Human Breast Cell-mediated Mutagenesis of Mammalian Cells by Polycyclic Aromatic Hydrocarbons

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ABSTRACT

A system has been developed in which human breast cells activate chemical procarcinogens to mutagenic compounds. The degree of activation is quantitated by the estimation of induction of 6-thioguanine-resistant specific locus mutants in a cocultured Chinese hamster V-79 cell population which does not activate carcinogens. Both mammary stromal and parenchymal cells could activate the procarcinogen 7,12-dimethylbenz(a)anthracene. In addition, it is shown that the two mammary cell populations converted both 7,12-dimethylbenz(a)-anthracene and benzo(a)pyrene to water-soluble metabolites. The stromal cells produced substantial amounts of glucuronic acid conjugates, but the parenchymal cells did not. Both cell types metabolize benzo(a)pyrene to the organic-soluble metabolites 9,10- and 7,8-dihydrodiol and both 9- and 3-hydroxybenzo(a)pyrene. These results suggest that the human breast may be a target for polycyclic aromatic hydrocarbon carcinogenesis.

INTRODUCTION

Breast cancer is the most prevalent cancer in females over age 40 (1) but even though one of 11 women will develop this disease, no major causative factor is apparent. Environmental and physiological factors are being investigated in both epidemiological and laboratory studies. While it is possible that the human breast is susceptible to both physical and chemical environmental carcinogens, to date, only ionizing radiation has definitely been linked to the etiology of the human disease (4). There is currently no clear epidemiological link between human breast cancer and environmental chemicals, but such studies are difficult to conduct and interpret due to the large number of risk factors that may contribute to breast cancer susceptibility. Environmental chemicals such as the PAHs have, however, been shown to be potent mammary carcinogens in the rat (5, 15). It is thus important to determine, through laboratory studies, if the human breast is also a target for neoplastic transformation by these compounds.

Carcinogenic PAHs such as B(a)P and DMBA require metabolic activation in order to exert biological effects, which include cytotoxicity, mutagenesis, and neoplastic transformation (12, 22). Both rodent (8, 9) and human (10) mammary cell populations converted both 7,12-dimethylbenz(a)-anthracene and benzo(a)pyrene to water-soluble metabolites. The degree of activation is quantitated by the estimation of specific locus mutants in the in vivo rodent model (7). DMBA, a weak mammary carcinogen, is efficiently activated by both mammary stromal and parenchymal cells. Aflatoxin B1, a strong hepatocarcinogen which is not a mammary carcinogen in the rodent, cannot be activated by either mammary cell type, while it is activated by liver cells (19). B(a)P, a weak mammary carcinogen, was activated to mutagenic metabolites only by the stromal cells and not by the parenchymal cells in this assay. This may in part explain why B(a)P is a weaker mammary carcinogen than DMBA and also why aflatoxin B1 is not a mammary carcinogen.

An extension of these results with rodent cells to a human cell system would be useful in order to quantitate the activation of carcinogens in the human breast. Here, we report the development of a human mammary cell-mediated mutagenesis assay and demonstrate that human mammary cells can metabolize PAHs to mutagenic compounds.

MATERIALS AND METHODS

Media and Chemicals. The mammary tissue was placed into sterile medium in the operating room. This medium was Ham’s F-12 (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% fetal bovine serum (Sterile Systems, Logan, Utah), glucose (4.5 g/liter), amphotericin B (5 µg/ml), and antibiotics. The tissue was digested in Ham’s F-12 containing 5% fetal bovine serum, collagenase (type III; Worthington Biochemical Corp., Freehold, N. Y.) (2 mg/ml), hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) (0.2 mg/ml), insulin (Sigma, 5 µg/ml), amphotericin B (5 µg/ml), and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 µg/ml; gentamicin sulfate, 50 µg/ml). The mammary epithelial cells were cultured in a medium...
adapted from that of Smith et al. (28). The Dulbecco’s minimal essential medium was replaced with a-minimal essential medium (Flow Laboratories, McLean, Va.) and was buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The stromal cells were cultured in the same basic medium as that for the epithelial cells, with supplements of 10% fetal bovine serum, insulin (5 μg/ml), and glucose (4.5 g/liter). The growth of V-79 cells for mutation expression was in a-minimal essential medium with 10% fetal bovine serum. For selection of hypoxanthine-guanine phosphoribosyltransferase mutants, this medium was supplemented with 6-thioguanine (2 μg/ml).

