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Modulating Factors in the Expression of Radiation-Induced Oncogenic Transformation

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Many assays for oncogenic transformation have been developed ranging from those in established rodent cell lines where morphological alteration is scored, to those in human cells growing in nude mice where tumor invasiveness is scored. In general, systems that are most quantitative are also the least relevant in terms of human carcinogenesis and human risk estimation. The development of cell culture systems has made it possible to assess at the cellular level the oncogenic potential of a variety of chemical, physical and viral agents. Cell culture systems afford the opportunity to identify factors and conditions that may prevent or enhance cellular transformation by radiation and chemicals. Permissive and protective factors in radiation-induced transformation include thyroid hormone and the tumor promoter TPA that increase the transformation incidence for a given dose of radiation, and retinoids, selenium, vitamin E, and 5-aminobenzamide that inhibit the expression of transformation. Densely ionizing α-particles, similar to those emitted by radon daughters, are highly effective in inducing transformations and appear to interact in a supra-additive fashion with asbestos fibers. The activation of a known dominant oncogene has not yet been demonstrated in radiation-induced oncogenic transformation. The most likely mechanism for radiation activation of an oncogene would be via the production of a chromosomal translocation. Radiation also efficiently induces deletions and may thus lead to the loss of a suppressor gene.

Introduction

Following the report by Berwald and Sachs on the induction of morphological transformants in Syrian hamster embryo cells by the chemical carcinogen benzo(*a*)pyrene more than two decades ago (*1*), a host of *in vitro* oncogenic transformation systems have been developed based on a variety of rodent and human cells (*2–5*). These *in vitro* model systems represent a powerful research tool in addressing two diverse aspects of cancer research. First, they may be used to accumulate data that are essentially pragmatic in nature. These *in vitro* systems, free of host-mediated homeostatic influences, afford the opportunity to evaluate both qualitative and quantitative aspects of oncogenic transformation. For example, they are useful to compare and contrast the oncogenic potential of a variety of chemical and physical agents. As such, they occupy a useful intermediate position between the bacterial mutagenesis assays, which are quick and inexpensive, and animal studies, which are cumbersome and inordinately expensive.

Second, *in vitro* systems make it possible to study the cellular and molecular mechanisms involved in radiation or chemically induced carcinogenesis, inasmuch as they represent models for *in vitro* tumorigenesis in which the various steps can be manipulated and modified more easily and in a more controlled way. *In vitro* systems afford the opportunity to study dose-related and time-dependent interactions of radiation with single cells and identify factors and conditions that may prevent or enhance cellular transformation by radiation and chemicals.

The recent advances in molecular biology using cloned fragments of viral transforming genes and the subsequent discovery of such proto-oncogenes in many animal and human tumors using the transfection technique have highlighted the practical need for these model systems.

Criteria of Malignancy

A number of different end points can be scored in transformation assays. The possible end points in order of increasing relevance for carcinogenesis in the human are as follows: altered morphology; growth in soft agar; unlimited growth; transplantability in animals; invasiveness; and metastasis.

By scoring the altered morphology of a colony or focus, it is quite practical to identify one transformed cell in a population of 10^6 cells in an experiment of manage-

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able size. Assays that depend on this criteria, such as those with Syrian Hamster embryo cells (*1,2*), C3H 10T½ (*3*) or 3T3 (*4*) cells, can therefore be used in highly quantitative experiments with radiation to determine, for example, the effects of radiation quality or radiation dose rate on the influence of protective and permissive factors. Unfortunately, the altered morphology of a colony or focus in a Petri dish is far removed from an overt tumor in the human, and the limitations of the systems must be appreciated. At the other extreme, for systems that score invasiveness and/or metastatic potential (*6,7*), the assay of treated cells must be performed in animals with a consequent loss of resolution and quantification. For example, it would be out of the question to obtain detailed dose-response relationships for two types of radiation to allow an assessment of relative biological effectiveness when scoring tumor invasiveness as an end point.

