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RADIATION-INDUCED NEOPLASTIC TRANSFORMATION OF HUMAN CELLS

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Abstract

Ionizing radiation can induce cancers in humans and animals and can cause *in vitro* neoplastic transformation of various rodent cell systems. However, numerous attempts to achieve neoplastic transformation of human cells by radiation have generally proven unsuccessful. Neoplastic transformation of immortalized human epidermal keratinocytes by X-ray irradiation has recently been reported. The carcinogenic effect of radiation on cultured human cells will be briefly reviewed. The current state-of-the-art in radiation-induced transformation of human cells in culture is presented. This will provide insight into the molecular and cellular mechanisms in the conversion of normal cells to a neoplastic state of growth.

Key Words: Radiation, transformation, human cells.

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Introduction

The carcinogenic action of ionizing radiation of humans has been well recognized from epidemiological data (Rossi and Kellerer, 1974; BEIR, 1972; UNSCEAR, 1977). Ionizing radiation can induce tumors in various tissues and organs in animals (Upton *et al.*, 1986) and can transform rodent cells in culture (Borek and Sachs, 1966; Borek and Hall, 1973; Borek, 1982; Little, 1986; Yang and Tobias, 1980) There have been, however, very few studies on radiation-induced neoplastic transformation of human cells. Thus, the mechanism of radiation-induced neoplastic transformation of human cells is poorly understood.

Carcinogenesis is a multistage process, initiated by carcinogen-induced genetic and epigenetic damages in susceptible cells that have gained a selective growth advantage. These cells undergo clonal expansion as the result of activation of proto-oncogenes and/or inactivation of tumor suppressor genes and progress to a malignant state of growth. Traditionally, different terms are used for the multisteps including initiation, promotion, and progression. Epidemiological studies have suggested that five or six independent steps are required for acquisition of the malignant phenotype (Peto, 1977). A recent genetic model of colorectal carcinogenesis has shown that the complex multistep nature of human cancer in which at least four genetic alterations occur in both growth-stimulating oncogenes and growth inhibitory tumor suppressor genes before the onset of tumor formation (Fearon and Vogelstein, 1990).

The neoplastic transformation of human cells *in vitro* is also a complex, multistep process by which normal cells acquire the various phenotypic characteristics (Rhim, 1989, 1992). Four major steps appear to be involved: (**a**) development of morphological transformation, (**b**) growth in semisolid medium, (**c**) immortality, and (**d**) tumorigenicity. In contrast to rodent cells, normal human cells in culture do not or rarely undergo spontaneous transformation and have generally proved resistant to neoplastic transformation by carcinogens. The basis for differences in transformation sensitivity is not known, but several explanations, such as difference in natural life span and in genetic stability, can be

speculated.

During the past several years, remarkable progress has been made in the field of human cell transformation systems. Neoplastic transformation of human cells in culture has been achieved by a stepwise process: immortalization and conversion of the immortalized cells to tumorigenic cells (Rhim *et al.*, 1985). Cooperation between oncogenes increases the neoplastic progression of human cells (Rhim *et al.*, 1985) as shown in rodent cells (Land *et al.*, 1983; Ruley, 1983). One of the critical initial events in the progression of normal human cells to tumor cells is the escape from cellular senescence. With few exceptions, normal human cells require immortalization to provide a practical system for transformation studies. Neoplastic transformation of immortalized human epidermal keratinocytes by X-ray irradiation has recently been reported (Thraves *et al.*, 1990). Dominant-acting transforming genes from radiation-transformed human epidermal keratinocytes by tumorigenicity assay have been detected (Thraves *et al.*, 1991). We discuss here the current state-of-the-art in radiation-induced neoplastic transformation of human cells in culture. We hope this will provide further insight into the molecular and cellular mechanisms involved in the conversion of normal human cells to a neoplastic state of growth.

