Hypoxia and tumours

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Cells in tumours become hypoxic in either or both of two ways. First, the form of tumour growth may result in some cells lying so far from the capillaries that constitute their immediate source of oxygen that it is almost used up by the metabolism of the intervening cells. Secondly, the vascular system of the growing tumours may fail to deliver enough oxygen both because of inadequacies in the vessels themselves and in the quantity of blood that flows through them. The presence of hypoxic cells in tumours is of importance because it may affect the outcome of treatment by radiotherapy. Some two and a half to three times the dose of *x* radiation is needed to destroy the ability of cells to proliferate in the absence of oxygen as in its abundance (Alper and Howard Flanders, 1956). These three topics will be discussed, though a fourth, not pursued, should be associated with them: this is that the vascular system of a tumour is the pathway by which cytotoxic drugs used in therapy reach their site of action, and a degree of failure in transport may diminish their effectiveness in treatment.

Hypoxia and the structure of tumours

Tumours grow possibly from a single cell, probably from not more than a few. An increase in cell number must mean a rise in the demand for oxygen. Where a population of tumour cells is not penetrated by capillary blood vessels, there is a limit to the size to which it can grow that is partly determined by the diffusion of metabolites. Simple examples of this can be seen in cross sections of spheres of tumour cells grown *in vitro* (Sutherland *et al*, 1971). The centres of these spheres become necrotic because the cells in them become too far distant from the surrounding medium for metabolites to diffuse to and from across the outer rim of optically intact cells in adequate quantities to maintain an environment in which life can continue. Whilst we do not know exactly which substances impose the limit of thickness to the viable rim, of the common metabolites—oxygen, free fatty acids, carbon dioxide and glucose—oxygen has by far the shortest pathway through metabolizing tissue before it reaches concentrations incompatible with normal cellular activity (Evans,

1967). It is thus possible that cells do live in the innermost layers of such a viable zone under conditions that are hypoxic.

Many examples of this pattern of growth can be found amongst tumours *in vivo*. One example (fig 1) is a sarcoma growing subcutaneously in a mouse and surrounded by connective tissue but not penetrated by capillaries. This is similar in structure to tumours in which the cell population kinetics have been studied as they grow in the anterior chamber of the rabbit's eye (Gimbrone *et al*, 1972). Here it was found that cell proliferation at the periphery was rapid, but that the daughter cells migrated, or were forced, toward the centre where they died. The component parts of the cells were then observed to

Fig 1 An avascular sarcoma in subcutaneous tissue in the mouse (by courtesy of Dr H. B. Hewitt). The overall diameter is 800 μm , the width of the outer zone of intact cells 140 μm .

FIG 2

FIG 3

Figs 2 and 3 *Developing basal cell carcinoma of the human scalp (by courtesy of Dr I. Whimster).*

Fig 2 *The tumour is 200 μm diameter and contains about 2500 cells.*

Fig 3 *The tumour is about 500 μm in diameter. On the subcutaneous aspect of the necrotic area there is a layer of intact cells.*

be extruded. In this way a dynamic equilibrium was established so that, in the absence of a penetrating vascular system, tumours might remain the same size for many weeks and yet be capable of infiltrative growth if transplanted to a favourable site. It is important to remember that the histological picture is, as it were, a still photograph taken from a cinematographic film.

However, it is perhaps possible to gain some understanding of the story of the cine-film from a series of 'stills'. The examination of a series of basal cell carcinomas taken from within a few millimetres of each other in the same human scalp has shown how the structure of central necrosis in a proliferating mass may come about. Smaller tumours contained a range from about 500 to 2500 cells (fig 2) and form a series in which there must be an increasing demand for oxygen that can only reach the central cells in each case by diffusing through the layers of other cells that lie between them and the capillaries and are using it. The central necrosis in the larger tumours

(fig 3) is probably a consequence of the increasing number of these layers.

