In Vitro Transformation of Primary Cultures of Neonatal BALB/c Mouse Epidermal Cells with Ultraviolet-B Radiation

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ABSTRACT

Primary epidermal cultures from neonatal BALB/c mice were used to study the carcinogenic effects of ultraviolet radiation in vitro. These cultures were irradiated once through a Falcon plastic dish cover with an FS40 sunlamp [ultraviolet B, λ ~290 to 400 nm] for various lengths of time and maintained for 8 to 12 weeks without subculturing. During this period, most of the cells in the untreated control cultures showed signs of morphological differentiation and eventually died. The cultures irradiated with ultraviolet B radiation also behaved in the same manner except that, in some dishes, small populations of surviving cells began to proliferate and developed into morphologically distinct foci. Seven long-term cell lines were derived from these ultraviolet-irradiated primary epidermal cell cultures. Six of these cell lines produced tumors when injected s.c. into normal and/or immunosuppressed syngeneic recipients. These tumorigenic cell lines lacked definitive characteristics of differentiated epidermal cells, but the cells possessed intermediate junctions, suggesting that they were of epithelial origin. Some of these in vitro-transformed cell lines appeared to be highly antigenic inasmuch as they grew preferentially in immunosuppressed BALB/c mice as compared to their growth in normal syngeneic recipients.

INTRODUCTION

Epidemiological and clinical studies have indicated that UV radiation in the UV-B1 region (280 to 320 nm) of the spectrum of the sun may be responsible for the induction of skin cancer in humans (27, 31, 32). Extensive studies with laboratory animals have confirmed this observation (3, 13, 21, 31, 32). However, the precise wavelengths involved in UV radiation-induced carcinogenesis have not yet been determined because of the lengthy and tedious experiments required to resolve this issue using animal models. To this end, in vitro systems using cells in tissue culture offer great advantages for determining precisely the wavelengths involved in UV radiation-induced carcinogenesis. Several reports on cellular transformation induced by UV radiation have appeared recently. DiPaolo and Donovan (9) first reported that UV radiation induced morphological transformation of Syrian golden hamster embryonic fibroblasts. Chan and Little (5, 6), using 10T1/2 cells from C3H mice, showed that UV radiation could produce tumorigenic murine fibroblasts. In contrast, Mondal and Heidelberger (23) reported that UV radiation induced transformation in 10T½ cells only when followed by repeated treatment with a tumor-promoting agent. Morphological transformation of human fibroblasts by in vitro exposure to UV radiation has been reported recently by Sutherland et al. (30).

All of these in vitro transformation studies used fibroblasts and 254 nm (UV-C) radiation. This short-wave UV radiation is absorbed by ozone in the stratosphere and never reaches the surface of the earth. Therefore, it probably does not participate in sunlight-induced carcinogenesis (28). Recently, however, there has been one study on the transforming effects of the longer UV wavelengths (the UV-B waveband), which penetrate to the surface of the earth. Withrow et al. (34) have reported the morphological transformation of murine embryonic fibroblasts (BALB 3T3 cells) by UV-B radiation. Although these transformants exhibit anchorage-independent growth, it is not known whether they are tumorigenic when injected into animals. Additional experiments are needed to characterize the carcinogenic activity of the UV wavelengths that are present in natural sunlight and in artificial light sources encountered in the human environment. Furthermore, studies using epithelial cells would be desirable because most human cancers, including those of the skin, occur in epithelial tissues.

In these studies, we attempted to assess the carcinogenic potential of UV wavelengths >290 nm for murine epidermal cells, using filtered sunlamps and freshly prepared epidermal cells from newborn mice. Furthermore, we wished to determine whether tumors produced in this way would exhibit antigenic properties characteristic of the skin cancers induced in mice by in vivo UV irradiation (22).

MATERIALS AND METHODS

Cell Culture. Primary epidermal cell cultures were prepared from neonatal (1-day-old) BALB/c mouse skin by the trypsin flotation method, described by Yuspa and Harris (35). Medium 199 (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% FBS (Reheis Chemical Co., Kankakee, Ill.), penicillin (100 units/ml), and streptomycin (100 μg/ml), was used for growing the cell cultures.

