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Cellular Transformation by Radiation: Induction, Promotion, and Inhibition

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Mammalian cell cultures offer powerful tools for evaluating qualitatively and quantitatively the oncogenic potential of radiation over a wide range of doses with particular importance at the low dose range that is relevant to human exposure and risk. Our studies have shown that early events in the process of radiation induced transformation in both rodent and human cells requires initial replication for fixation of transformation as a hereditary property of the cells and further clonal expansion for full expression. Early events (fixation) are inhibited by cell–cell contact and high cell density but can be modified at low temperature where repair processes are slowed. Cell–cell contact and communication in tissue organization may be in part responsible for our findings that radiation oncogenesis induced in utero in hamsters is expressed at a lower frequency than that induced in vitro.

Quantitative studies carried out on hamster embryo cells indicate that neutrons are more effective in their carcinogenic potential than x-rays but also more toxic, that splitting the dose of x-rays at low doses leads to enhanced transformation, but that at high doses protracted radiation has a sparing effect. At all dose ranges survival was increased by protracting the radiation dose, thus suggesting that different repair processes must be involved for survival and transformation. Similar observations were seen when the protease inhibitor Antipain was found to enhance transformation in rodent and human cells when present at the time of radiation, but was protective when added after radiation. Survival was not modified under any of those conditions, and Antipain did not affect DNA replication and repair. In our qualitative studies, once cells are transformed by radiation, they exhibit a wide range of structural and functional phenotypic changes, some of which are membrane-associated and are expressed within days after induction.

Our current studies on nutritional and hormonal influences on radiation transformation indicate the following: Pyrolysate products from broiled protein foods act in synergism with radiation to produce transformation, whereas vitamin A analogs are powerful, preventive agents. Retinoids inhibit both x-ray-induced transformation and its promotion by TPA; these modifications (enhancement by TPA, inhibition by retinoids) are not reflected in sister chromatid exchanges, but are reflected in the level of membrane associated enzymes Na/K ATPase. Whereas retinoids modify late events (expression, promotion), we find that thyroid hormone plays a crucial

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role in the early phases of radiation and chemically induced transformation. Under hypothyroid conditions no transformation is observed. The addition of triiodothyronine at physiological levels results in a transformation rate that is dose-related.

Our recent success in transforming human skin fibroblasts will enable quantitative and qualitative studies of radiation carcinogenesis in a system relevant to man.

Key words: in vitro carcinogenesis, promotors, hormones, retinoids, radiation protease inhibitors, human cell transformation

In recent years epidemiological and animal data have brought forth ample evidence that environmental factors, including diet, play a crucial role in determining the incidence of cancer [1–6].

The awareness of the carcinogenic potential of chemicals and occupational carcinogenesis [7] can be traced back to Sir Percival Pott who in 1775 reported scrotal cancer among chimney sweepers [8]. The recognition of ionizing radiation as a carcinogen evolved soon after the discovery of x-rays by Roentgen, 1895, and is well documented in studies on cancer incidence in early radiation workers [9] and survivors of Hiroshima and Nagasaki [10].

Whereas animal data have contributed much to our knowledge of carcinogenesis, they have their limitations in evaluating the hazard of low dose exposure and in studying cellular and molecular mechanisms involved in the induction, development, and modulation of carcinogenesis.

MATERIALS AND METHODS

Oncogenic Transformation of Animal Cells In Vitro

Cell culture systems, where defined nutritional conditions prevail, offer powerful tools in cancer research for evaluating the oncogenic potential of various agents, for detecting potentiating and promoting factors and for discovering conditions and compounds that act to prevent the onset or development of neoplastic transformation. Our original demonstration that diploid mammalian cells can be transformed in vitro by x-rays into malignant cells [11–13] has enabled us to study the process both qualitatively and quantitatively. Using short-term cultures of hamster embryo cells, we have elucidated the dose-response relationship for transformation over a wide range of doses [14, 15], investigated the temporal events involved in fixation of the transformed state as a hereditary property of the cells and its expression [12, 13], evaluated phenotypic changes associated with the transformation state [11–16] and sought ways to modulate both induction and expression of radiation oncogenesis [7–21].

Being normal diploid cells, hamster embryo cells have a finite life span in contrast to the transformed which become immortal. Spontaneous transformation is less than 10^{-6} and the transformability of these cells decreases with passage in vitro [12]. Although these short-term cultures differ from established heteroploid cell lines such as 3T3 [22] and C3H 10T½ [23], many of their responses to initiating, promoting agents as well as to inhibitors of transformation are qualitatively similar to those cell lines [17–21]. The hamster embryo cell

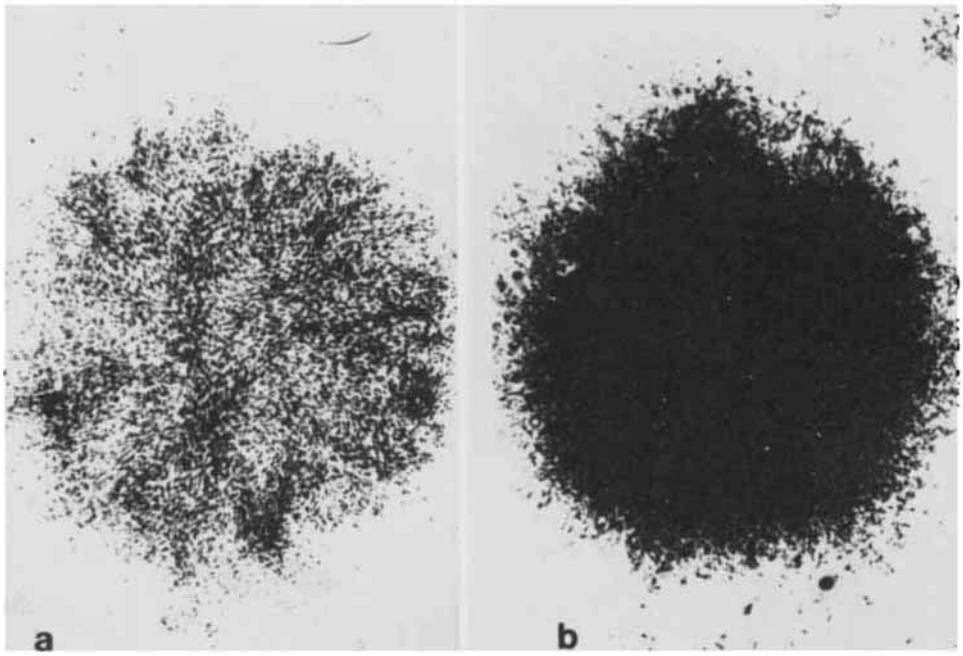

Fig. 1. a) An 8-day-old colony of normal hamster embryo cells. Note the regularity of cell–cell contact. b) An 8-day-old colony of hamster celss transformed by 1 rad of x-ray. Note the dense multilayering of the cells and the random cell–cell contact. Giemsa × 25.

system, like the 10T½ and 3T3, is composed of fibroblast-like cells. Transformants can be distinguished morphologically from controls by the characteristic multilayering and random cell–cell orientation [11–16] (Fig. 1), thus differing from epithelial cells where morphological distinction is not as reproducible [16] (Fig. 2, Table I).

Transformation in vitro consists of the following steps: 1) initiation, requiring exposure of the cultured cells to the carcinogen [12, 13, 15, 16]; 2) fixation of the transformed state requiring one to two doublings within hours after initiation [12, 13, 15, 16, 23]; 3) promotion and ultimate expression of the transformed state requiring several cell replications and a suitable milieu resulting in a colony or focus that in fibroblasts and some epithelial cultures is morphologically distinct from the control [12, 13, 15, 16, 23, 24] (Figs. 1, 2); 4) exhibition of the ability to grow in suspension in semisolid medium and to give rise to tumors in suitable hosts [11–16, 23].

Initiation, whereby the induction process takes place following exposure to the carcinogen, is an early event and is considered irreversible though under certain cellular physiological conditions such as hormonal milieu [20] it may be prevented. Fixation can be modified. When cells are exposed to x-rays as confluent cultures and held in liquid holding for 24 h and longer with no possibility to divide, fixation of transformation is prevented. However, if similar irradiated cultures are maintained at 25°C rather than at 37°C for as long as 5 days, fixation of transformation is retained and transformation fully expressed

TABLE I. Properties of Fibroblasts and Epithelial Cells Malignantly Transformed In Vitro Distinguishing Them From Normal Parental Cells*

Characteristic	Fibroblasts	Epithelial
Morphology (light microscopy)	Pleomorphic, refractile criss-cross orientation; irregular growth pattern	Often not dramatically different from normal; somewhat more pleomorphic in some cases (eg, liver)
Topography (scanning electron microscope)	Increase in surface features	Inconsistent changes; sometimes an increase in microvilli
Cell density	Increased saturation density, multilayering, and piling up of cells; loss of density-dependent inhibition of growth	Inconsistent; depending on the cell line and the tissue of origin; in some cell lines piling up of cells, in others maintenance of monolayer growth pattern
Serum requirement for growth	Decreased in rodent cells; less pronounced characteristic in transformed human cells, since normal human cells can grow in lower serum levels	Low as in the normal (in liver cells); has not been sufficiently studied in a variety of systems
Calcium dependence for growth	Reduced	Reduced
Altered cell surface glycoproteins	Yes	Yes
Agglutinability by low concentrations of lectins	Yes	Yes
Increased protease production	Yes	Inconsistent
Changes in cytoskeleton	Pronounced	Inconsistent
Growth in agar	Yes	Yes
Tumorogenicity	Yes	Yes

*Modified from C. Borek 1979 [16].

