more hypoxic, as defined by increasing percentages of pO_2 values $\leqslant 5.0$ mm Hg. This phenomenon was more obvious in the MCa-4 and SCC-VII tumors than in the OCa-I tumors. Since pO_2 values measured in areas of necrosis may not be biologically relevant and may artificially lower the tumor oxygenation profiles, a mathematical formula was used to correct the estimated relationship between tumor size and measured hypoxia. After correction, all three tumor types continued to demonstrate increasing hypoxia with increases in volume. However, in each case the slope of the logistic curve fitted to the corrected percentage pO_2 values $\leqslant 5.0$ mm Hg was shallower. A more significant effect of correction for necrosis was the inferior displacement of the fitted curve toward lower percentages for corresponding volumes, which was significant in two of the three tumors, MCa-4 and OCa-I.

Tumor necrosis was first reported at distances greater than 160 μm from capillaries, a distance corresponding to the theoretical diffusion limit of oxygen (20). Increasing necrosis is believed to be due to the progressive development of nutritional depletion and hypoxia (21, 22), which have been attributed to a deficiency in the functional vascular network as tumors grow (23). Murine tumors have also previously been shown to become more hypoxic as they increase in size: although most commonly this has been demonstrated by radiobiological assay (12, 13), it has also been established polarographically (14). While such a relationship might reasonably be expected for human tumors, a caveat must be expressed because of the more rapid growth of transplanted murine tumors and the influence of transplantation site and tumor histology on the development of both necrosis and hypoxia. Nonetheless, it is irrefutable that many, if not most, human tumors do contain substantial necrotic components.

Polarographic measurement of tumor pO_2 has long been possible in clinical radiation oncology (24, 25) but has become more prevalent recently, since improvements in microprobe and computer technology have been incorporated into a commercially available device (26, 27). Further development has been prompted by a number of clinical studies confirming the usefulness of pretreatment pO_2 as a predictor of treatment response (28–30). The potential, capabilities, and limitations of polarographic pO_2 measurement in rodent tumors is presently being defined. The technique has been used to document changes in tumor oxygenation following cytotoxic therapies (31, 32), and associated with the administration of nicotinamide, perflurocarbons, carbogen, and hemoglobin substitutes (33–35). A linear relationship between measured pO_2 and radiobiological hypoxia has been conclusively demonstrated using a mouse mammary tumor model (36), confirming an earlier correlation established between measured pO_2 in the murine fibrosarcoma FSa-II and the radiobiological hypoxic fraction determined from published studies (14). In parallel studies such issues as intratumor and intertumor variability of measured pO_2 (37, 38), and the effect of tumor site and of anesthesia (39) have been evaluated.

Considerable averaging occurs before arriving at a measure of the oxygen status of a given tumor by polarographic histography. Each individual measurement represents the oxygen tension of a finite volume of tumor tissue containing a limited number of cells. Furthermore, the number of actual measurements taken in a single tumor is usually limited by factors such as tumor size and accessibility. Therefore, the sample represents no more than a fraction of the total number of clonogens in even the smallest tumors. Clearly, inclusion of values from non-clonogenic areas would be expected to increase any disparity between measured and radiobiological hypoxia. Khalil et al. (17) reported increasing tumor necrosis with increasing tumor volume, concurrent with an increase in the percentage of pO_2 values $\leqslant 5.0$ mm Hg. Since pO_2 measurements taken in areas of necrosis, devoid of clonogenic cells, have no bearing on radiobiological hypoxia, these were eliminated from the sample, after which the apparent increase in the percent of pO_2 values $\leqslant 5.0$ was lost. The authors concluded that correction for the necrotic fraction was necessary in their tumor model when attempting to measure tumor oxygenation with electrodes (17). We were stimulated by their study to conduct a similar investigation. We have confirmed both an increase in necrosis and an increase in measured hypoxia as tumor size increases. In contrast to Khalil et al., however, we did not show that correction of pO_2 for necrosis abrogates the effect of tumor size on measured hypoxia.

A number of differences between the two studies should be discussed. The range of tumor sizes used was different. The mammary carcinomas used by Khalil et al. ranged from <100 to 1 000 mm^3 for estimation of necrosis and from approximately 100 to 700 mm^3 for pO_2 measurement, as opposed to 150 to 1 500 mm^3 in both cases for us. We did not identify any tumors with less than 5% necrosis as they did. Also we identified a plateau for MCa-4 and SCC-VII tumors with volumes greater than approximately 500 mm^3, above which more than 90% of the pO_2 values were $\leqslant 5.0$ mm Hg. Consequently, with correction there was less tendency for our measured pO_2 values to be shifted up at the small volume end or down at the large volume end of the tumor size range.

It could be argued that we have overestimated the amount of necrosis in these tumors by only examining a single section from the midplane, in contrast to multiple step-sections. However, our estimate for necrosis in the mammary carcinoma, across a comparable size range, agrees closely with that of Khalil et al. (17). Also, our estimates compare favorably with those we have previously

published for these tumors at 8 mm diameter (40) and with other published reports of necrosis in transplantable murine tumors (18, 35). Furthermore, the consequence of overestimating necrosis would be to eliminate too many hypoxic pO_2 measurements, and if that occurred, it was insufficient to remove the relationship between tumor size and measured hypoxia.

Clearly it would be wrong to conclude that correction of measured pO_2 for tumor necrosis is not neccesary since we have merely shown that such a correction did not abrogate the volume dependence of measured hypoxia in three transplantable murine tumors. On the contrary, in each case, elimination of pO_2 values estimated to have been taken in areas of necrosis lowered the percentage of pO_2 values ≤ 5.0 mm Hg. Thus we would conclude that uncorrected polarographic estimates of tumor oxygenation must be overestimates and as such less likely to be correlated with radiobiological hypoxia or have predictive potential.

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