UV Irradiation. Fresh cell suspensions were plated at a density of 2 x 10⁶ cells in 60-mm Falcon 3002 tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and incubated for about 24 hr in a 5% CO₂ humidified incubator. A total of 8 dishes (6 for the transformation experiment and 2 for the determination of cell survival) per treatment group were used. Before irradiation, the cells were washed twice with warm phosphate-buffered saline [KCl (0.2 g/l), NaCl (8.0 g/l), KH₂PO₄ (0.2 g/l), Na₂HPO₄, 7H₂O (2.16 g/l)] to remove dead cells. The cells were then irradiated in phosphate-buffered saline (1 ml), with the dish cover on, for various lengths of time using light from an FS40 fluorescent sunlamp (Westinghouse Electric Corp., Bloomfield, N. J.). The FS40 sunlamp emits light in the 255 to

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3 The abbreviations used are: UV-B, ultraviolet-B; FBS, fetal bovine serum; PE, plating efficiency; ATX, adult thymectomy and sublethal (450 R) whole-body X-irradiation.
400 nm range, with a peak at 313 nm. The plastic dish cover used in the experiment filtered out UV radiation below 290 nm (data not shown). The emission spectrum of the FS40 sunlamp filtered with the Falcon 3002 dish cover resembled that of an unfiltered FS40 sunlamp except that there was no measurable emission below 290 nm. The distance between the light source and the bottom of the culture dish was about 20 cm. The fluence of the filtered FS40 sunlamp was 0.2 Watt/sq m, as measured by a calibrated radiometer (Laser-Precision, Corp., Utica, N. Y.). After irradiation, the buffer was removed, the medium was added, and the plates were incubated at 37°C. The cells were refed with fresh medium 2 times/week. A set of unirradiated controls was treated in a similar manner, except that they were kept in the dark for the duration of the irradiation period.

**Cell Survival.** Duplicate plates containing $2 \times 10^6$ cells/plate were included in the experiment to assess cell survival. The cells were irradiated as described above and incubated at 37°C for 24 hr. The cells were then trypsinized, and the number of physiologically viable cells, measured by trypan blue exclusion, was counted in a hemacytometer. The PE is defined as follows:

$$PE = \frac{\text{No. of cells attached 24 hr after plating}}{\text{No. of cells plated}} \times 100$$

**Derivation of Long-Term Cell Lines.** The cell cultures were examined under an inverted light microscope 2 to 3 times/week. The cells from UV-irradiated and control plates were subcultured after 8 to 12 weeks following trypsinization. Initially, the cells were subcultured at a ratio of 1:5 and later at a ratio of 1:10 or 1:20. After 4 or 5 passages, the culture medium was changed from Medium 199 to Eagle's basal medium containing 10% FBS and antibiotics, because the cells grew faster in Eagle's medium than in Medium 199.

**Test for Keratin.** Keratin proteins were assayed by a radioimmunoassay method using a rabbit antibody elicited against a purified keratin of BALB/c mouse epidermal cells (36).

**Electron Microscopic Studies.** Confluent cells were fixed for 1 hr in 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer. The cells were washed in buffer and postfixed in osmium tetroxide according to the O-T-O procedure of Bucana et al. (4). The fixed cells were washed with distilled water and treated for 30 min with 1% aqueous uranyl acetate. Following dehydration, the cells were rapidly embedded using the procedure of Robbins (25). Thin sections were cut with glass knives in an LKB Ultratome III, stained with Reynolds's lead acetate for 3 min, washed, dried, and examined with a Hitachi HU-12 electron microscope at accelerating voltage of 75 kV.

**In Vivo Tumorigenicity.** The various cell lines were tested for tumorigenicity after their 15th passage by injecting the cells into 8- to 23-week-old normal and immunosuppressed BALB/c mice. The mice were immunosuppressed by ATX. Viable cells (5 to $10^5$) in 0.1 ml of Roswell Park Memorial Institute Medium 1640 without FBS were injected s.c. on the ventral side of the recipients. The mice were inspected weekly for at least 6 months for tumor growth, and the tumor sizes were recorded. Tumor biopsies were fixed in Bouin's solution, embedded, sectioned, and stained with hematoxylin and eosin for histological examination.

**RESULTS**

**Cell Survival.** Physiological cell viability was determined by the trypan blue dye exclusion method. Reproductive death could not be determined because most of the cells in the primary epidermal culture do not divide and form distinct colonies under the conditions used. Instead, they undergo terminal differentiation; thus, the number of cells decreases with time in culture. The PE of freshly explanted cells from mouse epidermis was about 60%. The cells were irradiated 24 hr after plating, and the cell viability was determined after another 24 hr of incubation. The number of viable cells in the unirradiated control group at the same time point was taken as 100% survival, and the relative decrease in survival in the UV-irradiated groups was calculated. The results presented in Chart 1 indicate that there is a UV dose-dependent decrease in cell survival. However, viability determinations at UV doses greater than 120 J/sq m could not be made because the number of viable cells was less than $10^5$ cells/plate.