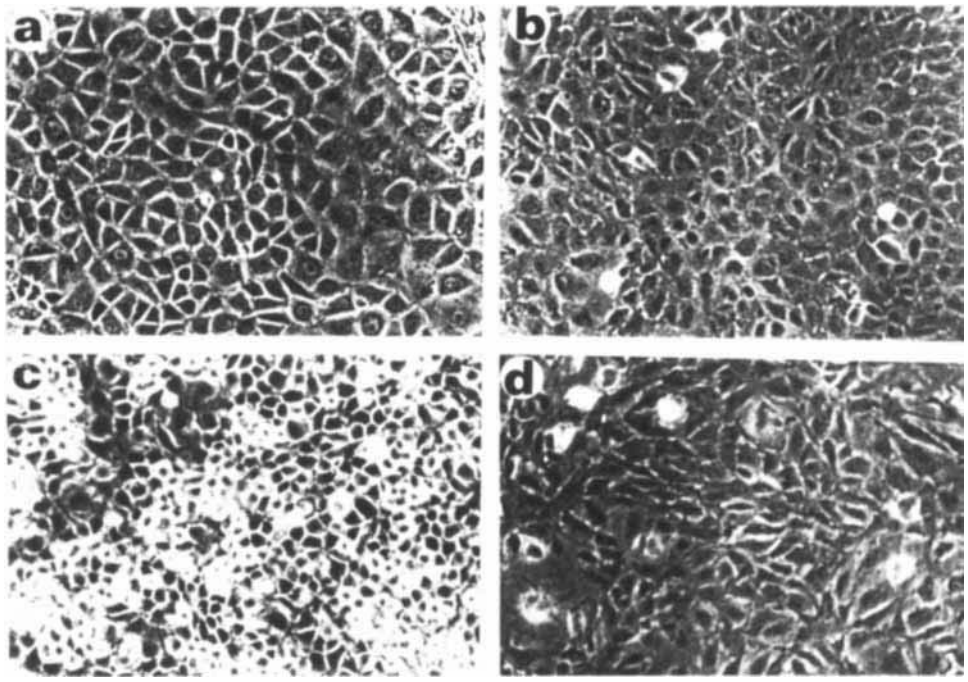

Fig. 2. Normal and transformed epithelial cells derived from liver [35], (a) and (b) are normal, and (c) and (d) are their transformed counterparts respectively. Note that the transformed cells in (c) form multilayers, whereas in (d) the transformed counterparts of (b) are flat. Phase × 100.

following subsequent subculture at 37°C [12] (Table II). This suggested that processes involved in cellular repair of damage associated with transformation, such as DNA repair [25–28], or possibly the induction of chromosomal instability [29] are slowed or inhibited at 25°C, thus preventing the loss of fixation and leading to ultimate expression of the transformed state. Complete induction, therefore, requires both initiation and fixation and an appropriate physiologic state of the cells. Whereas induction can be regarded as a cellular process involving direct and/or indirect interaction between the carcinogen and the cellular macromolecules, later events related to expression of transformation and involve clonal cell expansion and cell–cell interaction within the milieux in which they are proliferating. Normal cells in vitro can inhibit the replication of transformed counterparts whether these are transformed by radiation, chemicals, or viruses [30]; ultimate expression of transformation in vitro as a morphologically indentifiable end point is closely related to the density of cells at the time of exposure to the carcinogen [31, 32], transformation yield being lower at high cell density [31, 32].

The influence of cell–cell interaction on the expression of transformation is further indicated by the strikingly lower transformation rate observed when embryos are irradiated in utero, where cellular and tissue organization prevail as compared to in vitro where these arrangements are disrupted [33]. Although we are still ignorant of the exact mechanisms involved, our work has indicated

TABLE II. Transformation in Confluent Cultures Incubated at 37°C or 24°C

Incubation temperature of confluent cultures	Treatment of cells	Days after X-irradiation when cloned	Total no. colonies counted	Cloning efficiency (%)	Transformed colonies (%)
37°C	300 r	0	636	4.1	0.8
		5	1,536	5.3	0
	Control	0	529	6.6	0
		5	1,793	6.3	0
24°C	300 r	0	432	2.7	0.7
		5	576	3.7	0.6
	Control	0	704	4.4	0
		5	415	4.8	0

Confluent primary cultures 3 days after seeding were incubated for 1 day at 37°C or 24°C, the cultures irradiated or left as controls, and the cells either cloned immediately after irradiation or incubated another 5 days at 37°C or 24°C as confluent cultures and then cloned. Medium was changed in the confluent cultures both before and after irradiation. The mass cultures were grown in EM with nonfetal calf serum, and the cells cloned in EM with fetal calf serum. Colonies were scored after 10 days of incubation at 37°C. Similar results were obtained in two other experiments at 24°C, which gave 1.5 and 0.6% transformed colonies.

TABLE III. Membrane Enzyme Activities in Normal and Transformed Fibroblasts* (mean ± SE (μmoles Pi/hr/mg protein))

	(Na^+/K^+)-ATPase	Mg^{2+}-ATPase	5′ Nucleotidase
C3H/10T½	0.68 ± 0.19	1.12 ± 0.18	0.54 ± 0.07
X-ray-transformed C3H/10T½	1.08 ± 0.17[a]	1.03 ± 0.15	0.60 ± 0.05
Hamster embryo (HE)	0.59 ± 0.9	1.91 ± 0.29	5.65 ± 1.10
X-ray-transformed HE	1.02 ± 0.14[a]	1.59 ± 0.21	15.25 ± 2.61[a]

*Modified from [36].
[a]$P < 0.5$ significantly different from normal cells.

that intercellular ionic communication is modified upon transformation [34]; that in certain cell types there is a loss of ionic communication at permeable membrane junctions between normal and transformed cells [34, 35]; that Na/K ATPase, the membrane associated transport enzyme, is modified in the transformed cells [36] (Table III); and that those same transformed cells that have modified their communication with normal counterparts exhibit an absence of higher molecular weight gangliosides owing to a defect in biosynthesis [37] (Fig. 3). Recently, the effect of tumor promotors have been shown to modify cell–cell interaction [38].

Once cells have expressed their transformed state in vitro, neoplastic colonies can be isolated and propagated to yield large populations of cells for further characterization [15, 16, 33] (Table I). While karyotypically radiation transformed cells are near diploid at early stages following transformation and show normal binding patterns [16], their surface topography is markedly altered within 8 days following exposure to the carcinogen [39] (Fig. 4). These surface features, which include pleomorphism and an abundance of extrescences, prevail through out the cell cycle and are retained for the life of the cell line [39].

Of all the phenotypic changes associated with transformation (Table I), those most correlated with the malignant nature of the cells are able to proliferate in some solid medium such as agar, and to give rise to tumors on the appropriate host (Fig. 5). These properties in rodent cell systems have been associated with a late stage in neoplastic development [40]. However, recent evidence from human cell transformation [41] suggests that this may not always be the case since the property of growth in agar seems to appear concurrently with morphological changes.

