Distribution of Covalent DNA Adducts in Mouse Epidermal Subpopulations after Topical Application of Benzo(a)pyrene and 7,12-Dimethylbenz(a)anthracene

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ABSTRACT

The distribution of benzo(a)pyrene [B(a)P] and 7,12-dimethylbenz(a)anthracene (DMBA);DNA adducts was examined in five different subpopulations of SENCAR mouse epidermal cells separated based on buoyant density in continuous gradients of 61.5% Percoll. Three fractions consisted of primarily basal cells (Fractions 3 to 5), while two less dense fractions (Fractions 1 and 2) consisted of primarily differentiating keratinocytes. The levels of B(a)P and DMBA;DNA adducts were examined at 1 h, 6 h, 24 h, 72 h (except DMBA), and 28 days after a single topical application of an initiating dose. Among the basal cell subpopulations, the level of covalent B(a)P;DNA adducts in Fraction 5 cells was significantly higher (P < 0.05) than Fractions 3 and 4 at every time point examined. On the other hand, B(a)P;DNA adduct levels in Fraction 5 were only significantly higher than Fraction 2 at 6 h and 72 h and not significantly different from Fraction 1 at any time point. With DMBA, no significant differences were initially observed in the levels of covalent DNA adducts among the various Percoll fractions at 1 h and 6 h after treatment. However, at 24 h and at 28 days, Fraction 5 cells had significantly higher (P < 0.05) levels of covalent DMBA;DNA adducts than Fractions 1 to 4. To explore whether the observed differences in DNA adduct levels were due to differences in metabolic activation, we examined the levels of covalent adducts among epidermal subpopulations after topical application of (±)-a/αβiβ-β-lip-7,8-diol-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; dGuo, dcoxyguanosine; AAF, 2-acetylaminofluorene; HPLC, high-pressure liquid chromatography; MEM, minimal essential medium; PBS, phosphate-buffered saline.

INTRODUCTION

PAHs are widely distributed in our environment and are implicated in cancer of the skin and lung. Based on extensive evidence it is known that PAH must be metabolically activated to electrophilic intermediates which bind covalently to DNA in order to exert their mutagenic, cell-transforming, and tumorigenic effects (reviewed in Ref. 1). It is well established that good correlations exist between tumorigenic potential and mutagenic activity in both bacterial and mammalian mutagenesis systems for a wide range of chemicals (2, 3). In addition, many studies with diverse classes of chemical carcinogens have revealed a good relationship between the degree of carcinogenicity and overall extent of reaction with DNA (4).

A considerable body of information exists regarding the metabolic reactions leading up to and including the reactions of electrophilic metabolites with DNA (1, 5, 6). The most widely studied PAH carcinogen, B(a)P, forms covalent adducts with dGuo, dAdo, and possibly other bases in DNA in vivo (5). Although much attention has focused on the binding of B(a)P diol-epoxides to dGuo as the critical interaction, a more potent skin carcinogen, DMBA, binds extensively to dAdo residues in DNA (6). Dipple and coworkers (6, 7) have suggested that the total binding to adenine correlates most closely with the biological activity of DMBA. Studies performed in our laboratory (8) have further demonstrated a correlation between the formation of a specific DNA adduct (i.e., tentatively identified as DMBA-anti-3,4-diol-1,2-epoxide-dAdo) and the skin tumor-initiating activity of DMBA and its 9- and 10-fluoroderivatives.

