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Relationship between x-ray exposure and malignant transformation in C3H 10T½ cells

(carcinogenesis/epigenetic mechanism/transformation frequency)

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ABSTRACT **The appearance of transformed foci after x-irradiation of the C3H 10T½ line of murine cells requires extensive proliferation followed by prolonged incubation under conditions of confluence. When the progeny of irradiated cells are resuspended and plated to determine the number of potential transformed foci, the absolute yield is constant over a wide range of dilutions and is similar to that observed in cultures that have not been resuspended. In addition, for cells exposed to a given x-ray dose, the number of transformed foci per dish is independent of the number of irradiated cells. These observations suggest that few, if any, of the transformed clones occur as a direct consequence of the x-ray exposure and challenge the hypothesis that transformed foci are the clonal products of occasional cells that have experienced an x-ray-induced mutational change. Rather, it appears that at least two steps are involved. We suggest that exposure to x-rays results in a change, for example, the induction or expression of some cell function, in many or all of the cells and that this change is transmitted to the progeny of the surviving cells; a consequence of this change is an enhanced probability of the occurrence of a second step, transformation, when these cells are maintained under conditions of confluence.**

The mouse-embryo-derived cell line C3H 10T½ is widely used to measure the effects of radiation and chemical carcinogens on the formation of foci of transformed cells. When cells that have been exposed to these damaging agents are allowed to grow to confluence and then further incubated for several weeks, clones of cells that have lost contact inhibition become apparent on the background of confluent cells. When cells cloned from these transformed foci are inoculated into syngeneic mice, they are found to be tumorigenic (1).

The yield of transformed foci increases as a function of x-ray exposure, up to a dose of about 400 rads (1 rad = 1×10^{-2} gray) (1, 2). Further increases in x-ray exposure (to as much as 1400 rads) result in little or no increase in the yield of transformed foci (1, 2).

Extensive cellular proliferation is critical for the appearance of recognizable foci of transformed cells (1–3). In addition, the cell density at plating of the exposed cells has been reported to play a role in the expression of transformation (4–8).

In this paper, we describe experiments designed to examine the requirement for cellular proliferation and the effects of cell density on the development of malignantly transformed foci after an x-ray exposure of 400–600 rads. To investigate the mode of multiplication of cells having the potential to form transformed foci, x-ray-exposed cells were allowed to grow to confluence, about 13 generations, and resuspended; various dilutions of these resuspended cells were seeded again to permit growth and assessment of the yield of transformed foci. In addition, we investigated the influence of varying the number of irradiated cells on the ultimate yield of transformed foci.

We found that, with the progeny of irradiated cells, the number of transformed foci is independent of the number of progeny cells seeded and that the number of transformed foci detected per confluent dish does not increase with the number of irradiated cells giving rise to the confluent population. We interpret these results in terms of a two-step process. The first step involves a change that is induced in all of the surviving cells that are exposed to x-rays in this dose range. This change is transmitted to the progeny of the irradiated cells and makes them more prone to undergo the second step, transformation, many generations later.

MATERIALS AND METHODS

We used the C3H mouse-embryo-derived cell line (10T½, clone 8) isolated and characterized by Reznikoff *et al.* (8, 9) and adapted in our laboratory for studies of radiation-induced transformation (1–3). Stock cultures were maintained in 60-mm petri dishes and passaged by subculturing at a 1:20 dilution every 7 days. The cells used were in passages 9 to 14. They were grown in a humidified 5% CO_2/95% air atmosphere at 37°C in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were seeded on replicate 100-mm petri dishes (1–400 viable cells each) and irradiated 24 hr later. Irradiation was carried out at room temperature with a 100-kV Philips MG-100 industrial x-ray generator operating at 9.6 mA and having a dose rate of 78 rads/min. The transformation frequencies were the same for a given radiation exposure, whether the cells remained in the dishes in which they were irradiated or were resuspended immediately after irradiation and seeded in fresh dishes (2), thus excluding any possible persisting contribution from a radiation effect on the plastic of the petri dish. Plating efficiencies in each group of routine experiments were determined from dishes seeded at a cell density one-fifth of that used for the transformation assay and counted 10 days after irradiation. Types 2 and 3 foci were scored as transformants; type 3 cells are tumorigenic in 80–100% of inoculated mice, and type 2 cells are tumorigenic in 60–75% of inoculated mice (1).

