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ULTRAVIOLET-LIGHT-INDUCED TRANSFORMATION OF HUMAN PRIMARY CELLS *

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INTRODUCTION

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Understanding solar induction of skin cancer in man at the molecular level demands the development of experimental systems for probing this, the most frequent of all human cancers. A good model system should fulfill at least three criteria: first, the response should be light-dependent, with a low background (light-independent) response. Second, the system should be based on human cells, preferably primary skin cells from normal individuals. Third, after the oncogenic treatment, the responding cells should have properties similar to those cancers produced in man. Such systems should provide an excellent complement to the experimental UV-oncogenesis systems in rodents, which allow experimental manipulation of the intact animal but are subject to differences between rodents and humans in response to UV damage. They will also be useful in conjunction with the human epidemiological studies, which provide both prospective and predictive information on human populations, but are not amenable to experimental manipulation.

Two major problems impeded the development of such systems. First, human cells were recalcitrant to transformation by agents other than oncogenic viruses. Second, ultraviolet light had failed to produce transformation in any mammalian cultured cell, whether human or rodent.

In the mid 1970's, several systems were developed which met at least two of these criteria, Chan and Little (1976) reported a system for UV transformation of mouse C3H/10T $1/2$ cells. [Although note that Mondal and Heidelberger (1978) were unable to obtain

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transformation of the same cells by UV light unless a promoter was present.] DiPaolo and Donovan (1978) developed a system for UV transformation of Syrian hamster cells. Kakunaga (1978) obtained transformants of human cells using chemical carcinogens. Sutherland (1978) reported transformation of human diploid fibroblasts by ultraviolet light; I shall discuss the latter system below, since because it involves both human cells and ultraviolet light, and because I am most familiar with it.		1
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The system for UV transformation of human cells was developed from three major premises. First, most sunlight-induced human skin cancers (squamous cell and basal cell carcinomas) arise in sunlight-exposed areas in individuals with histories of chronic sunlight exposures. The experimental protocol thus subjects cultured human skin fibroblasts to multiple exposures of UV radiation. Second, in many bacterial mutagenesis experiments, it is necessary to allow growth to allow gene expression before requiring the mutagenized cells to use the new (acquired through mutation) gene product. The treated cells will therefore be allowed to grow before selection. (This requirement for growth before expression has also been found in other systems of oncogenesis.)		3
Third, one must choose a selection system for distinguishing transformed from non-transformed cells. Although many systems have been developed to distinguish transformed from non-transformed cells--ability to grow in low serum concentrations, loss of density-dependent growth control, etc.--the characteristic best correlated with tumorigenicity in rodent cells is the ability to grow without anchorage (Shin et al., 1975). The UV-treated cells will therefore be plated in soft agar; non-transformed cells will not grow, while cells transformed to anchorage-independent growth will develop into clones or colonies ranging from about one hundred to several thousand cells. In developing any systems, one must consider the type of cell to use as a basis for the assay. This system was originally developed with human embryonic skin and muscle fibroblasts (HESMs), which grew rapidly (generation time about 24 hr.), were available commercially, and were supplied as primary cells. Recently, the transformation system has been extended for use on other mammalian cells; differences and similarities among the various cell types will be discussed below.		4
THE UV TRANSFORMATION SYSTEM		5
I shall first describe the experimental protocol for induction of transformation of human cells to anchorage-independent growth. I shall then discuss factors affecting transformation frequencies, and shall describe properties of the		6

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transformants. I shall finally (briefly) note results obtained with other mammalian cells, and shall describe a use of the system in probing possible human health hazards.		1
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<u>Protocol for UV Transformation</u>		7
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This procedure is described in detail in Sutherland et al. (1980). Early passage, rapidly growing cells are maintained in a		9
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Fig. 1. (a) Unirradiated HESM cells 14 days after plating in soft agar: (b) anchorage-independent clone arising from UV-irradiated HESM cells, 14 days after plating in soft agar. Bar, 0.2 mm. Photograph taken from Sutherland et al., (1980) Cancer Res. 40:1934, with permission of the authors and publisher.

