Metabolism of Benzo(a)pyrene by Human Epithelial Cells in Vitro

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SUMMARY

Primary cell cultures derived from human skin epithelium metabolized benzo(a)pyrene to three classes of compounds: phenols, quinones, and dihydrodiols. The relative proportions of metabolites varied according to the skin donor but differed from the pattern of metabolites in rat liver microsomal preparations. While appreciable amounts of 7,8- and 9,10-dihydrodiol; 1,6-, 3,6-, and 6,12-quinone; and 3- and 9-hydroxy derivatives were found in the medium, no 4,5 (K-region)-dihydrodiol or epoxide was detected. Reduced amounts of quinones were produced when the cultures were pretreated with hydrocortisone before exposure to the hydrocarbon. The cultures did not require a period of enzyme induction for efficient metabolism of the hydrocarbon. Cultures of fibroblasts derived from the same skin samples as the epithelial cells metabolized the hydrocarbon but to a much different extent. Preexposure of the epithelial cell cultures to mixtures of polycyclic hydrocarbons resulted in a decrease in the amounts of carcinogen metabolized to phenols and dihydrodiols.

These findings suggest that the prevalence of carcinomatous disease in humans is due to the differential capacity of the epithelial cells to metabolize potential carcinogens to active forms, a capacity reduced in fibroblasts or other nonepithelial cells. This suggestion is supported by the observations that supposedly normal prostate cells also efficiently metabolize polycyclic hydrocarbons in a manner similar to that of epidermal cells. No evidence of neoplastic transformation was seen in cytological preparations of cells exfoliated into the medium.

INTRODUCTION

In the study of factors contributing to the formation of human carcinomas, the rodent fibroblast culture system appears to be severely limited as a model. Not only do rodent cells undergo unpredictable “spontaneous” transformation (5) but they are also subject to rapidly developing aneuploidy (11) and a variety of viral particles (6). Human fibroblasts, while relatively easy to cultivate, seem to be less affected by most chemical carcinogens than are other cell types (4).

We have grown human epidermis in primary culture and exposed it to the polycyclic hydrocarbon BP,1 a suspect human carcinogen which has been extensively studied in microsomal systems (7, 12, 13).

The use of human primary epithelial cultures for the study of neoplastic transformation is important, since these differentiated human cells derive from an organ (skin) that is a primary target tissue for polycyclic aromatic hydrocarbons.

MATERIALS AND METHODS

Cell Cultures. The epidermal cell cultures were from 2 patients presented for elective rhytidectomies. Neither patient had a history of neoplasia. Cultures were established by removing the s.c. fat from the skin samples (3 x 4 cm) and by dissection of as much of the dermis as was practical. The skin was then cut into approximately 1-mm explants and placed epidermal-side-up in plastic flasks (Falcon T25 Falcon Plastics, Oxnard, Calif.) that had been heavily coated with undiluted horse serum (obtained from KC Biologials, Lenexa, Kans.). The explants were allowed to incubate in the serum for 12 hr in closed flasks at 37°. Following the incubation period, 2 ml of NCTC 135 medium with 10% horse serum supplement were added per flask. The explants were allowed to remain in the flasks until a clear outgrowth of epithelial cells could be seen, after which the explants were removed. Tissue specimens showed an unexpectedly long survival rate when stored in the cold in phosphate-buffered saline (0.0067 M Sorensen sodium-potassium-phosphate with 0.85% NaCl solution). Some specimens yielded viable cultures of epithelium after 10 days of storage. For assurance of the growth of epithelium without overgrowth by fibroblasts, periodic trypsinizations (0.25% trypsin) were performed. The fibroblast populations could thus be selectively removed without disturbing the epithelial elements. No attempt was made to passage the cells to other flasks. Growth of confluent cell sheets required from 2 to 3 months.

