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Reconstituted 3-Dimensional Human Skin as a Novel *in Vitro* Model for Studies of Carcinogenesis

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EpiDerm (MatTek Co., MA) is a reconstituted human skin equivalent which exhibits morphological and growth characteristics similar to human skin. This model has previously been utilized to evaluate the cytotoxicity and irritant potential of various cosmetic and household products. In this study, we show for the first time that EpiDerm can be used successfully to evaluate the genotoxicity of different types of known carcinogenic agents such as benzo[*a*]pyrene (BaP), ultraviolet B radiation (UVB), ultraviolet A radiation (UVA), and psoralen-ultraviolet A radiation (PUVA) at the molecular level. The topical application of 50 μg/cm^2 BaP to EpiDerm resulted in the accumulation of BaP–DNA adducts and c-*fos* and *p53* proteins as evidenced by immunohistochemical localization. Similarly, exposure to UVB (50 mJ/cm^2) and UVA (2.5 J/cm^2) enhanced the epidermal expression of c-*fos* and *p53* proteins in the human skin equivalent. PUVA treatment of EpiDerm, however, resulted in the formation of both DNA–8-MOP adducts and augmented expression of c-*fos* and *p53* proteins. Most of these changes reached a peak 8 h after the treatments except in the case of UVA where maximum changes in the expression of c-*fos* and *p53* proteins were observed 24 h after treatment. These results are similar to those previously reported in human and murine skin following exposure to BaP, UVB, UVA, or PUVA indicating that human skin equivalents can be used as a convenient and cost-effective alternative to animal testing for assessing the genotoxicity and mechanism of action of mutagens/carcinogens in human skin. © 1999 Academic Press

Due to public concern regarding the protection of animals *in vitro* methods for toxicity testing are becoming increasingly important to the consumer product industry which must market only non-hazardous and safe products (1). Therefore, to evaluate the safety of skin care products, a number of *in vitro* test models have been developed. The assessment of the toxicity of material applied directly to the skin is dependent not only on the intrinsic nature of its chemical constituents, but also upon the degree of percutaneous absorption and xenobiotic metabolism. In regard to percutaneous absorption, skin is unique in having the outermost layer known as the stratum corneum, which serves as the major permeability barrier against topically applied compounds. This structure is not present in various *in vitro* skin models currently being employed for toxicity assessment such as monolayer or two dimensional keratinocyte cultures limiting the usefulness of these models (2). To overcome this deficiency several systems employing 3-dimensional reconstituted human epidermis (composed of keratinocytes and melanocytes) or reconstituted skin (composed of keratinocytes and fibroblasts) or cells on a collagen matrix have been developed (3). One such system is known as EpiDerm (MatTek Cor., MA) which is composed of neonatal foreskin-derived human keratinocytes seeded onto an inert nylon mesh. The cells are then allowed to differentiate and form an epidermis-like structure with basal, spinous, granular and stratum corneum layers. Biochemical and ultrastructural characterization of these human epidermal cultures have showed that they sustain differentiation and metabolic properties very similar to those of human skin (3, 4).

Numerous studies have shown that reconstituted human skin can be used for evaluating the cutaneous irritant potential of various consumer products, screening photo-protective agents, such as sunscreens, assessing cytotoxicity and studying biomarkers of inflammation (4–7). However, it is not known whether reconstituted human skin can be utilized as a suitable *in vitro* model system to assess the genotoxic potential

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Abbreviations used: 3D, 3-dimensional; UVB, ultraviolet B; UVA, ultraviolet A; PUVA, psoralen plus UVA; 8-MOP, 8-methoxypsoralen; BaP, benzo[*a*]pyrene; BPDE, benzo[*a*]pyrene diol-epoxide; MTT, 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltertyazoliumbromide.

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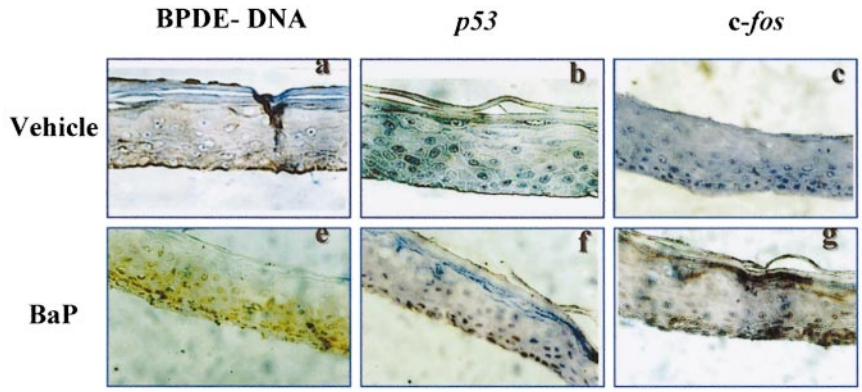

FIG. 1. Immunohistochemical staining of formalin-fixed, paraffin-embedded EpiDerm tissue cultured exposed to BaP (a) for BPDE-1–DNA adducts; (b) for p53; (c) for c-*fos* (magnification, ×400).

of chemical and physical agents thereby providing an alternative to animals for studying mutagenic/carcinogenic responses. The purpose of this study was to ascertain whether this 3-D human skin model could be useful for evaluating the mechanism of action of various skin carcinogens including chemicals and UV radiation. Our data indicate that human skin equivalents provide a convenient and cost effective alternative to animal testing for evaluating the genotoxicity of a variety of carcinogenic agents and for probing their mechanism of action leading to tumor induction.

