Changes in tumour morphology with alterations in oxygen availability: further evidence for oxygen as a limiting substrate

D.G. Hirst, V.K. Hirst, B. Joiner, V. Prise & K.M. Shaffi

CRC Gray Laboratory, Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK.

Summary The ability of cancer cells to survive at a distance from blood vessels should be dependent on the local supply of nutrients to each vessel. The corded growth of tumour cells around blood vessels within regions of necrosis in the RH carcinoma in the mouse allows the limit to which cells can be supported by individual vessels to be observed. The thickness of individual tumour cords was measured in conventionally stained tumour sections using a scanning technique to determine the distance between the blood vessel wall and the most distant viable cell adjacent to necrosis. Cord radius was found to vary with the oxygen supply conditions. Control animals had a mean radius of 105±2 µm while animals that had breathed 10% oxygen had significantly narrower cords (93±3 µm after 48 h) and animals breathing 100% oxygen had significantly wider cords (117±3 µm after 24 h). Mice made anaemic (mean hct. 28%) by phlebotomy and plasma transfusion had cord radii that were not significantly different from controls at any time up to 48 h. We conclude that this relatively slow growing mouse tumour is capable of rapid morphological adaptation (<3 h) to changes in nutrient availability and that oxygen is probably the limiting substrate.

The relationship between the availability of nutrients and the survival of cells in malignant tumours is complex and poorly understood. In the extreme cases where the tumour is deprived of all nutrients by clamping (Denevamp et al., 1983) the death of cells can be demonstrated histologically or through a delay in the growth of the tumour. An experiment of this kind tells us nothing, however, about the relative importance of different nutrients.

Some tumours in animals and in man, particularly carcinomas, grow in a pattern which allows us to obtain information about the consequences of modification of the limiting substrates. Thomlinson and Gray (1955) were the first to remark on the 'corded' structure of many human and rodent tumours and put forward the hypothesis that these structures comprising viable tumour cells arose around blood vessels because of the limited range of diffusion of nutrients (mainly oxygen) through metabolic depletion. Theoretical calculation of oxygen diffusion distances under the conditions prevailing in tumours produced a value of 150–200 µm, which is close to the radius of the corded structures seen in some human carcinomas. Can we assume then that oxygen is the limiting substrate for the survival of tumour cells? An obvious way to test this is to reduce the oxygen delivered to a tumour without altering the delivery of other nutrients. In theory, this could be achieved by exposing the host to a low oxygen atmosphere or by changing the oxygen transport characteristics of the blood, but we know from studies in spheroids (Mueller-Klieser et al., 1983; Tannock & Kopelyn, 1986) and in a 'sandwich' tumour system (Hlatky et al., 1988) that other factors, particularly glucose levels, will influence the survival of tumour cells deprived of oxygen. An experiment with low oxygen exposure was carried out by Tannock (1970). In mice exposed to 10% oxygen for 48 h the tumours (mammary carcinomas) had cords which were narrower than those in controls. The time course of cord shrinkage was not studied in these experiments though it is reasonable to assume that it would not be an instantaneous process and must proceed at a rate determined amongst other factors by the metabolism of the cells and their tolerance of hypoxia.

One of the practical implications for radiotherapy of these effects is that a reduction in oxygen supply conditions would be expected to produce only a transient change in the number of radiobiologically hypoxic cells. We have previously speculated (Hirst & Wood, 1987) that the adaptation of radiosensitivity that occurs over time when tumour-bearing animals are made anaemic could be accounted for by the death of cells at the periphery of corded structures, or at least those most distant from the supplying vessels, leading to a reduction in cord radius and the re-establishing of a lower hypoxic fraction similar to that before anaemia was induced. We have studied the effects of breathing oxygen, at both higher and lower than normal tensions, and of anaemia on the radius of cords in a slow growing mouse carcinoma. Our results show that PO2 in the inspired gas has a marked effect on cord radius though acute anaemia does not.

Materials and methods

Animals and tumour system

RH carcinomas were grown in their syngeneic host, the WHT/GyfBSVS mouse. Males 10–16 weeks old were used for tumour implantation. They were housed in an SPF animal colony and allowed free access to food and water. The RH carcinoma arose spontaneously in a WHT mouse at the Gray Laboratory in 1963. It has been serially passaged in the WHT mouse ever since with a return to the original frozen stock once a year. Experimental tumours were produced by implanting about 2 x 106 cells as a suspension in saline into the dorsal skin of the recipient mice. Tumours grew to the size required for the experiments (500–800 mg) in 2–3 months.

