Journal of Cellular Biochemistry 18:135–148 (1982)
Mechanisms of Chemical Carcinogenesis 243–256

Metabolism of Benzo(a)pyrene by Human Epithelial and Fibroblastic Cells: Metabolite Patterns and DNA Adduct Formation

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We demonstrate in cell culture that mammary epithelial cells from normal human breast specimens metabolize benzo(a)pyrene (BaP) and form adducts with the bases of their DNA more readily and at lower concentrations of BaP than do fibroblasts from the same specimens. BaP metabolism and adduct formation was determined in the same incubations with epithelial cells grown out in early passage from each of three specimens and with fibroblasts from one of these specimens. The metabolite pattern of the epithelial cells was indicative of preferential formation of 7, 8-dihydrodiol-9, 10-dihydroepoxybenzo(a)pyrene the ultimate carcinogen. In contrast, fibroblasts formed mainly mono- and dihydroxide derivatives of BaP. The metabolite pattern from epithelial cells was compatible with the ease in which adducts between DNA and the diolepoxide of benzo(a)pyrene were formed. These results provide evidence that chemical carcinogens should be considered as possible factors in the induction of breast cancer in women.

Key words: DNA adduct formation, benzo(a)pyrene metabolism, human cells, mammary fibroblasts, mammary epithelial cells, metabolite patterns, benzo(a)pyrene

Although experimental induction of mammary adenocarcinomas by chemical carcinogens in rodents is well documented [1–7], there is little information on the effects of such carcinogens on human mammary epithelial cells. The availability of a human mammary epithelial cell culture system that permits active growth of pure cell populations for 1–4 passages [8,9] offers the opportunity to investigate metabolism of chemical carcinogens directly in the cell involved in 99% of all breast malignancies [10]. Pure cultures of mammary fibroblastic cells from the same individuals are also available for comparison. We have chosen benzo(a)pyrene (BaP) for our initial studies because BaP is the most abundant environmental carcinogenic byproduct from the incomplete combustion of fossil fuel [11] and because the metabolic pathway to the ultimate carcinogen is relatively well understood. As with all naturally occurring chemical carcinogens, BaP must be converted enzymatically to the ultimate carcinogenic forms [12–14]. In the case of BaP, the

Received April 29, 1981; revised and accepted October 7, 1981.

0730-2312/82/1802-0135$04.00

tetrahydro-7,8-dihydroxy-9,10-epoxide of BaP (BPDE), particularly the 7R enantiomer of the anti steroisomer, correlates best with initiation of carcinogenic transformation in many system [15–19]. Most evidence points to covalent binding of BPDE to the bases of DNA as a crucial step in transformation [13,14, 19–22].

We have recently presented evidence (1) that normal human mammary epithelial cells grown out from breast specimens are much more sensitive to growth inhibition after exposure to BaP than are fibroblasts derived from the same individual, (2) that the formation of adducts between BPDE and DNA nucleosides occurs in these cells at a much lower substrate concentration than that in fibroblastic cells from the same specimens, and (3) that specific adducts between anti BPDE and deoxyguanosine and deoxycytidine are formed as early as 6 hours after exposure to BaP [23]. These results are compatible with chemical carcinogens as factors in the initiation of human breast cancer, if the breast epithelial cells are exposed to these agents under natural conditions. These results take on special significance when it is considered (1) that the etiology of breast cancer in women remains unknown, even though many factors influencing the incidence are suspected [24] and (2) that, among cancer of women in the US, breast cancer has the highest incidence by far and, even in the total population, ranks a close second to cancer of the respiratory tract [25,26]. We now ask the question: What is it about BaP metabolism by human mammary epithelial cells that results in modification of DNA more readily and at a much lower original concentration of BaP than that observed in mammary fibroblasts from humans? In partial answer to this question, we present here the complete metabolic yield from BaP by these breast epithelial cells and suggest how this metabolite pattern is reflected in DNA modification.

MATERIALS AND METHODS

Chemicals

Benzo(a)pyrene, generally labeled with tritium (19–40 Ci/mmole), was purchased from Amersham Corp. and unlabeled BaP from Aldrich Chemical. Both substrates were checked for purity by high pressure liquid chromatography (HPLC) prior to each use. All solvents for HPLC and isolation of BaP metabolites and DNA adducts were Omnisolve grade from Matheson, Coleman, Bell. Aryl sulfatase, β-glucuronidase, deoxyribonuclease, spleen phosphodiesterase, and alkaline phosphatase were purchased from Sigma Chemical Co. Standard adducts between BPDE and deoxycytidine, deoxyguanosine, and deoxyadenosine were formed by reacting calf thymus DNA with enough of a racemic mixture of BPDE to yield 1 adduct per 50 base pairs [27]. The DNA was digested to its constituent nucleosides as described below and those with BPDE adducts were purified by HPLC [27]. Standard metabolites of BaP (7,8-; 9,10-; and 4,5-dihydrodiols and monohydroxides at carbons 3,6, of 9 of BaP) were obtained from the National Cancer Institute Chemical Repository.