Another important determinant in the carcinogenicity of PAH derivatives is the extent to which their DNA adducts are repaired and/or persist in the target cell DNA. Mouse epidermis is a well known target tissue for PAH carcinogens (reviewed in Ref. 9). Several reports have shown that mouse epidermal cells have the capacity to actively remove PAH;DNA adducts, but this capacity appears to be limited, and some percentage of the total DNA adducts formed persists for many weeks after exposure (10–14). The detailed time courses of binding of B(a)P (15) and DMBA (16) to total epidermal DNA in vivo have demonstrated that, once maximal binding levels are reached (at ~24 h after application), PAH;DNA adducts disappear from this tissue as a result of a biphasic decay process. Mouse epidermis consists of a heterogeneous population of cells at different stages of terminal differentiation (3, 17, 18). Several studies have suggested that the differentiation state of epidermal cells may play a role in their ability to undergo a DNA repair response (12, 19–22) and also may affect their ability to metabolize xenobiotics (23, 24). One possible mechanism for the biphasic disappearance of PAH;DNA adducts observed in whole epidermis is differential adduct removal in subpopulations of epidermal cells. Furthermore, it is generally believed that the target cells for both the initiation and promotion stages of chemical carcinogenesis in mouse skin are epidermal stem cells (reviewed in Ref. 25). Therefore, it is also important to establish the relationship between covalent binding and the differentiation state in mouse epidermis after exposure to initiating agents.

In the present study, we examined the distribution of covalent B(a)P; and DMBA;DNA adducts as a function of time after application in different subpopulations of SENCAR mouse epidermal cells separated on continuous Percoll gradients (18, 26). Our results demonstrate that covalent DNA adducts were present in all epidermal subpopulations isolated on Percoll.
gradients for at least 28 days after treatment with the hydrocarbon. Furthermore, of the 3 Percoll fractions consisting of basal cells, the fraction containing the least differentiated (most dense) cells (Fraction 5) developed the highest levels of covalent adducts within 24 h of treatment and retained these higher DNA adduct levels over a 28-day time period. Since this epidermal subpopulation contains the highest proportion of clonogenic cells (18), these observations may have relevance to the process of tumor initiation.

MATERIALS AND METHODS

Chromatography. B(a)P (Gold Label) was purchased from the Aldrich Chemical Co. (Milwaukee, WI). DMBA was obtained from the Eastman Kodak Co. (Rochester, NY). [3H]B(a)P (specific activity, 58 to 82 Ci/mmol) and [3H]DMBA (specific activity, 29 Ci/mmol) were obtained from the Amersham Co. (Arlington Heights, IL). (+)-anti-[3H]BPDE (specific activity, 1.18 Ci/mmol) was provided by the Chemical Carcinogen Reference Standard Repository, a function of the Division of Cancer Cause and Prevention, National Cancer Institute, NIH, Bethesda, MD. Percoll was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Density marker beads, fluorescein-conjugated lectins Ulex europaeus and Griffonia simplicifolia, DNase I (bovine pancreas, EC 3.1.21.1), snake venom phosphodiesterase (Echis carinatus, EC 3.1.21.1), alkaline phosphatase (Escherichia coli, type III, EC 3.1.3.1), and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). RNase A (EC 3.1.14.22) was supplied by Worthington Biochemical Co. (Freehold, NJ). Newborn calf serum and trypsin type polycarbonate tube, and the gradients were generated in a Beckman TI 60 rotor for 35 min at 17,000 rpm at 4°C. Epidermal cells isolated by trypsinization were resuspended at 15 to 20 × 10^6 cells/ml in calcium-free MEM, and 2 ml of this suspension were added to the top of the gradients. The gradients consisted of 61.5% Percoll diluted to a density of 1.086 g/ml by the addition of 10% lOx calcium-free MEM, 10% fetal bovine serum, 17.5% sterile water, and 1% gentamicin. The pH was adjusted to 7 with sterile HCl, and density marker beads were added. A 24.5-ml aliquot of this solution was added to each 28-ml Oak-Ridge type polycarbonate tube, and the gradients were generated in a Beckman TI 60 rotor for 35 min at 17,000 rpm at 4°C. Epidermal cells isolated by trypsinization were resuspended at 15 to 20 × 10^6 cells/ml in calcium-free MEM, and 2 ml of this suspension were added to the top of the gradients. The gradients were slowly accelerated to 1500 rpm and centrifuged for 20 min at 4°C. Five epidermal fractions were collected from these gradients to include densities of 1.062, 1.076, 1.088, 1.100, and 1.141 g/ml as indicated by the density marker beads. The cells were washed with PBS and resuspended as required for counting and immediate analysis or frozen at −20°C until ready for DNA isolation.

