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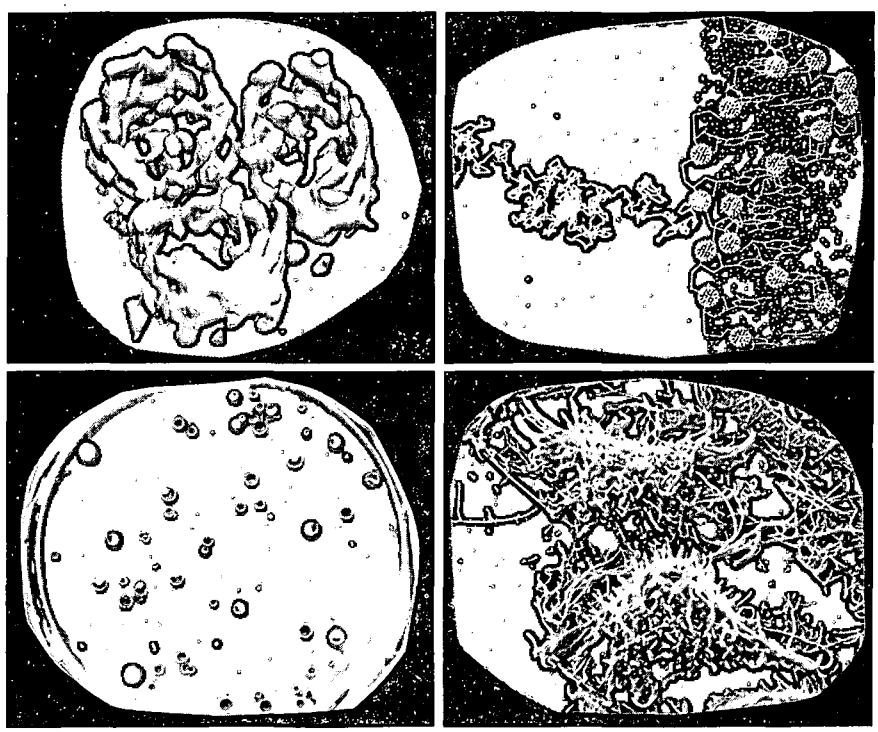

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CHROMOSOMAL CHANGES IN CULTURED HUMAN EPITHELIAL CELLS TRANSFORMED BY LOW- AND HIGH-LET RADIATION

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ABSTRACT

For a better assessment of radiation risk in space, an understanding of the responses of human cells, especially the epithelial cells, to low- and high-LET radiation is essential. In our laboratory, we have successfully developed techniques to study the neoplastic transformation of two human epithelial cell systems by ionizing radiation. These cell systems are human mammary epithelial cells (H184B5) and human epidermal keratinocytes (HEK). Both cell lines are immortal, anchorage dependent for growth, and nontumorigenic in athymic nude mice. Neoplastic transformation was achieved by irradiating cells successively. Our results showed that radiogenic cell transformation is a multistep process and that a single exposure of ionizing radiation can cause only one step of transformation. It requires, therefore, multihits to make human epithelial cells fully tumorigenic. Using a simple karyotyping method, we did chromosome analysis with cells cloned at various stages of transformation. We found no consistent large terminal deletion of chromosomes in radiation-induced transformants. Some changes of total number of chromosomes, however, were observed in the transformed cells. These transformants provide an unique opportunity for further genetic studies at a molecular level.

INTRODUCTION

Carcinogenic effect of radiation is of great concern for long-term manned spaceflight. Because of such concern, over the past several years, many investigators at various major laboratories have studied the neoplastic transformation of mammalian cells by ionizing radiation /1-9/. The cell systems used for these studies have been mainly rodent cells. Although it is generally recognized that radiation carcinogenesis studies with human epithelial cells are very much needed because of their direct relevance to radiation risk assessment, the difficulties in culturing human epithelial cells and in transforming human cells *in vitro* with radiation have severely impeded the progress.

In our laboratory, two established human epithelial cell lines are available for radiation cell transformation studies, and we have successfully transformed both human mammary epithelial cells and human epidermal keratinocytes with ionizing radiation and have performed some chromosome analysis with the transformants. Experimental results of radiation-induced transformation of these human epithelial cells and the data obtained from recent chromosome studies are given in this report.

