Transformation of Mammalian Cells *in vitro* by Low Doses of X-rays

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X-irradiation of hamster embryo cells in culture, followed by cloning of the cells, was used to obtain a dose–response curve in terms of cell transformation. Transformations were observed for doses as low as 1 rad.

PREVIOUS reports have described the induction of malignant cell transformation by a dose of 300 rad of X-rays in short term cultures of hamster embryonic cells[1–4]. The conditions required for the fixation of this transforming event as a hereditary property of the cells have also been reported[1,3,4], as well as some of the *in vitro* characteristics of the X-ray transformed cells[1–6] and their capacity to induce tumours *in vivo*[1,2]. Recently, some of these investigations have been repeated using established mouse cell lines[7]. A comprehensive study of the carcinogenic action of radiation must include a knowledge, at the cellular level, of the relationship between the absorbed dose and the probability of neoplastic conversion.

This report describes experiments designed to elucidate the dose–response relationship over a range of doses from 600 rad down to 1 rad.

Experimental Techniques

Minced midterm whole embryos from golden hamsters were used as the source of normal cells. Primary cultures were established by progressive dissociation of the minced fresh tissue[8]. Cells (10^7) were seeded into each 100-mm Petri dish (Falcon Co.) and incubated at 37° C in a humidified incubator with 5% CO_2 in air. Three-day-old primary cultures were prepared into a cell suspension by trypsinization, and cloned into 60-mm Petri dishes on X-irradiated (4,000 rad) feeder cells of the same type[9]. Seeding levels ranged from two thousand to ten thousand cells, depending on the dose to be administered, thus allowing for cell killing.

In the experiments to study transformations, the cells were irradiated at room temperature, 24 h after seeding, with a range of doses from 1 to 600 rad. The source of X-rays was a 210 kV Westinghouse constant potential generator, with added filters of 0.5 mm of copper and 1 mm of aluminium. For the higher doses (75–600 rad) a treatment distance of 50 cm was used, corresponding to a dose-rate of 70.6 rad min^{-1}. For the lower doses (1–10 rad) a longer treatment distance of 250 cm was used at which the dose-rate was 4.25 rad min^{-1}; the lower dose-rate was used to avoid shutter errors associated with very short exposure times. Doses were measured with a Victoreen R-meter, calibrated at the Bureau of Standards.

After a post-irradiation incubation period of 9 d the clones were fixed and stained[1]. A differential count was made of normal and transformed clones, the latter being identified by piled-up morphology, random cell orientation and loss of contact inhibition, characteristics not seen in the untreated control cultures[1–4].

In several experiments, transformed clones were isolated and grown into mass cultures. The ability of the cells to form clones in semi-soft agar[10,11] and their agglutinability by concanavalin A and wheat germ agglutinin (provided by M. M.

