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CONTRIBUTION OF LETHAL MUTATIONS TO EXCISION ASSAYS FOR TUMOUR CELL SURVIVAL

ANUPAM CHATTERJEE, RICHARD J. HODGKISS and ANAMARIA ROJAS

Conventional assays of cell survival determine only the proportion of colony-forming cells, assuming that all such cells are equivalent. However, cells surviving irradiation are reported to have lower plating efficiencies than unirradiated controls, suggesting an additional component of cellular damage that is ignored in conventional survival assays, but which could contribute to therapeutic outcome. Therefore we have examined the contribution of this additional form of damage to excision assays for cell survival in experimental tumours following both single dose and fractionated irradiation (10F/5 days) in vivo. Plating efficiencies were considerably lower for the long-term descendents of irradiated compared with non-irradiated cells. Expression of delayed reproductive death was reduced after fractionated radiation doses, only appearing after a substantial number of 3.4 Gy fractions had accumulated. Thus estimates of survival derived from single clonogenicity assays may underestimate the reduction in cell viability from a particular treatment. This could compromise assays for intrinsic radiosensitivity and mathematical modelling of the efficacy of treatment regimens.

It has been reported (1) that an increased death rate among the progeny of cells surviving acute radiation exposure persists for many generations after irradiation, as compared with that observed in non-irradiated cells. Colonies formed by CHO-K1 cells surviving irradiation contain a substantial proportion of non-viable cells which have segregated from the viable population at cell division (2). This reduction in cellular 'fitness' has been interpreted as a sign of genetic instability or heritable 'lethal mutations', which lead to the production of non-viable progeny at some cell divisions, and has been observed in several in vitro studies with mammalian cell lines (e.g. 1, 3–8), although not all studies have been in agreement with these findings (9). Expression of specific gene mutations (10) and neoplastic transformation (11) may also occur many cell generations after irradiation as a result of a general increase in genetic instability. Late expression of lethal mutations has also been observed after treatment with some, but not all, chemotherapeutic agents (12) and 6-thioguanine resistant mutants are induced for at least 10–14 cell generations after treatment with ethyl-methane sulphonate (13). By considering expression of these lethal mutations among the survivors of the irradiated cells, the conventional survival curve was corrected for the over-estimation of survival that occurs in the conventional Puck & Marcus assay (1, 4, 14). Such late survival curves differed from the conventional survival curves mainly by exhibiting a reduced shoulder, which is an indication of induction of lethal mutations within the dose range encompassing the shoulder.

The induction of lethal mutations by fractionated irradiation of CHO-K1 cells has been reported to occur to a lesser extent than with single dose irradiation (15). It was suggested that splitting the dose by more than two hours allowed for effective repair of lethal mutations, while a recovery interval of less than two hours allowed their expression at a level comparable to that seen after single dose irradiation. Until now, lethal mutations have been observed in careful experiments with both immortal and

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From Gray Laboratory of the Cancer Research Campaign, Mount Vernon Hospital, Northwood, Middlesex, U.K. (R. J. Hodgkiss, A. Rojas), and School of Life Sciences, North-Eastern Hill University, Shillong, India (A. Chatterjee).
Correspondence to: Dr R. J. Hodgkiss, Gray Laboratory of the Cancer Research Campaign, P.O. Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, U.K.

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primary cell lines. Their potential contribution to the in vivo response to radiation has not yet been quantified. We have, however, observed that the amount of cell kill in CaNT tumours after fractionated doses was less when using an excision assay than when the response to treatment was assessed in vivo with a local control assay (16). It has been suggested (1) that the accumulation of lethal mutations in vivo may partly explain the decreased tolerance of previously irradiated tissues. The relevance of lethal mutations to radiotherapy, and to excision assays for the intrinsic radiosensitivity of cells from tumours and normal tissues, will probably not be resolved using cell lines and requires the development of systems where lethal mutation induction can be observed in vivo. Excision and in vitro growth of experimental tumours is a useful method of assessing the effect of a treatment on the viability of cells within the tumours, and the induction of lethal mutations may affect the interpretation of the results of such assays. We have used excision assays of an experimental murine mammary carcinoma to evaluate the extent of induction of lethal mutations in cells surviving after irradiation of tumours with single and fractionated doses of radiation in vivo, and the propagation of lethal mutations in the progeny of these cells.