MATERIALS AND METHODS

3-D human skin culture. EpiDerm (MatTek Cor., Ashland, MA) is a living reconstituted human epidermis. The EpiDerm assay kit contains 24 units of 8-mm diameter tissue samples. In this study, we used only serum free medium. The epidermal equivalents were removed from the agarose containing 24-well plates and equilibrated to 37°C, 5% CO_2 for 1 h with the assay medium which was supplied with the kit**.** The epidermal equivalents were then transferred under sterile conditions to a petri dish containing phosphate-buffered saline (PBS) for subsequent treatment with benzo[*a*]pyrene (BaP) or 8-methoxypsoralen (8-MOP) and/or UV irradiation.

BaP treatment. BaP (Sigma Chemical Co., St. Louis, MO) was dissolved in corn oil and diluted with Tween 80 (20%) to make a final solution containing 0.5% BaP. BaP (10 μl) or vehicle was applied to the epidermis in triplicate and the tissue samples were kept in an incubator (37°C, 5% CO_2). Tissues were removed at 8, 24, and 48 h after treatment, cut into small pieces, and fixed in 10% buffered formalin.

UVB or UVA irradiation. Tissues were irradiated with UVB (50 mJ/cm^2) or UVA (2.5 J/cm^2) using a UV Irradiation Unit (Daavlin Co., Bryan, OH) equipped with an electronic controller to regulate the dosage. The UVB source consisted of eight FS72T12-UVB-HO lamps emitting UVB (290–320 nm, more than 75% of total energy); the UVA source consisted of eight F72T12-BL-HO lamps emitting UVA (320–380 nm, more than 90% of total energy). The dose of UV was quantitated with UV Spectra 305 Dosimeter obtained from the Daavlin Co. (Bryan, OH). The target distance was 38 cm. At this distance no measurable increase in skin temperature was detectable.

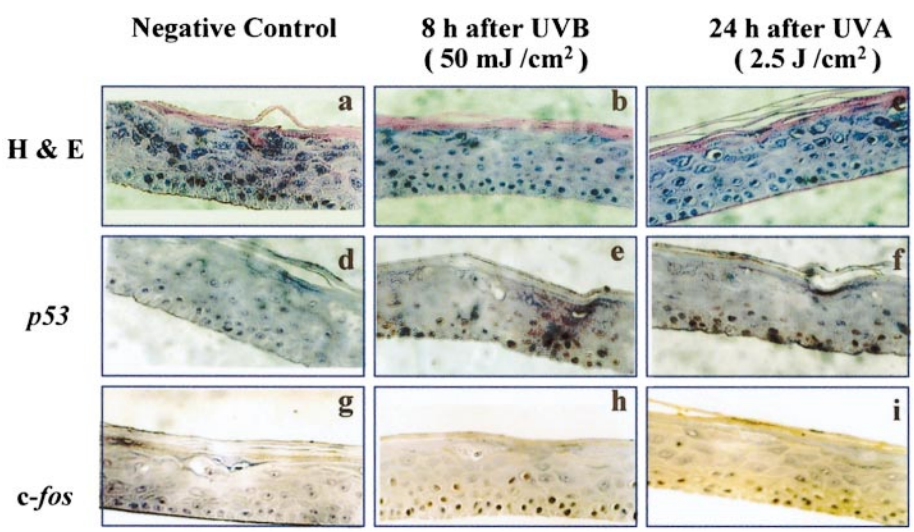

FIG. 2. H & E and immunohistochemical stainings of formalin-fixed, paraffin-embedded EpiDerm tissue exposed to UVB irradiation for p53 and c-*fos* (magnification, ×400).

