Non-constant Tumour Blood Flow

Implications for Therapy

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In the past few years, 'perfusion-limited' hypoxia caused by intermittent decreases in tumour blood flow has received increasing attention. Little effort, however, has gone into characterizing the nature, magnitude or duration of these changes, or their functional significance other than as modifiers of radiotherapy. We have therefore undertaken multiple, quantitative analyses of tumour blood flow in human tumour xenograft systems, and rigorously examined the ramifications of transient blood flow changes. Tumour blood flow in these experimental tumours is much less constant than has previously been assumed, and not only impacts on response to radiotherapy and chemotherapy, but also on the more fundamental processes of tumour growth and repopulation. Notably, responses entirely consistent with the laboratory results have been seen in our initial studies of human tumours sequentially biopsied during treatment.

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The past several years have seen dramatic changes in our understanding of not only the molecular biology of tumours, but also tumour 'physiology'. This may be best illustrated by the recent contribution from Juliana Denekamp and her colleagues (1) in which they suggested that it is time for a paradigm shift in the way we envisage tumour hypoxia and its therapeutic significance.

Tumour hypoxia has long been a concern in radiation oncology (2–4), and more recently in the chemotherapy field as well (5, 6). The classical picture of hypoxia is a consequence of the more rapid expansion of tumour cells than of the supporting vasculature, so that many tumour cells reside in areas largely inaccessible to oxygen, other metabolites, and presumably even many chemotherapeutic drugs (4, 7). Clearly, however, this inherently 'static' model of a tumour is an oversimplification. Research studies from our laboratory (8–10) and others (11–13) have shown that tumour blood flow in rodents can be subject to spontaneous and rather dramatic fluctuations. Similar results have been reported for tumour blood flow (14) and proliferation studies (15) in humans.

In model systems, dynamic changes in blood flow can readily be illustrated with repetitive injections of blood-born markers (9, 10), where differential delivery of the markers to a particular site provides an indication of the changing blood flow pattern. At the single vessel level, complete absence of one of the markers is convincing visual evidence that the vessel has either opened or closed in the time interval between administration of the markers. While dramatic, such changes are relatively infrequent in our experience, and arguably have diverted attention away from subtle, but potentially more important blood flow changes. Modest increases or decreases in blood flow can now be demonstrated by extending the classical visual 'mismatch' experiment with quantitative image analysis (10, 16).

We have had a long-standing concern that fluctuating tumour blood flow may well have consequences beyond the obvious ones pertaining to therapy. For example, consider the impact on proliferating cells if the nutrient supply is suddenly restricted or abolished. This is clearly quite a different situation than the classical model of tumour growth, in which proliferating cells near the vasculature persistently displace their precursors away from the nutrient supply and progressively into quiescence, hypoxia, and ultimately death via apoptosis or necrosis. If blood flow fluctuations are of sufficient magnitude, another order of complexity is imposed, such that (for simplicity) regions of the tumour actively proliferating at one point in time may not have cells actively synthesizing DNA at a different time. In the extreme situation, cells may cycle in and out of hypoxia, therefore potentially being protected during therapy but retaining the ability to proliferate rapidly once blood

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flow is sufficient. Ultimately, this suggests that any short-term assessment of tumour growth and growth fraction may significantly underestimate the number of cells with proliferative potential, as well as the regrowth capacity of the tumour.

These phenomena are addressed in this article by intercomparing estimates of tumour blood flow in murine hosts using consecutive administrations of fluorescent markers, using sequential administration of hypoxia probes (17–19) to address the question of constancy and magnitude of hypoxia, and questioning whether (previously) hypoxic cells re-enter the growth phase in unperturbed tumours. The studies are extended to tumours undergoing therapy, by assessing regrowth during multifraction studies in a xenograft tumour model system. Ultimately, we compare and contrast those results with representative data from sequential human tumour biopsies obtained during a course of radical radiotherapy.

METHODS

Most of the methods used in this work have previously been described in detail, so will be reviewed only briefly.

Two human tumour cell lines were used primarily: SiHa, a cervical squamous cell carcinoma (20), and WiDr, a colon adenocarcinoma (21). Both were obtained as cultured cell lines (ATCC, Rockville, Maryland) and then maintained in immunodeficient SCID or nude mice by intramuscular transplant. Although these tumour lines remained stable with repetitive transplants, they were nonetheless closely monitored for changes in growth or response, and routinely re-established from frozen stocks. Experimental tumours were grown as subcutaneous implants on the back or flank for mismatch, sorting and irradiation studies. Multifraction radiotherapy was performed using the SiHa tumours irradiated on a twice-daily schedule with 2.5 Gy/dose as previously described in detail (22, 23).

