Use of Human Epidermal Cells in the Study of Carcinogenesis

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Because of the importance of human cells, particularly human epithelial cells, in cancer research, we have studied certain phases or events of carcinogenesis using human epidermal cells in primary culture. 1) We found that human epidermal cells are capable of metabolizing benzo[a]pyrene. Large inter-individual variations are found in the basal and induced arylhydrocarbon-hydroxylase activities. 2) UV-induced unscheduled DNA synthesis was demonstrated in human epidermal cells on autoradiographs. We also found that DNA repair is defective in epidermal cells isolated from xeroderma pigmentosum by a new explant-outgrowth culture. 3) Human epidermal cells are unique in that there is a large number of binding sites to phorbol esters compared with mouse epidermal cells, but there is no down-regulation. Further, human epidermal cells show essentially negative responses to tumor promoters, i.e., no stimulation of DNA synthesis, sugar uptake, and no induction of ornithine decarboxylase activity. 4) Human epidermal cells contain 1.5×10^5 binding sites per cell for epidermal growth factor (EGF), whereas squamous cell carcinomas of skin and oral cavity have larger amounts of EGF receptors in the order of 10^6 per cell. 5) Based on the above results, we attempted to transform human epidermal cells by the treatment with chemical carcinogens, but until now no transformation was obtained.
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Increasing emphasis is being placed on the use of human materials in cancer research. Because human in vivo studies other than epidemiologic and clinical ones are impossible, the use of cultured human cells is the most promising way to obtain information on the etiology and biology of human cancers.

Because of the importance of human cells, particularly human epithelial cells, we have started a project on the use of human epidermal cells in studies of carcinogenesis. We chose epidermal cells because they can be obtained relatively easily during plastic surgery and because techniques for epidermal-cell culture have been well established. Furthermore, epidermal cells show terminal differentiation, reorganizing to form a multilayered epidermal sheet, and this process offers unique opportunities for investigating cellular differentiation and carcinogenesis.

Isolation and Cultivation of Epidermal and Dermal Cells from Human Skin Skin sections approximately 0.4-mm thick cut with a dermatome were obtained from material discarded during skin graftings. The skin layer was cut into small pieces and incubated with 0.25% trypsin solution overnight at 4°C. The epidermis was separated from the dermis with forceps. Epidermal cells were isolated from the epidermis sheets, while dermal fibroblasts were separated from the dermis by further trypsinization at 37°C for 30 min. The epidermal cells were grown in Eagle's minimum essential medium supplemented with 10% or 20% fetal calf serum at 37°C under 5% CO_2 in air. Further details were reported elsewhere [1]. We also used a new method of explant-outgrowth culture for biopsy specimens, as described later.

Cultures of epidermal cells consisted of tightly packed polygonal cells with a characteristic epithelial appearance. Some melanocytes were scattered singly in the sheets of epidermal cells, but scarcely any fibroblasts were found in the primary cultures (Fig 1*A*).

Many optimal culture conditions have been reported for the isolation and cultivation of epidermal cells. In our experience, the addition of cholera toxin and epidermal growth factor (EGF) greatly enhanced the growth of epidermal cells [2,3]. The growth of epidermal cells is considerably improved by plating on a feeder layer of irradiated 3T3 cells or C3H10T1/2 cells [3]. However, feeder cells were not used in the present study to avoid complications from the presence of living cells from another source.

Benzo[a]pyrene Metabolism in Human Epidermal Cells Because they are located on the surface of the body, epidermal cells are exposed to sunlight and environmental carcinogens. Benzo[a]pyrene (BP) and other polycyclic aromatic hydrocarbons are prevalent contaminants in the air, water, and soil.

We found that human epidermal cells are capable of metabolizing BP [1,4–6]. Analysis of BP metabolites by high-performance liquid chromatography indicated that epidermal cells metabolize BP preferentially at non-K-regions, such as positions 7–10, forming a precursor of the ultimate metabolite, BP-7, 8-dihydrodiol-9,10-epoxide. The metabolic activity was further demonstrated by a cell-mediated assay, in which V79 Chinese hamster cells were cocultured on top of sheets of human epidermal cells and treated with BP for 48 h for induction of mutagenesis [1,4,5].

