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Microregional blood flow in murine and human tumours assessed using laser Doppler microprobes

SA Hill[1], KH Pigott[2], MI Saunders[2], MEB Powell[2], S Arnold[1], A Obeid[3], G Ward[3], M Leahy[3], PJ Hoskin[2] and DJ Chaplin[1]

[1]*Tumour Microcirculation Group, Gray Laboratory Cancer Research Trust and* [2]*Marie Curie Research Wing, Mount Vernon Hospital, Northwood HA6 2JR, UK;* [3]*Oxford Optronix Ltd., Magdalen Centre, Oxford Science Park, Oxford OX4 4GA, UK.*

Summary A multichannel laser Doppler system has been used to measure microregional fluctuations in perfusion in the HT29 human tumour xenograft and in patients with advanced malignant disease. A comparison is made with previously obtained data for the SaF, a transplantable murine tumour. The 300 μm diameter probes recorded fluctuations in erythrocyte flux in tumour microregions with an estimated volume of 10^{-2} mm^3. Of the 66 human tumour microregions sampled, 26% showed a change in erythrocyte flux by a factor of 2 or more over the 60 min measurement period, compared with 37% of HT29 and 48% of SaF microregions. In each of the studies more than 50% of changes were completed within 20 min, although slower changes were more common in the human tumours than in the experimental systems. Within the 1 h monitoring period at least 30% of the changes were reversed (human tumours 30%, HT29 45%, SaF 31%). These findings demonstrate that microregional changes in erythrocyte flux, consistent with transient, perfusion-driven changes in oxygenation, are a feature of human malignancies as well as experimental transplanted tumours.

Keywords: hypoxia; erythrocyte flux; SaF murine tumour; HT29 tumour

The presence of hypoxic cells within tumours is considered to be an important factor in determining treatment outcome following radiotherapy (Dische, 1985, Overgaard, 1992, Höckel *et al.*, 1993). Since Thomlinson and Gray (1955) first proposed the existence of hypoxic cells beyond the diffusion distance of oxygen, efforts have continued to devise strategies to increase tumour oxygenation effectively. Hyperbaric or normobaric oxygen or carbogen breathing and the use of oxygen-mimetic radiosensitisers, while producing some successes, have proved generally disappointing in the clinic (Henk, 1981; Rubin *et al.*, 1979; Dische, 1985; Overgaard, 1992). Part of this failure may be because not all hypoxic cells arise as a result of diffusion limitations. More recently, attention has focused on overcoming both acute and chronic hypoxia. To this aim, based on the success of studies using murine tumour models (Kjellen *et al.*, 1991), clinical studies have been initiated to evaluate the combination of nicotinamide and carbogen breathing in accelerated radiotherapy regimes (ARCON) (Zackrisson *et al.*, 1994). While the use of carbogen is designed to overcome chronic, diffusion-limited hypoxia, the vitamin B derivative, nicotinamide, is believed to overcome acute hypoxia resulting from transient fluctuations in tumour blood flow (Horsman *et al.*, 1988; Chaplin *et al.*, 1990). Acute or perfusion-limited hypoxia was postulated to result from blood flow irregularities at the microvascular level by Brown (1979). More direct evidence was provided by observations of temporary non-perfusion of vessels in tumours grown as 'sandwich' preparations, between transparent plates (Reinhold *et al.*, 1977). Further studies using fluorescent perfusion markers combined with flow cytometric and histological techniques have confirmed that intermittent perfusion is a common feature of transplantable murine tumours and some human tumour xenografts (Chaplin *et al.*, 1987; Trotter *et al.*, 1989; Chaplin and Trotter, 1991). The techniques employed in experimental tumour systems are not applicable to the clinic however and to date, no evidence exists to indicate that transient perfusion and hence acute hypoxia, occurs in human tumours. Such information is clearly essential for the design of effective treatment strategies. The recent development of a multichannel laser Doppler system has made possible the real time monitoring of erythrocyte flux in tumour microregions, since each 300 μm probe has an estimated sampling volume of approximately 10^{-2} mm^3. We have recently reported the successful use of this system in measuring apparently spontaneous fluctuations in perfusion in transplantable murine tumours (Chaplin and Hill, 1995). We have also reported that nicotinamide can reduce the number of flow reductions measured in such a system (Hill and Chaplin, 1995). An initial study has already established the feasibility of using laser Doppler microprobes to detect changes in microregional erythrocyte flux in human tumours (Pigott *et al.*, 1996). The purpose of the current study was to use laser Doppler flowmetry to investigate the incidence of perfusion fluctuations in the xenografted human colonic carcinoma HT29 as well as a range of human malignancies, for comparison with the murine tumour studies.

