Metabolism and Formation of DNA Adducts of Benzo(a)pyrene in Human Diploid Fibroblasts

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ABSTRACT

Cultured human diploid skin fibroblasts incubated with [G-3H]benz(a)pyrene yielded about 10 times more H2O-soluble benzo(a)pyrene metabolites and DNA adducts at stationary growth phase than did proliferating cultures. This increased formation could be blocked by α-naphthoflavone. Trichloropropanoxide and cyclohexenoxide, inhibitors of the epoxide hydratase, inhibited predominantly the formation of DNA adducts. Cultures from older individuals formed significantly more benzo(a)pyrene metabolites and DNA adducts, but control cultures from patients with either lung cancer or melanoma did not. The age influence was not apparent when the ratio of DNA adducts to H2O-soluble metabolites was determined for each individual cell line. However, the proportion of DNA-bound material in the cells from patients with lung cancer was significantly increased compared to cells from melanoma patients or healthy individuals.

INTRODUCTION

Polycyclic hydrocarbons are thought to exert their carcinogenic influence by covalent binding of highly reactive metabolic intermediates to DNA (3). BP2 pyrene is the most abundant substance of this class. An ubiquitous pollutant produced by incomplete combustion (27), it is probably involved in the formation of certain types of human cancer, in particular of the lung and the urinary bladder (10, 26). The metabolism of benzopyrene is complex (28); more than 20 intermediary metabolites have been identified to date, and most probably they arise in varying proportions, depending on the type of monoxygenase present in the exposed tissue (20, 37, 40-43).

Conflicting results about the relationship between benzopyrene metabolism and the risk for human cancer, particularly bronchogenic carcinoma, have been reported. Studies with mitogen-stimulated lymphocytes have shown a trimodal distribution of the inducibility of the key enzyme aryl hydrocarbon hydroxylase, patients with lung cancer having an altered pattern (13, 21). Other investigators reported contradictory results, some also being unable to confirm a 2-allele model (1, 14, 15, 22, 33). Studies comparing either the monoxygenase activity or the formation of water-soluble metabolites in alveolar macrophages (25) or monocytes (32) also could not leave differences between tumor patients and controls that could have been attributed to a different, and possibly genetically determined, cancer risk resulting from exposure to benzopyrene.

This study describes the intra- and interindividual variability of water-soluble metabolites and DNA adducts of benzopyrene formed in skin fibroblast cultures from patients with bronchial carcinoma in comparison to healthy individuals and patients with melanoma as controls.

MATERIALS AND METHODS

Origin of Cultures. Skin biopsies from 29 patients with histologically or cytologically confirmed lung cancer were taken from the inner part of the upper arm prior to the onset of a specific therapy. The tumors were classified according to the recommendations of Kreyberg (23) as type I in 25 patients and type II in 4 patients. Eight patients of the lung cancer group had at least one first-degree relative with lung cancer. Informed consent was obtained from any donor of a skin biopsy. Twenty-eight apparently healthy male and female Caucasians served as controls. There was no history of lung cancer among their first-degree relatives.

For the determination of the intraindividual variability, 11 biopsies were taken at the same time from both upper arms and both upper legs of a normal 23-year-old female volunteer. Fibroblasts from 7 patients with histologically confirmed melanoma were cultured from apparently nonmela

Fibroblast Cultures. These were established from the skin biopsies by standard techniques (34), and after 3 to 5 generations the cells were stored frozen in liquid nitrogen. We used Dulbecco’s modified Eagle’s medium (Grand Island Biological Co., Grand Island, N. Y.) buffered with 20 mM 2,4-(2-hydroxyathyb)piperazmnyl-(1)-athanesulfonata (Sarva, Heidelberg, West Germany) and supplemented with 10% fetal bovine serum (Grand Island Biological; Lot K 155601 D), penicillin (120 mg/liter), and neomycin (100 mg/liter). For primary cultures, fetal bovine serum was added to a final concentration of 20%. Mycoplasma checks were performed by fluorescent microscopy after staining with 4',6-diamidino-2-phenylisodol (36). Growth factor-depleted fetal bovine serum (17) was prepared by adsorption with charcoal, 1 g/20 ml serum, at pH 4.0; the mixture was stirred for 60 min and then centrifuged twice for 1 hr at 20,000 × g to remove the adsorbant. Each batch of growth factor-depleted fetal bovine serum was checked prior to use according to Chart 2.