RESULTS

The protocol for x-ray transformation experiments is described schematically in Fig. 1. Because the capacity for cell proliferation is critical for the phenotypic expression of x-ray transformation in 10T½ cells (1, 2), the irradiated cells were incubated to allow about 12–13 rounds of cell division before they

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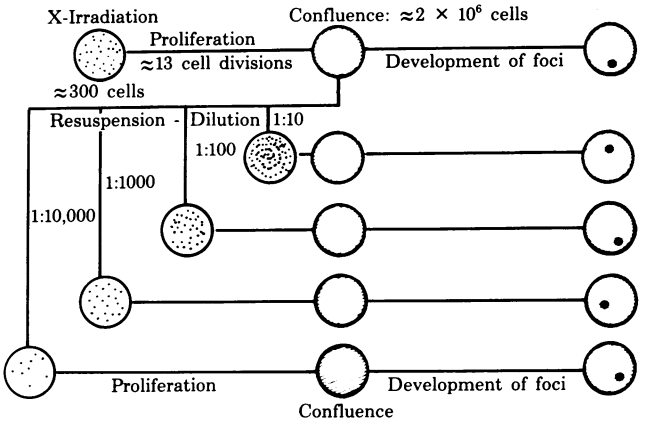

FIG. 1. Schematic description of development of malignant transformation *in vitro*. Specific time course shown in the top line is for 10T½ cells. The number of cells initially seeded was such that, taking into account the normal plating efficiency and the lethality of the x-ray treatment for 10T½ cells, about 300 viable cells per 100-mm petri dish (left) would result. The cells were irradiated the following day and then allowed to proliferate until confluence was reached (10–14 days later), when cell division ceased (center). Dense, piled-up, transformed foci appeared 4–5 weeks later (right) overlying the confluent monolayer (group A of Table 1). The lower lines describe the results of resuspension of individual dishes and reseeding at different dilutions. As each dish became nearly confluent, its contents were trypsinized, resuspended, diluted, and reseeded into new dishes (groups B–F of Table 1). The cells in each of the reseeded dishes then proliferated until confluence was reached a second time, and incubation was continued for 4 additional weeks until foci developed. In dilution experiments such as these each dish that is resuspended and diluted will give rise to one dish at each successive dilution (a total of as many as 4 dishes). Each of the 10 original irradiated dishes to be diluted was handled similarly.

reached confluence (about 2×10^6 cells per 100-mm dish), and this was followed by 4–5 weeks of incubation under confluent conditions.

Several of the experiments described here involved reseeding of the progeny of irradiated cells once they had reached the confluent state of growth. Cells were seeded in replicate dishes (usually 25–40 per experiment) at a density that would result in about 300 viable cells after exposure to 400 rads [surviving fraction about 20% after 400 rads (1) and plating efficiency about 25% for 10T½ cells]. The irradiated cells were allowed to proliferate until they became nearly confluent, about 10^6 cells per dish. At this point, some of the dishes were left undisturbed, while others were trypsinized and the cells in them were suspended and reseeded at various dilutions (Table 1). Cells harvested from each dish (irradiated) were treated separately to provide one reseeded dish at each successive dilution (see Fig. 1). The dishes containing various dilutions of reseeded cells were then returned to the incubator, and the cells were allowed to grow to confluence, followed by further incubation for the full period for the appearance of transformed foci (about 6 weeks). The frequency of appearance of transformants can be estimated from the number of foci per dish. Alternatively, if the appearance of foci is assumed to be Poisson distributed, the frequency of dishes on which no transformed foci appear can be used to calculate the mean yield. The latter procedure avoids the ambiguities that would derive from the occasional appearance of a transformed cell before a population of confluent cells is resuspended. Such a transformed cell may have proceeded through several divisions and thus be represented at a high frequency among the resuspended cells, like the "jackpot" clones of mutants described by Luria and Delbrück (10). Such presumed jackpot clones are evident in one or two of the sets of dishes derived from the 1:10 and 1:30 dilutions of resuspended confluent cells (groups B and C, Table 1).

From the fraction of dishes in each group that did not have transformants [P(0)], the average number (λ) of transformed foci per dish was calculated according to the Poisson distribution: $P(0) = e^{-\lambda}$. The 95% confidence interval for λ was estimated on the basis of a table of exact confidence intervals for a true binomial distribution of P(0). (The confidence limits are not symmetrical around λ because the Poisson distribution is skewed.)

