Clinical Relevance of Intermittent Tumour Blood Flow

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Acta Oncologica Vol. 40, No. 8, pp. 929–936, 2001

One of the goals of translational cancer research is to understand basic 'phenomena' so that tumour response to therapy can be improved. One such phenomenon is intermittent tumour blood flow. The impact of the transient hypoxia that results from decreased tumour blood flow is now beginning to be appreciated in preclinical systems, and also receiving some attention in clinical practise. Thus in this article we review the nature and frequency of microregional blood flow changes in preclinical and clinical tumours and examine the impact of those changes on response to both radiotherapy and chemotherapy. Additionally, the implications of non-constant blood flow for both the growth of the unperturbed tumour and the regrowth of surviving tumour clonogens during and after therapy are examined.

Received 6 August 2001
Accepted 22 August 2001

Of Julie Denekamp's many scientific endeavours, perhaps none was more satisfying to her than working toward the improvement of tumour treatment based on a better understanding of fundamental tumour biology and physiology. With this study, we seek to follow her example, where the implications of non-constant blood flow to solid tumours are examined with respect to tumour growth, regrowth, and response to therapy.

In the past several years, our understanding not only of the molecular biology of tumours but also of tumour physiology has increased markedly. Interestingly, many of those advances have resulted from the relentless study of tumour hypoxia, the best-known consequence of inadequate tumour blood flow. In concert with our improved insight into tumour physiology, our understanding of tumour hypoxia has also changed, as epitomized by the recent contribution from Julie Denekamp and her colleagues (1) in which they argued that it is time for a 'paradigm shift' in the way we envisage tumour hypoxia and its therapeutic significance.

The major change in our thinking about tumour blood flow and hypoxia has been an expansion of the classical model (2), where the more rapid growth of tumour cells than of the supporting vasculature results in areas of the tumour largely inaccessible to oxygen, metabolites, and even some chemotherapeutic drugs. That situation has now been recognized, however, as the extreme case of a much more dynamic process: an interplay of nutrient and oxygen supply and demand, modulated by a differential ability of tumour cells to survive in substandard conditions (3–5). In experimental tumours, blood flow changes can easily be illustrated with sequential injections of blood-borne dyes, where differential uptake provides an indication of the changing blood flow pattern (6, 7). Complete absence of one of the markers in a single vessel provides convincing visual evidence that the vessel either opened or closed in the time interval between administration of the two markers. While dramatic, such changes are relatively infrequent in our experience, and probably have diverted attention away from more subtle (but numerically much more frequent) blood-flow perturbations. Image analysis techniques to quantify stain delivery have therefore been developed to enumerate such microregional changes in blood flow (8–10).

While transient changes in blood flow and therefore oxygenation can have significant consequences for tumour radiosensitivity, our ongoing work suggests that other more general consequences can also be expected (11). Consider, for example, the impact on proliferating cells if the nutrient supply is suddenly restricted or abolished. Few investigations have directly addressed this issue, although published work on ischaemia (12) and hypoxia adaptation

 ISSN 0284-186X

(13, 14) provides some insights. Even more difficult is an assessment of such effects in tumours in patients, though some measurements with oxygen and blood flow probes have suggested that at least regional perfusion changes can occasionally be observed (15).

This article addresses these issues with data from both preclinical and clinical tumours. We have undertaken studies in human xenograft tumour systems in which blood flow changes and their impact on the multifraction radiation response and accelerated repopulation are directly measured. Additionally, in ongoing clinical protocols in Vancouver, both the responses of tumour cells to therapy as measured in sequential biopsies, and hypoxia assessment in selected cervix tumours are being examined. Data obtained from flow cytometry analyses of tumour cells from these biopsies are completely consistent with the fluctuating blood flow and consequences that can be demonstrated in the preclinical systems.

METHODS

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

Two human tumour cell lines were used: SiHa, a cervical squamous cell carcinoma (16), and WiDr, a colon adenocarcinoma (17). Both were obtained as cultured cell lines (ATCC, Rockville, Maryland), and then maintained in immunodeficient SCID or nude mice by intramuscular transplant. 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 (18, 19).

Short-term changes in blood flow in the xenograft systems were visualized using the dual staining mismatch technique (7, 8). The fluorescent bisbenzimide dye Hoechst 33342 (0.5 mg per mouse in 0.05 ml PBS) was administered by intravenous injection, followed 30 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 by locally developed software (8, 10).