METHODOLOGY

Cell Systems

Human mammary epithelial cells (H184B5) used for present studies were from primary cells treated with benzo(a)pyrene. They are immortal and nontumorigenic and require medium enriched with growth factors to grow /10/. The human epidermal keratinocytes (HEK) were immortalized by a transfection of pSV3-neo /11/. These HEK cells have a flat epithelial morphology, form monolayer with density inhibition, show no anchorage independent growth, and are nontumorigenic in athymic nude mice.

Irradiation of Cells

For neoplastic transformation studies, the irradiation was done with a 250 kVp Philips X-ray machine and heavy ions accelerated at BEVALAC in Lawrence Berkeley Laboratory. The dosimetry and exposure condition for X rays and heavy ions have been reported in detail /12/.

Cell Transformation Assay

The focus assay, similar to that for C3H10T1/2 cells, was used to study morphological transformation of human epidermal keratinocytes. The anchorage independent growth was determined by plating cells into 0.33% agar medium, and colonies containing more than 50 cells were counted as transformants. The tumorigenic test was done by injecting 10^6-10^7 cells in 0.2 ml serum free media subcutaneously on the back of nude mice. A result was considered as positive only when a nodule was formed at the site of injection and continued to grow into a size greater than 0.5 cm in diameter.

For transformation studies with human mammary epithelial cells *in vitro*, log-phase cells were irradiated and plated into dishes with enriched media (MCDB-170). At weekly interval, cells were subcultured and part of the cell population was seeded into MEM containing 10% new born calf serum to select for growth variants. The tests for anchorage independent growth and for the tumorigenic capacity of cells were the same as that used for human epidermal keratinocytes.

Chromosome Analysis

A standard karyotyping method was used for obtaining chromosome spread of human epithelial cells /13/. Briefly, exponential growing cells were treated with 1.0 μg/ml Colcemid for 2-3 hrs, followed by a hypotonic treatment of 0.075 M potassium chloride for 20 min, and then fixed with a solution of 3 parts methanol to 1 part glacial acid. After spreading the cells on a clean slide, the cells were air-dried. A Wright's stain was used to stain the chromosome.

RESULTS

Neoplastic Cell Transformation

We have successfully transformed human mammary epithelial cells from the stage of immortalization to the stage of anchorage independent growth. Figure 1 shows the sequence of stages of transformation. Immortalized cells (H184B5) were irradiated by 2.2 Gy of iron particles (600 MeV/u) and selected for growth variants in MEM supplemented with 10% serum. Growth variants were found at a frequency about 10^{-4}-10^{-3} per survivor, and were cloned. A growth rate comparison between H184B5 and a growth variant (H184B5-F5) is shown in Figure 2. In the MEM with H184B5 cells slowly died off. Although the growth variants can proliferate well in the medium with less growth factors, they cannot grow in soft agar media. A second exposure of radiation was found to be necessary to transform these growth variants into the next stage of progression, i.e., anchorage independent growth. The sequence of transformation stages appears to be definite. In spite of much effort, we have not been able to transform H184B5 cells, with a single exposure of radiation, into the stages of anchorage independent growth. Recently we have obtained transformants, which can grow in soft agar media, by irradiating the growth variants with 2.2 Gy iron beam (600 MeV/n). We are now in the process to test the tumorigenicity of cells that grew in soft agar media and received additional 2.2 Gy iron particles.

Primary	BaP →	cell line	radiation →	growth variant	radiation →	growing in soft agar	radiation →	tumorigenic
– defined medium with many growth factors		– immortal – defined medium with many growth factors		– growing in MEM with 10% serum		– anchorage independent growth		– 10^6-10^7 cells injection in athymic nude mice

Fig. 1. The sequence of transformation of human mammary epithelial cells. There are at least three steps involved in transforming H184B5 into tumorigenic by ionizing radiation.