This pattern is commonly found in both primary and metastatic tumours derived from cells which characteristically adhere rather to one another, notably non-keratinizing carcinomas of squamous epithelium in skin, cervix uteri and bronchus. Cells of these tumours tend to grow in cords, the appearance in histological section depending on the angle that the plane of the section has cut the cord. Where the cross section is most nearly circular it can be seen that in situations where there are no other patterns of necrosis the width of the zone of optically intact cells is independent of the radius of the whole cord and does not exceed approximately 180 μm (fig 4). An explanation of this appearance was offered in terms of diffusion by L. H. Gray (Thomlinson and Gray, 1955). Anoxic areas might be expected to appear in the centre of cords of metabolizing cells when their radii exceeded a critical size dependent on the metabolic rate of the cells, the oxygen tension around

Fig 4 Squamous cell carcinoma of bronchus. The tumour cells grow as cords here seen in cross-section. In small cords there is no necrosis, while larger cords have a necrotic centre and a rim of optically intact cells approximately 170 μm in width.

the cords and the coefficient of diffusion of oxygen in the tissue. Making some simple assumptions for these values, a close similarity was found between the predicted distribution and size of anoxic centres in

Fig 5 Calculations and measurements of cross sections of cords of tumour cells. R = radius of whole cord; r = radius of calculated anoxic centre or measured necrotic centre; $R - r$ = calculated width of viable tumour or measured width of optically intact cells (based on Thomlinson and Gray 1955); reproduced by courtesy of the Editor, Modern Trends in Radiotherapy).

cords of different radius and the measured pattern of necrosis (fig 5). From these observations it seemed likely that there would be cells that were hypoxic in the more central layers, but, of course, no conclusions about their capacity to proliferate could be drawn. It also seems likely that the normal circulation cannot maintain a thicker zone of this type of cell than was measured here. However, in many tumours thinner zones probably result from deficiencies in the circulation.

Hypoxia and the blood supply of tumours

Whatever the mechanism of angiogenesis in tumours (see Folkman and Cotran, 1976), there can be little doubt that a new capillary bed forms in many instances. This can be profitably studied in the preparations where tumour cells grow between windows inserted into the skin on the back of the rat (Reinhold, 1969) (fig 6). In relation to developing

Fig 6 The developing capillary network of a tumour implanted between transparent windows in the skin of the back of the rat. The main artery and vein from which the vascular bed of the tumour grows cross the photograph (by courtesy of Dr H. S. Reinhold).

necrotic areas in these 'tumours', a sequence of changes appears to take place in the nearby capillaries (personal observations by courtesy of Dr H. S. Reinhold). The first apparent change is dilatation of individual capillary loops. Since the red cells in these vessels can be observed and the passage of a white cell resolved, it is possible to draw some conclusions about the flow of blood. At first the volume passing through the vessel increases though its linear rate of flow is diminished. Over the next hour or two the flow of blood undergoes a variety of changes: sometimes it slows to a standstill and then accelerates again; occasionally it is suddenly reversed; for short periods the cells may oscillate within the vessel and then the flow is re-established. However, the net effect over this period is a slowing of flow leading eventually to stagnation. After this all the blood may leave the vessel so that it collapses and becomes invisible. In the case of one vessel after this had happened, occasional red cells passed along its course for a period exceeding an hour. Then, not only did the flow start again, but it was possible to demonstrate by the fluorescence of enzymes (Gosalvez *et al*, 1972) that nearby cells on the side of the capillary toward the necrosis were able to receive and to use oxygen. However, the flow in this vessel ceased soon afterwards.

The overall impression gained by a number of observers of the circulation in these preparations is one of a random network of capillaries of varied bore in which the flow is at the mercy of local pressure, in general the longest pathways transporting the least amounts of blood. The dilatation may be imagined to be some compensatory response to this situation.

These observations are consistent with the histological pattern commonly seen in primary and metastatic tumours in humans and rodents. An

example may be taken from a common situation: metastases in the human liver. As these tumours enlarge, if they do not abut more rigid structures, necrosis develops in their centres but the first change visible to the naked eye is a central pink flush due to capillary dilatation. With further enlargement of the tumour this expands to form a ring (fig 7). On a histological section the ring may be seen as a zone of dilated capillaries just within the central area of necrosis. Within this zone all tissue is dead and the blood vessels are empty. It may therefore be concluded that blood in the dilated capillaries has not undergone coagulation. It has been squeezed out, as in an arterial infarct, by the rise of hydrostatic pressure consequent upon an increase of osmotic pressure that results from autolysis of the dead tissue. If this be so, it is likely that the necrosis in these conditions is secondary to the slowing of blood flow through the longest capillary pathways, which, in this rather symmetrical situation, lie furthest from the periphery of the tumour. It is also probable that as the blood flow slows some tumour cells become hypoxic for a period before they die.

