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REPAIR MECHANISMS IN RADIATION-INDUCED CELL TRANSFORMATION

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Although mammalian cells in culture have been used for the past three decades for studies of radiation effects, it is only during the past 10 years or so that in vitro cell systems have been successfully applied to studies of radiation-induced neoplastic transformation. The initiation of such research must be regarded as a major advance since it made feasible, for the first time, studies of mechanism that had not been possible before then. It is evident that cells in culture cannot be expected to simulate cells in vivo in all regards. Still, as the work to be described will illustrate, cells in vitro permit an examination of questions that would be at best difficult to pursue with animals and also difficult, if not impossible, to address effectively via the application of epidemiological techniques.

Assay System

Advances that were made in the laboratory of the late Charles Heidelberger led in 1973 to the introduction of the 10T $\frac{1}{2}$ cell system (1). These cells were derived from a C3H mouse embryo, are fibroblast-like in appearance when growing attached to a substrate, and demonstrate contact inhibited or proximity-restricted growth which results in the cessation of division when the growth surface of a vessel is covered by a monolayer of cells. Morphologically transformed cells are identified as a colony of cells that overgrow the confluent sheet of cells, and two types of such cells produce fibrosarcomas when inoculated into appropriately prepared C3H mice (1, 2). Thus, the transformation of a cell by an initiator like ionizing radiation can be detected and can be expressed either as a frequency per cell exposed or a frequency per surviving cell. The distinction between these two probability statements is important only when cell killing is significant.

The details of the growth of 10T $\frac{1}{2}$ cells, and the analysis of the data from the experiments that we have performed with these cells, have been published (2-4). Suffice it to note here, that all experiments were performed with cells undergoing active asynchronous growth, Fig. 1. That is, first a stock culture of actively growing cells was used to make a suspension from which plastic dishes or flasks were inoculated at concentrations depending upon the dictates of a particular experiment. These cells were incubated for at least 24 hours, but usually for 44-48 hours, before they were irradiated to ensure further that they were in active asynchronous growth at the time of the initiation of treatment. Even when the experiment required irradiation over several days, cells were still in active growth before they were suspended for the survival and transformation assays. The migratory properties of 10T $\frac{1}{2}$ cells helped to ensure that they did not become locally crowded (5). Therefore, our results reflect the properties of cells undergoing active proliferation at the time of, and during, treatment. In contrast to the

Figure 1: Flow diagram of assays of transformation and survival, 10T½ cells.

approach that apparently has been adopted by others (see Fig. 5, ref. 6), cells in the stock cultures that we used were uncrowded and actively growing.

Transformation, Single Doses of High Dose Rate γ -Rays

As shown in Fig. 2, the survival curve of 10T½ cells exposed to a low linear energy transfer (low-LET) radiation, like ^{60}Co γ -rays, at a high dose rate is typical of what is observed with other lines of repair-competent cells. The transformation data were obtained from the same cultures and indicate the following: (a) the spontaneous frequency is $\sim 1 \times 10^{-5}$; (b) the frequency rises to a maximum when plotted per cell exposed as in Fig. 2; and (c) the transformation curve becomes parallel to the survival curve for doses larger than those at the maximum. We infer from point c that cells that are potentially transformed are equally susceptible to being killed by γ -rays as are nontransformed cells. Since a result similar to the one in Fig. 2 was obtained with fission-spectrum neutrons (2), a generalization seems reasonable that potentially transformed and nontransformed cells are equally at risk of being killed by ionizing radiation.

Because the final slopes of the curves in Fig. 2 are equal, factoring out the influence of cell killing results in a transformation curve that rises to a plateau. For γ -rays, the plateau is at a frequency of 3×10^{-5} per surviving cell, and for fission-spectrum neutrons, it is at 6×10^{-5} per surviving cell (2). The plateau for γ -rays could reflect a maximum and uniform probability of transformation of cells at all ages in the cell cycle, a probability of transformation that varies with cell age in the cycle, or a combination of these possibilities. Although studies with synchronized cells would be needed to settle this point, we favor a cell-age dependent probability since variations in response through the cell cycle are known for killing and mutation. Whatever might be the correct explanation for γ -rays will very likely not apply in all respects for fission-spectrum neutrons since, as noted, the plateau for this radiation is 2-fold higher.