For flow cytometry studies, single cell suspensions were prepared from excised xenografts or biopsies 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 a 30 μm nylon mesh to remove clumps, and the monodispersed cells washed by centrifugation and fixed in chilled 70% ethanol. The hypoxia marker Hydroxyprobe-1 (pimonidazole-HCl from NPI Inc., Belmont MA) was administered to mice at 100 mg/kg, and recognized (20) by a FITC-conjugated polyclonal antibody or with a 2-step approach using a conjugated rabbit anti-mouse secondary antibody (anti-pimonidazole antibodies were generously supplied by Dr James Raleigh of the University of North Carolina).

Iododeoxyuridine (IdUrd) or bromodeoxyuridine (BrdUrd) incorporation was interchangeably 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 vitro labelling used 5 μM for 15 min. Before analysis, the fixed cells were denatured in 2N HCl 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. Cellular DNA was counterstained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride hydrate) at 1 μg/ml. Standard flow cytometry techniques were used (18, 19, 21); 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).

Ongoing clinical protocols at the Vancouver Cancer Centre have been designed to assess response to fractionated external beam radiation therapy with weekly tissue biopsies, and to use pimonidazole as a hypoxia probe in selected carcinomas. The BC Cancer Agency, and University of British Columbia Ethics Committees approved both protocols. After obtaining informed consent from the patients, biopsies of cervical tumours were obtained prior to the prescribed course of radiation therapy, and when feasible, weekly during treatment. 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.

RESULTS

Demonstrating and/or quantifying blood flow changes in experimental tumour systems are subject to many fewer restrictions than in the clinical situation. An example can be found in Fig. 1, showing quantitative assessments of stain delivery using the dual staining 'mismatch' technique in two human tumour lines grown as xenografts in SCID mice. The WiDr (colon carcinoma) tumours show the greatest short-term fluctuations in blood flow of any of our xenograft systems, whereas blood flow in the SiHa (uterine cervix) tumours is among the most stable in short (2–3 h) intervals.

Data for the two tumour types are shown as 'cumulative incidence' plots, where the percentage of vessels showing more than a given decrease or increase in blood flow in a 0.5 h period can immediately be determined in panels a) and b), respectively. Mechanistically, data like these are obtained by quantifying the normalized intensity ratio of

the two stains (8–10) and plotting the cumulative percentage of vessels showing a given level of 'mismatch' (10). For reference, the vertical dotted lines show the approximate threshold for the visual methodology where vessels are scored when one of the stains is markedly greater in intensity than the other (7, 22). Reading from panel a), the dotted line shows that at this threshold level, less than 0.1% of the SiHa tumour vessels showed at least this degree of mismatch, whereas over 1% of the WiDr vasculature was subject to fluctuations of this magnitude or larger in the 30-min interval. Obviously, as the amount of fluctuation decreases, the number of vessels showing at least that amount of fluctuation systematically increases.

In addition to the quantitative changes that can be gleaned from Fig. 1, the qualitative shape of the curves is also instructive. The 'upswing' for larger blood flow changes seen in the WiDr system is an immediate indication of the greater short-term heterogeneity of blood supply in this particular tumour type. In all cases in Fig. 1, the data were for control tumours, and include in excess of 15 tumours for each tumour type. Note that the time interval between dye injections was constant at 30 min for all of these data; furthermore, it is apparent that quite a large fraction of vessels in either tumour type shows a twofold or greater variation in blood flow over this time interval.

The functional significance of such blood flow changes in the same two tumour systems is shown in Fig. 2, where cellular status is indicated by IdUrd uptake in cells actively undergoing DNA synthesis, or by binding of pimonidazole in hypoxic cells. The variables in this case were time and number of pimonidazole injections (using hourly injections of the hypoxia probe). To further quantify possible changes in blood flow impacting on IdUrd delivery, iododeoxyuridine was administered to all animals in vivo, and again in vitro after tumour disaggregation. The latter procedure identifies cells that were capable of synthesizing DNA, but to which IdUrd apparently could not be delivered while the cells were in situ (the result of either perfusion or diffusion/consumption limitations).