Preparation of [G-3H]Benzopyrene Solution. [G-3H]Benzopyrene (specific activity, 40 Ci/mmol; 5 nCi in 1 ml benzene) was obtained from Amershams-Buchler (Braunschweig, West Germany). The same batch was used for all determinations described here. Solution (0.1 to 0.2 ml) was purified by thin-layer chromatography on silica plates.
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(Merck, Darmstadt, West Germany) with benzene as a solvent. Two spots of unlabeled benzopyrene (Sigma Chemical Co., St. Louis, Mo.) were run on the same plate as a reference because its light yellow color facilitated localization of the labeled material after the run. The radiactive material was eluted from the silica matrix with acetone and mixed with a 40-fold excess of growth factor-depleted fetal bovine serum. The whole procedure was carried out under minimal light. Due to the rapid decomposition of the [3H]benzopyrene, chromatographic purification was necessary prior to each set of experiments to retain a sufficiently low background.

Determination of Water-soluble Metabolites. Cells (2 × 10^5) were seeded into an individual well of a cluster dish (Nunc, Roskilde, Denmark) and were allowed to settle for 12 to 15 hr. Then the cell layer was washed 3 times with Dulbecco’s phosphate-buffered saline (9), 1 ml of medium containing 10% growth factor-depleted fetal bovine serum was added, and the cells were incubated for another 48 hr. After this time, the medium, now containing 150 nM [G-^3H]benzopyrene, was replaced, and incubation was continued for another 24 hr.

During the entire incubation time, the cells were kept sterile and maintained in a moist incubator equilibrated with an atmosphere containing 2.5% CO_2 in air. H_2O-soluble BP metabolites were determined after the cells were extracted with organic solvent (19). Culture medium of each well (0.4 ml) was transferred into a 1.5-ml microtube (Netheler and Hinz, Hamburg, West Germany) together with 0.1 ml of 0.5 M Tris-HCl buffer, pH 8.0, containing 1% SDS. One ml of chloroform:methanol (2:1) was added to each tube, the sample was mixed thoroughly for 5 mm on a Modal 3300 mixer (Netheler and Hinz), and the phases were subsequently separated with a Model 3200 microcentrifuge (Netheler and Hinz). Supernatant (0.4 ml) was used to determine radioactivity with Instagel (Packard Instruments, Frankfurt, West Germany) in a Packard Model 2450 B liquid scintillation counter. The total content of H_2O-soluble radioactivity in one individual well was represented by cpm × 2.5. Blanks without cells were run on the same plate.

Determination of DNA Adducts. After 24 hr of incubation in the presence of [3H]BP, the cell layer was washed intensively 3 times with Dulbecco’s phosphate-buffered saline. The DNA was isolated by a modified method based on the use of protease K (12). The cells were dissolved with 0.5 ml of 0.01 M Tris buffer, pH 8.0, containing 1% SDS, 0.01 M EDTA, 0.01 M NaCl, and protease K, 50 μg/ml (Merck, Darmstadt, West Germany), for at least 6 hr in a moist atmosphere at 37°C. Then each mixture was transferred into an individual microtube. Distilled phenol (0.5 ml), saturated with 0.5 M Tris buffer, pH 8.0, containing 1% SDS, 0.01 M EDTA, and 0.01 M NaCl was added. After continuous mixing for 5 min, the samples were centrifuged. 0.4 ml of each supernatant was transferred into a new microtube and combined with 0.02 ml of RNA solution containing baker’s yeast RNA (Boehringer, Mannheim, West Germany), 20 mg/ml. One ml of ice-cold ethanol was added to each microtube. After storage of the samples at −20°C overnight, the precipitate was collected by centrifugation. It contained 75 ± 5% (S.D.) of the DNA initially present in the cells as determined by parallel runs with [4C]DNA (Amersham-Buchler). The RNA adducts of the sample were about 15% of the amount of DNA adducts as determined by parallel runs in the presence of RNase. The DNA precipitate was dissolved in 0.5 M perchloric acid, and its radioactivity was determined in 10 ml of Instagel with a liquid scintillation spectrometer.

DNA and Protein Determinations. DNA was isolated from cells derived from cultures handled at the same time and from the same cluster dish and quantitatively determined by the diphenylamine method (5). Calf thymus DNA was used as a standard. Protein was determined by the method of Lowry et al. (24) using crystalline bovine albumin as a standard.