Altered oxygen environment

Cages of mice were placed in translucent plastic bags which were flushed with either 100% oxygen or with 10% oxygen in nitrogen. The cages were then sealed except for an inlet and outlet port, permitting a flow rate of 21 min−1 to be maintained. Oxygen concentrations were checked at the beginning and end of exposure with a Thermox oxygen meter (Thermo Lab Inc., Pittsburgh, PA). After the required exposure the animals were killed within a few minutes and their tumours excised and immersed in 10% neutral buffered formalin. Conventional 4 µm sections were made from each tumour and stained with haematoxylin and eosin.
Induction of anaemia

The procedure for producing anaemia in the tumour-bearing mice was the same as previously described (Hirst et al., 1984). Plasma was obtained from donors of the same strain by bleeding under metofane anaesthesia from the suborbital sinus. About 1 ml of blood could be obtained from each animal, which was then killed before it recovered from the anaesthetic. The blood was then spun at 3,500 r.p.m. for 15 min to separate the plasma which was either used immediately or refrigerated for up to 48 h for later use. The haematocrit of tumour-bearing recipient mice were first measured in a 10 μl sample from the tail vein, then they were bled under anaesthesia from the suborbital sinus (~0.75 ml), their blood was also centrifuged and the plasma pooled with that from the donors. Within 10 min the recipients were transfused via a tail vein with 0.75 ml of warmed plasma and a haematocrit measurement again taken. The haematocrit of control mice was 48.9 ± 2.5% (mean ± 1 s.d.) and of transfused mice 27.1 ± 3.4%. The mice were killed after various durations of anaemia and their tumours excised and processed for histology as already described.

Measurement of cord radius

Tumours sections were scanned in a raster pattern at a magnification of 400 × until an interface between healthy and necrotic tissue was encountered. As can be seen from Figure 1 this interface is clearly delineated in the RH carcinoma. The distance from that point to the nearest blood vessel was measured with a concentric ring graticule in the eyepiece. This procedure is illustrated diagramatically by the overlaid arrows in Figure 1. An average of about 100 measurements of this distance, termed the cord radius, were made in each tumour section. The mean radius was calculated and the data from between 10 and 23 tumours was combined to give an overall value (mean ± 1 s.d.) for each treatment condition. The data were also analysed as histograms in which case all the individual measurements of cord radius for each treatment (1,500–3,000 per treatment) were pooled.

Results

The RH carcinoma shows corded structures of viable cells surrounding blood vessels with clearly defined boundaries between morphologically intact cells and necrosis. An example of this in a control tumour is shown in Figure 1. The mean radii after exposure to higher or lower than normal oxygen concentrations for up to 68 h are shown in Figure 2. The mean radius in control animals was 105 ± 2 μm (n = 23). Breathing 10% oxygen did not change cord radius for at least 6 h, but by 24 h the radius was significantly lower (P < 0.005) at 96 ± 2 μm (n = 15) and reached a minimum value of 93 ± 3 μm (n = 10) after 48 h of exposure. The opposite effect was observed in the tumours of animals breathing 100% O2. Cord radius increased significantly (P < 0.05) by <3 h; the large error on the 7 h value resulted from a single tumour (out of nine) with a very low cord radius (89 μm). Cord radius reached a maximum value of 117 ± 3 μm (n = 10) by 24 h and there was no further increase by 48 h (114 ± 2 μm; n = 10). The induction of anaemia by removing blood (0.75 ml) from the suborbital sinus and replacing it i.v. with an equal volume of mouse plasma had no significant effect on cord radius at any time up to 48 h (Figure 4).

The interanimal variation within groups receiving the same treatment, as represented by the standard errors in Figures 2 and 4 was quite small, but within each tumour there was considerable heterogeneity of cord radii. This is represented by the frequency distributions shown in Figure 3a–c. The distribution of values in control or anaemic animals was not significantly different from normal (data not shown). Radii as high as 200 μm and as low as 10 μm were seen. The distributions in high and low oxygen groups were, however, significantly different from normal being skewed to lower (10% O2) and higher (100% O2) values. The extremes of cord radius were not different in any of the groups.

Discussion

The mean cord radius measured in control tumours in the present study (105 ± 2 μm) is not significantly different from the value of 97 ± 4 μm reported for the same tumour 10 years ago (Hirst et al., 1982). The small discrepancy can easily be accounted for by differences in the routine used to scan the sections, which was random in the present study but selective in the previous one. The random technique has obvious advantages in that it does not require the operator to select the cords to be counted, and it does include distances from areas where no corded structure is visible, such as where a small area of necrosis is surrounded by viable tumour cells. Areas of this kind yield larger oxygen diffusion
Figure 3 Frequency distributions for individual cord radius measurements from the same animals as those used in Figure 2.