Material and Methods

The poorly differentiated mammary adenocarcinoma CaNT of spontaneous origin was maintained by serial passage in CBA mice, and for this work was implanted as a cell suspension subcutaneously in the rear dorsum of 10–12-week-old male mice. The CaNT tumour has a volume doubling time of less than 3 days. Tumours were used at a geometric mean diameter of 6 ± 1 mm calculated from three orthogonal measurements. These studies were performed under the regulations stipulated by the Animals (Scientific Procedures) Act (1986).

Irradiations

Unanaesthetized tumour-bearing mice were irradiated in special lead jigs with 240 kVp x-rays filtered with 0.25 mm Cu and 1.0 mm Al (HVL 1.3 mm Cu) at a dose rate of 3.9 Gy min^{-1}. Groups of 6 mice were used for each dose point, and irradiated with either single doses or with two fractions per day of 3.4 Gy per fraction, separated by an interfraction interval of 6 h to allow complete repair of reparable damage to occur between fractions. The mice were supplied with free-flowing air during the irradiation.

Excision assay

The effect of irradiating tumours in vivo was assessed by an in vitro colony-forming assay on cells isolated from tumours. Animals were killed 6 h after irradiation to allow repair of potentially lethal damage and sub-lethal damage to occur, and tumours aseptically excised, weighed and finely minced with scissors. The material was stirred for 30 min at 37°C with 1 mg ml^{-1} pronase, 0.5 mg ml^{-1} collagenase and 0.5 mg ml^{-1} DNAase, dissolved in growth medium without serum. The cell suspension was then diluted with Eagles Minimum Essential Medium (MEM) with 10% foetal calf serum (fcs), and the enzymes separated by centrifugation. The cells were resuspended in fresh growth medium and any remaining cell clumps broken up by syringing 5 times through a 19-gauge needle. The cells were counted with a Coulter Counter and appropriate numbers plated, at two cell densities, onto 9 cm Petri dishes that had been prepared with a feeder layer of 2×10^5 lethally irradiated (30 Gy, aerobic conditions) V79 379A Chinese hamster cells. Typically, 4 dishes were plated for each cell density, except for unirradiated controls where 6 dishes were plated at one cell density. Dishes were incubated under an atmosphere of 5% CO_2 + 5% O_2 at 37°C for 10 days before fixing and staining the colonies with 0.2% crystal violet in 70% ethanol. Colonies with a minimum of 50 cells were counted as the progeny of a viable cell.

Delayed plating of tumour cells

In addition to immediate plating of tumour cells for survival estimation, cells from each tumour were propagated in flasks, with feeder layers, for subsequent plating efficiency assays. At the time of each plating efficiency assay, the flasks in which cells were being propagated were trypsinised (0.05% trypsin, 0.02% EDTA) and cells plated at two cell densities on 9 cm dishes and into 25 cm^2 flasks, at the higher cell density, to propagate them for subsequent assays. Each passage was of sufficient time (12–14 days) for colony formation to have occurred on the dishes plated in parallel at the start of each passage, and the flasks of cells were sub-confluent.

The persistence of dead cells in the culture system was measured by comparing the growth of irradiated CaNT cells with that of untreated cells. Stirred suspensions of CaNT cells were irradiated with 12 Gy x-rays under aerobic conditions and 2.4×10^5 cells were plated into a number of 25 cm^2 flasks. Untreated cells were also plated into flasks at a similar density. At various times after plating, the medium was removed, the cell monolayer washed with physiological buffered saline, trypsinised into a total volume of 10–30 ml and counted with a Coulter Counter.