Assay Systems for Oncogenic Transformation

The following list of available assay systems is representative but by no means exhaustive. *a*) Syrian hamster embryo (SHE), C3H 10T½, and NIH 3T3 cell systems are the most quantitative, though by the same token the most removed from relevance to cancer risk assessment in the human. *b*) The HeLa-normal human fibroblast hybrid-cell line may be as quantitative as those in group *a* and it has the advantage that these are human cells. *c*) The RTE (respiratory tract epithelium) system in the rat lends itself to mechanistic studies and is also a quantitative assay system for carcinogenic agents including radiation, though it is not as quantitative or reproducible as the systems of group *a*. *d*) The thyroid and mammary cell systems in the rat, which are assayed for both cell survival and oncogenicity by transplantation into a fat pad of the animal, represent attractive model systems for the study of oncogenic transformation that is quantitative and amenable to a number of studies. *e*) Mouse skeletoblasts may be studied in a semi-quantitative way after being infected with retroviruses, but quantitative studies comparable to those reported for groups *a* and *b* have not been reported. *f*) Human fibroblasts have been studied extensively, but they must be immortalized in one of three ways: infection with the SV40 virus, irradiation with multiple doses of gamma rays to a total dose of 3 Gy or more, or transfection with the *myc* oncogene. Following immortalization by one of these procedures, human fibroblasts represent a system potentially suitable for quantitative studies. *g*) Human keratinocytes, immortalized by a combination of viruses, have been used for a variety of studies. The disadvantage is that the karyotype of the cell has been drastically altered, as is also true for C3H 10T½, 3T3, and all virally transformed or oncogene transfected fibroblast lines. *h*) Human uroepithelial cells have been immortalized by the chemical carcinogen 3-methylcholanthrene (MCA) and their properties characterized extensively, but up to the present time they have not been used for studies involving ionizing radiation. *i*) Human epithelial cells have been immortalized with the retrovirus HPV and insertion of the *ras* oncogene and may then be used both as a quantitative assay system and for mechanistic studies. *j*) Human keratinocytes spontaneously immortalized, or immortalized with the *ras* oncogene, may subsequently be grown on a collagen base in the animal. This system and the assay system described in *i* enjoy the advantage that the invasive capabilities of the induced tumor may be studied. Up to the present time, these systems have not been used for quantitative radiation studies.

In the list above, 10 assays of oncogenic transformation have been arranged in increasing order of complexity and possible relevance to the assessment of cancer risk estimates in the human. The simplest systems are short-term explants of SHE cells and the established cell lines designated, C3H 10T½ and Balb/c 3T3, which are highly quantitative and reproducible involving fibroblasts rather than epithelial cells, and are far removed from the real life situation of carcinogenesis in the human. On the other hand, increasing relevance in this list is matched by a decreasing level of quantification. While assays involving the established cell lines are highly repeatable and quantitative, systems involving the human epithelial cells that allow an observation of tumor invasion, for example, are difficult to use in a quantitative fashion. All of the quantitative studies described in the remainder of this paper involve short-term explants of SHE cells or the established cell line C3H 10T½.

Modulation of Radiation-Induced Transformation

A variety of agents have been identified that can dramatically influence the oncogenic transformation potential of a given dose of radiation. Some of these agents need not be present during irradiation, but may be added some time later. Results of a series of experiments along these lines are summarized in Figures 1 and 2.

It is interesting to note that manipulation of hormonal levels can dramatically alter the transformation incidence (Fig. 2), demonstrating in the Petri dish what is evident from experiments with both humans and animals (*8,9*). Under hypothyroid conditions, neither X-ray nor benzo(*a*)pyrene-induced transformations are expressed (*10,11*). When thyroid hormone (triiodothyronine) is added to the medium, oncogenic transformation is expressed in a dose-dependent fashion. The induction of cellular transformation protein by thyroid hormone and its effect on the oxidative state of cells is thought to influence the transformation induction in cells (*11*).