Quantitative Studies in Radiation Transformation

Whereas radiation is a weak carcinogen as compared to chemical carcinogens, it is the most universal and can interact synergistically with chemicals both environmental [42] and in the diet [43]. As members of the public we are

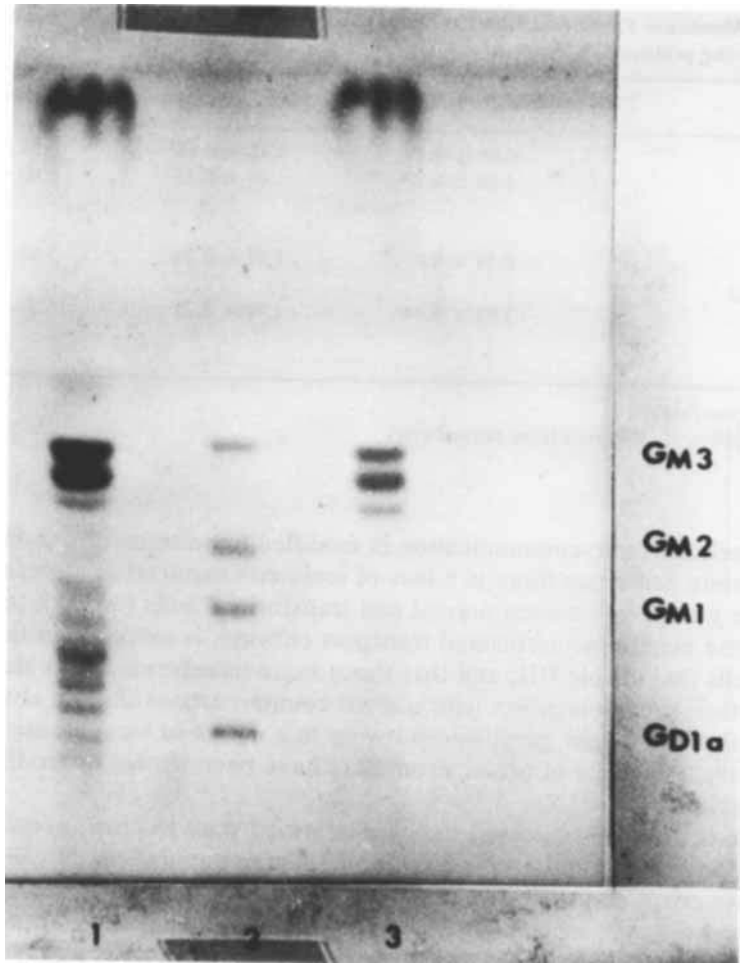

Fig 3 Thin-layer chromatogram of gangliosides from normal and x-ray-transformed hamster embryo cells Lane 1 control, lane 2 standard, lane 3 x-ray-transformed Note that in the transformed cells gangliosides higher than GM_3 are absent [from 33]

concerned with the effect of low doses. Since we are subjected to protracted radiation in our environment or in diagnosis, it is of great importance to know risk estimates under these conditions.

Using the hamster embryo diploid cell system, we have elucidated the dose response relationship for radiogenic transformation for x-rays as well as neutrons [14, 15] (Fig. 6). We found that doses as low as 1 rad of x-rays and 0.1 rad of neutrons can effectively transform the cells, and that whereas the efficiency of neutrons in inducing transformation is greater than that of x-rays, so is their effectiveness in cell killing (Fig. 7). We also demonstrated that splitting x-ray doses at low doses of 50 or 75 rad into two equal fractions can enhance transformation as compared to the same total radiation given as a single dose. In the higher dose range a sparing effect for fractionation and lower

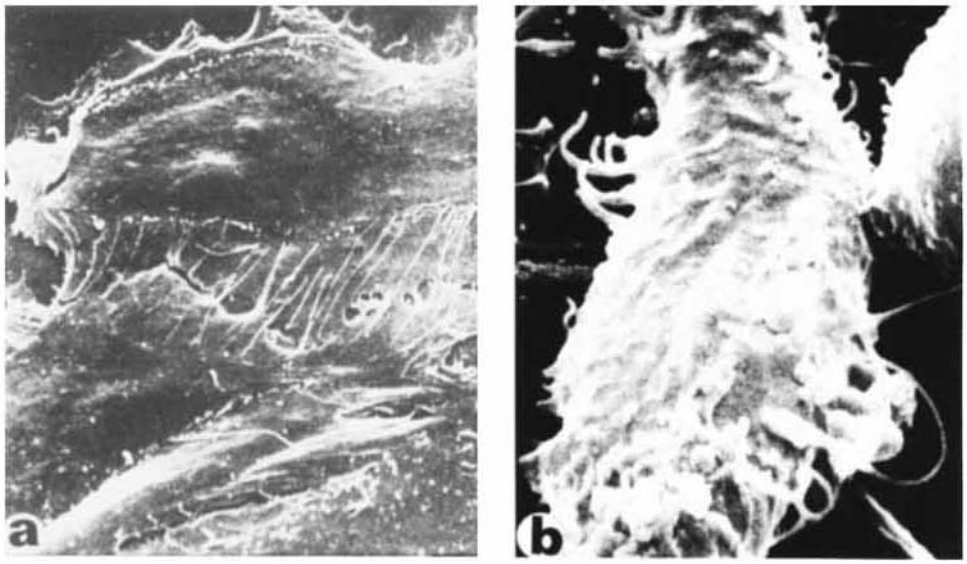

Fig 4 a) Normal hamster embryo cells Note tight cell–cell contact at membrane junction Cells are flat and devoid of surface features SEM × 8,000 b) Hamster embryo cells transformed by x-rays Note the abundant surface features, the pleomorphism of the cells and the striking change in cell–cell contact SEM × 10,000

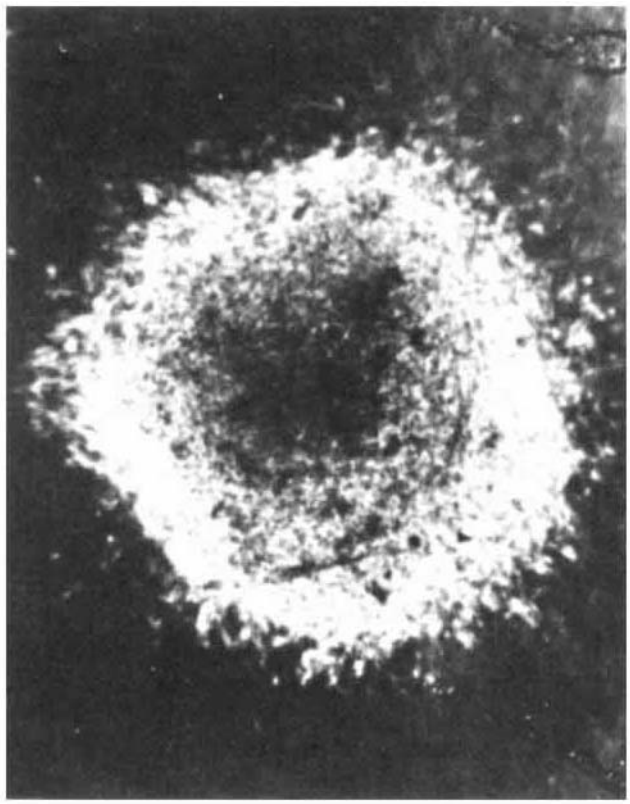

Fig 5 A colony of x-ray-transformed human cells growing in agar × 25

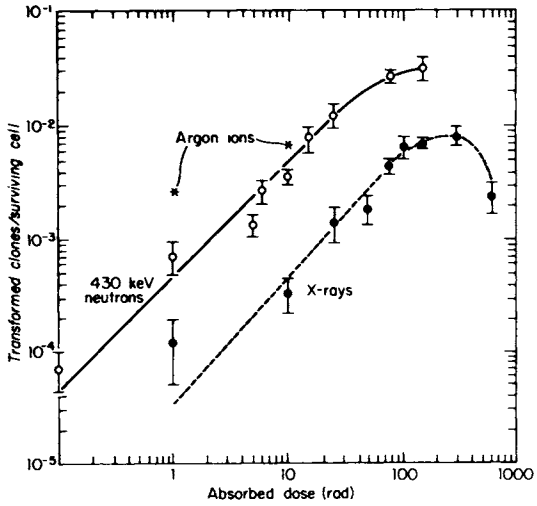

Fig. 6. Dose-response relationship for cell transformation by x-rays, neutrons, and Argonne ions. Note the high transforming efficiency of neutrons. (Reproduced with permission [15].)

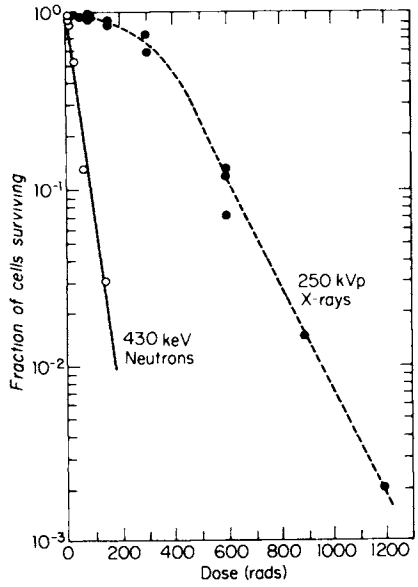

Fig. 7. Cell survival curve for x-rays and neutrons. Note the high killing efficiency of neutrons. (Reproduced with permission [15].)

transformation rate were observed [44] (Table IV). A sparing effect for cell survival was observed at all split doses as compared to the single dose. These data indicated that the use of linear interpolation from high to low dose levels may lead to cancer risk estimates that are neither conservative nor prudent depending on the distribution of dose in time. In addition these data suggest that there exist different repair mechanisms for survival and transformation.

