ABSTRACT

10-Fluoro-7,12-dimethylbenz(a)anthracene (10-F-DMBA) is a more potent skin tumor initiator in SENCAR mice when compared with the parent hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA). To elucidate the mechanism for this difference, the covalent binding of these two hydrocarbons to the DNA of mouse epidermal cells in vivo and in vitro was compared. The quantity of 10-F-DMBA covalently bound to mouse epidermal DNA in vivo was greater than that of DMBA at all doses tested over the range of 4 to 200 nmol/mouse. The magnitude of this binding difference between 10-F-DMBA and DMBA was greater at the higher doses (e.g., 1.5-fold at 4 nmol/mouse versus 3.4-fold at 200 nmol/mouse). These results correlated closely with the dose-response relationships for tumor initiation by the two hydrocarbons. Analysis of the isolated DNA samples by Servacel DHB chromatography revealed the relative proportion of syn-diol-epoxide:DNA adducts derived from DMBA increased dramatically as a function of dose (~30% at 4 nmol/mouse versus ~55% at 200 nmol/mouse). Conversely, the relative proportion of syn-diol-epoxide adducts derived from 10-F-DMBA was low and remained essentially constant over the same dose range.

High-pressure liquid chromatographic analyses of the DNA adducts from DMBA- and 10-F-DMBA-treated mice revealed qualitatively similar profiles. However, as expected, there was a marked reduction in the relative proportion of syn-diol-epoxide:DNA adducts derived from the epidermal samples from 10-F-DMBA-treated mice. The major syn-diol-epoxide:deoxyadenosine adduct was present at a level only 30% that found in high-pressure liquid chromatographic profiles of DMBA samples. Similar results were obtained when primary cultures of mouse epidermal cells were treated with the hydrocarbons. The results suggest that the increased total binding and possibly the decreased proportion of syn-diol-epoxide:DNA adducts confer greater tumor-initiating potency on 10-F-DMBA.

INTRODUCTION

The initiation stage of mouse skin tumorigenesis is believed to result from the interaction of reactive electrophilic intermediates of carcinogenic chemicals, including PAH, with DNA (reviewed in Refs. 19, 30, and 33). For example, excellent correlations have been observed between the differing carcinogenicities of a series of PAH and their covalent binding to mouse epidermal DNA (3, 28, 29). Based on extensive evidence accumulated in the last 15 years, it is known that PAH must be metabolically activated to these electrophilic intermediates which then bind to DNA and exert their carcinogenic effects (26). For example, following topical application of B(a)P to mouse skin in vivo, the major DNA-bound product formed is (+)-anti-BPDE, bound through a trans-addition to the exocyclic amino group of deoxyguanosine [(+)-anti-BPDE-dGuo] (reviewed in Ref. 30).

Much less is known about other PAH carcinogens and their DNA interactions, especially DMBA, which is one of the most potent skin carcinogens known (25, 35). Current evidence indicates that the metabolic activation of DMBA occurs primarily through the formation of a 3,4-diol,1,2-epoxide (reviewed in Ref. 34). This thesis is based primarily on fluorescence spectral studies of the hydrocarbon:DNA adducts formed in rodent embryo cells (14, 17, 20–22) or mouse skin (36, 37), indicating that DMBA is bound through a diol-epoxide in the A-ring (Chart 1). Recently, Bigger et al. (2) and Sawicki et al. (31) have demonstrated that, in mouse embryo cell cultures and mouse epidermis in vivo, both anti- and syn-3,4-diol,1,2-epoxide metabolites contribute significantly to the total binding of DMBA to the DNA of these cells. In addition, they have provided evidence that DMBA affects extensive modification of both dAdo and dGuo residues in the DNA (13).

One approach to understanding the mechanism(s) of PAH carcinogenesis is through the use of structural analogues (i.e., structure-activity relationships). We recently reported the tumor initiating activity of several fluorosubstituted derivatives of DMBA (8). One derivative, 10-F-DMBA, was found to be more potent than DMBA as a skin tumor initiator. In the present study, we have compared the covalent binding of DMBA and 10-F-DMBA to the DNA of mouse epidermal cells in vivo and in culture. The results suggest a mechanism for the increased biological activity of 10-F-DMBA and also provide clues about the DMBA:DNA adducts that may be important for tumor initiation in mouse skin.

