Interspecies Differences in the Major DNA Adducts Formed from Benzo(a)pyrene but not 7,12-Dimethylbenz(a)anthracene in Rat and Human Mammary Cell Cultures

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ABSTRACT

Mammary epithelial cells from rats and humans show both quantitative and qualitative species- and carcinogen-specific differences in their abilities to activate benzo(a)pyrene (B(a)P) and 7,12-dimethylbenz(a)anthracene (DMBA). Previous studies of the DNA binding of these compounds in mammary epithelial cells demonstrated that rat cells bound relatively more DMBA than B(a)P to DNA under identical treatment conditions, while the opposite pattern was exhibited by human mammary epithelial cells. The specific DNA adducts formed in these cells after 24-h incubations with [3H]DMBA and [3H]B(a)P were analyzed to determine if there were qualitative as well as quantitative differences in the amounts of individual adducts. Similar proportions of specific DMBA and B(a)P DNA adducts were found in both rat and human mammary cells, although the total amount of adducts formed was significantly higher in the rat cells. In contrast, an essentially qualitative species-specific difference was observed in the major B(a)P-DNA adduct present in the rat and human cells. The major B(a)P adduct formed in the human mammary epithelial cells was identified as the (+)-anti-B(a)P-7,8a-dihydroxy-9,10-epoxide(BPDE)-deoxyguanosine adduct. However, this adduct was formed at very low levels in the rat mammary epithelial cells. The rat cells contained a large proportion of syn-BPDE adducts, and other unidentified B(a)P-DNA adducts. The high level of the (+)-anti-BPDE-deoxyguanosine adduct in the human but not the rat mammary cells is consistent with the potential role of (+)-anti-BPDE in the high mutagenic activity of B(a)P in the cell-mediated mutagenesis assays using the human mammary cells as activators, and the low mutagenic activity of B(a)P when rat cells were used as activators. The quantitative differences in the activation of DMBA by cells from these two species are also consistent with the cell-mediated mutagenic activities of DMBA using these cells as activators. These results suggest that the higher carcinogenic activity of DMBA compared to B(a)P in the rat mammary gland may not be indicative of the relative carcinogenic potencies of these compounds for human mammary cells.

INTRODUCTION

The ability of many PAHs to induce mammary tumors in rodents has been well documented (1, 2). Relative tumorigenicity varies widely among these compounds, however. For example, DMBA is a potent mammary carcinogen in rats, while B(a)P exhibits lower tumorigenic activity (2). Since PAHs such as B(a)P are ubiquitous environmental contaminants, it is of great interest to determine their potential human carcinogenicity. In the absence of clear-cut in vivo human data, the development of a rodent model which accurately reflects human risk is desirable.

One approach to this problem is the use of organ-specific epithelial cells in culture for interspecies comparisons. Methods are currently available for the successful isolation and in vitro growth of both human (3–5) and rat (6–8) mammary epithelial cells. The abilities of these cells in culture to metabolize PAHs (5, 8–12), as well as form PAH-DNA adducts (8, 12–16), have been demonstrated. Results of B(a)P-DNA binding studies in either rat or human cells have suggested that significant interspecies differences may be present in this organ system. Several laboratories have characterized B(a)P binding in human mammary cells and have identified (+)-anti-BPDE-DGuo as the major adduct formed (11, 12, 16). However, Phillips et al. did not detect this adduct in their study of B(a)P-DNA adducts formed by rat mammary epithelial cells treated in vitro with B(a)P (17). These results indicate the need for a direct interspecies comparison.

Our laboratory has recently optimized methods for the isolation and culture of rat and human mammary epithelial cells under identical conditions (18, 19). These cells have been directly compared for their abilities to convert DMBA and B(a)P to mutagenic forms in a mammalian cell-mediated assay (18). We have also examined and compared the metabolism of these PAHs by the mammary cells (19), as well as quantitated DNA binding under identical conditions (20). In these studies human and rat mammary epithelial cells exhibited clear interspecies differences in their relative abilities to activate DMBA and B(a)P. DMBA was found to be highly mutagenic in the rat mammary cell-mediated mutagenesis assay, while B(a)P exhibited little or no mutagenic activity. The opposite pattern was found in human mammary cell-mediated assays (18). These results are not easily explained by the results of a comparison of Phase I PAH metabolism in these cells. The only major species-specific metabolic difference demonstrated was the ability of the rat, but not human, mammary epithelial cells to convert both DMBA and B(a)P to g-hcruoric acid conjugates (19).