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Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (Sutherland and Oliver, 1976) and tested before each experiment for mycoplasma. Cells are seeded into 60 mm dishes at densities ranging from $1-3 \times 10^5$ cells/dish. (All cell handling is performed under yellow fluorescent room lights, with no additional lighting in the sterile hood). From the first irradiation till the end of the experiment, all cell handling (including opening of the incubator) is done only in red fluorescent lights ($\lambda > 600$ nm). After overnight growth of the cells, the medium is removed from the cells, they are washed twice with 1-ml portions of phosphate buffered saline (PBS), and 1 ml PBS is layered over the cells. They are exposed to ultraviolet radiation, the PBS is removed and fresh medium placed over the cells. After 24 and 48 hr. growth, the cells are washed, irradiated and fed. (Thus each radiation treatment contains one-third of the total UV exposure). After the last treatment, cells are allowed to grow for five days. The cells are detached from the dishes by trypsin treatment, counted, and 10^5 cells are placed in a 3-ml layer of 0.33% agar over a 10 ml base of 0.5% agar (MacPherson, 1973). After the upper agar layer has solidified, the dishes are scored for cell clumps (which might grow into aggregates resembling

Fig. 2. Production of anchorage-independent clones by 9.6 J/m^2 of 254 nm radiation as a function of cell passage number. The length of the horizontal bars represents the span of passages for cells used in an individual experiment. Data taken from Sutherland et al., (1980), Cancer Res. 40:1934, with permission of the authors and publisher.

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transformed clones). The cells are fed once every 3-4 days with 0.5 ml medium to prevent drying of the agar layers. After 14 days' growth, the dishes are counted for clones not previously marked as the sites of clumps. Figure 1a shows the appearance of unirradiated HESM cells 14 days after plating in soft agar and Figure 1b shows an anchorage-independent clone from UV-irradiated cells (also photographed 14 days after plating).

Factors affecting the Transformation Frequency

We have explored four major factors which affect the frequency of transformation to anchorage-independent growth of the HESM cells. The first factor is UV dose. Sutherland et al. (1980) showed that in dose-ranges where there was > 95% viability, the number of transformants increased with increasing UV dose. They found, however, that although the shapes of the dose-response curves were similar from experiment to experiment, the absolute number varied from 25 to 80 transformants per 10^5 cells produced by 10 J/m^2 of 254 nm radiation. They showed that this variation reflected the passage number of the cells: low passage (in the sense of population doubling) cells gave high transformation rates, while the rate of transformation decreased sharply with increasing passage number. Figure 2 shows that HESM cells of greater than passage 15 gave many fewer transformants; it is therefore of great advantage with these cells to obtain cells at the earliest passage possible.

The frequency of transformation also depends on the wavelength of the UV. This provides a powerful tool for determining the cellular target for absorption of the transforming photon: if wavelengths absorbed by nucleic acids are most efficient in producing transformation, one has evidence for the role of DNA in UV light absorption in the cell. If, on the other hand, the most efficient wavelengths are in the 280 nm region (where most proteins absorb strongly because of their tryptophan content) it would be likely that absorption by aromatic amino acids in proteins was important in the transformation process. Jagger (1967) has outlined several criteria which must be met for the establishment of valid action spectra from dose-response data at different wavelengths. First, reciprocity of time and dose must hold. Second, the dose-response curves at the different wavelengths must be similar, indicating the existence of a common molecular process underlying the observed biological effect. Sutherland et al. (1981) found that the reciprocity of time and dose held in the HESM system, and that the dose-response curves at the various wavelengths (248, 265, 270, 280, 289 and 297 nm) were similar. These data allowed them to compute an action spectrum (shown as the solid symbols in Figure 3; the filled triangles show the reciprocals of photon fluences required to produce 150 transformants per 10^6 cells, and the filled circles those for the