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Abbreviations:
AHH: arylhydrocarbon hydroxylase
BDBU: phorbol-12-13-dibutyrate
BP: benzo[a]pyrene
EGF: epidermal growth factor
ODC: ornithine decarboxylase
SCC: squamous cell carcinoma
TPA: 12-O-tetradecanoylphorbol-13-acetate
UDS: unscheduled DNA synthesis
XP: xeroderma pigmentosum

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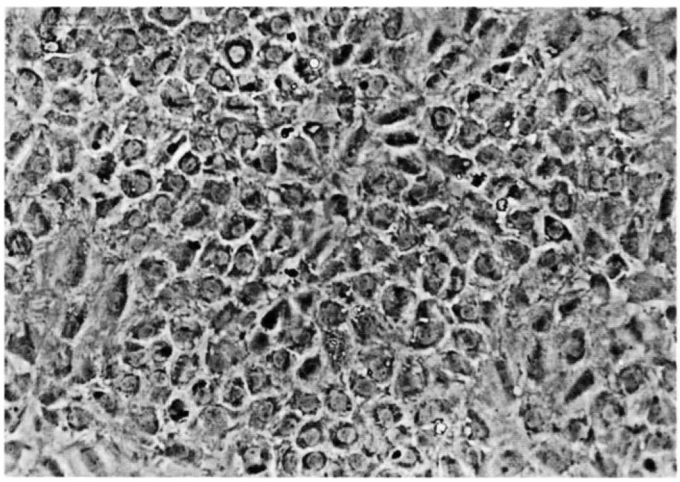

Figure 1. Phase-contrast picture of human epidermal cells.

The most striking observation was that human epidermal cells did activate BP to a form that was mutagenic to co-cultured V79 cells, but not dermal fibroblasts (Fig 2). In contrast, mouse fibroblasts activated BP and induced mutation in co-cultured V79 cells, indicating interspecies variation in metabolic activation of BP between human and mouse fibroblasts [5].

Subsequently, we measured activity of arylhydrocarbon hydroxylase (AHH), which metabolizes BP, of cultured epidermal and dermal cells with and without induction by 13 μM benz[a]anthracene for 24 h [6]. As shown in Fig 3, large inter-individual variations were found in the basal (mean: 3.9 units; SD: 4.2; range: 0 – 16.3) and induced AHH activities (mean: 97.4 units; SD: 69.4; range: 2.7 – 216.9) and the induction ratio (mean: 51.1; SD: 63.0; range: 0.6 – 262.0) (one unit was defined as 1 pmol of 3-hydroxy BP formed/mg protein/h). The basal and induced AHH activities of dermal fibroblasts were both lower than epidermal cells and distributed in a narrow range.

A major question about AHH activity and its inducibility is the relation of these factors to carcinogenesis. Kellermann et al [7] reported that the increased inducibility in cultured lymphocytes is associated with susceptibility to bronchogenic cancer, although this observation evoked much controversy. Follow-up of the donors in the present study will be interesting.

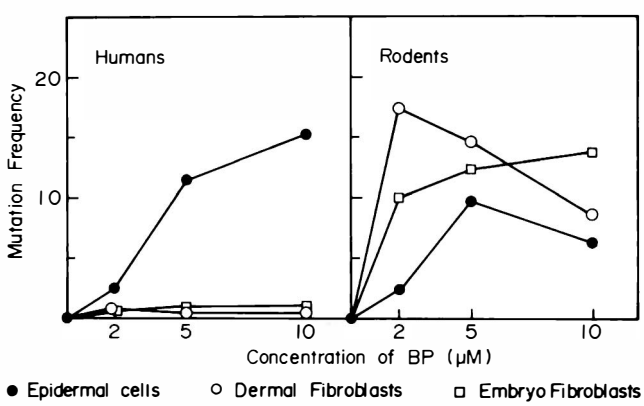

Figure 2. Induction of mutations in cell-mediated mutation assay in which BP was activated by human cells *(left)* or rodent cells *(right)* [5]. *A*: Human cell mediated assays in which epidermal cells *(closed circle)*, dermal fibroblasts *(open circle)*, and IMR-90 fibroblasts *(open square)* were used as an activating layer. *B*: Rodent cell-mediated assays in which epidermal cells *(closed circle)* and dermal fibroblasts *(open circle)* isolated from newborn C3H mice and rat embryo fibroblasts *(open square)* were used as the activating layer. Mutation in co-cultured V79 Chinese hamster cells, measured by resistance to 1 mM ouabain, is expressed as the number of resistant colonies per 10^5 survivors.

