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Cell Biology

Oncogenic transformation of mammalian cells *in vitro* with split doses of x-rays

(dose–response relationship/low-dose irradiation/estimation of cancer risk)

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ABSTRACT **An established line of mouse fibroblasts, C3H/10T½ cells, was used for the assessment *in vitro* of oncogenic transformations caused by single and split doses of x-rays. The shape of the dose–response relationship was determined over the range from 0.1 to 10 Gy. It was found that splitting the x-ray dose into two equal fractions, separated by 5 hr, led to a reduction in transformation frequency at doses above 1.5–2 Gy but to an enhancement of transformation at lower doses. The observations reported cast doubt on the assessment of human cancer risk at low dose levels by a linear extrapolation from available high-dose data from the Japanese atomic bomb survivors or from persons exposed for medical purposes.**

In a previous paper (1), we reported preliminary data showing that, at low dose levels, splitting an x-ray dose into two equal fractions enhanced transformation frequency compared with the same total dose delivered in a single exposure, whereas at higher dose levels fractionation produced the more conventional sparing effect. The effect on transformation of fractionating low x-ray doses is of such fundamental and practical importance that we considered it imperative to accumulate much more data, to subject them to a rigorous statistical analysis in order to allow unequivocal conclusions to be drawn, and to extend the scope of the experiments to even lower dose levels in order to elucidate the shape of the dose–response relationship.

The importance of the observations reported here lies in their possible implication to the development of human cancer risk estimates at low doses, by extrapolation from available data relating to high dose levels. Both major reports to appear in recent years, the UNSCEAR report of the United Nations (2) and the BEIR report of the U.S. National Academy of Sciences (3), use a linear extrapolation. The risk estimates assumed to apply at low doses are calculated from the slope of a straight line drawn from the origin through the data points for excess cancer incidence for higher doses, usually in excess of 100 rem. Furthermore, it is assumed in both reports that the linear extrapolation leads to an upper limit for the risk estimation at low doses that is "conservative" and "prudent," because most high-dose data in the human relate to single acute exposures, while the low-dose exposure of the public from man-made radiations is the result of multiple small exposures, which are assumed to be less effective.

MATERIALS AND METHODS

The C3H/10T½ mouse fibroblast cell line was used for these experiments. Isolated in the laboratory of Charles Heidelberger, these cells exhibit good contact inhibition after confluence, unless treated with chemicals or radiation, in which case a small proportion of the cells grow into dense piled-up clones that are capable of producing tumors when injected into compatible animals (4, 5). The details of the procedures have been published (6). Briefly, cells were seeded at low density into 50-cm^2 petri dishes such that an estimated 400 reproductively viable cells would survive the subsequent irradiation. Cells were allowed to attach overnight at 37°C for about 18 hr before being exposed to x-rays. The cells were at room temperature during irradiation, but were returned to a 37°C incubator between split doses. After all x-ray treatments the cells were incubated for 6 weeks, with the growth medium changed twice weekly, to allow the transformations to be expressed and to grow into visible clones. At the end of this period the cells were fixed with formalin and stained with Giemsa stain; type II and type III foci were scored as transformed, using the criteria described by Reznikoff *et al.* (5).

