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Neoplastic transformation of human diploid fibroblast cells by chemical carcinogens

(morphological alteration/growth in soft agar/transplantability in *nude* mice/4-nitroquinoline 1-oxide)

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ABSTRACT **Cultured fibroblast cells derived from a skin biopsy sample taken from normal human adult were exposed to a potent carcinogen, 4-nitroquinoline 1-oxide. Alterations of cell growth pattern such as higher density and piling up of cells were noticed in some fractions of cultures that were successively subcultured after nitroquinoline oxide treatment. Morphologically altered cells retained this growth pattern and became established lines of transformed cells without showing the limited life-span characteristic of normal cells in culture. The transformed cells showed a higher saturation density and the ability to grow in soft agar, properties that are usually correlated with neoplastic transformation of cells in culture. Selection of preexisting transformed human cells as a mechanism of this observed transformation seemed unlikely because clones of these normal cells could also be used to assess the transforming effect of nitroquinoline oxide. Preliminary results suggest that numerous cell divisions were required for the development of the transformation after nitroquinoline oxide treatment of these human cells.**

When the transformed cell lines were injected subcutaneously into *nude* (athymic) mice, solid tumors were produced at the site of inoculation.

Treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine also induced cell transformation, in a manner similar to treatment with nitroquinoline oxide. However, transformation was not induced with (*i*) 4-aminoquinoline 1-oxide (a noncarcinogenic derivative of 4-nitroquinoline 1-oxide), (*ii*) 3-methylcholanthrene (a carcinogen that cannot be metabolically activated by the target cells employed), or (*iii*) the solvent dimethyl sulfoxide.

The human risk from environmental carcinogens has been assessed mainly by epidemiological methods and by carcinogenesis experiments in animals. In recent years rapid assay systems have been developed using various species, including prokaryotes, and various test conditions. However, considering the evolutionary aspects of biological functions such as repair activity, endogenous virus expression, metabolic capacity, etc., it is likely that there may be some discrepancy between the results obtained with animals or prokaryotes and the potential carcinogenicity of environmental substances for humans. To resolve the problem of extrapolation of experimental results into human risk, it is of great importance to understand the relationship of the response to chemical carcinogens among different species and assay systems. It would be very useful to develop a system for the transformation of human diploid cells by chemical substances in order to establish a better method for the assessment of human risk from environmental carcinogens as well as for understanding the mechanisms of human carcinogenesis.

The use of cultured human diploid cells has many advantages for genetic and biochemical studies. The most important of these advantages is the stability of the ploidy and the availability of well-characterized cell mutants, i.e., the cultured cells derived from genetic disease patients. These features are very useful for the study of the mechanism of cellular carcinogenesis.

Many attempts have been made to transform human diploid cells by chemical carcinogens. Although three cases of chemical transformation of human cells have been reported, two of them used the cell strains derived from "tumor tissues" (1–3), and the third study lacks evidence of malignancy of the cells treated with chemicals (4). There have been no reports of the successful *in vitro* neoplastic transformation of normal human diploid cells by chemical carcinogens. This paper communicates the success of the chemical transformation of cultured human diploid cells derived from the skin biopsy of a normal adult.

MATERIALS AND METHODS

Chemicals. 4-Nitroquinoline 1-oxide (4NQO) and 4-aminoquinoline 1-oxide (4AQO) were obtained from the Daiichi Pure Chemical Co. (Tokyo), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) from the City Chemical Co. (New York), and 3-methylcholanthrene (MCA) from the Eastman Kodak Co. (Rochester, NY). All chemicals were dissolved in dimethyl sulfoxide (Me_2SO) (spectranalyzed grade, Fisher Scientific Co., Pittsburg, PA) at the appropriate concentrations and then added to culture medium in the dark. The chemical solution was prepared immediately before use. The final concentration of Me_2SO in culture medium was 0.2–1.0%.