Short-term changes in blood flow were visualized using the dual staining mismatch technique (9, 10). The fluorescent bisbenzimide dye Hoechst 33342 (0.5 mg per mouse in 0.05 ml PBS) was administered by intravenous injection to tumour-bearing mice, followed 25 min later by i.v. injection of the carbocyanine derivative $DiOC_7(3)$ (0.1 mg per mouse in 0.05 ml 75% DMSO in PBS). The animals were sacrificed 5 min after the carbocyanine injection and the tumours were excised, embedded, frozen, sectioned, and analysed using locally developed software (10, 16).

Iododeoxyuridine (IdUrd) incorporation was used as the indicator of DNA synthesis and therefore proliferation. Mice were injected intraperitoneally (90 mg/kg from a 6 mg/ml stock solution in 1.0 mM Tris buffer, pH 10). Single-cell suspensions were prepared from excised tumours that were finely minced then agitated in an enzyme suspension containing 0.5% trypsin and 0.08% collagenase for 40 min before adding 0.06% DNAase. The cell suspension was gently vortexed and filtered through 30 μm nylon mesh to remove clumps, and the monodispersed cells washed by centrifugation and fixed in chilled 70% ethanol. When convenient, the fixed cells were denatured in 2N HCI containing 0.1% Triton-X, and cycling cells visualized with the B-44 antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) at a 1 : 50 dilution (75 μl). DNA was counterstained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride hydrate) at 1 μg/ml. Standard flow cytometry techniques were used (22–24); list mode files were collected using a dual laser Epics Elite-ESP flow cytometer (Coulter Corporation, Hialeah, FL), and subsequently reprocessed for analysis. Doublet correction and bit-map-gating were used to select the cell populations of interest with the 'WINLIST' software package (Verity Software House, Inc., Topsham, ME).

The hypoxia markers pimonidazole and NITP were recognized by anti-pimonidazole antibodies generously supplied by Dr James Raleigh (University of North Carolina) or anti-theophylline (Fitzgerald Industries International Inc.), respectively. Once single-cell suspensions had been prepared and fixed, pimonidazole adducts in DNA were visualized using a FITC-conjugated polyclonal antibody, or with a 2-step approach using a conjugated rabbit anti-mouse secondary antibody.

At the Vancouver Cancer Centre there is an on-going clinical protocol assessing response to fractionated external beam radiation therapy, with weekly tissue biopsies. The University of British Columbia Ethics Committee has approved this protocol. After obtaining informed consent from the patients, biopsies of cervical tumours were obtained prior to the prescribed course of radiation therapy, and weekly during treatment when feasible (feasibility has been limited only by rapid tumour regressions in some cases; no infections or other complications from the biopsy procedure have been noted to date). Small excision biopsies were performed with a view to limiting the biopsy size, yet obtaining enough material for both flow cytometric and parallel pathologic assessment. For flow cytometry analyses, the biopsies were minced and enzymatically disaggregated to a single-cell suspension using procedures similar to those described above for the tumour model systems. In addition to DAPI staining for quantifying DNA content, proliferating cell nuclear antigen (PCNA-FITC, Sigma Immunochemicals, 1 : 100 dilution) was also used. Chicken red blood cells were added to the samples to provide an internal control for DNA content. Data were acquired in list mode, and post-processed to selectively display information from the presumed tumour cell population.

RESULTS

Changes in blood flow using the dual stain mismatch technique in 'control' experimental tumours (the WiDr xenografts) are visually demonstrated in Fig. 1, where each horizontal group of images is from a different tumour. The

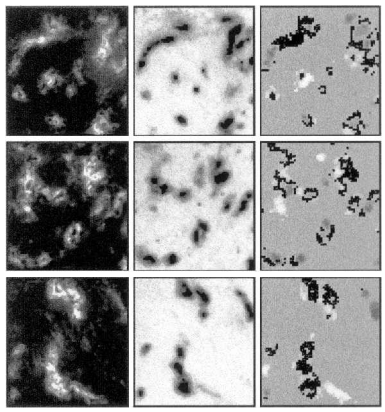

Fig. 1. Digitally reprocessed images of sections from three WiDr tumours excised from animals injected with Hoechst (left column) and then 30 min later, with carbocyanine (centre column). The right column shows analysed 'merged' images highlighting similar (black areas) or dissimilar stain delivery, against the background of uninformative pixels, shaded grey. Note that areas of obvious mismatch were seen, and that surprisingly few vessels showed similar perfusion even at this relatively short time interval.