Dose Rate Dependencies

To inquire if repair processes play a role in transformation, high dose rate fractionation experiments were performed with both X-rays and with fission-spectrum neutrons (2). From the results that were obtained, it was concluded that cells can repair both subeffective transformation and sublethal damage between two doses of a low-LET radiation but not of a high-LET radiation (2). To test further these conclusions, low dose rate experiments

were performed with γ -rays and with fission-spectrum neutrons. With γ -rays, the survival curve was shifted progressively upward as the dose rate was decreased from 100, to 2.5, to 0.5, and to 0.1 rad/min (3). In contrast, when the dose rate of fission-spectrum neutrons was dropped from 10.3-38 rad/min to 0.43 and to 0.086 rad/min, essentially no change in the survival curve resulted (4). The foregoing is consistent with what was observed when fractionated doses at high dose rates were used; namely, with γ -rays there was a significant increase in 2-dose survival whereas with fission-spectrum neutrons there was essentially no change in survival (2).

Figure 2: Survival and transformation curves of 10T_{1/2} cells exposed to ⁶⁰Co γ -rays. The transformation curve represents the probability per cell exposed, i.e., the probability with no accounting for cell killing. P.E. stands for plating efficiency; N for multiplicity. Uncertainties are standard errors. (From ref. 3.)

For the extremes of dose rates noted above, we plot the corresponding transformation frequencies, Fig. 3. Although data for the low dose regions are not shown, it is clear that reducing the dose rate has a qualitative effect for fission-spectrum neutrons opposite to that for γ -rays.

Repair and Misrepair of Transformation Damage

To discuss more fully the qualitative differences obtained with low- and high-LET radiations, we present first a biophysical model to explain the results obtained with γ -rays. The essential elements of this model, which have already been described in detail (3), are shown in Fig. 4.

Figure 3: The dose rate dependencies of the transformation of 10T½ cells exposed to ^{60}Co γ -rays or to JANUS fission-spectrum neutrons. Up to a total neutron dose of 100 rads at 0.086 rad/min, no change from that for unirradiated cells was observed in the distribution of cells in their cycle (4). At 0.1 rad/min of γ -rays, a similar result was obtained and, after a total dose of 400 rads, the cycle time was increased by only 20%. Other details as for Fig. 2.

In 1964, L. H. Gray received the Bertner Foundation Award from the M. D. Anderson Hospital. During the course of his lecture, Gray proposed that radiation-induced tumorigenesis resulted from the competition between two processes (7). The first was the increase in potentially transformed cells with dose, and the second was the dose dependence of cell killing. The net effect according to Gray was an induction curve for a given type of tumor that rose to a maximum and then declined approaching zero.

Gray applied his model to data on myeloid leukemia induction in mice from the work of A. C. Upton and associates. A simple power function of the dose multiplied by the expression for a multitarget/single-hit survival curve (i.e., a shoulder-type curve) was shown by Gray to fit the leukemia data quite well. Thus, Gray's model predicts a bell shaped curve for tumorigenesis (when linear-linear coordinates are used). A bell shaped induction curve for myeloid leukemia induction in mice has also been published by Mole et al. (8) who also showed that their results could be fitted by a polynomial of the dose for the induction curve multiplied by one or more expressions for a conventional survival curve.

It is possible, as discussed by Mole (9), that tumor expression may be influenced by progressive changes in the host in addition to cell initiation. Among these changes immune defenses may play a role and if they do, their effectiveness may depend upon the dose, dose rate, and quality of

Figure 4: Model for the combined effects of repair of sublethal and subtransformation damage (see text). D = dose, D' = dose rate. (From ref. 3.)

the radiation particularly when whole-body exposure is used (see discussion to come). For this reason and possibly other factors, the bell shaped curve of tumorigenesis may be distorted from that predicted simply by an expression that accounts for the competition between the induction of neoplastic transformation and cell killing. Since our biophysical model is intended to explain the qualitative features of the dependence of tumorigenesis on the dose rate of γ -rays and since, in general, systemic effects are reduced when the dose rate is decreased, it seems likely to us that Gray's model can be taken as a valid starting point.

To apply Gray's model in the context of low dose rates, we have to account for the reduced killing effectiveness of the radiation. In Fig. 4, the upper panel shows the shift in the survival curve to be expected (3). In the lower panels, we show how the frequency of transformation would be changed: (a) if only the repair of sublethal damage influences the net frequency (left panel); and (b) if in addition to the repair of sublethal damage, cells at a reduced dose rate can repair subeffective transformation damage, i.e., subtransformation damage, (right panel).

In the lower panels of Fig. 4, the ordinates are the frequency of transformation per cell exposed. If we assume, as did Gray, that a susceptible tissue consists of a fixed number of cells of which at least one cell must be transformed (but not killed), then the transformation curve in Fig. 2 would plot as a bell shaped curve (lower dashed curves). These curves, which show the progressive effect of cell killing, can be converted into an induction curve without cell killing by dividing through, as a

function of the dose, by the dose dependence of survival, upper panel. The upper dashed curves in the lower panels, designated "high D' (-killing)," represent the result.

