Inducible Repair and the Two Forms of Tumour Hypoxia—Time for a Paradigm Shift

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Acta Oncologica Vol. 38, No. 7, pp. 903–918, 1999

Clinical experience shows that there is a therapeutic window between 60 and 70 Gy where many tumours are eradicated, but the function of the adjacent normal tissues is preserved. This implies much more cell kill in the tumour than is acceptable in the normal tissue. An SF_2 of 0.5 or lower is needed to account for the eradication of all tumour cells, while an SF_2 of 0.8 or higher is needed to explain why these doses are tolerated by normal tissues. No such systematic difference is known between the intrinsic sensitivity of well-oxygenated normal and tumour cells. The presence of radioresistant hypoxic cells in tumours makes it even more difficult to understand the clinical success. However, there is experimental evidence that starved cells lose their repair competence as a result of the depletion of cellular energy charge. MRS studies have shown that low ATP levels are a characteristic feature of solid tumours in rodents and man. In this paper we incorporate the concept of repair incompetence in starving, chronically hypoxic cells. The increased sensitivity of such cells has been derived from an analysis of mammalian cell lines showing inducible repair. It is proportional to the SF_2 and highest in resistant cells. The distinction between acutely hypoxic radioresistant cells and chronically hypoxic radiosensitive cells provides the key to the realistic modelling of successful radiotherapy. It also opens new conceptual approaches to radiotherapy. We conclude that it is essential to distinguish between these two kinds of hypoxic cells in predictive assays and models.

Received 10 March 1999
Accepted 14 July 1999

It is now over a century since the first cancer patient was treated with ionizing radiation. During this time radiation oncology has been developed into a very effective branch of medicine. It permits the eradication of many malignant tumours without disfiguring the patient and with the preservation of adequate function of the adjacent organs. There is an accompanying body of radiobiological data that shows the principles of radiation-induced cell kill (clonogenic sterilization) and the multiple factors that influence the response between successive treatments in a fractionated schedule. The clinical outcome is often illustrated in radiobiology textbooks in the manner shown in Fig. 1. Steep dose-response curves are observed clinically for both tumour and normal tissue responses. The tumour control probability curve (TCP) is shown as being displaced to the left of the normal tissue complication curve (NTCP), in accordance with clinical experience for most solid tumours. The dose prescribed is usually intended to cause a small percentage (e.g. 5%) of patients to have moderately severe side effects, which will develop within 5 years. This is called the tolerance dose or $TD_{5/5}$. In this way the clinician can ensure that the patient is given the maximum tolerable dose. Unless there are some side effects against which to monitor the treatment efficacy, it is impossible to know whether the maximum dose has been given and thus the tumour might be underdosed. This diagram, in one form or another, is included in most radiobiology textbooks and forms the basis of modelling studies aimed at optimizing radiotherapy by modified fractionation, by the use of chemical modifiers, by alternative types of radiation, or by tighter conformation of the high-dose volume.

The aim of any novel approach is to increase the tumour control rate without a corresponding increase in morbidity, i.e. to increase the displacement between the two curves shown in Fig. 1 and thereby enlarge the therapeutic window. With conventional treatments, in many tumour sites, 50% or more local control rates can already be achieved with the normal tissue tolerance dose $TD_{5/5}$. In order to maximize the likelihood of achieving a therapeutic gain with any new modality, we need to understand the reasons behind the relative position of these two curves for conventional therapy. What makes tumours more radiosensitive than the normal tissues that are inevitably included in the high-dose volume? The inset in Fig. 1 illustrates what is known about the levels of cell kill needed for the tumour and normal tissue effects. In order to obtain complete tumour eradication the last clonogenic cell in a tumour has to be sterilized, i.e. at least 9 decades

 ISSN 0284-186X

of cells must be killed (see below for details). By contrast, a normal tissue will only continue to function if one or more cells is left to repopulate each of the functional subunits in the tissue, e.g. the nephrons in a kidney or the crypts in the intestine. These subunits may contain several thousand cells and only a fraction of the subunits can be lost (1–3). Therefore much less cell kill can be tolerated by the normal tissue than the cell kill needed to eradicate a tumour. Only if there is a large differential in intrinsic cellular radiosensitivity could one predict that the dose needed to kill 9 decades of tumour cells would kill only about 4 decades of normal tissue cells.

Intrinsic radiosensitivity. Many studies have been performed in culture to study the intrinsic cellular radiosensitivity, which give insight as to whether such a systematic difference in radiosensitivity exists between normal and malignant cells (4–13). Established cell lines have been used for decades, and more recently these have been supplemented by freshly explanted cells from patients. The cells have been studied by clonogenic survival, by measures of DNA damage or chromosome injury. They have been investigated growing in free suspensions, in soft agar, as monolayers attached to glass or plastic dishes, on specially prepared surfaces, as aggregates, spheroids or even as xenografts. Many features of the radiation response have been described, including the variation in sensitivity around the cell cycle, the effect of exponential versus plateau growth, the protective effect of contact between cells, the genetic composition, and so on. A wide range of radiation response has been seen, with survival of single cells at 2 Gy ranging from 0.1 to 0.9 in different cell lines and different culture conditions. However, no consistent difference between the sensitivity of tumour cells compared with their normal counterparts has been detected. Hence studies of cells in culture do not provide the universal reason that would account for the relative positions of the TCP and NTCP curves in Fig. 1.

We can then look at the more complex picture of the response to fractionated irradiation of a whole organ, tissue or tumour in vivo and seek an explanation there. Unfortunately the pathophysiology and architecture of solid tumours provide further reasons to suppose that the whole tumour might be more radioresistant than the normal tissues, and none to suggest an increased sensitivity.

Repopulation. Rapid proliferation between fractions can lead to sparing of the tumour by the addition of extra cells during the 6–7 weeks of radiation therapy, moving the TCP curve to higher doses. Tumour cell potential doubling times of a few days have been measured in many human tumours before treatment (14, 15). Analyses of clinical data for treatments spread over periods of longer than 6 weeks show that there is on average a 14% loss of local control for each added week (16). Thus proliferation causes extra cells to be born within the treatment schedule and requires extra doses to eradicate them. Although compensatory accelerated proliferation within the treatment schedule can also spare early reacting epithelial tissues, this compensation does not occur within the overall treatment time of 6 or 7 weeks in late reacting tissues (17). Most of the deep essential organs in the body are late reacting and these are therefore further disadvantaged relative to tumours.

Hypoxia. The existence of hypoxic cells as a result of the inadequate microvasculature and large intercapillary distances has long been postulated to increase the radioresistance of tumour cell populations (18). A complete absence of oxygen at the time of irradiation can increase the cellular resistance by a dose-modifying factor of 3. This translates into an enormous reduction in cell kill at the dose levels needed for tumour cure, e.g. by a factor of 10^6 for a completely hypoxic population. Even a small fraction of hypoxic cells requires a large increase in the dose needed for cure, depending critically on the pattern of reoxygenation (19). There is evidence from microelectrode studies and from the recent use of bioreductive stains that hypoxia exists in both experimental and human tumours and that it has an impact on the outcome of radiotherapy, though not as large as that predicted from the radiobiological studies (20–22).

Two distinct forms of hypoxia are recognized. Chronic (diffusion-limited) hypoxia occurs at the bottom of the nutrient gradient around each blood vessel (18, 23–26). It develops slowly and progressively worsens, leading ultimately to starvation-induced cell death. Superimposed upon this architectural feature is a rapidly changing form of hypoxia (termed intermittent, perfusion-limited or acute hypoxia). This is caused by the closure of individual

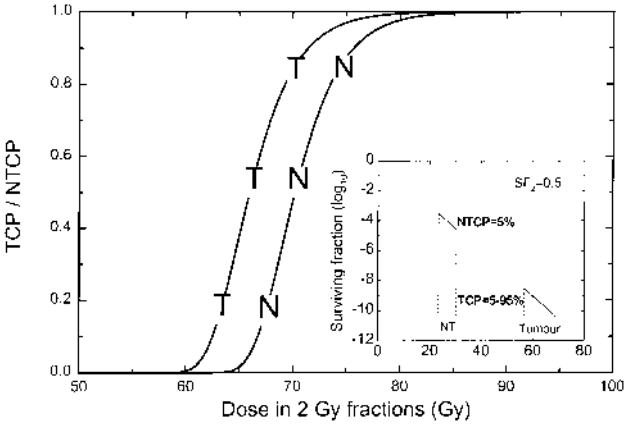

Fig. 1. Dose-response curves for tumour control probability (TCP) and normal tissue complication probability (NTCP) as they are commonly depicted in radiobiology textbooks. Fifty percent tumour control is depicted at the dose that gives 5% normal tissue complications. The inset panels show the difficulty of explaining this if all the cells have the same intrinsic radiosensitivity. Normal tissues would be destroyed at much lower doses than those needed to kill all cells in a tumour if there is no difference in intrinsic radiosensitivity.

capillaries or by blood flow ceasing for some minutes or hours (27–29). These two forms of hypoxia are usually discussed as if they have the same effect on the cellular response. We believe this has been a gross oversimplification, which has obscured some important aspects of cellular sensitivity as described below.

Energy charge. Another characteristic feature of malignant tumours is the reduction in energy charge. This is detectable by magnetic resonance spectroscopy (MRS), as the reduction of the peaks representing high-energy phosphates (ATP and ADP), and a resultant increase in the levels of low-energy phosphate (AMP) and inorganic phosphate (30–34). These characteristic spectra have been investigated as a technique for indirectly identifying hypoxia in tumours, though they are actually showing energy charge reduction as a result of overall nutrient deprivation, not simply oxygen reduction. This distinction may be critical and has so far not been included in any of the many modelling studies to predict treatment outcome.

Mathematical modelling in translational research. The most commonly used mathematical model to describe the response of cells to radiation is described by the linear quadratic equation, which provides a reasonable fit to many sets of experimental data over a wide dose range (35–37). However, many recent investigations of the very low-dose region have shown that the LQ model fails below doses of 1 Gy. There is a complex hyperfine structure to the survival curve, both in vitro and in vivo that is not predicted by the LQ model (38–47) This has forced a re-evaluation of the biophysical or biochemical basis of the shouldered shape of the survival curve. It appears that most untreated cells have little capacity to deal with DNA damage inflicted by ionizing radiation and are therefore exquisitely sensitive to very low doses of x-rays. However, if these cells are stimulated by genotoxic damage and recognize the danger to their DNA, they can activate or manufacture more enzymes that can then repair large numbers of DNA lesions. This form of stress response is analogous to the production of heat-shock proteins and hypoxia-inducible stress proteins, although in this case, the identity of the proteins has not yet been established. Intrinsically, radiosensitive cells are those that are incapable of mounting the adaptive response. The greater the capacity for inducing this repair, the more radioresistant the cell line and vice versa (12, 48, 49).