images in the left (Hoechst) and centre columns (carbocyanine 30 min later) are digital images obtained using excitation specific to each stain, presented as positive and negative images, respectively, so that the staining patterns and distributions can be intercompared. Different grey levels were used in the reconstructed images (right panels) to indicate relative differences in stain delivery and therefore blood flow. The uniform grey background in the right column of images shows areas from which no meaningful information was obtained; the black areas show where the relative intensities of the stains differed by less than two-fold. Those areas shaded lighter or darker than the background represent quantifiable differences between the dye intensities in the left and middle panels; lighter shading indicates greater delivery of the first stain. Conversely, if the blood flow had been greater at the time the second dye was administered, that area would be a darker grey than the background. Note that even with quite conservative criteria used for defining perfusion changes, a large fraction of the tumour volume showed significant perfusion differences in times as short as a few minutes.

In Fig. 2, we show a different method of functionally assessing tumour blood flow: 'hypoxia probes' that provide information over a circulating lifetime of 1–2 h in these murine systems (13, 19). In addition to the longer (day-to-day) variation in hypoxia measured in Fig. 2, we have introduced another time factor by subjecting the disaggregated tumour to cell sorting based on the distribution of Hoechst 33342. By sorting and then reanalysing cells as a function of the Hoechst dye delivery (where fraction 1 was the brightest Hoechst-stained subpopulation), we have assessed not only the fraction of cells that had taken up the hypoxia probes at different times, but also the perfusion status of the tumour shortly before its excision for analysis.

The primary questions addressed in Fig. 2 are thus the locations and numbers of cells sufficiently hypoxic to take

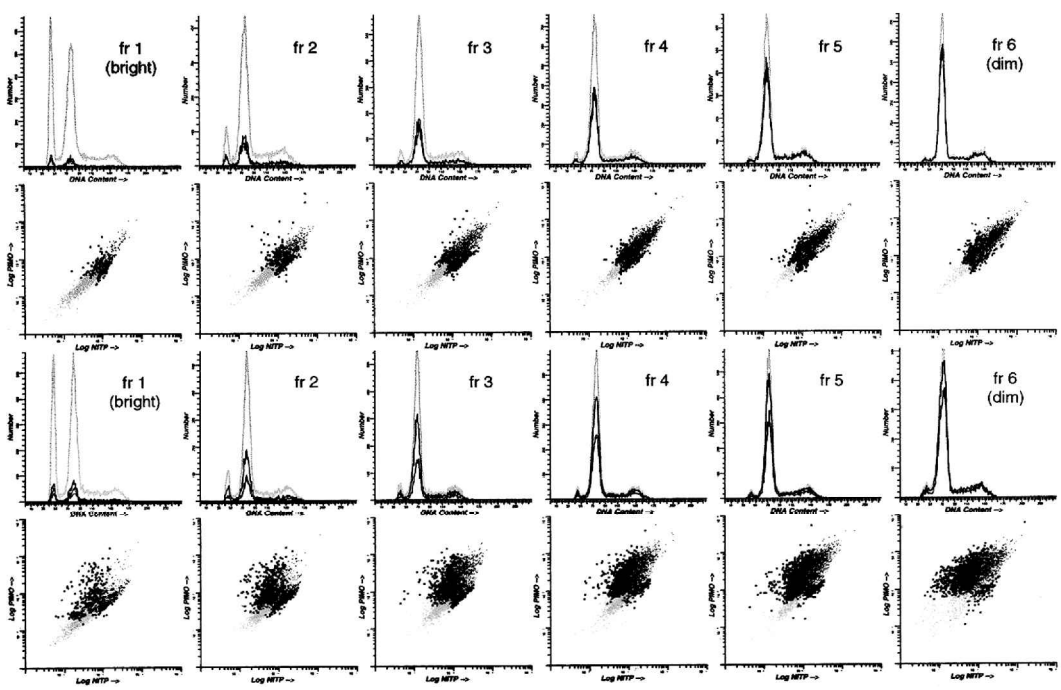

Fig. 2. Flow cytometry analyses illustrating the uptake of two hypoxia markers, pimonidazole and NITP in WiDr xenografts. The markers were either administered simultaneously (top 2 rows), or pimonidazole was administered several times during the day previous to NITP administration (bottom 2 rows). Hoechst was used 20 min prior to tumour excision to identify well (fraction 1) to poorly (fraction 6) perfused cell subpopulations. Cells labelled with pimonidazole, but not NITP (i.e. those that were hypoxic in the past but not at the time of NITP administration) are indicated by the darkest dots in the multivariate panels; similar shading in the univariate histogram lines indicates the DNA distribution of each subpopulation.