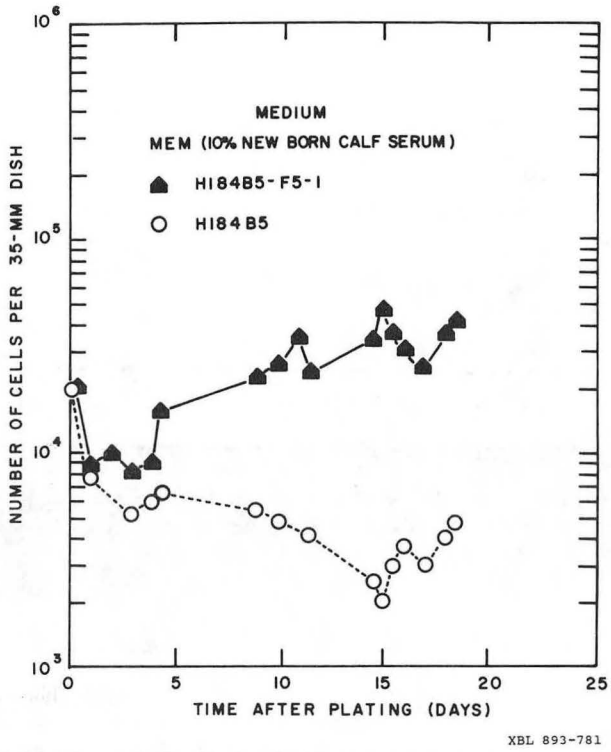

Fig. 2. A comparison of growth in MEM between H184B5 and a growth variant (H184B5-F5).

Human epidermal keratinocytes immortalized by pSV3-neo can grow in regular MEM supplemented with serum and 2 μg/ml hydrocortisone. Ionizing radiation can cause morphological transformation of these cells. In general, after 5-6 weeks incubation, foci can be found in the dishes of irradiated cells. There is an extensive piling up of cells in the focus, as shown in Figure 3. These transformed cells can grow in soft agar media, but do not form a tumor in athymic nude mice. When these transformed cells were given another exposure of radiation, they became tumorigenic in nude mice, as shown in Figure 4. The sequence of stages of transformation for human epidermal keratinocytes is shown in Figure 5.

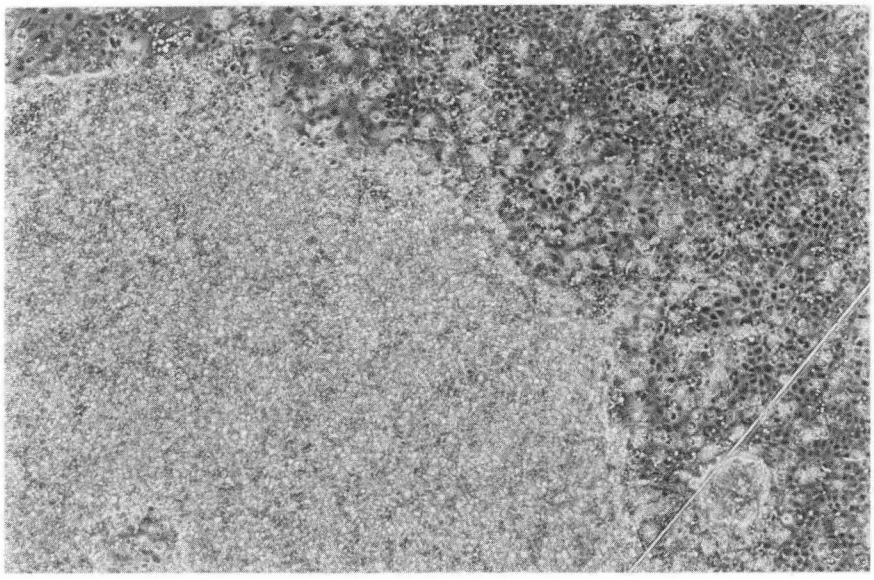

Fig. 3. A close-up picture of a transformed focus of human epidermal keratinocytes. There is extensive pilling up of cells in the focus.

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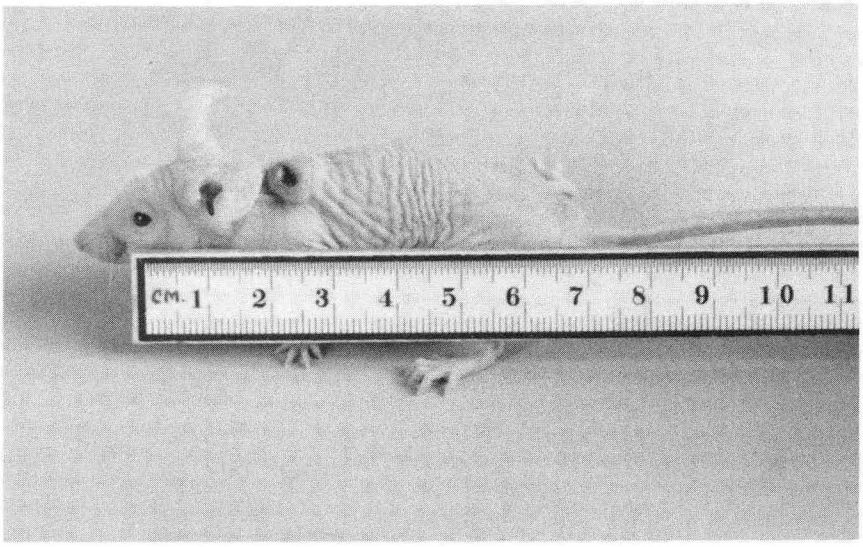

