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The Use of Non-clonogenic Assays in Measuring the Response of Cells *In vitro* to Ionising Radiation

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THERE HAS been an enthusiastic return to the *in vitro* assessment of response of tumour cells to ionising radiation following the appreciation that the intrinsic radiosensitivity of tumour cells may be a major determinant of clinical outcome in some tumours [1, 2], and that normal tissue cellular radiosensitivity may determine the observed normal tissue reactions *in vivo* [3]. Such focusing of radiobiological thought has led to the development of an ever-increasing number of *in vitro* assays that are reported to rapidly assess cellular response to ionising radiation. But do they?

Clonogenic assays are the established method of measuring the response of cells to ionising radiation. Survival is assessed by comparing the number of colonies that develop from treated and untreated single cell suspensions. A colony is defined as a group of 50 or more cells that have developed from a single cell. Clonogenic assays thus measure the proportion of cells that have not only survived, but have retained their reproductive capacity following treatment. Clonogenic survival is a useful endpoint for measuring response to ionising radiation for three reasons. Firstly, the regenerative capacity of cells is thought to be the most important factor in determining response both of renewing normal tissue and tumour cells. Secondly, clonogenic assays are usually highly reproducible, suitable for many *in vitro* cell lines and have been related to gross tumour response in a few experimental systems [4]. Finally, analysis and interpretation of the shape and composition of clonogenic survival curves have provided suggestions as to the underlying mechanisms of cellular response to ionising irradiation.

Non-clonogenic assays, however, are becoming increasingly popular for measuring the response of cells to ionising radiation as they provide a result in a shorter time and can be used for cells which do not grow as colonies. Such assays include growth assays and DNA damage assays. How accurately do they measure the cellular response to ionising radiation?

GROWTH ASSAYS

Growth assays estimate survival by comparing the total number of viable cells in treated and untreated cultures [5]. The recent development of new systems for primary culture of human tumours [6] and the hope of measurement of *in vitro* radiosensitivity of individual tumours that may be used to predict outcome of clinical radiotherapy treatment [7], have renewed interest in growth assays for the assessment of response of cells to ionising radiation. However, little attention has been given to the validity and interpretation of growth assays when adopted for assessing survival of a primary mass culture of human tumour cells following treatment with ionising radiation and, subsequently, the literature is becoming full of spurious values of the intrinsic radiosensitivity of human tumour cells.

THE GENERAL PRINCIPLES OF GROWTH ASSAYS

A mass culture of tumour cells are treated and then allowed to proliferate. Measurement of viable cell numbers in control and treated cultures is made at frequent (usually daily) intervals. Growth curves are then constructed (Figure 1). For untreated cells, after a short lag period growth is exponential and then later growth rate decreases when the nutrients in the medium are depleted or when the culture becomes confluent, i.e. saturation density is reached. Cells treated with ionising radiation show an initial dose-dependent lag phase and reduction in cell number followed by exponential regrowth at the same rate as the unirradiated controls. The efficacy of treatment is indicated by quantifying the difference in growth of the treated and untreated cultures.

Various methods of measuring the number of viable cells in culture have been developed and are summarised in Table 1.

Growth curves are constructed from serial measurements of cell numbers (Figure 1). Simply comparing cell numbers at any fixed time after irradiation will not produce meaningful surviving fractions. This is due both to the dose-dependent lag period after irradiation before regrowth is obtained and for the time taken by control cultures to reach confluence. The endpoint in a growth

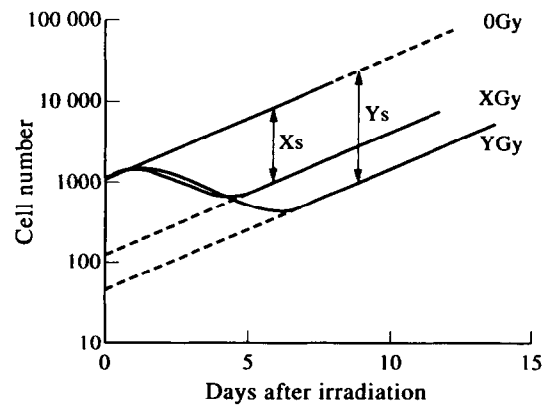

Figure 1. Calculation of radiation-induced cell kill from cell growth curves. Cell numbers are counted at regular intervals and growth curves (——) are constructed for untreated cells (0Gy) and cells treated with increasing doses of irradiation (XGy and YGy). Survival fractions at XGy and YGy are measured as Xs and Ys.