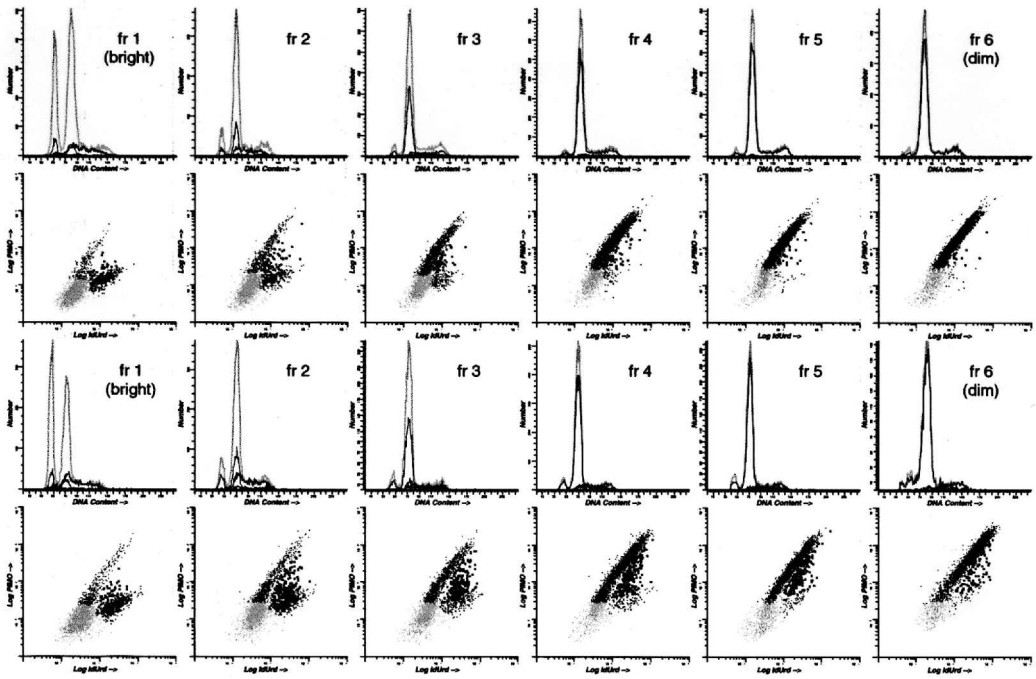

Fig. 3. A similar experiment to that of Fig. 2, but using pimonidazole to identify hypoxic WiDr tumour cells and IdUrd to mark perfused, DNA-synthesizing cells. Cells having incorporated both markers (i.e. those that were hypoxic in the past and actively synthesizing DNA at the time of analysis) are indicated by the darkest dots in the multivariate panels. Little dual labelling was seen with near-simultaneous administration in the upper two rows of panels, whereas many cells that had been sufficiently hypoxic to incorporate pimonidazole in the preceding 38 h were actively cycling at the time of IdUrd administration (bottom two rows).

up pimonidazole sometime in the previous day and a half, and whether all of those cells were (still) hypoxic at the time of NITP administration. As can be seen in the lower two panels, the fraction of (currently) aerobic cells that had been hypoxic was substantial, and principally located in the least well-perfused areas of the tumour (heavy black dots in the lower panels, and the black histogram traces in the third row). Conversely, when pimonidazole and NITP were administered simultaneously as in the first two rows, the only cells exhibiting one label in the absence of the other were those at the very lowest labelling intensities for either probe, i.e., those subject to the greatest measurement uncertainties. Thus, the data in Fig. 2 show conclusively that a significant number of cells that had been seriously hypoxic sometime in the past 38 h were no longer hypoxic at the time of NITP administration.