Immortalization of Human Fibroblasts by Repeated Gamma-Ray Irradiation

Namba *et al.* (1985) reported the successful immortalization of human diploid fibroblasts (KMST-6) by exposure to multiple doses of ^{60}Co gamma rays (12X, 2800 rads in total). However, this was a rare occurrence; only one immortalized cell line arose in a large number of experiments. This is the only case in which human diploid fibroblast has been successfully immortalized by exposure to a physical carcinogen. However, Little *et al.* (1991) have shown their failure to induce immortalization by exposure to single or multiple doses of X-irradiation in a total of 46 separate experiments with human diploid fibroblasts. Thus, these findings suggest that immortalization is a rare event and a rate-limiting step in transformation of human diploid cells. Radiation did induce persistent genetic changes in surviving cells, characterized by chromosomal rearrangements that were transmitted to progeny cells over many generations of replication. The cells possessing such rearrangements sometimes gained a selective growth advantage and emerged as abnormal clones. Some abnormal clones showed a markedly increased lifespan, but none became immortal. Unstable chromosomal abnormalities, such as rings, dicentrics and fragments, disappeared rapidly from the cultures with the first two passages after irradiation.

Radiation-Induced Neoplastic Transformation of Human Skin Fibroblasts

X-ray induced *in vitro* neoplastic transformation of human skin fibroblast (KD) has been reported (Borek, 1980). The transformed cells formed colonies in soft agar and induced tumors when injected into nude mice. However, cells cultured from the tumors induced by the X-ray transformed cells have not been characterized. Thus, there have been no studies on neoplastic transformation of human skin fibroblasts by X-ray irradiation.

Table 1. Biological properties of the RHEK-1 human epidermal line exposed to X-ray irradiation.

Total dose of X-rays	Morphological alteration	Soft agar colony formation*	Tumorigenicity in nude mice**
4Gy (2x2Gy)	+	+	+
4Gy (2x2Gy)SA†	+	+	+
8Gy (2x4Gy)	+	+	+
8Gy (2x4Gy)SA	+	+	+
12Gy (2x6Gy)	-	-	-
16Gy (2x8Gy)	-	-	-
None	-	-	-

*Cell suspension (1 x 10^5 cells/ml) were plated on 0.33% soft agar medium containing 10% fetal bovine serum.

**Inoculation with 10^7 cells. Tumors were reestablished in tissue culture and confirmed as human; their resemblance in the cells of origin was determined by karyological analysis.

†SA, lines derived from soft agar colonies.

Recently, reproducible neoplastic transformation of human fibroblasts *in vitro* has been achieved by a stepwise process as shown previously in human epithelial cell systems (Rhim *et al.*, 1985). There have been few experimental reports on successful malignant transformation of human fibroblasts by chemical or physical agents or oncogene alone (Little, 1986; Milo and Castro, 1986; McCormick and Mahr, 1988). In most cases, immortalized, nontumorigenic human fibroblast cell lines, induced by various methods, have been converted to tumorigenic lines using *ras* oncogene infection or transfection.

Namba *et al.* (1986, 1988) have shown that nontumorigenic human fibroblast cell line (KMST-6) immortalized by exposure to multiple doses of ^{60}Co gamma rays (37) was converted into neoplastic cells by H-*ras* oncogene infection and transfection. The H-*ras* oncogene alone did not convert normal human fibroblasts into either immortal or tumorigenic cells. Therefore, the immortalization of normal human cells may be an indispensable step for their tumorigenic transformation.

It is interesting to note that extensive *in vitro* passaging (547 passages for 2800 days after initial culture) of human fibroblasts (KMST-6) that were previously immortalized by ^{60}Co gamma ray-irradiation (Namba *et al.*, 1985) resulted in cells with tumorigenic potential. In addition, the acquisition of a malignant phenotype was not associated with the activation of *ras* oncogenes

(Mihara *et al.*, 1992). It was concluded that the immortalization process is a critical, initial and rate-limiting step in culture and that the subsequent malignant transformation involves multiple genetic events.