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Table 1.

Method of measuring growth of cells	Cell function being measured	Advantages	Disadvantages
1. Measurement of cell number	Direct e.g. Coulter counter or haemocytometer		1. Dead cells not always distinguished 2. Expensive automated systems
2. Dye exclusion	Membrane integrity	Cell morphology visualised	Labour intensive
3. Isotope precursor uptake (e.g. [^{3}H-] thymidine [^{35}S-] methionine	DNA or protein synthesis	1. Automated 2. Few false positives	DNA synthesis delay post-treatment
4. Measurement of cell metabolism	E.g. glucose or tetrazolium reduction	Automated	Non-linear relationship with cell number

assay when adopted to measure cell survival is the ability of the total cell population to regain the growth rate of the control population. Surviving fractions are thus obtained when treated cultures attain exponential regrowth at the control growth rate. When cell number is counted on just one day using one innoculum size, spurious results are obtained. If the timing is too soon after treatment, doomed cells will have not been cleared from the culture and an overestimate of survival will be obtained, and if too late, control cells will have reached confluence again leading to an over-estimate of surviving cell number. Figure 2a shows an example of the survival curves which can be obtained in these situations.

To overcome this, the following three approaches can be used to derive cell survival curves:

Back extrapolation of growth curves

Growth of control and treated cultures is measured over time. Cell number (log) is plotted as a function of time. Once doomed cells have been lost from the population and treated cells are proliferating exponentially, back extrapolation of growth curves provides an estimate of the number of cells that were lethally treated (Figure 1). This method is quite sensitive to small changes in growth rate. Culture conditions can be adapted to encourage the maintenance of exponential growth, for example, regular dilution of cell suspensions to allow unrestricted cell proliferation.

Vertical displacement of growth curves

When treated cultures regrow at the same rate as controls, evaluation of the vertical displacement of the curves can provide an estimate of the level of cell kill (Figure 1). Multiple estimations of survival can be made at various time intervals during the exponential growth phase. Problems arise with this method if treated cultures have not achieved exponential growth at the time when the rate of growth of control cultures is decreasing due to confluency. Forward extrapolation of control curves may be required to overcome this. Also, a single day may not be sufficient to obtain survival fractions for the full range of doses: the lower dose cultures may have regained exponential regrowth, but the higher dose cultures are still in the lag phase, so that the first part of the curve will be an accurate measure of survival but the latter part will not.

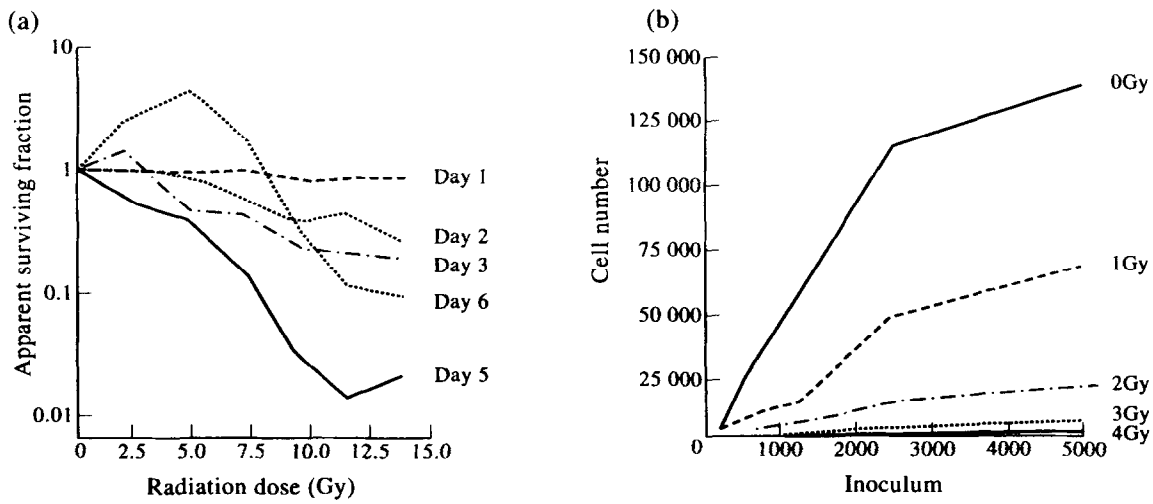

Figure 2. (a) Apparent survival curves for analysis of growth data at different times. An example of the radiation effect on a human bladder carcinoma cell line, MGH-U1, where the ratio of cell numbers has been calculated on several days. (b) Method of graded inocula. The plating of multiple cell numbers obviates the need for analysis on several days. (Redrawn from Price and McMillan, 1990.)

