Quantitation of Benzo(a)pyrene and 7,12-Dimethylbenz(a)anthracene Binding to Nuclear Macromolecules in Human and Rat Mammary Epithelial Cells

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ABSTRACT

Our laboratory has developed virtually identical techniques for the isolation and culture of mammary epithelial cells (MEC) from rats and humans. In a cell-mediated mutagenesis assay, rat MEC activated 7,12-dimethylbenz(a)anthracene (DMBA) but not benzo(a)pyrene [B(a)P] to mutagenic forms, and the opposite pattern was found with human MEC. These species-specific patterns were not readily explained by either qualitative or quantitative differences in Phase I metabolism of these compounds. In contrast, relative levels of covalent binding of these compounds to DNA in the human and rat cells under identical assay conditions generally parallel the pattern of the mutagenesis results, while not reflecting the absolute levels of metabolism in each system. The ability of the rat MEC to bind relatively higher levels of DMBA than B(a)P to nuclear DNA, and the reversed pattern in human MEC, was found at all incubation times tested between 6 and 48 h. Culture density was found to exert a greater effect on the levels of PAH-DNA binding in rat than in human cells, but in neither case did it affect the ratio of DMBA to B(a)P binding within a species. C5SO4 gradient separation of nuclear macromolecules from PAH-treated MEC revealed that the relative DNA binding levels of DMBA and B(a)P did not correlate with relative levels of nuclear protein binding. For both species, nuclear (DNA + protein) binding levels of B(a)P were approximately 2-fold higher than DMBA. However, these binding levels were 4 to 5-fold higher for both carcinogens in the human than in the rat MEC. The species-specific patterns of PAH activation shown by these cells suggest that caution should be used in extrapolating rodent carcinogenesis data to humans, for either quantitative or qualitative purposes.

INTRODUCTION

The ability of environmental xenobiotics such as the PAHs to induce mammary cancer in rodents is well documented (1, 2). It is generally assumed that those chemicals which are carcinogenic in rodent models will also prove to be human carcinogens. Like other environmental contaminants (3), the PAHs, which are lipophilic, have been shown to be stored in human fat (4), suggesting that breast tissue might be constantly exposed to these potential carcinogens. Given the ubiquity of a PAH in the environment, it is important to determine whether these chemicals are indeed carcinogenic to humans. Since in vivo human tumorogenesis studies are clearly not feasible, the question of how relevant in vivo rodent studies are to human carcinogenesis becomes a major concern. One approach to this question is to develop in vitro models for the biological activities of PAHs using rodent and human cells. Results from the in vivo and in vitro rodent systems can then be correlated, and this correlation extrapolated from the in vitro human model to the in vivo human situation.

In order to facilitate in vitro species comparisons, our laboratory has developed parallel methods for the isolation, culture, and characterization of rat and human MEC. Using virtually identical conditions, the abilities of these organ-specific cells to mediate PAH-induced mutagenesis in cocultured V79 cells were measured (5). In these studies, rat MEC were found to activate DMBA, but not B(a)P, to mutagenic forms. The opposite pattern was found with human MEC. In order to determine the biochemical basis for this species-specific difference, the metabolism of B(a)P and DMBA by the MEC was also studied (6). While minor qualitative and quantitative differences in metabolite profiles were observed, the only major species-specific difference in PAH metabolism observed was the ability of the rat but not the human MEC to convert both B(a)P and DMBA to glucuronic acid conjugates. Since these data, addressing primary metabolism and glucuronidation, did not yield any clear cut explanation for the mutagenesis results, our studies have been extended to another biochemical parameter, DNA binding, associated with the activation of PAH in cultured cells. Formation of covalent PAH-DNA adducts requires initial metabolic activation of the PAH (7) and has been documented in both human (8–10) and rat (11–13) MEC in culture. We have undertaken a quantitative study to compare nuclear DNA and protein binding levels of B(a)P and DMBA in rat and human MEC under identical conditions, and have found that the carcinogen-specific patterns of DNA binding qualitatively parallel the ability of these cells to mediate mutagenesis by the two compounds.

