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CONTRIBUTION OF TRANSIENT BLOOD FLOW TO TUMOUR HYPOXIA IN MICE

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Tumours grown in mice typically exhibit regions of hypoxia believed to result from two different processes: chronic oxygen deprivation due to consumption/diffusion limitations, and periodic deprivation resulting from transient reductions in tumour blood flow. The relative contribution of each is, however, not generally known. We have addressed this issue in transplanted SCCVII squamous cell carcinomas in C3H mice, using a quantitative extension of the fluorescence 'mismatch' technique coupled with cell sorting from irradiated tumours. At least half of the vessels in these tumours exhibit transient perfusion changes. Additionally, a majority of the 15–20% of cells that are sufficiently hypoxic to be resistant to radiation in the SCCVII tumours appear to result from cyclic, not continuous (diffusion-limited) hypoxia. Since different strategies may be necessary to counteract cyclic hypoxia in tumours, the possibility of transient blood flow changes should not be ignored when planning cancer therapy for humans.

The fact that solid tumours in all species generally develop regions of reduced blood flow, decreased pH, and ultimately, regional hypoxia has long been recognized. Unfortunately, most of these changes have a negative impact on the potential for successful anticancer therapy. Considerable effort has thus been expended in recent years to deal with the hypoxic cell problem in solid tumours, with occasional—but usually modest—successes.

An interesting paradox in the study of tumour hypoxia is that relatively little effort has been expended in attempting to understand the basis (or nature) of that hypoxia. The classic work of Thomlinson & Gray (1), supplemented by detailed laboratory information (2–4), led to the 'diffusion limited' model, whereby cells that are sufficiently displaced from blood vessels are continuously deprived of oxygen. The suggestion that transient blood flow changes (varying oxygen delivery) may occur in solid tumours was made much later (5), and definitive evidence for hypoxia resulting from this process has emerged only in the last decade (6, 7).

The realization that both continuous and cyclic hypoxia are likely to occur in many if not most solid tumours has led to a rethinking of the hypoxic cell problem, and of methods to overcome it (8–10). In fact, as argued in several of the recent reviews just cited, tumour hypoxia is now often envisaged as a therapeutic advantage, rather than a liability. The validity of that hypothesis undoubtedly will be tested as these newer approaches reach the clinic; we nonetheless believe that the chance of success in these endeavours will be markedly increased by a more detailed understanding of tumour hypoxia itself.

We are developing techniques that may resolve this question, and in this report, will illustrate their utility using the SCCVII squamous cell carcinoma line grown by transplantation in C3H mice. Using a quantitative extension of the fluorescence 'mismatch' technique (7, 11), coupled with cell sorting after irradiation (7, 12–14), we now report estimates of the proportions of hypoxic cells resulting from cyclic versus continuous oxygen deprivation in these tumours.

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Material and Methods

Mice and tumours

The murine SCCVII squamous cell carcinoma line was maintained by serial subcutaneous transplant in C3H/HeN animals, bred in our facility. Tumours were used for mismatch experiments when 50–900 mg in weight, and for sorting studies in the 300–600 mg range.

Mismatch studies

Microregional tumour perfusion was investigated by intravenous injection or infusion of Hoechst 33342 (Sigma Chemical Co., St. Louis, Missouri) at quantities up to 1.0 mg/mouse (range 4–52 mg/kg), and a carbocyanine derivative ($DiOC_7$, Molecular Probes, Eugene, Oregon) at 0.25 mg/mouse or less (range 2–14 mg/kg). Due to the significant vasoactivity (11, 15) of the carbocyanine derivative formulated in DMSO, it was injected last whenever the stains were not simultaneously administered. Tumours were typically excised 10 min (range 0–60 min) after carbocyanine administration, then embedded, frozen and sectioned.

Sections imaged using either a Zeiss axioplan or Zeiss photomicroscope were analyzed with our imaging systems using locally-developed software. Briefly, where the fluorescence intensity of either stain adequately exceeded background, the relative intensity of the $DiOC_7$ staining was compared with that of Hoechst. Variation by less than a factor of 2 was defined to be '0% change'; a 2–3 fold increase in carbocyanine staining relative to Hoechst was called a + 100% change, etc., whereas relative decreases in $DiOC_7$ intensity were similarly expressed as negative percentage changes. Changes exceeding ± 300% (> 4-fold increases or decreases in relative carbocyanine intensity respectively) correspond to our previous visual criteria (11) for 'mismatch'.