The hypersensitive response of cells to low doses of radiation was first mathematically described by Joiner & Johns (39). They put forward the inducible repair model (LQ/IR) that is a modification of the classical linear quadratic (LQ) model. This takes into account the progressive increase of the relative radioresistance of cells with increasing radiation exposure. An alternative model has been proposed by Wouters & Skarsgard (44), which predicts that a certain number of lesions in the DNA are needed as a threshold to trigger the repair. This model is more mechanistic and intellectually appealing, but has not been widely adopted because it predicts very sharp transitions from sensitive to resistant slopes and fits the data less well than the Joiner & Johns' (39) equation.

Incorporating chronic hypoxia. We recently compared the LQ and the LQ/IR mathematical models for simulating the response to fractionated radiotherapy of different cell populations in tumours and normal tissue. We have shown that quite different conclusions are drawn depending on the choice of model (49–53). It is crucial therefore for translational research activities to determine experimentally which model is correct. The use of the newer LQ/IR model provided strikingly different predictions for the impact of hypoxia on cells in tumours. Furthermore, this led us to investigate the clinical consequences if repair induction could be diminished or abolished in chronically hypoxic, starving cells. We predicted that such cells might provide an explanation of the effectiveness of radiotherapy (51). We subsequently found that this 'new concept' of chronic hypoxic sensitivity is supported by several comprehensive sets of experimental data in the literature, acquired by very experienced investigators over the past three decades (54–61). This experimental evidence that nutrient deprivation and/or chronic oxygen deprivation reduces the cell's repair competence has been widely ignored, especially by the computer modelling fraternity. It is the magnitude of sensitization by the loss of repair in chronically hypoxic cells that we seek to address in the present paper. We believe that the incorporation of these data into preclinical modelling results in the need for a complete paradigm shift in relation to the impact of hypoxia on radiosensitivity.

MATERIAL AND METHODS

We used simple mathematical models to predict the response of uniform or heterogeneous cell populations to single dose and fractionated schedules of treatment, leading through calculations of the surviving fraction to the construction of TCP and NTCP curves. The classical LQ model (35–37) (equation 1) relates the radiation dose D to the loss of clonogenic ability (usually designated Surviving Fraction or SF) through a quadratic equation containing a linear dose term with the constant α and a squared dose term with the constant β.

$$SF_{OXIC} = \exp(-\alpha \cdot D - \beta \cdot D^2) \quad [1]$$

The inducible repair variant of the LQ model proposed by Joiner & Johns (39) assumes little or no repair before irradiation commences and a gradual, exponential induction of repair capacity with increasing dose (equation 2). This model contains two extra terms compared to the LQ model:

1. the sensitivity at very low doses, designated α_S, that transforms gradually to the resistant α (the classical LQ shoulder slope), which is now termed α_R;

2. an 'induction dose' term, designated D_C, at which 1–1/e (i.e. 67%) of the transition to the maximum repair capacity has occurred.

$$SF_{OXIC} = \exp\left\{ -\alpha_R \cdot \left[1 + \left(\frac{\alpha_S}{\alpha_R} - 1 \right) \cdot e^{-\frac{D}{D_C}} \right] \cdot D - \beta \cdot D^2 \right\} \quad [2]$$

The maximum extent of inducible repair is expressed as the ratio of the initial pre-induction hypersensitive slope (α_S) to the final post-induction resistant slope (α_R), i.e. α_S/α_R, which we will term the inducible repair ratio (IRR). An IRR of unity corresponds to no inducible repair and is thus equivalent to the classical LQ model. Increasing values of IRR correspond to more repair induction and are linked with more radioresistance.

When considering the modification of the response by acute hypoxia, we have assumed that oxygen deprivation modifies all the parameters by an oxygen enhancement ratio (OER) (equation 3). In the modelling we have assumed a uniform dose modification factor of three. This is an oversimplification, but does not change the general conclusions, as discussed elsewhere (50, 53).

$$SF_{\text{ACUTELY HYPOXIC}} = \exp\left\{ -\frac{\alpha_R}{OER_\alpha} \cdot \left[1 + \left(\frac{\alpha_S}{\alpha_R} - 1 \right) \cdot e^{-\frac{D}{D_C \cdot OER_{D_C}}} \right] \cdot D - \frac{\beta}{OER_\beta} \cdot D^2 \right\} \quad [3]$$

The effect of prolonged hypoxia together with energy charge depletion (corresponding to chronic hypoxia) has been assumed to give the same chemical protection factor of three compared to an oxygenated state, but in addition it prevents repair induction. The response of such cells is therefore composed only of the initial hypersensitive slope (α_S) and the beta term, both modified by the OER as in equation 4.

$$SF_{\text{CHRONICALLY HYPOXIC}} = \exp\left(-\frac{\alpha_S}{OER_\alpha} \cdot D - \frac{\beta}{OER_\beta} \cdot D^2 \right) \quad [4]$$

Finally, the response of mixed populations of oxic cells with either acutely hypoxic cells or chronically hypoxic cells, or both, is obtained by summing the separate elements as in equation 5.

$$SF_{\text{MIXED}} = (1 - AHF - CHF) \cdot SF_{\text{OXIC}} + AHF \cdot SF_{\text{ACUTELY HYPOXIC}} + CHF \cdot SF_{\text{CHRONICALLY HYPOXIC}} \quad [5]$$

AHF and CHF are the fractions of acutely hypoxic and chronically hypoxic cells, respectively.

When a particular combination of subpopulations of oxic and hypoxic tumour cells are considered, we have assumed for the present calculations that they re-assort into the same fractions after each treatment. This corresponds to a moderate level of reoxygenation and assumes no progressive accumulation of cells in subcompartments that have a greater resistance. The response to a series of fractions can then be obtained by simply raising the effect of a single small fraction, e.g. SF_2, to a power equal to the number of fractions, e.g. $(SF_2)^{35}$ for 35 fractions.

Tumour control probabilities (TCP) and normal tissue complication probabilities (NTCP) were obtained from the cell survival (SF) through Poisson statistics (equation 6).

$$\text{TCP or NTCP} = \exp(-K \cdot SF) \quad [6]$$

K is the number of tissue rescuing units or tumour clonogens existing in the irradiated region, using the notation of Thames & Hendry (1). For the normal tissue we have used K values ranging from 10^4 to 10^5, which may be an overestimate, but can be seen as representing the spectrum for tissues or organs. These values of K are based on comparisons of clonogenic assays and dose-response curves for tissue damage, together with measurements of cell density and functional architecture (1–3). For tumours we have started from the common assumption that 1 g tumour tissue on average contains 10^9 cells, which is based on a tumour cell diameter of 12 μm and a high packing fraction, and matches yields of cell extracts. However, tumours are a mixture of potentially dangerous clonogenic cells, differentiated non-clonogenic cells, normal stromal cells and necrotic regions. We have therefore assumed that there are only 10% of clonogenic cells, i.e. 10^8 cells per gram in a tumour. This may be a slight underestimation. According to this assumption, a fairly small tumour of 3 cm diameter (10 g) would contain about 10^9 clonogenic cells. Many authors have discussed whether there is a very low stem-cell fraction in tumours but we think this is improbable (62, 63).

Using the equations above, we have made a series of calculations of the effect of fractionated irradiation on mixed populations of oxic and hypoxic cells. For simplicity, we have chosen to consider a 'black and white' mixture of oxic and hypoxic cells, although we are aware that there is in fact a gradient of radiosensitivities, representing a 'grey scale'. The effect of the gradient will be taken into account in future modelling studies, but it increases the complications of the mathematical modelling considerably. Our aim here is simply to explore the consequence of including acute and chronic hypoxic cells in the mixture and to compare the response of normal tissues and tumours. We have chosen the parameters for the cell survival curves based on a recent comprehensive review of all the in vitro mammalian cell survival data that give insight into the low-dose hypersensitivity (49). We have used the correlation between SF_2 and IRR derived in that paper to link

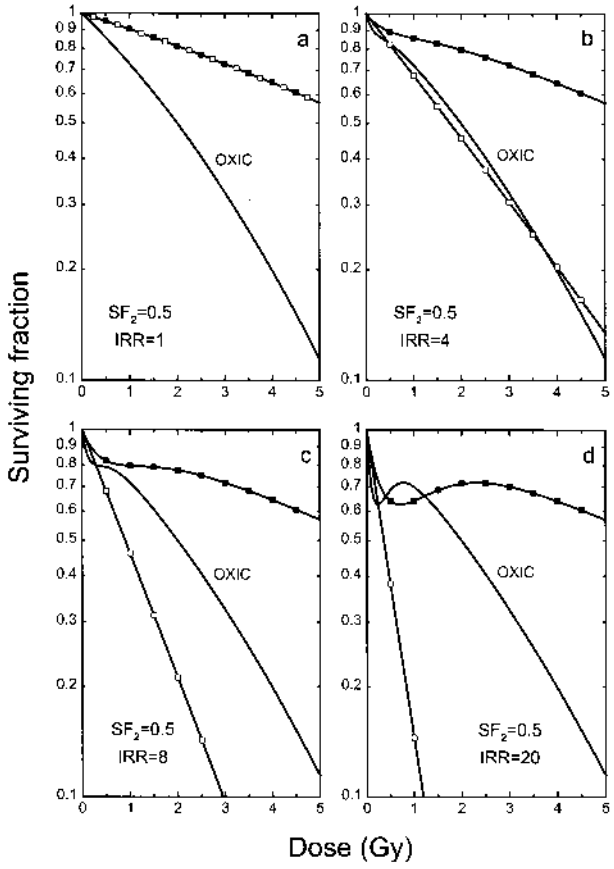

Fig. 2. Examples of the survival curves for oxic (solid line), acutely hypoxic (■) and chronically hypoxic (□) cells. The four panels represent cells with the SF_2 fixed at 0.5 and the indicated levels of inducible repair (IRR). Cells with more inducible repair show a greater differential between the response of resistant, acutely hypoxic cells and sensitive, chronically hypoxic cells.

the values of radioresistance for 'theoretical cell lines' with the appropriate values of inducible repair.