MATERIALS AND METHODS

Chemicals. DMBA was purchased from the Eastman Kodak Co. (Rochester, NY) and purified as described previously (9). Alkaline phos-
10-F-DMBA:DNA ADDUCTS IN MOUSE SKIN

Chart 1. Chemical structure of DMBA. Arrowhead indicates the carbon atom to which the fluorne (f) atom is attached in 10-F-DMBA. It should be noted that C-10 in DMBA is in a position which is distal to the "bay region" (between C-12 and C-1).

phatase (Escherichia coli type III, EC 3.1.3.1), snake venom phosphodiesterase (Crotalus atrox, EC 3.1.5.1), and DNase I (bovine pancreas, EC 3.1.4.5) were obtained from the Sigma Chemical Co. (St. Louis, MO). [G-H]DMBA (42 to 48 Ci/mmol) was acquired through Amersham Corp. (Arlington Heights, IL). RNase A (EC 3.1.4.22) was supplied by Worthing-thon Biochemical Corp. (Freehold, NJ). 10-F-DMBA was a generous gift from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, a function of the Division of Cancer Cause and Prevention, National Cancer Institute, NIH, Bethesda, MD. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). Serv-avel DHB was obtained from the Accurate Chemical Co. (Westbury, NY). All other chemicals and reagents used were of the highest purity deemed necessary.

Cell Cultures. Pure primary epidermal cell cultures were derived from newborn Sencar mice. The primary cultures were prepared as described previously for hydrocarbon metabolism experiments (10) and grown at 31°C in an enriched Waymouth's medium containing 10% fetal bovine serum (18). For DNA-binding experiments, approximately 10^7 cells/dish were seeded into 100-mm culture dishes. Twenty-four hr after plating, the medium was removed and replaced with fresh medium containing 25 nmol DMBA (a) or 10-F-DMBA (b) and sacrificed 24 hr later. The methanol-soluble material obtained from the short LH-20 cleanup step and containing the hydrocarbon DNA adducts represented 69 ± 11 and 83 ± 9% of the total DNA-bound radioactivity for DMBA and 10-F-DMBA, respectively. In agreement with our previous results with the mouse skin system (11) and those of others (2), 3 major DMBA:DNA adducts, Peaks H, I, and J, were present representing 22.4, 26.2, and 22.2%, respectively, of the total radioactivity chromatographed in Chart 3A (also see Table 1). As noted in the "Introduction," these adducts are believed to arise through reaction of both the syn- (Peak I) and anti- (Peaks H and J) 3,4-diol-1,2-epoxides of DMBA with DNA (2, 31), Dipple et al. (13), and Sawicki et al. (31) indicates that Peak H arises through reaction with dGuo residues, whereas Peaks I and J arise through reaction with dAdo residues. In addition, a number of other DNA adduct peaks were routinely detected (Peaks A to G and K to M) in our chromatograms. We have shown previously that Peak B is chromatographically indistinguishable from the major DNA adduct formed when 7-OHM-12-MBA is applied to mouse epidermis (11).

RESULTS

Dose-Response Relationship for Covalent Binding of DMBA and 10-F-DMBA to Epidermal DNA. The covalent binding of DMBA and 10-F-DMBA was monitored over a dose range similar to that used in tumor experiments published elsewhere (8): 4 to 200 nmol/mouse. A dose-dependent increase in gross covalent binding of DMBA and 10-F-DMBA was observed (Chart 2). Interestingly, the level of 10-F-DMBA bound to epidermal DNA was greater than DMBA at all doses tested. In addition, the difference in covalent binding between DMBA and 10-F-DMBA was dependent on the dose. At low doses (i.e., 4 or 10 nmol/mouse), 10-F-DMBA bound to epidermal DNA about 1.5 times more than did DMBA. At higher doses (between 25 and 200 nmol/mouse), the difference gradually increased from 2.5 times at the 25-nmol dose to 3.4 times at the 200-nmol dose.