Media and serum for cell culture were purchased from Grand Island Biological Co. (GIBCO) and hormones and growth factors from Sigma Chemical Co. and Collaborative Research Inc.

Cell Culture

The normal mammary epithelial cells and fibroblastic cells were isolated and subcultured as described by Stampfer et al [8]. Fibroblastic cells were grown in a

basal medium consisting of equal parts of Ham's F-12 and Dulbecco's modification of Eagle's medium (DME), with 5% fetal calf serum, 10 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. The epithelial cells were grown in an enriched medium, designated MM, containing conditioned media from human epithelial cell lines, 10 μg/ml insulin, 0.1 μg/ml hydrocortisone, 5 ng/ml epidermal growth factor, 1 ng/ml cholera toxin, 10^{-9} M estradiol, 10^{-8} M triiodothyronine, 100 U/ml penicillin, and 100 μg/ml streptomycin [8,9,].

These normal mammary epithelial cells have been characterized as epithelial based on ultrastructure, dome formation [8], and fibronectin pattern (9,28]. Their mammary origin was verified by the presence of antigens specific for human mammary epithelial cells [8,29,30]. On the basis of the fibronectin pattern, the second and third passage epithelial cell populations used in these experiments were 98% pure, the remaining cells present had the morphological appearance of myoepithelial cells [8,9]. The fibroblastic cells were used at fourth or fifth passage. Because epithelial cells are incapable of sustained growth in the basal medium, the fibroblastic cell populations were free of epithelial cells.

Exposure to ^{3}H-BaP flasks (T75, Corning) containing proliferating cells in culture 24 hours from visual confluence were exposed to ^{3}H-BaP at a concentration of 0.4 μM (specific activity, 19 Ci/mm) in MM for epithelial cells and at 0.4 and 4.0 μM (specific activity, 8 Ci/mm) in basal medium and in one case in MM for experiments with fibroblasts. The cell density (number of cells/flask) at the time of initial exposure to BaP was approximately the same for the epithelial and fibroblastic incubations and for each of the incubations with cells from the various specimens.

At the times indicated, medium was collected and frozen until analysis. The cells were washed twice with BaP-free medium and harvested by trypsinization. Persistence of the DNA adducts was tested by exposing cultures to labeled BaP for 24 hours, washing twice with BaP-free medium, and then continuing cultivation for an additional 72 hours in the absence of BaP with a media change 24 hours before harvesting the cells and media.

Assay of BaP and Metabolites in Media

The concentrations of BaP and its metabolites in the media were determined by extracting a portion of the media several times with a mixture of ethyl acetate and acetone (2:1, by volume) containing either α-tocopherol or hydroquinone as an antioxidant, and assaying the radiactive content of the BaP fraction isolated by HPLC (see below). The original media were also analyzed in this manner. In all cases, over 95% of the radioactivity added to the original media eluted in the BaP fraction off HPLC.

The radiolabeled material remaining in the extracted media was taken as the water soluble conjugates of BaP metabolites. To verify this value and to determine the quantity formed of each type of conjugate, portions of the extracted media were deproteinized and applied to alumina (Biorad) columns and the metabolites eluted as described by Autrup [31]. By this technique [31], the least polar components elute with 100% methanol; sulfate conjugates, with water; glucuronide conjugates, with ammonium phosphate (pH 3.0); and the most polar components including glutathione conjugates, with formic acid (25%). In all cases, a small amount of radiolabeled material eluted with methanol indicating that metabolites other than conjugates remained after extraction. The values for organic solvent-soluble metabolites

and for water-soluble metabolites were corrected by this amount so that the water-soluble material is the equivalent of the sum of the three classes of conjugates.

In the case of the experiment with cells from one specimen (H97), portions of the deproteinized media were also applied directly to the alumina columns which were eluted as above [31]. The metabolites in the methanol fraction were subjected to HPLC and the pattern compared to the separation of the metabolites extracted into ethyl acetate. The material eluted with water was subjected to sulfatase digestion and that eluted with ammonium phosphate was treated with glucuronidase [32]. The formic acid-eluting material was subjected to acid hydrolysis (1 M HCl for 2 hours at 50°C). The hydrolyzed material in all three cases was extracted with the ethyl acetate:acetone mixture and the radioactivity in the aqueous and organic phases were assayed.