In the context of chemoprevention, i.e., giving a compound to a group of people at high risk of developing cancer, such as patients cured of their first malignancies by a combination of radiation and chemotherapy, reti-

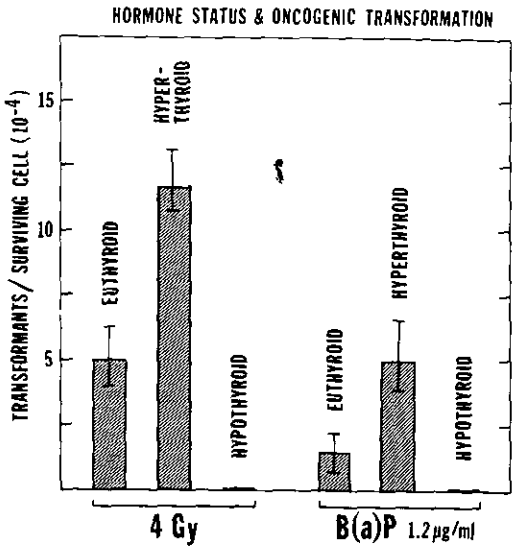

FIGURE 1. Effects of thyroid hormone status on radiation and chemically induced transformation frequencies in C3H 10T½ cells.

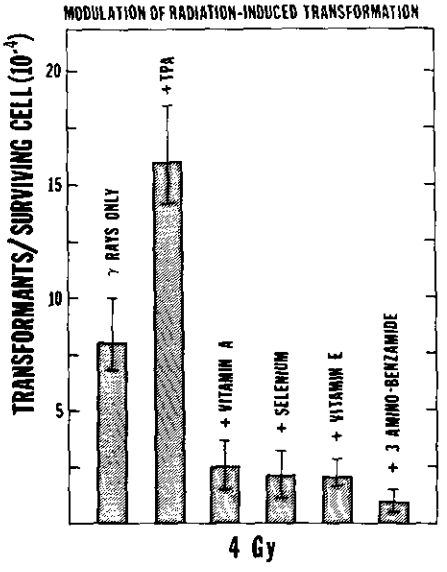

FIGURE 2. Transformation incidence in C3H 10T½ cells by 4 Gy of γ-rays and its modulation by various chemical and dietary factors.

noids would appear to be particularly promising and appropriate. The term retinoid has been used for a group of compounds that include vitamin A and its natural and synthetic analogues. Retinoids have been found frequently to block the phenotypic expression of tumor cells *in vitro* (*12*); they also inhibit growth and induce differentiation in many animal and human malignant cell types (*12*). Proposed mechanisms of retinoid action involve the protein kinase-C cascade system. This system may mediate the many diverse actions of retinoids including their effects on enzyme synthesis, membrane properties, growth factors, binding proteins, genomic and postgenomic expression, the extracellular matrix, and immunologic response. Figure 1 illustrates the suppression of radiation-induced transformation by vitamin A analogues, first shown more than 10 years ago (*13*). A large body of data has accumulated with regard to the importance of retinoids for chemoprevention of cancer, whether it be caused by radiation, chemical carcinogens, or viral transforming factors (*12,14*). Experimental studies have shown that chemoprevention with retinoids inhibits the development of neoplastic or transformed cell populations *in vitro* as well as in tumor models such as skin, bladder, and mammary glands in mice and rats (*15*). Some of the retinoids have been found to be effective in inhibiting the recurrence of these tumors. More work is needed to determine whether retinoids inhibit the transformation process *per se* or whether they simply inhibit the clonal expansion of initiated cells.

The modulation of transformation by other compounds is also illustrated in Figure 2. Selenium is a microcomponent of the diet and a cofactor for the enzyme glutathione peroxidase, illustrating again the substantial impact that dietary factors may have in cancer incidence resulting from physical or chemical insults. Vitamin E is principally an antioxidant, while 3-aminobenzamide is an inhibitor for the synthesis of poly-ADP-ribose and prevents DNA repair by interfering with the ligation process.

Recent data point increasingly to free radicals that cause a variety of cascading events associated with lipid peroxidation and play an important role in carcinogenesis, particularly the promotion phase. Superoxide dismutase, a free radical scavenger, inhibits transformation induced by either radiation or bleomycin if it is in contact with cells for a prolonged period of time after treatment with the oncogenic agent.