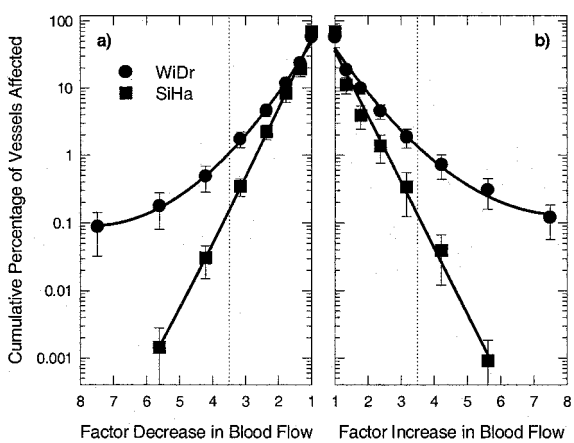

Fig. 1. Data from quantitative image analysis of sections of WiDr and SiHa xenografts sequentially injected with Hoechst and carbocyanine dyes. The ratios of the normalized staining intensities were used to indicate increasing (carbocyanine brighter) or decreasing (Hoechst brighter) perfusion, with the data plotted as cumulative distributions; note the use of the log scale to provide sensitivity at fairly low numbers of observations. Uncertainties are SEM from a minimum of 15 tumours per group.

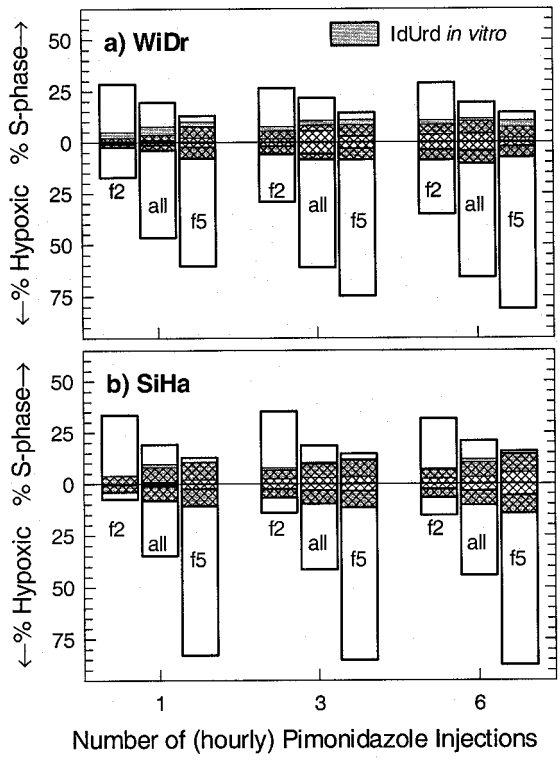

Fig. 2. Flow cytometry analyses of pimonidazole (hypoxia) and/or IdUrd (S-phase) incorporation in cells from Widr and SiHa xenografts in SCID mice that had been injected hourly with pimonidazole. Cross-hatching indicates uptake of both probes in the same cells; shading indicates the excess fraction of cells incorporating IdUrd in vitro during the analysis. In addition to average values (all), cell sorting on the basis of infused Hoechst33342 was used to identify fairly well (f2) and poorly (f5) perfused tumour cells. Several qualitative and quantitative differences are evident, as highlighted in the text.

A number of unexpected observations are evident in Fig. 2. First, exposure to IdUrd in vitro increased the number of IdUrd-labelled cells by at least 30% in WiDr tumours, and often by more than 50% in the SiHa xenografts. Concurrently, the fraction of cells showing both IdUrd and pimonidazole uptake also increased. Numerically, more than half of the cells capable of incorporating iododeoxyuridine in both tumour types had been hypoxic (bound pimonidazole) in the previous 3 to 6 h.

The overall responses just described were refined by using cell sorting (23, 24) in order selectively to examine well-perfused (Fraction 2, labelled f2) and poorly perfused (Fraction 5) subpopulations (the entire tumour cell popu-

lation was divided into 6 subpopulations based on Hoechst delivery just after the IdUrd injection). As expected, Fraction 2 contained more proliferating and fewer hypoxic cells for both tumours. Also, consistent with the more constant blood flow expected in the SiHa tumours over this relatively short time interval (6 h or less), cell sorting based on Hoechst perfusion produced a better discrimination of S-phase and hypoxic cells in SiHa than in the WiDr tumours.