Isolation of DNA

The DNA from the harvested cells was isolated, purified, and digested to constituent nucleosides as described by Straub et al [27]. The mixture of nucleosides covalently bound to BaP metabolites was isolated from free nucleosides by elution of a Sephadex LH 20 column first with water and then with methanol. The material eluting with methanol was separated into individual derivatized nucleosides by HPLC (see below). The extent of adduct formation is expressed as pmole BaP converted to material associated with bases recovered from the LH 20 column and isolated and identified with HPLC (see below). DNA concentration was determined by absorption at 260 nm. The mean value of the 260A/280A ratio for purified DNA was 1.89.

High Pressure Liquid Chromatography

A Varian chromatograph, model number 5000, fitted with an Altex ultrasphere column (250 × 4.6 mm) of octadecyltrimethoxysilane (ODS), was used. For separation of adducts, the column was eluted isocratically with 52% methanol in water (v/v) at room temperature for 80 minutes and then linearly, increasing the methanol gradient to 100% methanol over the next 20 minutes. The flow rate was 0.7 ml/min. Fractions were collected every 60 seconds throughout the run for determination of radioactivity. Fluorescence of the eluate was monitored by measuring emission above 370 nm with a Schoffel detector after excitation at 246 nm. For isolation and assay of BaP and its metabolites in the media, the column was eluted with a linearly increasing gradient of methanol from 60–100% over an hour using the same flow rate, detection system, and fraction collection protocol as with the adduct elutions.

RESULTS

The results presented here for the conversion of BaP to major metabolites are derived from the same experiments in which formation of adducts between BPDE, the ultimate carcinogen, and dG of DNA was examined. The mammary epithelial cells were grown out from three human breast specimens from reduction mammoplasties with no detectable cell pathology. The same measurements on fibroblasts grown out from two of the specimens act as a basis for comparison. The cells were exposed to BaP under growing conditions optimal for each cell type, medium MM

for epithelial cells, and the basal medium with serum for fibroblasts. To test that differences in BaP metabolism were not due to the different media, fibroblasts grown out from specimen H48 were incubated for 16 hours in the presence of BaP in each medium (Fig. 1B). The overall conversion of BaP was little affected by medium MM (decreased by 12%), but the partitioning into water-soluble metabolites was increased at the expense of organosoluble products. The profile of organosoluble metabolites was unchanged (Table II). On the basis of these results, we concluded that use of the optimal growing conditions for each, although different, was a valid basis for comparison.

Because of the inhibition of growth of human mammary epithelial cells by the metabolites of BaP [23], the concentration of BaP used in the epithelial cell cultures was 0.4 μM. Experiments at various concentrations of BaP revealed 4.0 μM BaP was required to detect DNA adduct formation in fibroblastic cultures [23]. Studies with fibroblasts exposed to 0.4 μM and 4.0 μM concentrations of BaP revealed no substrate inhibition of metabolism at 4.0 μM nor any significant change in the pattern of metabolites formed (Fig. 1, Table II).

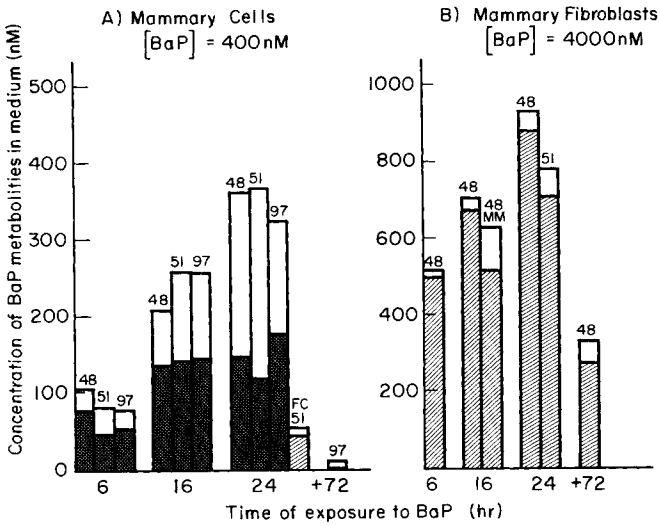

Fig. 1. BaP metabolite yield of human mammary epithelial cells (stipples) and fibroblasts (chevrons). Each bar denotes the total yield at the times designated; the shaded area, the yield of organosoluble metabolites; and the clear area, the quantity of water soluble conjugates produced. Number at the top of each bar designates specimen from which cells were derived. Epithelial cells in medium MM and H51 fibroblasts in a basal medium were exposed to 400 nM BaP (A). All other fibroblastic cultures were treated with 4000 nM BaP (B) in a basal medium plus serum with the exception of that from specimen H48 which was treated for 16 hours with BaP in medium MM (bar designated MM, Panel B). Bars labeled +72 hr designates those cultures exposed to BaP for 24 hours plus an additional 72 hours in medium to which no BaP was added. Note difference in the scales for panels A and B.