We have recently examined the levels of total DNA binding of DMBA and B(a)P in rat and human mammary cells, and have found that under assay conditions identical to those used in the mutagenesis studies, higher levels of cell-mediated mutagenesis were associated with higher levels of intracellular hydrocarbon-DNA binding in the mammary cells (20). A quantitative effect of cell culture density was found with the rat mammary cells, although DNA binding in the human cells appeared to be less affected by this factor. As long as cell cultures of the same density were compared, however, the ratio of DMBA to B(a)P binding within a species was not altered by the degree of cell confluence. After 42-h incubations with PAH, rat mammary cells bound approximately twice as much DMBA.
INTERSPECIES DIFFERENCES IN PAH-DNA BINDING

per mg cell DNA as B(a)P, with the reverse ratio found in human mammary cells.

The present study continues the interspecies comparison of DNA binding in human and rat mammary epithelial cells by comparison of the profiles of specific DNA adducts formed from both DMBA and B(a)P in these cells. This study was designed to confirm and extend the observation of an atypical B(a)P adduct pattern in rat mammary cells (17), as well as to determine whether qualitative as well as quantitative interspecies differences exist in PAH-DNA binding patterns in human and rat mammary cells.

MATERIALS AND METHODS

Cell Cultures. Human and rat mammary epithelial cells were isolated and cultured by identical methods as previously described (18, 19). Rat mammary tissue was obtained from virgin female Sprague-Dawley rats, 50–55 days old. Epithelial cells were isolated from the inguinal mammary glands of donor rats. After lymph node removal, mammary tissue was finely minced and placed in a solution of collagenase (2 mg/ml; Cooper Biomedical, Freehold, NJ, Type III) in minimum essential medium. This tissue suspension was incubated for 3 h with shaking at 37°C.

Human breast tissue was obtained from the residual surgical material from reduction mammoplasties. Donors were healthy women 18–30 years old. Tissue was collected aseptically in the operating room and immediately transported in 4°C medium to the laboratory. The tissue was grossly dissected, and skin and obvious mammary cell-free fat discarded. The remaining tissue, composed mainly of mammary parenchyma surrounded by dense connective tissue and some white fat, was finely minced and digested for 16 h at 37°C in Ham's F-12 medium containing 5% fetal bovine serum, collagenase (2 mg/ml; Type III), and hyaluronidase (0.2 mg/ml).

After this enzymatic dissociation, both rat and human tissue suspensions were processed identically to isolate epithelial cells (18, 19). DNase I (20 μg/ml) was added during the final 10 min of enzymatic incubation. Tissue suspensions were centrifuged and washed with medium to remove dissociated fat. Pellets containing mammary epithelial and stromal cells as well as blood elements were diluted and passed over a 53-μm pore size filter. This procedure traps most of the epithelial cells located in small ductal fragments on the filter, while most monodispersed stromal and blood cells pass through the filter. The trapped ductal fragments were rinsed, collected by reverse washing of the filter, and counted by phase microscopy, and plated into primary culture. After 3–5 days, cells were trypsinized and plated into secondary culture for treatment.

Preparation of [3H]PAH:DNA Adducts. Secondary passage cells were grown to 50–80% confluency in 90-mm culture plates. [G-H]B(a)P (Amer sham, Arlington, Heights, IL) was diluted with unlabeled B(a)P (Aldrich Chemical Co., Milwaukee, WI) in DMSO to a specific activity of 16.3–18.0 Ci/mmole and was added to the cultures in fresh medium at a final concentration of 2 μM B(a)P (0.5 μM/ml) and 0.1% DMSO. [G-H]DMBA (Amer sham) was diluted with unlabeled DMBA (Aldrich Chemical Co.) in DMSO and added to the cultures in fresh medium to yield the same final concentration of 2 μM. After 1 h, the cells were harvested by brief treatment with trypsin:EDTA followed by gentle scraping. Cells were washed twice and cell pellets were stored at −80°C.