Method of graded inocula

An alternative approach which does not require measurements to be made over a wide range of time intervals is for the treated and control cell suspensions to be plated out at a wide range of cell numbers and then at a chosen time interval to determine growth as a function of size of inoculum. Results obtained in this way are shown in Figure 2b. A sighting experiment to define the time-course of growth of treated and control cultures is necessary to choose the optimum time at which to carry out the measurements, that is when the majority of cultures show active regrowth. Curves of estimated cell number against inoculum size usually show a roughly linear initial region, followed by a tendency to saturate. The slope of this initial region provides a good measure of surviving cell number and the ratio of treated slope to control slope gives a reliable indication of surviving fraction. This method is slightly less reliable than that of vertical displacement of growth curves [8], probably because only one point at each dose level is obtained, although this point does incorporate a number of points relating cell number to cell inoculum. This method may be attractive as only recordings on 1 day are required, but it will be totally unreliable if carried out blind, and the time course of treated and control cultures has not already been determined and the day when growth curves are parallel known.

Importantly, growth assays need to be used in this way to obtain absolute levels of fractional cell kill. They are frequently used in comparing levels of cell kill *in vitro* with ranges of chemotherapeutic drugs. In this instance, a comparison of level of cell kill on 1 day may be adequate if only a relative comparison of cell kill is required.

COMPARISON OF GROWTH ASSAY AND CLONOGENIC ASSAY

Many groups report the unreliability of growth assays in assessing the response of cells to ionising irradiation which is explained by Figure 2a. However, if full construction of survival curves is performed as outlined above instead of fractional cell kill determined at an arbitrary time after irradiation, good correlation is seen between growth assays and clonogenic assays [5,8]. In a study on tumour biopsy material [9] a discrepancy was found between the absolute values for sensitivity as assessed by clonogenic and growth assays but the ranking of the samples by both assays was quite similar. This may be sufficient for some studies (e.g. predicting tumour cell radiosensitivity) but because of the various factors discussed in this paper the clonogenic assay is likely to be the most reproducible so it is generally desirable to reflect this end-point accurately.

ADVANTAGES AND DISADVANTAGES OF GROWTH ASSAYS

Growth assays may provide advantages for measurement of response of cells to ionising radiation. Firstly, they are useful when cells do not grow as colonies. Measurement of the response of tumour cells grown in suspension, on monolayer and in spheroids is possible. This may, in fact, find its greatest usefulness in the asessment of the radiosensitivity of non-tumour cells, e.g. lymphocytes and fibroblasts, where clonogenic assays are difficult as colony formation can be inconsistent. Secondly, growth assays are not greatly affected by the inability to produce reliable single cell suspensions. As long as the suspensions can be aliquoted evenly the growth of clumps of cells does not affect the results. Thirdly, growth assays can often provide a result in a much shorter time than clonogenic assays, with approximately 2 weeks usually being sufficient as compared with 3–4 weeks for clonogenic assays. This is useful for screening for variation in clonal sensitivity and screening for radiation response modifiers, and may be useful for predictive testing of radiosensitivity in human tumours.

The disadvantages of growth assays include firstly the timing of the assay following treatment—incorrect timing can lead to very misleading results. It is not possible to stipulate a single time which will be suitable for all situations as it depends not only on the treatment modality used, but also on the particular cells examined and to some extent on the level of treatment (i.e. dose). Secondly, achieving satisfactory growth of cells, especially from human tumour biopsy material still represents a major problem. Contamination with normal cells, which can quickly outgrow the tumour cells is a common finding in such assays (see below). This is not a problem with clonogenic assays using agar as generally fibroblasts do not grow under these conditions. Attempts to preferentially grow tumour cells have met with a little success but are still far from ideal [10,11]. Even if growth of tumour cells is achieved it can be very slow and this in itself may prove to be a problem in growth assays. Finally, the most serious disadvantage is that if growth assays are not interpreted correctly they may give erroneous results.