Data analysis

Logarithmically transformed survival data were fitted to the linear-quadratic equation

$$\ln S = -\alpha \times D - \beta \times D^2 \qquad \text{[Eq. 1]}$$

Table 1

Number of cells plated into flasks and numbers of cells obtained from flasks after each passage, following single-dose irradiations in vivo

	Dose (Gy)	Start Cell No.[a]	Start P.E.	End Cell No.[b]	End P.E.
Passage 1					
	0	1347	0.310	1.40×10^5	
	5	2356	0.206	6.63×10^6	
	10	10709	0.0550	9.46×10^5	
	15	32798	0.0140	1.76×10^6	
	20	104362	0.00143	3.26×10^5	
	25	106375	0.000149	6.40×10^5	
Passage 2					
	0	1246	0.301	6.82×10^5	
	5	2259	0.137	8.11×10^5	
	10	2432	0.197	9.60×10^5	
	15	3154	0.0788	3.10×10^5	
	20	4266	0.0273	1.70×10^5	
	25	5339	0.00289	1.13×10^5	
Passage 3					
	0	1307	0.213	3.72×10^6	0.203
	5	2239	0.186	3.17×10^6	0.129
	10	2014	0.191	3.96×10^6	0.189
	15	3367	0.109	8.82×10^5	0.176
	20	4161	0.101	5.97×10^5	0.142
	25	5502	0.0503	4.51×10^5	0.0974

[a] mean number of cells plated from 6 individual tumours 6 hours after irradiation in vivo.
[b] mean number of cells obtained from 6 near confluent replicate flasks.

Table 2

Number of cells plated into flasks and numbers of cells obtained from flasks after each passage, following fractionated irradiation in vivo

	Dose (Gy)	Start Cell No.[a]	Start P.E.	End Cell No.[b]	End P.E.
Passage 1					
	0	1121	0.217	3.74×10^5	
	3.4	962	0.183	3.29×10^5	
	6.8	2265	0.102	5.46×10^5	
	20.4	10542	0.00925	2.52×10^5	
	23.8	11272	0.00248	1.53×10^5	
	27.2	19693	0.000620	6.98×10^4	
	30.6	49700	0.000343	7.98×10^4	
	34.0	103070	0.000088	8.69×10^4	
Passage 2					
	0	1120	0.241	8.26×10^5	
	3.4	1084	0.303	8.23×10^5	
	6.8	1037	0.348	8.20×10^5	
	20.4	2007	0.153	3.06×10^5	
	23.8	3274	0.0893	2.35×10^5	
	27.2	3852	0.0702	5.92×10^5	
	30.6	4958	0.0510	5.32×10^5	
	34.0	4347	0.0105	2.55×10^5	
Passage 3					
	0	938	0.248	6.47×10^5	0.237
	3.4	1124	0.258	7.87×10^5	0.260
	6.8	1073	0.336	5.05×10^5	0.280
	20.4	2214	0.193	7.87×10^5	0.185
	23.8	3045	0.199	6.96×10^5	0.169
	27.2	3912	0.176	5.86×10^5	0.175
	30.6	5011	0.132	7.30×10^5	0.069
	34.0	5307	0.133	7.18×10^5	0.0868

[a] mean number of cells plated from 6 individual tumours 6 hours after irradiation in vivo.
[b] mean number of cells obtained from 6 near confluent replicate flasks.

by the method of unweighted non-linear least-squares using the RSI program (BBN Software Products Corporation) implemented on a Microvax computer. The data derived from the immediate plating of tumour cells was corrected for the contribution of the reduced clonogenicity of their progeny by multiplying surviving fractions from day 0 by the surviving fractions obtained from subsequent passages, e.g., corrected:

$$SF_{0-p3} = SF_0 \times SF_{p1} \times SF_{p2} \times SF_{p3} \qquad \text{[Eq. 2]}$$

These corrected survival data were then plotted and analysed in the same way as data from immediate plating.