The DNA was isolated from the cell pellets by homogenization in Tris buffer (10 mM Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulfate, pH = 8) and incubation at 37°C for 30 min with proteinase K as described previously (22). The mixture was extracted with chloroform:isoamyl alcohol (24:1, v/v) and the DNA precipitated with ethanol. The enzyme treatments, chloroform:isoamyl alcohol extraction, and ethanol precipitation were repeated. The DNA was then treated with RNase A, extracted, and precipitated with ethanol. The DNA was redissolved in 0.01 M Tris, pH = 7.2, and precipitated, then rinsed with ethanol and redissolved in 0.01 M Tris, pH = 7.2.

The isolated DNA was enzymatically degraded to deoxyribonucleosides (23). The DNA hydrolysates were applied to Sep-Pak C18 cartridges (Waters Associates, Milford, MA). Unreacted deoxyribonucleosides were eluted in 20 ml water and the PAH:deoxyribonucleoside adducts were eluted in methanol by washing the Sep-Pak with 1 ml methanol:water (40:60), followed by 6 ml methanol. Aliquots of each fraction were removed and the radioactivity in each was determined by liquid scintillation counting in aqueous counting solution (Amersham, Arlington Heights, IL). The PAH:deoxyribonucleoside adducts were then analyzed.

Immobilized Boronate Chromatography. The PAH:deoxyribonucleosides that contained cis-vincical hydroxyl groups were separated from the other PAH:deoxyribonucleoside adducts by chromatography on a column of (N-[(S)-m-dihydroxyboryl]phenyl)succinamylaminoethyl cellulose as described previously (22, 24). The PAH:deoxyribonucleoside adducts not containing cis-vincical hydroxyl groups were eluted with 10 mM morpholine, pH 9, buffer. The PAH:deoxyribonucleoside adducts containing cis-vincical hydroxyl groups were then released from the column with morpholine buffer containing 10% sorbitol. The [3H]-PAH:deoxyribonucleoside adducts eluted with each buffer were pooled and concentrated by chromatography on a Sep-Pak C18 cartridge. After elution of the buffer components with water, the PAH:deoxyribonucleosides were eluted from the Sep-Pak cartridges with methanol and the individual PAH:deoxyribonucleoside adducts were analyzed by reversed-phase HPLC.

HPLC Analysis of PAH:Deoxyribonucleoside Adducts. The B(a)P:deoxyribonucleoside adducts were chromatographed by HPLC on a 25-cm x 4.6-mm UltraspHERE ODS column (Waters Associates, Inc., St. Louis, MO). The column was eluted with methanol:water (46:54) for 34 min at a flow rate of 1.0 ml/min, then for 10 min with a linear gradient of methanol:water (46:54 to 55:45), followed by 24 min with methanol:water (55:45). Fifteen 0.3-ml fractions followed by 145 0.3-ml fractions were collected in scintillation vials. The radioactivity in each fraction was determined by liquid scintillation counting. An aliquot of [14C]-(+)-anti-BPDE:dGuo, prepared as described previously (22), was added to each sample prior to HPLC analysis.

Hydrolysis of B(a)P:Deoxyribonucleoside Adducts. Individual B(a)P:deoxyribonucleoside adducts for acid hydrolysis studies were prepared by immobilized boronate chromatography and reversed-phase HPLC as above, without addition of the [14C]-(+)-anti-BPDE:dGuo marker. A 20-μl aliquot of each HPLC fraction was analyzed for radioactivity by liquid scintillation counting and the fractions containing each peak of radioactivity were pooled. B(a)P:deoxyribonucleosides were hydrolyzed to tetraols as described previously (25) using a modification of the procedure described by Shugart et al. (26). A portion of the pooled HPLC fractions containing the B(a)P:deoxyribonucleoside adduct peak was evaporated under a N2 stream to 5 μl, 50 μl 0.1 N HCl was added, and the sample was incubated for 6 h at 80°C. After neutralization, the samples were chromatographed as above by reversed-phase HPLC with a mixture of the 7,10/8,9-, 7,9/8,10-, 7/8,9,10-, and 7,9,10-tetraols of benz(a)pyrene purchased from the Chemical Repository, Division of Cancer Etiology, National Cancer Institute.