Rate of BaP Utilization

The sum of the BaP metabolites recovered in the media revealed that the rate of BaP conversion to metabolites by the human mammary epithelial cells was relatively constant, 150–285 pm/flask/hr. Recovery of BaP metabolites based on BaP disappearance from the medium was 82–94% for epithelial cultures after 24-hour exposures, but only about one-third of the BaP disappearing was recovered in media at 6 hours and about 60–70% at 16 hours. These differences likely reflect an initial uptake into the cells that was relatively rapid followed by uptake dependent upon the rate of conversion to metabolites that proceeds at about 200 pm/flask/hr under the conditions used here. Less than 5% of the original BaP remained in the media from epithelial cell cultures 24 hours after exposure.

BaP conversion rates at similar time spans for mammary fibroblasts exposed to the tenfold higher concentration of BaP were about twofold higher than those in the mammary epithelial cells (Fig. 1). Fibroblasts exposed to 0.4 μM BaP metabolized only one-tenth of the BaP in 24 hours, a rate well below that for epithelial cells, or for fibroblasts at the higher concentration. At the end of 24 hours of exposure to 4.0 μM BaP, the fibroblastic cultures retained about 40% of the original substrate, and even though the fibroblastic monolayers were washed twice and BaP-free medium was added for the next 72 hours, the BaP concentration in the media at the end of that time was almost 0.2 μM.

Production of Water Soluble Conjugates

Conjugation of BaP metabolites by epithelial cells was less than the formation of organic-solvent soluble metabolites for the first 16 hours. By 24 hours, accumulation of water-soluble metabolites exceeded that in the other category (Fig. 1A). The rates of total conjugate formation for epithelial cells from all three specimens for the first two time periods were within the range of 67–130 pm/flask/hr, but the cells exhibited rates in excess of twice this range for the 16–24 hours time span (Fig. 1A). The reason for this increase in the rate of derivatization at this time was likely due to the increased availability of BaP metabolites for conjugation. Total conjugate accumulation, during the 24-hour exposure to BaP, ranged from 2200–3720 pm/flask (Fig. 1A).

Conversion of BaP to water-soluble metabolites by mammary fibroblasts was 10% or less of the total metabolite yield, while that by breast epithelial cells was 34–61% (Fig. 1B). Even in those fibroblasts exposed to BaP in MM (16-hour column, Fig. 1B, only 19% of the total metabolite yield in the medium was water-soluble material.

Identification of Conjugates Formed by Mammary Epithelial Cells

The pattern of conjugates formed was similar for epithelial cells from all three specimens: Conjugation of BaP metabolites with sulfate and glucuronide were approximately equivalent at all times, and these two together were usually similar to, or slightly less than conjugation with glutathione (Table I). The agreement between specimens is worth noting. With few exceptions the range among specimens of incorporation of BaP metabolites into a given conjugate at a specific time rarely exceeded twofold.

When the material in each conjugate class from the alumina columns was hydrolyzed, either enzymatically in the cases of putative sulfate and glucuronide con-

TABLE I. Distribution of BaP Metabolites Among Conjugates Formed by Human Mammary Epithelial Cells*

Conjugation with:	Specimen no.	Percentage distribution among conjugates at 6 hr	16 hr	24 hr	24 + 72(−) hr[a]
Sulfate[c]	H48	27	26	19	–[b]
	H51	22	20	21	–
	H97	11	23	18	14
Glucuronide[c]	H48	27	34	26	–
	H51	26	24	24	–
	H97	11	24	14	14
Glutathione[d]	H48	45	40	55	–
	H51	52	55	55	–
	H97	78	60	67	71

*The quantity in pmole/flask in each conjugate class for each time period can be calculated from the percentage distribution and the total conversion to conjugates (Fig. 1). Conjugate classes were separated on alumina columns as described by Autrup [31]. Original concentration of BaP was 400 nM. The results are from the same experiment as those presented in Tables I and II.

[a]Incubations 24 hours in the presence of BaP plus an additional 72 hours in BaP-free medium (see Table I and text).

[b]Designates none detected.

[c]Identified primarily as designated by enzymatic hydrolysis.

[d]Tentative identification based on work of others [31,36].

jugates or with acid for the fraction designated glutathione, over 70% of the original radioactivity was now extractable into ethyl acetate/acetone. These results verified that the elutions reputed to be sulfate and glucuronide conjugates were for the most part accurately designated. The identity of the last eluting fraction must remain unverified for now because sensitivity to acid hydrolysis indicates a conjugate but not which one.