It was found that the total number of foci per dish was approximately constant even though the dilution range was more than three orders of magnitude. Furthermore, the number of transformed foci per dish was similar to that observed in the dishes containing the undisturbed progeny of irradiated cells. Thus, irradiated cells that had been allowed to grow through 13 generations did not appear to contain an increased number of cells capable of yielding transformed foci.

Next, we investigated the transformation yield when the initial cell densities were less than the usual number (300–400) of viable cells per dish. The effects of radiation doses of 600 rads on cultures having initial cell densities ranging from ≈1 to ≈200 cells per dish are shown in Table 2. The mean yield of transformed foci per dish was constant over a range of two orders of magnitude of initial cell densities. Thus, a plot of the relationship between the average number (λ) of transformed foci per dish and the number of viable cells initially seeded or reseeded per dish shows that there is no significant change in the number of transformed cells per dish as a function of the number of irradiated cells per dish (Fig. 2). Rather, the yield appears to be related to the numbers of cells present on the plate when confluence is reached. The implications of this observation are discussed below.

At x-ray doses of less than 400–600 rads, the yield of transformed foci depends on the dose. Thus, in an experiment in which various cell densities were exposed to doses of 100 rads, the mean number of foci per dish was about 0.1 (seven foci among 85 dishes at cell densities of 3–86 cells per dish), substantially less than was observed after a dose of 600 rads.

Unirradiated controls for these low cell density or subcultured cell populations included dishes seeded with 300 viable cells (10 dishes per experiment) and dishes seeded with 1–10 cells (two experiments involving 10 dishes each). In addition, dishes seeded with 300 cells allowed to grow to confluence were then resuspended and seeded at a 1:1000 dilution, 300 cells per dish (two experiments, 10 dishes each), and allowed the full expression period for detection of transformed foci (6 weeks). No transformation was observed in any of these control cultures.

DISCUSSION

We have previously reported that, when irradiated cells (about 300 viable cells per dish) are permitted to grow to confluence (about 2×10^6 cells per dish) and are resuspended and reseeded at 300 cells per dish and incubated for 6 weeks to permit regrowth to confluence and the development of transformed foci, the yield of transformed foci per dish is the same as that on dishes containing undisturbed cells (11, 12). This observation does not contradict the notion that an occasional cell among the survivors of the x-ray exposure could have been altered in a heritable way and that the frequency of its progeny among all cells on the dish had remained unchanged during the growth to confluence. However, the observations reported in this paper show that the yield of transformed foci per dish is constant, even when the confluent cells are resuspended and reseeded over a range of ≈25 to 35,000 cells per dish. Similarly, in experiments in which the initial cell density at the time of x-ray exposure was

Table 1. Transformation as a function of dilution at confluence: 400 rads

Experiment	Dilution at confluence	Viable cells per dish, no.	Dishes, no.	Fraction of dishes containing transformants	Total foci observed, no. Type 3	Total foci observed, no. Types 2 and 3	Ratio of foci to dishes	Fraction of dishes without transformants, P(0)	λ (95% confidence interval*)
Group A†									
1	—	420	14	8/14 (0.57)	5	10			
2	—	440	27	21/27 (0.78)	4	33			
3	—	247	16	4/16 (0.25)	4	6			
4	—	96	10	5/10 (0.50)	2	6			
5	—	300	17	7/17 (0.41)	8	11			
6	—	120	20	6/20 (0.30)	3	6	72/104 (0.7)	53/104 (0.51)	0.7 (0.92–0.49)
Group B‡									
1	1:10	35,000	10	8/10 (0.80)	0	83§			
2	1:10	27,000	10	4/10 (0.40)	0	11	94/20 (4.7)	8/20 (0.40)	0.9 (1.61–0.45)
Group C									
2	1:30	9,000	10	3/10 (0.30)	2	21	21/10 (2.1)	7/10 (0.70)	0.4 (1.05–0.07)
Group D									
1	1:100	3,500	10	5/10 (0.50)	4	7			
2	1:100	2,700	10	4/10 (0.40)	0	9	16/20 (0.8)	11/20 (0.55)	0.6 (1.17–0.26)
Group E									
1	1:1,000	350	10	3/10 (0.30)	3	5			
3	1:1,000	216	20	5/20 (0.25)	3	5			
4	1:1,000	60	10	4/10 (0.40)	4	4			
5	1:1,000	325	19	3/19 (0.16)	2	3			
6	1:1,000	240	15	5/15 (0.33)	3	5	22/74 (0.3)	54/74 (0.73)	0.3 (0.48–0.17)
Group F	1:10,000	25¶	10	2/10 (0.20)	2	4	4/10 (0.4)	8/10 (0.80)	0.2 (0.82–0.03)

