*Effect~~ion~~ of LET and Microdistribution
of radiation on the transformation ⁱⁿvitro
and ⁱⁿvivo³* **COMPREHENSIVE PROGRESS REPORT**

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A. Overall Summary

Work during the past contract period has involved the three areas described in the previous application: 1) An investigation of the mechanisms of radiation carcinogenesis by studying the events involved in the process of malignant transformation of mouse 10T½ cells; 2) An investigation of the effects of promoting agents on radiation-induced transformation in vitro; and 3) An investigation of the induction of transformation by internally emitting radionuclides incorporated into cellular DNA. The latter area has been extended to include studies of mutagenesis by these radionuclides in human lymphoblasts, and molecular measurements of DNA strand breaks. During the past year, our research has focused on the first area, as well as on studies of the mutagenic effects of incorporated radionuclides.

Studies of malignant transformation in vitro suggest that this process involves at least two steps: the first is an immediate consequence of the radiation exposure. It is a frequent alteration occurring in a large fraction of the irradiated cells. Thus, though it appears to involve DNA damage, it does not appear to reflect a mutation in a specific structural gene or group of genes. The second step, the malignant transformation of one or more of the progeny of the irradiated cell, is a rare event occurring with a frequency of approximately 10^{-6} which occurs randomly during the growth of the irradiated progeny to confluence. In these characteristics the second step resembles a mutation. We suggest the hypothesis that cells respond to non-specific DNA damage by the induction of a process which enhances the spontaneous transformation frequency of their progeny during subsequent growth.

The most notable result in the studies of potential promoting agents is the observation that Epidermal Growth Factor (EGF) can act as a potent promoter of transformation initiated by low doses of radiation. To our knowledge, this result provides the first evidence that a naturally occurring, endogenous polypeptide growth factor can enhance malignant transformation. The endogenous steroid hormone 17- β estradiol induced transformation by itself, but did not appear to act as a true promoting agent. A stimulator of prostaglandin synthesis, melittin, was inactive either as a carcinogen or a promoter.

The Auger emitting radionuclide ^{125}I was extremely potent in inducing malignant transformation and 6-Thioguanine resistant mutations when incorporated into cellular DNA as ^{125}I -Iododeoxyuridine ($^{125}\text{IdUrd}$). Decay of this isotope leads to an intense deposition of energy in the DNA immediately surrounding the site of incorporation, leading to a high LET-like effect. Each disintegration produced at least one DNA double strand break, and the probability for a mutation arising from a decay anywhere in the HPRT gene was approximately 1.0. The most striking finding is the marked efficiency with which $^{125}\text{IdUrd}$ induced both transformation and mutations at dose levels of only a few total radioactive disintegrations per cell; doses which produced little or no cell killing.

In the detailed reports below, the references refer to the Publication List at the end of the Report.

B. Detailed Report

1. Mechanisms of Radiation Transformation

We have previously hypothesized that radiation transformation in vitro is a two-step process (Proc. Natl. Acad. Sci. 77: 762-766, 1980; and Carcinogenesis 1: 1039-1047, 1980); the first step is a frequent alteration occurring in a large fraction of irradiated cells, while the second step, malignant transformation, is a rare event with an approximate frequency of 10^{-6} . In the current contract period we have examined in more detail the events and steps associated with the development of malignant transformation in x-irradiated 10T½ cells as outlined in the previous application. In particular, we have directed our attention to studies of the nature of the second event, and the role of environmental factors on the expression of transformation.

The initial set of experiments was designed to determine more specifically when the proposed second event occurs (11, 12). In these experiments, cells seeded at low density were irradiated and allowed to reach confluence. At this time they were suspended and reseeded in their entirety into one or two dishes. These dishes were returned to the incubator for 4 weeks, and the distribution of transformed foci within a large number of such dishes was determined. The results of a typical experiment are shown in Table 1. The distribution of foci amongst dishes in this experiment is consistent with random occurrence of the second transforming event during the growth of the irradiated cells to confluence. If this is the case, the distribution of foci should follow that calculated for the distribution of mutants under similar conditions by Lea and Coulson (J. Genetics 49: 264-285, 1949). As can be seen in Table 2, the experimental data conform very closely to the prediction. This was true of five separate large scale experiments of this type (12). As 75% of the total number of mitoses occurs during the two rounds of cell division prior to reaching confluence, most transforming events would take place just prior to confluence. However, occasional progeny may transform much earlier.