To predict the effect, on the net transformation frequency per cell exposed, of the repair of sublethal damage alone, it is only necessary to modify the induction curve without cell killing by the survival curve at the reduced dose rate. The continuous line in the lower left panel is the result from which the following generalization may be inferred with minor qualifications (which have been discussed, 3); if the repair of sublethal damage, at a reduced dose rate, is the only process that modifies the frequency of transformation, the net induction curve will be bounded by the two dashed curves shown in Fig. 4, left panel. The essential biophysical basis for this last statement is that, to express the transformed state, a cell must remain viable. Since a reduced rate of delivery of a low-LET radiation usually results in higher survival, we would expect the induction curve to be shifted upward at all doses and to approach the upper dashed curve in the limit of no cell killing.

In the lower right panel of Fig. 4, we consider how the repair of subtransformation damage would be evident in addition to the repair of sublethal damage. Repair of subtransformation damage should cause the net transformation per cell exposed to drop. If the degree of such repair is large enough, in the small-to-mid dose region, the transformation curve could lie below the curve for a high dose rate as shown. However, the "leverage" due to the repair of sublethal damage may be sufficiently large in the high dose region to keep the net transformation above that observed for a high dose rate. The result is a transformation curve at a low dose rate that crosses the curve at a high dose rate as shown. The continuous line in the right panel is, therefore, a collapsed version of the continuous line in the left panel. Compared to the bell shaped curve for transformation at a high dose rate, the curve at a low dose rate remains bell shaped but is broader and has a reduced maximum. In the limit, we would expect the latter curve to become a horizontal line.

Net Transformation, Reduced Dose Rates of ^{60}Co γ -Rays

Using progressively lower dose rates, transformation induction as well as survival was measured as a function of dose (3). Panel A in Fig. 5 shows the same transformation data as in Fig. 2 except that both the ordinate and abscissa are linear. The curve "absolute frequency" was obtained from the lower curve by dividing through by the surviving fraction the dose dependence of which is also shown in Fig. 2. In Panel B, the curve designated "(abs. freq.)·(sur. frac.)" shows the effect of only the increased survival due to irradiating at 2.5 rad/min whereas the observed transformation frequencies per cell exposed are shown by the line through the closed circles. The drop from the former to the latter curve we attribute to the repair of subtransformation damage. Panels C and D show a further progressive increase in the separation between the predicted curves, if only the repair of sublethal damage were to influence the net transformation frequency, and the curves through the observed frequencies. In addition to a progressive "collapsing" of the transformation curve with decreasing dose rate, the crossing of the low and high dose rate curves is evident.

Figure 5: Transformation frequencies per $10T_{1/2}$ cell exposed for different dose rates of ^{60}Co γ -rays. The data in Panel A are from Fig. 2. (From ref. 3.)

Net Transformation, Protracted Low Doses, ^{60}Co γ -Rays

Figure 6 shows a comparison of the initial parts of the induction curves at 100 rad/min and at 0.1 rad/min, as well as the effect of five high dose rate exposures of ^{60}Co γ -rays each separated by 24 hours. In no instance were the cell cultures crowded at the end of a protracted exposure (see Fig. 1). Furthermore, the following checks were made relative to the multifractionation results (10). Cells that were respread after the first and third fractions of a five fraction sequence yielded the same frequencies as shown in Fig. 6. Also, cells inoculated and incubated in the same manner as for Fig. 6, but given a single high dose rate exposure of 300 rads at a time corresponding to the end of a 5-fraction sequence (i.e., after seven days of cell growth) yielded the same transformation frequency per surviving cell as cells exposed to a single high dose rate exposure of 300 rads after two days of growth (e.g., as in Fig. 2). Delaying further the resuspension of the cells for up to 24 hours after the single 300 rads dose had no effect on the survival or the transformation frequency.

In Fig. 6, each curve is fitted well by a straight line. In spite of the "single-hit," linear dose dependence of the high dose rate curve, it is evident that the steepness of the slope of the curve depends upon dose rate as well as multidose protraction. The low dose rate is 1000-fold lower than the high dose rate, but still the dose rate was biologically high, about 120 rads

Figure 6: A comparison, for total doses covering the initial low dose region, of the transformation frequencies of $10T_2$ cells exposed to ^{60}Co γ -rays at different dose rates and to five daily fractions (see text). Uncertainties are standard errors.

per cell cycle. The slope of the low dose rate curve decreases still further at lower dose rates (data to be reported). We infer from the results in Fig. 6 that cells can repair subtransformation damage even in the so-called "single-hit" domain of a low-LET induction curve. As discussed in reference to cell survival, the foregoing result is, in fact, consistent with radiobiological theory (11, 12).

