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ONLY ONE TYPE OF BENZO(A)PYRENE-DNA ADDUCT IS DETECTED IN TRANSFORMABLE MOUSE CELLS

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Summary

When highly transformable BALB/3T3-A31 clone 1-13 cells are exposed to benzo(a)pyrene for various lengths of time, only one type of carcinogen-DNA adduct is detected. No minor adducts were found as reported in other cell systems. High pressure liquid chromatography identified this persistent adduct as 10-trans-7R-benzo(a)pyrene diolepoxide I-deoxyguanosine. Formation of benzo(a)pyrene-deoxyguanosine adduct, therefore, appears sufficient to initiate the transformation process induced by benzo(a)pyrene in 1-13 cells.

Introduction

The prevailing genetic theory in chemical carcinogenesis argues that covalent binding of carcinogens to DNA is a crucial step in the initiation of neoplasia. A growing number of reports have indicated that only those covalent adducts which remain bound to DNA for extended periods could be essential for such process. For examples, with liver carcinogen, aromatic amide, both arylamidated N^2-and C8-deoxyguanosine adducts were formed in rat liver DNA; however, only the N^2-adduct has been found to be the persistent lesion in vivo (1-4). The persistence of O^6-alkyl deoxyguanosine in certain tissues has been correlated with the susceptibility of these tissues to carcinogenesis (5-8).

Abbreviations used: BP, benzo(a)pyrene; BPDE I,(±)-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, diol epoxide I, r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BPDE II, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, diol epoxide II, r-7,t-8-dihydroxy-c-9,10-oxy-7,8,9,10-tetrahydroxybenzo(a)pyrene, 10-trans-7R-BPDE I-dGua, N^2-(10S-[7R,8S,9R-trihydroxy-7,8,9,10-tetrahydrobenzo(a)-pyrene]yl-deoxyguanosine; dGua, deoxyguanosine; HPLC, high-pressure liquid chromatography; PBS, phosphate buffered saline; HEC, hamster embryo cells; MEF, secondary mouse embryo cell; BHK, baby hamster kidney cells.

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Benzo(a)pyrene (BP) is a common environmental contaminant and a strong carcinogen. Formation of BP-DNA nucleoside adducts has been detected in mammalian tissue cultures (9-18) and in intact animals (19). Although BP-deoxyguanosine adducts were the major products formed in all the examined systems, which ranged from the transformable rodent cells to the resistant human cells, some minor BP adducts in nucleosites other than deoxyguanosine were also produced (13,18). At the present time it is not known which types of these DNA modifications are correlated most strongly with the carcinogenic process.

We chose to investigate the covalent BP-DNA adducts produced in BALB/3T3-A31 clone 1-13 for the following reasons: (1) This cell line gives high plating efficiency; (2) Since this cell line is highly susceptible to the transformation induced by carcinogens (20), it provides a sensitive system to detect the carcinogen-DNA adducts, especially those adducts produced in minute amount, which may play an important role in the initiation process of cell transformation.

We report here that only one type of persistent BP-DNA adduct (i.e. BPDE I-dGua) is formed in this highly transformable mouse cell line, suggesting that BPDE I-dGua itself is sufficient to initiate the transformation process.

Materials and Methods.

Cell Cultures: Clone 1-13 isolated from mass culture of BALB/3T3-A31 was used (20). Cells were plated at $1x10^5$ cells per 10-cm tissue culture dish and were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum.

Binding of [6-^{3}H]BP to DNA: [6-^{3}H] BP (Amersham-Searle) was diluted with cold BP (Chemical Repository, NCI) to specific activity at 6-20 ci/mmole, and purified by a gravity flow silica gel column (21) before use. Cells at 70% confluency were treated with [6-^{3}H] BP for the lengths of time specified. At the end of incubation, cells were washed once with ice-cold phosphate buffered saline (PBS) and collected by scraping. After harvesting, cells were again washed with PBS, suspended in 10mM Tris-HCl (pH7.5), 10mM EDTA, 10mM NaCl, lysed by 0.5% sodium dodecyl sulfate (SDS) and treated with proteinase K at

50μg/ml overnight. DNA was extracted twice with buffered saturated phenol containing 0.1% 8-hydroxyquinoline, and was then precipitated with two volumes of cold 95% ethanol. The precipitated DNA was further purified by digestion with RNase, phenol extraction and ethanol precipitation. DNA solutions were then heated to 100°C for 15 min to remove the possible intercalated hydrocarbon, passed through a short Sephadex LH20 (pharmacia) column (1x15cm) and lyophilyzed to dryness. The amount of BP bound to DNA was determined by counting of samples of treated DNA in a Beckman LS8100 scintillation counter. Concentration of samples was determined by Burton reaction (22).