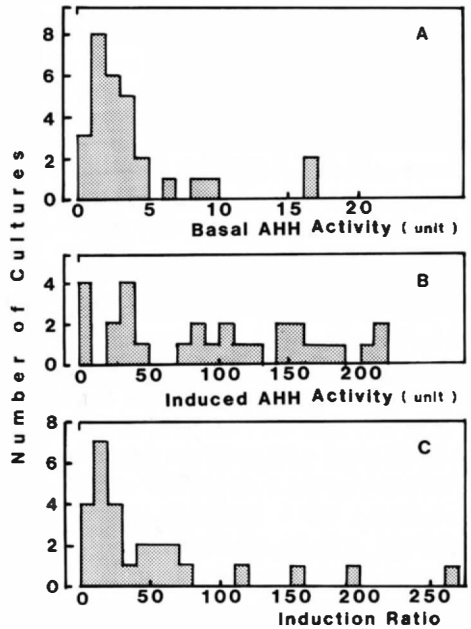

Figure 3. Frequency distribution of basal AHH activity (*A*), induced AHH activity (*B*), and the induction ratio (*C*) in 29 human epidermal cell cultures [6].

UV-Induced Unscheduled DNA Synthesis in Human Epidermal and Dermal Cells Sunlight is the primary stimulus for most human skin cancers. Despite the fact that epidermal cells are a target of UV-carcinogenesis, most information on DNA repair has been obtained from studies on cultured dermal fibroblasts. We measured UV-induced unscheduled DNA synthesis (UDS) of human epidermal cells on autoradiographs [8]. UDS was induced dose-dependently at doses of 5 to 20 J/m² to almost the same extent as mouse epidermal cells. In human dermal cells, the number of grains was about 3.3 times that in epidermal cells.

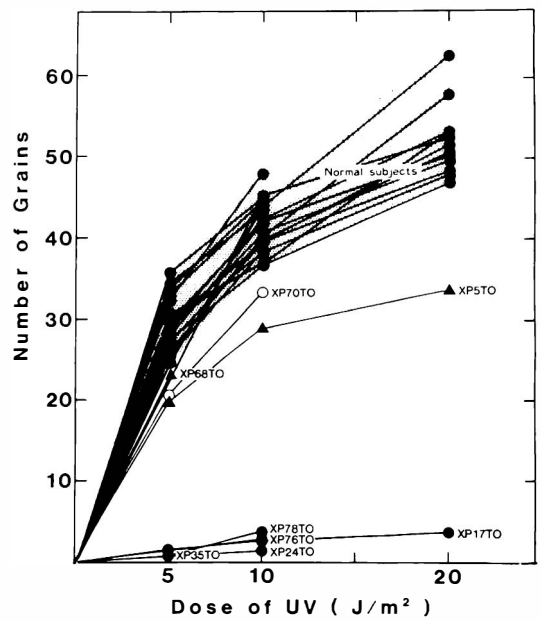

Figure 4. Dose-dependent induction of UDS by UV irradiation in normal and XP epidermal cells [9]. The range of UDS in normal epidermal cells is indicated by the *shaded area*. XP epidermal cells were isolated from patients of XP-A *(closed circle)*, XP-E *(open circle)*, and the XP-variant *(closed triangle)*.

Xeroderma pigmentosum (XP) is a rare, autosomal recessive disease characterized by a high frequency of skin cancers and defect in excision repair of UV-induced DNA damages. We developed a new method for explant-outgrowth culture of epidermal cells from small biopsy specimens by which we could measure DNA repair of XP epidermal cells [9]. We found that cultured epidermal cells from XP patients had a defect in UDS induced by UV-irradiation, the extent being dependent on a complementary group of XP, but being the same as their dermal counterparts within the group excepting XP variants (Fig 4).

Binding and Actions of Phorbol Ester Tumor Promoters in Human Epidermal Cells The process of tumor promotion has been studied in detail in mouse skin with phorbol ester tumor promoters isolated from croton oil, e.g., 12-O-tetradecanoylphorbol-13-acetate (TPA). Although there have been many studies in TPA and other tumor promoters, in almost all of them nonhuman materials, mostly mouse cells, were used.

We demonstrated the presence of specific binding sites for phorbol esters in human epidermal and dermal cells in culture by assay of binding of [^{3}H] phorbol-12,13-dibutyrate (PDBU) to intact cells [10]. Human epidermal cells bound PDBU with Kd of 28 nM and a Nmax of 3.7×10^6 molecules per cell, while human dermal cells bound PDBU with a Kd of 27 nM and a Nmax of 2.1×10^6 molecules per cell (Fig 5). Although mouse cells showed the same affinity as human cells, mouse epidermal cells bound one-third as much as human epidermal cells, and mouse dermal cells bound one-fifth as much as human dermal cells. When precultured with unlabeled PDBU for 24 h, [^{3}H]PDBU binding decreased time dependently in all cells except human epidermal cells (Fig 6). Thus, the binding of phorbol esters to human epidermal cells is unique in that there is a large number of binding sites compared with mouse epidermal cells, and there is no down regulation.