Neoplastic Transformation of Human Epithelial Cells by Irradiation

Human epithelial cells are particularly important for analyzing steps in cancer development, since most human tumors are of epithelial cell origin. Until recently, the inability to grow epithelial cells in culture had made it difficult to define the process of neoplastic transformation of these cells. Recently, we have developed an *in vitro* human keratinocyte multistep model suitable for the study of human epithelial cell carcinogenesis (Rhim *et al.*, 1985). This was developed following an infection of primary human epidermal keratinocytes with Ad12-SV40 virus leading to the acquisition of an indefinite lifespan in culture, but not the development of malignant phenotype. These immortalized human keratinocytes (RHEK-1), when treated subsequently with either Kirsten murine sarcoma virus (Ki-MSV) (Rhim *et al.*, 1985) or chemical carcinogens (Rhim *et al.*, 1986) led to the induction of morphological alterations, the colony formation in soft agar, and the development of a malignancy. The availability of this human keratinocyte system led us to determine the potential of X-rays as a carcinogenic agent in human epithelial cells and to characterize the molecular events involved in the development of a radiation-induced malignancy.

Neoplastic Transformation of Immortalized Human Epidermal Keratinocytes by Ionizing Radiation

We have recently shown that nontumorigenic RHEK-1 cells can be transformed malignantly by exposure twice to X-ray irradiation (Thraves *et al.*, 1990). These transformed cells showed morphological alterations, formation of colonies in soft agar, and induced carcinoma when transplanted into nude mice (Fig. 1 and Table 1). Primary human epidermal keratinocytes exposed to radiation in this manner failed to show any evidence of transformation. These findings demonstrate the malignant transformation of human primary epithelial cells in culture by the combined action of a DNA tumor virus and radiation, indicating a multistep process for radiation-induced neoplastic conversion.

Ras Oncogenes were not Activated in the Radiation-Transformed RHEK-1 Cell Lines

Since RHEK-1 cells could be transformed by Ki-MSV infection and become tumorigenic (Rhim *et al.*, 1985), we analyzed the *ras* p21 product in the radiation-transformed as well as in the Ki-MSV-transformed RHEK-1 cells by using antibody to p21 and SDS-PAGE. In comparison to the Ki-MSV transformed RHEK-1 cells, the radiation-transformed cells showed neither altered mobility nor increased expression of the p21 protein (Fig. 2). Moreover, the DNA from these X-ray altered cells failed to induce detectable transformed foci upon transfection of NIH/3T3 cells. These results indicate that the activation of a *ras* gene was not involved in the radiation-induced transformation of immortalized human epidermal keratinocytes (Thraves *et al.*, 1990).

While the activation of cellular *ras* oncogenes has been demonstrated in rodent tumors induced by ionizing radiation (Guerrero *et al.*, 1984a, 1984b; Sawey *et al.*, 1987), the activation of unique non-*ras* oncogenes has been shown in malignant radiogenic transformed rodent cells (Borek *et al.*, 1987). The reproducible neoplastic transformation of the RHEK-1 human epithelial cell line by X-ray irradiation suggests that cellular oncogenes may be activated as part of the process. Our evidence further indicates that *ras* oncogenes, which have been commonly implicated in radiation-induced animal tumors (Guerrero *et al.*, 1984a, 1984b; Sawey *et al.*, 1987) and spontaneous human tumors (Weinberg, 1982) were not activated in the transformation. Thus, this system may be useful in effort to detect and characterize other cellular genes that can contribute to the neoplastic phenotype of human epithelial cells.