Intracellular BP Pools: BP treated cells from two 10-cm culture dishes were rinsed twice with MEM, harvested with trypsin and resuspended in PBS at $2x10^6$ cells/ml. 2 ml of cell suspension were lysed with 2ml of acetone and then extracted with 4 ml of ethyl acetate. Following removal of the organic phase, the aqueous phase was extracted with equal volume of acetone/ethyl acetate (1:2 v/v). The organic phases were combined, dehydrated, dried, and finally dissolved in 0.5 ml of benzene. BP was separated from its metabolites on a silica gel column as described by Okano et al (23).

Analysis of [6-^{3}H]BP-DNA Adducts: BP modified DNA samples were enzymatically hydrolyzed and the nucleoside mixture was chromatographed on Sephadex LH20 (24). The isolated nucleoside adducts were analyzed by high pressure liquid chromatography (HPLC, Waters Associated) fitted with a Zorbax ODS column (6.2 x 250mm, DuPont). Samples were eluted with a linear gradient from 40-88% Methanol-water over a 50-min period.

Preparation of BP-7,8-dihydrodiol 9,10-oxide (BPDE)-polynucleotide products: [^{3}H]BPDE I (Chemical Repository, NCI) was reacted with poly d(G) and poly d(A) (P-L Biochemicals) for 18 hrs at 37°C in a tetrahydrofuran-water mixture (1:5, v/v, PH 7.0). After extraction with ethyl acetate, the polynucleotides were precipitated twice with ethanol. The BPDE I-polynucleotide products were enzymatically hydrolyzed, separated on Sephadex LH20 column, and analyzed by HPLC as authentic markers.

Results and Discussion

Previously, we reported that treatment of 1-13 cells with 0.5μM of BP produced 25 transformed foci per 10^4 surviving cells (25). We then examined, at this concentration of carcinogen, the amount of BP bound to DNA and types of BP adducts formed. The time course of binding of BP to 1-13 cellular DNA is shown in Fig.1. When cells were exposed to low level of carcinogen (0.5μM), the amount of BP bound to DNA reached a plateau at (or even before) 18 hr of incubation with the carcinogen. This could suggest that no remaining BP or BP metabolites were available for DNA binding at later time points. Alternatively, the steady state level of DNA binding seen in Fig.1 could be due to equal rates of forma-

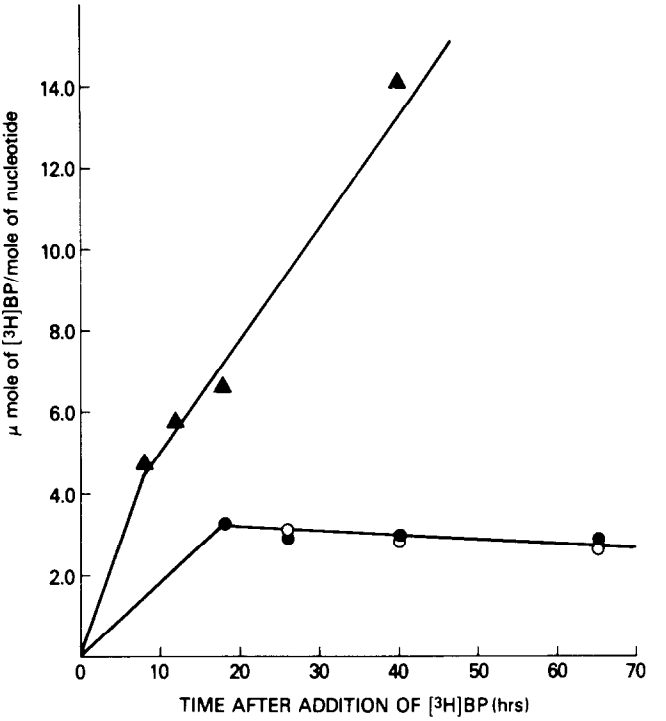

Figure 1. Time course of binding of [6-^{3}H]BP to DNA in BALB/3T3-A31 clone 1-13 cultures. -●-●-, cells were exposed to 0.5μM of [6-^{3}H]BP. After 18 hr incubation with the carcinogen, hydroxyurea was added to medium (final concentration, 1mM) to inhibit semiconservative DNA synthesis. -o-o-, "posttreatment incubation". Cells were exposed to 0.5μM of [6-^{3}H]BP for 18 hr, after which cells were rinsed with warm medium and then incubated in fresh BP free medium containing 1mM of hydroxyurea. -▲-▲-, cells were exposed to 2.0μM of [6-^{3}H]BP. DNA was isolated and the amount of BP bound to DNA was determined as described in Materials and Methods.