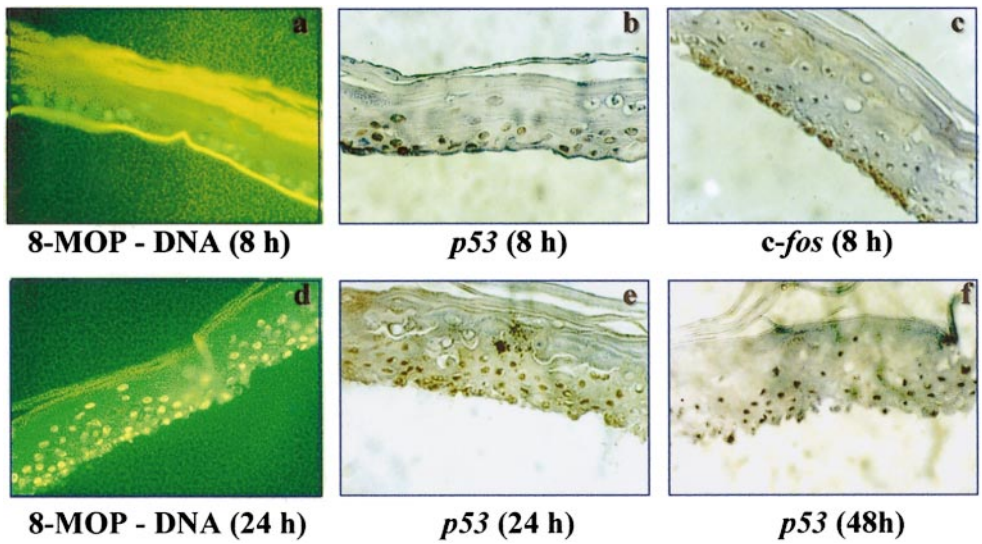

FIG. 3. Immunohistochemical staining of formalin-fixed, paraffin-embedded EpiDerm tissue culture treated with PUVA for MOP-DNA adducts, p53 and c-*fos* proteins (magnification, ×400) At different time points after PUVA treatment: a, detection of 8-OH-MOP-DNA adduct at 8 h; b, *p53* protein at 8 h; c, c-*fos* protein at 8 h; d, 8-OH-MOP-DNA adduct at 8 h; e, p53 protein at 24 h, and f, p53 protein at 48 h.

After irradiation, the tissues were again incubated and then removed at 8, 24, and 48 h. Thereafter, they were cut into small pieces and fixed in 10% buffered formalin.

PUVA (psoralen plus UVA) treatment. 8-MOP, purchased from Sigma Chemical Co., was dissolved in Tween 80 (20%) to prepare a final concentration of 0.5% MOP. 8-MOP (10 μl) or vehicle was applied to the epidermis in triplicate. Thirty minutes after treatment, the tissues were irradiated with 2.5 J/cm^2 of UVA and returned to the incubator. As described above, samples were removed at 8, 24, and 48 h following irradiation, cut into small pieces and fixed in 10% buffered formalin.

Immunohistochemical staining. Immunofluorescence staining for detecting 8-MOP-DNA adducts was performed as described previously (8–10). In brief, slides prepared from 5 μm paraffin-embedded sections treated with RNAse, proteinase K, hydrochloric acid (4N), and methanol, respectively. Non-specific binding was blocked with 1.5% normal horse serum and then the slides were incubated with anti 8-MOP–DNA monoclonal antibody 8G1 (1:10 dilution) overnight at 4°C. After washing with PBS, the tissue was incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate at 37°C for 30 min. Similarly, the immunoperoxidase staining for BPDE–DNA adducts, c-*fos* or *p53* proteins was performed as described previously (11) or as suggested by the manufacturers of the antibodies used. Briefly, after deparaffinization and dehydration in xylene and rehydration using a series of graded ethanol concentrations, the sections were treated with H_2O_2/methanol/distilled water solution in 1:50:50 ratio for 20 min. After microwave treatment, the sections were blocked in 2% (for BPDE–DNA adducts) and 10% normal mouse serum (for *p53*), or 1.5% normal horse serum (for c-*fos*) in PBS for 20 min, and incubated with the anti-BPDE-1–DNA monoclonal antibody No. 5D11, anti-p53 polyclonal antiserum CM-5 (Novocast Laboratories, Burlingame, CA) or anti-c-*fos* monoclonal antibody Ab-3 (Oncogene Research Products, Cambridge, MA) in PBS overnight at 4°C. ABC staining was performed using Vectastain (ABC elite kit, Vector Laboratory, Burlingame, CA) followed by diaminobenzidine as the chromogen (DAB kit, Vector Laboratory). Two independent observers scored the sections based on the intensity of staining and proportion of cells stained.

RESULTS

Effect of exposure of EpiDerm to BaP. BaP, a widely studied human mutagen and carcinogen, is metabolized by P450-dependent monooxygenase enzymes to highly reactive electrophilic species that bind to DNA (11, 12) or induce oxidative damage to DNA (13, 14). In this study, we observed that application of BaP to EpiDerm resulted in detectable BPDE–DNA adducts, and in enhanced expression of *p53,* and c-*fos* proteins as demonstrated by positive nuclear staining for BPDE-DNA adducts, and for the *p53,* and c-*fos* proteins (Fig. 1). The study of the kinetics of appearance of DNA adducts and p53 protein expression revealed that maximum staining occurred at 8 h and persisted up to 24 h but diminished after 48 h. c-*fos* expression was maximal at 8 h, decreased thereafter, and disappeared by 48 h. Staining for c-*fos* was confined to the basal layer only.