Hyperthermia is about the only cancer therapy agent, apart from the surgeon's knife, that has not been implicated in the induction of cancer as well as its cure. Hyperthermia has been shown to reduce the incidence of transformed foci induced by X-rays (*16*) and also by a number of chemotherapy agents (*17,18*).

Transformation by Simulated Radon Daughter Alphas and the Interaction with Asbestos

Recent evidence suggests that about half of the total radiation exposure received by the public results from α-particles emitted by radon daughter products deposited in the lungs. Densely ionizing charged particles that simulate the emission from radon can be accelerated in a van de Graaff machine and used to irradiate C3H 10T½ cells in culture. Figure 3 shows survival and transformation data for cells treated with γ-rays or high LET helium-3 ions with an LET of 120 keV/μm. The cell survival curve for γ-rays has a broad initial shoulder, while that for helium-3 ions is an exponential function of dose. For both types of radiation, the transformation incidence increases with dose, tending towards a pla-

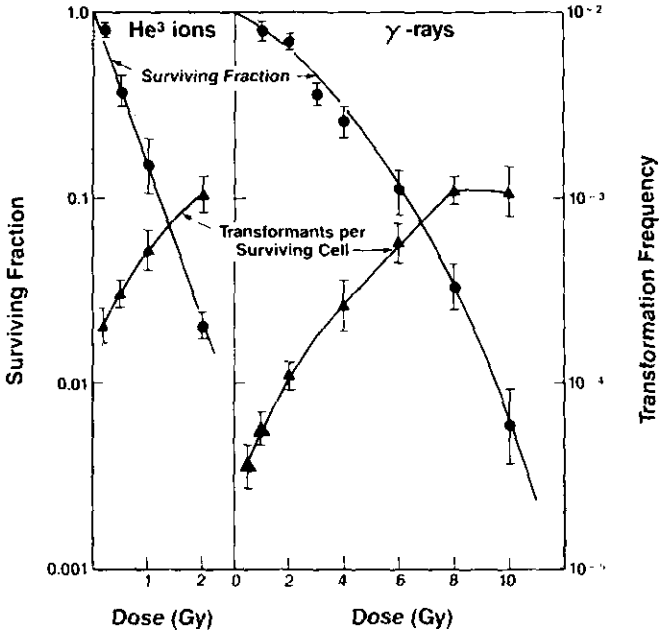

FIGURE 3. Dose-response relationships for cell survival and oncogenic transformation in C3H 10T½ cells exposed to graded doses of γ-rays or helium-3 ions with an LET of 120 keV/μm.

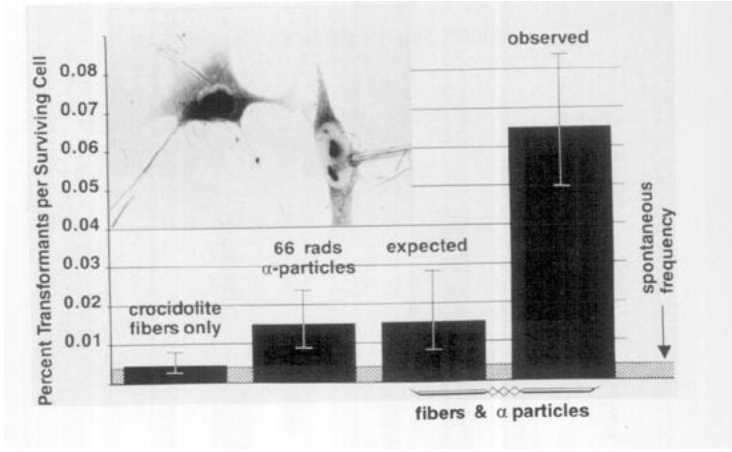

FIGURE 4. Transformation incidence in C3H 10T½ cells for asbestos fibers and helium-3 ions, delivered alone or in combination. Transformation appears to be supra-additive when asbestos and high-LET particles are delivered together. (Insert) Cells become impaled on fibers of the appropriate length.

teau at a dose of a few GY for γ-rays and at about 0.5 Gy for helium-3 ions.