A similar experiment is illustrated in Fig. 3, where the use of different probes allowed evaluation of the proliferative potential of (previously) transiently hypoxic cells. As in Fig. 2, tumours in the bottom two rows of Fig. 3 were subjected to numerous pimonidazole administrations over a 38-h period, and then iododeoxyuridine (IdUrd) was administered to identify those cells currently synthesizing DNA. The population of greatest interest (those cells that were previously hypoxic but now capable of DNA synthesis and therefore presumed to have been aerobic just before assessment) is shown as black dots. Again, substantial numbers of dual-labelled cells were seen, though their location was somewhat different from that of the 'reoxygenated' cells depicted in Fig. 2. In the case of DNA synthesis, most activity in reoxygenated cells occurred in areas of medium perfusion with few double-labelled cells found in the poorly perfused regions. As expected, when IdUrd and pimonidazole were injected much closer together in time (a 2-h pimonidazole exposure with IdUrd added half an hour before tumour excision), few cells were double-labelled (top two rows of Fig. 3) although occasional pimonidazole-labelled cells in the intermediately perfused subpopulations also incorporated IdUrd. Fig. 3 therefore shows that transiently hypoxic cells can maintain viability and functionality in terms of DNA synthesis.

These observations are extended in Fig. 4 by accelerating the time course and quantifying the number of cells capable of reoxygenating. In this case, pimonidazole was repeatedly injected at one-hour intervals; as expected, hourly pimonidazole injections had no apparent impact on the number of tumour cells in S-phase (panel a) as the labelling index was consistently around 30%. A step-wise increase in the numbers of pimonidazole-labelled hypoxic cells was observed with multiple injections, with a concurrent increase in the number of dual-labelled cells. For the tumours receiving 3 or more hourly pimonidazole injections, about 30% of the tumour cells were in S-phase at the time of analysis and some 10% overall had been hypoxic some time in the previous 3 to 6 h. Thus, as many as one-third of the S-phase cells had been hypoxic for as little as 3 h previously.

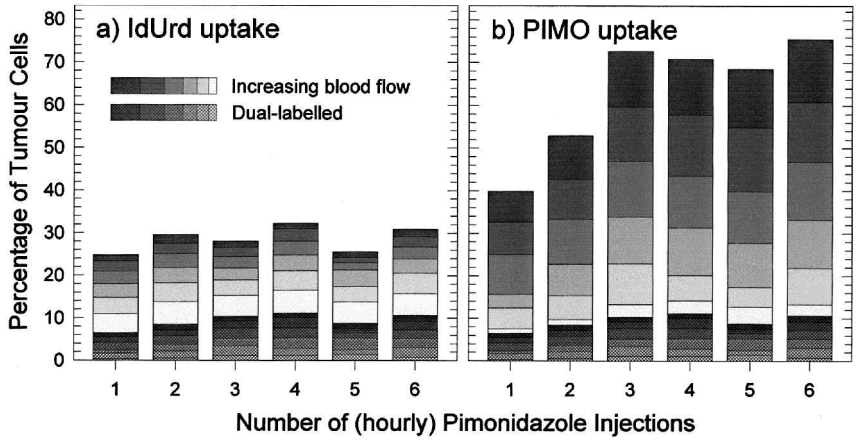

Fig. 4. Again, illustration of hypoxic WiDr tumour cells subsequently able to synthesize DNA. Note the much faster 'transition', in that pimonidazole was administered hourly, with IdUrd 30 min prior to tumour excision, followed 10 min later by Hoechst. Darker shades of grey indicate less perfusion, with the fraction of cells incorporating both labels shown in the crosshatched regions. Note that a substantial fraction of the S-phase cells had been hypoxic within the previous few hours.

To examine the role of transient blood flow changes on response to multifraction irradiation, we used our xenografts that show the least short-term blood flow variation, the SiHa system, and assessed DNA content distributions and cell kinetics daily during a multifraction radiotherapy regimen of twice-daily 2.5 Gy exposures. The DNA histogram distributions in Fig. 5 show the appearance of a significant 'G_2 block' even after one day (2 treatments), with a progressive increase in that population (G_2-blocked and/or mitotically-dying cells) through days 3, 5, and 7. By day 9, however, an increased proliferation rate (cells incorporating BrdUrd immediately prior to assay are shown as the superimposed white population under the black histograms) resulted in a partial resolution of the 'G_2 block' and the start of a return to more normal DNA distribution profiles. More complete analyses of this and comparable fractionated radiotherapy protocols for this tumour model have previously been described in detail (22, 23).