Several other types of experiments have also been performed in order to further study this phenomenon (12). In one series, 350-475 total viable cells were initially seeded representing an approximately 1:2 mixture of irradiated and non-irradiated cells. The resultant yield of transformants was directly proportional to the number of irradiated cells present in the mixture rather than to the total number of cells seeded. This result suggests that transformation occurs only amongst the progeny of irradiated cells, and speaks against the hypothesis that radiation may have activated a virus which spreads throughout the culture when the cells become confluent leading to a constant yield of transformants. It also excludes the possibility that irradiated cells may release into the medium some factor which causes the transformation of the progeny of irradiated or non-irradiated cells.

TABLE 1. DISTRIBUTION OF TRANSFORMED CLONE SIZES IN MOUSE 10T½ CELLS
8 AND 15 DAYS AFTER IRRADIATION¹

Treatment Group	Number of Transformed Foci in Each Dish	Fraction of Dishes Containing Transformed Foci	Among Dishes Containing Foci Average Number of Foci per Dish S.E.
1. 200 rads not reseeded	0,0,0,0,0,0,0,0,0,1,1,2	3/13 = 0.23	1.3 0.33
2. 200 rads reseeded at day 8	0,0,0,0,0,0,0,0,0,0,0,0,0,0, 0,0,0,0,0,0,0,0,0,0,1,1,2, 2,2,3,5,7,7,12,15,22	12/37 = 0.32	6.6 2.7
3. 200 rads reseeded at day 15	0,0,0,0,0,0,0,0,0,0,0,0,0,0, 0,0,0,0,0,0,0,0,1,1,1,1,1,1, 2,2,2,3,4,9,11,12,15,30,35	17/40 = 0.42	7.7 3.1

¹Data from Kennedy and Little (12).

TABLE 2. COMPARISON OF EXPECTED AND OBSERVED DISTRIBUTIONS OF CLONE SIZES
FOR THE DATA PRESENTED IN TABLE 2 (pooled results)¹

	Numbers of Transformed Colonies in Reseeded Groups (r) ²								
	0	1	2	3-4	5-8	9-16	17-32	33-64	64
Probability that a culture has r transformants (mutants) when the average number (m) of mutants (mutations) per culture is 0.5 (P(0) = 0.62) (Lea and Coulson (16), Table 2)	.6065	.1516	.0695	.0648	.0475	.0288	.0155	.0079	.0079

In 77 Dishes Observed	48	8	6	3	3	6	2	1	0
			9		12				
Expected	46.7	11.7	5.4	5.0	3.7	2.2	1.2	.61	.61
			10.4		8.3				

¹Data from Kennedy and Little (12).

²For the chi-square analysis, the categories were grouped as indicated such that no expected or observed numbers were less than 5; with 2 degrees of freedom, $\chi^2 = 3.3$, $p = 0.10$.

In other experiments, a constant number of irradiated cells was seeded into dishes of varying sizes ranging from 8 to 144 cm² in surface area. The yield of transformants was directly proportional to the surface area of the dish and therefore the number of progeny cells present at confluence, rather than to the initial number of irradiated cells. This finding further supports the two event hypothesis.

Finally, cells from mass cultures were removed at various times during a classical transformation experiment and examined for their ability to grow in soft agar under anchorage independent conditions. No soft agar colonies were observed during the first five days after irradiation, but the ability such to produce colonies occurred as the cultures became confluent, and reached a maximum a few days later. These results are consistent with a transformational event occurring near confluence as hypothesized above, at which time the ability to grow under anchorage independent conditions appears. When non-transformed cells were selected with a cloning cylinder from irradiated dishes at the end of an experiment (6-7 weeks post-irradiation) and reassayed for transformation, the frequency was not elevated over background levels, suggesting that transformation was complete by that time. The use of techniques to suppress proliferation during the confluent phase of expression had no influence on transformation, further supporting the earlier observations (Tables 1 and 2) which suggest that the second event occurred just prior to confluence.