In addition to the quantitative differences between the tumour types, some interesting qualitative differences also emerged. Most notable was the systematic increase in the number of cells labelled with both pimonidazole and in vivo IdUrd in the SiHa tumours, whereas dual labelling was more random in the WiDr xenografts. This is again consistent with the data in Fig. 1, confirming that the WiDr tumours experience more frequent blood flow fluctuations of shorter duration. Similarly, IdUrd exposure in vitro consistently provided a larger incremental increase in labelling in the poorly perfused cells of SiHa tumours than in the well-perfused fraction, unlike the quite inconsistent increases seen in the WiDr xenografts.

The data in Fig. 2 suggest that blood flow fluctuations (and/or the resulting hypoxia and pH changes) play a significant role in regulating DNA synthesis and therefore cell proliferation in these tumours. Quite large fractions of cells appear to be viable and capable of DNA synthesis, but non-proliferating in vivo at any particular point in time. This has significant implications for numerous aspects of tumour response to therapy (11), including accelerated repopulation during/after cytotoxic treatments (25).

In Fig. 3 the indications are that cell proliferation indeed plays a pivotal role in the multifraction response of the SiHa xenografts to radiotherapy. In these experiments, twice daily (7-h interval) exposures to 2.5 Gy/fraction were administered for 10 consecutive days, with tumours from each animal cohort consecutively harvested and analysed for DNA content and the distribution of bromodeoxyuridine that had been administered 4 h prior to sacrifice. Interestingly, Fig. 3 demonstrates that simple DNA distribution histograms provide considerable information regarding tumour response to therapy. Over the first 8 days of treatment (up to 40 Gy accumulated dose) there was a progressive increase in the fraction of G_2/M cells (reflecting a G_2 block and/or mitotic death) at the expense of the G_1 population. However, the increased proliferation first seen at 8 days and very evident 10 days into treatment indicates the expansion of a subpopulation of cells capable of repopulating even during this radiotherapy protocol. Both the overall S-phase population and those S-phase cells that were viable (able to incorporate bromodeoxyuridine) first decreased, and then increased in percentage; the relative changes were much more dramatic using the functional assay (BrdUrd incorporation).

To add additional perspective to these data, it is perhaps worth noting that 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. 3 is approximately 4 days (18, 19, 26). This represents a growth potential not very different from the median values typically observed in T_{pot} measurements from human tumour biopsies (27–29). Despite these unremarkable cell kinetics and rather high radiosensitivity, SiHa xenografts cannot be successfully controlled by multifraction radiation even with regimens as aggressive as that described in Fig. 3. The reason for this lack of responsiveness is clearly the regrowth potential of the tumour, which may be due in part to the fluctuating blood flow and consequent production of subpopulations of cells 'poised' to grow, but usually unable to do so because of the local microenvironmental conditions.