Fluorescence Microscopy. Separated epidermal cells were resuspended in PBS and fixed with 2% paraformaldehyde in PBS. The fluorescein isothiocyanate-conjugated lectins, G. simplicifolia 1 (specific for α-d-galactopyranosyl groups present on basal cells) and U. europaeus 1 (specific for α-L-fucose groups present on suprabasal cells), were used at a 1:1000 dilution (27). The nuclei were counterstained with 0.3 μg/ml of propidium iodide, and consecutive single cells were scored.

DNA Isolation. DNA was isolated from the cells by lysis with 0.6 M guanidine isothiocyanate and dialyzed against 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM NaCl for 24 h. DNA was purified by proteinase K and RNase A treatment followed by sequential extraction with phenol and chloroform-isooamyl alcohol (24:1). DNA was quantitated fluorometrically (28) using calf thymus DNA as a standard. The specific activity of binding is expressed as pmol of hydrocarbon bound per mg of epidermal DNA. Statistical analyses of the differences between mean DNA adduct levels were accomplished using the Student t test. The level of significance was set at P < 0.05.

Analysis of DNA Adducts. Isolated DNA samples were hydrolyzed to deoxyribonucleosides with DNase I, snake venom phosphodiesterase, and alkaline phosphatase (29). Prior to injection into the HPLC column, DNA hydrolysates were processed through a short column of Sephadex LH-20 as described (15). HPLC analyses were performed using a Dupont Series 8820 HPLC equipped with an Altex Ultrasphere ODS column (46 mm x 25 cm). The gradient system for [3H]B(a)P and anti- [3H]BPDE:DNA adducts was as follows: (a) 45% methanol in water (over 50 min); (b) 45 to 60% methanol in water gradient (linear, 40 min); and (c) 60 to 100% methanol in water gradient (linear, 15 min). The gradient system for [3H]DMBA was as follows: (a) 40 to 50% methanol in water gradient (linear, 50 min); (b) 50% methanol in water (over 10 min); (c) 50 to 60% methanol in water gradient (linear, 40 min); and (d) 60 to 100% methanol in water gradient (linear, 15 min). The column flow rate was 1 ml/min. Immediately after injection of samples into the HPLC, 0.5-min fractions were collected directly into scintillation vials. Radioactivity in each fraction was determined as previously described (11, 15).

RESULTS

Table 1 shows the sedimentation pattern and fraction designation of the mouse epidermal cells isolated in our present study by the trypsinization and Percoll gradient sedimentation procedures (Refs. 18 and 26; Footnote 5). Three basal cell fractions, each containing ≥93% viable basal cells, were recovered (Fractions 3 to 5). All three basal cell fractions demonstrated some heterogeneity with regard to cell size and morphology as determined by light microscopy. To further characterize the origin of the separated cells, two well-established cell surface determinants on basal and suprabasal cells were assessed by fluorescence microscopy (Table 2). The lower three fractions represented mainly basal cells. Cells of suprabasal morphology were identified primarily in the upper two fractions, which is in agreement with previous studies (Refs. 18 and 26; Footnote 5). It should be noted that only a small percentage of the cells making up Fraction 1 (at the top of the gradient) were viable (15 ± 6%). In the present study it was necessary to determine whether topical treatment with either B(a)P or DMBA altered the distribution of cells through the Percoll gradients. As shown in Table 2, no significant differences were found in the distribution of cells among the 5 fractions when analyzed 24 h after treatment with either acetone, B(a)P (200 nmol), or DMBA (10 nmol).