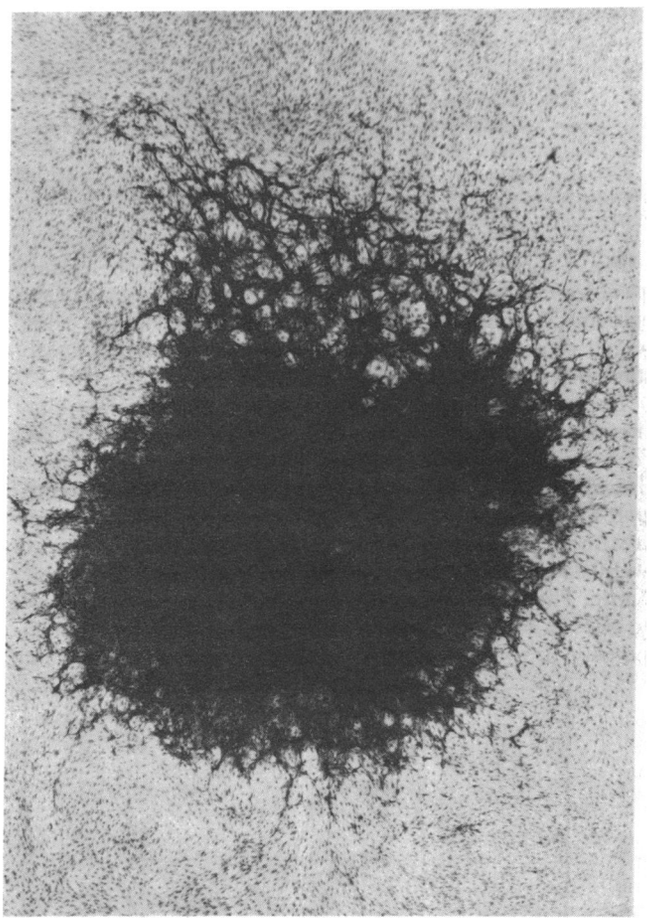

FIG. 1. Type III clone of transformed cells showing the dense piled-up cells and the criss-cross pattern at the edges. The confluent layer of contact-inhibited untransformed cells can be seen in the background.

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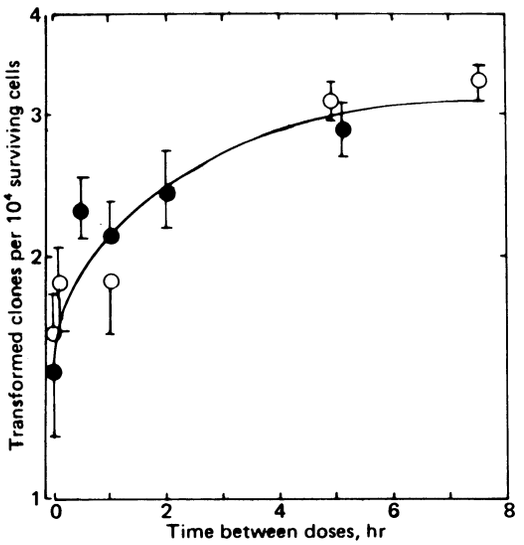

FIG. 2. Influence on transformation rate of the time interval between two dose fractions of 0.5 Gy of x-rays. The results from two experiments are shown, ±SD.

Fig. 1 shows a typical type III clone that can be readily identified by the densely stained piled-up appearance of the cells and the criss-cross pattern at the periphery of the clone, which shows up clearly against the background of lightly stained contact-inhibited untransformed cells.

Irradiations were performed with a Siemens Stabilipan x-ray therapy unit, operated at 300 kV (peak), 12 mA, with added filtration of 0.2 mm Cu. For the higher x-ray doses, a treatment distance of 50 cm was used, at which the dose rate was computed to be 1.8 Gy/min. For the lower x-ray doses, a longer treatment distance of 118 cm was used, at which the dose rate was 0.32 Gy/min. In all cases the exposure time was less than 5 min. The longer treatment distance was used at lower doses to allow a larger number of dishes to be irradiated simultaneously; this was necessary because, as can be seen from Table 1, a typical experiment at low dose levels involved over 1000 petri dishes.