Irradiation and cell sorting

Like our standard procedure (13, 14), cell sorting after 'anoxic' or in vitro irradiations used 1.0 mg Hoechst 33342 injected into the lateral tail vein. In both cases, animals were sacrificed 20 min later (nitrogen asphyxiation for anoxic controls, or cervical dislocation for the in vitro studies). Tumour irradiation with 250 keV x-rays at a dose rate of about 3 Gy/min was performed either in situ immediately after asphyxiation, or in vitro following tumour disaggregation as described subsequently. Air-breathing animals were irradiated at a reduced dose rate over a 10–12-min interval, while restrained and either simultaneously or subsequently infused with Hoechst (same total dose) using an automated infusion pump.

Once excised, tumours were rapidly cooled to ice temperature and finely minced, then treated with an enzyme suspension of 0.5% trypsin, 0.08% collagenase and 0.06% DNAase for 30 minutes, filtered through 30 μm nylon mesh to remove clumps, and the monodispersed cells resuspended in fresh medium for processing through the flow cytometer. Cells were defined on the basis of forward light scatter (cell size) and anti-mouse IgG negativity to exclude infiltrating immune cells (16); sort windows were

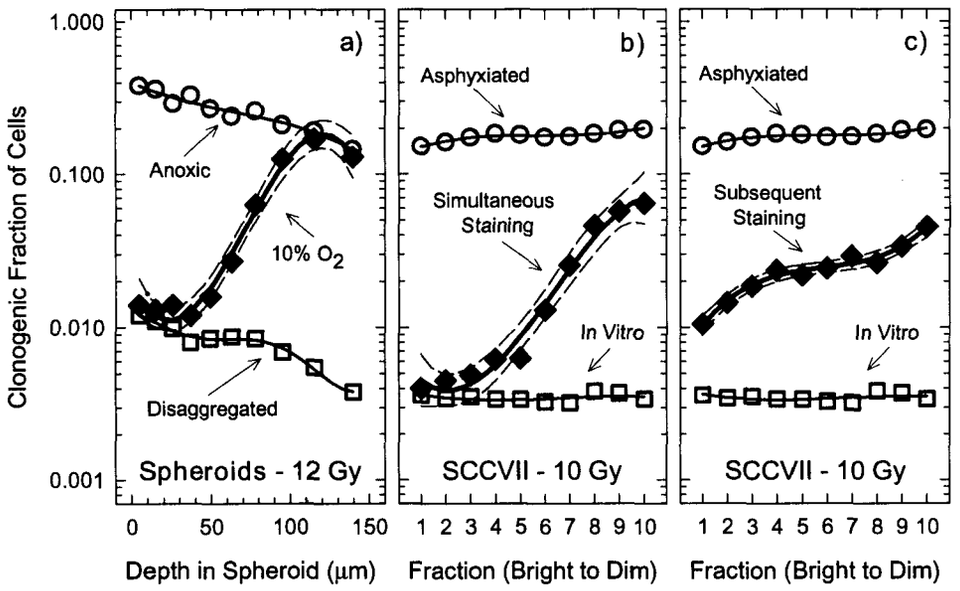

Fig. 1. Clonogenic fraction of cells as a function of cellular location following irradiation of V79 multicell spheroids (a) or SCCVII tumours (b and c) under the indicated conditions. In all cases, the expected survival for anoxic or fully reoxygenated cells from each subpopulation is indicated for reference (open symbols); the dashed lines show the 95% confidence intervals for the experimental curves for spheroids (n = 6), simultaneously perfused tumours (n = 5), and tumours stained following irradiation (n = 3). Note that the latter groups showed identical mean survivals, but quite different distributions of aerobic versus hypoxic cells.

automatically set to subdivide the cell population into 10 fractions (the most intensely Hoechst stained cells, labeled fraction 1, were proximal to functional vasculature).

Predetermined numbers of cells were sorted into test tubes containing culture medium (17); these tubes were then poured and rinsed into conventional Petri dishes and incubated in 94% air + 6% CO_2 for 12 days for colony formation. All in vitro techniques used minimal essential medium containing 10% fetal bovine serum; no special additives were used for tumour cell culture, nor were feeder cells, gel cultures, or low oxygen tensions found to significantly improve cell growth or viability.