Production of Unconjugated Metabolites

Epithelial cells from all three specimens converted BaP to organosoluble material to a similar extent (Fig. 1A). The rates of conversion by epithelial cells to organic-solvent soluble material ranged from 120–195 pm/flask/hr during the first 6 hours of exposure to BaP, and 90–135 pm/flask/hr for the next 10 hours. These rates of conversion were approximately twice those for conjugate formation for the same periods. The difference in the final conversions was due to the continued formation of water-soluble metabolites, but not organic-soluble, for the final 8 hours in the presence of BaP. As with the conjugates, only traces of metabolites could be detected in the media at the end of the 72-hour period in BaP-free medium.

After the first 6 hours of exposure to BaP, the fibroblasts, receiving 4 μM BaP, exhibited virtually linear rates of formation of organosoluble metabolites of 300 pm/flask/hr (Fig. 1B). This rate was barely twice that of the epithelial cells exposed to one-tenth the concentration of BaP. Because of the large amount of BaP remaining in the media at the end of the 24-hour exposure (2.4 μM), plus that likely adsorbed to the cells and to the plastic, not all the BaP could be removed prior to the additional 72-hour incubation in BaP-free medium. For this reason, at the end of the next 72 hours, 269 pm/flask of organosoluble metabolites were present. If all of this material were formed during the additional 72 hours, it would represent a

TABLE II. Distribution of BaP Metabolites in Organosoluble Extracts of Medium From Human Mammary Epithelial and Fibroblast Cells*

Peak no.[a] (metabolite)	Specimen no. cell type	Initial [BaP] (nM)	Percentage distribution at 6 hr	16 hr	24 hr	+72 hr[b]
1.	H48-EC[c]	400	18	27	48	0
	H51-EC	400	23	39	40	0
(Conjugates	H97-EC	400	15	35	40	79
tetrols)	H51-FC[c]	400	ND[e]	ND	6	ND
	H48-FC	4000	0	0	0,0[d]	0
2.	H48-EC	400	3	7	27[f]	0
	H51-EC	400	2	8	24[f]	0
(Tetrols)	H97-EC	400	3	15[f]	20[f]	17
	H51-FC	400	ND	ND	0	ND
	H48-FC	4000	6	8	0,5	4
3.	H48-EC	400	37	33	12[f]	0
	H51-EC	400	41	29	22	0
(9,10 Diol)	H97-EC	400	42	34	27	trace
tetrol, triol)	H51-FC	400	ND	ND	44	ND
	H48-FC	4000	15	16	46,42	55
4.	H48-EC	400	3	0	3	0
	H51-EC	400	0	0	5	0
(4,5 Diol)	H97-EC	400	0	2	2	0
	H51-FC	400	ND	ND	0	0
	H48-FC	4000	0	12	14,0	0
5.	H48-EC	400	19	16	5	0
	H51-EC	400	14	10	3	0
(7,8 Diol)	H97-EC	400	18	7	6	4
	H51-FC	400	ND	ND	28	ND
	H48-FC	4000	12	27	23,26	24
Other	H48-EC	400	20	17	6	0
peaks	H51-EC	400	20	14	5	0
(phenols)	H97-EC	400	21	7	6	trace
	H51-FC	400	ND	ND	22	ND
	H48-FC	4000	67	36	17,26	17

*The nanomolar concentration of each metabolite in the medium can be calculated from the percentages and the conversion to organosoluble metabolites given in Figure 1 for each specimen at the specific times of incubation. Results are from same experiments as those presented in other tables and in figures.

[a]Retention times for each peak are those denoted in Figure 2.

[b]Incubations 24 hours in the presence of BaP followed by an additional 72 hours in BaP-free medium.

[c]EC designates epithelial cells; FC, fibroblastic cells.

[d]Second value for FC, 4000 nM, is for 24-hour cultures in medium MM.

[e]ND, not determined.

[f]Value made up of two incompletely resolved peaks.

formation rate of about 60 pm/flask/hr (Fig 1B). Fibroblasts exposed to 0.4 μM BaP produced organosoluble metabolites at 20–25% of the rate of comparably treated epithelial cells (Fig 1A).

Identification of Unconjugated Metabolites

The patterns of unconjugated metabolites produced by the epithelial cells were remarkably similar to one another at all time points. The epithelial pattern was clearly different from that produced by the fibroblastic cells under all conditions tested (Table II). The major metabolites produced from BaP by the epithelial cells eluted within 12 minutes in the first three peaks; the second was often two peaks merged together. Retention times of these peaks in our system corresponds to tetrols (Fig 2). The third major peak from epithelial cells co-elutes with the standard 9,10-dihydrodiol of BaP and, based on the work of others [35,36], could also contain a triol. Formation of this latter metabolite seems unlikely by epithelial cells as no evidence for other isomeric forms of the trihydroxy-BaP derivatives was noted (Fig 2). The 7,9,10/8-tetrahydrotetrol elutes immediately prior to the 9,10-diol standard [35,36] and was not resolved in our system.