RESULTS

Fig. 2 illustrates the shape of the survival curves for four different theoretical cell lines with increasing levels of repair induction from panel a to d. The response is only illustrated over the clinically interesting dose range up to 5 Gy. In each panel, three curves are shown:

- for cells in well-oxygenated conditions, capable of repair induction (solid line);
- acutely hypoxic cells, capable of repair induction, but protected by an OER of 3 (■);
- chronically hypoxic cells, protected by an OER of 3, but with no capacity for repair induction (□).

For simplicity, in this first example, in illustrating the influence of inducible repair on the curve shapes, the surviving fraction at 2 Gy (SF_2) has been kept constant (0.5) for the four cell lines.

In panel 2a only two curves can be seen, for oxic and hypoxic cells. The curves for acute and chronic hypoxia are identical since there is no inducible repair (IRR = 1), corresponding to the classical LQ model. In panels b–d we see the more complex shapes that emerge, and the progressive separation of the lines for the two forms of hypoxia, as the IRR value increases. The greater the IRR, the more pronounced the hypersensitivity at low doses and the more obvious the transition from α_S to α_R in the oxic and acutely hypoxic cells. The induction of DNA repair competence requires a three times higher dose in acute hypoxia than in oxygen (42, 64, 65). This results in a complex relationship of these two curves at doses of about 0.5–1.5 Gy in the cells with lower IRR values and a crossover of the oxic and acutely hypoxic curves for the highest IRR. The chronically hypoxic cells are even more sensitive than fully oxic cells for all the examples shown.

We have next incorporated the link between IRR and SF_2 that has been shown in the literature (12, 48, 49). All the low-dose hypersensitivity studies are shown in Fig. 3, as they have been included in the recent review (49) of the relationship between IRR and SF_2. Eighteen sets of mammalian cell data are included. The line derived from a curvilinear equation which relates the IRR to the reciprocal log survival is shown. The data points are shown with different symbols according to whether they have been obtained experimentally using a flow cytometer (○) or a computerized microscope (●). This relationship allows any particular level of SF_2 to be automatically linked to a value of IRR (49). Using this information we have then constructed the curves shown in the following figures.

Fig. 4 shows a similar set of panels to that in Fig. 2, but now the cell kill that is achieved with 2 Gy is directly

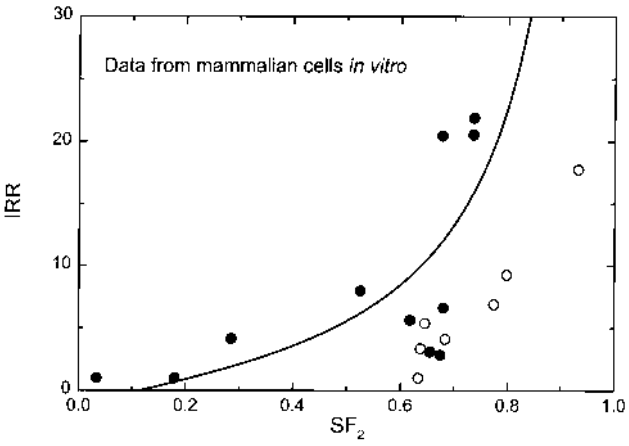

Fig. 3. Correlation between the extent of inducible repair (IRR) and the radiosensitivity (SF_2) for 18 sets of data from the literature. The curve fitted to the DMIPS data has been derived after progressive dissection of the factors that contribute to these parameters (see Daşu & Denekamp (49) for sources of data and details of the analysis). The curve relating IRR and SF_2 has been used for the calculations in the rest of this paper.

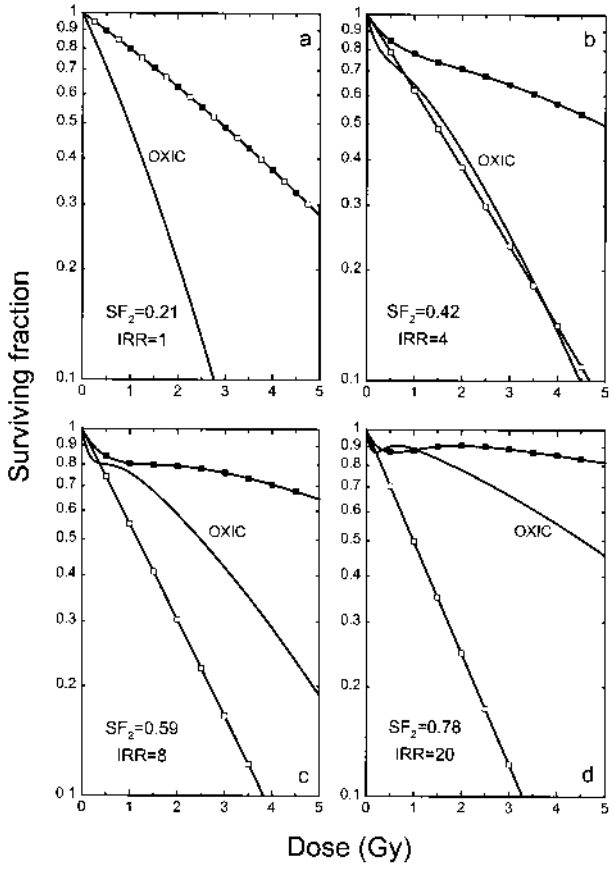

Fig. 4. Examples of the survival curves obtained when the radiosensitivity and repair capacity are linked by the relationship in Fig. 3. The four panels represent cells with the indicated levels of sensitivity (SF_2) and inducible repair (IRR). Curves are shown in each panel for oxic (solid line), acutely hypoxic (■) and chronically hypoxic (□) cells. The acute and chronic hypoxic cells show grossly different levels of survival at 2 Gy.

linked to the amount of inducible repair, using the equation for the curve in Fig. 3. A similar pattern emerges to that presented in Fig. 2 but, in this more realistic simulation of cell lines with varying radioresistance, the absolute levels of kill of oxic cells are now reduced in the cell lines with more inducible repair. It is still evident that the greatest differential between acute and chronic hypoxia occurs in the most resistant lines and that the chronically hypoxic cells are more easily killed than the oxic cells when IRR is above 1.0. This illustrates the difference in the predictions of the LQ and LQ/IR model.

In order to consider the potential clinical impact of these differences in cell survival for the three categories of oxic and acute or chronically hypoxic cells, we have simulated (Fig. 5) the effect of a fractionated schedule of 35×2 Gy fractions for the four cell lines shown in Fig. 4. We have first calculated the cell kill that would be achieved in a fully oxygenated population of cells. This is represented by the horizontal bar in each panel, ranging from 24 decades to 4 decades of cell kill for the same total dose. Of course, progressively less cell kill is seen in the increasingly resistant cell lines. This illustrates the immense impact of a four-fold change in SF_2 when it is raised to the 35th power. Only in panel 5d do we see a cell kill in the oxic compartment that is compatible with preservation of function of the normal tissues included in the high-dose volume.

In panels 5b–d there are two other curves shown which come from the computations of admixtures of increasing fractions of hypoxic cells. If any acutely hypoxic cells are added, in all panels we see an upward curve reflecting their radioresistance. If, instead, we add chronically hypoxic cells to the oxic cells, we see a progressive increase in the extent of cell kill below that in a purely oxic population, in proportion to the IRR value. Mixtures of acute and chronic hypoxia with the oxic cells lie between these two

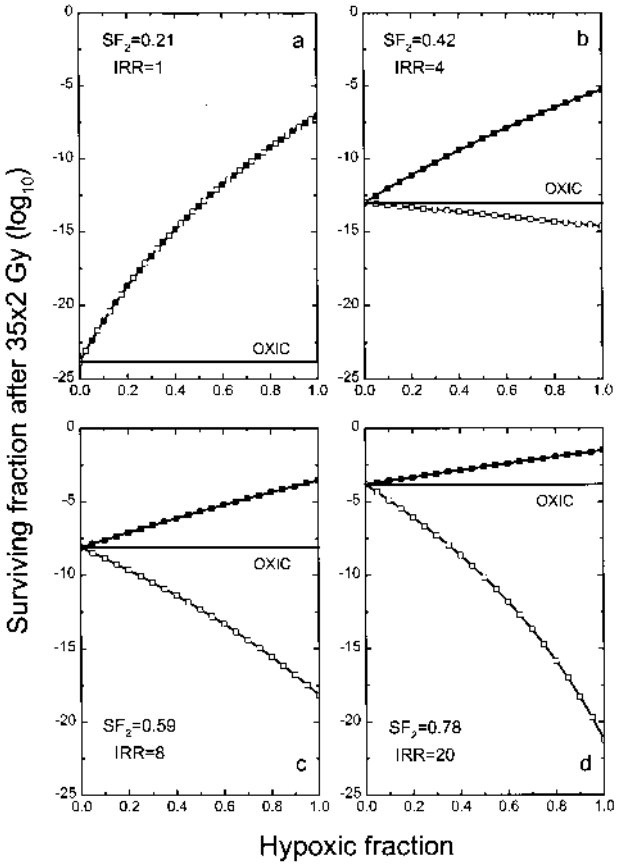

Fig. 5. Estimates of the surviving fraction after a series of 35 repeated fractions of 2 Gy to a fully oxic population (solid line, shown for reference) or with the addition of increments of acutely hypoxic (■), or chronically hypoxic cells (□). It has been assumed that there is a return to the specified levels of cell mixtures between fractions. There is a big difference in the cell kill achieved in a fully oxic population with different SF_2 values. Any acute hypoxia reduces the overall cell kill in all four panels. Adding chronically hypoxic cells increases the total cell kill, except in panel a) where the cells have no capacity for inducible repair (i.e equivalent to the classical LQ model). The sensitizing effect of chronic hypoxia is most marked in the most resistant cell lines.