Results

The basis for our interest in the nature of hypoxic tumour cells is illustrated in Fig. 1, where cell sorting was used to relate radiosensitivity (oxygenation) to accessibility of the fluorescent stain, Hoechst 33342. In Fig. 1a, cells from spheroids (the ultimate model of static, diffusion limited, continuous hypoxia) clearly demonstrate the expected range of radiosensitivities varying from that of well-oxygenated conditions to severe anoxia as cells were selected from regions progressively deeper into the spheroids. Conversely, SCCVII tumour cells irradiated in air-breathing animals at the same time that the Hoechst dye was being infused showed the expected response only for the well-perfused cell subpopulations (Fig. 1b), and when irradiation and Hoechst infusion were not coincident in time, the staining procedure was again (7) unable to reliably identify either aerobic or hypoxic cells (Fig. 1c). Interestingly, however, mean tumour cell survival was virtually identical in Fig. 1b and c; only the distributions of radiosensitivity changed.

In our laboratory, the SCCVII tumour grows with a hypoxic fraction of about 20% (6, 13, 18). When tumours are processed with our sorting techniques, that should result in hypoxic cells being recovered only from the two dimmest cell fractions (numbers 9 and 10) if hypoxia were continuous and exclusive to poorly perfused regions of the tumour. Further, if tumour perfusion and oxygenation were 'static', both in situ curves in Fig. 1b and c should have been identical. Neither was observed; significant numbers of hypoxic (radioresistant) cells were present even in some intermediate subpopulations (fractions 4–8) that were Hoechst stained in Fig. 1b, and in all subpopulations when staining and irradiation were not coincident (Fig. 1c). Hence, it appears that transient changes in tumour perfusion affect quite poorly-perfused regions, as well as the better-perfused regions of the tumour.

Numerical analyses allow several additional inferences to be drawn from the data in Fig. 1b. An estimate of tumour hypoxic fraction can be made in a manner analogous to the usual 'paired survival curve' method (19, 20); the fraction by fraction ratio of survival from tumour cells in the normal host to that in the asphyxiated animal

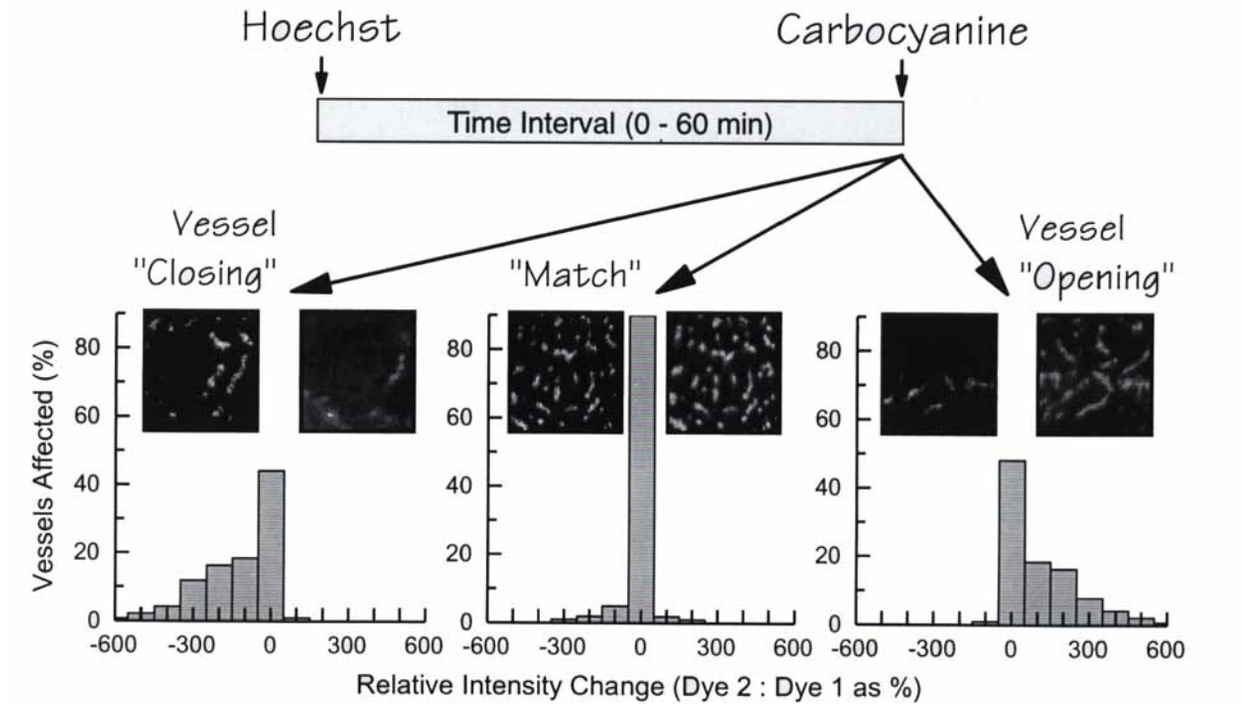

Fig. 2. A schematic representation of the quantitative fluorescent dye mismatch technique used in our laboratories. The digitized images show representative tumour sections illustrating each pattern of response; in all cases the left image indicates the Hoechst 33342 distribution, and the right image shows $DiOC_7$. See text for further details regarding quantification of the images.