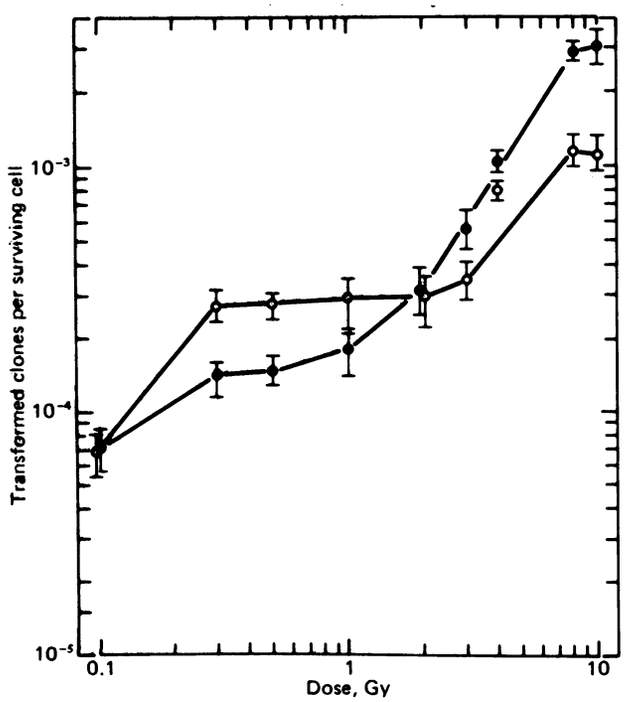

FIG. 3. Pooled data from many experiments for the transformation rate for single (●) and split (○) doses of x-rays. The time interval between split doses was 5 hr.

RESULTS

Transformation frequencies produced by two doses of x-rays of 0.5 Gy, separated by a time interval from 0 to 7.5 hr, are shown in Fig. 2. Fractionation leads to an elevated incidence of transformation, which increases with increasing time interval between the doses up to a maximum at about 4–5 hr.

The accumulated data from experiments designed to compare the transformation frequencies after single and split x-ray doses are summarized in Table 1 and plotted in Fig. 3. The design of the experiments was such that single and split doses were always compared within a given experiment. Because of the sheer size of the experiments, and the number of dishes involved, only a limited number of doses could be used in a given experiment. This was particularly true at the lower end of the dose scale. An interesting pattern emerges. At doses above about 2.0 Gy, fractionation leads to a reduction in transformation frequency compared with a single exposure of the same total dose. Between 0.3 and 1.5 Gy, fractionation enhances the incidence of transformation.

The cell survival data for single and split doses, resulting from the same experiments in which transformation was scored, are shown in Fig. 4. In all cases, fractionation leads to a sparing as far as cell lethality is concerned.

DISCUSSION

Two interesting and potentially important results emerge from the present investigation, one a direct consequence of the other: (*i*), the shape of the dose–response relationship for transformation; (*ii*) the effect of fractionation on the frequency of transformation, which varies with dose. These need to be discussed in turn.

First, the shape of the dose–response relationship. In Fig. 3,

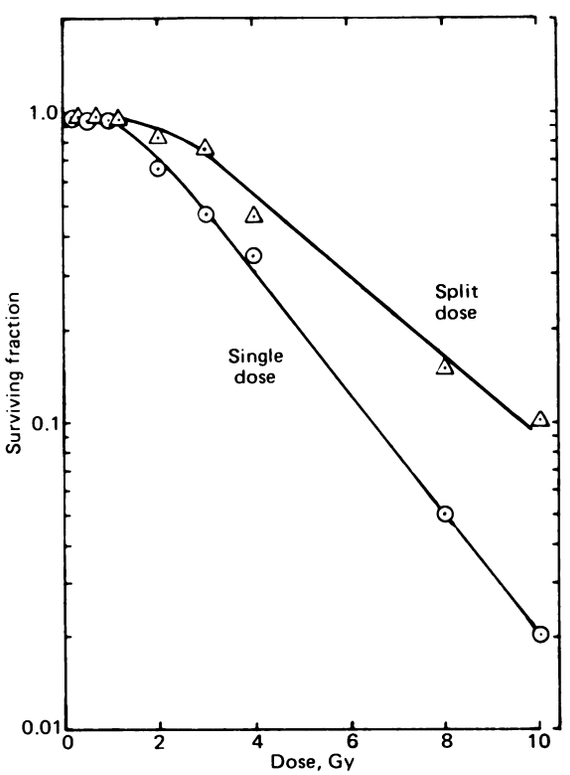

FIG. 4. Survival data for cells exposed to single and split doses of x-rays. The time interval between split doses was 5 hr.