Results

The cell numbers at the start and end of each passage are summarised in Tables 1 and 2. Microscopic inspection of tumour cells, plated into flasks 6 h after irradiation in vivo, showed that many of the dead cells never attached to the flask and that, among those cells that did attach, considerable cell growth and colony formation occurred during the first and subsequent passages (Tables 1, 2). Many of the dead cells were therefore eliminated when the flasks were washed prior to trypsinisation and were not included in estimates of cell numbers and plating efficiencies at the end of each passage. These observations were confirmed by counting the numbers of cells present in flasks at various times after plating. As shown in Fig. 1, only a small proportion of cells irradiated with 12 Gy attached to the flasks and were recovered by trypsinisation 24 h after plating, while the majority of the dead cells were removed during washing of the monolayers prior to trypsinisation. Subsequent growth of those cells that did attach was at a slightly slower rate to that of untreated cells, possibly reflecting continuing loss of lethally irradiated cells.

Microscopic inspection of the colonies grown from the CaNT tumour suggests that they are all of the same epithelial cell morphology. Colonies of skin fibroblasts from these mice are markedly different from the colonies grown from tumours. The cells in this tumour have an approximately tetraploid DNA content, and are thus readily distinguished from the normal diploid cells by flow cytometry of propidium iodide-stained cells. We have observed that the diploid stromal cell component of the

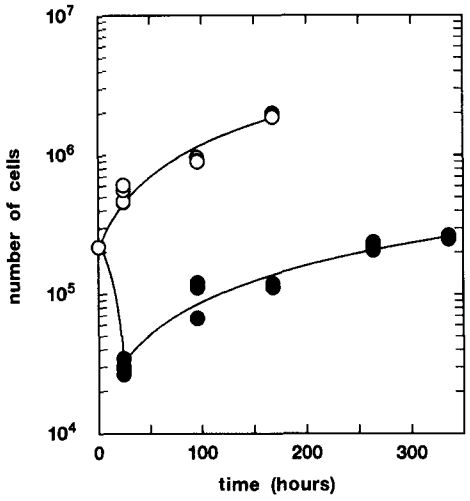

Fig. 1. In vitro growth curves for CaNT cells. (○) untreated cells; (●) irradiated with 12 Gy x-rays in well oxygenated conditions.

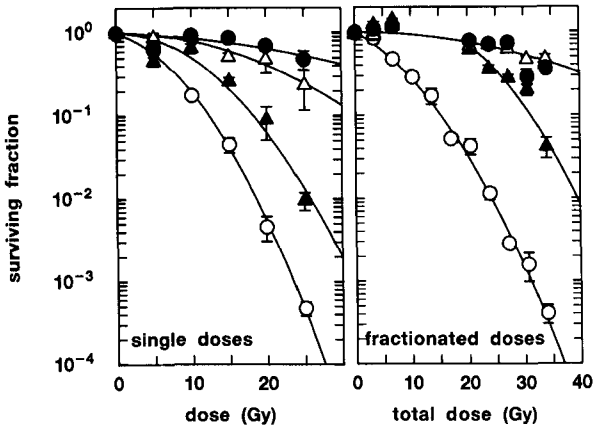

Fig. 2. Survival curves for cells from CaNT tumours irradiated in vivo with (a) single doses or (b) fractionated doses of 3.4 Gy per fraction. ○ = cells plated 6 h after irradiation, ▲ = cells plated 13 days after irradiation, △ = cells plated 24 days after irradiation, ● = cells plated 35 days after irradiation. The data plotted represent means and standard errors for 6 tumours per dose group.

tumour does not grow and is lost when the cells are cultured.

Survival curves were generated for cells from CaNT tumours irradiated with single doses of x-rays in vivo, plated 6 h post-irradiation and 13, 24 and 35 days later (Fig. 2a). Although a progressive recovery in the plating efficiency of the progeny of the irradiated cells with time was observed at all levels of survival (Table 1, Fig. 2a), a dose-dependent reduction in plating efficiencies was observed as late as 35 days after irradiation, leading to surviving fractions, corrected for zero dose plating efficiency, of less than 1.0.