Fig. 1 summarizes a large number of experiments to examine the distribution of B(a)P:DNA (a) and DMBA:DNA (b) adducts in mouse epidermal cell subpopulations after topical application of initiating doses of these hydrocarbons. The data are presented as total binding, i.e., pmol of covalently bound hydrocarbon per mg of DNA (± SD), although essentially identical results were obtained if absolute DNA adduct levels were calculated from the radioactivity in methanol phases after passage of digested DNA samples through LH-20 columns (data not shown). Interestingly, throughout the 28-day time course B(a)P and DMBA:DNA adducts were present in all epidermal sub-

populations isolated from the Percoll gradients. At every time point examined, the highest levels of covalent B(a)P:DNA adducts (Fig. 1a) were found in the most dense subpopulation, Fraction 5, and in the least dense subpopulation, Fraction 1. The level of covalent B(a)P:DNA adducts in Fraction 5 cells was significantly higher (P < 0.05) than Fractions 3 and 4 at every time point examined, while only significantly higher (P < 0.05) than Fractions 3 and 4 at 6 h; and thereafter, significantly higher DMBA:DNA adduct levels (P < 0.05) were found in the DNA of Fraction 5 cells compared with Fractions 1 to 4. It should be noted that the doses of both hydrocarbons chosen for the present studies were within the linear portion of the dose-response relationship for tumor initiation and, in addition, these doses of DMBA and B(a)P produce a comparable skin tumor initiation response (8).

To determine whether any differences existed in the types of DNA adducts formed in the various epidermal subpopulations, we examined the formation of specific B(a)P:DNA and DMBA:DNA adducts at 6 and 24 h after application of the hydrocarbons. Fig. 2 illustrates the HPLC profiles of DNA adducts in epidermal subpopulations.
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B(a)P:DNA adducts formed in the 5 different subpopulations of mouse epidermal cells at 6 h after application of the compound. In addition, the B(a)P:DNA adduct profile from unseparated epidermal cells, prior to sedimentation through Percoll gradients, is shown in Fig. 2, Panel 6. With the experimental procedures used in these studies, three B(a)P:DNA adducts were detected with Peak II representing the majority of DNA-bound material. The identities of these peaks are based on criteria which are described in detail elsewhere (11, 30) and are for Peak I, 9-hydroxy-B(a)P-4,5-oxide-dGuo; Peak II, (+)-anti-BPDE-N²-dGuo; and Peak III, syn-BPDE-dGuo. No differences in the B(a)P:DNA adduct profiles from the 5 epidermal cell subpopulations were found at either of the time points examined, nor did these profiles differ from that of the unseparated epidermal cells. Insufficient radioactivity was present in the samples from the other time points to allow further analyses using the current methodology.

Fig. 3 illustrates the HPLC profiles of DMBA:DNA adducts found at 6 h after application of this hydrocarbon. Three major DNA adducts were found in all 5 epidermal subpopulations, similar to results obtained with whole epidermis (16) and unseparated epidermal cells isolated in the present study (Fig. 3, Panel 6). Characterization of these adducts has shown that they arise through reaction of the 3,4-diol-1,2-epoxides of DMBA with DNA (31–33). **Fig. 3.** HPLC profiles of B(a)P:DNA adducts formed in SENCAR mouse epidermal subpopulations 6 h after topical application of 200 nmol of [³H]B(a)P. Samples were injected into the HPLC and, immediately after injection, 0.5-min fractions were collected into scintillation vials. The radioactivity in each fraction was determined and is expressed as a percentage of the total radioactivity eluted from the column (i.e., % dpm) as previously described (8, 11). Peak I, 9-hydroxy-B(a)P-4,5-oxide-dGuo; Peak II, (+)-anti-BPDE-N²-dGuo; Peak III, syn-BPDE-dGuo. Panels 1 to 5, Percoll Fractions 1 to 5; Panel 6, unseparated epidermal cells.

**DISCUSSION**

The existence of different subpopulations of keratinocytes has been known for some time (reviewed in Refs. 18 and 25).