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Fig. 3. Action spectra for transformation to anchorage-independent growth (filled symbols), killing of human fibroblasts (open circles; Kantor *et al.*, 1980) and pyrimidine dimer formation in V79 cells (open triangles; Rothman and Setlow, 1978). The upper curve (filled circles) represents the reciprocals of photon fluences required to produce 75 transformants per 10⁶ viable cells, and the solid triangles those for the production of 150 transformants per 10⁶ cells. Data taken from Sutherland *et al.*, (1981), Cancer Res. 41:2211, with permission of the authors and publisher.

production of 75 transformants/10⁶ cells). These action spectra are compared with data of Kantor *et al.* (1980) for killing of human fibroblasts, shown as open circles, and of Rothman and Setlow (1978), shown as the open triangles, for formation of pyrimidine dimers in isolated V-79 hamster DNA. All these spectra have maxima at 265 nm, and do not provide evidence for absorbing moieties other than nucleic acids. Recently action spectra for transformation and pyrimidine dimer formation in the Syrian hamster embryo system were reported to have a rather sharp maximum at 270 nm (Doniger *et al.*, 1981). The sharpness of this peak is rather surprising, since the absorption spectra of biological molecules are broad in this wavelength range. The possibility of

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an instrumental artifact should be excluded [since a similar sharp peak at 270 nm was obtained in an action spectrum for cell killing and mutagenesis in mouse cells by Jacobson et al. (1981) using the same monochromator and dosimetry apparatus] before the molecular cause of this peak be sought.

A further modification of the transformation rate results from repair processes. One repair process, photoreactivation, results in the light-dependent, enzyme-mediated reversal of cyclobutyl pyrimidine dimers in DNA. Sutherland et al. (1980) found that exposure of UV-irradiated HESM cells to photoreactivating light immediately after each UV treatment resulted in the reduction of the yield of transformants. As a light-mediated process, photoreactivation has a strong wavelength dependence; the action spectrum for photoreactivation of dimers in human cells, and for dimer photoreactivation by isolated human enzyme in vitro shows a broad maximum at 400 nm, and extends to shorter wavelengths of about 310 nm, and to wavelengths as long as 577 nm (Sutherland and Sutherland, 1975). If the photoreversal observed by Sutherland et al., (1980) results from true photo-enzymatic repair, its action spectrum should resemble that for action by the enzyme. This identification will allow direct assessment of the role of pyrimidine dimers, the only known substrate for the photoreactivating enzyme, in UV transformation.

Repair processes may also be responsible for the differences in the UV dose-response curves seen in the various rodent transformation systems and in human system. At low doses, DiPaolo and Donovan (1978) and Doniger et al. (1981) found a linear UV dose-transformation relation in the Syrian hamster system, and Chan and Little (1976) also reported a linear dose-response relationship in the mouse embryo C3H/10T 1/2 system. However, the data of Sutherland et al (1980, 1981) can not be adequately represented by a straight line, showing a paucity of transformants at very low doses. Since rodent cells in culture undergo much slower excision repair than do human cells (Regan and Setlow, 1973), the paucity of human transformants at low UV doses may result from repair by excision of lesions in the normal human, but not in the rodent cells.

Characterization of the Transformants to Anchorage-Independent Growth

Before one can characterize the transformed cells, one must be able to grow sufficient quantities of each clone for study. On the human cell system, development of suitable conditions (choice of serum lot, quantity of medium, isolation conditions) required some year's work. Figure 4 shows cells growing on a plastic surface from a clone transformed to anchorage-independent growth by UV-treatment. These cells grow rapidly in culture and can be used for characterizations.