That this is not an uncommon sequence of events in human tumours is suggested by a typical histological appearance seen in two adjacent areas of carcinoma of the bronchus (figs 8, 9). In one, a cross-section of a cord of stroma bearing capillary blood vessels is surrounded by a cuff of six to eight layers of intact tumour cells. This, in the section, lies as it were an island in a sea of necrosis. In the other, the pattern is similar, but the intact cells form a cuff only one, or in places, two layers thick. Yet these two cords were within a few millimetres of one another. The probable explanation is a slowing of the blood flow through the second.

The changes in blood vessels, and consequently of circulation, that accompany the growth of tumours are not, however, confined to the capillaries. As many tumours get larger they displace the surrounding structures. This they can only do by generating within themselves pressures that overcome the resistance of these tissues. Such pressures, up to 80 mm of water (unpublished observations), may distort the low-pressure vascular systems—the veins and lymphatics. Studies of tumours in the rat (Falk, 1977) demonstrate the drawing out and narrowing of circumferential veins and kinking, with possible obstruction to the flow of blood, in the tissue immediately beyond. Such changes must alter the intracapillary hydrostatic pressure or the arterio-venous pressure gradient on which the flow of blood through the capillary bed depends.

Nor are the arteries unaffected. The bore of even large arteries going to an organ bearing a tumour may be increased as an accompaniment of its growth

Fig 7 Cut surface of a metastasis in the liver 14.3 mm diameter. Dilated capillaries form a ring at the periphery of the necrotic central region.

Fig 8 Carcinoma of human bronchus. A cord of stroma containing blood vessels, surrounded by a cuff of optically intact cells beyond which is necrosis.

Fig 9 Carcinoma of human bronchus. A structure similar to that in fig 8 and lying 5.8 mm from it in the same tumour. The difference in thickness of the cuff of viable cells indicates that the circulation through the capillaries is impaired.

Fig 10 Retrograde arteriogram of human aorta and renal arteries. The renal vessels on the right side are normal. On the left, there is a carcinoma of the lower pole of the kidney. The main renal artery on the left is nine times the bore of that on the right (by courtesy of Professor R. Steiner).

This is difficult to measure except in relation to a paired organ, but the left renal artery in an example of a carcinoma of the kidney (fig 10) has a diameter three times that of the right and may consequently have been carrying nine times the flow of blood. How this should have come about does not appear to be known, but, if it be a response to tumour growth, a tumour dependent on an artery which, because of age or disease, cannot hypertrophy quickly enough may well become hypoxic for this reason alone.

However, none of these investigations of patterns of growth of tumours or of their vascular system and the flow of blood through it can give information about hypoxia affecting cells in a significant manner. If tumour cells were to die of malnutrition rather than from therapy, they would be of no more consequence to the patient. If, in becoming hypoxic, they were to become resistant to therapy, this would be another matter and one that could only be investigated by measuring their response to treatment.

Hypoxia and the response of tumours to irradiation

Ionizing radiations produce their effects on tissues by damaging the ability of cells to reproduce. The laws relating dose and effect are well understood (Hall, 1973) and will not be discussed here. The overall effect of the irradiation of tumours is to delay their growth. The more effective the treatment, the greater is the delay of growth, in terms of the growth rate of the particular tumour, until it becomes infinite with the cure of the patient. Of course, radiotherapy is a

local form of treatment and does only produce these effects within the volume that is treated.