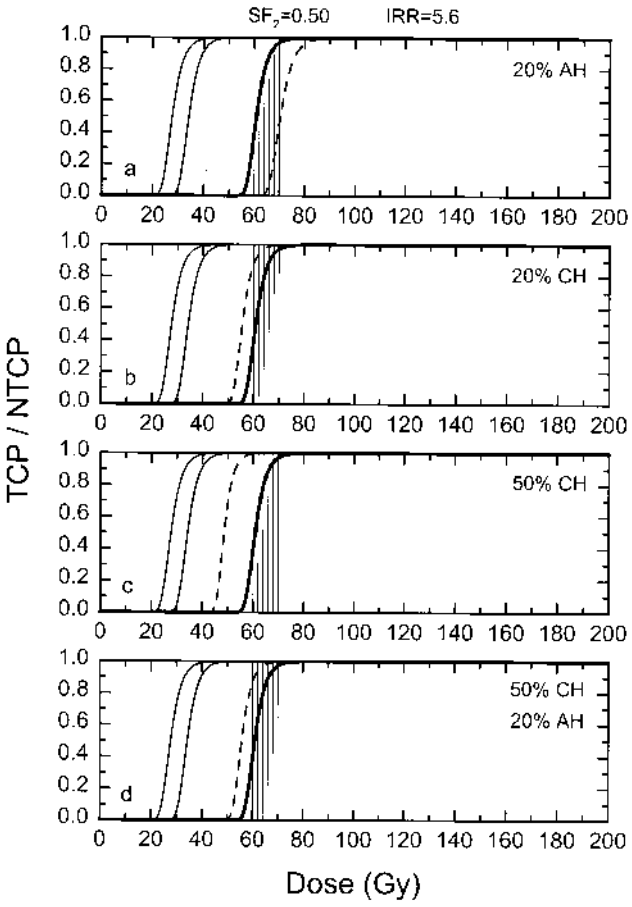

Fig. 6. Dose-response curves representing NTCP and TCP as a function of the dose administered as a series of 2 Gy fractions. An oxic SF_2 of 0.5 with an associated IRR of 5.6 have been assumed. The normal tissue complications (shaded area) occur in a much lower dose range than that used clinically. The tumour response (solid line) appears in the clinically relevant dose range (60–70 Gy), but it cannot be achieved without disastrous complications, even if chronically hypoxic cells are present (dashed lines). The percentage of acute or chronic hypoxic cells used to compute the dashed line is indicated in each panel.

extremes, as shown in detail elsewhere (51). Fig. 5 illustrates how the chronically hypoxic cells may provide the therapeutic window that is needed to explain the relative positions of the TCP and NTCP curves in Fig. 1. A mixture containing a proportion of chronically hypoxic, repair incompetent cells can be much more sensitive than a fully oxic population.

The next step in these modelling studies was to translate such cell survival calculations into dose-response curves. In Figs. 6–8 we show sample sets of data created using the same relationship between SF_2 and IRR as in Fig. 3 but using a more restricted range of SF_2 values. We have varied the number of 2 Gy fractions to create a broad range of doses against which to plot the TCP and NTCP curves. The range of doses used with curative intent in most radiotherapy departments is indicated by the vertical lines representing additional 2 Gy fractions between 60 and 70 Gy total dose.

In each figure, the four panels show NTCP and TCP curves for oxic populations and one mixed population of oxic and hypoxic cells. A shaded band is shown representing $K = 10^4$ to 10^5 for the normal tissue and a single solid line is shown for $K = 10^9$ for the fully oxic 10 g tumour. The displacement of these two curves results entirely from the difference in the level of cell kill needed for cure and tissue failure and is in the opposite sequence compared with clinical experience. The dashed line in each panel represents the addition of the specified fraction of acute (AH) or chronic (CH) hypoxic cells. The normal tissue curves always reach 100% complications before any single tumour is cured if all cells are oxic and there is no difference in their intrinsic sensitivity. The negative differential is even greater if acutely hypoxic cells are added (upper panel in each figure).

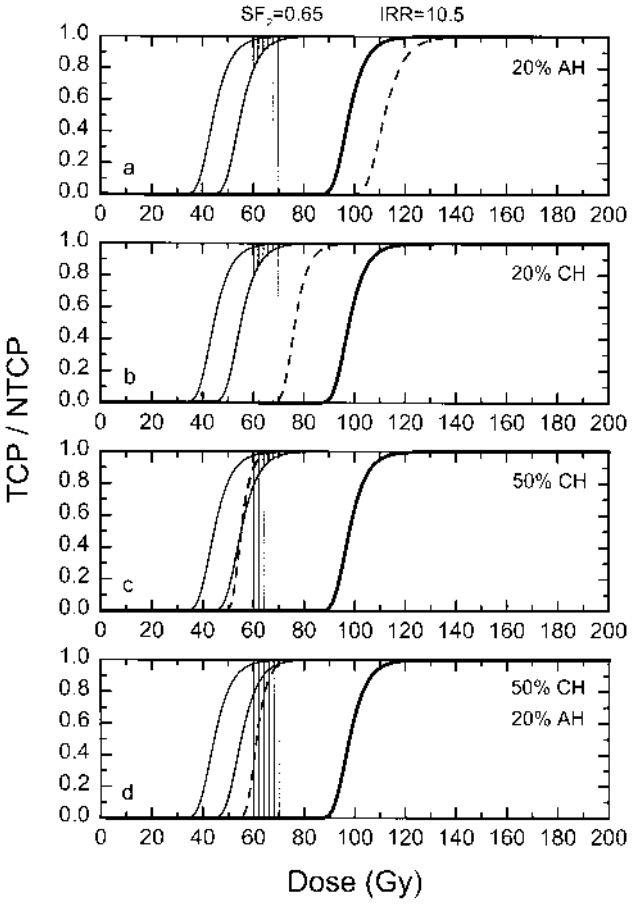

Fig. 7. Dose-response curves representing NTCP and TCP as a function of the dose administered as a series of 2 Gy fractions. Symbols are as described in the legend in Fig. 6. An oxic SF_2 of 0.65 with an associated IRR of 10.5 have been assumed. The NTCP and TCP curves are in the 'wrong' sequence in all four panels although the negative differential is somewhat reduced compared with $SF_2 = 0.5$.

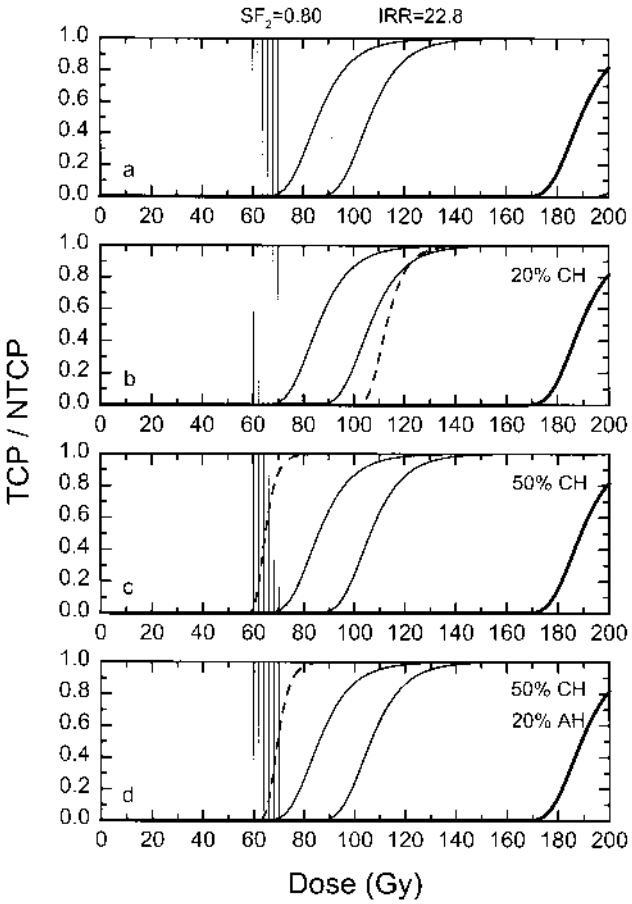

Fig. 8. Dose-response curves representing NTCP and TCP as a function of the dose administered as a series of 2 Gy fractions. An oxic SF_2 of 0.8 with an associated IRR of 22.8 have been assumed. The NTCP curves now fit the clinical expectation of a small fraction of complications in the therapeutic dose range. The oxic tumour is incurable and the addition of acutely hypoxic cells requires more than 200 Gy for an observable response. However, with 50% chronically hypoxic cells, with or without acutely hypoxic cells, it is curable and the desirable therapeutic window of cure with few complications has been achieved.

It is possible from these figures to determine what values of IRR are compatible with the clinical experience of 5% complications in the dose range 60–70 Gy. The results for an SF_2 of 0.5 are shown in Fig. 6. This is the most common assumption in mathematical modelling studies. It shows how disastrous such a scheme is predicted to be for the normal tissue, which should be totally destroyed in all patients at doses below 45 Gy. In Fig. 7 we show a similar series for a slightly more resistant cell line (SF_2 of 0.65). The normal tissue complication curve is approaching 100% in the therapeutic dose range. In Fig. 8, for the most resistant cell line in these particular simulations (SF_2 of 0.8), we finally achieve the desired therapeutic window, i.e. a very low incidence of normal tissue complications in the 60–70 Gy dose range

In each figure the addition of radioresistant, acutely hypoxic cells pushes the two curves of NTCP and TCP further apart and in the 'wrong' direction. By contrast, the addition of 20% or 50% of chronically hypoxic cells brings the normal tissue and tumour curves closer together. Even the admixture of both types of hypoxic cells (panel d) shows curative tumour response, in the dose range of therapeutic interest. This illustrates the potential importance of chronically hypoxic, repair deficient cells, but we must also look for the conditions that allow a reversal of the normal tissue and tumour response in the relevant dose range. Only in the most resistant cell line (Fig. 8) do we get the reversal of the TCP and NTCP curves that is needed to explain the everyday clinical experience, i.e. very few complications but a high tumour control probability. The TCP is reduced somewhat but still in the therapeutic range if some acutely hypoxic cells are added.