Tritiated B(a)P (40 Ci/mmol) and DMBA (45.6 Ci/mmol) were obtained from Amersham Corp. (Arlington Hts., Ill.). Unlabeled compounds were obtained from Aldrich Chemical Co. (Milwaukee, Wis.) in the purest form available. For metabolism experiments, labeled stock solutions were prepared in DMSO and added to the appropriate medium for a final carcinogen concentration of 1 μg/ml and a specific activity of 210 mCi/mmol. Final DMSO concentration was 0.1%. Stock solutions were at least 98% pure as determined by HPLC. Authentic standards of B(a)P metabolites were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository.

Human Mammary Cells. These studies used primary human mammary cells that were dispersed from residual surgical material from reduction mammoplasties of healthy women 18 to 30 years old, using a modification of methods developed by Stamper et al. (30). The tissue was cut into fragments <1 mm and incubated in standard medium (4°) to the laboratory within 30 min of its removal. Tissue was then grossly dissected, and skin and apparent fat were discarded. The remaining tissue was finely minced and digested for approximately 16 hr, in the above collagenase-hyaluronidase enzyme solution at 37°. Next, 1 ml 0.05% DNase was added to each 35-ml aliquot of tissue suspension, and the mixture was incubated for an additional 10 min at 37°. After centrifugation, the supernatant containing the fat was discarded. The pellet was then resuspended in fresh enzyme medium as described above and digested at 37° until most cells were in clusters of 50 to 300. This was determined microscopically and usually required 1 to 3 hr. The suspension was centrifuged and the pellet was washed, resuspended in medium, and plated into tissue-culture dishes to separate the rapidly adhering (stromal) cells. At this stage, the amount of material plated was estimated by the weight of dissociated tissue, and cells were plated at a concentration of 5 tissue g, equivalent per 100-mm plate unless otherwise noted. The cells were then incubated for 2 hr. After this time, the unattached cells were removed, and the adhering cells were washed. The nonadhering cells were centrifuged twice at low speed, and the supernatants containing the majority of RBC and other monodispersed cells were discarded. The pellets containing mammary ductal fragments and small cell aggregates were resuspended and filtered through a nylon mesh filter with a pore size of 53 μm. The ductal fragments, which usually contained 25 to 100 cells, were washed from the filter. The number of fragments per ml was determined from several representative samples, and the average number of cells per fragment was estimated by phase microscopy (×320). From this information, the number of cells per ml of suspension was estimated and has proven to be ±50% when verified by more reliable but destructive techniques. Finally, the desired number of cells were plated at densities shown in Chart 4.

Evaluation of Mammary Cultures. Mammary cultures were evaluated for epithelial content by light microscopic criteria previously reported (6). In addition, both epithelial- and stromal-enriched cultures were examined by electron microscopy to confirm our light microscopy findings. For electron microscopy, cultures were fixed with 3% glutaraldehyde at room temperature for 45 min and postfixed in 2% osmium tetroxide at 4° for 45 min. The cells were then embedded in Epon 812 while still in their dishes. Liquid nitrogen was used to cleave the Epon- embedded cells from their dishes. Thin sections were cut, stained, and examined with a Hitachi H500 electron microscope.

Mediated Mutagenesis Assay. This assay was modified from one previously described in detail (6). Approximately 36 hr after plating, the mammary cells were irradiated with 5000 rads of 137Cs γ-rays. Cells from a Chinese hamster fibroblast-like line, V-79, were added to the mammary cultures at a density of 8 × 10^5/cm^2. After the V-79 cells attached (3 to 4 hr), DMBA (1 μg/ml) dissolved in DMSO was added to the cultures. Control cultures were treated with equivalent amounts of DMSO (0.1%). The cocultures were exposed to carcinogen for 42 hr, and washed, and the V-79 cells were then removed by selective trypsinization. They were then cultured for expression of hypoxanthine-guanine phosphoribosyltransferase mutants. After an optimal 6-day expression period, the V-79 cells were either plated in selection medium containing 6-thioguanine (2 μg/ml) for detection of mutants or were plated in standard medium for an estimation of survival. Mutant colonies were grown for 12 to 14 days, viability colonies were grown for 7 to 9 days, and numbers of induced mutations were calculated on a per-surviving-cell basis. Mutations obtained in these cells have been shown to be heritable for at least 3 months (6).