The results cited in the paragraphs above would be consistent with a mutational hypothesis for the second event in transformation of 10T½ cells. By this hypothesis, the initial radiation exposure would have the effect of enhancing the probability of spontaneous mutations (the second event) occurring amongst the irradiated cell progeny. By a classical mutation model, the probability of such mutations would be a function of the number of cell divisions occurring in the culture. Radiation would thus enhance the probability of mutational events which lead to transformation. In order to test this hypothesis, a number of experiments have been performed to determine first whether the spontaneous mutation frequency was enhanced in irradiated 10T½ cells seeded at low density and allowed to reach confluence, and secondly, whether such cells were hypersensitive to the induction of mutations by classical mutagens. Studies carried out to date in 10T½ cells using ouabain resistance as a marker have all been negative; higher spontaneous or MNNG- induced mutations to ouabain resistance were not observed at various post irradiation intervals. Similar experiments were also carried out with Chinese hamster V-79 cells with 6-thioguanine resistance as a mutational marker. We also found no evidence for higher mutation frequencies in pre-irradiated V-79 cells.

The observation that the initial event in malignant transformation in vitro appears to be a common one involving a large fraction of cells speaks against this event representing a mutation in a specific structural gene or group of genes. Experiments described below with ¹²⁵I dUrd indicate, however, that DNA damage is clearly involved in the initiation of transformation. Based on our results to date, we hypothesize that cells respond to non-specific DNA damage by the induction of a process which enhances the spontaneous transformation frequency. This second event acts like a mutational one in that it occurs with very low frequency and at random during the growth of the cells. It differs from the usual SOS process in that it cannot be driven by mutagen exposure. Modifiers of transformation such as tumor promoters or protease inhibitors appear to influence the probability of occurrence of this second event.

These observations and the hypotheses proposed to explain them are certainly unusual in terms of the classical view of mechanisms of transformation. The question arises as to whether they are peculiar to this particular cell system, or whether they might have more general application to radiation carcinogenesis. None of our current knowledge is inconsistent with such a mechanism. To the contrary, there is at least one piece of experimental evidence *in vivo* which supports such an interpretation. Terzaghi and Nettesheim (Cancer Res. 39: 4003-4010, 1979) have presented evidence in an animal tumor model that though only an occasional tumor arises in a large population of carcinogen exposed cells *in vivo*, a large fraction of such cells have tumorigenic potential if allowed to grow under the appropriate conditions *in vitro*. This potential is expressed in terms of the actual transformation of one of their progeny. The unusual characteristics associated with the transformation of mouse embryo fibroblast derived cell lines which we have observed suggest that the general process of radiation transformation is not easily amenable to simple mechanistic models.

2. Effects of Tumor Promoting Agents on Radiation Transformation

In these experiments (1-4), we have examined several classes of agents for possible promoting effects on x-ray transformation in mouse 10T½ cells. These include: 1) the endogenous steroid hormone 17- β -estradiol; 2) the endogenous growth factor called Epidermal Growth Factor (EGF); 3) the bee venom toxin melittin, a stimulator of prostaglandin synthesis; and 4) the non-steroidal anti-inflammatory agent indomethacin. Anti-inflammatory agents have been reported to inhibit promotion *in vivo*. In all cases, the effects of these agents were compared with those of the known phorbol ester tumor promoter TPA, and with the protease inhibitor antipain—a potent suppressor of x-ray transformation. In most experiments, the cells were incubated with non-toxic or minimally toxic concentrations of these agents for the entire 6-7 week post-irradiation expression period. The results of some of these experiments are shown in Tables 3 and 4 on the following pages.