It could be argued that the most relevant parameter for extrapolation to carcinogenesis in the whole organism, or whole organ, is transformation per initial cell at risk, as this ratio represents a balance between transformation and cell killing. This quantity rises at low doses, reaches a maximum, and subsequently falls at higher doses as cell killing becomes dominant. For densely ionizing particles, the peak occurs at a much lower dose, and reaches a value five times higher than is the case for γ-rays. These high LET particles, which closely mimic the emission from radon daughters, are much more effective than γ-rays at cell killing, but even more effective at inducing transformation. The interesting point is that a dose of about 0.5 Gy of helium-3 ions produces an incidence of transformants per initial cell at risk that is not achieved by any dose of γ-rays.

Experiments have also been performed to investigate an interaction between densely ionizing particles and asbestos fibers (*19*). Figure 4 shows transformation data for C3H 10T½ cells resulting from a 24-hr pretreatment with 5 μg/mL crocidolite fibers, or a dose of 0.66 Gy helium-3 ions, or a combination of both. As shown previously, crocidolite fibers, at concentrations that resulted in only moderate cell killing, induced transformation frequencies indistinguishable from the spontaneous rate. However, cells pretreated with crocidolite fibers for 24 hr and subsequently irradiated with α-particles exhibited a significantly higher transformation incidence than after radiation alone. The insert in this figure shows the way in which cells become impaled on fibers of certain lengths. The combination of asbestos fibers and high-LET particles produces a transformation incidence higher than the simple sum of the two agents alone. This apparent supra-additivity has implications for the possible interaction of radon and asbestos in the environment, and possibly for combinations of other environmental pollutants too.

Identification of Oncogenes

Transformation assays play an essential role in identifying and characterizing oncogenes. The overall strategy is illustrated in Figure 5. Genomic DNA from cell clones transformed by an agent such as radiation can be transfected into recipient cells, the cultures can be expanded in culture, and implanted into animals to determine their tumorigenicity. DNA isolated from the tumors formed is compared with known DNA sequences to identify the existence of oncogenes by chromatographic techniques.

In the case of radiation transformation, the exact nature of the oncogenes involved has not yet been elucidated. However, a number of interesting experiments have been performed in this area in which DNA from a transformed focus, induced by radiation, can be transfected into recipient cells that subsequently show the characteristics of malignancy themselves—the ability to grow in soft agar and eventually produce tumors in animals (*20*). Furthermore, Southern blot analysis of NIH 3T3 and Rat II transfectants carrying oncogenes from radiation-transformed C3H 10T½ on hamster embryo cells indicates that the oncogene(s) responsible for the transformation of 3T3 cells are unique non-*ras* transforming genes (*20*). Recent studies by Sawey and Kennedy using a radiation-induced transformant as a source of transforming DNA have demonstrated no activation of *ras* oncogene in the resultant tumorigenic recipients; however, a rearrangement of the c-*myc* oncogene is detected as the dominant molecular change (*21*).