Since Fig. 5 shows an obvious dose response, we hypothesized that if comparable data could be obtained from tumour biopsies in humans early in treatment, it should be possible to project outcome (and possibly to modify therapy) based on that response. We have therefore begun to assess sequential biopsies obtained before and during radiotherapy for patients with cervix cancer. The data shown in Fig. 6 are from two patients, chosen to illustrate extremes of the responses in more than 45 tumours observed to date. The tumour repetitively assessed in the left panels initially had a relatively low S-phase fraction, and showed definite radiosensitivity (cumulative appearance of the 'G_2 block' and hyperdiploid non-PCNA staining and therefore presumably non-proliferative cells). Interestingly, virtually no tumour cells remained halfway through the prescribed treatment (lower left panels of Fig. 6). The bivariate histograms of Fig. 6 additionally show both the chicken red blood cell marker (from which the position of the normal diploid cells, arrows, was determined), and PCNA-positive cells (the black dots; cells actively proliferating).

The tumour shown in the right panels of Fig. 6 had a somewhat higher DNA index, but was unremarkable in terms of the pretreatment proliferative fraction. Rather than displaying any G_2 block (radiosensitivity/death) early in

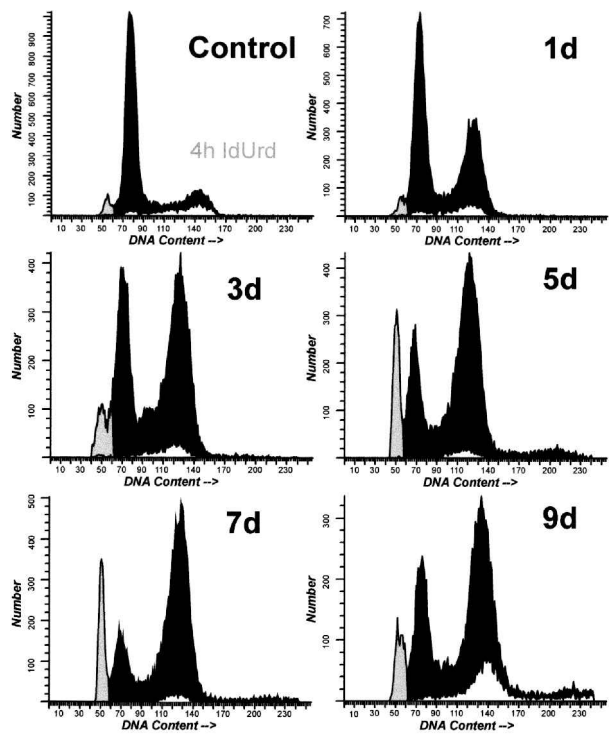

Fig. 5. Response of SiHa xenografts to continuous twice-daily x-ray exposures of 2.5 Gy. Note the rapid and progressive initial development of the 'G_2' tumour cell subpopulation representing blocked, and dead/dying cells. Normal cells are indicated in grey, tumour cells in black, and the distribution of tumour cells that incorporated IdUrd delivered 4 h prior to analysis is overlaid in white.