An inducing solution composed of crude MPH was prepared by dissolving commercial-purity BP, methylcholanthrene, dimethylbenzanthracene, pyrene, and pyrenequinone in acetone. This solution was diluted in medium to give a concentration of 5 μg of BP, 0.1 μg of dimethylbenzanthracene, 1 μg of methylcholanthrene, 1 μg of pyrene, and 0.05 μg of pyrenequinone per ml of medium. These reagents were used as: BP, benzo(a)pyrene; MPH, multiple polycyclic hydrocarbons.
gents were obtained from Aldrich Chemical Co., Milwau-
kee, Wis. Cells were exposed to inducing solution for 5 days
followed by 3 washes with culture medium and introduction of
labeled BP.

Hydrocortisone was obtained in “chromatographic” pu-
rity from Calbiochem, La Jolla, Calif.

**Labeled Compounds and Other Reagents.** Tritiated BP
(17 Ci/mmole) was purchased from Amersham/Searle
Radiochemical Centre, Amersham, England, and was di-
luted with cold BP to a specific activity of 400 mCi/mmole.
This was purified by elution with benzene from a mi-
crocolumn of silica gel (15 × 6 cm). The labeled compound
was recovered from the solvent and dissolved in dimethyl
sulfoxide to a concentration of 1 μg BP per μl. BP was
handled under red light, and cultures were incubated in the
dark.

Cultures were treated with BP by adding labeled com-
pound to give a concentration of 10 μg of BP per ml of
medium. The cultures were incubated for 4 days in the dark.
Equivalent amounts of dimethyl sulfoxide alone showed no
effects on control cultures, and commercial analytical-grade
solvent was apparently sterile because we found no mi-
crobial contamination in any of our cultures after addition
of carcinogen dissolved in this solvent.

**Chromatography of Products.** Following incubation of
cultures with labeled BP, the medium was removed and
stored frozen in the dark until extracted. The stored medium
was thawed, extracted 3 times with 4 volumes of ethyl
acetate, and dried over anhydrous magnesium sulfate. The
solvent was removed under vacuum, and the metabolites
were dissolved in 0.1 ml of methanol. Separation of tritiated
metabolites was performed on a DuPont Model 830
high-pressure liquid chromatograph fitted with a reverse-
phase Permaphase ODS 1-meter column. Peak identifica-
tion was by comparison with authentic compounds synthe-
sized on National Cancer Institute Contract NOI-CP-33387
and by 14C-labeled internal standards (12). The eluted
products were collected in 0.2-ml fractions and counted for
1H and 14C content in a Beckman Model 350 liquid
scintillation counter, using Aquasol (New England Nuclear,
Boston, Mass.) counting cocktail. Concentrations of me-
tabolites were calculated from the radioactivity. Results are
presented as the percentage of metabolites produced in
relation to total isotope recovered from the medium.

**Papanicoulaou Smears.** In addition to the metabolism
study, the medium was collected from each culture once a
week after carcinogen treatment for cytological examina-
tion of exfoliated cells. The medium was passed through a
Millipore type HA filter in a Swinnex filter holder (Mil-
lipore Corp., Bedford, Mass.). The filter was immediately
fixed in 95% ethanol, stained by a standard Papanicoulaou
technique (Ortho Diagnostics, Raritan, N. J.), and exam-
ined for evidence of neoplastic transformation.

**Electron Microscopy.** Cells were fixed with 2.5% glu-
taraldehyde in cacodylate-sucrose buffer (pH 7.3). Post-
fixation in chrome-osmium was followed by staining en bloc
with 1% uranyl acetate in 50% ethanol. Cells were dehy-
drated in graded ethanol, embedded in Epon-Araldite,
sectioned with a diamond knife, double stained with uranyl
acetate and lead citrate, and examined in a Siemens IA
electron microscope.

**Estimation of Cell Growth.** The numbers of cells per flask
were not accurately determined since the flasks were moni-
tored for transformation and cell sheets were not disrupted.
However, nuclei per unit of surface were counted to yield a
crude estimate of 8 × 10⁴ cells/flask.