Effect of exposure of EpiDerm to UVB and UVA irradiation. Our studies showed that 80 mJ/cm^2 of UVB or 30 J/cm^2 of UVA produced no discernible cytotoxicity as observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) incorporation and cytokine release assay experiments (data not shown). In this study, 50 mJ/cm^2 of UVB or 2.5 J/cm^2 of UVA caused no apparent cytotoxicity as shown in Fig. 2. However, at these doses of UVB and UVA , enhanced expression of *p53* and c-*fos* proteins was observed. UVB exposure resulted in increased expression of *p53* at 8 h whereas the response to UVA was detected at 24 h following irradiation. In both cases *p53* protein expression persisted up to 48 h. UVB induced c-*fos* protein expression by 8 h, whereas UVA induced it at 24 h. Levels slowly

decreased and finally disappeared by 48 h. Interestingly, immunostaining for c-*fos* was seen throughout the granular, spinous, and basal layers of epidermis after UVB exposure whereas UVA effects were limited to the basal cell layer.

Effect of exposure of EpiDerm to PUVA. PUVA photochemotherapy has been widely used for treating psoriasis and other skin diseases. However, long-term follow-up studies suggest that patients receiving chronic PUVA are at high risk for squamous cell carcinoma and melanoma (15, 16). It is known that PUVA is a potent mutagen and carcinogen (17, 18). Our results indicate that 8 h after PUVA treatment 8-MOP–DNA adducts as well as c-*fos* and *p53* protein expression was observed (Fig. 3a). A delayed phototoxic response occurred between 24 and 48 h following PUVA treatment. In fact, 48 h after PUVA treatment, the basal cell layer was almost completely destroyed although p53 expression was seen in these tissues (Fig. 3b).

DISCUSSION

Human skin equivalents offer a potentially useful approach for assessment of cutaneous toxicity as an alternative to animal testing, thereby reducing the cost involved in evaluating product safety and reducing the large time period required for data generation. However, these models have only been used to provide information regarding the cytotoxicity, irritant potential and immunotoxicity of an agent (4–7). It is imperative that the three-dimensional reconstituted normal human skin equivalents derived from human foreskin have various morphological and growth characteristics similar to human skin that are sustained at an air-medium interface (3, 4) thereby permitting the direct application of any liquid or solid test materials to the skin surface. Our data indicate that exposure of EpiDerm to a variety of different carcinogens, BaP, UVB, UVA, and PUVA produces DNA adducts and alterations in the expression of transcription regulatory genes similar to those observed in animal skin, suggesting that these models can also be utilized for assessment of genotoxicity. The presence of BPDE-DNA and 8-MOP-DNA adducts in EpiDerm exposed to BaP or PUVA respectively suggests that reconstituted normal human skin retains the metabolic integrity consistent with that of intact human skin. Another important advantage of using human skin equivalents over most other *in vitro* genotoxicity assay systems employing bacteria (*Salmonella typhimurium, Escherichia coli B/r* WP_2), yeast (*Saccharomyces cerevisiae*), or transformed mammalian cells (such as JB6, RTE, A431, SV40, etc.) is that the data generated in these systems should be more directly relevant to humans (19–21).

It is known that exposure of animals to chemical or physical agents induces expression of multiple early response genes in various organs including skin (22). For example, UV-induced- or chemical carcinogen-induced DNA damage gives rise to transient accumulation of wild-type p53 protein which ultimately leads to cell cycle arrest in G_1 allowing DNA repair prior to the cell's entry into S phase or elimination by apoptosis (23, 24). Similarly, exposure to these carcinogens leads to enhanced expression of various transcription factors such as c-*fos* and c-*jun*. Parallel to these observations, the induction of *p53* and c-*fos* in EpiDerm following exposure to a variety of different carcinogens, BaP, UVB, UVA, and PUVA indicates that the EpiDerm is a useful model system for studying molecular events related to skin carcinogenesis. Furthermore, the induction of expression of transcription regulatory proteins by non-cytotoxic concentrations of carcinogens and differences in the kinetics of these changes suggest that reconstituted human skin models are sufficiently sensitive for early assessment of the carcinogenic potential of various agents. We are also exploring the possibility of using EpiDerm for evaluating other molecular endpoints/markers for studying cell proliferation, differentiation, cell cycle events, signal transduction, and apoptosis. In summary, our studies indicate that human skin equivalents provide a convenient and cost effective alternative to animal testing for evaluating the genotoxicity and probing the mechanism of action leading to skin carcinogenesis.

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