Thus, repair incompetence resulting from chronic hypoxia can provide a possible explanation of the common clinical experience that TCP curves can lie to the left of NTCP curves, but only if all the cells are intrinsically radioresistant and a considerable fraction of the tumour cells is repair incompetent. The question we then posed is how many chronically hypoxic cells would be needed to obtain this therapeutic window for each of these levels of oxic radiosensitivity. We have next computed the dose needed to obtain 50% local control for a wide spectrum of IRR values and a wide variety of fractions of chronically hypoxic cells to give the array shown in Table 1. The parameters used for the calculations and the dose that would produce 5% complications in normal tissues are shown at the top of the table. In the lower four sections are shown the simulated tumour populations with no acute hypoxic cells or with an admixture of 5, 10 or 20%. The combinations of oxic and AH + CH cells that give a TCD_{50} within the normal therapy dose range (60 to 70 Gy) are highlighted. For the entire range of intrinsic radiosensitivities it is possible to model a combination of oxic and hypoxic cells that would allow 50% tumour cure to be obtained with clinically relevant doses. However, if the oxic cells have an SF_2 less than 0.7, the normal tissue tolerance is exceeded with doses much lower than the therapeutic dose range. An intrinsic radiosensitivity corresponding to $SF_2 = 0.75$ or greater is needed to explain the clinical results. In successive sections of the table we see that, even with the addition of radioresistant acute hypoxic cells, tumours can be cured if there are enough radiosensitive chronically hypoxic cells.

DISCUSSION

This paper has shown the importance of differentiating the two types of hypoxia in building models to predict treatment outcome. The inducible repair variant of the LQ model and the concept of starvation-induced loss of repair is needed instead of the simple LQ model and uniform

hypoxic protection. Only then can we predict the known response of both tumour and normal tissue to fractionated radiotherapy. These simulations provide a plausible explanation of the common clinical experience that some tumours can be cured without disastrous complications for the patients. Surprisingly little attention has been paid to the considerable discrepancy between the level of cell kill that has been shown to lead to normal tissue injury in animals and that needed for tumour eradication. This is probably because it has not been possible, using the LQ model, to incorporate these data and to come up with a clinically reasonable outcome. Likewise, little attention has been paid to the studies in the literature that have indicated that the biochemical repair capacity of cells may be reduced or abolished if the hypoxia is either very prolonged or accompanied by glucose depletion.

Until the magnitude of IRR was identified, it would have been difficult to predict that the potential increased sensitivity resulting from the loss of repair in energy-deprived cells would be so much larger than the simple acute hypoxic protection. The widely accepted existence of chronic hypoxia in tumours has therefore not previously been seen as important, or as a way of providing a differential that explains the current success of radiotherapy. The concept of a low stem-cell fraction has usually been put forward in order to explain how a cure can be achieved with a lower dose than that predicted to be necessary to kill every tumour cell. That argument has been dealt with elsewhere (62, 63). The low stem-cell concept does not need to be invoked if, instead, we incorporate the known facts about chronic hypoxia and if we allocate a value (IRR) for the increased sensitivity due to repair incompetence that is directly linked to radioresistance.

The question of how a normal tissue can tolerate the same level of dose as that needed for tumour cure has notbeen incorporated into most modelling studies. It has been

Table 1

Doses needed to achieve 5% NTCP or 50% TCP in mixed populations of cells. Bold numbers show the clinically relevant dose ranges that give tolerable normal tissue complication rates above the clinical dose range or at least 50% tumour control probabilitiesbeloe the clinical dose range

Cell parameters							
Oxic SF_2		0.50	0.60	0.70	0.75	0.80	0.90
IRR		5.6	8.5	13.3	17.1	22.8	51.2
		Dose in 2 Gy fractions needed to achieve 5% NTCP in populations of 10^4 or 10^5 cells					
	10^4	23	32	45	56	**73**	**154**
	10^5	30	41	58	**72**	**93**	**198**
AH	CH	Dose in 2 Gy fractions need to achieve 50% TCP in mixed populations of 10^9 cells					
0	0	**61**	83	118	147	189	400
0	0.2	**56**	**68**	86	98	113	157
0	0.4	**51**	**57**	**66**	71	77	91
0	0.5	**49**	**53**	**58**	**61**	**65**	73
0	0.6	**47**	**48**	**51**	**53**	**55**	**60**
0.05	0	**63**	85	122	152	195	415
0.05	0.2	**58**	**70**	89	101	116	160
0.05	0.4	**53**	**59**	**67**	72	78	92
0.05	0.5	**50**	**54**	**59**	**62**	**66**	74
0.05	0.6	**48**	**50**	**53**	**54**	**56**	**60**
0.1	0	**65**	88	126	157	202	430
0.1	0.2	**60**	73	91	103	118	162
0.1	0.4	**54**	**61**	**69**	74	80	93
0.1	0.5	**52**	**56**	**61**	**64**	**67**	75
0.1	0.6	**50**	**51**	**54**	**55**	**57**	**61**
0.2	0	**70**	95	135	168	217	464
0.2	0.2	**64**	77	96	109	124	168
0.2	0.4	**58**	**64**	72	77	83	95
0.2	0.5	**56**	**59**	**64**	**66**	**69**	76
0.2	0.6	**53**	**54**	**56**	**58**	**59**	**62**

assumed that optimized dose conformation, even in conventional therapy, means that most normal tissue is in the penumbra. The fact that a margin of at least 2 cm is included around the tumour and is prescribed to receive the same dose as the tumour (i.e. is within the prescribed treatment volume) means that up to 10 times more normal tissue than tumour is usually taken to the full prescribed dose. Indeed for some cancers, e.g. bladder, the whole organ is deliberately treated to the full dose, yet it continues to function for many years. It is difficult to understand why such treatments do not lead to huge necrotic masses, unless we postulate that SF_2 values higher than 0.7 are commonplace. Fig. 3 shows that 14/18 cell lines used in these studies have SF_2 values above 0.6. In addition, it was clearly shown in the 1970s that intracellular contact makes cells much more resistant than if they are irradiated as single cells (66–69). This is seldom considered in relation to predictive assays of single cells in culture.

Because of the importance of the therapeutic implications, we have recently reviewed in detail the evidence for the new inducible repair concept (49). This is based on cell-survival studies which allow the fine structure of the survival curve to be elucidated and on fractionated studies of normal tissue and tumour response using very small fractions (38–47, 70, 71). Specialized techniques have been developed, both in vivo and in vitro, to obtain the very high precision that is needed to demonstrate this low dose hypersensitivity. These are described in detail elsewhere, together with some of the other predictions about possible clinical consequences (49–53). In earlier publications we have focused on the possibility that 0.5 Gy fractions may be useful for specifically overcoming the resistance of the acutely hypoxic cells because of the crossover of the curves shown in Figs. 2 and 4. In the present paper we have focused instead on the implications of inducible repair for understanding the success of conventional therapy and have recognized the potential of chronic hypoxia as a means of explaining tumour radiosensitivity.

Hypoxia has been recognized for decades as one of the most characteristic features of solid tumours. Many different approaches to novel radiotherapy protocols have been based on the desire to remove the radioprotective effect of hypoxia: 100% or 95% oxygen levels, either at normal pressure or at hyperbaric pressure have been used. The whole field of oxygen mimetic sensitizers is based on the implicit assumption that all hypoxic cells are equally resistant. This includes the drugs that have gone into clinical trials (metronidazole, nimorazole, misonidazole, pimonidazole and tirapazamine), and also the later concept of bioreduction of prodrugs in hypoxic cells. The use of high LET particles, both neutrons and heavy ions, is also built on the concept that the hypoxic radioresistance with photons will be reduced with higher LET radiations. All of these approaches have given slightly encouraging results in some clinical trials but they have all been very disappointing compared to the high expectations from the radiobiological principles.

Most of the thinking in this field was built on the original model of Thomlinson & Gray (18) of chronic hypoxia. No fundamental change in thinking occurred when the two different forms of hypoxia were recognized. Reinhold first described intermittent closure of vessels using thin tumour sandwich preparations in the early 1970s (72, 73). This has subsequently been confirmed by many authors, in sandwich tumour preparations, by dual staining techniques and with Doppler measurements of changes in blood flow (27–29, 74–76). We believe that the clinical disappointments with methods aimed at overcoming hypoxic radioresistance may become more understandable if those strategies are re-evaluated using the new knowledge about the magnitude of inducible repair in different tumour lines and if a clear distinction is drawn between acute and chronic hypoxia.

In studies of the influence of modifying factors, most experimenters attempt to change only one factor at a time, thereby allowing the dissection of the importance of different factors. In this way the impact of dose rate, the time-scale of DNA repair, etc, are elucidated. In the studies of hypoxia, almost all experimenters have grown cells in ideal conditions. They have then changed the oxygen tension in the gas phase shortly before irradiation, either to pure nitrogen or to defined low oxygen tensions, but without altering the nutrient content of the medium. In this way, an understanding of the redox chemical aspects of free radical scavenging in the microseconds after the ionizations has been built up and the OER dependence on oxygen concentration has been obtained (77–82). In almost all studies the gas has been changed back to air immediately after the irradiation, allowing the cells to repair in ideal conditions. These experimental conditions may provide a reasonable model of acute hypoxia, but they mimic only cells with that particular sequence and time-scale of deoxygenation and reoxygenation, closely linked to the timing of the irradiation.

These conditions do not mimic at all closely the chronic hypoxia that Thomlinson & Gray (18) initially described from histological examinations of human lung cancers.

A few experimenters have attempted to simulate more closely the poor nutrient conditions that are believed to coexist with hypoxia at several cell diameters away from the capillaries in tumours. They have used contact inhibited plateau phase cultures, either fed or starved, and have reduced both the oxygen tension and the glucose and other nutrients that are available to the cells (54–61, 83–86). These studies are less easy to undertake because the culture conditions themselves are more toxic to the cells, yielding a lower plating efficiency in the control dishes. Nevertheless, this may be a much better model of the environment within tumours, where the cells have been predicted from labelling studies to survive for only 5–11 h in the last hypoxic layer next to necrosis (87, 88).

Experiments with both hypoxia and nutrient deprivation, or just prolonged hypoxia extending into the post-irradiation period, have shown that the shoulder on the single-dose survival curve is diminished or lost, and the ability to repair between fractions is also lost (54–61, 83–86). This has been matched to the fall in cellular energy charge. Thus hypoxia has been demonstrated to have two opposing effects, depending on the time-scale and/or the other nutrient conditions. There is a very fast chemical protective effect and a much slower biochemical sensitization effect. Surprisingly little attention has been paid to these very important observations. We believe that they are the key to understanding the differential between tumour and normal tissue response.