In order to determine the amounts and nature of the hydrocarbon:DNA adducts formed from DMBA and 10-F-DMBA, DNA samples were hydrolyzed to deoxyribonucleosides as described in "Materials and Methods." Chart 3 shows the HPLC elution profiles of epidermal DNA samples obtained from mice treated with 25 nmol DMBA (a) or 10-F-DMBA (b) and sacrificed 24 hr later. The methanol-soluble material obtained from the short LH-20 cleanup step and containing the hydrocarbon DNA adducts represented 69 ± 11 and 83 ± 9% of the total DNA-bound radioactivity for DMBA and 10-F-DMBA, respectively. In agreement with our previous results with the mouse skin system (11) and those of others (2), 3 major DMBA:DNA adducts, Peaks H, I, and J, were present representing 22.4, 26.2, and 22.2%, respectively, of the total radioactivity chromatographed in Chart 3A (also see Table 1). As noted in the "Introduction," these adducts are believed to arise through reaction of both the syn- (Peak I) and anti- (Peaks H and J) 3,4-diol-1,2-epoxides of DMBA with DNA (2, 31). Further characterization of these adducts by Bigger et al. (2), Dipple et al. (13), and Sawicki et al. (31) indicates that Peak H arises through reaction with dGuo residues, whereas Peaks I and J arise through reaction with dAdo residues. In addition, a number of other DNA adduct peaks were routinely detected (Peaks A to G and K to M) in our chromatograms. We have shown previously that Peak B is chromatographically indistinguishable from the major DNA adduct formed when 7-OHM-12-MBA is applied to mouse epidermis (11).

* J. DIGiovanni, unpublished observations.
10-F-DMBA:DNA ADDUCTS IN MOUSE SKIN

Chart 2. Semilogarithmic plot of the dose-response relationship with DMBA (•) and 10-F-DMBA (△) for covalent binding to mouse epidermal DNA. [3H]DMBA or [3H]-10-F-DMBA was applied topically to groups of 10 mice each at the various doses shown, and mice were sacrificed 24 hr later. Values represent gross covalent binding to epidermal DNA.

The elution profile of epidermal DNA samples from mice treated with 25 nmol 10-F-DMBA (Chart 3b) was qualitatively similar to the DMBA:DNA adduct elution profile (Chart 3a). It should be noted that individual DNA adducts derived from 10-F-DMBA eluted 1 to 2 fractions later in our HPLC system than did the corresponding DMBA:DNA adducts. The relative positions of the adducts, however, remained the same. The elution profile shown in Chart 3b has been corrected for this difference in retention times. Interestingly, there was a marked reduction in the relative proportion of several of the 10-F-DMBA:DNA adducts. This was especially pronounced with regard to DNA adduct peak I' which represented only 8.5% of the total radioactivity chromatographed in Chart 3b (see Table 1). The distribution of DNA adducts from mice treated with 25 nmol DMBA or 10-F-DMBA is summarized in Table 1. We have included only those adducts which clearly and reproducibly correspond in both the DMBA and 10-F-DMBA elution profiles of Chart 3.

Separation of Syn- and Anti-Diol-Epoxide Adducts of DMBA and 10-F-DMBA. The relative proportion of syn- and anti-diol-epoxide adducts derived from DMBA and 10-F-DMBA was monitored as a function of dose. Groups of animals were treated as described for the dose-response experiment in Chart 2.

Digested DNA samples were first passed through a short LH-20 column followed by separation of syn- and anti-diol-epoxide DNA adducts on a column of Servacel DHB. This procedure results in 2 fractions (Peak I, representing syn, and Peak II, representing anti) which then must be passed through another LH-20 column prior to HPLC. The results from these analyses are illustrated in Chart 4. As shown, the proportion of syn-diol-epoxide:DNA adducts was greater in DNA samples from DMBA-treated mice at all doses tested. The most striking observation was that the relative proportion of syn-diol-epoxide:DNA adducts from DMBA-treated mice increased dramatically with the dose applied whereas, with 10-F-DMBA, it remained relatively constant (or decreased slightly). We have also determined the relative proportion of syn- and anti-diol-epoxide adducts from these 2 hydrocarbons over a 7-day time course. Interestingly, the ratio of syn:anti-adducts was constant for both compounds (using a given dose) over the entire time course (data not shown).