We further studied biologic and biochemical responses of human epidermal cells exposed to TPA [11]. TPA inhibited DNA synthesis and sugar transport, and did not induce ornithine decarboxylase (ODC) activity, a key enzyme in polyamine synthesis. Because of these essentially negative responses, the effects of TPA on human dermal and mouse epidermal and dermal cells were also studied for comparison. These cells were found to behave differently from human epidermal cells in at least one way. These findings seem to cast some doubt on the possibility that phorbol esters act as a tumor promoter in human skin. In contrast, however, Verma et al [12] reported the induction in vitro of ODC by TPA in human-skin biopsy specimens incubated in serum-free Eagle's minimum essential medium. The possible cause of the difference in this and our studies is unclear.

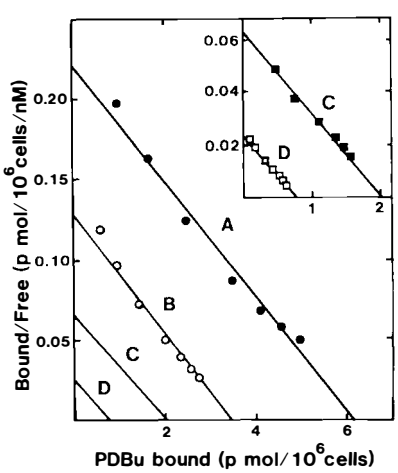

Figure 5. Scatchard plot analysis of specific binding of [^{3}H]PDBU to intact cells [10]. *Line A (closed circle):* human epidermal cells; *Line B (open circle):* human dermal cells; *Line C (closed square):* mouse epidermal cells; *Line D (open square):* mouse dermal cells. *Inset:* plots of *Lines C* and *D*.

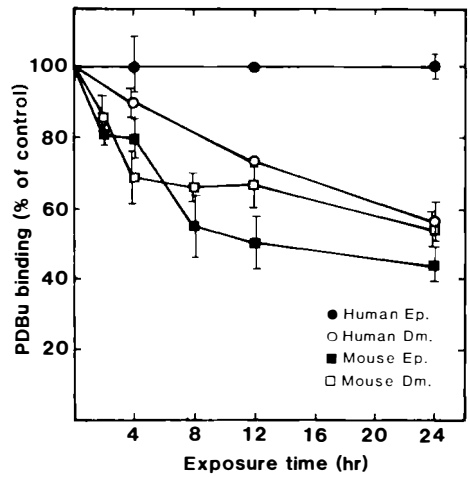

Figure 6. Down regulation of [^{3}H]PDBU binding in human epidermal cells *(closed circle)*, human dermal cells *(open circle)*, mouse epidermal cells *(closed square)*, and mouse dermal cells *(open square)* [10]. After preincubation with 30 nM unlabeled PDBU at 37°C for 2 to 24 h, bound PDBU was removed, followed by binding assay.

Possible Role of EGF Receptor in Carcinogenesis of Epidermal Cells Growth of human epidermal cells is enhanced by treatment with EGF. The effect of EGF is mediated by a cell surface receptor with a molecular weight of 170,000 that shows tyrosine-specific protein kinase activity and is coded by the proto-erb B oncogene.

We studied the role of EGF and its receptors in carcinogenesis using epidermal cells and their malignant counterparts, squamous cell carcinoma (SCC) cells [13,14]. EGF inhibited the growth and colony formation of all SCC cells at doses that are mitogenic in epidermal cells and dermal fibroblasts. This inhibitory effect was specific because EGF did not inhibit, and in some cases slightly stimulated, the growth of other tumor cells.

The amount of EGF receptors on SCC cells was measured by a binding assay of membrane preparations using ^{125}I-EGF (Table I). Of 13 SCC cell cultures tested, all except three of esophageal SCC showed higher levels of EGF receptor than normal epidermal cells, which contain 1.5×10^5 binding sites per cell. In general, SCCs of the skin and oral cavity had large amounts of EGF receptor in the order of 10^6 per cell. The values for the equilibrium dissociation constant (Kd) of these cells were on the order of nM. Amplification of erb B gene was also noted in SCC cell lines by "Southern" blotting assay. Sensitivity to the inhibitory effect of EGF correlated

Table I. EGF Receptors in Epidermal Cells and SCCs of the Skin, Oral Cavity, and Esophagus[a]