In order to illustrate the principles that determine the effects of hypoxic cells on the response of tumours to irradiation, some results of experiments with a transplantable sarcoma in the rat will be described. All tumours were irradiated at the same size, when growing in the same site, in a pure inbred strain of rat. From measurements of diameter it was possible to show that increasing the size of single doses of x rays produced a progressive delay in the growth of the tumours until cure was achieved (fig 11). These irradiations were carried out under barbiturate sedation and could be performed under various conditions of oxygenation: an 'anoxic condition' in which the arteries and veins supplying the tumour were occluded by clamping; an 'air-breathing condition' in which the circulation was free and the animal was breathing air; and other conditions in which the sole change was the replacement of air by gases with different concentrations of oxygen ranging from 10 per cent oxygen in nitrogen to pure oxygen at 45 psi pressure.

Closely similar effects were produced by irradiation under air-breathing and anoxic conditions by doubling the radiation dose for the latter (fig 11). An increase in the delay of growth was produced by the same dose of radiation by changing the conditions from anoxia to air breathing or hyperbaric oxygen (fig 12). This experiment demonstrates that in the air-breathing condition there are cells in this tumour that are protected from radiation injury by lack of oxygen and that they are able to survive and proliferate in the environment that follows treatment.

A more detailed examination of the effects of hypoxic cells may be carried out by measuring the delay imposed by treatment in terms of the time taken for tumours to grow from the standard diameter of 9 mm, at which they are treated, to an arbitrarily chosen diameter of 25 mm, which for the untreated tumours takes six days. The delay imposed by doses of radiation given under particular conditions of oxygenation may then be related graphically to the dose used (fig 13). When the radiation is given in anoxic conditions a smooth curve results with a lengthening delay as the dose is increased. The points depending on doses up to about 1500 rads given in oxygenated conditions can be fitted by a curve based on the anoxic curve, but with the anoxic doses divided by a constant factor of 3.4. This is the 'oxygen enhancement ratio' for the tumour system and it implies that 3.4 times the dose of x rays given in the absence of oxygen is needed to produce the same effect in its presence.

When doses above 1500 rads are given in air-breathing conditions, the points obtained for the

Fig 11 The response of the transplantable sarcoma RIB₅ in the rat to single doses of x rays. All tumours were treated on day 0 when growing in the same site and at the same size. Curve A, untreated control. Curves B to H, the response to radiation given with air-breathing conditions of doses of 0.5, 1, 2, 3, 4, 5, and 6 thousand rads respectively. Curve K, the response of tumours in animals that were cured. Curve X, 4000 rads given to anoxic tumours for comparison with curve D, 2000 rads given in 'air-breathing' conditions.

Fig 12 Curve A shows the response of the tumours irradiated with 2000 rads under anoxic conditions. This response is enhanced when the same dose is given with 'air breathing' (curve B) and to a greater degree with hyperbaric oxygen at 45 psi (curve C).

delay follow a curve of the same shape as the anoxic curve, but displaced upward on the ordinate by an amount corresponding to an additional 12.3 days. Thus the air-breathing curve seen as a whole has a biphasic character, the lower part expressing the effect of radiation on the well oxygenated component

Fig 13 Curves relating the time taken for tumours to grow from 9.0 mm (the standard size for irradiation) to 25 mm (an arbitrary size chosen as an endpoint), to the dose given. Doses have been given under different conditions of oxygenation. Curve A is the response of anoxic tumours. Curve O is derived from curve A by dividing doses by a constant chosen to give the best fit to experimental points obtained after irradiation in oxygenated conditions (see Thomlinson and Craddock, 1967, for details). This constant is 3.4 and represents the 'oxygen enhancement ratio' of the system. Curve C was obtained after irradiation under air-breathing conditions. It is biphasic, the lower dose portion following the 'oxic' curve and the larger dose part following an 'anoxic curve' displaced upward from curve A by a constant equivalent to 12.3 days additional delay in growth. Curves B, D, and E are similarly biphasic and were obtained under conditions of breathing 10% oxygen in nitrogen, 100% oxygen at atmospheric pressure and 100% oxygen at a pressure of four atmospheres absolute respectively. There is an 'anoxic' phase of each curve displaced from the 'wholly anoxic' curve by 6.4, 16.1, and 19.1 days respectively. These values of delay in regrowth, additional to the delay imposed by doses given under anoxic conditions, are inversely related to the proportion of hypoxic cells present at the time of irradiation.

of the population and the upper part that on the anoxic component. The displacement of the upper part from the anoxic curve is related to the proportion of hypoxic cells in the whole clonogenic cell population of the tumour.