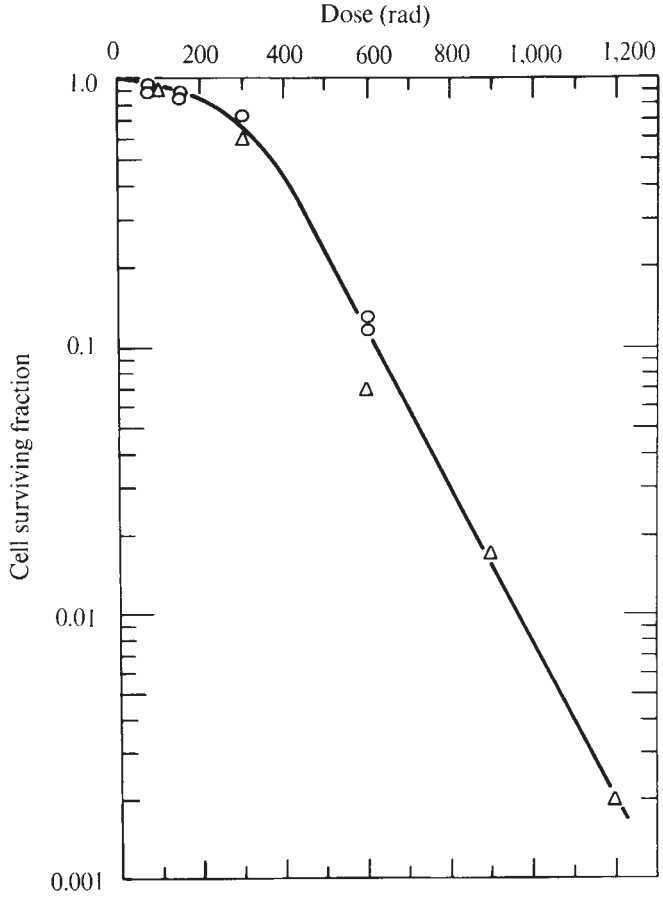

Fig. 1 Survival of reproductive integrity of hamster embryo cells as a function of X-ray dose. Circles describe points calculated from the transformation experiments (Table 1). Triangles describe points established from a separate cell survival experiment (see text). *N*, The extrapolation number, defined as the point in the surviving fraction axis to which the straight portion of the surviving curve extrapolates, =6. D_0, The dose required to reduce the population to a fraction of 37% (used as a measure of the slope in the linear portion of the survival curve), =147 rad.

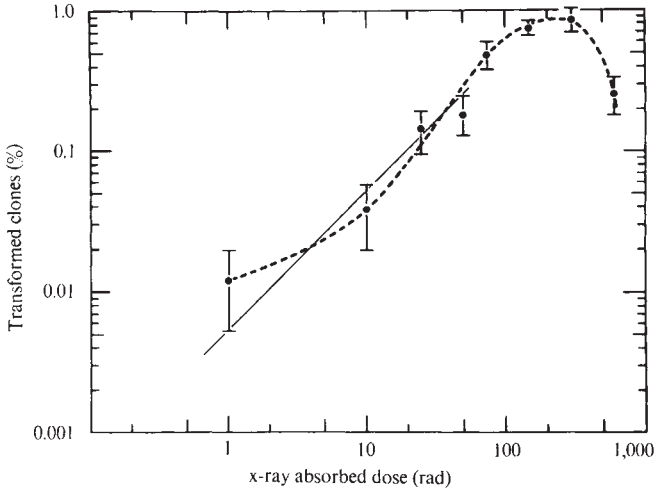

Fig. 2 Incidence of hamster embryo cell transformation following exposure *in vitro* to X-irradiation. For doses at which more than one experiment was performed, the data were pooled; the mean value together with the standard deviation is plotted in the figure (see Table 1 for results of individual experiments). The broken line is drawn by eye to the mean data points; the full line has a slope of +1 and passes through the error bars of each datum point.

Burger)[6,12,13] were used as further criteria for the transformed nature of the cells as compared with control cultures.

Dose–Response Relationship for Transformation

The data for cell survival are shown in Fig. 1, in which dose is plotted on a linear scale against surviving fraction on a logarithmic scale. The shape of the survival curve is characteristic of that commonly observed for mammalian cells exposed to sparsely ionizing radiations, such as X-rays[14]. At low doses there is a broad initial shoulder, implying that X-rays are relatively inefficient at killing cells in this region; for larger doses the survival curve becomes a straight line in this semi-logarithmic plot, implying that over this dose range cell survival is an exponential function of dose.

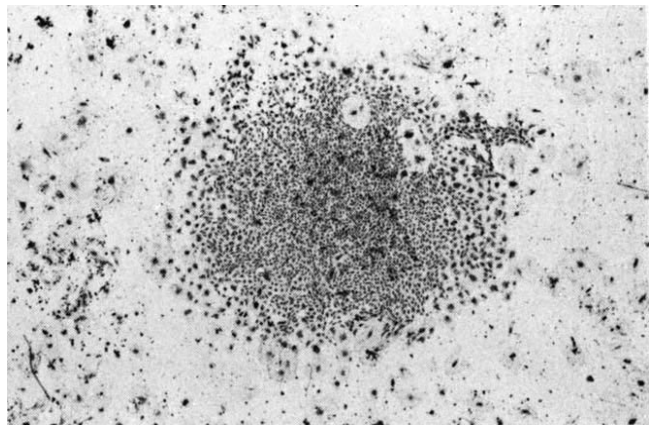

Fig. 3 A 9-d-old clone of normal embryo cells. Giemsa stain ×11.

The dose–response data for transformation are shown in Table 1 and Fig. 2, in which dose is plotted against transformation (%) on a double logarithmic plot. The percentage of cells transformed increases as the dose is increased up to a plateau of approximately 1%, which corresponds to doses of between 150 and 300 rad. A further increase in dose results in a marked decrease in the proportion of cells transformed. Photographs of representative clones, both normal and transformed, are shown in Figs 3–6.