The syn- and anti-diol-epoxide:DNA adduct fractions from mice treated with 25 nmol DMBA or 10-F-DMBA were analyzed by HPLC. Servacel Peak I, which represented syn-diol-epoxide:DNA adducts, contained 5 radioactive peaks in the samples from both DMBA- and 10-F-DMBA-treated mice (Charts 5b and 6b, respectively). Servacel Peak II, which represented anti-diol-epoxide:DNA adducts, contained 3 radioactive peaks in the samples from both DMBA and 10-F-DMBA-treated mice (Charts 5c and 6c, respectively).

Formation of DMBA:DNA and 10-F-DMBA:DNA Adducts in Primary Cultures of Mouse Epidermal Cells. To determine the ability of DMBA and 10-F-DMBA to bind covalently to the DNA of mouse epidermal cells grown in primary culture, confluent cultures were treated for 24 hr with the hydrocarbons (0.25 µg/
Table 1

Distribution of DNA adducts from mice treated with 25 nmol DMBA or 10-F-DMBA and sacrificed 24 hr later

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total binding (pmol hydrocarbon/mg epidermal DNA)</th>
<th>Relative % of adduct distribution&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA</td>
<td>6.11 ± 1.02&lt;sup&gt;b&lt;/sup&gt; (5)</td>
<td>B/B' 2.4</td>
</tr>
<tr>
<td>10-F-DMBA</td>
<td>15.8 ± 3.8 (10)</td>
<td>E/E' 2.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are presented as the percentage each peak represents of the total dpm eluted from the HPLC column after subtracting background. Maximum variation between DMBA and 10-F-DMBA for a given peak was 18%.

<sup>b</sup> Mean ± S.D.

<sup>c</sup> Numbers in parentheses, number of experiments.

Chart 4. Relative percentage of syn- and anti-diol-epoxide:DNA adducts as a function of dose applied to mouse epidermis in vivo. a, percentage of syn; b, percentage of anti. Values represent the percentage of total adducts applied to the Servacel DHB columns. Recoveries were 96 ± 5% (S.D.) for all of the samples applied to the Servacel DHB columns (i.e., both DMBA and 10-F-DMBA).

Chart 5. HPLC elution profiles of the syn- and anti-diol-epoxide:DNA adduct fractions from Servacel DHB column chromatography of DMBA:DNA samples. a, total DNA adducts formed in vivo after topical application of 25 nmol [3H]DMBA; b, Servacel DHB Peak I, representing syn-diol-epoxide:DNA adducts; and c, Servacel DHB Peak II, representing anti-diol-epoxide:DNA adducts. UV-absorbing markers are depicted as arrows as described in Chart 3.
10-F-DMBA:DNA ADDUCTS IN MOUSE SKIN

Chart 6. HPLC elution profiles of the syn- and anti-diol-epoxide:DNA adduct fractions from Servacel DHB chromatography of 10-F-DMBA DNA samples. a, total DNA adducts formed in mouse epidermal DNA in vivo after topical application of 25 nmol [3H]-10-F-DMBA; b, Servacel DHB Peak I, representing syn-diol-epoxide:DNA adducts; and c, Servacel DHB Peak II, representing anti-diol-epoxide:DNA adducts. UV-absorbing markers are depicted as arrows as described in Chart 3.