Isolation of Dominant Human Sequences from Radiation Transformed RHEK-1 Cells by a Tumorigenicity Assay

DNA-mediated gene transfer studies using rodent cells as recipients have demonstrated the presence of transformed genes in radiation-induced tumors and rodent cells transformed by radiation. As described above, we have been unsuccessful in isolating human sequences using the NIH/3T3-focus formation assay. Since the majority of tumor DNAs fail to induce transformed foci in the NIH/3T3 focus formation assay (Krontiris and Cooper, 1981; Perucho *et al.*, 1981; Pulciani *et al.*, 1982) probably due to this system having a bias for *ras* genes containing structural mutations, an alternative assay was required. Therefore, we have used, as an alternative, the NIH/3T3 DNA transfection-nude mouse tumorigenicity assay as previously described by Fasano *et al.* (1984). This approach has been shown to be more sensitive than the NIH/3T3 assay for detecting transforming genes (Ananthaswamy *et al.*, 1988; Blair *et al.*, 1982; Tainsky *et al.*, 1987). This system is a modification of the one described by Blair *et al.* (1982). It relies on the ability of transformed NIH/3T3 cells to form tumors in nude mice, but also incorporates the use of a co-transfection with a selectable marker to increase the sensitivity (Wigler *et al.*, 1979). More recently, Yuasa *et al.* (1990), using this tumorigenicity assay, have been able to isolate transforming genes from the cells of patients with familial adenomatous polyposis. The DNA from a highly tumorigenic soft agar clone-derived line (8 Gy clone 10) induced human Alu-positive tumors in nude mice by a tumorigenicity assay. These tumor DNAs were also Alu-positive in second