RESULTS

Both the time required to reach confluency and the final density of the cell layer differed considerably among cell lines, even when derived from the same individual. However, we observed no morphological differences between individual cell lines that could be attributed to the status of the donor, i.e., disease, age, or sex. The data presented in Charts 1 to 6 were obtained with different cell lines, each derived from a healthy donor. As shown in Chart 1, the cell growth markedly influenced the conversion of benzopyrene to H_2O-soluble products. In apparently confluent cultures, cell growth was about 4 times higher than in exponentially growing cultures. This increase could be enhanced after the cells had been arrested in G phase of the cell cycle by growth factor-depleted fetal bovine serum or aminophylline (6). In all instances, the BP metabolism could be completely inhibited by ANF. The effect of charcoal depletion of fetal bovine serum on the cell proliferation is demonstrated in Chart 2. In growth-arrested confluent cultures, both BP
metabolism and formation of DNA adducts proceeded linearly with time from 5 to at least 36 hr of incubation (Chart 3). Both parameters, however, increased exponentially during the first hours of incubation in the presence of BP. This is due to the well-known substrate-mediated metabolic induction of BP metabolism (27, 39). When induction by the substrate was inhibited by cycloheximide, 10 μg/ml, we observed only minimal residual metabolic activity (Chart 1). After the cells were preincubated with BP, the addition of cycloheximide progressively reduced their metabolic activity to 50% after 6 hr (Chart 4). ANF, an inhibitor of the cytochrome P-448-dependent monoxygenase (8), blocked the formation of water-soluble metabolites (Chart 1), and the formation of DNA adducts was dose dependent (Chart 5). In contrast, when we added CHO and trichloropropenoxide as inhibitors of the epoxide hydratase (4, 30) to the cells together with [³H]BP for 6 hr, predominantly the formation of DNA adducts was reduced. Chart 6 shows that the ratio between H₂O-soluble metabolites and DNA adducts rises in the presence of both inhibitors. This effect is less pronounced with CHO because partially it also inhibits the monoxygenase at the concentrations used (44) and because it is destroyed by epoxide hydratase (29, 31) and by rapid reaction with glutathione (30).

Table 1 summarizes our data for all fibroblast lines. Two cell lines with high and one with a low BP turnover were tested at weekly intervals during the 4th to 16th subculture. The variability of the parameters tested was about 10% or less when the same fibroblast line was used. On the other
Table 1

<table>
<thead>
<tr>
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<th>BP metabolites (pmol/10 μg DNA)</th>
<th>BP adducts (fmol/μg DNA)</th>
<th>Metabolites DNA adducts</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>S.E.</td>
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<tr>
<td>Multiple determinations (n) with 3 cell lines (A, B, C)</td>
<td></td>
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</tr>
<tr>
<td>A. n = 5</td>
<td>26.6</td>
<td>2.81</td>
<td>1.26</td>
</tr>
<tr>
<td>B. n = 5</td>
<td>5.61</td>
<td>0.54</td>
<td>0.24</td>
</tr>
<tr>
<td>C. n = 3</td>
<td>28.8</td>
<td>2.44</td>
<td>1.41</td>
</tr>
<tr>
<td>Different cell lines derived from the same donor (n = 11)</td>
<td>8.59</td>
<td>3.42</td>
<td>0.99</td>
</tr>
<tr>
<td>Controls (n = 28)</td>
<td>16.2</td>
<td>7.21</td>
<td>1.36</td>
</tr>
<tr>
<td>Lung cancer (non-familial) (n = 21)</td>
<td>17.9</td>
<td>4.44</td>
<td>0.97</td>
</tr>
<tr>
<td>Lung cancer (familial) (n = 8)</td>
<td>18.5</td>
<td>6.29</td>
<td>2.22</td>
</tr>
<tr>
<td>Melanoma (n = 7)</td>
<td>16.2</td>
<td>13.7</td>
<td>5.16</td>
</tr>
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CV, coefficient of variation.

hand, the 11 individual fibroblast cultures from the same donor had a coefficient of variation of about 40%, which is similar to cultures from different donors. However, the ratio of metabolism to DNA adducts in different cell strains from the same donor remained fairly constant (coefficient of variation, 6.6%). The BP turnover and formation of DNA adducts in cells derived from patients with bronchogenic carcinoma are slightly but not significantly increased compared to controls (t test). Age-matched individuals of both groups did not differ. However, the ratio of water-soluble metabolites to DNA adducts differs significantly in cultures derived from patients with bronchial carcinoma compared to controls (healthy individuals and melanoma patients). A significant difference (p = 0.05) was obtained whether the data were analyzed by a parametric test (t test) or a nonparametric test (Mann-Whitney). The data on the water-soluble metabolites:DNA adduct ratio are independent of the age of the donor (Chart 8). Cell cultures from 8 patients with familial lung cancer were evaluated separately but did not differ significantly from those of either nonfamilial tumors or controls.