The effects of potential tumor promoters on x-ray transformation are shown in Table 3. Indomethacin and 17- β -estradiol both induced a fairly high yield of transformants by themselves. In cultures treated with x-rays and indomethacin, however, the yield was reduced over that expected. EGF did not transform by itself, but consistently enhanced the yield of transformants induced by either x-rays (Table 3) or ultraviolet light exposure (data not shown) (2). The enhancement was similar in magnitude to that induced by TPA in the same experiments; it was not significantly inhibited by indomethacin. Melittin, on the other hand, appeared to suppress both x-ray and UV-induced transformation. Also included as a control in these experiments was the protease inhibitor antipain. Antipain induced no transformation by itself, but markedly suppressed transformation induced by x-rays (Table 3). As previously reported, it also completely inhibited the promotion of x-ray transformation by TPA (14). Indomethacin also inhibited the TPA enhancement of x-ray transformation.

As is also shown in Table 3, no promoting effect was observed when x-irradiated cells were incubated during the entire expression period in the presence of low concentrations of mitomycin-C. Some investigators have found that TPA induces a low level of sister chromatid exchanges (SCE) in mammalian cells, and it

TABLE 3. EFFECT OF POTENTIAL PROMOTING AGENTS ON X-RAY TRANSFORMATION

Treatment ^a		Total No. of Dishes	Total No. of Cells	No. Transformed Foci Observed	Average No. Foci per Dish
1	2				
-	-	120	28,640	1	0.01
-	EGF (50 ng/ml)	28	9,575	0	0
-	Mitomycin-C (10^{-9} M)	17	6,075	1	0.06
-	Indomethacin (0.1 μ g/ml)	33	11,645	37	1.12
-	17- β -estradiol (10^{-6} M)	82	24,600	21	0.26
100 rads	-	105	34,335	21	0.20
100 rads	EGF	29	11,010	26	0.90
100 rads	Melittin (2 μ g/ml)	18	6,210	0	0
100 rads	TPA (0.1 μ g/ml)	32	12,400	36	1.12
100 rads	Mitomycin-C (10^{-9} M)	30	10,530	4	0.13
100 rads	Indomethacin (0.1 μ g/ml)	11	5,885	2	0.18
600 rads	Antipain (50 μ g/ml)	26	9,625	1	0.04

^aSecond treatment begun 24 hrs after seeding (immediately after irradiation) and maintained for full 6-7 week expression period. Data pooled from several experiments.

TABLE 4. EFFECT OF 17- β -ESTRADIOL ON X-RAY TRANSFORMATION

Treatment ^a		Total No. of Dishes	No. Transformed Foci Observed	Average No. Foci per Dish
1	2			
-	-	56	0	0
-	17- β -estradiol (10^{-5} M)	72	14	0.19
400 rads	-	102	26	0.25
400 rads	17- β -estradiol (10^{-5} M)	70	31	0.44
400 rads	17- β -estradiol (10^{-5} M) antipain (600 μ g/ml)	20	2	0.10
-	17- β -estradiol (10^{-5} M) antipain (600 μ g/ml)	20	1	0.05

^aSecond treatment begun 24 hours after seeding (immediately after irradiation) and maintained for 5 days.
Data pooled from several experiments. Estradiol and antipain given concurrently.

has been hypothesized that this phenomenon may reflect mitotic recombinational activity which leads to promotion by facilitating the segregation of specific recessive mutations in daughter cell populations. Mitomycin-C is a potent inducer of SCE; at the concentration employed it enhanced the spontaneous SCE frequency by a factor of about 1.5, a similar enhancement to that produced in 10T½ cells by TPA.

The interactions between x-irradiation and the endogenous steroid hormone 17- β -estradiol are shown in Table 4 (1). This hormone induced transformation by itself, and increased x-ray transformation in an additive fashion. Thus, these two agents may act as carcinogens, but estradiol does not appear to act as a true promoter of x-ray transformation. As can be seen in Table 4, the protease inhibitor antipain also suppressed transformation induced by estradiol.

Perhaps the most important finding to arise so far from these experiments, in terms of hazards of low level radiation exposure, is the observation that EGF can act as a potent enhancer of x-ray transformation (2). EGF was not carcinogenic by itself (Table 3). To our knowledge, these results provide the first evidence that a purified polypeptide growth factor can enhance malignant transformation in vitro. On a molar basis, EGF is about 10 times more potent than the highly active plant-derived tumor promoter TPA. The concentration range in which it was effective in these experiments (10^{-9} - 10^{-10} M) is within physiologic levels. Thus, the origin of human tumors may result from a complex interaction between environmental factors including radiation and endogenous growth factors.