Table 1. Frequency of transformation induced by various single and split doses of x-rays

		Single dose				Split dose (5 hr)			
Exp.	Dose, Gy	Dishes	Surviving fraction	Transformation frequency*	Average transformation frequency × 10⁴†	Dishes	Surviving fraction	Transformation frequency*	Average transformation frequency × 10⁴†
1	0.10	119	1.00	4/ 63,300		100	0.97	4/ 57,000	
2		114	0.95	8/ 88,800	0.686 ± 0.135	110	0.99	7/ 89,300	0.637 ± 0.133
3		350	9.98	14/227,000		338	0.99	12/214,600	
4	0.30	62	0.91	5/ 40,400		94	0.91	15/ 60,000	
5		167	0.91	15/101,000	1.40 ± 0.23	166	0.94	31/109,000	2.83 ± 0.33
6		215	0.88	18/131,000		185	0.91	29/ 96,200	
7	0.50	59	0.91	4/ 29,200		93	0.90	14/ 49,300	
8		78	0.98	5/ 38,600		92	0.98	12/ 57,900	
9		191	0.87	14/ 96,500	1.52 ± 0.18	128	0.98	21/ 72,700	2.78 ± 0.28
10		198	0.89	25/155,000		135	0.97	36/115,000	
11		132	0.98	11/ 60,200		150	0.98	18/ 68,400	
6	1.00	150	0.92	10/ 61,700	1.76 ± 0.41	114	0.97	13/ 48,300	2.86 ± 0.62
11		100	0.93	8/ 40,500		69	0.91	8/ 25,100	
5	2.00	51	0.69	7/ 18,400		50	0.87	4/ 10,800	
12		33	0.66	3/ 11,400	3.27 ± 0.73	85	0.67	2/ 9,600	2.94 ± 0.74
14		87	0.59	10/ 31,100		102	0.62	10/ 34,000	
7	3.00	68	0.47	6/ 9,800		126	0.76	14/ 52,000	
8		131	0.39	28/ 55,000	4.55 ± 0.63	133	0.72	23/ 53,800	3.76 ± 0.52
10		130	0.50	18/ 49,500		92	0.71	16/ 35,200	
9	4.00	23	0.31	8/ 5,200		17	0.40	3/ 2,400	
12		81	0.30	34/ 32,800	9.96 ± 1.17	61	0.39	16/ 22,000	7.04 ± 1.07
13		43	0.43	13/ 14,700		30	0.49	9/ 13,300	
14		52	0.26	17/ 19,600		52	0.48	15/ 23,400	
12	8.00	17	0.051	9/ 2,500		21	0.13	3/ 3,700	
13		31	0.053	30/ 9,600	24.4 ± 2.7	36	0.16	17/ 10,900	12.0 ± 1.90
14		63	0.060	44/ 21,900		68	0.19	22/ 20,400	
12	10.00	27	0.021	31/ 9,700	31.9 ± 5.7	43	0.10	20/ 15,000	13.3 ± 3.00

* Number of transformed clones/total surviving cells.
† Averages ± 1 SD are given.

transformation frequency is plotted against dose on a double logarithmic scale. The slope clearly changes over the range of doses used. At higher doses, above about 2 Gy, the curve is steep and certainly consistent with a slope of 2, implying that transformation frequency may be related to the square of the absorbed dose. At lower doses, below about 0.3 Gy, the curve has a slope consistent with unity, implying that the transformation frequency may be directly proportional to dose. Over the intermediate dose range, the curve is shallow indeed, and within the confidence limits of the data points, transformation frequency barely changes at all between 0.3 and 1 Gy.