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Fig. 4. A tumor formed in an athymic nude mice at the site of injection of transformed cells which first received 5 Gy X rays and then 2 Gy iron ions (600 MeV/u).

Primary culture	pSV3-neo transfection →	cell line	radiation →	growing in soft agar	radiation →	tumorigenic
-- defined medium with many growth factors -- limited life span		-- immortal -- growing in 10%cs MEM with hydrocortisone -- density inhibition of growth		-- anchorage independent growth -- higher growth rate		-- 10^6-10^7 cells injection in athymic nude mice

Fig. 5. The sequence of radiation transformation of human epidermal keratinocytes.

Chromosome Analysis

Using a simple karyotyping technique, we studied the chromosome changes in radiation transformed cells. Figure 6 shows a metaphase spread of growth variants of human mammary epithelial cells. These chromosomes were stained by Wright's stain. There was a significant change of modal number of chromosomes in these transformed cells. Walen and Stampfer /14/ found that the modal number of chromosomes of H184B5 cells was 47 with a very narrow range (46-48). Our results showed a significant decrease of modal number of chromosomes in the growth variants and a wide range (42-49) of chromosome numbers (Table I).

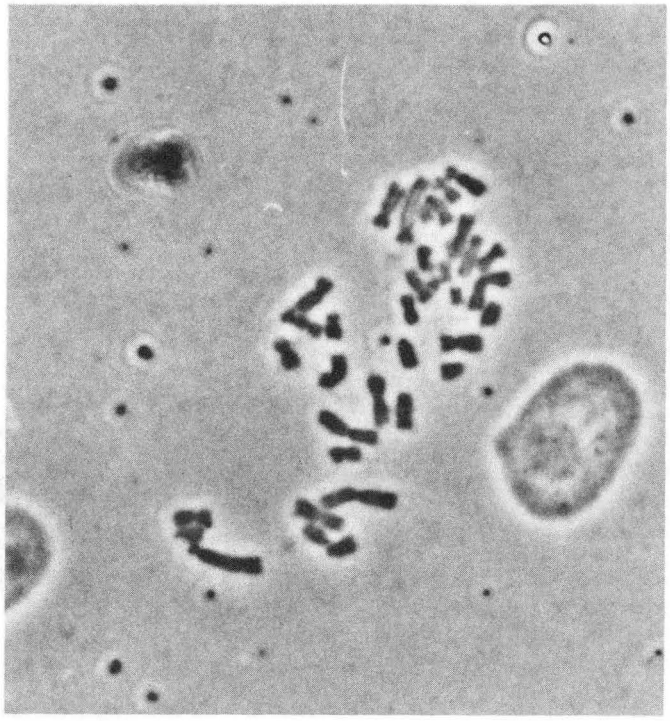

Fig. 6. A metaphase spread of chromosomes of human mammary epithelial cells (H184B5-F5).

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Table I. Karyotype Analysis of Human Mammary Epithelial Cells

Cell	Growth Properties	Number of Chromosomes	Chromosome Aberrations
H184E	normal; limited life span	48 (diploid)	none
H184B5	immortal; anchorage dependent; growth factors enriched medium	47 (46-48)	der (4) t (1:4) (q23:q35) der (8) t (1:8) (q24:q24) der (10) (p11) der (11) t (11:?) del (17) (p11) trisomy #20
H184B5-F5	immortal; anchorage dependent; MEM with 10% fetal calf serum	45 (42-49)	aneuploid

Human epidermal keratinocytes transformed by radiation to various stages of progression were obtained and analyzed for chromosome changes. The modal number of chromosomes of these cells was found to be 50 with a range from 49-50 (Figure 7). Similar analysis was done for cells transformed by X rays and/or heavy ions and experimental results are given in Table II. Compared with nontransformed cells (HEK), radiation transformed keratinocytes in general had less number of chromosomes and a broader range, as shown in Figure 8. There was no large terminal deletion of chromosomes in transformed cells when the karyotype of these cells was examined (Figure 9).