Chart 8 shows the wide interindividual variability of BP metabolism and formation of DNA adducts in control fibroblasts when plotted against the age of the donor at the time of biopsy. In spite of this wide variability, linear regression analysis of both parameters showed that they were correlated with the age of the probands in each cell line tested.

**DISCUSSION**

Our results indicate that it is necessary to define the growth phase in order to be able to compare the BP turnover between individual fibroblast cell lines. In contrast to fetal hamster cells, where the ability to induce the hydroxylase activity is greatest in cells entering logarithmic growth phase (27), human fibroblasts metabolize more BP in the stationary phase. Therefore, it seems reasonable to test growth-arrested cultures. This can be achieved either by drugs like aminophylline (influences the BP-metabolizing enzymes) or by growth factor depletion of the fetal bovine serum in the culture medium. The growth-promoting activity of fetal bovine serum differs from batch to batch (18). This could explain the influence of serum on the BP inducibility and turnover reported by others (11, 14). A coefficient of variation of about 10% was observed for the BP turnover and the formation of DNA adducts within the same cell line. Surprisingly, in different cell cultures derived...
from skin biopsies obtained from the same donor at the same time, the coefficient of variation for both parameters was in the same range as for cell lines from different donors, both being 40%. We assume that skin biopsies from different areas of the body may yield cell populations in culture that are composed of several morphologically undistinguishable cell types that differ in their capacity to metabolize BP. Similarly, the enhanced BP turnover observed in cultures from older probands can be explained as an age-dependent shift in the proportion of cell types originally present in the same culture. On the other hand, since lung cancer occurs in advanced age, one could speculate that the increased formation of reactive BP metabolites observed in cultured cells from older probands could reflect an enhanced predisposition in vivo for BP-induced cancer in older people.

In contrast to the wide variability of the formation of H$_2$O-soluble metabolites and DNA adducts observed in cultures from different biopsies of the same individual and from different individuals, the proportion of active metabolites (calculated as the ratio of H$_2$O-soluble to DNA-bound material) consistently showed a low intrapersonal variability both within the same cell line and in different cell lines from the same individual, whereas the variability was double in cells from different donors. Therefore, we consider that the increased proportion of active metabolites observed in the cells from patients with lung cancer is possibly related to an increased risk for BP-induced tumors in vivo. Apparently, more reactive intermediates are bound to the DNA of patients than to that of controls at a given rate of BP turnover. Such a relatively increased formation of DNA adducts in lung cancer patients could theoretically be based on either an altered pattern of BP metabolites or a reduced capacity to eliminate reactive BP metabolites from DNA. The observed difference in the proportion of DNA-bound material versus total metabolites in both groups, although relatively small, could be important for the development of lung cancer if one speculates that only a small proportion of the total DNA adducts is potentially mutagenic. Something similar has been demonstrated with ethylnitrosourea (35), where a high "noise level" of material unspecifically bound may conceal even large individual differences of specific DNA adducts. An analysis of the reaction products of BP metabolites with DNA (38) formed in the cells of lung cancer patients and controls may elucidate this problem.

The complete inhibition of BP metabolism by ANF indicates that in skin fibroblasts it is exclusively dependent on a cytochrome P-448 monooxygenase. Since trans-diol-epoxides, the most important reactive intermediates of this pathway (32), can be formed only in the presence of epoxide hydratase, one would expect that after inhibition of this enzyme the proportion of active metabolites is decreased. Indeed, this was obtained with the epoxide hydratase inhibitors trichloropropenoxide and CHO. Of course, comparing cultured diploid fibroblasts and human cells in vivo can be questioned, particularly if the possible target cells of inhaled carcinogens are in the bronchial system. Nevertheless, fibroblasts used for such studies afford an opportunity to test independently of the donor (16) and avoid uncontrolled influences such as the disease status or therapeutic materials that may be present in blood cells or biopsy material.

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