*Unpublished observations.*
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Fig. 3. HPLC profiles of DMBA:DNA adducts formed in SENCAR mouse epidermal subpopulations 6 h after topical application of 10 nmol of [3H]DMBA. Samples were injected into the HPLC and, immediately after injection, 0.5-min fractions were collected into scintillation vials. The radioactivity in each fraction was determined by scintillation counting and is expressed as a percentage of the total radioactivity eluted from the HPLC column (i.e., % dpm) as previously described (8, 11). Peak I, anti-DMBA-diol-epoxide-dGuo; Peak II, syn-DMBA-diol-epoxide-dAdo; Peak III, anti-DMBA-diol-epoxide-dAdo. Panels 1 to 5, Percoll Fractions 1 to 5; Panel 6, unseparated epidermal cells.

Fig. 4. Binding of anti-[3H]BPDE to DNA of mouse epidermal subpopulations 3 h after topical application. Groups of 10 to 20 mice were treated with 200 nmol of anti-[3H]BPDE each. Columns, average of three separate experiments; bars, SD. *, mean DNA adduct level significantly (P < 0.05) lower than that of Fraction 5.

In mouse epidermis, continuous Percoll gradients have been used to separate keratinocytes into 5 fractions (18, 26). Morphological, immunological, and autoradiographic studies have identified the 3 most dense fractions as consisting primarily of basal cells, while the uppermost fractions consist primarily of maturing keratinocytes (reviewed in Ref. 18 and Tables 1 and 2 of the present study). Further analyses have revealed that those cells making up the most dense Percoll fraction (i.e., Fraction 5) are enriched for clonogenic cells, DNA synthetic activity in vivo, and [3H]thymidine “label-retaining” cells. While the significance of these findings is not fully understood at the present time, such properties are believed to be characteristic of epidermal stem cells (17). Epidermal stem cells are believed to be important target cells for both the initiation and promotion stages of mouse skin carcinogenesis (reviewed in Refs. 25 and 35). An interesting observation in our present study was the finding that, of the 3 basal cell fractions obtained from similar Percoll gradients, Fraction 5 cells became enriched for B(a)P:DNA, DMBA:DNA, and BPDE:DNA adducts. These results, using biochemical techniques, confirm earlier preliminary observations (Refs. 18 and 36; Footnote 5) using autoradiographic techniques, demonstrating that the “carcinogen label” was higher in the slowly cycling population of epidermal cells (i.e., [3H]thymidine “label retaining” cells) 1 mo after application of radiolabeled B(a)P. Subsequent experiments have identified these slowly cycling epidermal cells as part of Fraction 5 cells from continuous Percoll gradients similar to those used in our present study (Ref. 36; Footnote 5). Our present results also extend these earlier observations and demonstrate that higher DNA adduct levels are actually present in Fraction 5 cells, compared with the other basal cell fractions (i.e., Fractions 3 and 4), as early as 24 h (or even earlier in the case of B(a)P and BPDE) after topical application, depending on the hydrocarbon.

Our current results also demonstrate the presence and per-
The presence of DNA adducts in epidermal subpopulations with little proliferative potential (i.e., Fractions 1 and 2), although not surprising, is probably of less significance for the process of tumor initiation than their presence in epidermal cells with high proliferative potential (Fractions 3 to 5). Nevertheless, DNA adducts were detectable in the more differentiated cell populations throughout the 28-day time course examined in the present study.

The exact mechanism(s) for the observed differences in adduct levels among epidermal subpopulations remains unknown at present; however, several possibilities exist. The two most obvious are (a) differential repair and (b) differential metabolism. With regard to the first possibility, several studies have suggested that the differentiation state of epidermal cells may play a role in their ability to undergo a DNA repair response. Nakayama et al. (12) reported that differentiating populations of newborn epidermal keratinocytes in culture efficiently removed DNA adducts formed from B(a)P. Furthermore, we recently reported (22) that cultures of differentiating mouse epidermal cells displayed significant DNA repair capacity when exposed to a variety of chemical carcinogens including B(a)P, DMBA, and BPDE. In contrast, Bowden et al. (19) suggested that epidermal basal cells, but not epidermal cells that have been committed to the differentiation pathway, were capable of repairing UV-induced DNA damage. Liu et al. (20) reported that the repair of UV light-induced DNA damage in human epidermal basal cells was much greater than in differentiated cells from the same skin preparation. Further experiments examining the DNA repair capacity of epidermal subpopulations will be necessary to determine the role of DNA repair in the different adduct levels observed among epidermal subpopulations in our present study. It should be noted that one of the original goals of the present study was to explore the possibility that the biphasic disappearance of B(a)P- and DMBA-DNA adducts in whole epidermis could be explained by different adduct disappearance rates among epidermal subpopulations. Clearly, this is not the case. In this regard, once total DNA adduct levels were maximal for B(a)P and DMBA, differences in adduct levels already existed among the epidermal subpopulations. Furthermore, the relative differences among these subpopulations were maintained even after 28 days, suggesting that adducts were subsequently disappearing from all epidermal subpopulations in a similar way.