Cell Cultures. A human fibroblast cell strain, termed KD, was used as the indicator cell. This strain was initiated by Day (5) from a skin biopsy sample taken from the lip of an adult female who has not shown evidence of any genetic diseases. The primary culture was made by plating the cells dissociated by stirring the biopsy material in trypsin/collagenase solution. The grown cells were subcultured at 1:2 dilution and stored in a liquid nitrogen freezer (−100°) until use. For transformation experiments, the cells were used at the 6th to 34th passage after isolation. Cells were cultured in 100-mm plastic dishes [Falcon Plastics (Oxnard, CA)] containing 10 ml of culture medium in a humidified CO_2 incubator at 37°. The regular culture medium consisted of Eagle's minimum essential medium [Grand Island Biological Co. (Grand Island, NY)] supplemented with 10% fetal calf serum [Flow Laboratories (Rockville, MD), lot 4055752]. The serum used was selected by prescreening the serum samples from various sources for cloning efficiency and the effect on the morphology of cultured KD cells. The medium used for transformation experiment (Tr-medium) was made of the regular culture medium plus 0.4–0.5% human serum

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Abbreviations: 4NQO, 4-nitroquinoline 1-oxide; 4AQO, 4-aminoquinoline 1-oxide; MCA, 3-methylcholanthrene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; Me_2SO, dimethyl sulfoxide.

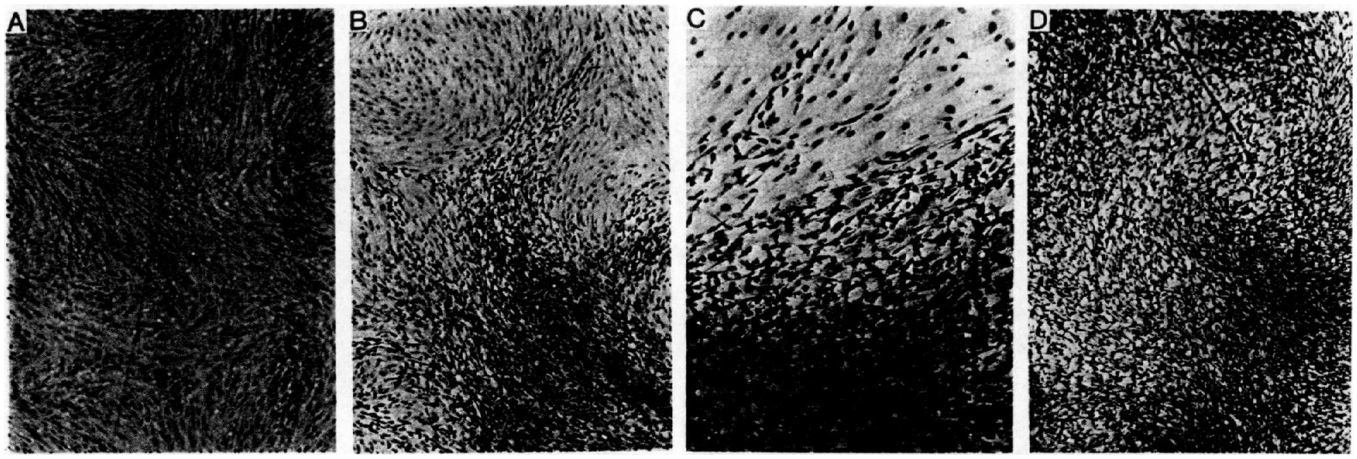

FIG. 1. Photomicrographs after fixation and Giemsa staining of (*A*) untreated KD cells (×65); (*B*) focus area of the morphologically altered cells in a culture treated with 4NQO (0.1 μg/ml) (×65); (*C*) a higher magnification (×165) of *B*; and (*D*) a transformed line, 4NQ T-3 (×165).