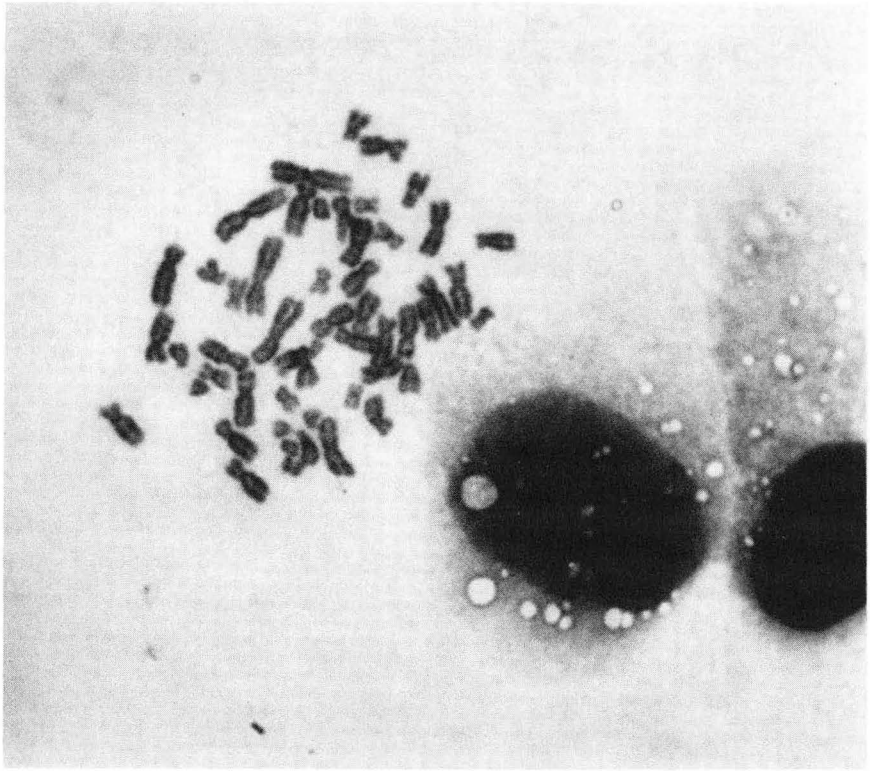

Fig. 7. A chromosome spread of human epidermal keratinocytes immortalized by pSV3-neo.

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Table II. Karyotype Analysis of Human Epidermal Keratinocytes

Cell	Growth Properties	Number of Chromosomes/Cell	% Cells
HEK	immortal; anchorage dependent growth	49	25.0
		50	75.0
HEK-A2	growing in soft agar	41	7.1
		42	7.1
		43	7.1
		44	7.1
		45	7.1
		46	14.3
		47	21.4
		48	21.4
		51	7.1
HEK-A3-Fe	tumorigenic in nude mice	48	20.0
		49	40.0
		50	40.0
HEK-Ar	growing in soft agar	47	16.7
		48	83.3

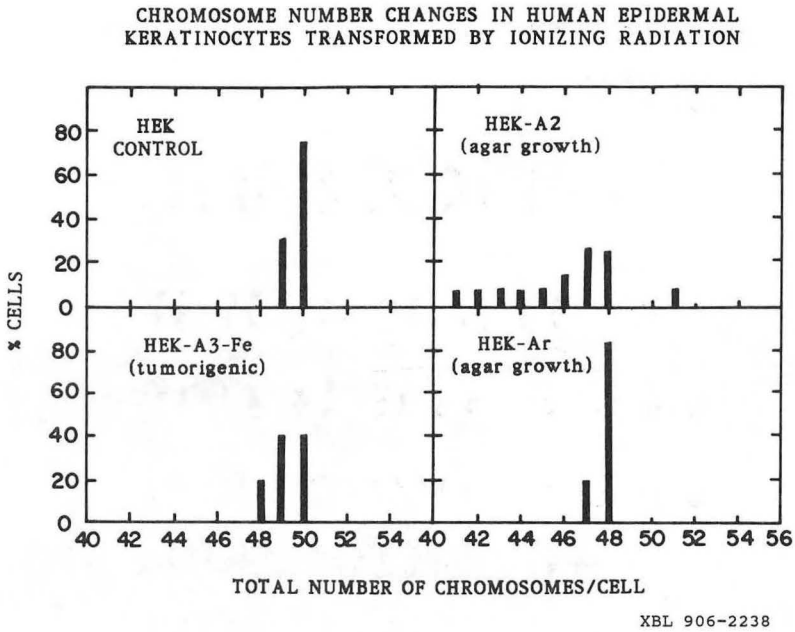


Fig. 8. Number of chromosomes in control and radiation-transformed human epidermal keratinocytes.