Second, a complex pattern emerges for the effect of fractionation on transformation. Above about 1.5–2 Gy, dividing a given dose into two equal fractions results in a reduction in transformation. Between 0.3 and 2 Gy, fractionation clearly results in an elevated frequency of transformation. This results directly from the changing slope of the dose–response relationship for single exposures. Indeed, the dose–response curve for split doses can be derived from that for single exposures if it is assumed that the two exposures, 5 hr apart, are totally independent and do not interact with one another in any way. On this basis, the transformation frequency for two doses of $D/2$ Gy, separated by 5 hr, should be twice that for a single exposure of D Gy. This is found to be approximately true over the entire range of doses tested. The maximum separation between the curves is a factor of 2 in transformation frequency between single and split exposures of the same total dose. The data reported here all involve C3H/10T½ cells, but the effects of fractionation have been reported by Borek and Hall (7) and by Borek (8) for cells derived from fresh explants of hamster embryos. The data are given in Fig. 5, in which, to facilitate comparison, the ratio of transformation frequencies for split to single doses is plotted as a function of dose. Compared in this way, there is a remarkable similarity in the effects of fractionation on transformation assessed by these different biological systems. Above 1.5–2 Gy, fractionation decreases transformation frequency, whereas below this dose level fractionation enhances it.

It is evident, then, that in the case of *in vitro* transformation, the dose–response relationship has a sufficiently complex shape, so that the transformation frequency for low doses cannot be predicted accurately by a linear extrapolation from data obtained at high doses. It can be seen from Fig. 3 that a linear extrapolation from high doses may either substantially overestimate, or equally well underestimate, the transformation incidence at low doses, depending upon the dose range in-

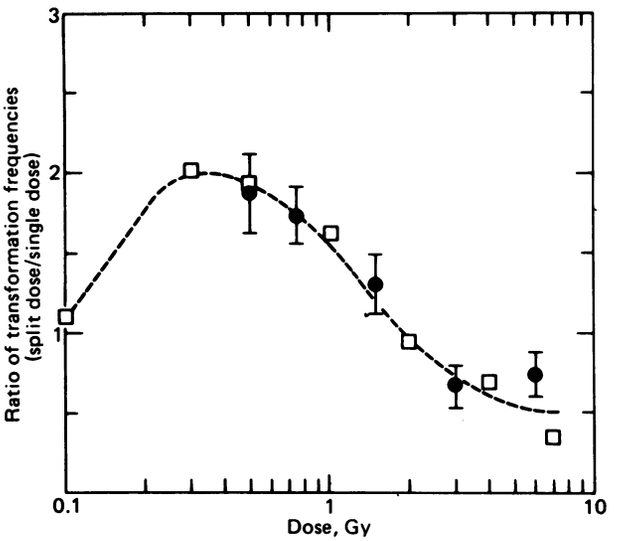

FIG. 5. Comparison of the data presented in this paper for C3H/10T½ cells (□) with the data of Borek and Hall (7) and Borek (8) for fresh explants of hamster embryo cells (●). The ratio of the transformation frequencies for split and single doses is plotted as a function of total dose. A ratio in excess of unity implies that fractionation enhances transformation; a ratio of less than unity implies that fractionation results in a reduction of the transformation rate. For both cell systems the crossover point between the enhancing and sparing effect of fractionation occurs at about 1.5–2.0 Gy.

volved. Furthermore, there is a broad and important range of doses over which fractionation *enhances* transformation frequency so that estimates from data relating to a single prompt exposure do not necessarily represent an upper limit to the transformations that could accrue from multiple small doses.

It must be admitted, of course, that morphologically identified transformed clones in a petri dish are a far cry from leukemia or solid tumors in humans; as a model system, transformation *in vitro* clearly has its limitations. Cell transformation is likely to be an initial step in carcinogenesis, but the transition from transformation of a cell to the development of a tumor is undoubtedly a complex process. It could be argued that dose and dose-rate characteristics of the basic transformation process may be obscured, or even reversed, in the final expression of carcinogenesis.

However, the attraction of the *in vitro* transformation system lies in its exquisite sensitivity, as a result of which it is possible to obtain a dose–response relationship over a range of doses, and with a precision, that is unlikely ever to be equaled in humans. It is clearly not prudent to ignore the possible implications of the shape of this dose–response relationship or the enhancement of transformation resulting from fractionation at low dose levels.

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