Correspondence: SA Hill

Materials and methods

Experimental tumour models

Two experimental tumour systems were used in this study: the undifferentiated murine sarcoma SaF and the human colonic adenocarcinoma HT29. Subcutaneous tumours were produced by injecting 0.05 ml of a crude SaF cell suspension or 5×10^5 tissue culture maintained HT29 cells dorsally into 12-16 week old CBA/Gy f TO (SaF) or SCID (HT29) mice. Animals were selected when their tumours reached 5-6.5 mm geometric mean diameter (150-300 mg), and were restrained, unanaesthetised, in Perspex jigs for the duration of the measurements, at the end of which a lethal dose of sodium pentobarbitone was injected via a previously inserted tail vein catheter.

Patients

Following written informed consent, 13 superficial tumours were entered into the study. The lesions, chosen for their accessibility, comprised primary, recurrent and metastatic deposits in different sites and of varying histological type. Patients lay comfortably on a couch and were requested to remain as still as possible during recording.

Laser Doppler flowmetry

Erythrocyte flux was monitored using the Oxford array multiple channel laser Doppler system (Oxford Optronix, Oxford, UK). Up to six custom-designed microprobes (300 μm diameter), each monitoring a nominal sampling volume of 10^{-2} mm^3, were inserted into each tumour, allowing simultaneous measurements of perfusion in several discrete locations. Once stable readings were obtained from each probe, erythrocyte flux was monitored for 60 min.

Data analysis

Each of the Doppler channels recorded 20 readings per second. From these values a single average was calculated for each 2 min interval, for each channel. In the animal studies, a final 2 min average was calculated for the readings recorded after the lethal injection. This value was then subtracted from all the preceding calculated averages: it is most likely due to the Brownian motion of free red blood cells in front of the probe. In order to allow for this 'background' component of the signal in the clinical studies, data from several murine experiments were examined. When the final, post mortem, average was expressed as a percentage of the last 2 min average calculated while the animal was alive, a mean value of 30% was calculated (Chaplin and Hill, 1995; Pigott *et al.*, 1996). Thus, for the clinical studies, 30% of the last average calculated for each channel was subtracted from the data. The final plots of erythrocyte flux against time were compared with the original recorded data in order to eliminate any changes associated either with animal/patient movement or probe movement (detected as an abrupt change in the backscatter signal).

Results

For comparison with the 36 SaF tumours previously studied (Hill and Chaplin, 1995), blood flow measurements were performed on 18 individual HT29 tumours and 13 human tumours. The latter included both primary and recurrent breast carcinomas as well as metastases to regional lymph nodes and skin from a variety of tumours of different histologies. Patients ranged in age from 47 to 80 years old and their tumours ranged in size from approximately 3×3 cm (see Figure 1) to 10×10 cm. Figure 1 shows five needle probes inserted in a metastatic colon adenocarcinoma skin nodule via 20 gauge cannulas. Depending on the size and morphology of the tumour, the probes were inserted such that their tips might be separated by anything from a few millimetres to 1 or 2 cm.