The first characteristics which must be determined for each strain are those which mark its integrity of origin and freedom from contamination. The HESM transformants cultures are first checked for mycoplasma contamination [MacPherson (1973) notes that mycoplasma contamination can lead to increased frequency of growth in soft agar.] and maintained in antibiotic-free medium to allow immediate detection of bacterial or fungal contamination. The species of origin is checked by analysis of isozymes of lactate dehydrogenase (Shannon and Macy, 1973), which are distinct and quite different among the human and rodent cells in our laboratory (Sutherland et al, 1980). The karyotype is determined to insure against contamination with other human cell lines, for example HeLa (HeLa cells are not permitted to be grown in our laboratory).

After ascertaining the integrity of the cultures of transformants, one can begin to characterize the cells. In the case of the human UV-induced transformants to anchorage-independent growth, transformation, the variation from one clone to another precludes characterization of "the" transformant, but instead requires description of a full range of cell types. The transformant clones differ in ability to attach to plastic surface (even under apparently optimal conditions), growth rate of the cultures as anchored cells growing on a plastic surface, and in ability to grow in soft agar (Sutherland et al., 1980). One clone was found to be non-tumorigenic when injected into immune

deficient mice (the mice died 3-4 months after cell injection, presumably of other causes.) Other clones from this system must be evaluated before a valid assessment of their tumorigenic potential can be made.

The UV Transformation System using other Mammalian Cells

The recent unavailability of HESMs from their commercial supplier has led us to test other mammalian cells in our UV-transformation system. We examined the UV-transformability of several strains of mouse 3T3 cells; like Withrow et al. (1980) who used single dose irradiation schedules--we obtained transformation to anchorage independent growth. However, we, like Withrow, found high background (anchorage independent clones appearing in unirradiated samples); for example, a maximum number of 30 clones/ 10^5 viable cells might be produced with 254 nm exposure over a background of 10 per 10^6 . This contrasts strongly with the HESM system, in which the irradiated samples might contain $80/10^5$ viable cells, while the unirradiated cell contained $< 1/10^6$ - 10^7 cells. We have now developed a system using neonatal foreskin fibroblasts cells; this system has only a slightly higher background (~ 1 per 10^6 cells) and a good transformation rate (~ 30 per 10^6 cells). The first results using this system are described in the following section.

Transformation by Sunlamp Exposure in the presence of Para-amino Benzoic Acid

Para-amino benzoic acid (paba) is the parent compound upon which many sunscreen preparations are based. This compound is similar in structure and energy levels to many compounds which sensitize pyrimidine dimer formation in DNA by wavelengths not absorbed by the DNA itself (Lamola, 1966; Sutherland and Griffin, 1981). Hodges et al. (1977) showed that paba-and-UV light induced damage in *E. coli* was photoreactivable, implying the formation of pyrimidine dimers. We thus examined the effects of sunlamp exposure on neonatal foreskin fibroblasts with and without 0.2% in the overlying PBS. The sunlamp alone was inefficient in producing transformation; 19 sec. of FS40 sunlamp exposure produced only 0.7 ± 0.3 transformant per cells. We showed that even this low rate of transformation resulted from the shorter wavelengths (less than about 310 nm) present in the sunlamp by interposing a thin mylar film between the cells and the sunlamp to exclude the shorter wavelength. At the range of doses we used, the filtered sunlamp produced no transformants to anchorage-independent growth. In the presence of paba, the number of transformants increased by a factor of ~ 10 . These results are compatible with those of K. Griffin and J. C. Sutherland (1981), who found that paba + 313 nm light produced dimers in DNA, and was mutagenic to *Salmonella* tested in the Ames system. They are also

compatible with the data of R. Daynes (personal communication) who showed that although paba reduced skin thickening and abnormal pigmentation induced by UV in mice, it did not affect the rate of UV-induced tumor formation in the mice.

These in vitro systems offer great promise for probing and understanding damage to human cells at the molecular level. They should complement both the systems for experimental oncogenesis in rodents and the human epidemiological studies. Development of these systems to provide a fuller understanding of their potential and limitations, strengths and blind spots, will allow a more accurate assessment of their adaptability to detection of potential carcinogens.

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