MATERIALS AND METHODS

Cell Isolation. Procedures for the isolation and culture of human and rat MEC have been previously described (5). Briefly, human MEC were derived from residual surgical material from reduction mammoplasties of healthy women 18–30 years of age. Tissue was grossly dissected, and skin and apparent fat discarded. The remaining tissue was minced, then incubated overnight in digestion mixture containing collagenase (Type III; Worthington, Freehold, NJ) and hyaluronidase (Sigma Chemical Co., St. Louis, MO) while shaking at 37°C. Rat MEC were derived from the pooled inguinal mammary glands of 6–12 female Sprague–Dawley rats, 50–60 days old. Tissue was cleared of lymph nodes, minced, and digested with collagenase (Type III; Cooper Biomedical, Freehold, NJ) for 3 h at 37°C.

Identical isolation techniques were then applied to the enzymatically digested preparations of human or rat MEC. D Natasha (Sigma) was added to samples during the final 10 min of incubation. The samples were centrifuged to remove fat, and the cell pellet was resuspended and plated into 90-mm plastic tissue culture dishes to separate out a rapidly adhering fraction containing stromal elements. The nonadhering cells were collected after 2 h and further fractionated by filtering through a 53-μm pore size nylon mesh filter (Tetko, Elmsford, NY). The filter was then inverted and washed to collect the fragments. The epithelial cells in the form of organoids (>10 cells/fragment) were counted and the numbers of cells were estimated. Primary cultures were plated at an approximate density of 1 × 10⁷ cells/90-mm dish. These were maintained for 7 days with regular feeding with "mammary medium" (see below), then trypsinized and replated into secondary cultures. Secondary cultures were maintained in culture for 3–7 days before addition of PAH.

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3 Abbreviations: PAH, polycyclic aromatic hydrocarbon; B(a)P, benzo(a)pyrene; MEC, mammary epithelial cells; DMBA, 7,12-dimethylbenz(a)anthracene; DMSO, dimethylsulfoxide.
Both human and rat mammary epithelial cells were cultured in mammary medium modified from Stampfer et al. (14). This medium consisted of 35% Ham’s F-12 medium (GIBCO, Grand Island, NY), 35% α modification of Eagle’s medium (GIBCO), and 30% medium conditioned by human umbilical vein endothelial cells. These cells were cultured in Medium 199 (GIBCO) supplemented with 20% fetal bovine serum and 150 μg/ml endothelial cell growth supplement (Collaborative Research, Waltham, MA). Thus, the final residual concentrations of fetal bovine serum and growth supplement in the mammary medium were 6% and 45 μg/ml, respectively. Mammary medium was supplemented with 10 μg/ml insulin (Collaborative Research), 5 ng/ml epidermal growth factor (Collaborative Research), 6.5 ng/ml triiodothyronine (Sigma), 0.27 ng/ml β-estradiol (Sigma) and 620 μg/ml gentamicin (United States Biochemical Corp., Cleveland, OH).

PAH Treatment of MEC. PAHs of the highest purity commercially available were purchased from Aldrich Chemical Co., Milwaukee, WI. Amersham Corp., Arlington Heights, IL, was the source of [3H]DMBA (>5 Ci/mmol) and [3H]B(a)P (150–210 Ci/mmol). [3H]PAH stocks were prepared in DMSO, then added to culture medium to provide a final PAH concentration of 2 or 4 μM, a final DMSO concentration of 0.05%, and a specific activity of 2–4 Ci/mmol. The purities of [3H]PAH stocks were regularly checked by high-performance liquid chromatography and maintained at >95%.

MEC were labeled by the addition of 8 ml of fresh medium containing [3H]PAH as described above to each 90-mm dish. These plates were incubated for the chosen time period at 37°C. Extracellular media were then collected and the cells washed with phosphate buffered saline. Aliquots of medium were assayed for the conversion of PAHs to water-soluble metabolites. Samples were extracted three times with two volumes of ethyl acetate and the distribution of radioactivity between the organic and aqueous phases was determined. Organic solvent-soluble fractions were analyzed by high-performance liquid chromatography as previously described (6) to determine the levels of metabolites present. The amounts of water- and organic solvent-soluble metabolites were then added to determine total levels of PAH metabolism.