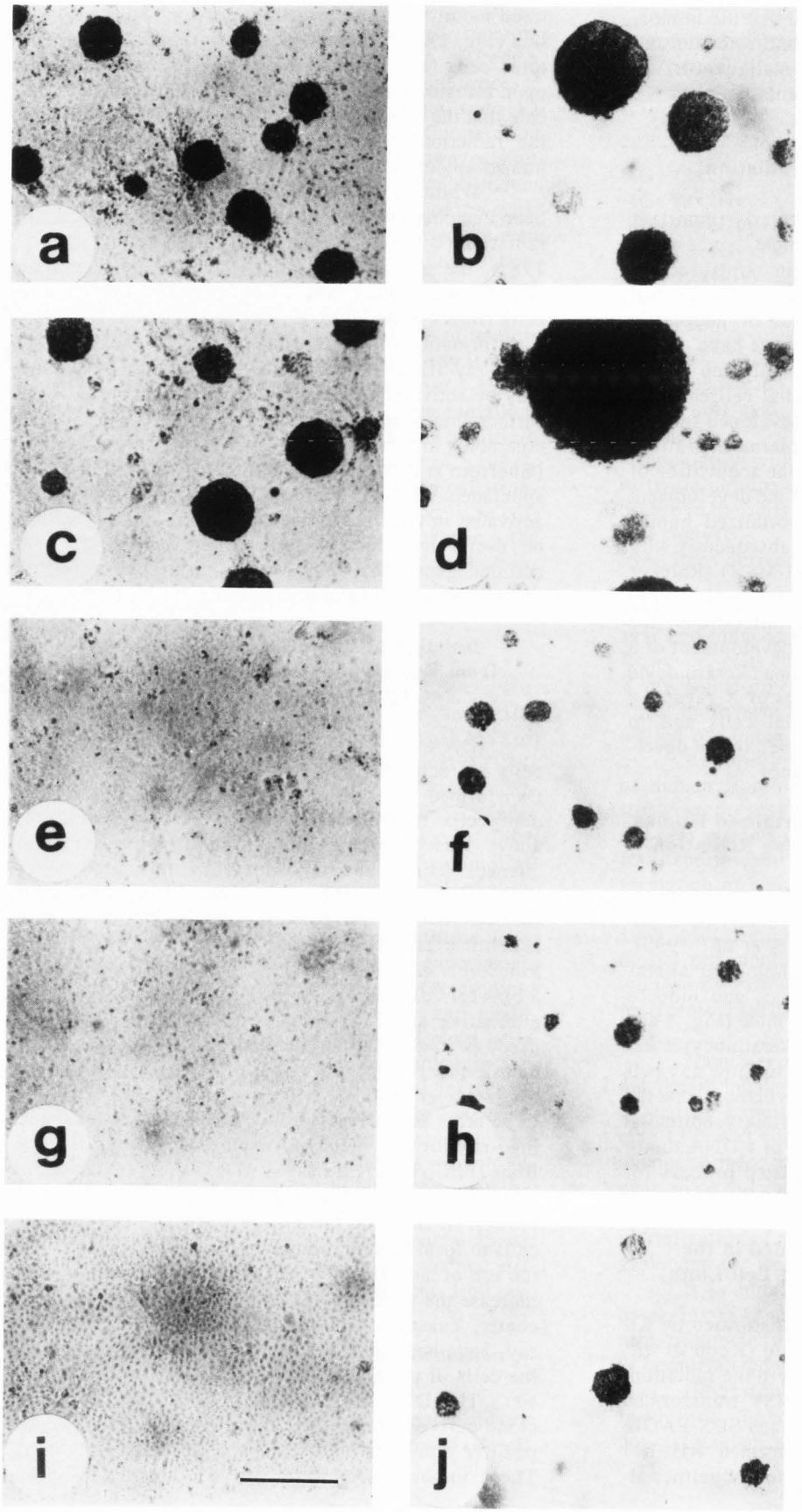


Figure 1. Morphology of human epidermal keratinocyte cells (RHEK-1) irradiated with X-rays twice, followed by a third subculture in nutrient medium: *a*, 4 Gy (2 Gy twice); *c*, 8 Gy (4 Gy twice); *e*, 12 Gy (6 Gy twice); *g*, 16 Gy (8 Gy twice); and *i*, unirradiated RHEK-1 cells. The colonies produced in soft agar by these cells: *b*, 4 Gy (2 Gy twice); *d*, 8 Gy (4 Gy twice); *f*, 12 Gy (6 Gy twice); *h*, 16 Gy (8 Gy twice); and *j*, unirradiated RHEK-1 cells. Bar = 500 μm (all micrographs are at the same magnification).

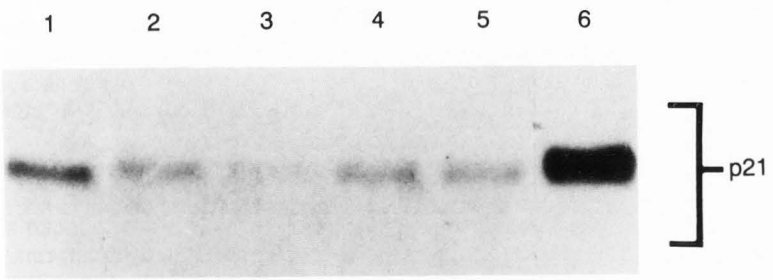
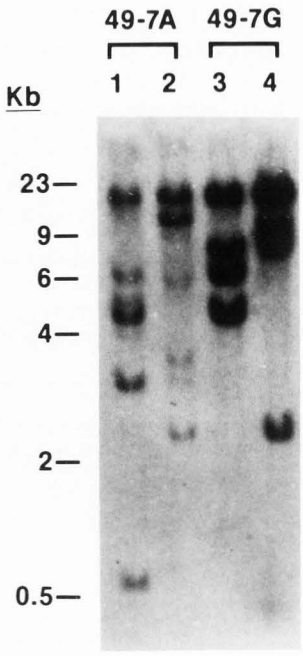


Figure 2 (above). Analysis of *ras* oncogene p21 product in RHEK-1 cells irradiated with X-rays. [^{35}S]Methionine-labeled cell extracts from unirradiated RHEK-1 cells (*Lane 1*), 4-Gy (2 Gy twice)-irradiated RHEK-1 cells (*Lane 2*), 8-Gy (4 Gy twice)-irradiated RHEK-1 cells (*Lane 3*), 12 Gy (6 Gy twice)-irradiated RHEK-1 cells (*Lane 4*), 16 Gy (8 Gy twice)-irradiated RHEK-1 cells (*Lane 5*), or Ki MSV-transformed RHEK-1 cells immunoprecipitated with anti-p21 monoclonal anti-body Y13-259 and analyzed by SDS-PAGE.