(Flow Laboratories, lot 4030063) and 2 mM L-glutamine (Grand Island Biological Co.). The culture medium was changed two or three times a week. The subcultivation was made whenever the cultures became confluent (approximately 3×10^6 cells per plate at confluency), by adding 0.25% trypsin solution (Grand Island Biological Co.) to the culture for 3–5 min, removing the trypsin solution, and letting the culture stand at room temperature until the cells showed some change in morphology (a sign of digestion). Cells were then suspended in fresh culture medium and dispensed into new dishes as quickly as possible. The dilution ratio of subculture was 1:2 before chemical treatment and 1:4 after the treatment. Plating efficiency was determined with Giemsa-stained dishes 10–14 days after plating by scoring the colonies that were composed of more than 40 cells.

Chemical Treatment. The cells were treated in suspension with 4NQO, 4AQO, or MNNG for 30 min, washed, suspended in Tr-medium, and then plated at 3×10^6 cells per plate for the transformation assays and at 1×10^2 and 5×10^2 cells per plate for plating efficiency determinations. When MCA was used as the carcinogen, it was added to culture medium 24 hr after the cells were plated. Three days later, the culture medium containing MCA was replaced by fresh MCA-free Tr-medium. The regular medium was used during exposure of cells to chemicals.

Biologic Properties of Cells. Saturation density was determined after inoculating 5×10^4 cells into 60-mm plastic dishes containing 5 ml of regular culture medium. The medium was changed every 3 days, and the number of cells was counted daily using a hemacytometer. The cell saturation density was expressed as the cell number per cm^2 when counts on 3 successive days did not change significantly. Growth of the suspended cells in soft agar was examined by the procedure described previously (6).

To examine the transplantability, the cells were suspended in 0.2 ml of serum-free Eagle's minimum essential medium and injected subcutaneously into 5- to 16-week-old female *nude* (athymic) mice, which were maintained under conditions of strict sanitation and segregation from other strains of rodents. Groups of 10 mice with the same distribution of ages in each group were designated. Mice were checked weekly for the development of tumors. Biopsy samples were removed surgically from half of the tumor-bearing mice in each experimental group. Each biopsy sample was divided into two portions, one portion being fixed for histological examination and the other portion being prepared for subsequent growth *in vitro*. Six months after injection, all surviving mice were sacrificed and autopsies were performed.

RESULTS

Morphological Transformation. The human diploid cell strain KD showed the typical morphology characteristic of fibroblasts in mass culture (Fig. 1*A*) and did not produce any colonies of epithelial morphology when plated so sparsely that it formed separate colonies. Thus, the major population of the KD cell stock seemed to be of fibroblastic morphology, although the original skin biopsy contained epidermal tissue as well.

Seven to 14 weeks or 5 to 10 passages after the treatment with 4NQO, morphologically altered cells were found in small areas of a very small number of dishes (Table 1). The major morphological alteration was the localized piling-up of cells into multi-cell layer foci (Fig. 2). Mitotic figures were observed in these foci. The shape of the focus was dependent on the pattern of the surrounding unaltered cell. The edge of the focus followed the direction of the surrounding cells (Fig. 1*B*). Disorganization and criss-cross pattern of cell arrangement, which is the typical pattern of most transformed cells in culture, was not distinctive, although some disorder of cell arrangement was recognized by careful observation (Fig. 1*C*). When the cells