Figure 4 The radius of viable cords around blood vessels (mean ± 1 s.d.) at different times after induction of acute anaemia. Radii in control animals breathing air are shown by the hatched area.

distances than for a cord of tumour within a large area of necrosis (Trott, 1983) so we would expect higher values to be obtained in the present study. It is, however, remarkable that the radii have remained so stable. This suggests that the cellular characteristics that determine survival at a distance from blood vessels, such as metabolic rate, glycolytic capacity and hypoxia tolerance are fundamental properties of a given cell line that are not subject to change under the selection pressures found in the tumour environment.

Before discussing further the implications of our observations we should consider the limitations of the method we have used. The radii measured must always represent the maximum possible distance between blood vessel and necrosis because there could always be another vessel or a con-

...
This suggests that it is areas of relatively good perfusion that are most vulnerable to Po2 reduction while cords in all size ranges appear to benefit from an increase in oxygenation. There is also a suggestion (Figure 3, top panel) that it may be possible to reach a maximum radius (in this tumour about 200 μm) beyond which cords will not grow even if more O2 is made available. This could represent the point at which the diffusion of other substances such as glucose become limiting for cell survival.

The impact of raised or lowered arterial Po2 on oxygen diffusion distances has also been modelled by Groebe and Vaupel (1988). The numbers they derive rely on the exact values chosen for oxygen consumption, Hb/O2 binding affinity, intercapillary distance and capillary length. Nevertheless, the diffusion radii they obtain for the normal situation (mean of arterial and venous end of vessel = 62 μm) or at an arterial Po2 of 50 mmHg which will be close to that found in mice breathing 10% O2 (mean = 49 μm) are considerably lower than those for cord radius in our study. We should be aware, however, that the point at which O2 is reduced to less than 1 mmHg (the cut off chosen by Groebe & Vaupel, 1988) and the point at which tumour cells die could be quite different even if oxygen is the critical nutrient. The discrepancy arises because cells will continue to survive in hypoxia for some time. Clamping experiments have shown that 50% of the cells in a mouse sarcoma are dead after 8 h of total nutrient deprivation (Denekamp et al., 1983) and preliminary data for the CaRH indicate that this survival time lies between 6 and 16 h (Hill, personal communication). It seems reasonable to assume that this survival time in an uncultured tumour will not be less than under this most extreme form of nutrient deprivation so, for the purpose of this discussion, we will assume that the CaRH cells remain morphologically intact for at least 8 h after they pass the O2 diffusion limit, during which time they will have migrated, based on our previous labelling studies with the RH carcinoma (Hirst et al., 1982), by about 16 μm. Thus we would always expect that cord radius will be determined by diffusion distance by a considerable margin, creating the population of ‘chronically’ hypoxic cells originally proposed by Tholinson and Gray (1955).

Our data clearly demonstrate that tumour cord radius is an indicator of oxygen availability within the tumour and may have some prognostic value, particularly if other characteristics such as hypoxia tolerance and O2 consumption rates can be established in vitro in cells from the same tumour. There have been the O2 attempts to correlate the morphological parameters of presumed significance to tissue oxygenation, with the outcome of radiotherapy of human tumours (Awwad et al., 1986; Lauk et al., 1989; Siracka et al., 1982). The conclusions from these studies were apparently inconsistent, one (Lauk et al., 1989) actually showing a highly significant direct correlation between the mean distance of tumour cells from the nearest blood vessel and local control by radiotherapy of oral squamous cell carcinomas. A large mean distance from tumour cells to blood vessel must mean either that the vessel was supplying a lot of oxygen (i.e. high blood flow), O2 consumption by the tumour cells was low or that the tumour cells have a high tolerance from hypoxia. This last possibility would create a high hypoxic fraction though the first two would tend to decrease it. Also for purely geometric reasons the hypoxic rim of cells around a large cord is smaller as a proportion of the total volume than that surrounding a small one. There are clearly difficulties in using purely morphologic parameters as prognostic indicators, but if we focus only on relative differences within the same tumour before and after a particular intervention, information useful to therapy could be obtained.

We have chosen in this analysis to focus on oxygen as the substrate whose manipulation has important consequences for tumour cell survival and diffusion distances. This is probably an oversimplification of the complexity of tumour metabolism in view of the fact that we did not measure glucose levels in the tumour or even in the blood. It has been shown, however, in a tumour spheroid model in vitro that the thickness of the viable rim of tumour cells surrounding necrosis is only sensitive to glucose levels when they fall to less than half the normal blood level of 60–130 mg 100 ml−1 (Tannock & Kopelyan, 1986). We conclude, therefore, that the changes in morphology reported in the present study can be attributed predominantly to the effects of oxygen, though experiments are in progress to determine the importance of glucose.

