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ABSTRACT

We have developed a system for ultraviolet light (UV) transformation of human embryonic cells to anchorage-independent growth. The procedure involves multiple UV irradiations, post irradiation growth, and plating in soft agar. Transformants are obtained at frequencies from 1 to 80 per 10⁶ cells at UV exposures to 25 J/sq m. The resulting transformants can be subcultured on solid surfaces. The cells show crisscrossing and piling up; they reach 2- to 5-fold higher saturation densities than the parental cells. Some subcultures show increased plating efficiency in soft agar and increased life span. The susceptibility of the UV transformation process to apparent photoenzymatic reversal implies that pyrimidine dimers play a role in its induction.

INTRODUCTION

UV induces skin cancer in experimental animals and in humans (3). However, it has been difficult to develop an in vitro model for human UV oncogenesis, e.g., UV transformation of human cells, due to the apparent refractory nature of human cells to in vitro transformation with agents other than viruses. [We here use "in vitro transformation" in accord with the nomenclature of the terminology committee of the Tissue Culture Association (13).] Recently, however, transformation of human cells by chemicals and by 60Co γ-irradiation has been reported by Kakunaga (6) and by Namba et al. (11), and preliminary reports of UV transformation of human cells have been presented (9, 18). Chan and Little (1) have also produced transformation of the mouse line C3H/10T½ by UV.

We thus sought to develop a system for UV transformation of human cells to anchorage-independent growth; our system involves multiple UV irradiations, cell growth before plating, and selection by growth in soft agar. The frequency of anchorage-independent growth increases as a function of increasing UV exposure. The frequency at a given UV exposure depends on the generation number of the cells.

An important tool in UV photobiology has been the "photoactivation test" (19). Photoreactivating enzyme carries out the specific, light-dependent monomerization of cyclobutyl pyrimidine dimers in DNA (14, 15). This specificity of the enzyme for dimers allows its use as an analytical tool. If UV-induced biological damage is reversible in a true photoenzymatic reaction, then pyrimidine dimers are important in production of that damage. Although UV transformation of rodent cells was reported in 1976 (1), the low level of photoreactivating enzyme in those cells, at best 10% that of normal human cells (23), and the dependence of photoreactivating enzyme levels on culture conditions (10, 21) have impeded its use in evaluating the role of dimers in UV transformation. We have used photoreactivation-competent cells grown under conditions favorable for photoreactivating enzyme production; in these cells, exposure to visible light immediately after UV greatly reduced the transformation frequency. These results imply that pyrimidine dimers are important in induction of UV transformation in human cells.

MATERIALS AND METHODS

Primary cultures of HESM's4 were obtained from Flow Laboratories, Rockville, Md. Cells were grown in a Dulbecco's modified Eagle's medium (21) prepared in this laboratory and supplemented with 20% fetal calf serum (Irvine Scientific, Irvine, Calif.) Cells were checked for distribution of LDH isozymes and for karyotype to check for species and normal human chromosomal complement. Cells were checked weekly and before each experiment for the presence of Mycoplasma by the method of Chen (2). Cells were plated in 60-mm plastic culture dishes and allowed to grow overnight. The medium was removed, the cells were washed with two 2-ml rinses of phosphate-buffered saline (171 mM NaCl-3.36 mM KCl-1 mM Na₂HPO₄-3.68 mM KH₂PO₄), and then 1 ml of the solution was layered over the cells.

The cells were exposed to UV radiation from a low-pressure mercury arc with its principal emission at 254 nm. UV fluences were determined using a Jagger meter (5) calibrated against a Yellow Springs Instruments Company radiometer. For photoreactivation, cells were exposed to a 60-watt incandescent bulb at a distance of 21 cm. A 12-mm Plexiglas plate was placed between the cells and the bulb. Dark controls were placed in a light-proof box and placed beside the cells being illuminated to assure similarity of temperature between the 2 groups. Pilot experiments indicated that the temperatures of the 2 groups varied less than 1°. All exposures were carried out at room temperature.

The method of Macpherson (8) was used in all soft agar plating experiments. In brief, a 10-ml agar layer [80 ml 1.25% Bacto-agar, 20 ml of tryptone phosphate (Difco Laboratories, Inc.) broth, 20 ml of fetal calf serum, and 80 ml of a double strength Dulbecco's medium prepared according to Sutherland and Oliver (21)] was placed in a 60-mm plastic Petri dish and allowed to solidify. Medium was removed from the cells, and the cells were washed with two 2-ml portions of phosphate-

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1 This research was supported by Grants CA 23096 from the National Cancer Institute and NP-154B from the American Cancer Society and by the United States Department of Energy.