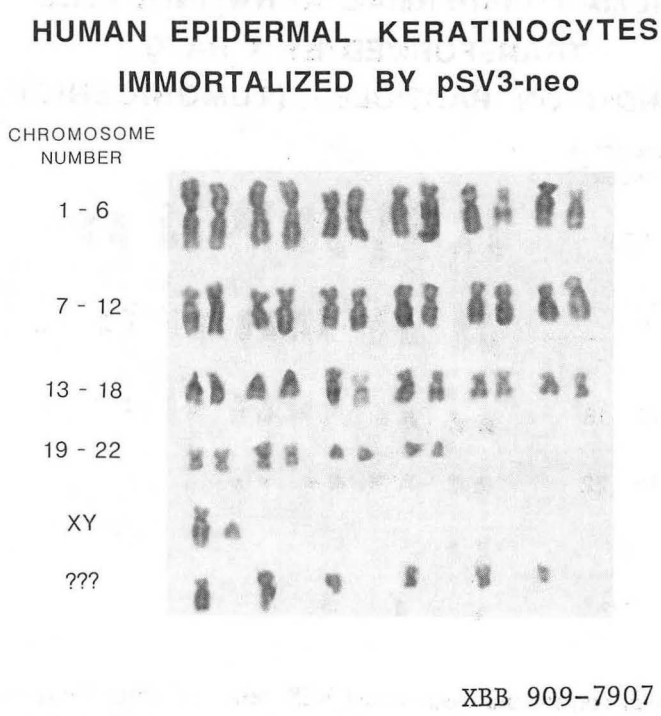
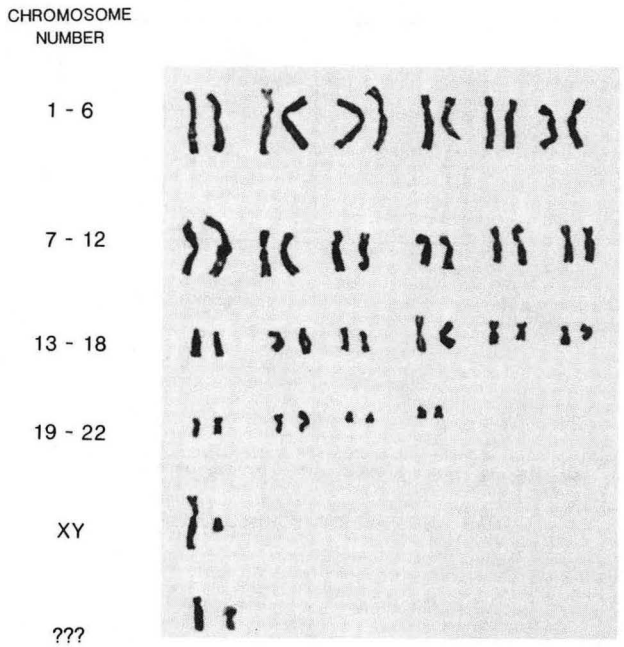


Fig. 9. Karyotyping of control and transformed HEK cells: (A) control;

HUMAN EPIDERMAL KERATINOCYTES
TRANSFORMED BY X RAYS
(GROWING IN SOFT AGAR, NON-TUMORIGENIC)



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HUMAN EPIDERMAL KERATINOCYTES
TRANSFORMED BY X RAYS
AND IRON PARTICLES (TUMORIGENIC)