The initial peak on HPLC was a major peak at all time periods with epithelial cells, and the predominate one at 24 hours (Table II). Molecules with this retention time (3–7 minutes) in our system include the 7,10/8,9-tetrahydroxy tetrol of BaP [35] and the conjugates of BaP metabolites. When the conjugates produced by epithelial cells from H-97 were first separated from the other metabolites on alumina columns and the metabolites eluted with methanol were subjected to HPLC, this first peak was still a major one, indicating that the majority of this material was tetrol. Hence, in the ethyl acetate extracts subjected to HPLC (H48 and H51), most of the material in this first peak likely represented the tetrol, also.

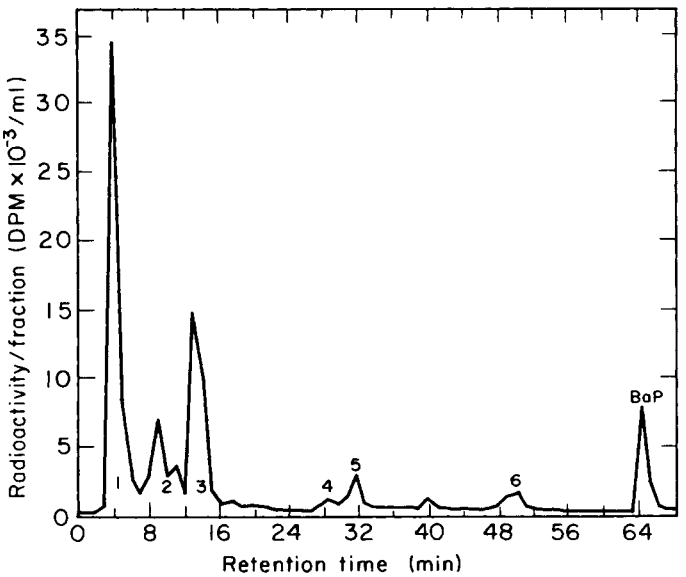

Fig. 2. Elution pattern of organosoluble BaP metabolites produced by human mammary epithelial cells grown out from specimen H97. Mammary epithelial cells were exposed to 0.4 μM BaP for 24 hours. The BaP metabolites were isolated by alumina column chromatography [30], and those eluted with methanol were subjected to HPLC. See text for further details. The retention time of peak 1 corresponds to that of a tetrol and the water soluble conjugates; peak 2 to that of tetrols; peak 3 to that of 9, 10-diol, a tetrol and a triol [35,36]; peak 4 to that of 4,5-diol; peak 5 to that of 7,8-diol; and peak 6 to that of the monohydroxide derivatives of BaP.

Formation of the 7,8-dihydrodiol of BaP (peak 5) could be detected with certainty at all time periods for epithelial cells, but was always a minor peak. These results indicate that epithelial cells rapidly converted 7,8-diol to tetrols, presumably with the ultimate carcinogen, the 7,8-diol-9,10-epoxide of BaP, as an intermediate. The 4,5-diol (peak 4) was also formed by epithelial cells, but in lesser amounts (Fig. 2, Table II). Other metabolites recovered from epithelial cell media included mainly various combinations of the phenolic derivatives of BaP.

Not only was the rate of formation of organosoluble metabolites by fibroblasts different from that by breast epithelial cells, but the pattern was also distinctly different. Fibroblastic cells exposed to either concentration of BaP formed mainly the monohydroxy derivatives of BaP and diols, both the 7,8-dihydro (peak 5) and the 9,10-dihydrodiols (peak 3), rather than tetrols (Table II). Although peak 3 was observed in extracts of fibroblastic media, the symmetry of this peak, which may contain both the 9,10-diol and a tetrol, and the fact that no other tetrol isomers were recovered (Table II), led us to conclude from the fibroblast tracings that the 9,10-dihydrodiol was the major eluant in this fraction. Formation of the 4,5-dihydrodiol by fibroblasts was also detected by 24 hours in the presence of BaP. After an additional 72 hours in media originally free of BaP, the recovered metabolites from fibroblastic cultures were essentially the same as those in the first 24 hours: Phenols plus the 7,8- and 9,10-diols (Table II).

DNA Adduct Formation

Isolation of the nucleoside derivatives of BaP metabolites accumulated in the DNA of mammary epithelial cells revealed that the major adducts were between BPDE, both the anti and syn stereoisomers and deoxyguanosine. These results have been published in a different form elsewhere [23] and are presented here to provide the complete analysis of the metabolic fate of BaP in human mammary epithelial cells. With all the human epithelial samples, two major peaks preceded the dG derivatives. The first of these peaks co-eluted with a tetrol standard. Certainly, the epithelial cells formed large quantities of tetrols and a relatively small fraction of this amount intercalated into DNA might be carried through the nucleoside isolation procedure. This explanation is supported by the fact that this peak was not detected in samples from mammary fibroblastic cultures [23] that produced only small quantities of tetrols. The retention time of the second major peak was similar to that for the deoxycytidine adduct with anti BPDE. Adducts of this form have been reported in other systems metabolizing BaP [19].