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Received July 30, 1979; accepted February 28, 1980.
buffered saline. One ml of 0.05% trypsin and 0.51 mM EDTA in Hank's balanced salt solution [containing, per liter, 0.4 g KCl, 0.006 g KH₂PO₄, 8 g NaCl, 0.35 g NaHCO₃, and 0.09 g Na₂HPO₄·7H₂O (pH 7.4 to 7.6)] were layered over the cells and removed as soon as the cells began to round up. A 2-ml portion of medium was added, and the cells were resuspended and counted in a hemocytometer for total cell number or for viable cells by their ability to exclude erythrosin B (6 × 10⁻⁵ g/ml). Cells were diluted so that 10⁶ cells in 1 ml of medium were added to 2 ml of the agar mixture, and the resulting suspension (3 ml) was plated on a 60-mm dish containing the agar base. Dishes were scored after plating for the presence of cell clumps; each dish and lid was marked with a vertical orientation mark, and the location of each clump was marked on the dish lid with a green felt-tipped pen. Such clumps occurred rarely (0 to 2 per plate). Cells were allowed to grow for 14 days and then were scored for the presence of colonies which did not arise from cell clumps. (Clones were unambiguously distinct from the clumps, which were identified from the overlying green mark.)

Photoreactivating enzyme was determined by the rapid dimer assay of Sutherland and Chamberlin (20). In brief, cell extracts prepared by a 45-sec sonication of washed cells suspended in Buffer E (10 mM Tris, pH 7.0-0.1 mM EDTA, 0.1 mM dithiothreitol) were added to 0.2 ml 20 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM dithiothreitol, 0.1 mM EDTA, and 30 to 100 pmol of UV-irradiated ³²P-labeled T-7 DNA. For each determination, one assay tube was placed in the dark, and another was exposed to photoreactivating light from a General Electric 150-watt spot lamp; both samples were maintained at 37° by immersion in a circulating water bath. The dimer content of the samples was then determined by nuclease digestion, adsorption of dimer-containing oligonucleotides to Norit, filtration, and counting. Photoreactivating activity is the difference in dimer content of the light and dark samples; units of photoreactivation are expressed as pmol/mg/hr. Protein concentrations were determined by the method of Lowry (7) using bovine serum albumin as the protein standard.

RESULTS

Production of Transformants. We have developed a protocol for UV induction of anchorage-independent growth by human cells. This procedure involved plating 1.0 to 3 × 10⁶ cells/60-mm dish (Day 1) and allowing them to grow overnight at 37° in a 5% CO₂ atmosphere. The cells were washed, UV irradiated (one-third of the total exposure), and then supplied with fresh growth medium on Days 2, 3, and 4. The cells were allowed to grow (Days 5 to 9), and then they were plated in 0.33% soft agar and scored for cell clumps (Day 9). The cells were allowed to grow for an additional 14 days, and the resulting clones were counted on Day 23. Cells exposed to 254-nm radiation by this protocol gave rise to clones capable of anchorage-independent growth (Fig. 1b). Clones ranged from about 100 to several thousand cells. Fig. 1a shows a typical dish of unirradiated HESM cells 14 days after plating in soft agar. These cells did not divide in the soft agar, although cellular metabolism continued, as judged by a slow drop in the pH of the agar overlay. Since we usually plated 10⁶ cells/dish, we could have easily detected one clone in 10⁶ cells; we estimate that these cells gave rise to clones at a frequency of approximately one clone per 20 to 50 plates of unirradiated cells, i.e., one clone per 2 × 10⁶ to 5 × 10⁶ cells. Experiments in which 1, 2, or 5 × 10⁶ cells were plated in one dish showed no clones; however, the presence of such high cell numbers might be unfavorable for growth.

We examined 2 features of this procedure to determine their necessity in the production or observation of transformants, namely, the multiple irradiation schedule and the growth period before plating in agar. Results of 2 independent experiments indicated that, although transformants were obtained when the UV was administered in a single dose, the transformation frequency was increased by a factor of about 4 when the same total UV dose was given in 2 (one-half the total dose for each exposure) or 3 (one-third the total dose for each exposure) exposures. Thus, all experiments in this series were carried out using 3 exposures. We also examined the requirement for growth of the irradiated cells before plating in agar. In an experiment in which the 5-day growth period yielded 20 transformed clones/10⁶ cells (for 10 J/sq m total UV exposure), cells plated immediately after UV gave less than 2 clones/10⁶ cells, and cells plated after growth for 3 days gave 6.5 clones/...
10^5 cells. We thus allowed the cells to grow in the plastic culture dishes for 5 days before plating in agar.