Four individual traces from different probes inserted into a primary breast carcinoma are shown in Figure 2 and four traces from a recurrent non-Hodgkin's lymphoma neck node are shown in Figure 3. It can be seen that within the same tumour, fluctuations in erythrocyte flux occurred independently in two of the microregions sampled. Individual traces showed great variation, some showed no change over the whole 60 min measurement period, while others showed increases or decreases in erythrocyte flux. Some traces showed more than one change. The apparent rapid decrease in perfusion indicated in one of the traces shown in Figure 2

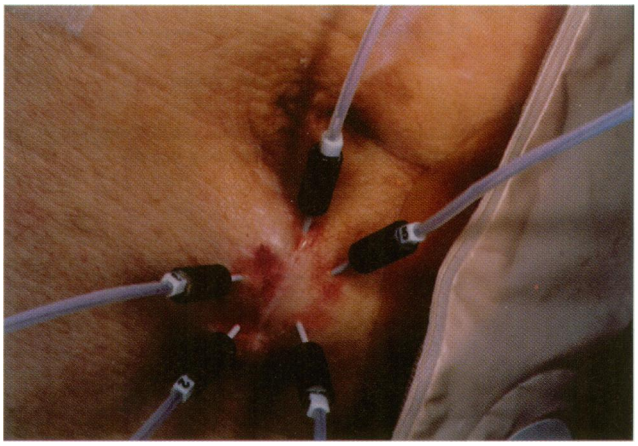

Figure 1 Five needles probes positioned within a colon carcinoma metastatic skin nodule. The 20 gauge cannulas through which the probes were introduced are also visible.

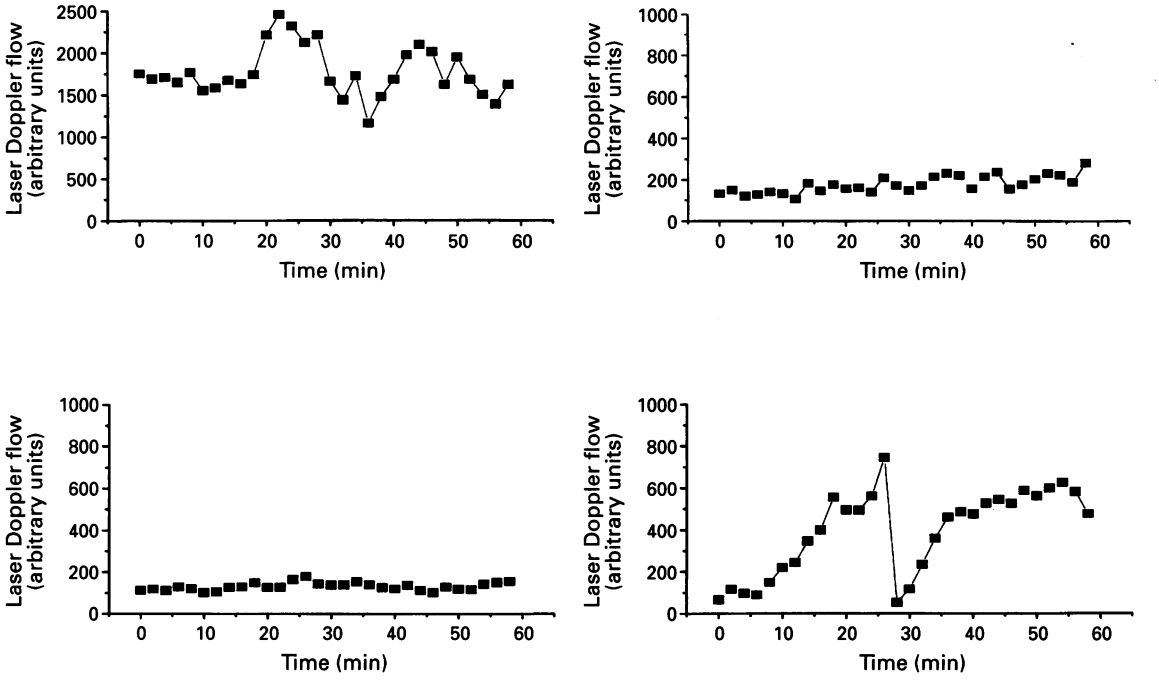

Figure 2 An example of four recorded traces from separate probes positioned in different microregions of a primary breast carcinoma. Each point represents the average of 2400 readings taken over a 2 min sampling period.