Isolation of Nuclear Macromolecules. Washed MEC were removed from culture dishes with 0.25% trypsin-0.02% EDTA. Aliquots of the washed cell suspensions were taken to determine radioactivity, DNA, and protein contents (15). Cells were resuspended in 10 mM Tris buffer, pH 7.0, containing 1.5 mM MgCl₂, 0.5% Triton X-100, and 0.5% sodium deoxycholate. Outer cell membranes were then disrupted with several strokes of a Potter Elvejhem homogenizer as previously described (6). Nuclei were collected by centrifugation and the completion of homogenization was microscopically checked. Nuclear pellets were frozen at −80°C for later analysis.

The binding of PAHs to nuclear DNA was analyzed by the methods of MacLeod et al. (16). Nuclei from carcinogen-treated MEC were resuspended in 6 mM guanidine-10 mM EDTA, pH 7.0, and sonicated on ice until nuclear lysis was judged microscopically to be complete. The lysates were extracted twice with three volumes of ethyl acetate to remove unbound carcinogen, and then layered onto 2.2 M Cs₂SO₄-10 mM EDTA, pH 7.0, in 9% DMSO in polyallomer tubes. Samples were centrifuged at 35,000 rpm for 41 h at 10°C. Tubes were then pierced from the bottom and 10 to 12 drop fractions collected. Aliquots of the original sample and each fraction were assayed for radioactivity, DNA, and protein content. DNA and protein were determined by the method of Erwin et al. (15). Specific activities of binding (pmol PAH bound/mg nuclear DNA) were calculated from the pregradient sample data in the case of total nuclear binding and from the pooled DNA- and protein-containing fractions for the specific macromolecular binding quantitations. The protein + DNA-containing fractions accounted for 95–98% of the total radioactivity in the pregradient samples.

Significance levels for interspecies comparisons were determined using a two-sample t test as described by Snedecor and Cochran (17).

RESULTS

Secondary cultures of MEC from rats and humans were prepared under identical conditions for studies of PAH metabolism (6) and DNA binding. These cultures were examined by immunofluorescent staining for keratin, and were determined to be approximately 90% epithelial. This method may underestimate culture purity, since some epithelial cells may remain unstained (5). No reproducible differences were found in the purities of primary versus secondary MEC cultures, or in rat versus human MEC. We have previously compared the abilities of freshly isolated MEC versus primary, secondary, and tertiary passage MEC cultures to metabolize PAHs and have found no loss of metabolic capacity in either human or rat cells under our current culture conditions (6 and Moore and Gould, data not shown).

Due to the differing relative levels of mutations induced in the cell-mediated mutagenesis assay, we wished to determine the corresponding levels of binding of B(a)P and DMBA to the DNA of human and rat MEC. Under the same dose (4 μM) and time (42 h incubation) conditions used for the cell-mediated mutagenesis (5) and PAH metabolism (6) studies previously reported, the results shown in Table 1 were obtained. These data, obtained from multiple determinations with MEC cultures of equivalent densities (see below), showed little variation between samples and reiterated the species differences found in the mutagenesis assay. In the case of B(a)P, the human MEC bound significantly higher levels of PAH to DNA than the rat cells (P < 0.005 as determined by application of a two-sample t test), while the opposite pattern was found with DMBA (P < 0.005).

In order to determine whether the species-specific patterns seen were unique to the relatively long incubation period (42 h) used in the mutagenesis assay, DNA binding levels were measured at various time points between 6 and 48 h. The results shown in Fig. 1 indicate that differences in B(a)P and DMBA binding levels within each species could be found at all time points tested (P ≤ 0.01 in all cases). In the case of DMBA binding in the rat and B(a)P binding in the human MEC, incubation periods longer than 48 h did not result in significantly higher levels of DNA binding per mg nuclear DNA (data not shown). Identical time courses of binding were found with either 2 or 4 μM PAH concentrations in the medium. All subsequent experiments measured binding after 24 h exposures to 2 μM PAH.