TABLE IV. Neoplastic Transformation of Hamster Embryo Cells Following Single and Split Dose of X-Rays

Dose (rad)	Total no. clones counted	Number of experiments	Mean survival fraction[a]	Total transformed clones counted	Mean % cell transformation[b] ± standard error	Split dose/single dose ± standard error
Control	51,699	4	1.0	0	0	0
25 + 25	15,582	4	0.96	55	0.35 ± 0.05	1.84 ± 0.24
50	14,272	4	0.92	27	0.19 ± 0.04	
35.5 ± 37.5	23,762	5	0.92	255	1.07 ± 0.07	1.72 ± 0.17
75	25,882	5	0.89	160	0.62 ± 0.05	
75 + 75	13,118	3	0.73	110	0.83 ± 0.08	1.30 ± 0.18
150	15,348	3	0.65	98	0.64 ± 0.06	
150 + 150	11,925	3	0.58	48	0.40 ± 0.06	0.67 ± 0.13
300	11,450	3	0.47	69	0.60 ± 0.07	
300 + 300	11,037	3	0.15	48	0.43 ± 0.06	0.74 ± 0.14
600	11,818	3	0.10	69	0.58 ± 0.07	

[a]$\text{Survival fraction} = \dfrac{\text{Number of surviving clones counted}}{\text{Number of cells seeded} \times \text{plating efficiency}}$.

[b]$\text{Cell transformation (\%)} = \dfrac{\text{Number of transformed clones}}{\text{Number of clones counted}} \times 100.$

$\text{Plating efficiency} = \dfrac{\text{Number of clones counted in control plates}}{\text{Number of cells seeded in control plates}}$.

Modification of Transformation by Antipain

Further confirmation for differences in these two mechanisms of coping with cellular damage eminated from our work with the protease inhibitor Antipain [17]. Addition of 6 μg/ml Antipain before irradiation resulted in enhanced transformation, whereas adding the compound after irradiation resulted in protection and lower transformation rate [17] (Table V). Although transformation was modified in such opposing ways cell survival was similar, suggesting that repair for survival and transformation takes place by different mechanisms. The complexity of the protease inhibitor is not yet understood, but apparently its action is unrelated to the modification of DNA repair [45] (Fig. 8).

Nutritional and Hormonal Factors in Carcinogenesis

Because of the fact that we are still unable unequivocally to associate particular molecular changes with initiation, we must evaluate the effects of modulators in carcinogenesis by their function in modifying the expression of neoplastic transformation.

TABLE V. Cell Transformation In Vitro After Treatment With X-Rays and Antipain

	Hamster embryo		C3H/10T½	
Treatment	Surviving fraction	Transformation incidence (B/A × 10^5)	Surviving fraction	Transformation incidence (B/A × 10^4)
Antipain added before x-irradiation with 300 rad				
24 h				
Control	1.00	0	1.00	0
Antipain	0.94	0	0.98	0
X-irradiation	0.60	6.6 ± 1.0	0.41	4.9 ± 0.6
X-irradiation and Antipain	0.55	12.3 ± 1.2	0.38	7.4 ± 1.1
Antipain added after x-irradiation with 300 rad				
10 min				
Control	1.00	0	1.00	0
Antipain	0.96	0	0.94	0
X-irradiation	0.53	8.4 ± 0.7	0.38	3.1 ± 0.4
X-irradiation and Antipain	0.44	3.7 ± 0.5	0.35	1.5 ± 0.4
24 h				
Control	1.00	0	1.00	0
Antipain	0.92	0	0.96	0
X-irradiation	0.60	6.5 ± 0.7	0.40	1.1 ± 0.1
X-irradiation and Antipain	0.62	5.9 ± 1.2	0.41	1.0 ± 0.3
48 h				
Control	1.00	0		
Antipain	0.89	0		
X-irradiation	0.65	6.4 ± 1.3		
X-irradiation and Antipain	0.62	5.9 ± 1.2		

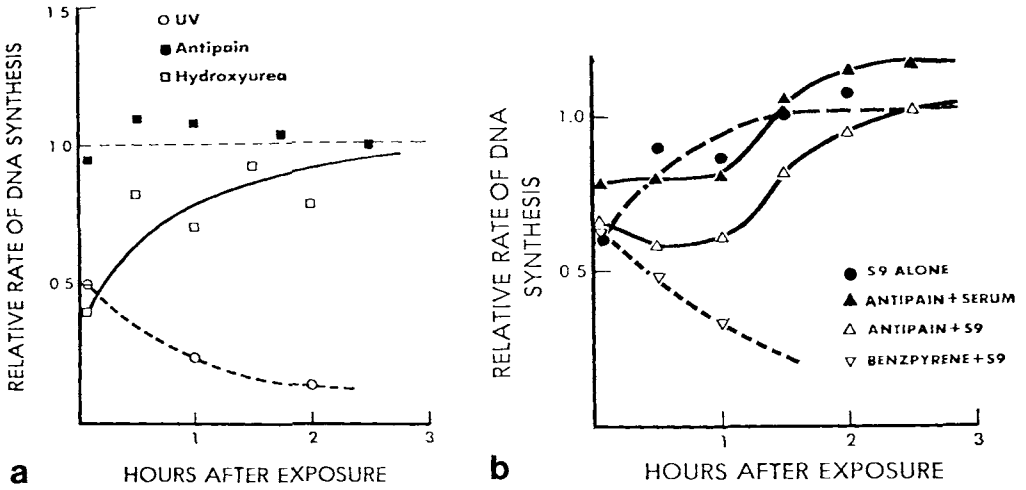

Fig. 8. a) Relative rate of DNA synthesis in human fibroblasts exposed to 1.7 mM Antipain for 30 min, 13 J/m² of 254 nm UV light or 2 mM hydroxyurea for 30 min, and pulse-labeled for 15 min with 10 μCi/ml (^{3}H)dThd (60 Ci/mmole) at various times after exposure. b) Relative rate of DNA synthesis in human fibroblasts exposed for 30 min to S9 mix, 1.7 mM Antipain plus 10% serum, 1.7 mM Antipain plus S9 mix, or 30 μg/ml benzpyrene plus S9 mix, and pulse-labeled for 15 min with 10 μCi/ml (^{3}H)dThd (60 Ci/mmole) at various times after exposure. (From [45].)

Nutritional and hormonal factors can modify cancer incidence in the following manner [46]: They can act 1) as auxiliary to other environmental factors, as potentiators and promotors (examples of these would be estrogens [41] or food pyrolysates [43]; 2) as themselves causes of cancer [47]; 3) in quite the reverse manner as cancer-preventive factors such as retinoids, and selenium [18, 19, 21] or hypothyroid conditions [20]. Although we are slowly becoming conscious of (1) and (2), we are constantly in search of (3).

Since in cancer development an inducing agent can be its own promoter when delivered at the appropriately high dose to the right target tissue, the effectiveness of preventive agents in suppressing carcinogenesis may also be related to dose and target tissues.

Although fibroblasts certainly do not represent the large range and variety of differentiated tissues, they do offer useful cellular tools to study the effects of the various compounds and conditions.

Nutritional Factors

Retinoids. Using the hamster embryo cell system described above as well as the C3H 10T½ heteroploid mouse cell line, we have studied the effectiveness of retinoids [18, 19, 21] in inhibiting radiogenic transformation and in suppressing the effect of tumor promotor TPA.

Our early findings that a vitamin A analogue is capable of inhibiting radiation-induced transformation in fibroblasts indicated that indeed, the effectiveness of retinoids in chemoprevention is not confined to in vivo induced chemical carcinogenesis or to cells of epithelial origin. This and our finding that vit-

TABLE VI. TPA and Retinoid Modification of X-Ray-Induced Transformation in Hamster Embryo and C3H/10T½ Cells

	Hamster embryo		C3H/10T½	
Treatment	Surviving fraction	Mean rate of transformation (10^{-3}) ± standard error	Surviving fraction	Mean rate of transformation (10^{-4}) ± standard error
Control	1.00	0	1.00	0
TPA (0.16 μm)	0.70	0	0.93	0
Retinoid (7.1 μm)	0.62	0	0.75	0
Retinoid, TPA	0.89	0	0.67	0
X-Rays	0.42	6.99 ± 1.65	0.32	8.78 ± 1.29
X-Rays, TPA	0.53	12.52 ± 1.81	0.52	16.15 ± 1.59
X-Rays, retinoid	0.38	2.94 ± 0.79	0.30	4.37 ± 0.93
X-Rays, retinoid, TPA	0.41	2.41 ± 0.85	0.36	2.46 ± 0.54

amin A will antagonize TPA effect on inhibiting vitamin A binding protein [48, 49] prompted us to investigate the following: 1) Will vitamin A inhibit TPA enhancement of radiation transformation? 2) If it does, is the action reflected in DNA damage as ascertained by sister chromatid exchanges [50] or via other cellular and molecular events for example at the level of the cell membrane?

Our protocol, which is detailed elsewhere [18, 19], consisted of the following schedule: Cells were plated in the presence of the retinoid all-trans-retinoic acid (hamster cells) or trimethyl methoxyphenyl analogue of N-ethyl retinamide (10T½ cells). They were irradiated 24 h later with 300 rad (hamster cells), a dose that yields maximum transformation in this system, or 400 rad for the 10T½. TPA was added immediately after irradiation and kept on for the duration of the experiment (2 weeks for HE, 6 weeks for the 10T½). The retinoids were removed from the culture dishes 4 days after irradiation. Thus, if retinoids could exert an inhibitory influence on cell transformation and interfere with the promotional effect of TPA, they had to do so within 4 days. As seen in Table VI this was indeed the case. Whereas TPA significantly increased radiation-induced oncogenesis, retinoids inhibited the radiation effect and eliminated any promotional and enhancing effect exerted by the tumor promotor.