From an examination of the stained cultures it was apparent that the dominant type of transformed clone consisted of cells which were piled up, one on another, having lost contact inhibition[15], but were less randomly orientated than hamster cells transformed by some oncogenic viruses or chemical agents[1]. This morphology has also been observed when tumours induced by X-ray transformed cells were cultured and cloned *in vitro* (C. B., unpublished). Transformed clones of epithelioid morphology[8,13] were found at a very low frequency. In contrast to controls, the X-ray transformed cells derived from all of the ten isolated clones were agglutinable by 50 μg ml^{-1} of concanavalin A or wheat germ agglutinin. The ability to form colonies in 0.33% agar was limited to the cells of the two isolated transformed clones of epithelioid morphology.

Table 1 Neoplastic Cell Transformation of Hamster Embryo Cells following X-Irradiation *in vitro*

	Control				Irradiated			
Experiment No.	No. of clones counted	Plating * efficiency	No. of transformed clones	Dose (rad)	No. of clones counted	No. of transformed clones	Transformed † clones (%)	Surviving ‡ fraction
1	1,600	1.6	0	600	920	2	0.22	0.12
2	1,002	2.2	0	600	3,020	8	0.27	0.13
3	1,012	1.3	0	300	1,130	8	0.71	0.75
4	1,220	1.6	0	300	1,960	18	0.92	0.75
5	2,500	4.5	0	150	6,200	44	0.71	0.89
6	4,020	2.1	0	150	3,268	25	0.77	0.86
7	1,620	2.0	0	75	2,132	11	0.52	0.92
8	4,100	1.2	0	75	2,200	10	0.46	0.95
9	1,025	1.05	0	50	5,402	10	0.185	0.95
9	1,025	1.05		25	5,503	8	0.145	1.00
6				10	10,200	4	0.039	1.00
8				1	6,800	1	0.015	1.00
10	9,600	2.5	0	1	9,200	1	0.011	1.00
10	7,200	1.9	0	1	7,900	1	0.013	1.00

$$* \text{ Plating efficiency (P.E.)} = \frac{\text{Number of cells plated}}{\text{Number of clones counted}} \times 100$$

$$\dagger \text{ Cell transformation (\%)} = \frac{\text{Number of transformed clones}}{\text{Number of clones counted}} \times 100$$

$$\ddagger \text{ Surviving fraction} = \frac{\text{Number of cells plated}}{\text{Number of clones counted} \times \text{P.E.}}$$

A preliminary study of the karyotypes of the X-ray transformed cells using the Giemsa banding method[16] indicates that the cells tend to be aneuploid but there are, apparently, no gross chromosomal aberrations. Experiments kindly conducted by Dr I. B. Weinstein, using reported techniques[17], demonstrated that there were no C-type virus particles present in X-ray transformed cells which had been proved to be able to induce tumours[1].

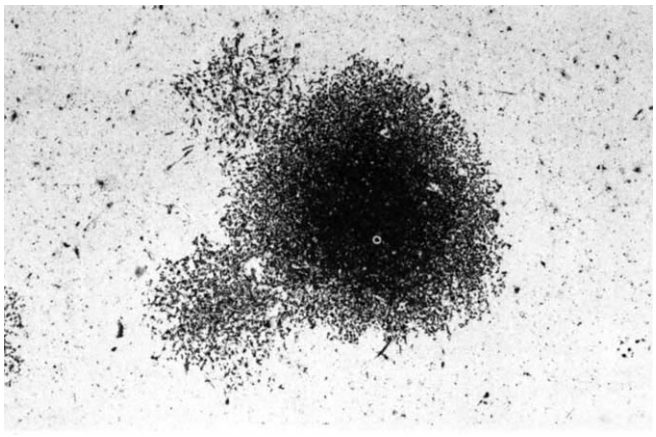

Fig. 4 A 9-d-old clone of hamster embryo cells transformed *in vitro* by 300 rad of X-irradiation. Giemsa × 11.

These results demonstrate that neoplastic transformation can be induced in single cells with doses of X-rays as low as 1 rad. At any of the doses used, the fraction of cells killed is small, and this, coupled with the fact that no transformed clones have been observed in control cultures, strongly supports the view that transformation is directly induced by X-irradiation, and is not a result of a selection of spontaneously occurring transformed cells.