Since previous studies indicate that cell density has a significant effect on the quantitative ability of these MEC to metabolize PAHs (6), both rat and human cells were plated into secondary cultures at varying densities and the levels of nuclear DNA binding of the PAHs were measured. Fig. 2 shows the results of these studies. Density was measured as μg nuclear DNA/90-mm dish, which was found to give a more accurate estimate of cell density than cell counts (6). When mg nuclear protein/dish was measured, there were no consistent differences

<table>
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<th>Carcinogen</th>
<th>Species</th>
<th>pmol Carcinogen bound per mg DNA</th>
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<tr>
<td>B(a)P</td>
<td>Human</td>
<td>21.98 ± 2.08*</td>
</tr>
<tr>
<td>DMBA</td>
<td>Human</td>
<td>9.93 ± 0.98</td>
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<td></td>
<td>Rat</td>
<td>20.33 ± 0.83</td>
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* Mean ± SE.
in the DNA to protein ratios found in human and rat MEC cultures. Culture density appeared to have little or no effect on DNA binding levels of either PAH in the human MEC. In the rat cells, however, increased cell density was associated with increased levels of DNA binding measured per mg nuclear DNA. Parallel effects were found with B(a)P and DMBA. This occurs concurrently with a relative decrease in the metabolism of these compounds, measured on a per mg nuclear DNA basis, as culture density increases (6). Due to this density effect, only data obtained with cell cultures at equivalent densities were directly compared in these studies.

Relative levels of PAH binding to nuclear DNA and protein were examined by separation of these macromolecules from the nuclei of treated cells by isopycnic centrifugation on CsSO₄ gradients. The profiles of B(a)P and DMBA binding in rat MEC nuclei are shown in Figs. 3 and 4, respectively. Since these assays were not performed under RNase-free conditions, only low levels of binding to RNA (eluting in fractions 6–8) were observed and were not quantitated. The ratio of protein to DNA binding in the rat cells for B(a)P was approximately 5:1, while for DMBA the ratio was about 1:1. A set of representative quantitative data for the nuclear binding levels of B(a)P and DMBA in both rat and human MEC, obtained with cell cultures of equivalent densities, are presented in Table 2. These cultures were compared for levels of PAH metabolites formed, total nuclear binding (protein + DNA) and DNA binding levels in cells treated for 24 h with 2 μM PAH. Overall levels of nuclear binding were approximately 2-fold higher for B(a)P than DMBA in both species. In general, the human MEC bound 4 to 5-fold higher levels of both PAHs to total nuclear macromolecules than the rat MEC. Unlike the absolute levels of binding, the ratio of protein to DNA binding of each PAH remained constant at all cell densities tested. Of the total nuclear binding of B(a)P, 14.7 ± 0.8% (mean ± SE for four experiments performed at different cell densities) in the rat and 3.8 ± 0.5% in the human, measured by radioactivity, represented DNA binding and >92% of the remainder was protein binding. For DMBA, however, 50.3 ± 4.0% of the rat and 4.1 ± 0.1% of the human nuclear binding constituted DNA adducts.

**Table 2.**

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As indicated by Figs. 3 and 4, DNA binding in the rat MEC represented a significantly greater proportion of the total binding of DMBA than B(a)P (P < 0.001).

**DISCUSSION**

The purpose of these experiments was to clarify the interspecies carcinogen-specific differences in cell-mediated mutagenesis observed with the human and rat MEC (5). In those studies, incubation of rat MEC with DMBA, but not B(a)P, resulted in measurable mutations in cocultured V79 cells, while an opposite pattern of PAH-induced mutagenesis was found with human MEC. It has been demonstrated that B(a)P-DNA adducts formed in V79 cells cocultured with carcinogen-activating cells for a mediated mutagenesis assay are similar to those formed in the activating cells themselves, suggesting that the transfer of active metabolites is a reasonably efficient process (18). We have therefore measured the relative levels of binding of B(a)P and DMBA to the DNA of our activating MEC and have found that the mutagenesis data qualitatively parallel the relative levels of PAH-DNA binding in the MEC themselves under treatment conditions similar to the mutagenesis assays, indicating that the species differences in mutagenesis results were not simply a unique characteristic of the cell-mediated system.