RESULTS

The binding of DMBA to DNA was compared in rat and human mammary epithelial cell cultures in order to determine...
if either the amount of DMBA bound to the DNA or the formation of specific DMBA-DNA adducts was related to the difference in the induction of biological effects by DMBA in these cell cultures (18). As previously reported (20), the level of DMBA binding to DNA was much greater in the rat mammary cell cultures (86.4 pmol DMBA bound/mg DNA) than in the human mammary cell cultures (12.0 pmol DMBA/mg DNA) of the same density after 24 h of exposure. The individual DMBA:deoxyribonucleoside adducts present were analyzed by enzymatic degradation of the DNA to deoxyribonucleosides and HPLC separation of the DMBA-modified deoxyribonucleoside adducts as described in “Materials and Methods.” The HPLC elution profiles of the human and rat mammary cell DNA samples are shown in Fig. 1, A and B, respectively. Both samples contain a large adduct peak (Fig. 1, A and B, peak 6) and a number of smaller adducts. In both samples the large adduct peak (peak 6) has been tentatively identified, based upon the studies of Dipple et al. (27, 28) and our own analyses (29), as a deoxyadenosine adduct. The DMBA-modified DNA from rat cells was also analyzed by immobilized boronate chromatography, peaks 2, 4, and 7 were formed from reactions of anti-DMBADE with DNA. Peak 4 has tentatively been identified as a deoxyguanosine adduct and peak 7 as a deoxyadenosine adduct of anti-DMBADE (27, 28). Peaks 1, 3, 5, 6, 8, and 9 were not retained by the boronate column and are probably syn-DMBADE:deoxyribonucleoside adducts. Peak 6 was therefore tentatively identified as the deoxyadenosine adduct of syn-DMBADE. The proportions of the individual DMBA:deoxyribonucleoside adducts formed in the rat and human cells were essentially the same, with only minor differences in some of the smaller adduct peaks. Thus, the proportions of adducts formed were virtually identical, but the rat cultures contained a larger total amount of DMBA-DNA adducts than did the human cell cultures.

In contrast to the results with DMBA, the levels of B(a)P-modified deoxyribonucleosides were higher in human mammary cell cultures than in rat cell cultures exposed to [3H]B(a)P for 24 h (16.6 pmol versus 10.1 B(a)P bound/mg DNA, respectively). The B(a)P-DNA adducts from both species were further analyzed by immobilized boronate chromatography to determine if the adducts resulted from reaction of a metabolite of B(a)P containing cis-vicinal hydroxyl groups, such as anti-BPDE (22). In the DNA sample from the human cell cultures, almost 80% of the B(a)P-DNA adducts contained cis-vicinal hydroxyl groups, but in the rat cell cultures the percentage was less than 50. Thus, there was a difference in the proportions of the metabolites responsible for the binding of B(a)P to DNA in the mammary cell cultures from rats and humans.

The individual B(a)P:deoxyribonucleoside adducts in the pooled morpholine and morpholine-sorbitol fractions from the immobilized boronate chromatography were then analyzed by HPLC (Fig. 2). The morpholine buffer fractions from the human mammary cell cultures (Fig. 2A) contained some material that eluted at the breakthrough volume of the column and two small peaks of adducts that eluted in the same relative positions as syn-BPDE-deoxyribonucleoside adduct markers (Fig. 2A, peaks M1 and M2). The rat mammary cell cultures

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Fig. 1. DMBA-DNA adduct profiles from human and rat mammary cells. HPLC elution profiles of the DMBA:deoxyribonucleoside adducts present in the DNA of human (A) and rat (B) mammary epithelial cell cultures after 24 h exposure to 2 μM [3H]DMBA. The DNA was degraded to deoxyribonucleosides and chromatographed by HPLC as described in “Materials and Methods.” The human cell sample contained 0.058 mg of DNA and 12.0 pmol DMBA bound/mg DNA. The rat cell sample contained 0.013 mg of DNA and 86.4 pmol DMBA bound/mg DNA.

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Fig. 2. B(a)P-DNA adduct profiles from human and rat mammary cells. HPLC elution profiles of the B(a)P:deoxyribonucleoside adducts present in the morpholine (A and C) and morpholine-sorbitol (B and D) elution fractions obtained by immobilized boronate chromatography of enzyme-digested DNA samples from human (A and B) and rat (C and D) mammary epithelial cell cultures exposed to 2 μM [3H]B(a)P. The DNA samples were degraded and analyzed by immobilized boronate chromatography and HPLC as described in “Materials and Methods.” The human cell sample contained 16.6 pmol B(a)P bound/mg DNA and the HPLC profiles were obtained from A, 0.23 mg DNA and B, 0.06 mg DNA. The rat cell sample contained 10.1 pmol B(a)P bound/mg DNA and the HPLC profiles were obtained from C, 0.29 mg DNA and D, 0.29 mg DNA. Arrow, elution position of a [14C]-(+)-anti-BPDE:3'Guo adduct marker.
contained several relatively large peaks of adducts which eluted in the morpholine buffer fractions (Fig. 2C, peaks M1 and M2), as well as a small amount of material that eluted at the breakthrough volume of the column. The largest peak (M1) eluted in the same relative position as syn-BPDE:dGuo and deoxyguanosine markers, M2 eluted in the same position as a syn-BPDE:dGuo adduct marker, and later peaks eluted in the region of deoxyadenosine adducts.