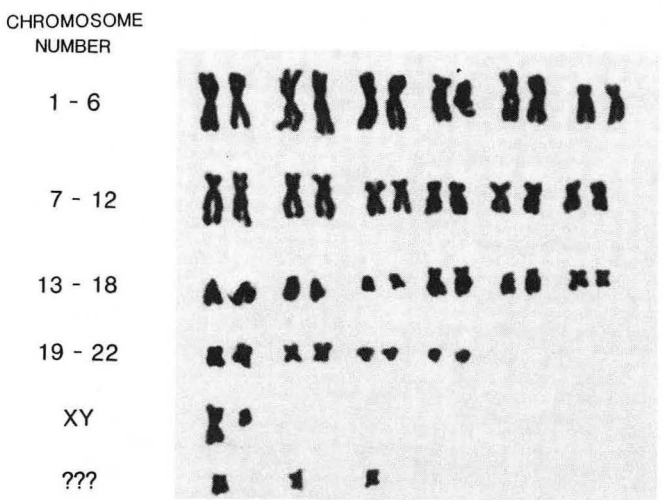

Fig. 9. Karyotyping of control and transformed HEK cells: (B) cells transformed by 5 Gy X rays, growing in soft agar media; (C) tumorigenic cells obtained by giving 2.2 Gy iron particles (600 MeV/u) to cells growing in soft agar media.

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DISCUSSION

When astronauts travel in deep space for a long period of time, they will unavoidably receive a significant amount of cosmic rays, which consist of low- and high-LET radiation. Among various biological damages which can be induced by cosmic rays, the carcinogenic effect is of most concern. One of the important approaches to determine the potential carcinogenic effect of heavy ions is *in vitro* cell transformation. There are many advantages of using cultured cells to study the radiation carcinogenic effect, including direct control of various factors which can suppress or enhance the carcinogenic processes /15/. In general, the data obtained from cell transformation studies have been consistent with that from animal work, indicating that a cellular change of growth behavior is essential for tumor development *in vivo*. In the past ten years, many studies have been done to determine the relative effectiveness of high-LET radiation in causing neoplastic transformation. Most of these studies used rodent cells, including primary cultures and cell lines. Although data from these experiments are valuable, it remains to be proved that human cells, especially epithelial cells which form most of the tumors in man, will respond to space radiation in a similar way. In view of this need, we have worked for several years to develop the methods which can yield information on the carcinogenic effect of radiation in human epithelial cells. Our experimental results indicate that both human mammary epithelial cells immortalized by benzo(a)pyrene and human epidermal keratinocytes immortalized by pSV3-neo can be useful systems for studying the neoplastic transformation by radiation.

Unlike many rodent cells, which can be transformed to tumorigenic stage by a single radiation dose, human epithelial cells can only be transformed one step after each exposure to radiation. There appears to be a definite sequence of steps in the multistage process of transformation. The sequence of these steps are growth variant, anchorage independent growth, and tumorigenic. Our experimental results strongly suggest that multi hits are necessary for neoplastic transformation of human epithelial cells by ionizing radiation. 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 tumorigenic and that protracted irradiation can be much more hazardous than acute exposure. In space, astronauts are exposed to radiation chronically. The health risk of radiation in space is, therefore, particularly important to know. However, if the total dose received during a spaceflight is small, e.g., one particle per cell nucleus, the chance that a normal human cell will be transformed to malignant stage will be very small if not zero. The effect of a small dose of space radiation, nevertheless, cannot be ignored, because in reality many of the cells in the body may have been initiated already before the flight by other physical or chemical carcinogens present in the environment. One further step change by radiation may be sufficient to make the cells tumorigenic. For a given radiation dose, thus, the risk may be quite different for different individuals.

Shortly after the discovery of X rays, the carcinogenic effect of radiation was noticed. Since then, the question how radiation causes cancer in man has been a major interest in radiobiology. With the success of transforming human epithelial cells *in vitro*, we now have an unprecedented opportunity to search for the answer at cell and molecular level. In our laboratory, we have just begun to study systematically the genetic changes in transformed human epithelial cells. It has been shown that ionizing radiation, especially high-LET heavy ions, can cause large deletions in DNA. For this very reason, we did simple chromosome preparations and expected to find some large terminal deletion. Contrary to our expectation, the results showed no consistent large terminal deletions in transformed cells. A decrease of total chromosome number, however, was obtained in the transformants. The significance of the changes of total chromosome number is unclear at present.

Because no banding of chromosome was done in these studies, it is impossible to tell if there are any small deletions and translocations in the chromosomes. Further studies with these transformed cells should be done with chromosome banding technique and with molecular analysis to uncover the genetic changes important for radiation transformation.

ACKNOWLEDGMENTS

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