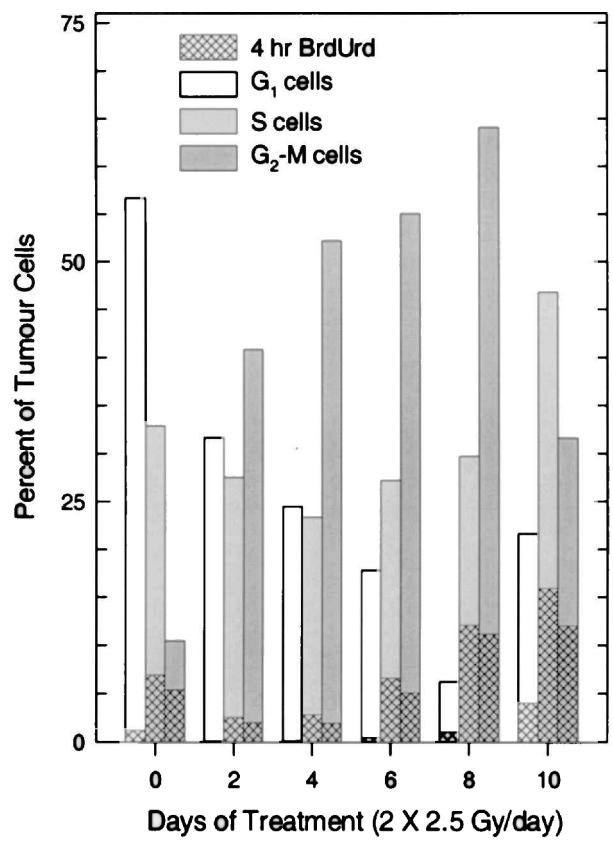

Fig. 3. The response of tumour cells recovered from SiHa xenografts to continuous, twice-daily x-ray exposures. Note the rapid and progressive initial development of the 'G_2/M' population, representing blocked and dead/dying cells. Concurrently, the G_1/G_0 subpopulation rapidly diminished, indicating very rapid recruitment of cells into the proliferative compartment. The distribution of cells that incorporated BrdUrd delivered 4 h prior to excision is shown by cross-hatching; note that this was a more informative index of response than the S-phase fraction determined by DNA content alone. Repopulation was evident near the end of treatment, as discussed in the text.

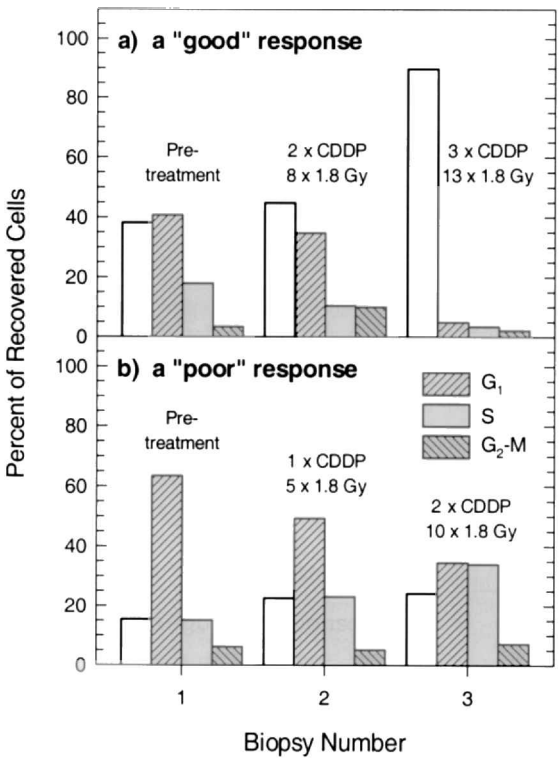

Fig. 4. Representative data from patients with cancer of the uterine cervix, where biopsies were taken before and approximately weekly during treatment. These are examples of the usual response (panel a), where the accumulation of G_2/M cells in the second week of treatment is suggestive of radiosensitivity and cell death, and the proportion of tumour cells recoverable in the third and later weeks was low. Conversely, the tumour in panel b) showed only a modest decrease in tumour to normal cell ratio during treatment, with a progressive increase in the fraction of S-phase cells (tumour proliferation). For reference, the open bars indicate the fraction of diploid (non-malignant) cells recovered in each biopsy.

Since the data in Fig. 3 show that 'simple' tumour cell DNA histograms obtained during the course of multifraction treatments provided a general indication of both radiosensitivity (the pronounced G_2 block) and regrowth of surviving cells, we are now examining biopsies obtained during consecutive weeks of radiotherapy for human tumours, the majority of which are primary cervical malignancies. Our experience is now approaching 40 patients, and we have found that 2 to 3 biopsies can be obtained *during* treatment for the vast majority of patients.

In Fig. 4 we present 'extreme' examples of the responses seen for these patients. Panel a) reflects the response seen in about two-thirds of the patients, where tumour radiosensitivity is implied by an increase in the G_2/M peak of tumour cells during the first week of radiotherapy. By the second to third week, these biopsies are largely devoid of malignant cells. To add perspective on the number of 'normal' versus 'tumour' cells recovered from the biopsies, the data in Fig. 4 include the normal cell compartment (i.e., the cells with diploid DNA content). Normal/tumour discrimination was defined by DNA content alone in Fig. 4 where hyper-diploid tumours were analysed; in diploid tumours, discrimination is more difficult but can sometimes be improved with cytokeratin and other in vitro probes. For perspective, both patients illustrated in this figure were treated with curative intent based on their clinical assessments (a planned radiotherapy course of 25×1.8 Gy external beam treatment delivered in 5 weeks with 40 mg/m^2 cisplatin weekly, followed by intracavitary brachytherapy).