Dose–response relationships for the induction of neoplasms by ionizing radiation have been studied and discussed previously, using human and animal data[18–21]. The shape of the dose–response curve obtained in the present *in vitro* experiments is not unlike that characteristic of *in vivo* data. It consists of three portions; an ascending limb, a plateau and a descending limb.

In the *in vivo* situation there are insuperable difficulties involved in obtaining significant data for low doses of radiation in the range of 1–25 rad, where tumour incidence is low and inordinately large numbers of animals would be required to detect it. Consequently there has been much speculation concerning the actual shape of the ascending limb of the curve. Linear extrapolation from higher doses, on the one hand, has been the conservative approach, while the existence of a threshold dose below which there is no effect cannot be ruled out[22,23]. One of the principal assets of the *in vitro* system is that a very low frequency of transformation can be detected by irradiating large numbers of isolated single cells and scoring thousands of clones. A cell which has received a "hit" at a crucial site and been converted to a neoplastic state can replicate freely and thereby fix this event as a hereditary property[2], and grow into a transformed clone.

If a curve is naively drawn through the data points in Fig. 2, it would have a slope of less than unity for doses between 1 and 10 rad, which on this double logarithmic plot would imply that the transformation rate increases with a power of the dose that is less than one. This kind of dependence has been observed for the induction of mammary neoplasms by neutrons. Rossi and Kellerer[24] have pointed out that in that instance it was necessary to assume a multicellular mechanism of tumour induction because the number of neutron secondaries per cell was considerably less than one. In our experiments the number of X-ray secondaries (electrons) per cell, or even per cell nucleus, is considerably larger than one and the shallow slope which appears to fit the data best may be due to a range of susceptibility among the cells. But the confidence limits on the data points, particularly at the lower doses, are such that one can draw a line with slope +1 that does not fall outside the error bars of any of the points. A linear dependence of induction on dose therefore cannot be ruled out.

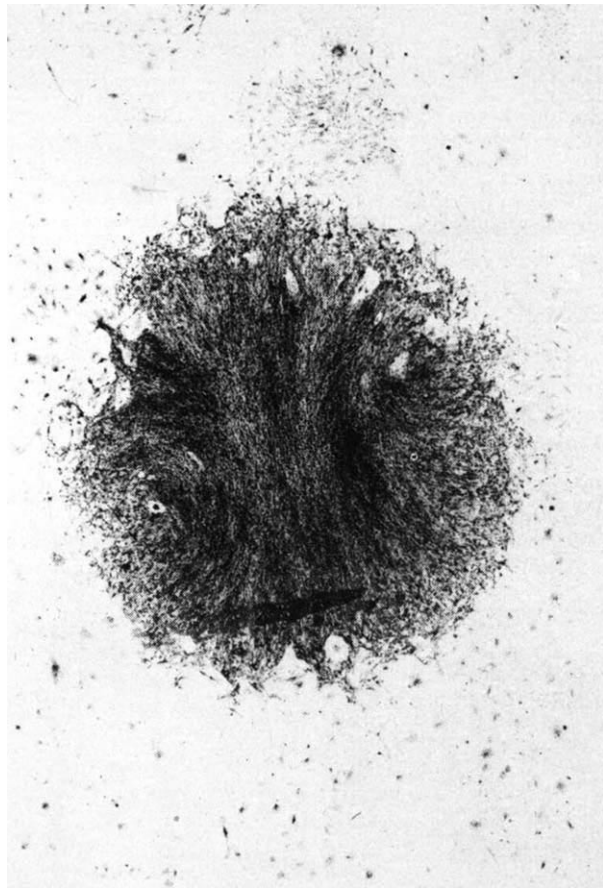

Fig. 5 A 9-d-old clone of hamster embryo cells transformed *in vitro* by 1 rad of X-irradiation. Note the random orientation on the circumference. Giemsa × 15.

The plateau between about 150 and 300 rad closely resembles that often reported for *in vivo* experiments[20]. For a change in dose by a factor of two, the frequency of neoplastic conversion, which is about 1%, remains essentially unaltered. A further increase in dose above 300 rad results in a marked decrease in the frequency of transformation. Similar observations made in humans and animals[18,21] have resulted in speculative interpretations suggesting that the overall high cell killing occurring in these multi-hit dose ranges, and reflected in the survival curves, is responsible for the sharp decline in capacity to induce tumours.