Comparisons of the cytotoxicity of PAH doses used for these experiments (2–4 μM) further indicate carcinogen-specific patterns of activation of B(a)P and DMBA in the rat and human MEC. Neither B(a)P nor DMBA induced morphological alterations in cultured MEC at up to 8-μM concentrations in culture medium. Both compounds do affect reproductive viability of these cells, however. DMBA was mutotoxic to human and rat cells after in vitro treatment with 4 μM DMBA for 46 h. In contrast, under identical treatment conditions, B(a)P was at least five times more cytotoxic to human than rat MEC. Since identical levels of DNA binding are obtained in human MEC cultures treated with B(a)P at doses ranging from 1 to 4 μM, even though reproductive cytotoxicity increases over this dose range, we do not believe that loss of reproductive capacity plays a significant role in the DNA binding patterns observed.

DNA binding of PAHs is known to require prior metabolic activation of these compounds (7). However, this binding occurs at relatively low levels even under idealized in vitro conditions (19). Protein binding usually represents a major proportion of overall macromolecular binding of PAHs in cells (20), at least in part because of the greater amounts of protein than DNA present. Our data suggest that the relationship in MEC between PAH metabolism and each of these two forms of macromolecular binding are quite different. Levels of both metabolism and protein binding were up to 2-fold higher for B(a)P than DMBA in MEC from both rats and humans. However, protein binding of both PAHs in the human MEC represented a much greater proportion of the overall nuclear binding than in the rat cells, although the ratios of protein to DNA in the MEC from both species were similar. Levels of organic solvent-soluble relative to water-soluble PAH metabolites are also higher in human than rat MEC (Table 2), suggesting that protein binding in the MEC of both species may correlate with the levels of unconjugated PAH metabolites.

In the case of DNA binding, however, no such correlation between metabolic and relative DNA binding levels was found. While a relatively nonspecific model for bulk levels of protein binding is suggested by the above data, DNA binding levels followed a distinct species-specific pattern, with rat MEC exhibiting a greater capacity for DNA binding of DMBA than B(a)P, and the reverse for human MEC. This is the first direct quantitative comparison of carcinogen-specific DNA and protein binding levels in MEC from the two species, although several groups have studied PAH-DNA binding in human (8–10) or rat (11–13) MEC separately under differing conditions. Tay and Russo have found higher levels of DMBA binding to DNA in rat MEC from animals with increased physiological susceptibility to DMBA-induced carcinogenesis, which may be an effect of both increased binding efficiency and lower levels of DNA repair (12). However, quantitative binding levels may not be the key factor involved in the interspecies differences in mutagenesis observed; formation of specific adducts may also play a key role in interspecies differences. Phillips et al. have reported that the major DNA adducts of B(a)P formed in the rat MEC are not derived from the anti-B(a)P-7,8-dihydrodiol-9,10-epoxide (21), which has generally been considered the most active carcinogenic and mutagenic metabolite of B(a)P (22). This is consistent with our finding of little or no mutagenic activation of B(a)P by rat MEC (5). Tay and Russo have reported the apparent formation of DMBA bay region diol-epoxide-DNA adducts in rat MEC (12), which would also be consistent with our mutagenesis results (5). Other studies indicate that human MEC may form bay region diol-epoxide adducts of both DMBA (10) and B(a)P (8, 10), although the cell-mediated mutagenesis results indicate that only B(a)P is a strong mutagen in the human MEC system (5).

Our data indicate a qualitative correlation between relative in vitro mutagenicity and DNA binding levels in rat MEC, and in vivo carcinogenicity of B(a)P and DMBA in the rat mammary gland. This finding is in agreement with the general correlation observed in these parameters (23). However, when human MEC are tested for the same in vitro biological parameters, a pattern of PAH activation opposite to the rat is obtained. These data indicate that caution should be applied when extrapolating in

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* Kamiya, Eldridge, Clifton, and Gould, manuscript submitted for publication.
vivo rodent data to humans, and that further studies of the potential involvement of B(a)P in human breast cancer should be conducted.

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REFERENCES

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