**RESULTS**

**Growth of Epithelial Cultures.** After explantation, a lag
period in epithelial cell growth of from 3 to 15 days
occurred, a period highly variable from explant to explant.
Usually, epithelial cells in fan-shaped growth pattern would
appear to “flow” off the explant away from the tissue
fragment. At the growth front of this confluent sheet, a
“wave” of cells invariably heaped up into a ridge that
bordered the cell sheet (Fig. 1a). Epidermal cells always
grew cohesively and not as single isolated cells or as small
clumps.

**Effects of Exposure to BP.** Some cultures were exposed to
unlabeled BP (10 μg/ml) for 2 to 8 days without medium
renewal in an effort to provoke neoplastic transformation.
No obvious effects appeared for about 6 days, after which a
marked change occurred characterized by sloughing of
epithelial sheets and the appearance of floating detritus.
The lag period preceding these toxic effects suggested that a
period of enzyme induction might be required to activate the
carcinogen; accordingly, the MPH-inducing mixture was
used. As with BP alone a period of inappropriate toxicity was
followed by a widespread response.

In the Pananicoulaou-stained preparations, 2 principal
types of cells were found: epidermal cells in various stages of
keratinization (Fig. 1b) and histiocyte-like cells, presumably
of fibroblastoid origin. Most of the cells in the supernatants
are dead with pyknotic degenerated nuclei, making quanti-
tation of cell types impossible. “Tadpole”-like cells and
epithelial “pearls” (Fig. 1c), frequently found in clinical
material, were also observed in the culture fluids (9).

Fig. 1d shows a clump of intensely hyperchromic cells
with densely stained nuclei and cytoplasm. These types of
cells were found typically in cultures after carcinogen
treatments.

**Electron Microscopy.** The cells studied had most features
usually found in epithelial cells, including abundant desmo-
somes, prominent tonofilaments, and intricate interlocking
cell attachments. In some cells there are indications of
keratinization (Fig. 2).

**Metabolism of BP.** In cultures that received labeled BP,
there was abundant carcinogen metabolism even without
prior enzyme induction (Chart 1, A and E). Some differ-
ences appeared between cultures from the 2 donors with
respect to amounts of phenols, quinones, dihydrodiols, and
total metabolites produced. However, with both donors
little or no 4,5-dihydrodiol was produced; this pattern
occurred, a period highly variable from explant to explant.

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little or no 4,5-dihydrodiol was produced; this pattern
contrast with those of the metabolites produced by rat
liver microsomes (Chart 1H). Cultures from both donors
that were pretreated with the MPH mixture for 5 days
We also investigated the possibility that hydrocortisone could ameliorate the toxicity produced by either BP or the MPH mixture. In cultures from both of the patients reported here, pretreatment with hydrocortisone (2 μg/ml) did not affect toxicity but did alter metabolism, particularly inhibiting production of quinones (Chart 1F). With both donors simultaneous treatment with MPH and hydrocortisone had no effect on toxicity and only slightly lowered dihydrodiols as compared with MPH alone (Chart 1C).

To confirm the events in human epidermal cell cultures, we also exposed a culture of human epithelial cells from normal prostate to labeled BP and isolated the metabolic products. These epithelial cells also exhibited abundant carcinogen metabolism without prior enzyme induction. The pattern of metabolism (Chart 1G) is similar to that obtained with skin, although there was an increase in the amount of 9,10-dihydrodiol.

Cultures of fibroblasts isolated from the epidermal cultures of Patient 1 showed a reduced metabolism compared to that of epithelial cells (Chart 1D). In addition, there was no cytotoxic response as occurred with the epithelial cells from the same patient. The principal products were 7,8- and 9,10-dihydrodiol while the amounts of 9- and 3-hydroxy-BP were very small. No increase in quinones occurred over the blank nor was there formation of 4,5-dihydrodiol. An identical pattern of metabolism was observed with fibroblasts from a normal 3-year-old male child (American Type Culture Collection, CRL 1121, Pat Bru) in the 8th passage.