The maximal accumulation of BaP adducts in epithelial cell DNA was at the end of the 24-hour incubation in the presence of BaP (Fig. 3). At this time, the sum of the BPDE-dG adducts ranged from 0.56–1.32 adducts/10^6 base pairs. There was greater variation in the quantity of dC adducts with BDPE, ranging from 0.05–0.29 adducts/10^6 base pairs. Analysis of DNA from epithelial cells incubated for an additional 72 hours in the absence of BaP revealed that the quantity of DNA adducts was 64–90% of that after 24 hours in the presence of BaP.

The rate of formation of both types of BPDE-dG adducts increased slightly with time (Fig. 3). The rate of accumulation of the adduct between dG and syn BPDE decreased relative to the other type as the incubation progressed. Because both types of dG adducts exhibited equivalent persistence during the 72 hours in a

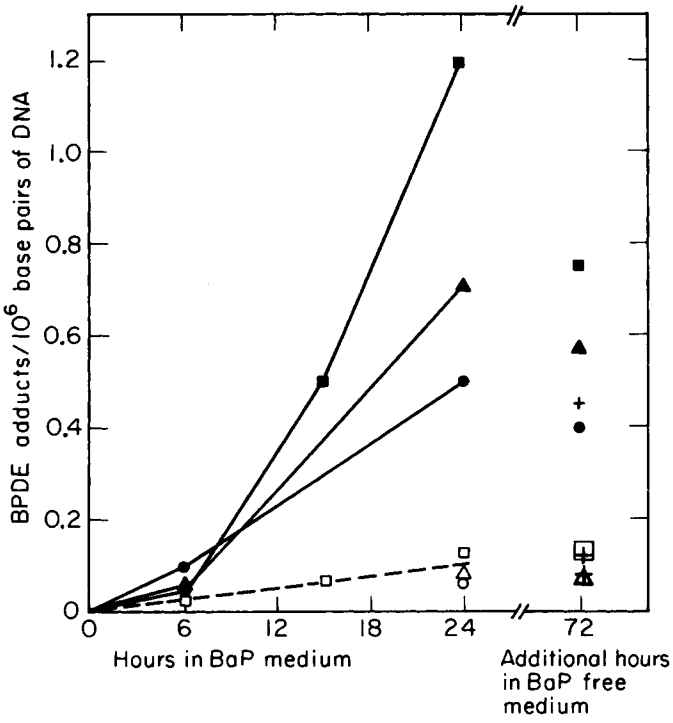

Fig. 3. Extent of adduct formation between deoxyguanosine and anti and syn forms of BPDE. Human mammary epithelial cells from three specimens were exposed in cell culture to BaP containing BaP-free media for the times shown. The nucleoside derivatives of BaP metabolites were isolated from purified DNA and subjected to HPLC analysis as described in the text. The results are expressed as BPDE adducts with dG/10^6 base pairs of DNA. Broken lines and open symbols indicate extent of adduct formation between dG and syn BPDE and the solid lines and symbols that between dG and anti BPDE in epithelial cells. Values from specimen H48 are denoted by triangles; those for H51, by circles, and those for H97, by squares. The extent of anti BPDE/dG adducts in fibroblasts at the only time detected (last time point) is indicated by +; that for syn BPDE/dG adducts in fibroblasts by ‡.

BaP-free medium (Fig. 3), the difference was likely due to changes in the rate of formation rather than to preferential loss of the syn BPDE adducts of dG.

BPDE adduct formation with specific bases of DNA could not be detected in the mammary fibroblastic cells after a 24-hour exposure to a BaP concentration ten times that added to epithelial cell cultures [23]. At the end of an additional 72 hours, 96 hours of total incubation, the extent and type of adduct formation by the fibroblasts was similar to that in the mammary epithelial cells (Fig. 3). It must be kept in mind that for fibroblasts, the concentration of BaP at the end of the 96-hour experimental period was still one-half that for epithelial cells at commencement. The inability to remove all of the BaP and BaP metabolites from the fibroblastic cultures would allow these cells an additional 72 hours to form substrate for macromolecular adduct formation. Hence, if given enough time at the higher concentration of BaP, DNA adducts with BPDE can be produced in human mammary fibroblasts.