The extent of this displacement can be altered at will by causing the animals to breathe different concentrations of oxygen. With 10 per cent oxygen in

nitrogen it is lowered to 7.4 days, indicating a higher proportion of hypoxic cells, and with pure oxygen at one and four atmospheres' pressure, it is increased to 16.1 and 19.1 days respectively, indicating a lower proportion of hypoxic cells (Thomlinson and Craddock, 1967).

The result of such irradiation is the killing of clonogenic cells in the tumour. This inhibits growth and may lead to regression. Such changes alter the state of oxygenation of a tumour because they tend to put in reverse the processes that lead to hypoxia as tumours grow. The first effect is that cells die, so that the consumption of oxygen falls leaving more available for the remainder. Secondly, any pressure that may have been exerted on veins and lymphatics is relieved when the tumour begins to shrink. Thirdly, as the process of shrinkage continues the capacity of the capillary bed in relation to the arteries that fill it is reduced, the longest pathways taken by blood from artery through capillary to vein are shortened, and the distribution of the flow of blood within the tumour is evened out. The occurrence of these changes is reflected in the response of tumours to irradiation.

Just as a change in the proportion of hypoxic cells induced by altering the respired gas is shown by the displacement in the upper component of the curves relating to dose (fig 13), so a similar displacement that occurs with air breathing, when a second dose of radiation is given after a varied interval, reflects the change in the proportion of hypoxic cells that has occurred between doses.

A dose of radiation of any size can be regarded as being given in two fractions with a zero time interval between them. A single dose of 1500 rads with air breathing eliminates the effects of the oxygenated cells in these tumours. Therefore the second part of any single dose greater than 1500 rads is given in these conditions to a practically wholly hypoxic population. The same result can be obtained by giving one dose of 1500 rads under air-breathing conditions and a second after some time has elapsed under anoxic conditions. However, if the second dose is also given under air-breathing conditions the resultant curve is displaced from that obtained in the anoxic conditions by an amount that depends on the length of the interval between doses. This displacement with time is plotted in figure 14. Because the displacement is inversely related to the proportion of hypoxic cells present at the time of the second dose, a curve indicating the changes in this proportion that occur with time after a single dose of 1500 rads can be calculated (fig 15).

A rise and fall in the proportion of hypoxic cells surviving a dose of radiation given to a tumour had been predicted (Thomlinson, 1967), but the second

Fig 14 The response to doses greater than 1500 rads given with air breathing is determined by the hypoxic cells (fig 13, curve C). If 1500 rads are given as a first dose and additional doses given as a second dose after a variable lapse of time, the second doses give a biphasic curve with an 'anoxic' phase further displaced from the wholly anoxic curve. This displacement is expressed as 'additional days delay' above the single dose response and is related to the time interval between doses. The fluctuations are inversely related to the proportion of hypoxic cells present at the time of, and therefore protected from, the second dose.

rise that occurs in this particular case was, when first observed, somewhat of a surprise. Whilst no explanation is yet fully supported by experimental evidence, the one that is most likely does throw some light on the events that probably take place in most tumours and carries implications of possible practical value. The initial rise that accompanies the first dose can be accounted for by the selective killing of the more sensitive oxygenated population, leaving amongst the surviving cells a higher proportion of the less sensitive hypoxic population. The first fall in the proportion of hypoxic cells is due to an excess of cell deaths over cell birth and is accompanied by a fall in the oxygen consumption of the tumour as a whole (N. T. S. Evans, personal communication). This effect would tend to make more oxygen available to the remaining cells were the supply to remain constant. However, the second rise in proportion of hypoxic cells accompanies an enlargement of the tumour that invariably occurs even when curative doses are given. Both the enlargement of the tumour and the rise in the proportion of hypoxic cells reach a maximum at 48 hours and the one probably causes the other by compressing the capillary bed in much