2° Nude Mouse Tumor DNA's digested with EcoRI (1 and 3) with BamHI (2 and 4)

Figure 3 (at right). Twenty micrograms of genomic DNA from secondary nude mouse tumor DNAs, 49-7A and 49-7G, were digested with restriction endonucleases EcoRI and BamHI both at 5 units/mg DNA; *Lanes 1 and 2*, 49-7A, digested with EcoRI and BamHI, respectively; *Lanes 3 and 4*, 49-7G digested with EcoRI and BamHI, respectively. Following digestion, the products were electrophoresed on a 1% agarose gel, blotted onto nylon, and probed with 32-labeled human Alu (Blur-8) probe.

round analysis of the tumorigenicity assay. Restriction enzyme analysis of these secondary nude mouse tumor DNAs with EcoRI yielded four strong Alu-positive bands with approximate molecular weights of 20, 8, 6 and 5 kb (Fig. 3). The DNA from these Alu-positive secondary nude mouse tumors were also screened for homology with probes for the *ras* and *myc* gene families. None of the Alu-positive bands were found to have homology with N$^-$, K$^-$ or H-*ras*. No homology was observed with probes for the *myc* family of genes (c-*myc*, N-*myc* or L-*myc*). Subsequent analysis has also eliminated the c-*raf* gene. Further characterization and cloning of these transforming sequences is in progress (Thraves *et al.*, 1991).

Evidence for the Multistep Nature of *In Vitro* Radiation-Induced Human Cell Carcinogenesis

In addition to the Ad12-SV40 immortalized human epidermal (RHEK-1) model already described, we and others have demonstrated the potential of other multistep models for human epithelial cell transformation.

1. Tumorigenic conversion of a normal epidermal (11367) line established by pSV$_3$-*neo* transfection was achieved by repeated irradiation (Yang *et al.*, 1991). Immortalized 11367 cells irradiated with 5 Gy X-ray exhibited morphological alterations and grew in soft agar but did not form tumors in nude mice. Another irradiation (2 Gy iron ions (600 MeV/u) led the cells to become tumorigenic. No large terminal deletion of chromosomes in transformed cells was observed, but fewer and a more broad range of chromosomes were observed in these cells.

2. Benzo(a)pyrene-immortalized human mammary epithelial (H 184B5) cells were successfully transformed from the state of immortalization to the stage of anchorage independent growth (Yang *et al.*, 1991). Human mammary epithelial cells (H 184B5) irradiated with 2.2 Gy ion particles (600 MeV/ui, LET = 200 keV/Um) and selected for growth varients in MEM + 10% calf serum vs MCDB-170 medium died in MEM medium and did not grow in soft agar. However, the H 185B5 cells irradiated twice grew in soft agar but did not form tumors in nude mice.

3. The effect of radiation on the human papilloma virus type 16 or 18 immortalized human bronchial epithelial lines (BEP 2D and BEP 3D) was examined (Willey *et al.*, 1991). Morphological alterations were observed in the 8 or 10 Gy (2X) low LET irradiated BEP 2D line and 8 Gy (3X) irradiated BEP 3D line. However, the formation of colonies in soft agar was observed in the 8 or 10 Gy (2X) irradiated BEP 2D line. New chromosomal alteration (aberration of chromosomes 9 and 11 p15) was noted in the 10 Gy (2X) irradiated cells. Similar karyological characteristics were observed in nonirradiated and 8 Gy (2X) irradiated cells.