DISCUSSION

We have demonstrated previously that the type and rate of DNA adduct formation with the metabolites of BaP in human mammary epithelial cells makes them more likely candidates for transformation by chemical carcinogens than their fibroblastic counterparts [23]. We demonstrate here that the greater ease of DNA adduct formation in the epithelial cells is likely related to the metabolites produced from BaP by these cells. If, as it appears, that the glucuronide and sulfate conjugates were with the monohydroxides and 7,8-diol of BaP [33,34,37] and those with glutathione were with epoxides [37], then the metabolite results taken as a whole indicate mammary epithelial cells preferably converted BaP to diols, some of which were conjugated with glutathione at the first epoxidation step [37]. The remaining dihydrodiols were readily converted to BPDE, a large proportion of which ended up as tetrols and glutathione conjugates, but in the process BPDE was available to react with macromolecules, notably DNA. In contrast, mammary fibroblasts preferentially produced phenols and diols. The accumulation of diols in the later stages of the fibroblastic incubations indicates that these cells had a much lower capacity to convert the diols to the diolepoxide form than did human mammary epithelial cells.

Evidence for a metabolite pattern of chemical carcinogens involving a large conversion to water-soluble metabolites has been reported previously in organ cultures of rodent mammary gland[39]. Presumably, the epithelial cells present in these mixed cultures were principally responsible for this production. The fact that carcinogen-treated explants transplanted into syngeneic hosts produced mammary adenocarcinomas provides evidence in the rodent model system that mammary epithelial cells in organ culture are transformed by chemical carcinogens [39]. This finding is, of course, consistent with the observation originally made many years ago that feeding chemical carcinogens to rodents results in production of mammary adenocarcinomas [1,2]. The ability of mammary epithelial cells to form adducts between DNA and metabolites of chemical carcinogens and a correlation between this adduct formation and mammary carcinogenesis have also been demonstrated in a rodent model system [7]. Recently, other workers have demonstrated that mammary epithelial cells grown out in culture from human specimens metabolize chemical carcinogens in a manner yielding adducts with DNA [40]. The massive amount of work demonstrating that chemical carcinogens act to transform mammary epithelial cells in the rodent system [1–7] and the recent demonstrations that the human mammary epithelial cell metabolizes chemical carcinogens in a manner compatible with carcinogenic transformation [23,40], provide ample evidence that a role for chemical carcinogens should be taken seriously in the development of breast cancer in women.

The wide interspecies variation revealed by studies on chemical carcinogens [41] has emphasized the need for human cells and tissues for studies on the mechanisms of cancer initiation and promotion. In addition, the fact that 85–90 per cent of all human cancer is of epithelial cell origin makes apparent the importance of an epithelial system for carcinogenic investigations. Studies of chemical carcinogenesis have been conducted in a wide variety of human epithelial organs in culture [14,36,41–46]. Such studies are hampered by wide sample variation and, more important to our understanding of carcinogenic mechanisms, by the mixture of cell types present. Indeed, some of the sample variation observed is likely related to

cellular heterogeneity. Furthermore, the mixture of cell types precludes study of carcinogenic metabolism and DNA adduct formation, as well as the relationship between these two, in a specific cell type. Pure epithelial cell cultures derived from human specimens are available in cell lines. However, the selective pressure of repeated passage in culture and the fact that these cells are "immortal" makes the relationship between the metabolic activity of these cells and that occurring in normal epithelial cells questionable.

The system described here overcomes the disadvantages of both the organ culture and cell line culture systems. Some individual variation in the metabolic characteristics was retained but it was not as great as that observed in organ cultures of human tissues [36,44–46]. Hence, it would appear that the ability to passage the human mammary epithelial cells allowed us to maintain the cells for a time sufficient to minimize environmental, nutritional, and hormonal influences impinging on the cells while in the donor. On the other hand, the passage number was low enough to avoid much of the selective pressure of cell line development. The variation among epithelial cells from three specimens was small enough so that the metabolic pattern of chemical carcinogen metabolism by these cells was clearly distinct from that of fibroblasts. Therefore, the extent of specimen variation in human mammary epithelial cells is apparently sufficiently limited to allow studies under the controlled conditions of cell culture of factors thought to influence the initiation and promotion of breast cancer in women.

ACKNOWLEDGMENTS

We gratefully acknowledge Annie Pang and Cheryl Namy for excellent technical assistance, Dr. Dezider Grunberger for helpful discussions, and Dr. Adeline Hackett for her support and encouragement. This research was supported by USPHS grant CA-24844 from the National Cancer Institute, by grant CD-61B from the American Cancer Society, by contract 4425/003298 from the U.S. Department of Energy, by the Peralta Hospital Association, and by the Assistant Secretary for Environment, Office of Environmental Research and Development, Biomedical and Environmental Research Division of the US Department of Energy under contract W-7405-ENG-48.

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