DNA DAMAGE ASSAYS AS AN ALTERNATIVE MEASURE OF RADIOSENSITIVITY

As more is learnt about the sub-cellular processes which can lead to cell death following irradiation, new end-points can be utilised to assess radiosensitivity. Perhaps the best correlations between cell death and DNA damage are at the level of chromosomal damage. Detailed analysis of radiation-induced chromosomal aberrations can relate very accurately to cell death in some cells [12]. As a predictive clinical test of radiosensitivity this has the advantages that it requires limited cell proliferation and relatively few cells. The main disadvantage is that it is extremely time-consuming and requires great expertise. Micronucleus and premature chromosome condensation assays detect chromosomal fragments and, at least in normal cells, this correlates well with cell death. Micronuclei are relatively easy to score and have been assessed directly in biopsy material taken from irradiated tumours [13]. Unfortunately, the relationship between micronuclei and cell death cell can be lost in cells which have an abnormal karyotype [14,15] so that this assay may only be useful in the assessment of normal cell sensitivity.

The use of DNA damage assays, specifically those which measure DNA double strand breaks (dsb), have also been explored for their potential as measures of cellular radiosensitivity. The assays for this have been discussed previously [16]. Unfortunately, it is becoming evident that a number of different factors may determine the cellular response to irradiation [17]. Thus it is not surprising that a single end-point from DNA damage assays cannot be used as a measure of sensitivity. The level of DNA damage induced ([18], Whitaker and McMillan, submitted), the rate or repair ([19], Whitaker and McMillan, submitted) and the final level of residual damage [20, 21] have been shown to be related in some way to cell survival in some cell systems. While a single end-point may prove to be dominant so that it can be used alone, it is likely that a combination of end-points will need to be used.

A review of studies which have examined the molecular basis of radiation sensitivity inevitably comes to the conclusion, as described above, that no single part of the post-irradiation DNA damage process is the sole determinant of this phenotype.

However, the feeling is developing that there may be a feature of DNA packaging and chromatin structure which may be influential at several of the post-irradiation steps [22]. In the context of predictive testing this is exemplified by the study of Lynch *et al.* [23] who found a correlation between the extent of relaxation of nuclear supercoiling after irradiation and the response to radiotherapy of patients with transitional cell carcinoma of the bladder. The endpoint used, nucleoid expansion measured by flow cytometry, was influenced by the level of initial damage inflicted and the extent of DNA supercoiling. Thus these two endpoints may be linked and the possibility of assessing radiosensitivity by the measurement of an aspect of chromatin structure is raised. A similar endpoint incorporating a repair time has found a relationship between repair in lymphocytes and the incidence of bowel complications following radiotherapy [24].

As with other assays of tumour cell sensitivity from biopsy material DNA damage assays have to contend with the problem of normal cell contamination. With growth assays this problem is potentially great since normal cells can often out-grow tumour cells very quickly in culture. In clonogenic assays these can be a problem if the cloning efficiency of the tumour cells is low so that even a low proportion of normal cells can influence the results significantly. An advantage of clonogenic assays over DNA damage assays is that the normal cells potentially can be identified at the end of the assay and removed from the analysis. In growth assays normal cells can be identified but it is not so easy to eliminate these from the final result. Normal cell contamination cannot be identified at the end of damage assays; however, if the proportion of normal cells can be reduced initially to low levels (<5–10%) their influence on the results would be much less than the other assays. Various approaches have been tried to reduce the initial normal cell number including flow cytometry and differential attachment to substrates and in some cases this has been successful.

The potential advantages of DNA damage assays in clinical predictive testing are that they can be rapid, some need few cells, they may not need cell growth in tissue culture and some of them may allow the separation of tumour and normal cells in a single sample.

CONCLUSIONS

Interpreted correctly, growth assays can provide a reproducible measure of survival which compares well with clonogenic cell survival measurements. The assay can be used to obtain radiation cell survival curves faster than clonogenic assays and has distinct advantages when measuring radiation response in those cells which do not form colonies. The main disadvantage of growth assays is that an overestimate of survival is made if the assay is misinterpreted and survival measured when the cells are not in exponential growth. It is for this reason that growth assays have fallen into disrepute. However, if performed and interpreted correctly they provide a useful alternative to clonogenic assay.

DNA damage assays provide a hope for assessment of radiosensitivity due to their speed and requirment for few cells. Much work still needs to be performed, however, to identify the appropriate assay and end-point(s) for DNA damage measurement in this context.

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