Cells	SCC of	Kd (nM)	N max: Sites per Cell ($\times 10^5$)	N max: Ratio to Epidermal Cells
Epidermal cells	—	2.5	1.5	1.0
A431	Vulva	5.0	36	24.0
HSC-1	Skin	5.3	79	52.6
HSC-2	Oral cavity	5.4	73	48.7
HSC-3	Oral cavity	4.1	5.6	3.7
HSC-4	Oral cavity	3.2	21	14.0
Ca9-22	Oral cavity	4.3	28	18.7
NA	Oral cavity	5.8	67	44.6
ZA	Oral cavity	4.0	10	6.7
TE-1	Esophagus	3.2	6.3	4.2
TE-2	Esophagus	2.1	1.0	0.7
TE-3	Esophagus	6.1	1.4	0.9
TE-4	Esophagus	5.8	1.2	0.8
TE-8	Esophagus	4.2	6.2	4.1

[a] Reference 14.

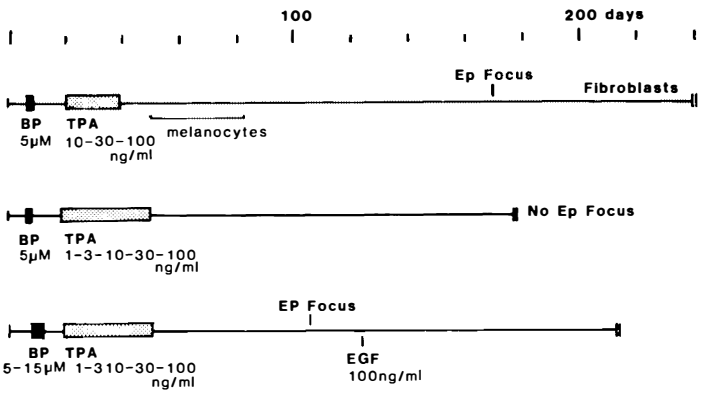

Figure 7. Attempts to transform human epidermal cells by the treatment with BP and TPA. Concentration of TPA was increased stepwise up to 100 ng/ml. *EP Focus:* focus of epithelial cells.

well with the elevated level of EGF receptors in 12 SCC cell lines. The present observations suggest that EGF and EGF receptors play a role in carcinogenesis of epidermal cells.

Attempts to Transform Human Epidermal Cells by Chemical Carcinogens One object of our studies is to transform human epidermal cells by chemical carcinogens. On the basis of the above knowledge, we have attempted to transform human epidermal cells in vitro with BP and TPA (Fig 7). We used TPA because it stimulates terminal differentiation, thus allowing the growth of undifferentiated cells, although TPA itself may have no promoting activity on human epidermal cells, as described above. With time of cultivation, most epidermal cells were sloughed off into the medium as a result of terminal differentiation, and foci of slowly growing epidermal cells appeared in two of three cultures after 110 or 170 d in culture. However, these epithelial foci were eventually occupied by fibroblastic cells. Thus, we have not yet succeeded in obtaining chemical transformation in vitro of human epidermal cells (unpublished data).

CONCLUDING REMARKS

Carcinogenesis studies using human tissues and cells offer unique opportunities. Extrapolation of carcinogenesis data and knowledge of its mechanisms from laboratory animal to human may be achieved by comparative studies using cells derived from humans and rodents. In the present series of studies, we found that human epidermal cells are very similar to mouse epidermal cells in terms of metabolism of BP and DNA repair of UV-induced damages, but they are unique in the binding and the responses to phorbol ester tumor promoters. These differences may be explained in the near future by regulation of gene expression.

Another characteristic of human cells is the lack of spontaneous transformation in vitro, which is possibly related to their chromosomal stability. In vitro transformation of human cells has proved to be much more difficult than that of rodent cells. Although several groups succeeded in obtaining neoplastic transformation of human fibroblasts, these experiments are not necessarily repeatable. Furthermore, fewer investigators have studied transformation of human epithelial cells (for review, see Ref 15). Yoakum et al [16] reported that transfection of v-Ha-ras oncogene transformed human bronchial epithelial cells, which may be due to enhanced genetic instability mediated by transfected ras, rather than direct actions of the ras gene product [15]. In our study, we found amplification and overexpression of erb B gene in SCC cell lines, suggesting possible involvement of erb B gene in carcinogenesis of epidermal and epithelial cells. We expect that human epithelial cells will be used for studies of oncogene-induced transformation, thus providing experimental evidence for causes of human cancers.

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