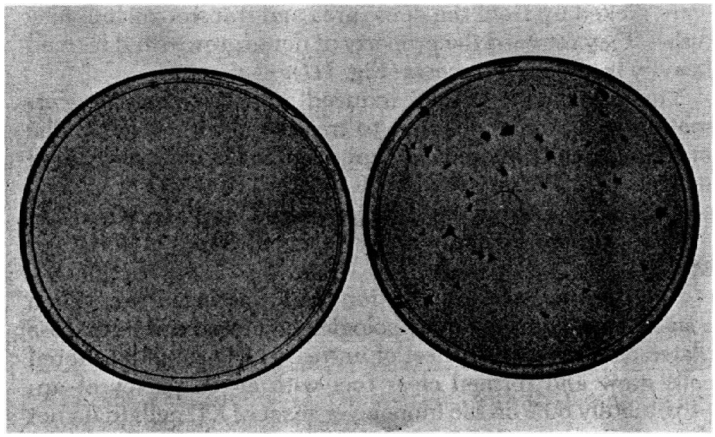

FIG. 2. Photograph after fixation and Giemsa staining of dishes as an example of the focus formation by the morphologically altered cells. They were derived from the cultures that were treated for 30 min with 0.3% Me_2SO (*Left*) and 4NQO at 0.1 μg/ml (*Right*), then subcultured 11 times at 1:4 dilution ratio whenever they became confluent, and maintained for 24 days without further subculturing.

Table 1. Morphological transformation of human fibroblast cultures by 4-nitroquinoline 1-oxide and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine*

Experiment	Treatment: Passage level of culture	Treatment: Compound	Treatment: Concentration, μg/ml	Relative plating efficiency,† %	Time of transformation,‡ days	Dishes showing transformation§
1	6	Me_2SO		100	—	0/10
		4-NQO	0.02	90	—	0/10
			0.1	32	76	1/10
			0.3	2	—	0/10
		4AQO	0.5	27	—	0/10
		MCA	1.0	93	—	0/10
			10.0	80	—	0/10
2	10	Me_2SO		100	—	0/10
		4NQO	0.02	88	—	0/7
			0.1	25	50, 60, 97	3/10
			0.3	1	82	1/9
		4AQO	0.1	94	—	0/9
			0.5	38	—	0/10
		MCA	10.0	89	—	0/10
3	12	Me_2SO		100	—	0/10
		MNNG	0.02	101	—	0/10
			0.2	28	73, 88	2/10
4	13	Me_2SO		100	—	0/10
		4NQO	0.1	39	—	0/10
5¶	33	Me_2SO		100	—	0/10
		4NQO	0.1	30	50, 50, 67	3/10
6¶	34	Me_2SO		100	—	0/10
		4NQO	0.1	22	63, 81	2/10
7¶	34	Me_2SO		100	—	0/10
		4NQO	0.1	35	61, 76	2/10

* The cultures of growing KD cells were treated with chemicals as described in *Materials and Methods*. The dishes for assay of plating efficiency were fixed 10–14 days after chemical treatment. The cultures for assay of transformation were subcultured at 1:4 whenever they became confluent. One of four subcultured dishes was used for the subsequent subculturing.

† Actual plating efficiencies of cells treated with Me_2SO alone were 92, 100, 83, 80, 70, 61, 54, and 64% in experiments 1–7, respectively.

‡ Days after treatment when transformation was first noticed.

§ Number of dishes showing transformation after subculture, per number of dishes treated.

¶ Clo- d KD cells were used in experiments 5, 6, and 7.

were picked up from the dense area and transferred into new dishes, they retained the property of dense growth and formed heavily multilayered sheets (Fig. 1*D*).

Treatment with MNNG induced morphological transformation in a similar manner to treatment with 4NQO. The morphologically altered cells appeared in the cultures treated with about a 30% survival dose (0.1 μg/ml) of 4NQO or MNNG and did not appear in the cultures treated with Me_2SO, MCA, 4AQO, or those treated with low concentrations of 4NQO or MNNG.

Requirement of Cell Division for Transformation. When a small number of the morphologically altered cell lines were plated on a monolayer sheet of untreated KD cells, the altered cells grew and formed clear foci with an efficiency of approximately 80% on the monolayer sheet of KD cells (data not shown). Because 4NQO- or MNNG-induced transformation appeared only after extensive subcultivation, this finding suggests the possibility that a large number of cell divisions is necessary for the development of the transformation of human diploid cells. To test this possibility and confirm the chemically induced transformation, experiments were done by using cloned KC cells. Several clones were isolated from the original KD cell strain. All the clones isolated showed a fibroblastic morphology. Each clone was treated with 4NQO at 0.1 μg/ml and continuously subcultured (see legend to Fig. 3). Figs. 3 and 4 show two representative results. Regardless of the duration of time that the cultures were maintained in the confluent state, the morphologically altered cells were found only after many repeated subcultivations. The average number of cell generations required for the development of morphological alteration was calculated to be at least 13. No morphological alteration was observed in the control cultures in which the same clone was treated with Me_2SO only and subcultured in the same manner as the treated cultures. This supports the idea that morphological alteration was induced by treatment with 4NQO in the human diploid fibroblast. Susceptibility of different clones argues against selection as being a mechanism for this transformation.