The morpholine-sorbitol buffer fractions from the human mammary cell culture DNA contained one major adduct peak (Fig. 2B, peak MS2). This peak coeluted with a [14C]-(+)-anti-BPDE:dGuo adduct marker. In contrast, the rat mammary cell DNA sample contained an adduct peak that eluted more than 20 fractions before the [14C]-(+)-anti-BPDE:dGuo adduct marker (Fig. 2D, peak MS1) and another that eluted almost 20 fractions later than this marker (Fig. 2D, peak MS3). Only a small peak of radioactivity eluted with the (+)-anti-BPDE:dGuo adduct marker (Fig. 2D, peak MS2). Based upon previous studies in rat embryo cell cultures, the early eluting peak (MS1) is an unidentified adduct that contains cis-vicinal hydroxyl groups but does not result from unmodified anti-BPDE, and the later peak (MS3) is a tetraol that results from spontaneous decomposition of syn-BPDE:DNA adducts (25). Thus, the DNA from human mammary cell cultures contained a significant amount of (+)-anti-BPDE:dGuo adduct (MS2) (13.6 pmol adduct/mg DNA), but the DNA from the rat cell cultures contains very little of this adduct (0.49 pmol adduct/mg DNA).

To confirm that the larger adduct peak (MS2) present in the human mammary cell DNA sample (Fig. 2B) was actually (+)-anti-BPDE:dGuo, this material was further characterized after conversion to tetraols by acid hydrolysis at 80°C, followed by HPLC (data not shown). The majority of the radioactivity coeluted in a single peak with synthetic 7,10/8,9-tetraol, a flavin adduct marker (Fig. 2Z, peak MS2). Based upon previous fractions later than this marker (Fig. 2D, peak MSB). Only a few of these compounds (30). This has been demonstrated for both levels of binding observed in the present studies as well as the specific identities of these adducts may be important for the biological effects of PAHs.

Species-specific differences in the abilities of human and rat mammary epithelial cells to activate DMBA and B(a)P to cytotoxic and mutagenic (18) derivatives have been demonstrated. This has been demonstrated as the most carcinogetic of the four enantiomeric bay region diol-epoxides of B(a)P (32, 33), and the deoxyguanosine adduct of this metabolite constitute the major B(a)P-DNA adduct formed in many biological systems, such as cultured cells (16, 25) and mouse skin (34). Persistence of the (+)-anti-BPDE:dGuo adduct has also been correlated with mutagenesis in human fibroblasts (35, 36). Current evidence thus suggests that both levels of total adduct formation as well as the specific identities of these adducts may be important for the biological effects of PAHs.

DISCUSSION

Covalent binding of PAHs to cellular DNA has frequently been correlated with the mutagenic and carcinogenic properties of these compounds (30). This has been demonstrated for both total binding levels of various PAHs (31) and for specific adducts. (+)-anti-BPDE has been demonstrated as the most carcinogetic of the four enantiomeric bay region diol-epoxides of B(a)P (32, 33), and the deoxyguanosine adduct of this metabolite constitute the major B(a)P-DNA adduct formed in many biological systems, such as cultured cells (16, 25) and mouse skin (34). Persistence of the (+)-anti-BPDE:dGuo adduct has also been correlated with mutagenesis in human fibroblasts (35, 36). Current evidence thus suggests that both levels of total adduct formation as well as the specific identities of these adducts may be important for the biological effects of PAHs.

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method to analyze B(a)P DNA adducts in mammary epithelial cells indicate that the patterns of adducts formed by rat mammary cells exposed to B(a)P in situ and in vitro are different, and neither is identical to that formed by human cells treated in vitro (43). It will be important to determine the biological significance of the metabolic activation of B(a)P by the pathways found in the rat mammary cells in vitro and in situ.

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