**Frequency of Transformation.** In a series of experiments designed to determine a dose-response curve for the frequency of UV transformants as a function of UV exposure, we found that the shapes of the dose-response curves were similar but that the frequency of transformation varied from experiment to experiment. Chart 1 shows 6 experimental dose-response curves for UV transformation of HESM's. The maximum frequency of transformation in each case occurred at about 10 J/sq m, but the value of the maximum varied from approximately 80 to 3 clones per 10^6 cells. We noted that the highest transformation frequencies seemed to occur in cells of the lowest passage number; Chart 2 shows that there is a strong dependence of transformation frequency on passage number, with almost no transformants evident after passage 25.

The data in Charts 1 and 2 represent frequency of transformants per 10^6 cells plated in agar. It was likely that the decreased transformation frequencies at higher UV exposures resulted from cell killing and that transformation frequencies based on a measure of viable cells might indicate if this were true. Since even unirradiated HESM cells form colonies very poorly on plastic or glass surfaces (10%), we regard such data as giving information only on a small fraction of the population. We have thus used vital dye exclusion (6 x 10^-5 g of erythrosin B per ml); this method gives good correlation with colony-forming ability in V-79 cells, which form colonies efficiently on solid surfaces. Chart 3 shows that this is indeed the case and that the frequency of transformation increases up to at least 25 J/sq m. Under the multiple exposure protocol, virtually no loss of viable cells was observed at 5 J/sq m, with about 10 to 25% loss at 10 J/sq m.

**Photoreversal of Induction of Transformation.** Normal human cells grown on the Dulbecco's modified Eagle's medium used in these experiments contain photoreactivating enzyme and can photoreactivate dimers in cellular DNA. We tested HESM's for photoreactivating enzyme activity and found the specific activity of the enzyme (605 pmol/mg/hr) to be about the same as other normal human cells (600 to 700 pmol/mg/hr). We then examined the photoreversibility of the induction of transformants by UV. For each of the 3 irradiation
to the stock cells; no differences were found between the stock and the 5 transformants tested. Stock HESM's undergo approximately 50 to 55 doublings under our conditions. We examined 20 isolates from clones in agar for life span under normal growth conditions. One of 20 underwent no more doublings than did the stock HESM's. Three isolates underwent 70 doublings before the generation time increased greatly, and 2 had reached their 91st and 95th generation, respectively, when they were lost due to contamination resulting from sterile hood malfunction.

We assessed the ability of cultures derived from the clones in agar to plate in soft agar. Table 2 shows that the individual isolates varied greatly in replating efficiency in agar, ranging from a 1.32-fold increase, essentially the same as stock cells, to a 84-fold increase, almost 100 times that of the HESM stocks. In collaboration with S-I. Shin, Albert Einstein College of Medicine, we also tested one isolate for tumorigenicity in immune-deficient mice. Cells were injected into each of 3 nude

procedures, cells were exposed to UV and then kept in the dark or illuminated for 15 min with light from a 60-watt incandescent bulb. Chart 4 shows the results of such an experiment; post-UV exposure of the cells to photoreactivating illumination results in a marked decrease in the frequency of transformation produced by a given UV exposure. Photoreactivating illumination alone had no effect on the cells.

Characterization of the Transformants. Clones growing in the soft agar were picked individually, pipetted vigorously to remove the clone from the agar, and placed in 1 ml of medium in a T-25 plastic culture flask. Clones attached to the plastic surface and individual cells growing out from the clone were visible after overnight growth. The resulting cultures were first tested for contamination by bacteria, fungi, or Mycoplasma. We also determined the pattern of electrophoretic mobility of the isozymes of LDH to assure that the transformants were indeed human cells. Shannon and Macy (16) have pointed out that "... isoenzyme analysis has been useful in determining the species of presumably 'transformed' cells that have been submitted [to the American Type Culture Collection] for identification. In all cases thus far, determination of the G6PD [glucose-6-phosphate dehydrogenase] and LDH patterns have shown that such cultures contained cells predominantly (if not all) from another species." Table 1 shows the LDH isozyme patterns for HESM cells (parental stock) and for cells from transformed clone 1868 in comparison with the standards of human lung fibroblast, WI-38, normal human skin fibroblast, rat heart, rat muscle, Chinese hamster (V-79), and Chinese hamster ovary cells. It is clear that both the HESM stock and the 1868 transformant show LDH isozyme patterns characteristic of human cells.