**Derivation of Long-Term Cell Lines.** The untreated cells from freshly explanted neonatal BALB/c mouse epidermis began to enlarge by the end of the first week after plating and then showed signs of differentiation. Cells in the untreated control group were sloughed off into the medium continuously, and eventually all died after about 8 to 12 weeks in culture. Many of the UV-irradiated cells also detached from the plates during the first 2 weeks, partly as a consequence of UV-induced killing. After 6 to 8 weeks in culture, some of the surviving cells began to multiply and eventually gave rise to dense foci. Typical colonies, fixed and stained at 3 months after irradiation, are shown in Fig. 1. Most of the foci were composed of cells arranged in multiple layers. In one of the untreated plates, the cells also proliferated into colonies. These colonies were less dense than those observed in the UV-irradiated plates, but they eventually reached confluence. However, the cells collected from this untreated plate did not survive beyond the sixth in vitro subculture. In this regard, it is worth mentioning that Slaga et al. (29) also did not obtain continuously growing or spontaneously transformed cell lines from untreated primary epidermal cell cultures.

When the proliferating cells reached confluence, they were
removed from the plate by trypsinization and were subcultured. (The foci were not counted because it was difficult to do so without staining the dishes.) This procedure was repeated for at least 15 passages before the cell lines were tested further. Table 1 shows that about 40% of the plates developed foci of proliferating cells, but only 7 of the 16 cultures passed survival beyond the 15th subculturing. These 7 cell lines have been continuously subcultured for 50 to 60 passages thus far without evidence of senescence.

The morphology and growth characteristics in culture of the 7 cell lines were quite varied. All the cell lines except UVB-1 grew to a high cell density. The heterogeneity of cell size and shape among the different cell lines is illustrated in Fig. 2. None of the cell lines exhibited morphological characteristics of primary epidermal cells, and none reacted with antibody against keratin proteins. However, ultrastructural examination of the cell lines by electron microscopy revealed that all the cell lines except UVB-15b had intermediate junctions between cells (Fig. 3). These junctional complexes are characteristic of epithelial cells (15).

**In Vivo Tumorigenicity of UV Radiation-transformed Cell Lines.** The ultimate criterion of neoplastic transformation is the ability of transformed cells to produce tumors in syngeneic or immunodeficient recipients. The data presented in Table 2 show that at passages 15 to 17 only UVB-10 and UVB-15b cell lines produced tumors in normal and immunosuppressed recipients. In contrast, UVB-2a, UVB-5, and UVB-5a cell lines induced tumors only in immunosuppressed recipients but not in normal mice, whereas UVB-1 and UVB-2b cell lines did not produce tumors in either host. An untreated primary culture of epidermal cells from newborn BALB/c mice also did not produce tumors in either recipient.

All of the cell lines transformed by UV radiation were tested again for tumorigenicity at higher passages (passages 36 to 48) in order to determine whether (a) the nontumorigenic cell lines underwent progression towards the neoplastic state and (b) the incidence and latency of tumor formation in normal and immunosuppressed hosts were altered after several passages in the absence of further UV treatment. The results presented in Table 3 indicate that by 40 passages, the previously nontumorigenic cell line UVB-2b had become tumorigenic and produced tumors preferentially in immunosuppressed recipients. In addition, the UVB-5a cell line, which did not induce tumors in normal mice at passage 16, did induce tumors in normal mice at passage 48, although the cells still grew preferentially in immunosuppressed mice.

In general, the cell lines transformed by the higher doses of UV radiation (UVB-10 and UVB-15b) exhibited shorter latent periods (~3 weeks) than the other cell lines (7 to 19 weeks). This suggests that the cell lines transformed by the higher doses of UV radiation may exhibit more aggressive behavior than do the cell lines exposed to lower doses of UV. In fact, the injection of as few as 100 cells of UVB-10 and UVB-15b resulted in tumor development in ATX recipients (tumor incidence, 2 of 5; latent period, ~4 weeks).

Histological sections of the tumors produced by injection of UV radiation-transformed cell lines are shown in Fig. 4. The tumor cells were generally spindle-shaped, undifferentiated cells. None exhibited morphological characteristics of differentiated epidermal cells.

**DISCUSSION**

The results presented here demonstrate that UV-B radiation from FS40 sunlamps induces neoplastic transformation of freshly prepared cells from the epidermis of neonatal BALB/c mice. Although a substantial proportion of the cultures exposed to a filtered FS40 sunlamp gave rise to morphologically altered foci, only a few long-term cell lines were derived from them. Because unique morphological features of transformed primary cultures have not been defined rigorously, escape from senescence was used as an initial criterion for transformation. Primary cultures generally have a finite life span, the length of...
which differs among various cell types (2, 14, 16, 18), and several investigators have taken advantage of this property in order to select transformed cells (1, 7, 8, 10, 12, 14, 17, 29).