DISCUSSION

The major DMBA:DNA adducts, found in our present study in mouse epidermal DNA after topical application of the hydrocarbon, were similar to those which we (11) and others (2) have reported recently. Bigger et al. (2), Dipple et al. (13), and Sawicki et al. (31) have shown that the 3 major DNA adducts derived from DMBA are an anti-diol-epoxide reacted with dGuo and dAdo (Chart 3a, Peaks H and J, respectively) and a syn-diol-epoxide reacted with dAdo (Chart 3a, Peak I). As noted, our present results are consistent with these assignments. In a preliminary experiment (data not shown), we have prelabeled the DNA of mouse epidermal cells in primary culture with [14C]-guanine and then treated them with either [3H]-DMBA or [3H]-10-F-DMBA (0.25 µg/ml) for 24 hr. HPLC analyses of the isolated DNA samples indicated that Peaks H and H' (from DMBA- and 10-F-DMBA-treated cultures, respectively) contained the 14C label and therefore were the result of reaction with dGuo. These preliminary results further illustrate the similarity in adduct identification between our study and that of Bigger et al. (2).

In addition, we have further compared the binding of 10-F-DMBA to mouse epidermal DNA both in vivo and in vitro with that of DMBA. These experiments were undertaken to: (a) ascertain the mechanism for increased tumor-initiating activity of 10-F-DMBA compared with DMBA; and (b) to learn more about the DMBA:DNA-adducts critical for tumor initiation in a target tissue through use of a structural analogue with different biological activity. We showed previously that 10-F-DMBA was a more potent tumor initiator than was DMBA (8). An interesting observation in that study was that the magnitude of the difference in initiating activity between 10-F-DMBA and DMBA was less at low doses and became greater as the dose increased. In our present study, we compared the gross covalent binding of 10-F-DMBA with that of DMBA. Surprisingly, a similar relationship was found; i.e., 10-F-DMBA bound more extensively than DMBA to epidermal DNA (Chart 2), and the magnitude of this difference became greater as the dose of hydrocarbon was increased. For example, at the 4-nmol dose, this difference was ~1.5-fold whereas, at the 200-nmol dose, this difference was ~3.4-fold. Thus, the dose-response curves for covalent binding of 10-F-DMBA and DMBA to mouse epidermal DNA in vivo closely paralleled the dose-response curves for tumor initiation in the same tissue.

The above results alone could explain the difference in tumor-initiating activity between 10-F-DMBA and DMBA, if one assumes everything else to be equal between the 2 hydrocarbons. One must also assume, however, that the magnitude of gross covalent binding correlates exactly with the magnitude of the observed difference in biological activity. Studies from our laboratories (12, 27) as well as those from others (4, 5) clearly indicate that this is not often the case. We therefore examined the nature of the DNA adducts derived from 10-F-DMBA. Bigger et al. (2) demonstrated that the relative proportion of syn-diol-epoxide adducts derived from DMBA in mouse epidermal DNA increased with increasing dose of the hydrocarbon applied to mouse skin. We determined the relative percentage of syn-diol-epoxide adducts derived from 10-F-DMBA compared with DMBA as a function of dose and found a similar increase in the propor-
tion of syn-diol-epoxide adducts with the latter hydrocarbon. Interestingly, no dose-dependent increase in the proportion of syn-diol-epoxide adducts was observed with 10-F-DMBA. These results suggest that the increased total binding and concomitant decrease in proportion of syn-diol-epoxides may account for the greater biological activity of 10-F-DMBA. These results also suggest that syn-diol-epoxide adducts of DMBA are no more important than anti-diol-epoxide adducts for mouse skin tumor initiation by this PAH. Based on our data, it is interesting to speculate that syn-diol-epoxide adducts may be less important for DMBA tumor initiation, however, further work is necessary to prove or disprove this hypothesis.