DNA instability has been implicated in the process of carcinogenesis [29, 51], and the technique of sister chromatid exchange analysis reflects some aspects of DNA damage [50]. It was thus of interest to establish whether the effective modification of transformation by the retinoids is reflected in the pattern of SCE in the treated cells.

We found, as seen in Figure 9, that whereas the inducing agent, x-rays, enhanced SCE 2-fold, the agents that modulated transformation did not express their modifying activity at the chromosomal level. The promoting TPA and the inhibitory retinoids both gave rise to increased SCEs.

We therefore proceeded to evaluate the effects of the retinoids and TPA on the level of membrane-associated enzymes Na/K ATPase, Mg/ATPase, and 5′ nucleotidase. We found that regulation of cell transformation by the retinoids and TPA are reflected in the level of the membrane-associated enzyme Na/K ATPase [18, 21,49]. As seen in Table V, the effects are specifically on Na/K ATPase, TPA enhances the level of the enzyme, and retinoids inhibit it. When cells are treated with both agents concomitantly, enzyme levels return to control level. This indicates that the effectiveness of retinoids in modifying transformation is at the level of gene expression mediated via the membrane at the later stage of expression and not in the early phase of initiation. Once cells are transformed and neoplastic, a condition where preventive measures can no longer be effective, the retinoids do not modify the Na/K ATPase (Table V).

At present we do not know the exact mechanism underlying the effects of the retinoids at the membrane level. Retinoids alter membrane glycoproteins [52]. In their absence differentiation is modified, and cellular interaction with carcinogens is enhanced [53, 54]. Retinoids can also trap free radicals and such species are produced following radiation. Thus the retinoids could be acting in part by deactivating these chemical species, by inhibiting peroxidation of the cell membrane [55]. These membranes differ biochemically in the transformed

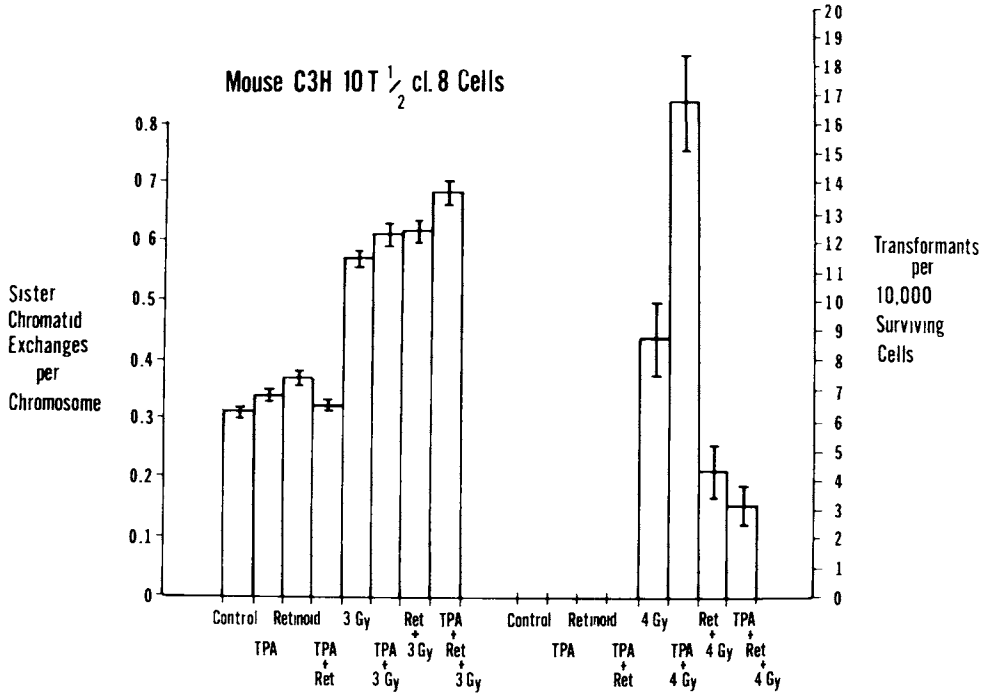

Fig. 9. Histogram describing the effects of x-rays, retinoid, and TPA on cell transformation in C3H 10T½ cells and on sister chromatid exchanges. Note the lack of correlation between the modification of transformation by the agents and their effect on SCE. (Reproduced with permission [18].)

state [56], which may account for the differences in the influence of the retinoids on the membrane associated enzymes.

Thyroid Hormone as Modulator of Carcinogenesis

Early studies have indicated that the process of neoplastic transformation precludes thyroid control, in that triiodothyronine failed to induce Na/K ATPase in hepatoma HTC cells but did induce the enzyme in normal hepatocytes. (I.S. Edleman, personal communication). In addition, animal studies have shown that altered thyroid status modifies the growth and metastatic potential of implanted tumors [57, 58]. Tumors transplanted to hypothyroid animals show less metastatic potential, whereas in the hyperthyroid state metastasis increases.

Because of the complex homeostatic which prevail in vivo, it is impossible to evaluate the direct role of thyroid hormones in carcinogenesis. Moreover, because conditions of hypo- and hyperthyroid may result in multiple physiological changes, it is hard to evaluate whether thyroid hormones play a role in the process of *initiation* or *potentiation* of carcinogenesis *induced* by oncogenic agents. In vitro, because of the defined conditions of cell culture, it is possible to evaluate these points.

In recent studies, we have used the mouse 10T½ cells and diploid hamster embryo cells to investigate the role of thyroid hormone in radiation induced transformation. We set out to study whether in vitro oncogenesis can be modulated in cells that had been subjected to and maintained under hypohyper-, or euthyroid conditions [20, 58, 59].

Thyroid hormone, both triiodothyronine-T_3 and thyroxin-T_4, was removed from fetal bovine serum as described. This constituted the hypothyroid serum ($-T_3$). Addition of 10^{-7} T_3 to the hypothyroid serum constituted the hyperthyroid condition. Thus, in each experiment, six culture conditions were used where medium was fortified with a particular serum: 1) resin-treated serum ($-T_3$) unirradiated; 2) serum-treated serum ($-T_3$) irradiated; 3) serum-treated T_3, added at 10^{-7}M, ($+T_3$) unirradiated; 4) serum-treated, with T_3 added ($+T_3$) irradiated; 5) untreated serum unirradiated; 6) untreated serum irradiated.

A dose-response relationship for cell survival under these various conditions as well as growth curves indicated that neither cell survival nor cell growth were modified by the hypo- or hyperthyroid conditions. Thus, any differences in cell transformation following irradiation would be attributable to a direct effect of the thyroid conditions in the cellular millieux.

The results described in Table VIII indicate that when cells are maintained in hypothyroid conditions ($-T_3$) x-ray-induced transformation is in-

TABLE VII. Effect of All-Transretinoic Acid (RA) and TPA on Membrane Enzyme Activities in Hamster Embryo and C3H/10T½ Cells*†

Treatment	Na^+/K^+-ATPase	Mg^+-ATPase	5'-Nucleotidase
Hamster embryo			
Control	1.21 ± 0.21	1.35 ± 0.17	1.73 ± 0.28
TPA (0.16 μm)	1.53 ± 0.43	1.44 ± 0.45	1.89 ± 0.31
Retinoid (7.1 μm)	0.78 ± 0.19	1.32 ± 0.36	1.75 ± 0.21
Retinoid, TPA	1.13 ± 0.27	1.19 ± 0.41	1.91 ± 0.32
C3H/10T½			
Control	1.79 ± 0.32	1.26 ± 0.31	0.56 ± 0.08
TPA (0.16 μm)	2.18 ± 0.31	1.26 ± 0.26	0.60 ± 0.12
Retinoid (7.1 μm)	1.13 ± 0.20	1.32 ± 0.26	0.68 ± 0.05
Retinoid, TPA	1.73 ± 0.23	1.23 ± 0.28	0.64 ± 0.10
Transformed hamster embryo			
Control	2.46 ± 0.27	1.54 ± 0.31	5.75 ± 1.21
TPA (0.16 μm)	2.31 ± 0.21	1.38 ± 0.21	5.90 ± 1.38
Retinoid (7.1 μm)	2.50 ± 0.29	1.42 ± 0.21	5.83 ± 1.10
Retinoid, TPA	2.34 ± 0.31	1.50 ± 0.32	5.92 ± 1.23
Transformed C3H/10T½			
Control	0.97 ± 0.09	0.87 ± 0.08	0.40 ± 0.07
TPA (0.16 μm)	1.21 ± 0.12	1.38 ± 0.22	0.46 ± 0.06
Retinoid (7.1 μm)	0.82 ± 0.21	8.99 ± 0.06	0.59 ± 0.02
Retinoid, TPA	1.04 ± 0.18	1.05 ± 0.10	0.47 ± 0.16

*All values are mean ± standard error in moles Pi/h/mg protein.
†From [52].