We should then ask what time-scales will dictate whether a cell should be resistant due to hypoxia or sensitized by it. Rapid mixing of streams of oxic and hypoxic cells and very fast kinetic studies using a gas explosion technique have been used in vitro to define the ultrafast time-scale associated with radiochemical protective effects. The level of oxygenation at the instant of irradiation is crucial. However, the oxygen tension can be increased microseconds later and not influence the hypoxic radioprotection, because of the short lifetime of free radicals (80, 82). In animals, it is a little more difficult to predict how rapidly cells in a tissue or tumour can become sufficiently hypoxic to be fully resistant to radiation or to lose their energy charge. In the 1970s an in vivo clonal assay for epidermis was used as an artificially hypoxic model to investigate oxygen-mimetic radiosensitizers (89). This involved rendering the skin acutely hypoxic by giving the mice pure nitrogen to breathe before irradiation. An increase in the radioresistance could be achieved by a factor of 2.5–2.8. An experiment to determine how long they needed to breathe nitrogen showed that full resistance developed within 20 seconds of changing the gas supply. In that time all the oxygen in the 1 litre irradiation container, in the lungs, in the blood stream and in the cells could be depleted. Thus we can assume that in tumours complete radioresistance will also occur within a very short time (seconds to minutes) of cessation of flow through any individual blood vessel.

What, then, of the time-scale of hypoxia leading to energy charge depletion? Studies in vitro have shown that if all oxygen and glucose is removed, the energy charge drops within an hour or two (59, 86). Studies with MRS have shown that the time-scale is faster in vivo, though it is slower than the time-scale of developing hypoxic radioresistance. The use of a clamp to occlude the blood supply or of hydralazine or BW12C pharmacologically to shut down the tumour vasculature led to reductions in ATP 15–30 min after the hypoxia was initiated, although radioresistance was demonstrated within minutes (32). This time-scale of tens of minutes to hours to deplete the cellular energy reserves means that cells that have been pushed down a nutrient gradient over many hours or days, especially in an environment that is low in glucose, are very likely to be energy deprived.

^{31}P-MRS and bioluminescence studies of both animal and human tumours have shown that low ATP is a common phenomenon (30–34, 90–96). It is not possible to quantify from the MRI images exactly how many of the cells are ATP deficient due to chronic hypoxia, though it may be possible to do this with bioluminescence. The extent of energy charge depletion varies from one tumour type to another, being less marked, for example, in gliomas, which are known to be characterized by an extremely rich capillary network.

The main techniques used to quantify hypoxia in tumours in vivo have been the micro-electrode and more recently bioreductive stains (20–22, 95–98). We deemed it important to consider which type of cell is identified by these two techniques. The Eppendorf electrode is the most widely used tool and often referred to as the 'gold standard'. It contains a recessed electrode, which in the early model was 12 μm diameter and in more recent models is 17 μm. A hemisphere of cells contributes to the oxygen measurement and is proportional to the diameter of the electrode. We estimate that the detection volume includes about 70 cells for the older 12 μm electrode and 500 cells for the most recent 17 μm model. We have modelled in the computer a regular array of tumour cords in which we have been able systematically to investigate the relationship between the architecture and the average oxygen tension registered by the electrode (99). We have mimicked the diffusion gradient, and have positioned the electrode tip on cells with known pO_2 values. We have systematically altered whether the electrode is looking up a gradient towards a capillary or down a gradient towards necrosis. These modelling exercises show that such an electrode can never determine the absence of oxygen in a single anoxic cell layer or even two adjacent cell layers because of contamination by better-oxygenated layers that are within its detection volume. We therefore conclude that such probes, when they do detect very low pO_2 values, are more likely to be measuring areas in which flow has temporarily ceased, i.e. areas of acute hypoxia.

The correlations that have been seen between hypoxia measured in this way and clinical outcome would then be in agreement with the predictions of the model used in this paper (100–102). However, the acutely hypoxic cells detected around a closed vessel are mixed with the chronically hypoxic cells that pre-existed when the vessel was open because of diffusion distances. Thus, the fraction of radioresistant hypoxic cells will be overestimated. Furthermore, if any individual vessel has been closed for more than 15–30 min, all the cells may have already fallen in energy reserves. It is easy to imagine that they could therefore transiently change from a radioresistant to a radiosensitive state and back again if flow then resumes.

The bioreductive stains are also difficult to interpret. Three of those that are currently available, NITP, EO5 and Pimonidazole, are nitro-imidazoles with side chains that are detectable by antibodies (23–26). These are metabolized by an enzyme requiring process, but only in conditions of low oxygenation. They are then converted to a bound product that can be detected by immunohistochemistry. The pro-stain is normally kept in contact with the cells for at least an hour or two in order to get a good distinction between the signal and noise from background staining. Indeed, with Pimonidazole, which is currently undergoing clinical tests, exposure times of at least a day are being used between injection and removal of the biopsy (97, 98). Since no distinction has been considered necessary between acute and chronic hypoxia, this long interval has not been considered important. We predict that this may lead to difficulties in interpreting the predictive value of the data. A cell will bind the stain when it is at a point in the nutrient gradient where it is still biochemically active, though it has reached a critically low level of oxygen. It seems to us unlikely that such a cell will be in the same state many hours or even days later.

The modelling in this paper emphasizes the importance of having more information about the kinetics of hypoxia, about the availability of other nutrients and the critical levels of both oxygen and glucose that are needed to tip a cell from hypoxic protection to hypoxic radiosensitization. These data are needed in order to interpret the results that are coming from current tests of predictive assays, which seek correlations with clinical responses. A measurement of energy charge may have more prognostic value. If a technique can be developed to distinguish clearly between acute and chronic hypoxia, it will then be possible to test the predictions we have made. At present it seems to us unlikely that composite estimates of hypoxic fractions will have good prognostic or predictive value, since they may contain different contributions from resistant acute and sensitive chronically hypoxic cells.

The present modelling also invites an alternative way of considering the estimates of SF_2 that are being made in individual tumours. Some authors have found these to have predictive value, whereas others have not (5–13). Our modelling confirms that very sensitive cells should be more readily cured, but only at the expense of unacceptable levels of normal tissue injury. This has already been encountered in several clinics when Ataxia Telangiectasia patients have been treated. For the more usual range of radiosensitivities ($SF_2 = 0.5$–0.8) we have shown that there may be a close link between the outcome of treatment and the *combination* of SF_2 and chronic hypoxia, which will not be anticipated from either assay alone. Indeed these modelling studies provide an explanation for the poor absolute correlation between the SF_2 and the dose predicted to cure the tumours. Those studies in which a wide range of SF_2 values have been found, show a similar rank order to the clinical tumour outcome, but no direct quantitative link. A 10 g tumour with an SF_2 of 0.1 should have a TCD_{50} of 18 Gy, whereas one with an SF_2 of 0.9 would require 400 Gy. In reality, most of these tumours show between 5 and 95% local control in the commonly used clinical range of 60–70 Gy. This would not be predicted from classical radiobiology but would be predicted from these new modelling studies (Table 1). It is intriguing to note that glioblastomas have been reported to have MRS spectra and bioluminescence patterns that are more like normal brain than most tumours (93, 94), and are known to have a very rich capillary network. This could be part of the explanation for their exceptional radioresistance, since they may not be benefiting from the sensitizing effect of chronic hypoxia. If they have SF_2 values in the region of 0.8, we would predict that doses in excess of 200 Gy would be needed to cure them, unless some way can be found of increasing their level of energy depletion. This could explain why no benefit has been seen with altered fractionation or chemical modifiers that offer a gain of only 10–50% in dose effectiveness.

Further modelling and experimental studies are needed to consider these predictions in detail. The computations, so far, indicate that increasing the level of chronic hypoxia before each radiation treatment might be a very effective strategy for cancer therapy with both radiation and chemotherapeutic drugs. Cells resist the effects of all DNA-targeted cytotoxic agents by DNA repair mechanisms and it is highly likely that a similar form of inducible repair occurs in response to low doses of common chemotherapeutic agents. Increased energy depletion (chronic hypoxia) could be achieved with mild hyperthermia, non-metabolizable glucose substitutes, reduced inhaled oxygen for half an hour before therapy, or drugs such as hydralazine or BW12C. If these were combined with reoxygenation immediately before and during irradiation, we would predict a further beneficial result. On the other hand, simply increasing the access to oxygen, especially for a prolonged period that allows cellular energy stores to be replenished, would be predicted to be counterproductive. It is interesting to note that little benefit of hyperbaric oxygen or normobaric carbogen was seen when prolonged pre-irradiation breathing times were used, either in laboratory animals or in the clinic (103). We can now speculate that these studies may have permitted restoration of the energy charge in some of the previously chronically hypoxic tumour cells and thereby abolished their biochemical radiosensitivity. By contrast, short pre-irradiation breathing times have been shown to be an advantage in experimental animals (103) and have recently led to surprisingly good clinical results in advanced head and neck tumours when combined with nicotinamide (104). Since chemical substitutes for oxygen cannot be used in metabolism, the offsetting of energy charge against radiosensitization should not be such a problem as with

oxygen itself (105). This may explain why the non-toxic but weakly electron-affinic sensitizers, metronidazole and nimorazole, have been so effective in several clinical series (106–109).

CONCLUSION

We believe that the concepts that have emerged from the adoption of the inducible repair variant of the Linear Quadratic model have opened many new horizons in the field of applied radiobiology. It is time for a paradigm shift. The central dogma of considering all hypoxic cells to have the same radioresistance is over-simplistic and not supported by laboratory data. The present calculations highlight the danger of oversimplified models. They indicate the urgent need for a re-evaluation of some of the most fundamental principles and concepts in radiobiology. When this takes place, a number of puzzling anomalies will perhaps be better explained and new approaches to utilizing chronic hypoxia as a weapon to cure radioresistant tumours, perhaps even gliomas, may be recognized. More complex modelling now needs to be undertaken to incorporate gradients of oxygen and hence of radioresistance, reoxygenation kinetics and the addition of cells due to proliferation during fractionated radiotherapy.

ACKNOWLEDGEMENTS

We are grateful to the Swedish Cancer Society and the Scott of Yews for providing the resources to initiate the Translational Research Group activities in Umeå. We also thank Lions Cancer Foundation and Umeå University for financial support. We are grateful to various colleagues for stimulating discussions, especially Jack Fowler, Bo Littbrand, Anamaria Rojas, Oliver Scott, Adolf Wainson and Tony Waites.