* Estimated by using exact confidence intervals for a binomial distribution of P(0).
† Group A not reseeded; initial cell density shown.
‡ Cell density shown is for after reseeding.
§ More than half of the 83 colonies were on 2 dishes (distribution among 8 dishes: 1, 1, 4, 8, 8, 11, 19, 31).
¶ For this group, number of cells per dish was determined on the cell suspension used for the transformation assay itself.

varied, the yield of transformants per dish appeared to be constant and independent of the number of cells that were initially exposed to x-irradiation.

When irradiated cells have grown to confluence, their progeny have frequencies of transformation (number of transformants per dish) independent of dilution and equal to the frequencies observed on dishes in which the cells have remained undisturbed. This suggests that the cell alteration that results in the formation of a clone of transformed cells is not the immediate, direct consequence of the exposure to x-rays (e.g.,

Table 2. Transformation as a function of cell density: 600 rads

Cells per dish, no.	Viable cells per dish, no.	Dishes, no.	Fraction of dishes containing transformants	Total foci observed, no. Type 3	Total foci observed, no. Types 2 and 3	Ratio of foci to dishes	Fraction of dishes without transformants, P(0)	λ (95% confidence interval*)
100–400	260	19	7/19 (0.37)	4	8			
	165	20	10/20 (0.50)	6	12			
	135	20	5/20 (0.25)	4	5			
	114	20	6/20 (0.30)	2	6			
	113	20	6/20 (0.30)	3	6	37/99 (0.37)	65/99 (0.66)	0.41 (0.58–0.29)
50–100†	100	17	9/17 (0.53)	6	10			
	90	14	4/14 (0.29)	2	4			
	82	13	3/13 (0.23)	1	3			
	52	20	4/20 (0.20)	3	4	21/64 (0.33)	44/64 (0.69)	0.37 (0.59–0.24)
10–30†	26	20	9/20 (0.45)	7	13			
	20	15	5/15 (0.33)	4	6	19/35 (0.54)	21/35 (0.60)	0.51 (0.87–0.27)
<5†‡	4	13	6/13 (0.46)	4	6			
	4	11	4/11 (0.36)	3	5			
	3	16	5/16 (0.31)	2	6			
	3	8	4/8 (0.50)	2	4			
	2	16	5/16 (0.31)§	2	5			
	1	5	2/5 = 0.40§	1	2			
	1	6	1/6 = 0.17§	0	1	29/75 (0.40)	48/75 (0.64)	0.45 (0.65–0.29)

* Estimated by using exact confidence intervals for a binomial distribution of P(0).
† Number of cells per dish was determined from the actual dishes used for the transformation assay and terminated at 10 days (i.e., not from a normal plating efficiency—a 1/5 dilution of the cell suspension used for the transformation assay).
‡ A few dishes that appeared to be confluent with transformed cells were counted as one focus. At this cell density, several of the foci were larger than normally observed, sometimes covering the entire dish.
§ Based on the number of dishes that reached confluence (i.e., had at least one cell per dish).

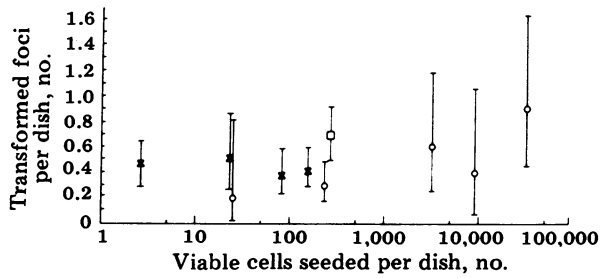

FIG. 2. Relationship between average number of transformed foci per dish and average number of viable cells seeded per dish. □, 400 rads, no dilution (group A of Table 1); ○, 400 rads, various dilution groups (groups B–F of Table 1); x, 600 rads, initial cell density (Table 2). Bars indicate 95% confidence interval.

a mutational event). If the occasional transformed colony were the result of a change induced by x-rays in an occasional cell, resuspension of the descendants of that cell would be expected to give rise to many transformed foci when the cells were reseeded at high cell densities. The number of transformed foci per dish would be expected to decrease when the cells were reseeded at progressively lower densities. Neither expectation was realized. The further observation that the yield of transformants (measured in terms of the number of transformants per dish) is insensitive to the initial cell density similarly contradicts the expectation that x-ray damage is the determining event in the formation of a transformed cell.