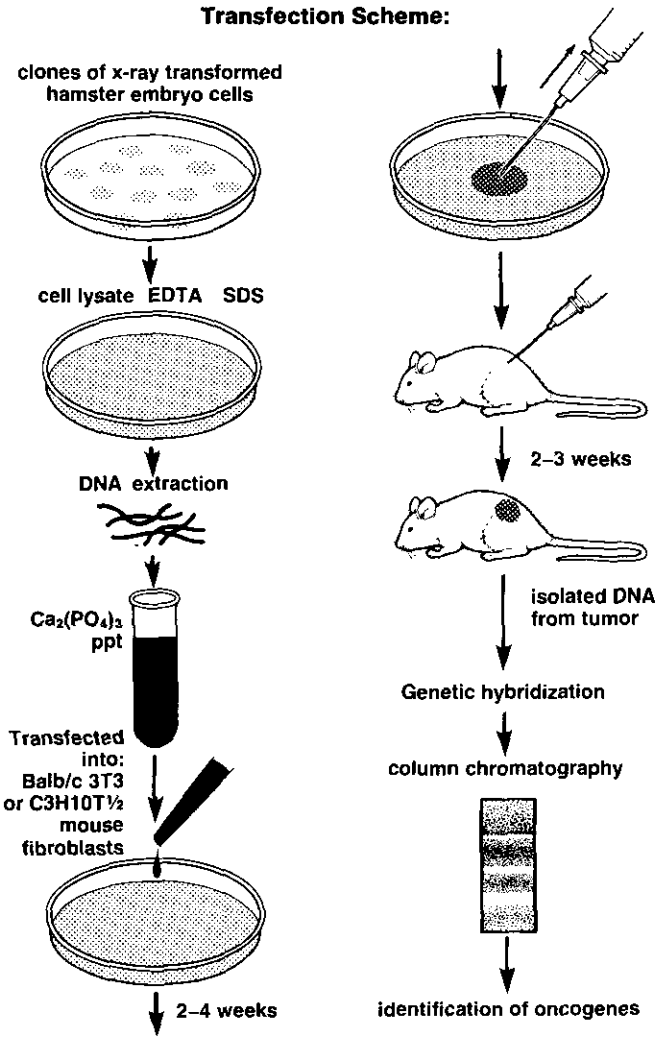

FIGURE 5. Schematic diagram of a typical DNA transfection protocol where oncogenes can be isolated from cells transformed *in vitro* by either radiation or chemical carcinogens. DNA sequences are then characterized by genetic hybridization.

Oncogenes in Human Cancer

To date, about 24 oncogenes have been identified in human cancer with the transfection assay. Among them, the most frequently found are members of the *ras* gene family: H-*ras*, K-*ras*, and N-*ras*. H-*ras* and K-*ras* are the cellular homologues of the oncogenes present in the Harvey and Kirsten strains of murine sarcoma viruses. The N-*ras* locus has so far not been transduced by retroviruses. Cellular *ras* genes encode the p21 ras protein (*22*). They acquire transforming properties by single point mutations along their coding sequence. The most frequent mutated sites involve the codons for amino acids 12, 13, or 61 (*23*). Identified in almost every form of human cancer, *ras* oncogenes have an overall incidence of 10% to 15%. In some types of tumor this percentage is significantly lower (e.g., breast carcinomas), whereas in others (e.g., acute myelocytic leukemia) it could be as high as 25%. More than 80% of the transforming genes detected in gene transfer assays have been found to be members of the *ras* gene family.

Other human oncogenes identified by different experimental approaches were also found to represent cellular homologues of well-characterized retroviral oncogenes. For instance, the oncogene involved in the chromosomal translocations characteristic of Burkitt's lymphoma, c-*myc*, was first identified as the oncogene of avian myelocytomatosis virus. Similarly, the oncogene implicated in the development of chronic myelogenous leukemia, c-*abl*, turned out to be the cellular homologue of v-*abl*, the oncogene of Abelson murine leukemia virus. These findings clearly illustrate the existence in human tumors of dominant oncogenes, some of which correspond to those previously characterized as responsible for the carcinogenic effect of acute transforming retroviruses.

Cooperation of Cellular Oncogenes

The multistage nature of carcinogenesis has also been demonstrated with cellular oncogenes. The first pair of cellular oncogenes found to cooperate were H-*ras* and *myc* (*24*). Figure 6 illustrates these very important experiments. The experiments showed that if the *ras* gene or the *myc* gene was transfected into primary cultures of embryo cells, no transformed foci were produced. On