gives the fraction of hypoxic cells in each cell subpopulation recovered, and can be summed to estimate the net hypoxic fraction. This exercise yields a number of 13%—lower than the value of 20% previously mentioned for acute irradiations, but as expected if a part of the normal hypoxic fraction is due to transient changes in blood flow (by protracting the irradiation interval, cells in regions subject to transient flow should be aerobic for at least part of the exposure).

While the sorting data of Fig. 1 suggest that transient hypoxia may be a significant factor in the SCCVII tumours, these data alone do not resolve the issue. In particular, Fig. 1 shows no direct evidence for blood flow variations; that conclusion is entirely inferential. We have consequently invested considerable effort in developing and quantifying techniques for the direct demonstration of changes in tumour perfusion. The fluorescent stain 'mismatch' technique originally developed in our laboratory (11, 15) has now been extended to allow quantitative estimates of both the degree and extent of blood flow changes, as briefly described in 'Material and Methods' and shown schematically in Fig. 2.

Many limitations of the 'mismatch' technique have become evident during our continuing studies. Perhaps the most important of these can be appreciated from the representative staining images included in Fig. 2, where dissimilar diffusion patterns for different stains outward from blood vessels can potentially lead to problems of interpretation and quantification. As a consequence, even under conditions where total 'match' between the stains is expected (for example, simultaneous administration), a mismatch profile skewed somewhat toward excess Hoechst dye is routinely observed, as illustrated in the central panel of Fig. 2 labeled 'Match'.

In the course of developing these techniques, we have now processed more than 200 'control' SCCVII tumours, where control denotes no treatment other than dye administration. Some results particularly pertinent to the question of quantifying cyclic hypoxia in this tumour system are summarized in Fig. 3; in each case, for ease and consistency of data presentation, we show only the percentage of image pixels (which, due to the numbers of sections analyzed per tumour essentially represents the percentage of tumour vessels) that showed relative staining