Both sets of observations can, however, be accounted for by assuming that a two-stage process is responsible for radiation-induced transformation. We assume that exposure to x-rays, in the dose range of 400–600 rads, is sufficient to produce (in all or nearly all of the surviving cells) a functional change that is inherited by their progeny. We further assume that a consequence of this functional change is an increased probability that the cells, when maintained under conditions of confluence, will sport a cell capable of forming a transformed focus. The reduced yield of transformants evident at lower x-ray doses could reflect a reduced fraction of cells manifesting this functional change.

Other investigators, calculating the transformation yield on the basis of the frequency per surviving cell, have reported an apparent increase in yield with decreasing cell densities for 10T½ cells (4, 5, 8). This observation is similar to that reported here. It has also been suggested that a minimum colony size is necessary for potentially transformed cells to escape the suppressive effects of normal cells and express themselves as transformants (13). To account for our observations by such an effect would require a remarkable coincidence—i.e., that the suppressing effect be precisely balanced by the dilution effect over a wide range of dilutions and of growth experience (13 to 29 cellular generations).

Other investigators, examining the transformation yield from small numbers of treated cells have found large fractions of dishes having transformed foci. In transformation experiments using one cell at risk per dish and carcinogenic chemicals, Mondal and Heidelberger showed that, for C3H mouse prostate cells (14, 15), most or all of the dishes manifested transformed foci. A similar finding has recently been reported by Terzaghi and Nettesheim (16) for the development of tumorigenic potential in carcinogen-exposed cells of the rat tracheal mucosa. Although a large fraction of cultures containing tracheal epithelial cells exposed *in situ* to dimethylbenzanthracene had neoplastic potential on explantation and growth *in vitro*, only a small number of tumors developed from carcinogen-exposed cells left in the host animals (16).

We wish to suggest that the change that is detected as a transformed focus in these experiments is not a direct consequence of the exposure to radiation or chemical carcinogens. Rather, we suggest that, for the dose range that has been used, the exposure results in a cellular alteration such as a functional or metabolic change common to most or all of the surviving cells and that this change is transmitted to their progeny during subsequent growth. One consequence of this change is an enhanced probability of a second event, perhaps mutational, that is expressed as a transformed clone. This second event appears to occur for the most part during maintenance of the cultures under confluent conditions.

Evidence suggesting that the cellular alteration leading to malignancy may involve an epigenetic change has been discussed by Braun (17). The primary consequence of radiation exposure could be such an epigenetic change. The biochemical nature of this change remains, of course, as yet unspecified.

Although the suggested first step could also reflect a genetic change, a mutation common to most or all of the cells exposed to these radiation doses seems unlikely. On the other hand, physiological changes inherited through many cell generations are characteristic of differentiation processes in higher organisms. The mechanisms involved are obscure, but possible models in which an induced physiological change is inherited during clonal growth in the absence of any genetic change (18) have been described for bacterial systems.

In one such case, Novick and Weiner (19) showed that there is a concentration range of an inducer of the β-galactosidase operon of *Escherichia coli* insufficient to induce the operon genes but sufficient to maintain, for many generations, a maximum induced level of β-galactosidase in bacteria previously induced by exposure to a high concentration of inducer. A second product of the β-galactosidase operon, the β-galactoside permease, provides an explanation for the inheritance of this physiological change—i.e., that, when induced levels of the permease are present, the bacteria can concentrate the inducer even when its external concentration is too low to produce induction in uninduced bacteria. Another well-analyzed case of a persistent nongenetic change has been reported for the bacteriophage λ regulatory system in lysogens of *E. coli* (20).

The x-ray-induced functional change postulated as the initial alteration in irradiated 10T½ cells may be a novel example of a persistent nongenetic change in a mammalian cell line.

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