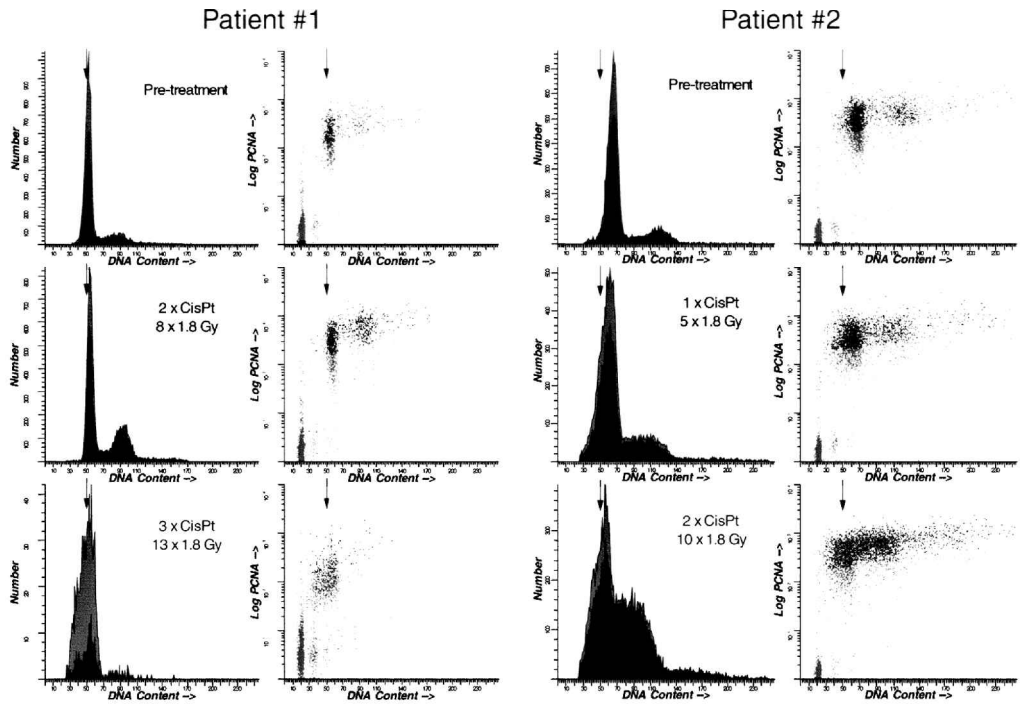

Fig. 6. DNA histograms (gated to eliminate the DNA marker) and bivariate plots (including normal cells and marker) from 2 patients, selected to show a presumably favourable outcome (left panels) versus emergence of a rapidly proliferating subpopulation (right panels). PCNA-positive cells are indicated by larger, black dots in the bivariate plots, and the black overlay in the histograms. Arrows show the expected position of cells with diploid DNA content.

treatment, the tumour appeared to proliferate rapidly, and in the lower panel showed a very large S-phase and PCNA-positive population. Consequently, Fig. 6 illustrates two features in common with the xenograft systems: 1) radiosensitivity can be demonstrated in situ during clinical treatment (about 70% of the tumours we have seen are like those of Patient #1), and 2) proliferation kinetics can change markedly even early into the course of treatment (responses like those for Patient #2 are seen in about 10%).

DISCUSSION

The data in this manuscript illustrate a number of features of experimental and, inferentially, clinical tumours that are not entirely consistent with conventional dogma. First, as a solid tumour grows and outstrips the blood supply, one typically thinks of an ordered progression of cells from the aerobic to hypoxic to necrotic (or apoptotic) compartments, with that progression generally visualized as 'one way' only. While there has been increasing appreciation of a role for transient changes in blood flow, it has also typically been thought that such changes are merely infrequent anomalies superimposed on the normal order just described. Our data challenge that supposition, suggesting instead that tumours can be much more dynamic.

The visual presentation of staining 'mismatch' in Fig. 1 shows that a large fraction of the tumour vessels experienced variations in stain delivery over relatively short periods of time. The bottom three panels of Fig. 1 clearly show examples of 'gross mismatch' (a vessel which received a high concentration of the first dye but minimal delivery of the second is seen in the lower centre of the image, whereas a vessel in the upper right corner received little of the first dye but was more substantially perfused at the time the second was administered). However, a numerically more important feature is the relative area of the rightmost images coloured black (no significant difference in blood flow) versus the lighter or darker shaded areas (visually, up to half of the assessable image). Thus, while any degree of 'mismatch' could be reported (within the dynamic range of the imaging system), even very conservative criteria driven by the different circulating lifetimes and different uptake patterns of the two probes used (25) resulted in the conclusion that very few tumour cells experienced 'constant' blood flow over a one-half-hour interval.

Figs. 2–4 also challenge conventional dogma by showing that substantial numbers of tumour cells can be hypoxic at one point in time, yet fully functional and proliferating subsequently. Interestingly, the WiDr and SiHa tumour systems are generally described as 'acutely' and 'chronically' hypoxic respectively (26). However, our results reported here (and additional studies to be reported elsewhere) suggest that the real difference is not an absence of measurable blood flow changes, but rather the frequency of their occurrence (much faster in the 'acutely hypoxic' WiDr tumours). Consequently, cell proliferation is dependent not

only on proximity to a blood vessel, but additionally on temporal changes in blood flow and nutrient delivery. While this is perhaps obvious, it is undoubtedly less obvious that only a small percentage of tumour cells may *not* be subjected to blood flow fluctuations. This small population is the diffusion-limited, chronically hypoxic population; in most cases, those cells are probably destined to die irrespective of treatment (27).

The pivotal role of adequate blood flow (nutrient delivery) in controlling tumour proliferation has perhaps not been generally appreciated. At any point in time, it appears that the majority of tumour cells could not only proliferate (traverse the division cycle) if given the opportunity, but also could do so without any extended 'start-up' period. In fact, blood flow decreases may transiently arrest even S-phase cells, as suggested by the cells with S-phase DNA content that did not take up IdUrd in Fig. 3 and Fig. 5, yet the virtually universal PCNA labelling of cells with S-phase DNA content in Fig. 6. This has negative implications for therapeutic manipulations, insofar as it implies both a reduction in sensitivity to anti-proliferative drugs, and repopulation initiating much earlier into a therapeutic regimen than previously may have been thought.