Carcinogen Metabolism. Stromal- and parenchymal-enriched cell cultures were grown in 100-mm dishes and after 36 hr in culture were labeled with medium containing either [3H]B(a)P or [3H]DMBA as described above. Control plates without cells were also treated with carcinogens. Medium was removed from the cells after 48 hr, unless otherwise noted, and extracted 3 times with 2 volumes of ethyl acetate to separate organic- and water-soluble extracellular metabolites. The radioactivity of multiple aliquots of both extraction phases was measured by liquid scintillation counting. To determine cell numbers, cells were removed from plates with trypsin-EDTA, diluted, and counted by hemocytometer. Samples for HPLC were prepared by drying the ethyl acetate phases with magnesium sulfate, flash evaporating the samples to dryness, and redisolving them in 50 μl of acetonitrile. Samples were centrifuged and filtered before injection.

HPLC analysis of the ethyl acetate-extractable metabolites of B(a)P formed by the mammary cells was performed on a Waters RCISS Radial PAK PAH C4 column using a gradient of 40 to 100% acetonitrile in water. Profiles were determined by the radioactivity of aliquots which was determined by liquid-scintillation counting. Quantitation of glucuronic acid conjugates of B(a)P and DMBA was determined by treating aqueous phases with β-glucuronidase (Sigma) for 4 hr at 37° (pH 5.0). The resulting mixture was then extracted with ethyl acetate as above. Control samples of medium were similarly incubated without β-glucuronidase and extracted in the same manner. Sulfate conjugates were assayed by treatment of aqueous phases with a-lysulfatase (Sigma) at pH 5.0 in the presence of saccharolactone. Samples were incubated overnight at 37°, neutralized, and extracted with ethyl acetate. Control samples without enzyme were similarly treated.

RESULTS

Evaluation of Cell Cultures. The attachment efficiency of the mammary epithelial clusters was >80% based on microscopic examination. The clusters spread rapidly on the dishes, forming epithelial-like cell islands that contained an average 10 to 100 cells. By light microscopy, >95% of the cells in the epithelial-enriched cultures were epithelial-like by morphological criteria. Electron microscopy confirmed the epithelial nature of these cultures (Fig. 1). Most cells possessed specific epithelial characteristics such as junctional complexes, desmosomes (Fig. 2), and microvilli (Fig. 1). We occasionally observed other cell types which included fibroblast-like cells and macrophages (Fig. 1). The paucity of nonepithelial cells at the electron microscope level was in agreement with our light microscopic observations.

The stromal-enriched fraction contained <5% morphologically identifiable epithelial cells at the light microscope level, and the lack of epithelial cells was confirmed at the electron microscope level. Most of the cells were fibroblast-like (Fig. 3),
although a few macrophages could also be identified from the electron micrographs.

**Carcinogen Metabolism.** To confirm the ability of these mammary cells to metabolize PAHs, Stromal- and epithelial-enriched cultures were treated with [3H]B(a)P (1 μg/ml), and medium was collected at various time points. The rates of conversion of B(a)P to WSM for both cell types are compared in Chart 1. Epithelial cells treated for 4 days converted 75% of the B(a)P to WSM, which was the maximum value obtained even in longer incubations of up to 8 days. The epithelial cultures did not show significant fibroblast contamination after up to 10 days. Stromal populations converted a maximum of 20% of the B(a)P to WSM over a 8-day incubation period. In all cases, <1% B(a)P incubated in the absence of cells for up to 8 days was found in the form of WSM.

Metabolism of DMBA was quantitated and compared to B(a)P in a similar fashion, using 48-hr incubations of cells with carcinogen. To determine whether the plateau in B(a)P metabolism seen in the previous data was due to loss of cell viability in long-term culture, cells were labeled with carcinogen at various time points after plating, ranging from 2 to 8 days. In all cases, levels of WSM production from B(a)P and DMBA and the HPLC profiles of B(a)P metabolites were identical, indicating that neither stromal nor epithelial cell cultures lost the ability to metabolize PAHs within the first 10 days in culture. Multiple experiments on cells from a single donor were performed; the results are shown in Table 1. DMBA was metabolized to a significantly lower extent than was B(a)P, on both a percentage of total carcinogen and a per cell basis. Both PAHs were converted to WSM more rapidly by epithelial- than stromal-enriched cell populations, even with compensation for differences in cell density. Similar experiments measuring B(a)P metabolism in mammary cells from another donor gave values of 5.1 ± 0.5% (S.E.) WSM production for stromal cells and 52.6 ± 2.5% WSM from epithelial cell populations.