REFERENCES

1. Thames HD, Hendry JH. Fractionation in radiotherapy. London: Taylor & Francis, 1987.
2. Steel GG, ed. The radiobiology of human cells and tissues. Proc 15th L. H. Gray Conference. London: Taylor & Francis, 1989.
3. Potten CS, Hendry JH, eds. Cell clones: manual of mammalian cell techniques. Edinburgh: Churchill Livingstone, 1985.
4. Courtenay VD, Selby PJ, Smith IE, Mills J, Peckham MJ. Growth of human tumour cell colonies from biopsies using two soft-agar techniques. Br J Cancer 1978; 38: 77–81.
5. Arlett CF, Harcourt SA. Survey of radiosensitivity in a variety of human cell strains. Cancer Res 1980; 40: 926–32.
6. Fertil B, Malaise E-P. Inherent cellular radiosensitivity as a basic concept for human tumor radiotherapy. Int J Radiat Oncol Biol Phys 1981; 7: 621–9.
7. Duchesne GM, Peacock JH, Steel GG. The acute in vitro and in vivo radiosensitivity of human lung tumour lines. Radiother Oncol 1986; 7: 353–61.
8. Malaise E-P, Fertil B, Deschavanne PJ, Chavaudra N, Brock WA. Initial slope of radiation survival curves is characteristic of the origin of primary and established cultures of human tumor cells and fibroblasts. Radiat Res 1987; 111: 319–33.
9. Brock WA, Baker FL, Peters LJ. Radiosensitivity of human head and neck squamous cell carcinomas in primary culture and its potential as a predictive assay of tumor radiocurability. Int J Radiat Biol 1989; 56: 751–60.
10. West CM, Scott D, Peacock JH. The Association for Radiation Research First Workshop: report. Normal cell radiosensitivity: clinical application in predicting response to radiotherapy and cancer predisposition 21–23 March, 1994, Westlakes Research Institute, Cumbria, UK. Int J Radiat Biol 1994; 66: 231–4.
11. West CM. Invited review: intrinsic radiosensitivity as a predictor of patient response to radiotherapy. Br J Radiol 1995; 68: 827–37.
12. Lambin P, Malaise EP, Joiner MC. Might intrinsic radioresistance of human tumour cells be induced by radiation? Int J Radiat Biol 1996; 69: 279–90.
13. Bjork-Eriksson T, West CM, Karlsson E, et al. The in vitro radiosensitivity of human head and neck cancers. Br J Cancer 1998; 77: 2371–5.
14. Begg AC, Hofland I, Moonen L, et al. The predictive value of cell kinetic measurements in a European trial of accelerated fractionation in advanced head and neck tumors: an interim report. Int J Radiat Oncol Biol Phys 1990; 19: 1449–53.
15. Wilson GD, Dische S, Saunders MI. Studies with bromodeoxyuridine in head and neck cancer and accelerated radiotherapy. Radiother Oncol 1995; 36: 189–97.
16. Fowler JF, Lindstrom MJ. Loss of local control with prolongation in radiotherapy. Int J Radiat Oncol Biol Phys 1992; 23: 457–67.
17. Gray LH, Conger AD, Ebert M, Hornsey S, Scott OCA. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol 1953; 26: 638–48.
18. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer 1955; 9: 539–49.
19. Denekamp J, Joiner MC. The potential benefit from a perfect radiosensitizer and its dependence on reoxygenation. Br J Radiol 1982; 55: 657–63.
20. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 1989; 49: 644–65.
21. Stone HB, Brown JM, Phillips TL, Sutherland RM. Oxygen in human tumors: correlations between methods of measurement and response to therapy. Summary of a workshop held November 19–20, 1992, at the National Cancer Institute, Bethesda, Maryland. Radiat Res 1993; 136: 422–34.
22. Vaupel P, Thews O, Kelleher DK, Hoeckel M. Current status of knowledge and critical issues in tumor oxygenation. Results from 25 years' research in tumor pathophysiology. Adv Exp Med Biol 1998; 454: 591–602.
23. Hodgkiss RJ, Begg AC, Middleton RW, et al. Fluorescent markers for hypoxic cells. A study of novel heterocyclic compounds that undergo bio-reductive binding. Biochem Pharmacol 1991; 41: 533–41.
24. Hodgkiss RJ, Webster L, Wilson. GD. Development of bioreductive markers for tumour hypoxia. Acta Oncol 1995; 34: 351–5.
25. Evans SM, Jenkins WT, Joiner B, Lord EM, Koch CJ. 2-Nitroimidazole (EF5) binding predicts radiation resistance in individual 9L s.c. tumors. Cancer Res 1996; 56: 405–11.
26. Raleigh JA, Miller GG, Franko AJ, Koch CJ, Fuciarelli AF, Kelly DA. Fluorescence immunohistochemical detection of hypoxic cells in spheroids and tumours. Br J Cancer 1987; 56: 395–400.

27. Brown JM. Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. Br J Radiol 1979; 52: 650–6.
28. Chaplin DJ, Durand RE, Olive PL. Acute hypoxia in tumors: implications for modifiers of radiation effects. Int J Radiat Oncol Biol Phys 1986; 12: 1279–82.
29. Pigott KH, Hill SA, Chaplin DJ, Saunders MI. Microregional fluctuations in perfusion within human tumours detected using laser Doppler flowmetry. Radiother Oncol 1996; 40: 45–50.
30. Rofstad EK, DeMuth P, Fenton BM, Ceckler TL, Sutherland RM. ^{31}P NMR spectroscopy and HbO_2 cryospectrophotometry in prediction of tumor radioresistance caused by hypoxia. Int J Radiat Oncol Biol Phys 1989; 16: 919–23.
31. Okunieff P, McFarland E, Rummeny E, et al. Effects of oxygen on the metabolism of murine tumors using in vivo phosphorus-31 NMR. Am J Clin Oncol 1987; 10: 475–82.
32. Adams GE, Bremner JC, Stratford IJ, Wood PJ. Can ^{31}P magnetic resonance spectroscopy measurements of changes in experimental tumour metabolism be related to modification of oxygenation status. Br J Radiol 1992; 23 (Suppl 24): 137–41.
33. Gerweck LE, Koutcher JA, Zaidi ST, Seneviratne T. Energy status in the murine FSaII and MCaIV tumors under aerobic and hypoxic conditions: an in vivo and in vitro analysis. Int J Radiat Oncol Biol Phys 1992; 23: 557–61.
34. Gerweck LE, Koutcher J, Zaidi ST. Energy status parameters, hypoxia fraction and radiocurability across tumor types. Acta Oncol 1995; 34: 335–8.
35. Chadwick KH, Leenhouts HP. A molecular theory of cell survival. Phys Med Biol 1973; 18: 78–87.
36. Douglas BG, Fowler JF. Fractionation schedules and a quadratic dose-effect relationship. Br J Radiol 1975; 48: 502–4.
37. Barendsen GW. Dose fractionation, dose rate and iso-effect relationships for normal tissue responses. Int J Radiat Oncol Biol Phys 1982; 8: 1981–97.
38. Joiner MC, Denekamp J, Maughan RL. The use of 'top-up' experiments to investigate the effect of very small doses per fraction in mouse skin. Int J Radiat Biol 1986; 49: 565–80.
39. Joiner MC, Johns H. Renal damage in the mouse: the response to very small doses per fraction. Radiat Res 1988; 114: 385–98.
40. Marples B, Joiner MC. The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. Radiat Res 1993; 133: 41–51.
41. Lambin P, Marples B, Fertil B, Malaise EP, Joiner MC. Hypersensitivity of a human tumour cell line to very low radiation doses. Int J Radiat Biol 1993; 63: 639–50.
42. Marples B, Joiner MC, Skov KA. The effect of oxygen on low-dose hypersensitivity and increased radioresistance in Chinese hamster V79–379A cells. Radiat Res 1994; 138: S17–20.
43. Singh B, Arrand JE, Joiner MC. Hypersensitive response of normal human lung epithelial cells at low radiation doses. Int J Radiat Biol 1994; 65: 457–64.
44. Wouters BG, Skarsgard LD. The response of a human tumor cell line to low radiation doses: evidence of enhanced sensitivity. Radiat Res 1994; 138: S76–80.
45. Joiner MC, Lambin P, Malaise EP, et al. Hypersensitivity to very-low single radiation doses: its relationship to the adaptive response and induced radioresistance. Mutat Res 1996; 358: 171–83.
46. Wouters BG, Sy AM, Skarsgard LD. Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. Radiat Res 1996; 146: 399–413.
47. Wouters BG, Skarsgard LD. Low-dose radiation sensitivity and induced radioresistance to cell killing in HT-29 cells is distinct from the 'adaptive response' and cannot be explained by a subpopulation of sensitive cells. Radiat Res 1997; 148: 435–42.
48. Alsbeih G, Raaphorst GP. Radiosensitive human cells at the crossroads: high split-dose recovery and diminished inducible response. Radiation Research Society Meeting, Rhode Island, May 1997: 162. (abst).
49. Daşu A, Denekamp J. Inducible repair and intrinsic radiosensitivity: a complex but predictable relationship. Radiat Res 1999 (in press).
50. Daşu A, Denekamp J. New insights into factors influencing the clinically relevant oxygen enhancement ratio. Radiother Oncol 1998; 46: 269–77.
51. Denekamp J, Daşu A, Waites A. Vasculature and microenvironmental gradients: the missing links in novel approaches to cancer therapy? Adv Enzyme Regul 1998; 38: 281–99.
52. Denekamp J, Daşu A, Waites A, Littbrand B. Hyperfractionation as an effective way of overcoming radioresistance. Int J Radiat Oncol Biol Phys 1998; 42: 705–9.
53. Daşu A, Denekamp J. Superfractionation as a potential hypoxic cell radiosensitiser: prediction of an optimum dose per fraction. Int J Radiat Oncol Biol Phys 1999; 43: 1083–94.
54. Hall EJ, Bedford JS, Oliver R. Extreme hypoxia; its effect on the survival of mammalian cells irradiated at high and low dose-rates. Br J Radiol 1966; 39: 302–7.
55. Howard A. The oxygen requirement for recovery in split-dose experiments with Oedogonium. Int J Radiat Biol 1968; 14: 341–50.
56. Berry RJ, Hall EJ, Cavanagh J. Survival of mammalian cells in confluent culture ('stationary phase') irradiated under aerobic and hypoxic conditions: its implications for radiotherapy. Br J Radiol 1969; 42: 719.
57. Hall EJ, Cavanagh J. The effect of hypoxia on recovery of sublethal radiation damage in Vicia seedlings. Br J Radiol 1969; 42: 270–7.
58. Hall EJ. The effect of hypoxia on the repair of sublethal radiation damage in cultured mammalian cells. Radiat Res 1972; 49: 405–15.
59. Nagle WA, Moss Jr. AJ, Roberts Jr. HG, Baker-Max L. Effects of 5-thio-D-glucose on cellular adenosine triphosphate levels and deoxyribonucleic acid rejoining in hypoxic and aerobic Chinese hamster cells. Radiology 1980; 137: 203–11.
60. Spiro IJ, Kennedy KA, Stickler R, Ling CC. Cellular and molecular repair of x-ray-induced damage: dependence on oxygen tension and nutritional status. Radiat Res 1985; 101: 144–55.
61. Ling CC, Robinson E, Shrieve DC. Repair of radiation induced damage—dependence on oxygen and energy status. Int J Radiat Oncol Biol Phys 1988; 15: 1179–86.
62. Trott KR. Tumour stem cells: the biological concept and its application in cancer treatment. Radiother Oncol 1994; 30: 1–5.
63. Denekamp J. Tumour stem cells: facts, interpretation and consequences. Radiother Oncol 1994; 30: 6–10.
64. Koval TM. Multiphasic survival response of a radioresistant lepidopteran insect cell line. Radiat Res 1984; 98: 642–8.
65. Marples B, Joiner MC, Skov KA. An x-ray inducible repair response: evidence from high resolution survival measurements in air and hypoxia. In: Sugahara T, Sagan LA, Aoyama T, eds. Low dose irradiation and biological defense mechanisms. Amsterdam: Elsevier, 1992: 295–8.