One of the most interesting responses seen to date is shown in panel 4b). Instead of a relative disappearance of tumour cells from the biopsy, there was a suggestion in the first week and definite evidence in the second week that a highly proliferative hyperdiploid population of cells had emerged. Such a response is seen in a minority of patients, and we do not as yet have sufficient follow-up time to be entirely confident that the observed kinetic changes will be prognostic for failure. Irrespective of their predictive potential, however, the data in Fig. 4 indicate that the response of some tumours can be quantified during the course of therapy. Given that none of the traditional variables in the clinical assessment suggested that such differences would be seen, it is perhaps not completely inappropriate to suggest that a currently neglected parameter, blood flow fluctuation, may play a role. This suggestion is consistent with both the potential for rapid repopulation after debulking, as well as the fact that the pretreatment S-phase fraction and other indices of proliferation were not obviously different between the two tumours.

The data in Fig. 4, however, only indirectly address the possibility of transient blood flow in human cervix tumours in situ. A somewhat more definitive approach is shown in Fig. 5, where tumour cells (hyperdiploid cells in all samples) from patients that had received pimonidazole about 24 h prior to biopsy were also subjected to in vitro exposure to iododeoxyuridine during the tumour disaggregation procedure. In three of the five tumours in Fig. 5, more than 5% of the cells incorporating IdUrd had previously been hypoxic, i.e., were pimonidazole-labelled (note the log scale, which emphasizes differences even in the relatively small numbers of cells showing binding of either pimonidazole, iododeoxyuridine, or both). Interestingly, when the biopsies were ordered in terms of increasing iododeoxyuridine uptake in vitro, as in Fig. 5, there appeared to be some correlation between decreasing hypoxic fractions and increasing numbers of functional S-phase cells. However, no apparent correlation was observed between either the amount of hypoxia or amount of iododeoxyuridine uptake, and dual labelling. While these data unequivocally show that tumour cells capable of undergoing DNA synthesis in vitro had incorporated the hypoxia stain pimonidazole in vivo, it must be stressed that this

does not guarantee that such cells would ever have become reoxygenated and/or synthesized DNA in situ.

DISCUSSION

The data presented here indicate that transient changes in blood flow can have significant ramifications in xenografted human tumours and are implicated as having a role in clinical responses to therapy. Perhaps most significantly, these results suggest that a number of features of experimental, and inferentially, clinical tumours may deviate to some extent with conventional dogma. One typically thinks of a solid tumour as growing more rapidly than its blood supply, resulting in an ordered progression of cells from the aerobic to hypoxic to necrotic (or apoptotic) compartments, with that progression being 'one-way' only. When changes in blood flow are also considered, they are typically regarded as infrequent 'anomalies' superimposed upon the logical progression just described.

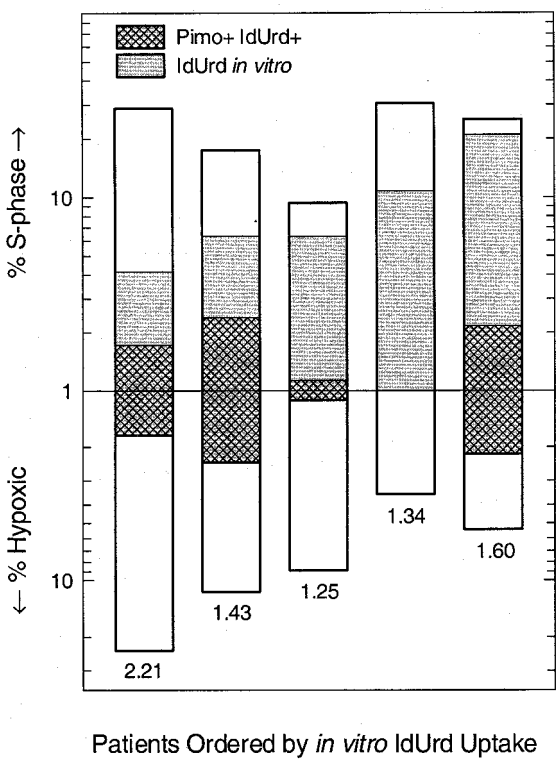

Fig. 5. Examples of hypoxic cells that were capable of DNA synthesis in vitro, from biopsies of cancers of the uterine cervix where pimonidazole had been administered about 24 h prior to biopsy. During the biopsy disaggregation procedure, cells were exposed to 5 μM IdUrd in vitro; analyses were done by flow cytometry and positive cells defined as those brighter than the unstained cohorts. Cross-hatching represents double labelling; shading is the S-phase fraction defined by IdUrd uptake (note that the S-phase fraction defined by DNA content was always larger). The DNA index of the major tumour cell population is shown below each sample.