Net Transformation, Fission-Spectrum Neutrons versus ^{60}Co γ -Rays

It is evident in Fig. 3 that a reduction in the dose rate of fission-spectrum neutrons has an opposite qualitative effect compared to γ -rays. More recent results (see Hill, Han, and Elkind, these Proceedings) show that the neutron induction curve between zero and 10 rads at 0.086 rad/min is linear and has a slope some 9-fold steeper than the slope of the initial part of the high dose rate neutron curve. Also, we have found that multiple daily fractions of high dose rate neutrons induce transformation at a rate consistent with the low dose rate curve in Fig. 3. If the final expression of subeffective transformation damage due to γ -rays is influenced by repair, clearly that due to fission-spectrum neutrons must be subject to misrepair (see ref. 13 for discussion). An alternate explanation for the results with neutrons is that at a low dose rate the expression of damage, which is not ordinarily expressed, is facilitated.

Since the effective LET of a fission-spectrum neutron beam is quite high, the microdosimetry of energy absorption must be considered in the small dose

region. If the smallest dose in the low dose region can result only, with high probability, from a single primary event (e.g., a single recoil proton), then it would not be likely that any dependence on dose protraction could be observed. The smallest dose that we have used at 0.086 rad/min is 2.4 rads. An analysis based upon the microdosimetric approach taken by Goodhead (14) leads us to conclude that multiple primary absorption events could be involved in transformation induction at a dose as small as 1 rad of fission-spectrum neutrons when the following factors are taken into account: (a) the neutron energy distribution of the fission-spectrum beam (15); (b) the average volume of a $10T\frac{1}{2}$ cell, $\sim 10\mu\text{m}$ diameter; (c) the flattened shape of the nuclei of cells during irradiation (2, 16); (d) the likelihood of some γ -ray contamination of the neutron beam (15); and (e) the low expected frequency of cell transformation for doses in the low dose region (Fig. 3).

Summary, Conclusions, and Projections

Our data with both low- and high-LET radiations are qualitatively similar to results obtained in vivo. This is evident, for example, in the reductions in cell transformation for protracted exposures of γ -rays and similar reductions in tumorigenesis as shown by Upton et al. (17), Ullrich and Storer (18), and Mole and coauthors (8, 9). Also, enhanced tumor induction, or life shortening, has been reported in several instances when exposures to fission-spectrum neutrons are protracted (see references in 4). The foregoing similarities support the conclusion that the use of the $10T\frac{1}{2}$ cell system is a valid way of assessing radiation-dependent variables in tumorigenesis.

We are led, therefore, to the inference that since similar dependencies on dose protraction are observed in vitro and in vivo, similar cellular mechanisms are involved. As is evident in Fig. 1, the density of $10T\frac{1}{2}$ cells is very low, about 2-4 cells/cm², when the transformation assay is started. Hence, it is unlikely that cell-to-cell interaction is required to effect the transformed state. While it is possible that in tissues two or more cells must be affected in order that a so-called DNA fragment from one cell can lead to the transformation of another (9, 19), the low cell densities in our assay make such a mechanism unlikely. Moreover, the results in Fig. 6, for example, involve measurable transformation frequencies with little if any attendant cell killing, a presumptive requirement for the generation of the required DNA fragment (9, 19).

Lastly, the consistencies outlined between our results with cells and the data of others with animals lend support to Gray's hypothesis that tumorigenesis is the net effect of a low probability inductive process, and a high probability killing process. Mathematically, we express this relationship as follows:

$$T = (a_0 + \sum_{i=1}^n a_i D^i) \cdot S(D, D', L) \cdot H(D, D', L, \dots?),$$

where T is the probability of tumorigenesis, the induction process is expressed as a polynomial in the dose D , S is the probability of survival, and H stands for possible radiation-dependent host factors. (H could be expected to be a constant for small doses delivered at low dose rates of a low-LET radiation.) In the equation, a_0 is the spontaneous frequency, and S and H may depend on the dose rate D' , and the LET L . The a_i 's, of course, would also depend on D' and L .

From this expression, an important prediction can be made when a_0 is appreciable [e.g., 43% in the case of reticulum cell sarcoma in RFM mice

(20)]. For small doses, tumorigenesis would drop provided that: (a) the cells responsible for the spontaneous incidence are present at the time of exposure; and (b) the progenitor cells of the tumor are not resistant to cell killing. An example of where a and b apply may be found in the results of Ullrich and Storer (20). Acute doses of γ -rays cause the curve for reticulum cell sarcoma to drop from 43%. We may also infer from the equation that if cell killing is spared, e.g., because of dose protraction, tumorigenesis for small doses will increase. This too was observed for reticulum cell sarcoma when RFM mice were irradiated with low dose rate γ -rays (20) lending further support to the Gray model and the early presence of transformed cells in unirradiated mice that have tumors late in life.

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