An alternate hypothesis for the observed early differences in DNA adduct levels in epidermal subpopulations is that metabolic differences may lead to higher initial adduct levels in some epidermal subpopulations. Differences in metabolic capability among epidermal subpopulations as a function of differentiation have been reported (23, 24). In this regard, differentiating keratinocytes have been shown to have higher 7-ethoxyxoumarin-O-deethylase activities (23), as well as greater ability to metabolize both B(a)P and DMBA to DNA-binding metabolites (24). However, these differences do not appear responsible for the differences in DNA adduct levels we observed in the present study, since higher DNA adduct levels were observed in Fraction 5 cells compared with Fractions 1 to 4 following application of anti-BPDE (see Fig. 4).

Finally, cell specificity in accumulation of DNA adducts has been observed after exposure of other tissues to carcinogens. For example, in rat liver after exposure to methylating agents which induce hepatic angiosarcomas, the nonparenchymal cells accumulated higher levels of O6-methylguanine (38). These differences apparently reflect the ability of the two cell populations to repair O6-alkylguanine, since detailed investigations utilizing separated hepatocytes and nonparenchymal cells have indicated that rapid and enhanced removal of O6-alkylguanine occurred primarily in hepatocytes (39). AAF is hepatocarcinogenic in adult rats, but generally only after prolonged exposures. After both single and continuous exposure of AAF, more extensive adduct formation was observed in hepatocytes, the target cell for AAF, as compared with nonparenchymal cells (40). Following exposure of F344 rats to the lung carcinogen 4-(N-methyl-N-nitroamino)-1-(3-pyridyl)-1-butane, considerably higher O6-methylguanine was found in the DNA of Clara cells, the presumed target population for this carcinogen, compared with other lung cell populations (41). The higher O6-methylguanine levels observed in this study may be due, in part, to the levels of specific cytochrome(s) P-450 species in the Clara cells (42). It should be pointed out that these studies in liver and lung have compared distinctly different cell types, whereas in our present study we have examined cells of the same origin but at presumably different stages of terminal differentiation. Furthermore, the Percoll gradient procedure is an enrichment procedure and, as noted previously (Refs. 18 and 36; also see Tables 1 and 2 of the present study), there is some overlap of cells among the various fractions. It is not surprising therefore, that the differences observed in carcinogen-DNA adduct levels among epidermal subpopulations in our present study are not as striking as those observed in other tissues.

In summary, our present study, which is the first of its kind to examine the hydrocarbon-DNA adduct distribution in subpopulations of mouse epidermal cells, has demonstrated their presence and persistence in all subpopulations for at least a 28-day period. Furthermore, significantly different adduct levels were observed among the subpopulations of basal cells isolated on continuous Percoll gradients (Fraction 5 > Fractions 3 and 4). Since the presumed target cells for skin tumor initiation are believed to reside within the basal cell population and the process of tumor initiation is believed to occur relatively early after carcinogen application (11, 16), further work on the mechanism for the differences in DNA adduct levels in epidermal subpopulations seems warranted. Current studies are examining the DNA repair capacity of the 5 epidermal subpopulations obtained from the continuous Percoll gradients.

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