66. Durand RE, Sutherland RM. Effects of intercellular contact on repair of radiation damage. Exp Cell Res 1972; 71: 75–80.
67. Sutherland RM, Durand RE. Cell contact as a possible contribution to radiation resistance of some tumours. Br J Radiol 1972; 45: 788–9.
68. Durand RE, Sutherland RM. Radiation-resistant tumor cells may be more sensitive in vitro. Cancer Res 1972; 32: 2587–8.
69. Alper T. Cellular radiobiology. Cambridge: Cambridge University Press, 1979.
70. Beck-Bornholdt H-P, Maurer T, Becker S, Omniczynski M, Vogler H, Würschmidt F. Radiotherapy of the rhabdomyosarcoma R1H of the rat: hyperfractionation-126 fractions applied within 6 weeks. Int J Radiat Oncol Biol Phys 1989; 16: 701–5.
71. Hamilton CS, Denham JW, O'Brien M, et al. Underprediction of human skin erythema at low doses per fraction by the linear quadratic model. Radiother Oncol 1996; 40: 23–30.
72. Reinhold HS. The influence of radiation on blood vessels and circulation. Chapter IV. Structural changes in blood vessels. Curr Top Radiat Res Q 1974; 10: 58–74.
73. Reinhold HS, Blachiwiecz B, Blok A. Oxygenation and reoxygenation in 'sandwich' tumours. Bibl Anat 1977; 270–272.
74. Ward-Hartley KA, Jain RK. Effect of glucose and galactose on microcirculatory flow in normal and neoplastic tissues in rabbits. Cancer Res 1987; 47: 371–7.
75. Foltz RM, McLendon RE, Friedman HS, Dodge RK, Bigner DD, Dewhirst MW. A pial window model for the intracranial study of human glioma microvascular function. Neurosurgery 1995; 36: 976–84.
76. Kimura H, Braun RD, Ong ET, et al. Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. Cancer Res 1996; 56: 5522–8.
77. Alper T, Howard-Flanders P. The role of oxygen in modifying the radiosensitivity of E. coli B. Nature, Lond 1956; 178: 978–9.
78. Deschner E, Gray LH. Influence of oxygen tension on x-ray-induced chromosomal damage in Erlich ascites tumor cells irradiated in vitro and in vivo. Radiat Res 1959; 11: 115–46.
79. Koch CJ, Kruuv J, Frey HE. Variation in radiation response of mammalian cells as a function of oxygen tension. Radiat Res 1973; 53: 33–42.
80. Michael BD, Adams GE, Hewitt HB, Jones WB, Watts ME. A posteffect of oxygen in irradiated bacteria: a submillisecond fast mixing study. Radiat Res 1973; 54: 239–51.
81. Koch CJ, Meneses JJ, Harris JW. The effect of extreme hypoxia and glucose on the repair of potentially lethal and sublethal radiation damage by mammalian cells. Radiat Res 1977; 70: 542–51.
82. Watts ME, Maughan RL, Michael BD. Fast kinetics of the oxygen effect in irradiated mammalian cells. Int J Radiat Biol 1978; 33: 195–9.
83. Franko AJ, Sutherland RM. Rate of death of hypoxic cells in multicell spheroids. Radiat Res 1978; 76: 561–72.
84. Franko AJ, Sutherland RM. Radiation survival of cells from spheroids grown in different oxygen concentrations. Radiat Res 1979; 79: 454–67.
85. Kwok TT, Sutherland RM. Reoxygenation associated radiosensitization after chronic hypoxia: effect of temperature and oxygen tension. Int J Radiat Oncol Biol Phys 1992; 22: 411–4.
86. Gerweck LE, Seneviratne T, Gerweck KK. Energy status and radiobiological hypoxia at specified oxygen concentrations. Radiat Res 1993; 135: 69–74.
87. Hirst DG, Denekamp J. Tumour cell proliferation in relation to the vasculature. Cell Tissue Kinet 1979; 12: 31–42.
88. Hirst DG, Denekamp J, Hobson B. Proliferation kinetics of endothelial and tumour cells in three mouse mammary carcinomas. Cell Tissue Kinet 1982; 15: 251–61.
89. Denekamp J, Michael BD, Harris SR. Hypoxic cell radiosensitisers: Comparative tests of some electron affinic compounds using epidermal cell survival in vivo. Radiat Res 1974; 60: 119–32.
90. Mueller-Klieser W, Schaefer C, Walenta S, Rofstad EK, Fenton BM, Sutherland RM. Assessment of tumor energy and oxygenation status by bioluminescence, nuclear magnetic resonance spectroscopy, and cryospectrophotometry. Cancer Res 1990; 50: 1681–5.
91. Nordsmark M, Keller J, Nielsen OS, Lundorf E, Overgaard J. Tumour oxygenation assessed by polarographic needle electrodes and bioenergetic status measured by ^{31}P magnetic resonance spectroscopy in human soft tissue tumours. Acta Oncol 1997; 36: 565–71.
92. Robinson SP, Howe FA, Rodrigues LM, Stubbs M, Griffiths JR. Magnetic resonance imaging techniques for monitoring changes in tumor oxygenation and blood flow. Semin Radiat Oncol 1998; 8: 197–207.
93. Hossmann KA, Linn F, Okada Y. Bioluminescence and fluoroscopic imaging of tissue pH and metabolites in experimental brain tumors of cat. NMR Biomed 1992; 5: 259–64.
94. Oberhaensli RD, Hilton-Jones D, Bore PJ, Hands LJ, Rampling RP, Radda GK. Biochemical investigation of human tumours in vivo with phosphorus-31 magnetic resonance spectroscopy. Lancet 1986; 2: 8–11.
95. Rasey JS, Koh WJ, Evans ML, et al. Quantifying regional hypoxia in human tumors with positron emission tomography of [18F]fluoromisonidazole: a pretherapy study of 37 patients. Int J Radiat Oncol Biol Phys 1996; 36: 417–28.
96. Kallinowski F, Zander R, Hoeckel M, Vaupel P. Tumor tissue oxygenation as evaluated by computerized-pO_2-histography. Int J Radiat Oncol Biol Phys 1990; 19: 953–61.
97. Arteel GE, Thurman RG, Raleigh JA. Reductive metabolism of the hypoxia marker pimonidazole is regulated by oxygen tension independent of the pyridine nucleotide redox state. Eur J Biochem 1997; 253: 743–50.
98. Varia MA, Calkins-Adams DP, Rinker LH, et al. Pimonidazole: a novel hypoxia marker for complementary study of tumor hypoxia and cell proliferation in cervical carcinoma. Gynecol Oncol 1998; 71: 270–7.
99. Waites A, Denekamp J. Computer simulation of microelectrode measurements of regional oxygen tensions in a corded tumour model. 1999 (in preparation).
100. Höckel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996; 56: 4509–15.
101. Kolstad P. Intercapillary distance, oxygen tension and local recurrence in cervix cancer. Scand J Clin Lab Invest 1968; 106 (Suppl): 145–57.
102. Nordsmark M, Overgaard M, Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 1996; 41: 31–9.
103. Siemann DW, Hill RP, Bush RS. The importance of the pre-irradiation breathing times of oxygen and carbogen (5% CO_2: 95% O_2) on the in vivo radiation response of a murine sarcoma. Int J Radiat Oncol Biol Phys 1977; 2: 903–11.
104. Kaanders JH, Pop LA, Marres HA, et al. Accelerated radiotherapy with carbogen and nicotinamide (ARGON) for laryngeal cancer. Radiother Oncol 1998; 48: 115–22.

105. Suit HD, Brown JM. Relative efficacy of high-pressure oxygen and misonidazole to reduce TCD_{50} of a mouse mammary carcinoma. Br J Radiol 1979; 52: 159–60.
106. Balmukhanov SB, Aitkulova ZK, Mustafin ZS, Rismukhamedova RS, Filippenko VI. Intratumoral and parametric administration of metronidazole in the radiotherapy of cancer of the cervix. Med Radiol Mosk 1989; 34: 26–30.
107. Pereslegin IA, Mufazalov FF. The frequency of recurrences and metastases of bladder cancer following a radical course of radiotherapy. Vestn Rentgenol Radiol 1992; 57–8.
108. Andreev VG, Mardynskii IS, Molotkova NG, et al. Combined therapy of malignant tumors of the upper jaw and nasal cavity using pre- and post-operative radiation with metronidazole radiomodification. Vopr Onkol 1996; 42: 81–3.
109. Overgaard J, Hansen HS, Overgaard M, et al. A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5–85. Radiother Oncol 1998; 46: 135–46.