Our data support the view that cord shrinkage is a major component of the adaptation (Hirst, 1986) proposed to account for changes in radiosensitivity (Siemann et al., 1979) after reduced or elevated oxygen availability. They do not, however, support this as a mechanism for adaptation to reduce haematoctrit as previously suggested (Hirst, 1986; Hirst & Wood, 1987). We do not yet have data showing this radiobiological adaptation in the RH carcinoma used in the present study, though it is perhaps surprising that low haematocrit (<30%) blood can support the same cord radius as normal blood. It suggests that the reduced viscosity, and improved tissue perfusion, compensates for the reduced oxygen carrying capacity (Sevick & Jain, 1989). We have no evidence for this in the RH carcinoma but our studies of the NT carcinoma show a reduction in relative perfusion after anaemia compared with the expected increase seen in normal tissues (Sensky & Hirst, unpublished).

Finally, our data (Figure 2) show clearly that improved oxygenation very quickly (<3 h) leads to a small but significant (P<0.05) increase in cord radius, presumably through cell growth or proliferation. This rapid response is perhaps surprising in view of the relatively long potential doubling time (58 h) measured in this carcinoma (Hirst et al., 1982) and suggests that cell growth rather than simply proliferation may be involved in cord expansion. Whatever the mechanism, this observation emphasises that if methods are to be used to improve tumour oxygenation during radiotherapy they should be applied as briefly as possible.

This work is supported entirely by the Cancer Research Campaign. We wish to thank Peter Russell and his staff for production and care of the mice used in these experiments.

References

AWWAD, H.K., EL NAGGAR, M., MOCKTAR, N. & BARSOUM, M. (1986). Intercapillary distance measurement as an indicator of hypoxia in carcinoma of the cervix uteri. Int. J. Radiat. Oncol. Biol. Phys., 12, 1329.

DENEKAMP, J., HILL, S.A. & HOBSON, B. (1983). Vascular occlusion and tumour cell death. Eur. J. Cancer Clin. Oncol., 19, 271.

GROEBE, K. & VAUPEL, P. (1988). Evaluation of oxygen diffusion distances in human breast cancer xenografts using tumor-specific in vivo data: role of various mechanisms in the development of hypoxia. Int. J. Radiat. Oncol. Biol. Phys., 15, 1425.

HIRST, D.G. (1986). Anemia: a problem or an opportunity in radiotherapy? Int. J. Radiat. Oncol. Biol. Phys., 12, 2009.

HIRST, D.G., HAZELHURST, J.L. & BROWN, J.M. (1984). The effect of alterations in haematocrit on tumour sensitivity to X-rays. Int. J. Radiat. Biol., 46, 345.
MUELLER-KLIESER, W., FREYER, J.P. & SUTHERLAND, R.M. (1983). Evidence for a major role of glucose in controlling development of necrosis in EMT6/Ro multicell tumor spheroids. In Oxygen Transport to Tissue, Bicher, H. & Bruley, D.F. (eds), p. 487. Plenum Publishing Corp: New York.

SEVICK, E.M. & JAIN, R.K. (1989). Viscous resistance to blood flow in solid tumours: effect of hematocrit on intratumor blood viscosity. Cancer Res., 49, 3513.

SIEMANN, D.W., HILL, R.P., BUSH, R.S. & CHHABRA, P. (1979). The in vivo radiation response of an experimental tumour: the effect of exposing tumour-bearing mice to a reduced oxygen environment prior to but not during irradiation. Int. J. Radiat. Oncol. Biol. Phys., 5, 61.

SIRACKA, E., SIRACKY, J., POPPOVA, N. & REVESZ, L. (1982). Vascularization and radiocurability in cancer of the uterine cervix. A retrospective study. Neoplasma, 29, 183.

TANNOCK, I.F. (1970). Effects of pO₂ on cell proliferation kinetics. In Time and Dose Relationships in Radiation Biology as Applied to Therapy, Bond, V.P., Suit, H.D. & Marcial, V. (eds), p. 215. Brookhaven, National Laboratory: Upton.

TANNOCK, I.F. & KOPELYAN, I. (1986). Influence of glucose concentration on growth and formation of necrosis in spheroids derived from a human bladder cancer cell line. Cancer Res., 46, 3105.

THOMLINSON, R.H. & GRAY, I.H. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. Br. J. Cancer, 9, 539.

TROTT, K.-R. (1983). Die Bedeutung de Besonderheiten der Tumor-mikrozirkulation fuer die Strahlentherapie. Mikrozirk. Forsch. Klin., 2, 114.