Survival curves after fractionated irradiations were generated using 1–10 × 3.4 Gy fractions (Fig. 2b), with a similar range of cell survival, following immediate plating,

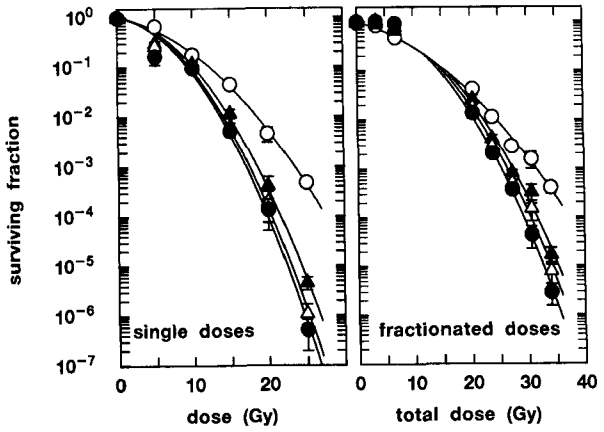

Fig. 3. Survival curves, corrected for the reduced viability of progeny, for cells from CaNT tumours irradiated in vivo with (a) single doses or (b) fractionated doses of 3.4 Gy per fraction. ○ = cells plated 6 h after irradiation, ▲ = cells plated 13 days after irradiation, △ = cells plated 24 days after irradiation, ● = cells plated 35 days after irradiation. The data plotted represent means and standard errors for 6 tumours per dose group.

to that obtained with the single dose irradiations. The cells from three of the dose groups (3, 4 and 5 fractions) in the immediate plating curve shown on Fig. 2b were lost during the first passage and are not therefore included in the delayed plating curves or in Table 2. A dose-dependent reduction in plating efficiencies was seen after 7–10 × 3.4 Gy fractions at times of up to 35 days after irradiation although, as observed in the single-dose experiment, considerable cell proliferation occurred at each passage in the flasks of cells used for the delayed plating (Table 2). However, delayed plating efficiencies were generally higher, for the same level of cell survival, after fractionated irradiation compared with single dose irradiation. Delayed plating efficiencies were similar to those of untreated cells after one and two fractions of radiation.

In Fig. 3 a series of survival curves, corrected to take into account residual damage, were constructed from the data in Fig. 2, as previously described (3) by multiplying the immediate surviving fraction by the delayed surviving fraction [see Eq. 2]. It is evident that conventional cell survival curves, both after a single (Fig. 3a) or a fractionated dose (Fig. 3b), plated soon after irradiation considerably underestimate the amount of lethal damage inflicted on the cells. The fractionated corrected survival curves shown in Fig. 3b support the idea that lethal mutations are expressed only after several fractions of radiation. There was little reduction in the delayed plating efficiency of the progeny of cells surviving small numbers of fractionated treatments.

Discussion

It has been reported that the progeny of x-irradiated surviving cells are less fit than untreated cells, and that

their plating efficiency is reduced for many cell-generations after the original irradiations (1, 3–8). However, these experiments have always been carried out on primary and established cell lines grown and irradiated in vitro, and the relevance of lethal mutations to irradiation of cells in vivo has been uncertain. Persistent changes in the biochemical properties of surviving cells have also been observed (e.g. (17)), and similar changes have also been found in irradiated normal tissues and tumours (18–20). Evidence has recently been obtained that considerable cytogenetic instability is observed in the progeny of cells irradiated with alpha particles (21), and late expression of specific mutations and transformation has been observed following irradiation with low LET radiation (10, 11). The potential persistence of cytogenetic instability in cells irradiated with lower LET radiation is of obvious concern and may provide a mechanism for the expression of lethal mutations.