3. Biological Effects of Internally Emitting Radionuclides

This research has focused on the effects of ^{125}I incorporated into the DNA of cells as ^{125}I -iododeoxyuridine ($^{125}\text{IdUrd}$) and tritium incorporated into DNA as ^3H -thymidine ($^3\text{H-TdR}$). Decay of ^{125}I is associated with the release of a shower of very low energy Auger electrons, leading to a highly localized distribution of radiation. There are thus two special characteristics of this radiation in terms of its biologic effect: Its LET is high; and its effects are localized within small regions of the DNA around the site of disintegration leading to a high probability of producing double strand breaks. Cells were incubated with the radiolabeled precursor for a single S phase of synchronous growth following their release from density inhibition as described in the proposal. Results have been expressed on a per disintegration basis by actual measurements of the incorporated radioactivity in each experiment, and parallel autoradiographic controls to assess the fraction of labeled cells.

Incorporated ^{125}I was found to be 12-16 times as lethal per decay as incorporated ^3H . For the induction of malignant transformation, however, ^{125}I was approximately 25 times as effective per decay as ^3H (5). When the frequency of transformation induced at various levels of survival by ^{125}I , ^3H , or X-rays was compared, lethality was found to correlate closely with transformation at doses that yielded significant cell killing. An exception occurred at very low doses where ^{125}I appeared much more effective than X-irradiation in inducing transformation: transformation frequencies equal to those induced by about 300 rads of X-rays resulted from ^{125}I exposures yielding little or no cell killing (5). This result is shown graphically in Figure 1 on the following page.

Figure 1: Transformation by $^{125}\text{IdUrd}$ (●) or X-Rays (X) normalized to surviving fractions.

In separate experiments, the induction of DNA single strand and double strand breaks by these two radionuclides has been studied under similar exposure conditions by use of alkaline and neutral elution techniques (9). The results indicate that ^{125}I is much more efficient than ^3H in inducing DNA double-strand breaks: Each ^3H disintegration yielded about 0.14 double strand breaks, whereas each ^{125}I disintegration yields approximately 1 double strand break. If double-strand breaks are lethal lesions, they must be repaired reasonably efficiently, as the mean lethal dose in 10T½ cells is approximately 50 disintegrations per cell. Based on these observations, we hypothesize that the high potency of ^{125}I for the induction of malignant transformation may be related to misrepair of induced double strand breaks (10). This hypothesis is supported by recent data of Martin and Haseltine (Science 213: 896-898, 1981) who used DNA sequencing techniques to demonstrate that energy deposition from ^{125}I decay was absorbed within a few base pairs of the site of disintegration.

In separate studies, the relative sensitivities of human diploid fibroblasts and mouse 10T½ cells to ^3H -TdR and ^{125}I dUrd were compared (6). Whereas these cells show similar sensitivities to acute X-irradiation, the D_0 for clonal survival of 10T½ cells was 5-10 fold higher than the D_0 for the human fibroblasts for both radionuclides. A large difference in sensitivity between these two cell types was also seen after protracted irradiation from tritiated water at 37°C, but this difference was markedly reduced when the cells were exposed to tritiated water at 0°C to simulate an "acute" irradiation. An established human tumor cell line was similarly hypersensitive to killing by tritiated water as compared with early passage diploid hamster embryo cells. These results indicate that human cells are more radiosensitive than rodent cells when a radiation dose is protracted over several days rather than given as an acute exposure. The results are thus consistent with the hypothesis that human cells are hypersensitive to low dose rate irradiation as compared with rodent cells.