TABLE VIII. Effect of Thyroid Hormone on X-Ray Irradiation-Induced Cell Transformation In Vitro of C3H/10T½ Mouse Cells and Hamster Embryo Cells in Culture*

Cells	Serum treatment/(T_3 conditions)	X-rays (rad)	Total surviving colonies	Transformation frequency[a]
C3H/10T½	Untreated FBS	0	23,313	0
	Untreated FBS	300	27,737	8.65×10^{-4}
	Resin-treated FBS/($-T_3$ $-T_4$)	0	23,154	0
	Resin-treated FBS/($-T_3$ $-T_4$)	300	33,747	0
	Resin-treated FBS $+T_3$/($+T_3$)	0	15,884	0
	Resin-treated FBS $+T_3$/($+T_3$)	300	21,040	7.60×10^{-4}
HE	Untreated FBS	0	2,800	0
	Untreated FBS	2.20	4,800	3.33×10^{-3}
	Resin-treated FBS/($-T_3$ $-T_4$)	0	2,400	0
	Resin-treated FBS/($-T_3$ $-T_4$)	2.20	1,500	0
	Resin-treated FBS $+T_3$/($+T_3$)	0	2,600	0
	Resin-treated FBS $+T_3$/($+T_3$)	2.20	3,600	4.17×10^{-3}

*Values are given totals of three separate experiments with C3H/10T½ cells and two separate experiments with hamster embryo cells. Stock 10^{-3}M T_3 in 50% n-propanol was diluted in media with 10% resin-treated fetal bovine serum (FBS) to give a final concentration of 10^{-7}M T_3 (designated $+T_3$). Media without thyroid hormone were prepared with 10% resin-treated FBS and an amount of diluent equal to that added in the $+T_3$ media. Stock cultures of C3H/10T½ cells were maintained in Eagle's basal medium + 10% heat-inactivated FBS; hamster embryo cells in Dulbecco's modified Eagle's medium + 10% FBS. Both cultures contained penicillin (50 U ml^{-1}) and streptomycin (50 μg ml^{-1}). All cells were maintained at 37°C with 5% CO_2 in air throughout the experiment. One week before seeding, stock cultures were placed in the experimental culture media, with and without T_3 (described in text) and maintained in these conditions for the duration of the experiment. Twenty-four hours after seeding, the cells were irradiated with x-rays at room temperature (2.20 rad for HE and 300 rad for C3H/10T½ cells), at a dose rate of 0.322 rad min^{-1}, thereafter receiving weekly media changes. After an appropriate period (6 wk for C3H/10T½ and 2 wk for HE), the cells were fixed and stained with Giemsa and scored for transformation.

[a]Transformation frequency = no. of transformed colonies/total surviving colonies.

hibited. Furthermore, when T_3 is readded ($+T_3$) the observed transformation frequency is similar to control, with irradiated cells maintained in untreated serum. When studying the dose-response relationship for transformation under varying doses of T_3, we find, as will be detailed elsewhere [58], that transformation rate increases linearly with T_3 doses, at physiological ranges of 10^{-12} to 10^{-10}, after which it decreases when doses rise to pharmacological levels. Furthermore, we find that for maximum transformation T_3 must be added to the hypothyroid cultures 12 h before irradiation, and that its action will persist even if removed 24 h later.

The action of T_3 is specific to the metabolically active hormone. If reverse T_3 (isomer of T_3 with no thyroid hormone activity) is added instead of T_3 to cultures under hypothyroid conditions, no transformation is observed. The action of T_3 in the transformation process requires synthesis as suggested by the fact that cyclohexamide inhibits the T_3 influence on x-ray-induced transformation. Recent work also indicates that when the carcinogen benzo-(a)-pyrene (1 μg/ml) is used as the oncogenic agent its effect on induction is inhibited under hypothyroid conditions, similar to x-irradiation [59]. Thyroid hormone therefore plays a crucial role in vitro in the induction and/or potentiation of carcinogenesis by both physical and chemical agents. It may play a significant and decisive role in vivo, and hyperthyroidism may provide a predisposing condition to susceptibility to carcinogens while hypothyroid conditions may serve as protective measures.

The effectiveness of the thyroid in regulating early events and the induction process of neoplastic transformation is in contrast to the retinoids, which seem to affect late events and the expression stage of malignant transformation.

We are currently evaluating whether the retinoids may interfere with the action of T_3 in its potentiating effect, and in addition whether hypothyroid can protect cells as did the retinoids from the promotional effects of tumor promotors on transformation in both rodent and human cells.

Human Cell Transformation

Although animal cell cultures serve well in the qualitative and quantitative evaluation of neoplastic development and in the study of the nature of the transformed cell, the question exists, How do these data pertain to the human situation especially in terms of risk? Thus, it seems clearly desirable to develop a human cell culture system and to carry out similar experiments as described above in cells of human origin.

Oncogenic Transformation of Human Cells In Vitro

Recently, we have succeeded in transforming human diploid skin cells, the KD cells by 400 rad of x-rays into cells that progressed in vitro to malignancy and were able to grow in agar and gave rise to tumors when injected into nude mice [41].

We used an early passage of the KD strain in which the diploid nature of the cells was ascertained by chromosomal G banding analysis. Cell doubling time was 30–32 h. Survival curve analysis indicated that survival fraction following a dose of 400 rad was close to 12% of the total population (Fig. 10).

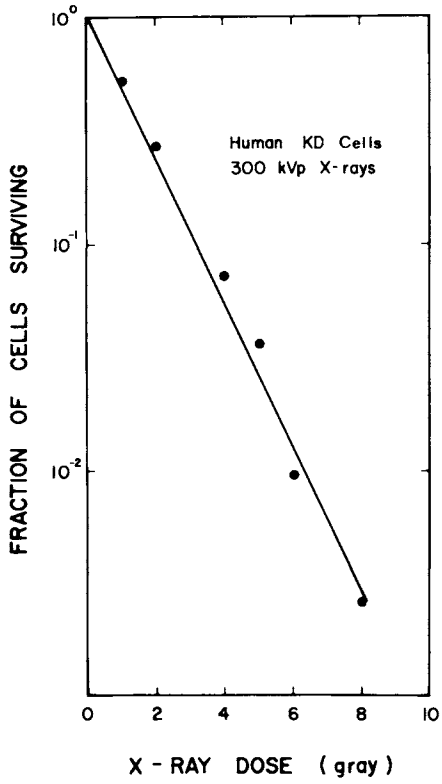

Fig. 10. Survival curve for the human KD cells following X irradiation. (Reproduced with permission [41].)

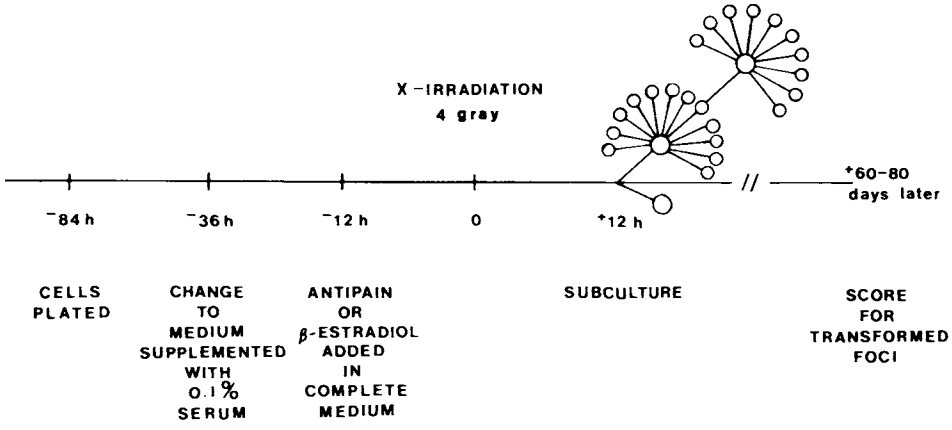

Fig. 11. The scheme for human transformation by x-rays. (Reproduced with permission [41].)

Cells were routinely maintained in MEM fortified with 10% fetal bovine and 1% human serum. The scheme for transformation is presented in Figure 11 and was as follows: Stock of KD cells were trypsinized (0.25% trypsin), suspended in complete medium, and plated in tissue culture flasks (75 cm^2) (Falcon) at 2.6×10^5 cells. Two days later, the medium was substituted by MEM containing 0.1% serum for 24 h, whereby cell proliferation was greatly reduced. After 24 h medium was again exchanged, this time for complete medium (11% serum) containing either 6 $\mu g/ml^{-1}$ of the protease inhibitor antipain 6 $\mu g/ml$ or 1 $\mu g/ml^{-1}$ β-estradiol. Experimental cultures were irradiated 12 h later with 400 rad of x-rays. All cultures were divided in two 10–12 h after irradiation and reseeded in fresh complete MEM without Antipain or estradiol. At near confluency, one of the two flasks was subcultured into 12 flasks, the other being left undisturbed. In each experiment, when these 12 flasks reached confluency, one flask was subcultured 1:10 and thus defined as "continuously passaged," and the remaining 11 flasks were left at high density.