In order to clarify some of the WSM, the aqueous phases were treated with β-glucuronidase to cleave any glucuronic acid conjugates that might be present. These results are also shown in Table 1. All medium samples treated with the same temperature and pH conditions without enzyme showed approximately 9% conversion of WSM to ethyl acetate-soluble forms, probably due to differences in partitioning of metabolites between the 2 phases due to low pH. This control value has been subtracted from the data shown. The epithelial cell cultures produced negligible levels of cleavable conjugates from the carcinogens, although significant amounts were produced by the stromal cells. In the case of B(a)P, HPLC analysis of the cleavage products from stromal cell populations indicated that these cells conjugated predominantly phenol derivatives to glucuronic acid, with a higher ratio of 9-hydroxybenzo(a)pyrene to 3-hydroxybenzo(a)pyrene (3.8) than is found in untreated extracellular medium (1.6). Aqueous phases from both fibroblasts and epithelial cells were treated with arylsulfatase to cleave any sulfate conjugates present. Less than 2% of the WSM were released in ethyl acetate-soluble forms in all cases, indicating that sulfates do not constitute a significant portion of the WSM formed by either cell type.

HPLC analysis of the ethyl acetate-soluble derivatives of B(a)P was performed in order to compare the metabolic patterns of each cell type. Profiles obtained from stromal- and epithelial-enriched populations are shown in Charts 2 and 3, respectively. The predominant metabolites in both cell types were the 9,10- and 7,8-dihydrodiol, with no detectable 4,5-dihydrodiol in either system. Both 9-hydroxy- and 3-hydroxybenzo(a)pyrene were detected as well. The identities of all metabolites were confirmed by addition of unlabeled authentic standards.

**Mediated Mutagenesis.** In order to determine if human mammary cells could metabolize the PAHs to active mutagens, we developed a human mammary cell-mediated specific locus mutagenesis assay. Chart 4 gives the results of an assay in which primary mammary epithelial cells activated DMBA (1 μg/ml) to metabolites that caused 6-thioguanine-resistant mutants.

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell type</th>
<th>No. of cells/sq cm</th>
<th>% of WSM</th>
<th>nmol WSM/10^6 cells</th>
<th>% of cleavage with β-glucuronidase</th>
<th>pmol cleaved/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA</td>
<td>Stromal</td>
<td>10^4</td>
<td>2.6 ± 0.3</td>
<td>12.3 ± 0.3</td>
<td>32.5 ± 2.5</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Epithelial</td>
<td>10^5</td>
<td>20.2 ± 1.7</td>
<td>24.1 ± 3.4</td>
<td>6.2 ± 0.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>B(a)P</td>
<td>Stromal</td>
<td>10^4</td>
<td>9.1 ± 1.1</td>
<td>39.8 ± 3.2</td>
<td>13.4 ± 2.7</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Epithelial</td>
<td>10^5</td>
<td>53.0 ± 0.3</td>
<td>71.8 ± 0.8</td>
<td>1.4 ± 0.1</td>
<td>3.4 ± 0.5</td>
</tr>
</tbody>
</table>

*a Cells were plated in 60-mm dishes as described in "Materials and Methods." *b Experiments were performed 4 times with 2 plates/data point.

* Percentage of total radioactivity added to the cultures that was found in the form of WSM.

* Calculated by determining the amount of radioactivity found in the aqueous phase after extraction. Cell counts indicated the attachment of 1.2x10^5 epithelial cells/sq cm and 2.6x10^4 stromal cells/sq cm in their respective media. 

* Percentage of total radioactivity found in the form of WSM and converting to nmol using the known specific activity of the compound.

* Percentage of total radioactivity found in the original aqueous phase converted to ethyl acetate-soluble form by β-glucuronidase treatment.

* pmol of conjugates released to the ethyl acetate phase was calculated as in Footnote c.
The results also demonstrate that there was an optimum density of mammary cells for the induction of mutants. Similar results were also observed with mammary epithelial cells obtained from 2 additional women.