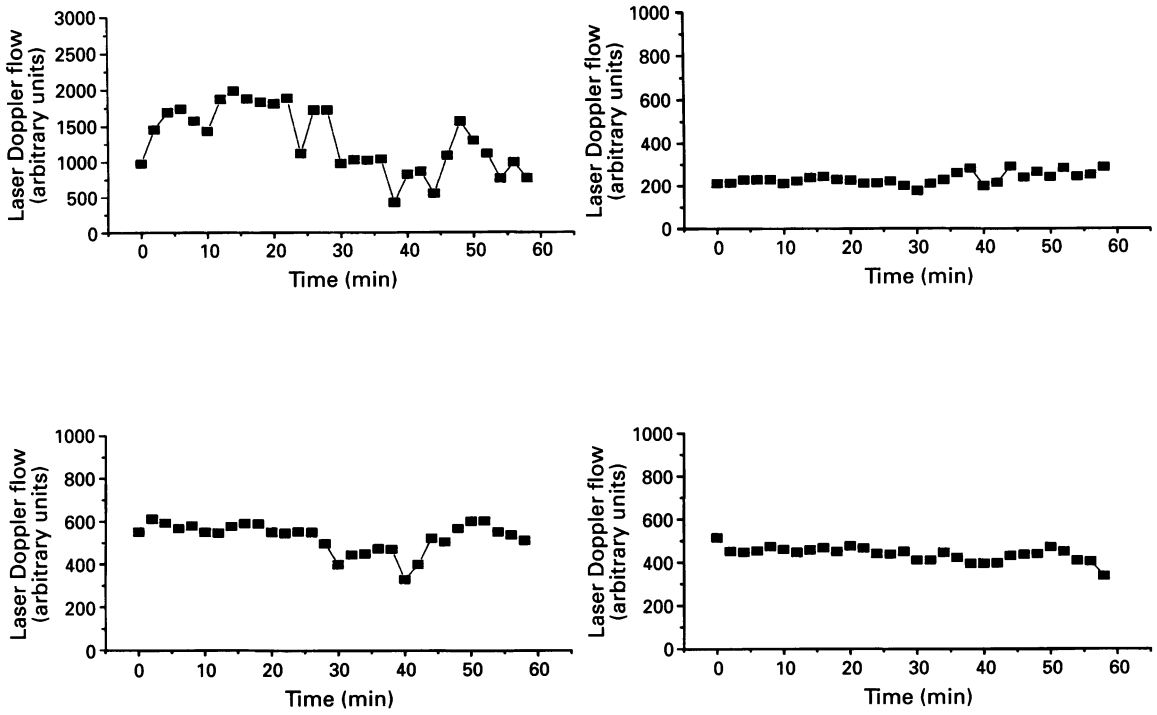

Figure 3 An example of four individual traces from a non-Hodgkin's lymphoma neck node. Each point represents the average of 2400 readings taken over a 2 min sampling period.

Table I Changes in microregional erythrocyte flux during 60 min observation periods

	Murine SaF			*Xenograft HT29*			*Human tumours* in situ *Various histologies*		
Factor of change	*Percentage of traces showing change (n-116)*	*Direction of change*		*Percentage of traces showing change (n=63)*	*Direction of change*		*Percentage of traces showing change (n=66)*	*Direction of change*	
≥1.5	–			–			58	38↑	22↓
≥2	48	39↑	49↓	37	20↑	22↓	26	15↑	9↓
≥5	16	10↑	14↓	10	4↑	6↓	3	3↑	0↓

was accompanied by an abrupt change in the recorded backscatter signal, indicating a change in the position of the probe and was therefore excluded from the analysis.