As noted earlier, the formation of quinones occurs spontaneously in culture medium alone. In epidermal and prostate cultures, the amounts of quinones are considerably increased over blanks indicating an active metabolic process. Formation of quinones, however, was decreased or undetectable in cultures of fibroblasts or in cultures pretreated with hydrocortisone.

**Evidence of Neoplastic Transformation.** There was no evidence to indicate that these cultures underwent neoplastic transformation as a result of the carcinogen treatment. There was a toxic reaction after carcinogen treatment, which was followed by a gradual decrease in the numbers of viable epithelial cells over the succeeding 3 months.

**DISCUSSION**

These experiments were an attempt to determine the effects of polycyclic hydrocarbon carcinogens on human epithelial cells in vitro, to investigate the differences in metabolism of BP in epithelial cells from different patients, and to contrast that metabolism with cultured fibroblasts from the same donor.

Contrary to our experience with cultivated human fibroblasts, epithelial cells in culture were quite sensitive to polycyclic hydrocarbons. There was a profound effect on cultures that resulted in extensive cell death and loss of epithelial sheets. In addition, epithelial cultures derived from epidermis underwent a rather constant rate of cell death and exfoliation in culture, an observation in keeping with the recent suggestions of Cairns (2) regarding the “mortality” of epithelial populations.

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**Chart 1.** Each clear bar represents the amount of metabolite recovered from the medium expressed as percentage of total metabolites recovered. Stippled portion of quinone bar, amounts of quinones found after incubation of cell-free, sterile medium with labeled BP. Hatched bar, percentage of total metabolites relative to total label recovered. All label not accounted for in the metabolites was contained in residual BP. The amounts of 4,5-diol are scarcely above the level of background. ?, unknown metabolite, 9,10, 9,10-dihydro-9,10-dihydroxy-BP; 7,8 7,8-dihydro-7,8-dihydroxy-BP; Q, 3 incompletely resolved quinones, BP-1,6-dione, BP-3,6-dione, and BP-6,12-dione; 9, 9-hydroxy-BP; 3, 3-hydroxy-BP; HC, hydrocortisone. A, metabolism of BP by epithelial cells from Patient 1. Cells received no pretreatment. B, metabolites produced after exposure of cell cultures from Patient 1 to MPH mixture. C, cells from Patient 1 treated with MPH and hydrocortisone prior to administration of labeled BP. D, distribution of metabolites found after incubation of tritiated BP with fibroblasts isolated from epithelial cultures from Patient 1. This pattern of metabolism was found in cultures of presumably normal fibroblasts from another source as well. E, profile of metabolites from Patient 2's epithelial cell cultures. Cells were not pretreated with xenobiotic substances prior to labeled BP. Compare differences in formation in quinones, phenols, dihydrodiols, and total metabolites (hatched bar) with those in A. F, metabolites formed in epithelial culture (Patient 2) that was treated with hydrocortisone prior to administration of labeled BP. Amounts of quinones were the same as those found in cell-free controls. G, products of incubation of tritiated BP with a culture of human prostate cells grown in primary culture. Note large amounts of dihydrodiols when compared with those of Patient 1. H, “normal” distribution of metabolites found on incubation of rat liver microsomes with tritiated BP.

showed a slight decrease in the production of dihydrodiols (Chart 1B). When 4 skin cultures from Patient 1 were treated with MPH followed by BP, the pattern of metabolites were almost identical (correlations at p < 0.01).
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While we found no evidence of a neoplastic transformation in any of our cultures, there was an injury reaction to the administration of the carcinogens similar to that seen in patients who have been irradiated or treated with chemotherapeutic agents (9).

Comparative differences in patterns of metabolism of BP were analyzed statistically. A significant correlation was found between increases in 7,8-diol and 9,10-diol (0.94 with \(p < 0.01\)), which indicates that epoxidation at these 2 positions may be linked in some way.