Fig. 15 A curve relating the proportion of hypoxic clonogenic cells in a tumour calculated to be present at different times after a dose of 1500 rads of x irradiation. The calculations are based on the response to second doses of radiation given under different conditions of oxygenation at each time. The curve shows the initial rise due to the selective killing of more sensitive well oxygenated cells; a first fall due to diminished demand following cell death; a second rise caused by swelling of the tumour; a second fall accompanying shrinkage of the tumour, and a final rise caused by regrowth.

the same way as the swelling of an arterial infarct can squeeze all the blood out of the capillaries within it and cause further death in the surrounding tissue in which an inflammatory reaction had been developing.

The second period of fall in the proportion of hypoxic cells accompanies regression of the tumour and is brought to an end when the tumour begins to grow again. It is probably caused by a greater supply of oxygen resulting from improvements in the circulation. This fall ends when the demand for oxygen increases because of the proliferation of the surviving tumour cells that leads to the renewed growth.

Thus the pattern of what is called 're-oxygenation' in tumours involves many factors and is extremely complicated. Were the sequence of events that occur known in individual type of tumour it would be possible to prescribe for them the optimal time intervals at which fractionated doses of radiation should be given. Recent measurements of changes in volume in carcinoma of the human breast have shown great variability of early response, but, on average, during schemes of prolonged fractionation, 40 per cent of

the total dose of radiation, calculated as refts, has been given before tumours have shrunk to less than their size at the start of treatment (Thomlinson and Errington, 1977). This has led to the use of cytotoxic agents in small quantities in order to turn an expanding mass into a shrinking mass so that the whole dose of radiation can be given when re-oxygenation is occurring. This is but one of several methods that are being used to try to overcome the effects of hypoxic cells in tumours. However, until the factors that cause cells to be hypoxic in individual types of tumour and the timing of the processes of re-oxygenation are understood, hypoxia in tumours will continue to be a topic of concern to all engaged in the therapy of cancer.

References

- Alper, T., and Howard-Flanders, P. (1956). The role of oxygen in modifying the radiosensitivity of *E. Boli*. *Nature*, **178**, 978-979.
- Evans, N. T. S. (1967). Personal communication. Quoted in oxygen therapy—biological considerations. In *Modern Trends in Radiotherapy*, edited by T. J. Deeley and C. A. P. Wood, p. 63. Butterworths, London.
- Falk, P. (1977). In preparation.
- Folkman, J., and Cotran, R. (1976). Relation of vascular proliferation to tumour growth. In *International Review of Experimental Pathology*, **16**, 207-248.
- Gimbrone, M. A., Jr., Leapman, S. B., Cotran, R. S., and Folkman, J. (1972). Tumor dormancy *in vivo* by prevention of neovascularization. *Journal of Experimental Medicine*, **136**, 261-276.
- Gosalvez, M., Thurman, R. G., Chance, B., and Reinhold, H. S. (1972). Indication of hypoxic areas in tumours from *in vivo* NADH fluorescence. *European Journal of Cancer*, **8**, 267-269.
- Hall, E. J. (1973). *Radiobiology for the Radiologist*. Harper and Row, Hagerstown Maryland and New York.
- Reinhold, H. S. (1969). The response of tumour blood supply to fractionated irradiation. In *Conference on Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, Carmel, California, pp. 210-213. (BNL 50203 (C-57) Brookhaven National Laboratory, Upton, New York.
- Sutherland, R. M., McCredie, J. A., and Inch, W. R. (1971). Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *Journal of the National Cancer Institute*, **46**, 113-117.
- Thomlinson, R. H. (1967). Oxygen therapy—biological considerations. In *Modern Trends in Radiotherapy*, edited by T. J. Deeley and C. A. P. Wood, pp. 52-72. Butterworths, London.
- Thomlinson, R. H., and Craddock, E. A. (1967). The gross response of an experimental tumour to single doses of X-rays. *British Journal of Cancer*, **21**, 108-123.
- Thomlinson, R. H., and Errington, L. M. (1977). In preparation.
- Thomlinson, R. H., and Gray, L. H. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. *British Journal of Cancer*, **9**, 539-549.