These findings demonstrate that radiation-induced neoplastic transformation of human epithelial cells is a multistep process and a single exposure of radiation can cause only one step of transformation. Recently,

Tuynder *et al.* (1991) have reported that repeated X-irradiation of normal human keratinocytes resulted in a nontumorigenic keratinocyte line that was differentiation-defective. Neoplastic transformation of human epithelial cells by ionizing radiation, thus, requires multiple hits, as shown recently by Thraves *et al.* (1991) and Yang *et al.* (1991). This finding has an important implication for radiation risk assessment. It suggests that a single exposure to radiation is unlikely to cause a normal human cell to become tumorigenic, and that protracted irradiation can be much more hazardous than acute exposure. This finding also suggests that several genes may have to be altered by radiation before a normal human epithelial cell becomes tumorigenic.

Our current knowledge of the importance of genetics, viruses, and chemical carcinogens in the induction of cancer suggests that may if not all cells in the body may be carrying one or more hits by the time human being reaches adulthood. Thus, it can be speculated that radiation is a second or third step rather than a primary step when thinking of risk.

Conclusions

The carcinogenic effect of radiation was widely recognized shortly after the discovery of X-ray in 1895 (Brown, 1936). Since then, the question of how radiation can cause cancers in humans has been a major interest in radiation biology. As discussed in this brief review, the success of transforming human cells malignantly in culture now provides an opportunity to search for answers at the cellular and molecular levels. Radiation-induced neoplastic transformation in nontumorigenic human epithelial cells has been shown to be due to a dominant-acting transforming gene. This gene has been isolated and is in the process of being characterized and sequenced. Even though some progress has been qualitatively made in radiation-induced malignant transformation of human cell systems, the difficulties still exist in trying to quantify human cell transformation which is an important task to be accomplished through future research.

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Discussion with Reviewers

T.M. Seed: Would the authors please comment/speculate on the possible radiation-induced interaction between selected proto-oncogene activation (e.g., *ras* family oncogenes) and inactivation of tumor suppressor genes (e.g., Rb gene) in terms of the neoplastic transformation sequence, *in vitro*?

Authors: We can speculate that the possible radiation-induced interaction between selected proto-oncogene activation (e.g., *ras* genes) and inactivation of tumor suppressor genes (e.g., Rb or p53 gene) could occur during the process of malignant transformation.

J.C. Willey: When evaluating genes that might be in-

volved in radiation-induced transformation, it is worth considering what types of genes involved in proliferation control would be more likely to be altered by radiation. Because radiation is more likely than other mutagens to cause DNA strand breakage, it would be advantageous to evaluate cells that have been tumorigenically transformed following radiation for tumor suppressor genes. Methods commonly used to screen tumors for tumor suppressor gene inactivation are cytogenetic and RFLP analysis for deletion of chromosome regions that contain known or putative tumor suppressor genes. In contrast, activation of oncogenes, in particular the *ras* oncogene, usually results from a point mutation. This type of mutation is induced by radiation, but is less common than strand breakage. Would it be worthwhile to evaluate your radiation transformed cells for deletional inactivation of tumor suppressor genes?
Authors: We are currently investigating this aspect.

J.C. Willey: In our experience irradiation of human papillomavirus-immortalized human bronchial epithelial cells using the same protocol described by Thraves *et al.* (text references) did not result in malignant transformation, although we are now observing some tumors following irradiation with high LET radiation. One possibility is that there is inter-tissue variation in susceptibility to transformation following ionizing radiation and that human bronchial epithelial cells are more resistant than keratinocytes. Would you agree?
Authors: There are too few studies to conclude this at present. Differential susceptibility of human cells to viral transformation has been shown.

J.C. Willey: You stated (in **Introduction**) that four major steps appear to be involved in *in vitro* neoplastic transformation of human cells: development of morphological transformation, growth in semisolid medium, immortality, and tumorigenicity. Is there any evidence as to the order in which the first three steps occur in different systems? Must the sequence of events be the same in every tissue?
Authors: There is no evidence yet since there are too few published studies. We state here in general "Four major steps appear to be involved in *in vitro* neoplastic transformation of human cells".