There seem to be 3 different chemically reactive sites for metabolic activity on the BP molecule (Chart 2). The most reactive site is at position 6 (10); the next site is at the 4,5 positions, the classical K region, which is a reactive locus for epoxidation and dihydrodiol formation by rat liver microsomes (12). Finally, there are a number of possible products formed in biological systems by reaction at the 7,8 and 9,10 positions. It is these latter products that have recently been suggested as an activated carcinogenic metabolite (14). Presumably, the phenols that are formed by biological metabolism are the result of epoxide rearrangement at the 9,10 position, or 2,3 position, or by an intermediary free radical mechanism initiated at carbon 6.

It is evident from these studies that there are differences between these 2 individuals in the ability of their epithelium to metabolize carcinogenic hydrocarbons, which is in agreement with the suggestion that there may be genetic variance in human lymphocyte induction of arylhydrocarbon hydroxylase (8). However, human carcinomas arise in epithelium and are the predominant forms of human cancer. Therefore, it is relevant to study the tissue of origin for the amounts of carcinogen metabolized and the types of products produced.

The absence of 4,5-dihydrodiol or 4,5-epoxide offers several interesting speculations. One possibility is that the products formed by oxidation at the 4,5 or K region are rapidly and efficiently converted to cell- or tissue-bound components and consequently do not appear free in the medium or that the epoxide is reduced back to starting material (1). While this is possible, whole-cell preparations of lymphocytes and microsomal preparations of human liver both form 4,5-dihydrodiol that is recoverable in the medium or incubation mixture (13).

Criteria for neoplastic transformation of epithelial cells are difficult to obtain. Establishment as long-term lines, alterations in growth habits, and formation of tumors in nude mice are all possible indicators of transformation. In addition, for epidermis, we believe that some information may be obtained from Paninicolaou smears of excised or exfoliated cells. In the cultures reported here, most of the epithelial cells were lost subsequent to carcinogen treatment. If transformation is to be demonstrated in vitro, it seems likely that less toxic carcinogens, or some means for reducing the toxicity, must be found to avoid complete removal of the epithelium.

Metabolism of carcinogens by cells not previously exposed to inducers of aryl hydrocarbon oxygenases may be a result of exposure to styrene polymers or monomers in the plastic culture vessels. We are now attempting to study this phenomenon using cells grown in glass flasks.

Metabolism of polycyclic hydrocarbons by cultured epithelial cells, derived from the epidermis and human prostate, indicates that the portion of human cancer. Therefore, it is relevant to study the tissue of origin for the amounts of carcinogen metabolized and the types of products produced.

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REFERENCES

Fig. 1. a, living epithelial cells in primary culture. The leading edge of the culture has a heaped-up “ridge” of cells. Phase contrast. initial magnification, × 100. (Stripes in flask are an artifact produced in manufacturing by the microwave molding process.) b, Papanicolaou-stained keratinized epithelial cells exfoliated into the medium. Cells were obtained from spent medium during regular medium replacement. No effort was made to dislodge cells, and the cells shown here are from a control culture that did not receive carcinogens. Initial magnification, × 200. c, an epithelial “pearl.” These whorled structures were frequently found in both treated and untreated cultures. Papanicolaou, initial magnification × 200. d, clumps of hyperchromic cells with dense nuclei and basophilic cytoplasm. Found typically as an indicator of toxic reactions to BP or MPH. Papanicolaou, initial magnification, × 100.

Fig. 2. a, desmosomes (D) with tonofilaments (arrowheads) typical of epithelium. Uranyl acetate and lead citrate, × 96,000. b, edge of cell with build-up of filaments into a cornified-like layer. Uranyl acetate and lead citrate, × 38,000. c, interdigitation of cells and their frequent connection by desmosomes (D). Uranyl acetate and lead citrate, × 30,000.
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