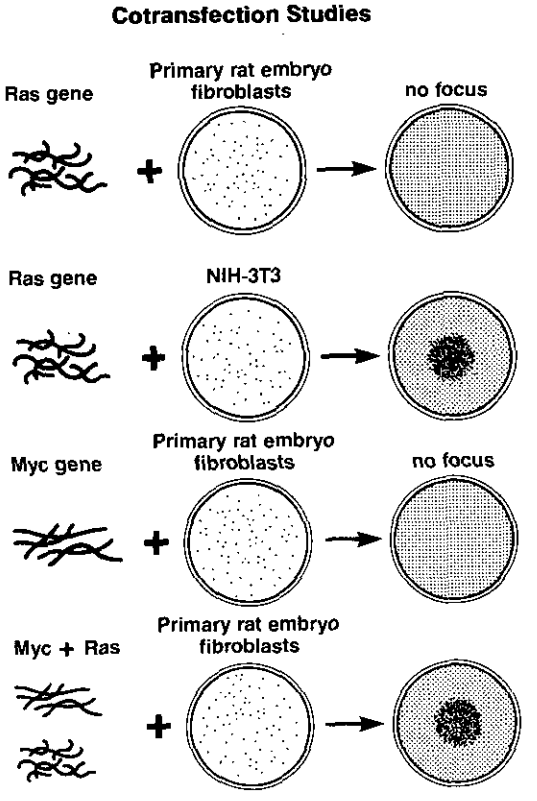

FIGURE 6. Schematic illustration of the co-transfection studies of Land et al. (*24*), in which a cooperation of both the *ras* and *myc* genes are required to transform primary, nonimmortalized cells.

the other hand, if the *ras* gene was transfected into the already immortalized NIH 3T3 cells, foci were then observed. To obtain transformed foci in fresh explants of embryo cells it was necessary to transfect both the *myc* and the *ras* oncogenes. It is generally considered that the *myc* gene confers immortality, and the *ras* gene produces the morphological changes characteristic of transformation. Other examples of cooperating oncogenes may involve oncogenes that have nuclear gene products such as *myc* and p53 with genes that have cytoplasmic gene products such as the *ras* and *src*.

More recent studies have suggested that the H-*ras* gene site may be the direct target of chemical carcinogens, and the H-*ras* oncogenes are activated during the initiation phase of carcinogenesis. The implications that the *myc* gene can suppress cellular differentiation in progenitor cells in experimental tumor models and is overexpressed in proliferating cells provide a functional explanation for the *ras-myc* cotransfection scheme. In the case of radiation-induced transformation, experiments parallel to those of Weinberg have been performed (*20*): DNA from X-ray-transformed C3H 10T½ cells were transfected into three different recipient cells, two of them established cell lines (10T½ and 3T3) and one of them a short embryo culture (hamster embryo cells). The result of the experiments indicated that foci could only be produced when the recipient cells were already immortalized, that is with the 3T3 or 10T½ cells, but no transformed clones were observed when the oncogenic DNA from the 10T½ cells was transfected into the short-term cultures of hamster embryo cells. These radiation experiments again suggest that only one oncogene is necessary when the recipient cell line is already immortalized, but that more than one is necessary to produce transformation in primary cell lines. The fact that focus formation can be induced in *ras*-transfected primary fibroblasts after application of the tumor promotor TPA (*25*) and that TPA can enhance c-*ras*-induced transformation in C3H 10T½ cells (*26*) suggest that protein kinase C may be a common pathway for the induction of c-*myc* expression by both TPA and platelet-derived growth factors.

Molecular Mechanisms of Radiation-Induced Transformation

In general, oncogenes can be activated by one of three processes; point mutation, a chromosome translocation that moves the gene from an inactive to an active site, and overexpression. Ionizing radiations are inefficient in producing point mutations, but extremely effective in inducing chromosomal translocations. Nothing is known of the mechanisms whereby radiation may result in gene amplification. Of the three principal mechanisms for oncogene activation, therefore, a chromosome translocation would be the most likely to be induced by radiation.

While the activation of a dominant oncogene is associated with some human malignancies, particularly the leukemias and lymphomas, it appears that an increasing number of solid tumors, such as small cell lung carcinoma, colon cancer, and glioblastoma, result directly or indirectly from the loss of a suppressor gene, or what some have termed an anti-oncogene. Ionizing radiations efficiently induce chromosome deletions, and it may well be that radiation carcinogenesis in some instances may be a consequence of the loss of a suppressor gene.

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