Examination of the HPLC elution profiles of 10-F-DMBA:DNA adducts compared with those of DMBA at one dose (25 nmol) indicated marked differences in the relative proportions of the 3 major adducts. The relative proportion of the major syn-diol-epoxide:dAdo adduct (Chart 3b, I) was reduced by approximately 60%, and the major anti-diol-epoxide:dAdo adduct (Chart 3b, Peak J) also was reduced, but to a lesser extent. Surprisingly, the major anti-diol-epoxide:dGuo adduct (Chart 3b, H') represented a substantially greater proportion of the total adducts (~46%). Moschel et al. (23) have shown recently that the major syn-diol-epoxide:dAdo adduct derived from DMBA is the least stable of the 3 major adducts. Under acidic conditions, this adduct was found to decompose to liberate a tetraol. Therefore, the possibility existed in our present study that this adduct derived from 10-F-DMBA (formed in vivo or in vitro) was even less stable than was the corresponding adduct derived from DMBA and was decomposing during enzymatic hydrolysis to give the HPLC profile shown in Chart 3b. The following observations, however, indicate that this was not the case: (a) all of our DNA samples were stored desiccated as dried pellets prior to beginning hydrolysis or were hydrolyzed immediately after isolation; (b) for a given dose of DMBA or 10-F-DMBA, there was little, if any, variability observed in the HPLC elution profiles among various samples; (c) <5% of the total radioactivity in DNA samples from 10-F-DMBA-treated mice could be extracted with water-saturated n-butyl alcohol, even after incubation for 24 hr at 37° in Tris-magnesium chloride (pH 7.3) buffer prior to adding DNase I and beginning the digestion process. The HPLC elution profiles of these DNA adduct samples were not significantly different from those shown in Chart 3b; (d) incubation of isolated DNA samples from either DMBA or 10-F-DMBA-treated mice in Tris-magnesium chloride (pH 5.0) buffer for 24 hr at 37° dramatically increased the water-saturated n-butyl alcohol-extractable radioactivity. HPLC elution profiles from these samples showed a marked reduction in the major syn-diol-epoxide:dAdo adduct from both DMBA and 10-F-DMBA (I and I', respectively). Thus, in our hands, mildly acidic conditions led to decomposition of the major syn-diol-epoxide:dAdo adduct derived from both hydrocarbons, but this did not occur under the conditions of our normal digestion.

In a previous study (8), we proposed several possible mechanisms to explain the increased tumor-initiating potency of 10-F-DMBA compared to the parent hydrocarbon, DMBA. We suggested a mechanism that involved an electronic effect due to one of the unshared pair of electrons (associated with the fluorine atom) participating in resonance with the ring system. The present study further supports this hypothesis and suggests that the fluorine atom in the 10-position alters the amounts or stabilities of the individual diol-epoxide metabolites, thus leading to an overall increase in covalent binding to DNA and a decreased proportion of syn-diol-epoxide adducts. Additional evidence is accumulating in support of this notion that fluorine substituents, in addition to blocking or reducing metabolism at the site of attachment (6, 7, 15, 16, 24), can affect the reactivity of other regions of the PAH molecule (32, 38). For example, Wong et al. (38) have shown that the presence of a methanolysis product of the metabolically formed 5,6-epoxide of DMBA is dramatically reduced if fluorine atoms are present in Positions 1, 2, 3, 5, 9, and 10. Shiokh et al. (32) have demonstrated that fluorine atoms at various positions in benz(a)anthracene can influence the metabolism at specific double bonds through a resonance effect similar to that described above.

In conclusion, we have presented a comparison of the covalent binding of DMBA and its 10-F-analogue in a target tissue where the biological activity was assessed previously. The increased biological potency of 10-F-DMBA correlated with a greater level of covalent binding to epidermal DNA and a marked reduction in the relative proportion of syn-diol-epoxide DNA adducts. Interestingly, with 10-F-DMBA, the major adduct is the anti-diol-epoxide dGuo product (Chart 3b, Peak H'). Therefore, the increased biological potency of 10-F-DMBA also correlates with increased binding to dGuo residues. Dipple et al. (13) and Moschel et al. (22) have recently speculated that the binding of DMBA to dAdo residues in DNA may be closely associated with its biological activity. While the mechanism of skin tumor initiation with 10-F-DMBA may differ from that of DMBA, we have found that this derivative (with greater biological potency) shows a marked increase in binding to dGuo residues, suggesting that binding to this base in DNA may also be important for the biological activity of DMBA.

REFERENCES


Covalent Binding of 7,12-Dimethylbenz(a)anthracene and 10-Fluoro-7,12-dimethylbenz( a)anthracene to Mouse Epidermal DNA and Its Relationship to Tumor-initiating Activity


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