There was some variation in the time of the first observation of morphological alteration between cultures of separate experiments in which different clones of KD cells were used as well as between cultures of the same series of experiments. It

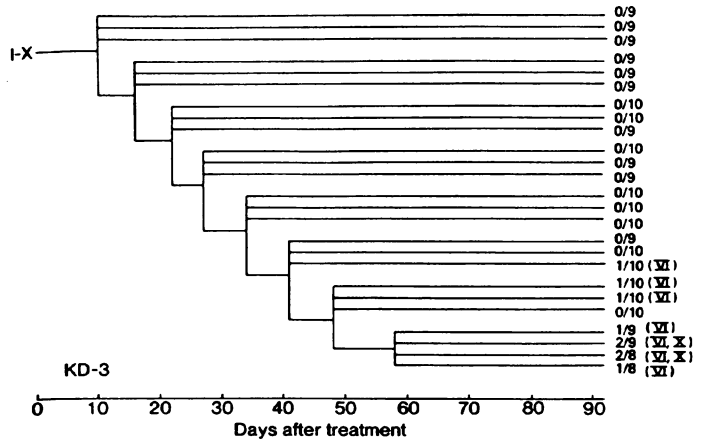

FIG. 3. History of the passage levels and the appearance of the morphologically altered cells in the 4NQO-treated human diploid cells, which corresponds to a part of experiment 6 in Table 1. Ten cultures (I–X) of a KD clone, KD-3, were treated with 4NQO (0.1 μg/ml) for 30 min and then subcultured at confluency at a dilution ratio of 1:4. From the second subcultivation, only one dish out of four was used for the subsequent subcultivation, while the other three dishes were maintained without further subculturing. All dishes were fixed after 35–82 days of maintenance without subculturing, stained with Giemsa, and examined for morphological changes. The number at the end of line indicates the number of dishes in which the morphological alteration was observed per the number of dishes scored. Cases in which the number of dishes scored was less than 10 indicate the accidental loss of dishes throughout the experiment. The number in parentheses indicates the initial dish number from which the culture containing the morphologically altered cells was derived. Only those cultures (two out of ten) that are identified in parentheses became morphologically transformed during the experiment.

is uncertain whether there were significant differences in susceptibility to alteration among the clones of KC cells.

Properties of the Transformed Cells. Saturation density and the ability to grow in soft agar were examined in the several lines of morphologically altered cells of which each line was derived from each focus area of chemically treated cultures. These cells were compared with the untreated and untransformed KD cells (Table 2). Saturation density was increased in all the morphologically altered cells. The ability to grow in soft agar was also increased in the morphologically altered cells, although the increase was slight in the lines derived from cloned KD cells. The increase in the saturation density was not quantitatively parallel to the increase in the ability to grow in soft agar.