In our experiments, about 40% of the cultures that contained proliferating foci at 8 to 12 weeks survived repeated subculturing. Hence, these cultures were considered to be long-term cell lines. The only definitive characteristic for neoplastic transformation of the cells, however, is their ability to produce tumors in syngeneic hosts. We found that the majority of the long-term cell lines derived from UV-irradiated epidermal cells from neonatal BALB/c mice were tumorigenic in syngeneic recipients. The cell strains transformed by the lower doses of UV-B radiation were either nontumorigenic in normal and ATX mice or grew only in ATX mice after a relatively long latent period. In contrast, the cell lines transformed by higher doses of UV-B radiation exhibited short latent periods and induced tumors in both normal and immunosuppressed mice. In addition, the cell lines transformed by higher doses of UV-B radiation were highly malignant, in that injection of as few as 100 cells could initiate tumor growth. It has been suggested that tumor formation after injection of such a small number of cells implies a clonal origin of the tumor cells (24). Although in these studies the cell lines developed from multiple foci, it is possible that cloning might have occurred spontaneously by a process of selection during passageing of the tumorigenic cells, due to a faster growth rate.

It is interesting to note that some of the cell lines transformed by UV-B irradiation in vitro appear to exhibit antigenic characteristics similar to those of skin tumors induced by in vivo UV irradiation of mice with FS40 sunlamps. Most UV radiation-induced skin tumors differ from chemical carcinogen-induced skin tumors in that the UV radiation-induced tumors are highly antigenic. Many of these tumors are rejected immunologically upon transplantation into normal syngeneic recipients, whereas tumors induced by chemical carcinogens usually grow progressively when transplanted into normal syngeneic mice (21). However, the skin tumors induced by in vivo UV irradiation are not rejected when transplanted into immunosuppressed hosts. Some of the in vitro UV-B-transformed cells also behaved in this manner; i.e., they grew into tumors preferentially in immunosuppressed BALB/c mice relative to their growth in normal mice. This suggests that the high degree of antigenicity exhibited by UV radiation-induced tumors produced in vivo may also be characteristic of the tumors produced by in vitro exposure of epidermal cells to UV-B radiation. Because the same type of light source (FS40 sunlamp) was used in the in vivo induction of skin tumors (21, 22) and the in vitro transformation of these primary cell cultures, it is possible that the same cellular events or pathways are involved in transformation in both instances.

These in vitro-transformed cell lines appear to have originated from the explanted epidermal cells because all the cell lines except UVB-15b showed the presence of intermediate junctions when examined under an electron microscope. The origin of UVB-15b, however, is uncertain. It is possible that these transformed cells arose from contaminating fibroblasts in the primary culture. On the other hand, loss of intermediate junctions as a result of UV radiation-induced transformation cannot be ruled out. This is supported by the fact that none of the cell lines transformed by UV radiation exhibited other epidermal markers such as keratin proteins, tonofilaments associated with desmosomes, etc. Similarly, Colburn et al. (8) and Slaga et al. (29) have reported that epidermal cells from neonatal mice transformed by chemical carcinogens also lost all epidermal characteristics except for intermediate junctions. Also, it is possible that these cell lines are derived from the undifferentiated precursors of keratinocytes and thus do not have the characteristics of differentiated epidermis.

In any case, the implications of the carcinogenic effects of UV-B radiation from light sources used in the human environment are obvious. Although the lethal and mutagenic effects of UV-B radiation have been demonstrated both in prokaryotes (11, 33) and in mammalian cells in culture (19, 20, 26), neoplastic transformation of epidermal cells by in vitro UV-B irradiation has not been demonstrated previously. Additional studies are needed to determine the precise wavelengths present in the sunlight that are responsible for the induction of skin cancer in humans. In this respect, in vitro transformation using cells in culture may serve as a model system for defining an action spectrum for UV radiation-induced carcinogenesis.

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Fig. 1. Fixed and stained dishes of normal and "transformed" primary epidermal cells. a, no UV, 1 day after plating $2 \times 10^6$ cells; b, no UV, after 3 months in culture; c, 60 J/sq m UV-B, after 3 months in culture. The cells were fixed and stained as described for electron microscopic studies in "Materials and Methods."
Fig. 2. Morphology of primary epidermal cells and UV radiation-induced long-term cell lines. a, primary epidermal cells, 48 hr after plating; b, UVB-1 at passage 25; c, UVB-2a at passage 43; d, UVB-2b at passage 33; e, UVB-5 at passage 45; f, UVB-5a at passage 43; g, UVB-10 at passage 48; h, UVB-15b at passage 47. Phase-contrast, × 200. Cells were fixed and stained as described in Fig. 1.
Fig. 3. Electron micrographs. a, primary epidermal cells after 2 days in culture showing the presence of tonofilaments (TF) associated with desmosomes (D). Inset, primary epidermal cells at a higher magnification. b, and c, cell lines UVB-5a (passage 43) and UVB-10 (passage 48) showing intermediate junctions (IJ) without associated tonofilaments.
Fig. 4. Tumor morphology of UV-B-transformed cell lines. a, UVB-2a; b, UVB-5a; c, UVB-10; d, UVB-15b. H & E.
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