The above experiment protocol (Fig. 11) took advantage of the following: 1) The cells that were well adapted to conditions of serum deprivation for 24 h reached a complete quiescence; 2) the addition of complete medium 24 h later led to a synchronous wave of DNA synthesis, and a treatment of the cells at that point enabled the capturing of cells entering S phase.

Within months of irradiation discrete foci were detected in the irradiated cultures pretreated with Antipain of β-estradiol (Fig. 12). The foci were composed of cells with subtle irregular orientation. By exchanging the medium-to-low-calcium-containing medium, the foci were altered dramatically while the normal cells degenerated within 24 h (Fig. 12c), several foci were isolated. Chromosome G banding indicated a near diploid range of chromosomes [46–49]. Saturation density was 2-fold over that of the normal KD cultures, and the transformed but not the normal cells were agglutinable by 25 $\mu g/ml$ concanavalin A and were able to form colonies in 0.33% agar. The ultimate proof of malignant transformation lies in the ability of the transformed cells to form tumors in the appropriate host.

Whereas the five isolated transformed cell lines grew in agar, three gave rise to fibrosarcomas when injected intradermally into immunodeficient nude mice. This indicated that growth in semisolid medium such as agar is only suggestive of a neoplastic state of the cells transformed in vitro, and that injection into animals is imperative for complete assurance of the malignant nature of the cells. No tumors arose upon the injection of the normal KD cells into nude mice.

Cultures that were treated and irradiated but not allowed to replicate more than four or five times before reaching confluency did not exhibit transformation. This indicated that in human cells as in hamster cells [12, 13] replication is required following radiation for the fixation and expression of the transformed state.

In pursuit of the mechanism by which Antipain potentiated the x-ray-induced transformation, we carried out a series of experiments on the effects of Antipain on DNA damage and repair in the human skin fibroblasts [46]. We assayed for DNA damage and repair following treatment with 2 mM Antipain by inhibition and recovery of DNA synthesis, presence of single strand breaks,

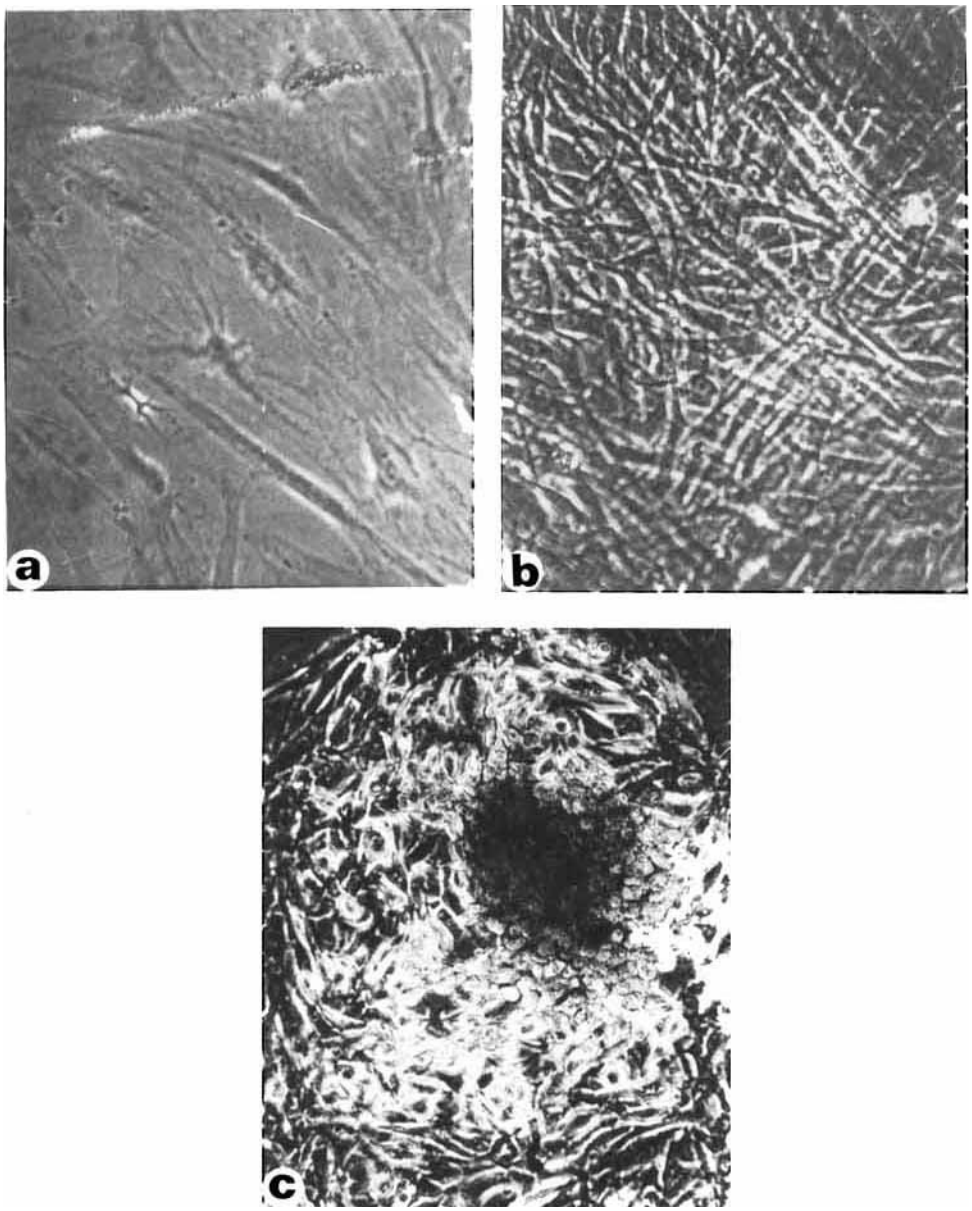

Fig. 12. a) Normal irradiated KD cells, untransformed. b) Irradiated transformed KD cells. c) The same cells as in (b), following 24-h treatment in medium containing low calcium content. Phase × 120. (In part from [41].)

accumulation of single strand breaks during growth in cytosine arabinoside, repair replication, and unscheduled DNA synthesis. Whereas in parallel experiments these assays readily detected damage and repair following ultraviolet light irradiation, they failed to reveal any change in DNA following Antipain treatment [46]. Thus, the Antipain effectiveness in potentiating radiation induced transformation with rodent cells and in human cells must occur by a mechanism that may not involve interaction of the compound with DNA.

We therefore remain in relative ignorance of the mechanism by which Antipain and the equally effective estradiol potentiate radiation-induced transformation in the human cells. Our recent findings (C. Borek, in preparation) indicate that the frequency of radiation-induced transformation in human cells in vitro is much lower than that observed in rodent cells given the dame dose of radiation. Whereas in the human cells the frequency per treated cells is approximately 10^{-6} at 400 rad, a frequency associated with mutational events, rodent cells show a significantly higher incidence of 10^{-4} at that dose level. It is of interest to note from initial observations that the number of doublings required for the expression of the transformed state of the human cells was approximately 10–13, similar to that observed in some rodent cells. The longer doubling period of the human cells (30–32 h) compared to that of rodent cells (16 h) may account in part for the increased length of time required to detect morphological transformation in the human diploid cultures as compared to that in diploid rodent cells. Other observations on the transformed human cells can be stressed. 1) Initial loss of contact inhibition is not as striking as that seen in rodent cells. 2) In contrast to rodent cells the ability to proliferate in medium with low serum (1%) is not confined to the transformed cells; our normal KD cells as well as the transformed proliferated in medium containing low serum. 3) The transformed state is associated with membrane changes and, as in rodent cells, agglutinability by plant lectins can be used as a distinguishing probe. 4) Surface topography in the x-ray-transformed human cells is altered but not as dramatically as in the rodent cells. Microvilli found in abundance on rodent cells were increased to a lesser degree in the transformed human cells. 5) The potential to grow in agar is acquired early along with morphological alteration. 6) As in the diploid hamster embryo cell systems, there were few karotypic changes associated with the transformed state of the human cells. 7) Transformability of human cells depends on the source of the cells and seems to be inversely proportional to the age of the donor, foreskin fibroblasts being more readily transformable and yielding higher rates than those derived from adults (C. Borek, in preparation).