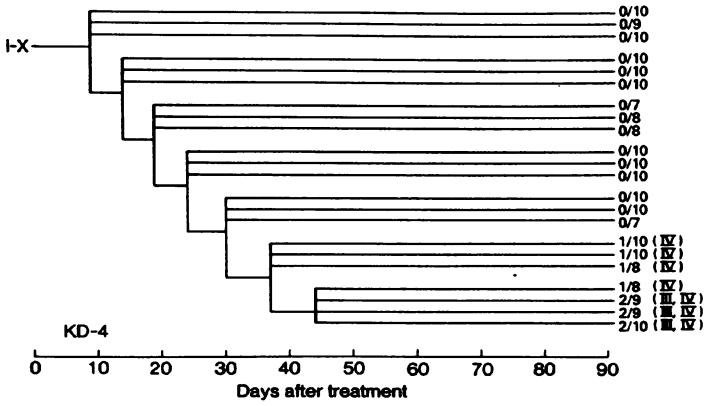

FIG. 4. History of the passage levels and the appearance of the morphologically altered cells in the 4NQO-treated human diploid cells, which corresponds to experiment 7 in Table 1. Ten cultures of a KD clone, KD-4, were treated in the same manner as clone KD-3 in Fig. 3.

Table 2. Saturation density and colony-forming ability in soft agar

Cells	Days in culture after treatment with chemical	Saturation density, (cells/cm^2) × 10^{-4}	Colony-forming efficiency in soft agar
Untreated	—	3.2	$<5 \times 10^{-7}$
Solvent-treated	4	3.0	$<1 \times 10^{-6}$
	95	2.6	$<1 \times 10^{-6}$
4NQ-NT*	95	2.7	$<1 \times 10^{-6}$
4NQ-T-1†	95	7.2	9×10^{-4}
4NQ-T-2†	95	5.4	1×10^{-3}
NG-T-3†	105	9.4	2×10^{-3}
4NQ-RT-1‡	97	12.7	2×10^{-5}
4NQ-RT-2‡	97	11.7	1×10^{-5}
4NQ-RT-3‡	97	10.3	1×10^{-6}

* The cells of the cultures that were treated with 4NQO and did not show any morphological change.
† The cell lines isolated from each focus area in the subcultures at 73 days (4NQO-T-1 and 2) or 90 days (NG-T-3) after the treatment with 4NQO (4NQ-T-1 and 2) or MNNG (NG-T-3).
‡ The cell lines isolated from each focus area in the subcultures at 83 days after the treatment of KD-1 clone with 4NQO.

Most of the morphologically altered cells isolated from foci grew exponentially and have not shown any sign of a limit to their life span (up to 2 years of continuous cultivation).

Two concentrations each of seven different morphologically altered and unaltered human cells were injected into 14 groups of 10 *nude* mice each. All the morphologically altered cells grew as tumors at the site of injection (Table 3). The latent period for tumor appearance varied from 2 to 21 weeks (average 6 weeks), depending on the cell lines and the number of cells inoculated. The tumors tended to grow progressively.

Table 3. Transplantability of the cultured cells into *nude* mice

Cells	Days in culture after chemical treatment	Cells injected ×10^{-6}	Mice with tumors/mice injected
Untreated	—	0.2	0/20
		2	0/20
4NQ-NT*	107	0.2	0/10
		2	0/10
4NQ-T-1†	107	0.2	3/10
		2	9/10
4NQ-T-2†	107	0.2	0/10
		2	7/10
NG-T-3†	125	0.2	6/10
		2	10/10
4NQ-RT-2‡	120	0.2	0/10
		2	4/10
4NQ-RT-3‡	120	0.2	0/10
		2	6/10
4NQ-RT-11‡	150	0.2	2/10
		2	3/9
4NQ-RT-15‡	150	0.2	1/8
		2	1/9

* The cells of the cultures that were treated with 4NQO and did not show any morphological change.
† The cell lines isolated from each focus area in the subcultures at 73 days (4NQ-T-1 and 2) or 90 days (NG-T-3) after the treatment with 4NQO (4NQ-T-1 and 2) or MNNG (NG-T-3).
‡ The cell lines isolated from each focus area in the subcultures at 83 days (4NQ-RT-2 and 3) or 101 days (4NQ-RT-11 and 15) after the treatment of KD-1 clone (4NQ-RT-2 and 3) or KD-3 clone (4NQ-RT-11 and 15) with 4NQO.