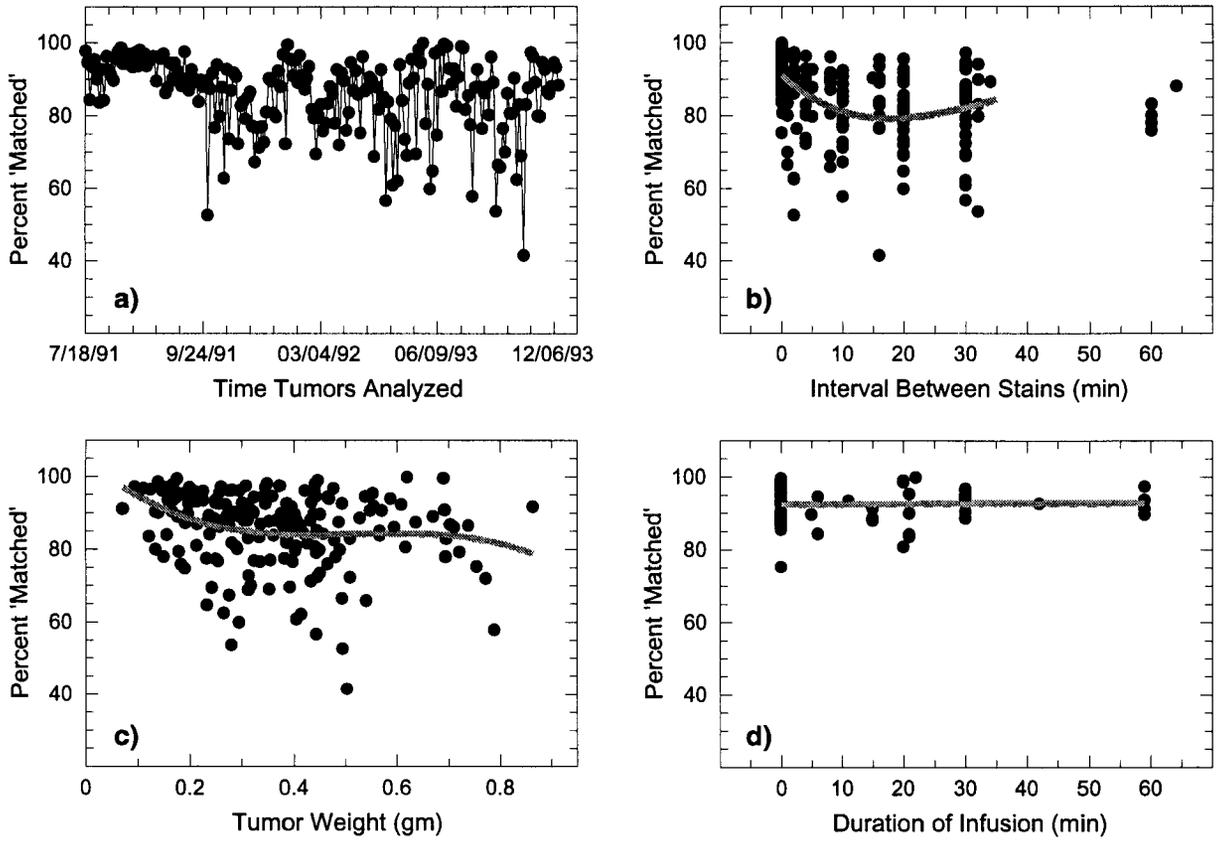

Fig. 3. A compendium of the data we have obtained for dye mismatch studies in control (dye-stained only) SCCVII tumours during development of our image quantization procedures. In all cases, the percent of matched pixels (vessels) is plotted; a) shows the sequential response over a three-year period; b) indicates mismatch as a function of the time interval between administration of the stains; c) shows staining agreement as a function of tumour weight; and d) shows the lack of complete concordance of the stains even when simultaneously injected (time = 0) or infused over increasing time intervals.

Table

Numerical analysis of selected data from Fig. 3b

Interval	0 min	1/2/4 min	8/10 min	16/20 min	30 min
0 min	93.08 ± 1.19 (n = 67)				
1/2/4 min	$\mathbf{p < 8 \times 10^{-13}}$	80.26 ± 3.97 (n = 27)			
8/10 min	$\mathbf{p < 2 \times 10^{-11}}$	p = 0.723	81.23 ± 3.94 (n = 26)		
16/20 min	$\mathbf{p < 9 \times 10^{-13}}$	p = 0.763	p = 0.525	79.38 ± 4.38 (n = 28)	
30 min	$\mathbf{p < 1 \times 10^{-7}}$	**p = 0.036**	p = 0.083	**p = 0.022**	85.44 ± 3.00 (n = 29)

The percentages of vessels showing perfusion that remained within 2-fold limits are shown with 95% confidence limits along the diagonal (for selected groupings of injection intervals). The probability of any two of these values being identical (Student's t-test) is indicated in the intersecting row and column, with those that are significantly different at the 95% level or greater highlighted.

levels differing by less than a factor of two. We define these to be vessels which were not subject to transient perfusion changes during the times of observation.

Fig. 3a shows our results plotted in temporal sequence, thereby illustrating two important points. First, depending upon the particular protocol under investigation, considerable amounts of 'mismatch' were observed (we again emphasize that these animals received no treatments other than dye injection). Second, despite the fact that these data were accumulated over a 4-year period and analyzed using different microscopes, video cameras and imaging boards, no systematic changes in blood flow alterations in this tumour system were seen (remembering, of course, that different types of experiments were performed as the project evolved). Fig. 3b presents the same data displayed as a function of the time interval between stain administrations (concurrent injection or infusion of the two dyes was plotted as 0 min between the stains). For the more extensive data spanning intervals up to 30 min, it is interesting to note that the plotted third order polynomial regression line suggests that the number of matched vessels was less than 100% even for simultaneous injection or infusion, and perfusion match was minimal for intervals of 15–20 min. These points are reinforced in the table, where selected observations were grouped for statistical analyses. It should also be noted that the somewhat lower than expected percentage of matched vessels for the shorter intervals between stains results in part from transient vasoconstriction (induced mismatch) by the Hoechst dye.