Data from each probe were analysed separately and a summary of the changes in erythrocyte flux measured in all of the individual traces obtained is shown in Table I for both the HT29 and the human tumours, together with previously published data for the murine SaF (Hill and Chaplin, 1995). In the human tumours, 26%, of the sampled microregions showed a change in erythrocyte flux by a factor of 2 or more over the 1 h measurement period, compared with 37% of HT29 microregions and 48% of SaF microregions. The incidence of changes by at least a factor of 5 is also indicated, as is a smaller change, by a factor of 1.5 or more for the human tumours. Approximately equal numbers of increases and decreases in flow were measured in the SaF and the HT29 compared with the human tumour measurements which indicated a greater number of flow increases. We believe this may reflect haemorrhage in front of the probe, since one patient with a very low platelet count showed increases in erythrocyte flux in five of his six tumour microregions monitored.

We are now able to monitor erythrocyte flux in skin, simultaneously with the tumour measurements. Limited data are available at this point, since the skin probes have so far been used for only three patients. However, only one of the nine evaluable traces shows a change in erythrocyte flux by a factor of 1.5.

Table II Kinetics of change in microregional erythrocyte flux

Time from max to min or min to max	*Murine SaF*	*Xenograft HT29*	*Human tumours*
Rate of change (%)			
Within 10 min	49	42	25
Within 20 min	75	69	56
Changes reversed within observation period (%)	31	45	30

As well as the incidence of perfusion fluctuations, the laser Doppler system provides kinetic information on the speed and duration of the changes measured. These data are summarised in Table II. Although some traces show a very slow rate of change, all three studies indicate that more than 50% of changes occur over a period of 20 min or less. The human tumours show a greater number of slow changes than the HT29 or the SaF. In each study, a significant proportion of the changes was reversed within the observation period.

Discussion

With the advent of the Eppendorf polarographic electrode system direct measurements of tumour tissue oxygenation are now possible in patients, and the existence of regions of low oxygen partial pressure has been demonstrated in a number

of studies (Kallinowski *et al.*, 1990; Vaupel *et al.*, 1991; Höckel *et al.*, 1993). However the global picture of pO_2 distribution throughout a tumour will not reflect changes in oxygen tension which may occur transiently in different regions of the tumour due to periods of impaired perfusion or vessel closure. At present no system is available to measure temporal changes in oxygenation directly at single or multiple fixed points within a tissue. However, since it has been demonstrated that clonogenically hypoxic cells can result from dynamic perfusion changes (Chaplin *et al.*, 1987) studies of acute hypoxia and its elimination have focused on the measurement of microregional fluctuations in tumour perfusion. Studies involving many rodent tumour models have indicated the widespread incidence of temporary flow reductions and the development of strategies designed to eliminate them remains a focus of experimental research. It is therefore important that the presence or absence of acute hypoxia, or perfusion changes leading to it, is established in human tumours.

This study demonstrates that microregional fluctuations in erythrocyte flux can be detected in human tumours using laser Doppler flowmetry. The temporal changes in flow measured in the HT29, a human tumour growing as a xenograft, and in primary and metastatic tumours in patients are similar to those reported previously for two transplantable murine tumours (Chaplin and Hill, 1995). Over the 1 h monitoring period, 37% of HT29 microregions, and 26% of the human tumour microregions sampled showed a change in erythrocyte flux by a factor of 2 or more. This compares with 48% in the SaF. The figure for human tumours could be an exaggeration, if some of the flow increases actually reflect haemorrhage in front of the probe. If the true number of increases was equal to the number of decreases measured, the percentage of traces showing a 2-fold change would still be approximately 20% however. A factor of 2 reduction in perfusion could correspond at one extreme to the complete closure of 50% of the vessels contained in the sampling volume or at the other to a 50% reduction in flow in all of the vessels in the region. Likewise, a factor of 1.5 decrease would result if flow ceased in 30% of the sampled vessels. In each case, an increased level of hypoxia would result, either by the creation of new foci of hypoxic cells or by decreasing the supply of oxygen and thereby causing an expansion of the rim of hypoxic cells. In the human tumours, as in the experimental models, a high proportion of the changes measured occurred within 20 min and in at least 30% of cases the change was reversed within the 60 min of recording.

In summary, the current study establishes that temporal changes in erythrocyte flux consistent with the occurrence of perfusion-limited hypoxia are a common feature of both human and murine tumours. This finding has important implications for tumour biology and therapy.

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