Our data support the idea that above about 300 rad, cell reproductive death[25] begins to be an important factor; the percentage of transformed clones declines above 300 rad in spite of the fact that they are scored from amongst the survivors of the irradiated population, implying that there is a preferential killing of potentially transformed cells. If normal and poten-

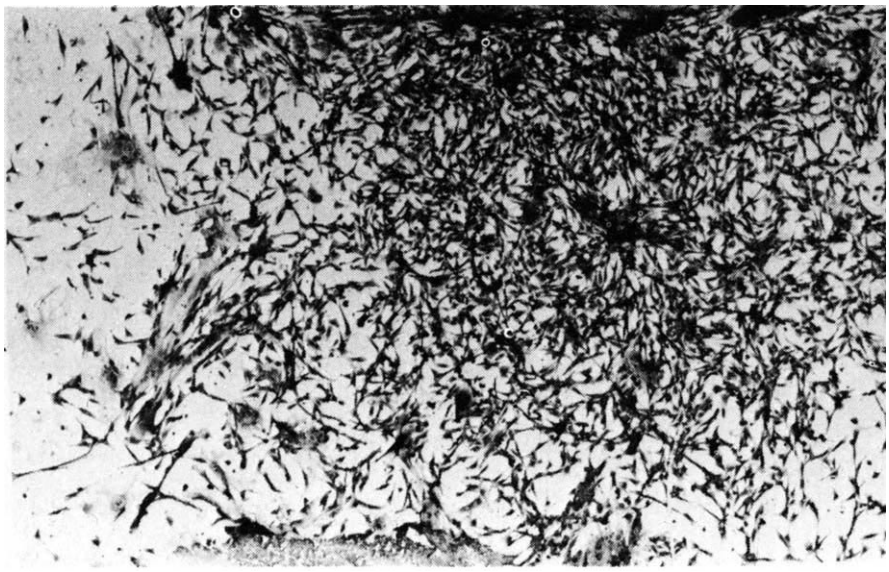

Fig. 6 A section of the circumference of the clone in Fig. 5. Giemsa ×45.

tially transformed cells were equally sensitive to radiation, then a decline in the proportion of transformed cells at higher doses would not be expected. These results support the ideas proposed by Gray[21] inasmuch as they suggest that cells that have been altered by the absorption of radiant energy and are destined to become transformed cells, are more susceptible to being killed by the accumulation of additional damage at higher doses. In other words, many of the potentially transformed cells never have the chance to form a colony because they die a reproductive death due to the extra damage produced by the additional "hits" received at larger doses.

One of the drawbacks of this technique stems from the use of the whole embryo, as a result of which there is a mixture of many cell types. Furthermore, the cell cultures are asynchronous at the time of irradiation, so not all cells are equally at risk. Differences in cell sensitivity to low doses of radiation at various stages of the cell cycle have been reported[25–27].

Our preliminary karyotype analysis of the cells transformed by a dose of 300 rad of X-rays revealed no gross chromosomal aberrations within the limitations of the banding technique used[16]. The event that leads to transformation, however, may well be a subtle genetic structural alteration, such as a gene mutation, which results in the loss of control of cell replication and alteration in the cell surface.

While all the X-ray transformed cells exhibited a microstructural surface change characteristic of the neoplastic state and detectable by plant agglutinins[6,12,13], not all of them form colonies in semisoft agar. As mentioned previously, most of the X-ray transformed cells which are fibroblast-like, and the cells grown from tumours induced by these transformed cells, are not as randomly oriented as those transformed by oncogenic viruses or chemicals. This suggests that these cells have not completely lost "anchorage dependence"[28] which is a requirement for growth in a semi-solid medium such as agar. The cell type which did grow in agar was of epithelioid origin with different growth patterns. They multilayered as rounded cells exhibiting very little adhesion to one another and to the surface on which they were grown.

Some types of leukaemia induced by X-irradiation have been found to be associated with the presence of viruses, implying an indirect action of the ionizing radiation[20,29]. Our findings indicate the absence of C-type virus particles in transformed cells which on injection into newborn hamsters give rise to fibrosarcomas.

In vivo studies on neoplasias induced by X-rays have stressed the wide range of tumour types which arise[18–21]. The fact that neoplastic transformation induced *in vitro* by X-rays cell, together with a striking similarity between the *in vivo* and *in vitro* dose–response relationships, make this system suitable for further studies on the carcinogenic action of X-rays and other ionizing radiations present and increasing in our environment.

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