As shown in Fig. 5, nutrient-deprived but growth-competent subpopulations of tumour cells can have a major impact on response to multifraction therapies. To add perspective, SiHa tumours as grown in our laboratory are quite radiosensitive, with the surviving fraction at 2 Gy (SF2) approaching 30%. Cell cycle analysis indicates that the cycle time is about 26 h, and the T_{pot} for 0.5 g tumours as used in Fig. 5 is approximately 4 days (22). This represents a growth potential not very different from the median values typically observed in T_{pot} measurements from human tumour biopsies (28, 29). Despite the unremarkable cell kinetics and significant radiosensitivity, SiHa xenografts *cannot* be successfully treated by multifraction radiation even with regimens as aggressive as that shown in Fig. 5 (twice daily exposures to 2.5 Gy fractions, delivered continuously with no weekend break)—an unequivocal result of rapid repopulation even during the multifraction treatments.

Although Fig. 5 does not directly present 'kinetic' data, several inferences can be drawn from the timing and extent of the 'G_2' peaks. First, their magnitude and sequential enlargement confirm the relative radiosensitivity of the tumours; conversely, in view of the T_{pot} values previously discussed, one would not expect the majority of tumour cells to pass through the S-phase so rapidly. With a cycle time of 26 h and a growth fraction of 30–40%, the proliferating cells would go through the requisite three doublings to ultimately double the tumour volume in no less than 3 days. Interestingly, however, the data in Fig. 5 suggest that about two-thirds of the cells had gone through at least one S-phase by the third day, and practically all had gone through at least one round of DNA replication by day 7. This is not consistent with 'accelerated repopulation' occurring only late in treatment (30), nor is it expected if a large fraction of the tumour is quiescent and recruited back into cycle only following substantial shrinkage of the tumour volume (31). It is, however, consistent with the idea that transient changes in perfusion place an additional control on tumour proliferation, such that both 'proliferation' and 'quiescence' are dynamic states. Transient perfusion changes therefore may not only protect substantial fractions of tumour cells from cytotoxic attack by most conventional anticancer agents, but additionally, keep them poised to grow if the situation improves.

Clinical demonstration of similar phenomena will be difficult; the number of experimental manipulations possible in the clinic is necessarily limited, and the interactions between cell cycle time, stimulation of repopulation, and response to therapy add further complexity. Circumstantial evidence supporting temporally variable nutrient supply to human tumour cells is, however, already available, and includes the observation of transient delivery of halogenated pyrimidines in human head and neck tumours (15), and the well-established principle that the S-phase index will always be higher if in vitro rather than in vivo labelling of human tumours is used (32, 33). We would similarly argue that the tumour response seen in the right panels of Fig. 6, with extremely rapid recruitment of proliferating cells, is entirely consistent with our postulate of intermittent changes in blood flow in some tumours.

Objectively, however, our data with the consecutive human tumour biopsies obtained during treatment, taken alone, provide little concrete information either supporting or refuting the concept of transient blood flow in human tumours. How well the highly selected, rapidly proliferating xenografted tumours may represent the clinical situation similarly must be questioned. Clearly, S-phase fractions of 30% or greater, and hypoxic fractions exceeding 50% as seen for the WiDr xenografts are not representative of most clinical tumours. Even the S-phase fraction of 20% seen for our SiHa tumours exceeds that typically seen in the clinic (34), as does the hypoxic fraction of 35% compared to oxygen electrode (35) or comet assay (36) measurements of hypoxic fractions in comparable human cervical tumours, or their pimonidazole labelling (Aquino-Parsons, unpublished data). Interestingly, we often see larger hypoxic and proliferating fractions in individual xenografted tumours with greater transient changes in blood flow (as in Fig. 4). Consequently, while the xenograft models may be quantitatively different, we nonetheless believe that they are qualitatively representative. This suggestion seems quite well justified by the tumour response seen in the right panels of Fig. 6, showing that extremely rapid recruitment of proliferating cells can be seen in at least some clinical tumours in a site thought to exhibit clinically relevant hypoxia.

We are particularly encouraged by our apparent ability to demonstrate human tumour radiosensitivity or resistance

in situ with simple DNA histograms, and also to note dramatic treatment-induced proliferation in some clinical samples. Naturally, we are anxiously awaiting the acquisition of more biopsies from 'repopulating' tumours, and a sufficient follow-up period to determine whether the responses seen are indeed prognostic and/or predictive of outcome.

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