We next examined the growth properties of cells resulting from the anchorage-independent clones. The transformants showed 2- to 5-fold higher density than did the stock cells and showed both crisscrossing and piling up of cells. We also examined the serum dependence of the transformants relative
mice, but no tumors had appeared 3 to 4 months later, when the mice died (presumably of other causes).

DISCUSSION

We have developed a system for the UV induction of anchorage-independent growth in human cells. The system involves multiple UV exposures, a growth period before plating, and selection by growth in soft agar. It is based on 3 principles: (a) there is a high incidence of sunlight-induced skin cancer in individuals with histories of chronic sunlight exposure (12); (b) in many bacterial mutagenesis experiments, cells must be allowed a growth period for expression of a mutant gene before they are subjected to selection procedures requiring expres-
sion of that gene; and (c) the ability to undergo anchorageindependent growth has been closely correlated with tumori-
genicity (17). We examined the first 2 points to determine their necessity for production of anchorage-independent growth. We found that division of the UV into 2 or more exposures increased the transformation frequency about 4-fold. Namba et al. (11) also found that multiple 60Co γ-irradiations were necessary for transformation of human (WI-38) cells. We also found that the frequency of transformation to anchorage-inde-
pendent growth was increased by allowing the cells to grow for several days before plating in agar. The growth under condi-
tions allowing anchorage may be analogous to growth under permissive conditions before selection under nonpermissive conditions. In addition to possible expression of genes permit-
ting anchorage-independent growth, the preplating growth pe-
riod may allow an increase in the number of such transformants relative to the anchorage-dependent population. For both 60Co and chemical transformation of human cells, growth periods after treatment were required for appearance of transformed cells.

Are the anchorage-independent clones we observe due to induction of transforming event(s) by the UV or to selection by UV of the radiation-resistant fraction of cells which are coinci-
dentially able to grow without anchorage? Because of the strong dependency of transformation frequency on passage number, it was not possible for us to test the transformation frequency in cultures derived from individually isolated cells, as Kakunaga (6) did for chemical transformation of human cells. However, 2 lines of evidence argue against selection as the source of the transformants: (a) we obtained transformants even at UV doses where there was little or no loss of cell viability; (b) we observed photoreactivation of induction of transformation even at these low UV exposures. This implies that the transformation event was separable from killing and thus that the anchorage inde-
pendent cells did not arise from selection of a radioreistant, anchorage-independent subpopulation.

The transformants to anchorage-independent growth show some characteristics of normal cells and some characteristics of human cells transformed by other agents. They are able to grow in soft agar; they do not maintain the monolayer config-
uration of the parental HESM’s but rather show crisscrossing and piling up (the rapidly growing cultures of the transformants are frequently characterized by the presence of macroscopi-
cally visible 3-dimensional protrusions of cells from the cell surface); they reach higher saturation densities than do the parental cells; and they show an extended life span. However, their plating frequency in soft agar is lower than for many oncogenic cell lines; some, at least, of the cultures derived from the anchorage-independent clones have a finite life span; and the cell isolate tested apparently was nontumorigenic. It is possible that these cells represent an intermediate on the way to oncogenic transformation or that oncogenic cells were pro-
duced but were overgrown by nononcogenic cells during the post-UV proliferative period.

Photoreactivating enzyme carries out the specific light-de-
pendent monomerization of cyclobutyl pyrimidine dimers in DNA. This specificity allows the use of photoreactivation as a test of the role of dimers in the production of biological damage. If the production of UV-induced biological damage can be reversed in a true photoenzymatic event, then pyrimidine di-
mers are important in the induction of that damage. Hart and Setlow (4) have presented evidence that dimers play a major role of induction of tumors by UV in the fish Poecilia formosa. However, the low level of photoreactivating enzyme in murine cells and the strong dependence of photoreactivating enzyme levels in human cells on culture conditions have deterred examination of photoreactivation of transformation in mamma-
lian cells. We have used photoreactivation-competent cells and culture conditions favorable for production of the photoreacti-
vating enzyme. The results of Chart 4 indicate that the induction of transformation is photoreversible. Photoreactivating light given alone or before UV irradiation did not affect the transfor-
mation frequency. These results imply that pyrimidine dimers are important in transformation of human cells UV.

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Ultraviolet Light-induced Transformation of Human Cells to Anchorage-independent Growth

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