In Chart 5, primary human mammary stromal cells were tested for their ability to activate DMBA (1 \(\mu g/ml\)). Like the epithelial cells, these cells metabolized DMBA to compounds which induced 6-thioguanine-resistant mutants in the cocultured V-79 cell populations. The number of mutants induced was related to the number of cells plated, which in turn was related to the number of g tissue from which they were derived. Again, comparable results were obtained with stromal cells from 2 other women. It should be noted that the DMBA was not totally metabolized in 48 hr at any of the cell densities used.

**DISCUSSION**

The ability of rat mammary cells to metabolize PAHs (8, 9, 23) and activate DMBA to DNA-binding intermediates (16) has been demonstrated. Recent studies have explored the modification and repair of this binding in rodent mammary cells in more detail (13, 31). Although the PAHs have not yet been proved human carcinogens, human mammary cells, a potential target of these compounds, have also been shown to convert PAHs to DNA-binding forms (20, 29). We have now shown that DMBA is metabolized by human mammary cells to mutagenic derivatives as well. All of these studies indicate the ability of human mammary tissue to metabolize the PAHs. Grover et al. (11) have examined the production of dihydrodiols of B(a)P and DMBA in human mammary epithelial cells and found production of the precursors to the highly reactive bay-region diol-epoxides of these carcinogens. Our examination of the ethyl acetate-extractable metabolites of B(a)P confirms this observation and extends it to the stromal cell population of human mammary tissue as well. These data suggest that human mammary tissues are indeed capable of metabolizing the PAHs to what is currently considered to be their ultimate carcinogenic forms (17).

In our experiments, both stromal and epithelial cell populations converted B(a)P and DMBA to WSM. These compounds include a number of conjugated forms which are generally considered to be biologically inactive (18). However, they do offer a good indication of overall rates of metabolism. We measured only small differences in the capacities of both mammary cell types to convert DMBA and B(a)P to WSM, and
similar values were obtained from tissues derived from 2 separate donors. The results of our treatment of the WSM with β-glucuronidase indicate a major difference in the ability of the stromal and epithelial cells to form glucuronic acid conjugates. The epithelial cells formed virtually no conjugates that could be detected in this assay system. Mehta and Cohen (21) also found little or no conjugation of B(a)P metabolites to glucuronic acid by human peripheral lung tissue. However, in their cell system, sulfates were major products, while none were detectable in our mammary cell systems.

While the study of the production of metabolites and DNA adducts from PAH-exposed specific cell types yields much interesting data, it does not provide sufficient information to quantitate biologically relevant damage. This is due in part to our lack of detailed information of the biological potency of many of the PAH metabolites and adducts. In studying the potential biological effects of xenobiotics, one ideally should be able to quantitate toxicity in an organ- and species-specific model. Here, we report the development of a human mammary cell-mediated specific-locus mutagenesis assay, and we demonstrate that both human mammary parenchymal and stromal cells can activate DMBA to forms which induce mutations in mammalian cells. While we have found this ability in mammary cells from several individuals, we do not yet have sufficient numbers to answer the question of individual variation in the capacity to activate carcinogens. When these human mutagenesis data are compared to our rat mammary mutagenesis data (6, 7), it appears that human mammary cells activate DMBA more slowly than do rat cells. We plan to confirm and quantitate this observation, accounting for both individual variability and minor differences in the experimental protocols that were used with human and rodent tissue.

Our results demonstrating the abilities of both human and rodent mammary cells to convert PAHs to mutagenic and possibly carcinogenic metabolites, coupled with the fact that these compounds are potent in vivo rodent carcinogens, suggest that the PAHs may be carcinogenic in the human breast. Obviously, many steps beyond metabolic activation are required for the production of a frank breast neoplasm. The question of susceptibility of the human breast to PAH-induced neoplastic transformation requires further laboratory and epidemiological investigations.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Electron micrograph of a primary culture of epithelial-enriched human breast cells. Note the darker cell which is a macrophage. × 3,850.

Fig. 2. Higher magnification of an epithelial culture showing the presence of many desmosomes. × 22,100.

Fig. 3. Electron micrograph of a fibroblast-like cell found in a mammary stromal-enriched culture. × 9,000.
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