Spontaneous regression of tumor and metastasis (spread to regional lymph nodes) were observed only in 2 cases out of 52 positive cases. Histological examination of the tumors formed *in vivo* revealed that the tumors were generally sarcomas, with variations from fibrosarcoma to undifferentiated sarcomas. No tumors were produced by injection of morphologically unaltered cells.

DISCUSSION

The results described above indicate that diploid human cells can be malignantly transformed by chemical carcinogens in culture. The difficulty of transforming human diploid cells has been long argued. Some of the explanations for this difficulty were based on the problems dealing with the methods and the conditions of the experiments such as the following. (*i*) Usual conditions for the culturing of mammalian cells are not suitable for the culturing of human diploid cells. For example, a low plating efficiency of human cells is generally observed. (*ii*) Human diploid cells, especially fibroblasts, lack the enzyme activity (or have diminished levels) needed to convert many chemicals into active forms. (*iii*) The transformed phenotype of human cells may be different from the transformed phenotypes of experimental animals.

We found that our culture conditions were quite suitable for routinely producing a cloning efficiency of 80–95% with most of the human diploid fibroblast strains available and that the majority of these strains could grow over 60 cell generations. The addition of a small amount of human serum of a selected lot into the culture medium enhanced the growth of human diploid cells as well as the transformed cells.

The second problem has been circumvented by using the chemical carcinogens that are activated in the cells of all species and tissues. Failure to obtain transformation by MCA in this report may be due to the low activity of KD cells in metabolizing polycyclic hydrocarbons, as shown by very low levels of induced aryl hydrocarbon hydroxylase activity (unpublished data).

The third problem does not seem to be a major one, because it has been previously reported that the cells in culture obtained from human tumor tissue show similar biological properties to the transformed cells of experimental animals (7, 8). Our results also indicate that cells that have the properties characteristic of most of the malignantly transformed animal cells could be obtained by treating normal human cells with carcinogens in culture, although the possible existence of the neoplastic cells that express alternative transformed phenotypes in culture cannot be excluded. It has been shown that human tumor transplants or cells freshly cultured from human malignant tumors give rise to tumors in *nude* mice (9–11). Tumorigenicity in *nude* mice has also been reported to be correlated with cell growth in semisolid medium (7). *Nude* mice are therefore considered to be one of the best available systems for testing the malignancy of human cells. All the morphologically altered cell lines produced tumors in *nude* mice in our experiments. Aneuploidy was found in the transformed cells, and it was confirmed that the tumor cells were of human origin by karyotype analysis of the initial cells, the transformed cells, and the cultured *in vivo*-derived tumors (T. Kakunaga, unpublished data).

The transformation frequency is calculated to be approximately $1–3.3 \times 10^{-7}$ per surviving cell and $0.33–1 \times 10^{-7}$ per treated cell, although the requirement of subculturing for the development of transformation makes this calculation somewhat ambiguous. The transformation frequency obtained with this human cell system is of quite low magnitude compared to the frequencies obtained in many transformation systems using rodent cells, which range from 6×10^{-1} to 1×10^{-4} per surviving cell (6, 12–15).

It has been reported that the approximate number of cell generations required for the development of transformation is 4 to 5 in 4NQO (16) and MCA (17) transformation of the A31-714 mouse cell line, 2 in x-ray transformation of hamster embryonic cells (18), and 11 in x-ray transformation of the 10T 1/2 mouse cell line (19). The average number of cell generations required for transformation was at least 13 in this experiment. This is based on the assumption that all the surviving cells divided equally. There is a possibility that the stability of ploidy of human cells in culture contributes to the low frequency of transformation and the requirement for a large number of cell generations for the development of transformation in the human cells. It is noteworthy that heteroploid conversion has been recently reported in MCA-treated human skin cells in culture (20).

Although there remain many points to be clarified, modified, and improved, the results in this paper seem to provide a step forward in establishing a system for studying neoplastic transformation of human cells by carcinogens and for assessment of human risk from environmental carcinogens.

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