In contrast to the initial report from our laboratory (11), our current dataset shows a distinct decrease in vessels showing constant perfusion as tumours enlarge to about 300 mg, with a more gradual but progressive decrease accompanying further growth. While Fig. 3c shows a fitted third order polynomial to indicate 'substructure' in the data, even a simple linear regression analysis results in a statistically-significant decrease in matched perfusion with tumour size (the regression equation is $y = 90.5 - 13.1x$, with 95% confidence limits of 3.1–22.7 for the slope). A feature of particular interest to us is plotted in Fig. 3d, which shows the subset of tumours in which the two dyes were administered simultaneously as injections (plotted as an infusion duration of 0 min), or infusions for the indicated periods. Clearly, mismatch was observed in all cases, and interestingly, was not reduced for long infusion times.

Several additional inferences can be drawn from the data in Fig. 3. Given that the half life of each stain is about 2 min in the murine circulatory system (15, 21), and that our analytical procedures are limited to a range of intensities spanning about 50-fold (the intensity decrease that would be seen after 5–6 stain half lives), it follows that over a 10–12 min time interval, as much as 20% mismatch was observed. This value, as discussed later, is entirely consistent with the inferences derived from the sorting data of Fig. 1.

Discussion

Based on the numbers and locations of hypoxic cells observed with our cell sorting techniques, and on direct analysis of tumour perfusion by fluorescent dyes, we conclude that transient blood flow in the SCCVII murine squamous cell carcinoma is not just a mechanistic curiosity, but rather, is the major cause of hypoxia in that tumour.

The basis for that statement is evident in both the sorting and mismatch data presented here. Delivering radiation over a 10–12 min interval (in an attempt to maximize the concordance between dye delivery and tumour oxygenation for simultaneous irradiation and staining) led to a reduction of the hypoxic fraction from 20% to 13%, immediately suggesting that at least a third of the cells normally classified as hypoxic in these tumours during acute radiation exposures are produced by transient perfusion changes. Further, at least half of the hypoxic cells recovered for sorting during the perfusion/irradiation experiments, and virtually all recovered when staining followed irradiation showed some staining with the Hoechst dye, again suggesting transient perfusion and increasing the total pool of such cells to at least two-thirds of all hypoxic cells. Direct analysis of perfusion mismatch with

the fluorescent dyes indicates that at least 15% of all vessels showed significant mismatch over a similar time frame; when coupled with other (unpublished) observations from our lab suggesting that the transient flow changes probably have a periodicity of 15–25 min, it follows that up to half of all tumour blood vessels may be subject to transient changes in perfusion (note the conservative nature of these arguments; the observed mismatch is actually more than adequate to explain all hypoxia in the tumours).

The qualitative differences seen for the sorting data using spheroids versus tumours in Fig. 1 add credibility to conclusions drawn in the tumour studies. Clearly, the 'resolution' seen for spheroid cell sorting argues that technical limitations in the sorting procedures cannot be entirely responsible for the tumour results. In the (static) spheroid system, the internal cells become nutrient depleted, and consequently accumulate in the more radiosensitive G_0 phase of the cell cycle (22, 23). This cycle-dependent increase in radiosensitivity is seen under both the normal hypoxic, and the artificially reoxygenated conditions. By inference, the absence of a similar sensitivity differential in the poorly perfused tumour cells is again indicative of a lack of corresponding, chronically-depleted cells.

A major question still to be resolved is the adequacy of dye delivery as a measure or indication of oxygenation status. Clearly, the lack of concordance between staining and hypoxia could be explained on the basis of imperfect dye delivery for either Fig. 1b or Fig. 1c. However, the differences between those panels cannot be discarded as easily. We are also concerned about the role of stress in the response (data like those in Fig. 3b and d suggest that the measurement techniques themselves can modify tumour blood flow). It should, however, be noted that the animals were handled almost identically for both irradiation and staining experiments, thus increasing our confidence that irrespective of whether or not trauma exacerbates transient blood flow, it is at least a constant in all our experiments (and, of course, a reality in all irradiations and/or other manipulations of experimental animals, or patients).

We find it interesting that there is now little opposition to the idea that transient blood flow is a potential contributor to tumour hypoxia (10, 24), yet much of the experimental work currently directed toward the hypoxic cell problem inherently assumes that only continuously hypoxic cells pose a problem for therapy. As a practical illustration of that point, consider the usual test for hypoxia-directed cytotoxicity of bioreductive agents: the ability of the bioreductive agent to kill cells which survive a large dose of radiation. Given our observations that at least half of the SCCVII cells which survive irradiation will be well perfused very quickly thereafter, the ability to kill such cells provides no information on hypoxic cell specificity. Consequently, our data highlight the need for an improved understanding of the nature of hypoxia in tumour models, as well as indicating the difficulties of extrapolating such data to human disease.

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