The data presented here shows that the plating efficiency of cells from tumours irradiated in vivo is reduced for many cell generations after irradiation, and is a factor which needs to be taken into account when assessing the effects of irradiation. Reduced plating efficiencies of survivors can be observed following both single dose and fractionated irradiation and this effect persists for many weeks after the original irradiation. Correcting conventional survival curves with delayed plating efficiencies has been shown to reduce the size of the shoulder of the survival curve of some cell lines (1). However, there is no evidence for a reduced shoulder in the corrected single dose survival curves from the CaNT tumour model. The reduction in the long-term viability of irradiated cell populations appears to be dose-dependent and is most noticeable after large doses of radiation. However, accumulation of lethal mutations in cells from tumours exposed to many small fractions indicates that this damage occurs at relatively low doses of radiation, and can build up over a period of time.

We have shown that the cells that can be grown from the CaNT tumour represent the aneuploid tumour cells and that the diploid stromal cells do not persist in culture, and are therefore not included in the data presented here. Microscopic observation of cultures suggested that the majority of lethally irradiated cells either never attach to the plastic flasks or detach after incubation. In addition, some may fail to survive trypsinisation intact. This was confirmed by counting the number of cells in irradiated and control populations (Fig. 1). Although the initial loss of cells over the first 24 h after irradiation only accounted for the first decade of cell killing, any further loss of dead cells was masked by the proliferation of survivors. The similarity in the rates of growth in the irradiated and control populations suggests that a high proportion of the residual cells in the irradiated population proliferate.

Although the numbers of cells at the beginning and end of the first passage (Table 1, 2) suggest that only 3–4 cell doublings could have occurred after the higher doses of radiation, this is at variance with our observation that considerable proliferation and colony formation occurred on these dishes. However, the overall cell numbers at the end of Passage 1 reflect a balance between loss of lethally irradiated cells and growth of the clonogenic cells (e.g., Fig. 1). Dilution of the dead cells by proliferation of the clonogens during Passage 1, means that the dead cells would be much less of a potential problem in the later passages, even in the unlikely event of a large proportion of them surviving trypsinisation and plating for Passage 2. For example, after a single dose of 25 Gy (Table 1), there is a 6-fold increase in cell numbers by the end of Passage 1. Even if this figure were to include all of the non-clonogenic cells, the newly produced cells would comprise at least 80% of the population. If the plating efficiency of the newly divided cells was similar to that of controls an overall plating efficiency of about 25% would be expected at the start of Passage 2, compared with the observed plating efficiency of 3%.

Tumour cells surviving one and two fractions of irradiation had normal plating efficiencies, within the resolution of the experiment, suggesting that inter-fraction recovery may mitigate the production of lethal mutations and that several fractions are required to build up enough damage for it to be expressed as reduced plating efficiency of the survivors. This is consistent with observations (15) that lethal mutations are not observed in split-dose experiments, although clearly some damage must accrue from each fraction.

Cell survival is a widely used experimental endpoint, and a significant amount of work has been invested in the development of in vitro predictive assays for the intrinsic sensitivity of cells from both tumours and normal tissues to radiotherapy and other cancer treatment modalities (22–24). Predictive assays for intrinsic radiosensitivity are often used to estimate the surviving fraction at 2 Gy (SF2) (25). Such data may be used to estimate the result of giving a number (n) of 2 Gy fractions as $SF2^n$, omitting in this simplified calculation the effects of inter-fraction recovery, cell proliferation and tumour hypoxia. Most radiosensitivity assays rely on a single clonogenic or other determination of cell survival, a short time after treatment of the cells with the agent of interest, and will not be informative about long-term changes in the proliferative potential of the cells. Expression of lethal mutations during a course of radiotherapy could reduce the rate at which proliferating cells repopulate the tumour with clonogenic cells. However, by ignoring the reduced viability of the cells surviving each radiation fraction, and therefore their reduced ability to proliferate, calculations of treatment efficacy based on SF2 measurements could underestimate the cell-killing potential of several fractions of

radiation (Fig. 3b), and may compromise attempts at carrying out more sophisticated predictive modelling of therapeutic regimes.

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