Our data suggest that this view is overly simplistic; tumours in model systems and patients would appear to be more dynamic and therefore much more complex. Substantial numbers of cells in both the SiHa and WiDr xenograft systems clearly can be hypoxic at one point in time, and subsequently viable and proliferating (able to incorporate thymidine analogues). In experiments with longer time frames (days rather than hours) not included here, we have observed that in both the WiDr and SiHa xenografts, cells having undergone DNA duplication and division are only marginally more likely to enter a subsequent S-phase than are 'quiescent' cells (11, 18, 19, 26). Taking all these observations together, perhaps the most interesting result is an 'inverse' conclusion: few cells in either xenograft system appear to be in a relatively static situation. Those cells are the chronically hypoxic subpopulation that never becomes reoxygenated—cells that presumably have a limited life expectancy (26).

Fluctuating blood flow appears to have unanticipated effects on tumour growth and regrowth potential, mediated by effects on tumour cell 'viability' and 'turnover'. It seems that a large fraction of cells in the xenograft systems (and, inferentially, in the human tumours) retain their viability and growth potential as a result of intermittent perfusion with oxygen and nutrients (11, 26). Consequently, it is not unreasonable to expect such cells rapidly to begin to proliferate if the local microenvironment permits this at any point during treatment. Our data indicate that even human tumour cells in S-phase can be (transiently?) arrested, yet capable of incorporating thymidine analogues and of proliferating given the chance. Whether the determining factor is decreases in pH due to glycolysis (11, 30–32), or simply lack of nutrient delivery, or other factors remains to be determined.

Transient growth arrest and a hypoxic microenvironment together have negative implications for most therapeutic manipulations, insofar as they imply 'protection' against anti-proliferative drugs as well as radiation, and yet the possibility of repopulation much earlier in a therapeutic regimen than may previously have been thought. Interestingly, other evidence more directly suggesting transient blood flow changes in human tumours is already available in the literature, including documentation of transient delivery of halogenated pyrimidines in human head and neck tumours (33), direct measurement of oxygen and blood flow changes with implanted probes (15), and the well established principle that the S-phase index is always higher for human tumour cells labelled in vitro compared to in vivo (34–36).

Our data with the consecutive human tumour biopsies obtained during treatment, when considered alone, provide little concrete information either supporting or refuting the concept of transient blood flow in human cervix tumours. Likewise, the highly selected, rapidly proliferating xenografted tumours in mice may not represent the clinical

situation. Obviously, the S-phase fractions reported here for the WiDr and SiHa tumours are higher than those measured by either flow cytometry or in vitro IdUrd uptake in our patients, or the comparable data for human cervical tumour S-phase fractions in the literature (28, 36). We find it interesting, however, that we often see larger hypoxic and proliferating fractions in those individual xenografted tumours that also show the greatest transient blood flow (10, 18). Even though magnitudes may vary, we would still argue that the phenomena seen in the xenograft systems may well provide a partial explanation for human tumour response in situ.

We are particularly encouraged by our ability to acquire and analyse human tumour biopsies during the course of radiotherapy, and to see measurable responses that we believe will ultimately be validated as prognostic if not predictive indicators. Furthermore, the demonstration that cells from human cervix tumours that incorporated pimonidazole remain functionally able to incorporate iododeoxyuridine in vitro is consistent with transient blood flow in human tumours, and unequivocally demonstrates the viability of at least some of the hypoxic cells identified with that hypoxia marker. While the clinical data at present are still too preliminary for firm conclusions, we nonetheless are optimistic that data such as these will contribute to Julie Denekamp's fondest hope: the ability of new insights from tumour biology to impact on the clinical management of human cancer.

ACKNOWLEDGEMENTS

This research was supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society, and by the Canadian Institutes of Health Research. Skilled technical assistance was provided by Nancy LePard, Denise McDougal, Angela Nicol and Darrell Trendall; Dr Edward Sham and Kevin Bennewith also contributed while undertaking fellowship training in our laboratory.

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