Finally, we have measured mutagenicity induced in human diploid lymphoblasts by various doses of x-rays, ^{125}I dUrd and ^3H -TdR (8). ^{125}I was much more effective than ^3H in killing cells and producing mutations to 6-thioguanine resistance (6-TG^R). No ouabain resistant mutants were induced by any of these agents. The slopes of the mutation curves were approximately 7.5×10^{-8} 6-TG^R mutants per cell per disintegration for ^{125}I dUrd and 2×10^{-8} for ^3H -TdR. X-rays induced 8×10^{-8} mutants per cell per rad. Normalizing for survival, ^{125}I remained much more mutagenic than x-rays at low doses (high survival levels) similar to its effects on transformation. This can be seen graphically in Figure 2 on the following page. As with transformation, a significantly increased frequency of mutations was observed with only a few total radioactive decays per cell, doses which produced little or no measureable lethal effects. Based on reasonable estimates of the effective size of the 6-TG^R gene, we calculate that each disintegration within the gene leads to a mutation; as stated above, each disintegration also leads to approximately one DNA double strand break. Treatment of cells at either 37°C or while frozen at -70°C yielded no difference in cytotoxicity or mutation for ^{125}I dUrd, whereas x-rays were six-times less effective in killing cells at -70°C.

Figure 2. Induction of 6-thioguanine resistant mutants in human diploid lymphoblasts by ¹²⁵IIdUrd (●) and X-rays (X) normalized to the surviving fraction.

C. Publications (1980-83)

- Removed
- *1. Kennedy, A.R. and R.R. Weichselbaum. Effects of $^{17}\beta$ -estradiol on radiation transformation in vitro; inhibition of effects by protease inhibitors. *Carcinogenesis* 2: 67-69, 1981.
 - *2. Fisher, P.B., R.A. Mufson, L.B. Weinstein and J.B. Little. Epidermal growth factor, like tumor promoters, enhances viral and radiation induced cell transformation. *Carcinogenesis* 2: 183-187, 1981.
 3. Little, J.B. Influence of noncarcinogenic secondary factors on radiation carcinogenesis. *Radiat. Res.* 87: 240-250, 1981.
 4. Little, J.B. and A.R. Kennedy. Promotion of X-ray transformation in vitro. In: *Carcinogenesis*, Vol. 7, *Cocarcinogenesis and Biological Effects of Tumor Promoters*, edited by E. Hecker, N.E. Fusenig, W. Kunz, F. Marks and H.W. Thielmann. Raven Press, New York, 1982, pp. 243-257.
 - *5. LeMotte, P.K., S.J. Adelstein and J.B. Little. Malignant transformation induced by incorporated radionuclides in BALB/3T3 mouse embryo fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 79: 7763-7767, 1982.
 - *6. LeMotte, P.K. and J.B. Little. A comparison of the lethal effects of intracellular radionuclides in human and rodent cells. *Radiation Res.* 95: 359-369, 1983.
 - *7. Robertson, J.B., A. Koehler, J. George and J.B. Little. Oncogenic Transformation of mouse BALB/3T3 cells by plutonium-238 alpha particles. *Radiation Res.* (in press, 1983).
 - *8. Liber, H.L., P.K. LeMotte and J.B. Little. Toxicity and mutagenicity of X-rays and ^{125}I Urd or ^3H -TdR incorporated in the DNA of human lymphoblast cells. *Mutation Res.* (in press, 1983).
 - *9. LeMotte, P.K. and Little, J.B. DNA damage induced in human diploid cells by decay of incorporated radionuclides. *Cancer Res.* (in press, 1983).
 10. Little, J.B., P.K. LeMotte, and H.L. Liber. Quantitative studies of cytotoxicity, mutagenesis and oncogenic transformation by radioisotopes incorporated into DNA. Chapter 22, In: *Human Carcinogenesis* (C.C. Harris and H.N. Autrup, eds.), Academic Press, New York, (in press, 1983).
 11. Kennedy, A.R., J. Cairns and J.B. Little. The timing of the steps in transformation of C3H 10T $\frac{1}{2}$ cells by x-irradiation. *Nature* (submitted).
 - *12. Kennedy, A.R. and J.B. Little. Evidence indicating that the second step in x-ray induced transformation in vitro occurs during cellular proliferation. *Radiation Res.* (Submitted).
- Removed

Copies of manuscripts and papers preceded by () are included in Appendix.