CONCLUSION

Although the phenotypic expression of neoplastic transformation is similar following induction by radiation, chemicals, or viruses, the primary events leading to this state may differ. Viruses introduce new genetic material. Chemicals can form aducts with macromolecules, some chemicals requiring metabolic processing and activation in order to become effective carcinogens. The initial action of radiation occurs within a fraction of a second and its effects result, not by introducing anything new, but by producing disturbances ("lesions"?) in

existing macromolecules, either directly (eg, on DNA or associated macromolecules) or indirectly through the mediation of free radicals and their subsequent effects on membranes and other cellular components. Radiation damage can be grave, resulting in reproductive death, but it can also initiate alterations that, if not repaired in restorative fashion, will ultimately be potentiated and lead to neoplastic transformation.

Transformation is therefore the end result of particular lesion(s) induced and the capacity of the cell to cope with the lesion(s) and repair the damage. It may differ from the cellular repair associated with survival since we have observed an enhancement of transformation under conditions where cell killing was reduced [17, 44]. Promoting agents such as TPA and β-estradiol may amplify the lesions or cripple the cell's capacity to repair the damage. Agents that inhibit transformation may do so by providing unsuitable physiological conditions for complete induction [20]. The details of those events are being studied [58, 59]. Alternately, inhibiting factors may act as a later stage in neoplastic development, and prevent full expression. Retinoids could fall into this category. Some agents may act as "double-edged swords", potentiating radiation action when added concomitantly, yet serving to inhibit transformation if added after radiation exposure. Antipain falls into this category [17].

We use in vitro cultures as defined simplified systems, yet these cells are derived from proliferating and nonproliferating tissues and are "forced" to grow freely in vitro. Whereas cell strains from freshly explanted cultures such as the hamster cell system [11] or human [41] senesce in vitro, cell lines such as the 10T½ are populations of selected cells that are no longer subject to the control of time clocks and finite life span.

Thus the transformation process in the 10T½ [32, 60] may differ from that observed in a cell strain consisting of normal diploid cells.

Normal embryos explanted in culture give rise to cell population in which some are in a competent state to undergo induction by an oncogenic agent. This frequency of cells in this state of competency may vary not only with the cell population explanted, but also with the donor (ie, there are differences in competency among cells of different embryos (C. Borek, unpublished).

The frequency of competent cells decreases with passage in vitro, [12, 13] in contrast to the situation in a variety of cell lines in which progressive culture in vitro, enhances the number of competent cells, some of which undergo spontaneous transformations.

Once cells are induced and are continuously cultured [11], transformation frequency is enhanced progressively, as determined by clonal procedures since the expression of transformed cells is progressively less hindered by the presence of normal cells, which are subject to a finite life span. This is in contrast to the cell lines like 10T½ in which the untransformed cells are immortal, thus serving as relatively constant comparison to the transformed.

Our findings that susceptibility to radiation decreases with cell passage in hamster embryo cultures is also true of cultured human embryo cells exposed to UV [61]. Human adult skin cells induced by radiation and allowed to grow to confluence and then recultured at high density showed enhanced number of foci as compared to the undisturbed cultures [41], in contrast to the 10T½ [32], where foci enhancement upon subculturing at high density was not observed.

Clearly, the road that begins in a particular state of competence to induction processes, cellular or environmental potentiation, and progression to neoplasm may differ in different cellular systems.

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REFERENCES

1. Nagao M, Sugimura T: Ann Rev Genet 12:117, 1978.
2. Proceeding of a symposium: Cancer Res 35:3231, 1975.
3. Weisburger JH, Cohen LA, Wydner EL: Cold Spring Harbor Laboratory 567, 1977.
4. Occupational carcinogenesis: Ann NY Acad Sci 271, 1976.
5. Host environment interaction in the etiology of cancer in man: IARC scientific publication No. 7. FIC Proc 18:297, 1973.
6. Doll R: Proc R Soc 235:47, 1979.
7. Heidelberger C: Adv Cancer Res 18:317, 1973.
8. Pott P: Chirurgical observations (England), 1776.
9. Brown P: "American Martyrs to Science Throughout Roentgen Ray." Springfield: Charles Towns, 1936.
11. Borek C, Sachs L: Nature 210:276, 1966.
12. Borek C, Sachs L: Proc Natl Acad Sci USA 57:1522, 1967.
13. Borek C, Sachs L: Proc Natl Acad Sci USA 59:83, 1968.
14. Borek C, Hall EJ: Nature 244:450, 1973.
15. Borek C, Hall EJ, Rossi HH: Cancer Res 38:2997, 1978.
16. Borek C: Rad Res 79:209, 1979.
17. Borek C, Miller RC, Pain C, Troll W: Proc Natl Acad Sci USA 76:1800, 1979.
18. Borek C, Miller RC, Geard CR, Guernsey DL, Osmak RS, Rutledge-Freeman M, Ong A, Mason H: Ann NY Acad Sci 359:237, 1981.
19. Miller RC, Geard CR, Osmak RS, Rutledge-Freeman M, Ong A, Mason H, Napholz A, Perez N, Harisiadis L, Borek C: Cancer Res 41:655, 1981.
20. Guernsey DL, Ong A, Borek C: Nature 288:591, 1980.
21. Borek C: "Proceedings of the 1981 MD Anderson Symp on Molecular Interrelation of Nutrition and Cancer" (in press).
22. Todaro GJ, Green H, Goldberg BD: Proc Natl Acad Sci USA 51:66, 1964.
23. Reznikoff CA, Bertram JS, Brankow DW, Heidelberger C: Cancer Res 33:3239, 1973.
24. Borek C: Proc Natl Acad Sci USA 69:956, 1972.
25. Hart RW, Setlow RB, Woodhead AD: Proc Natl Acad Sci USA 74:5574, 1977.
26. Goth R, Rajewsky MF: Proc Natl Acad Sci USA 71:639, 1974.
27. Cerutti PA: Photochem Photobiol Nucl Acids 2:375, 1976.
28. Painter RB, Young RB: Mutat Res 14:225, 1972.
29. Cairns J: Nature 289:353, 1981.
30. Borek C, Sachs L: Proc Natl Acad Sci USA 56:1705, 1966.
31. Borek C, Sachs L: Unpublished observations, 1967.
32. Kennedy AR, Fox M, Murphy G, Little JB: Proc Natl Acad Sci USA 77:7262, 1980.
33. Borek C, Pain C, Mason H: Nature 266:452, 1977.
34. Borek C, Higashino S, Loewenstein WR: J Memb Biol 1:274, 1969.
35. Borek C: In Gerschenson LE, Thompson EB (eds): "Gene Expression and Carcinogenesis in Cultured Liver." New York: Academic Press, 1975, p 62.
36. Borek C, Guernsey DL: Studia Biophys 84:53, 1981.

37. Brady RO, Borek C, Bradley KM: J Biol Chem 244:6552, 1969.
38. Yotti LP, Chang CC, Trosko JE: Science 206:1089, 1979.
39. Borek C, Fenoglio CM: Cancer Res 36:1325, 1976.
40. Barrett JC, Ts'O POP: Proc Natl Acad Sci USA 75:3761, 1978.
41. Borek C: Nature 283:776, 1980.
42. DiPaolo JA, Donovan PJ: Int J Radiat Biol 30:41, 1976.
43. Borek C, Ong A: Cancer Lett 12:61, 1981.
44. Borek C: Br J Radiol 50:845, 1979.
45. Borek C, Cleaver JE; Mutat Res 82:373, 1981.
46. Borek C: Adv Cancer Res 37 (in press).
47. Miller EC, Miller JA: Biochem Nutrit IA 27:123, 1979.
48. Borek C, Smith JE: J Cell Biol 79:78a, 1978.
49. Borek C, Miller RC, Geard C, Guernsey DL, Smith JE: In Hecker E, Slagat S (eds): "Carcinogenesis, Cocarcinogenesis, and Tumor Promoters." New York: Raven Press (in press).
50. Wolff S: Ann Rev Genet 11:183, 1977.
51. Shilo B, Weinberg RA: Nature 289:607, 1981.
52. DeLuca L, Maestri N, Bonanni F, Nelson D: Cancer 30:1326, 1972.
53. Harris CC, Sporn MB, Kaufman DG, Smith JM, Jackson FE, Saffiotti U: J Natl Cancer Inst 48:743, 1972.
54. Genta UM, Kaufman DG, Harris CC, Smith JM, Sporn MB, Saffiotti U: Nature 247:48, 1974.
55. Peto R, Doll R, Buckley JD, Sporn MB: Nature 290:201, 1981.
56. Borek C, Fenoglio CM, King DW (eds): "Advances in Pathobiology," Vol 7, "Aging, Cancer and Cell Membranes." New York: Thieme-Stratton, 1980.
57. Kumar MS, Chiang T, Deodhar SD: Cancer Res 39:3515, 1979.
58. Guernsey DL, Borek C, Edelman IS (in press).
59. Borek C, Guernsey DL, Ong A, Edelman IS: manuscript in preparation.
60. Fernandez A, Mondal S, Heidelberger C: Proc Natl Acad Sci USA 77:7272, 1980.
61. Sutherland BM, Cimino JS, Delihas N, Shih AG, Oliver RP: Cancer Res 40:1934, 1980.