tion and removal of BP-DNA adducts since excision of BP damaged DNA has been reported in many mammalian systems (11,13,18,26). To distinguish between these possibilities, we studied the fate of BP both in medium and in intracellular BP pools. We also monitored DNA repair by post-treatment incubation of cells in the absence of carcinogen. As shown in Table 1, after 18 hrs incubation of cells with 0.5uM of [6-^{3}H]BP, only 1% of BP remained in the medium. BP pool studies also suggested the sequestering of the carcinogen inside cells. Furthermore, when 1-13 cells were first exposed to BP for 18hr and then incubated with BP free medium for various lengths of times, it followed the same binding

Table I Intracellular BP pools and extent of BP binding to BALB/3T3-A31 clone 1-13 DNA.

Duration of treatment (hr)	% of BP remained in medium		Intracellular BP pools (p mole/10^6 cell)		Extent of binding[a] (μ mole BP/mole of nucleotides)	
	2 μM	0.5 μM	2 μM	0.5 μM	2 μM	0.5 μM
0	100.0	98.5	-	-	-	-
8	37.0	ND	ND	ND	4.7	ND
12	24.4	ND	14.7	ND	5.8	ND
18	13.4	1.0	6.5	0.24	6.6[b]	3.3
26	ND	0.7	ND	0.14	ND	2.9 (3.1)[c]
40	ND	0.3	ND	0.15	14.1[b]	3.0 (2.8)
65	ND	0.3	ND	0.12	ND	2.9 (2.6)

a values obtained from two determinations
b mean of 5 independent experiments
c values in parentheses represent BP bound to DNA after posttreatment incubation of culture with BP free medium

kinetics as that when BP containing medium was used (Fig. 1). These findings support the idea that the plateau of DNA binding is due to the lack of BP or its active metabolites for further reactions, and that the small decrease in specific activity of DNA during posttreatment incubation is attributable to the removal of BP adducts from DNA. Our results indicated that 1-13 cells excised carcinogen adducts with extremely low efficiency. More than 80% of the lesions remained intact in the cells over two and a half cell generations. Further studies are required to determine whether the low repair capacity of 1-13 cells play a crucial role in its high susceptibility to BP-induced transformation. On the other hand, when cells were exposed to high level of carcinogen (2.0μM), binding of BP to cellular DNA increased with time as long as BP was available in the medium or in the cells (Fig. 1, Table 1). These data suggest that in order to obtain relevant correlation between the study of chemical-biological interactions and the transformation process induced by this carcinogen, it is better to use the same concentration of carcinogen in both types of experiments. If a higher concentration of carcinogen has to be used to detect the biochemical products, the importance of

these products in relation to the transformation process should be established.

DNA isolated from 1-13 cells exposed to 0.5μM and 2.0μM of [6-^{3}H]BP was subjected to enzymatic hydrolysis and the nucleoside mixture was chromatographed on Sephadex LH20. The isolated nucleoside adducts were analyzed by high pressure liquid chromatography (HPLC). We have employed relativey large amount of samples for HPLC studies to prevent either overlooking or overestimating the minor peaks which may be produced by total cell population or might be produced by a negligible fraction of cells in total cell population. HPLC profiles of 1-13 DNA digests were compared with the profiles of BPDE I modified [^{14}C] purine DNA digest and of authentic markers (Fig. 2). The digests of all 1-13 samples revealed only one radioactive peak. Its elution position coincided with the second peak of deoxyguanosine adducts, which corresponded to 10-trans-7R-BPDE I-dGua found in hamster embryo cells (HEC,13). This predominant isomer contributed 97-99% of the total radioactivity recovered. The remaining 1-3% of radioactivity was associated with the first peak of deoxyguanosine adducts (7S enantiomer of BPDE I-dGua,13). The stereoselective synthesis of BPDE I isomer by 1-13 cells is in accord with the finding in mouse embryo 10T1/2 cells (18), Mouse skin (19), and in liver microsomes (27). We did not observed changes in the relative abundance of these two isomers as that reported in HEC (13), in which 7R-BPDE I-dGuo is removed at a faster rate than its 7S enantiomer. In the in vivo systems which have been studied, the types of BP-DNA adduct formed vary from system to system. 10-trans-7R-BPDE I-dGua was the only adduct produced in human and bovine bronchial explants (14). In addition to this major adduct, small amount of BPDE II-dGua was formed in the explants of human colon (15), lung (16),

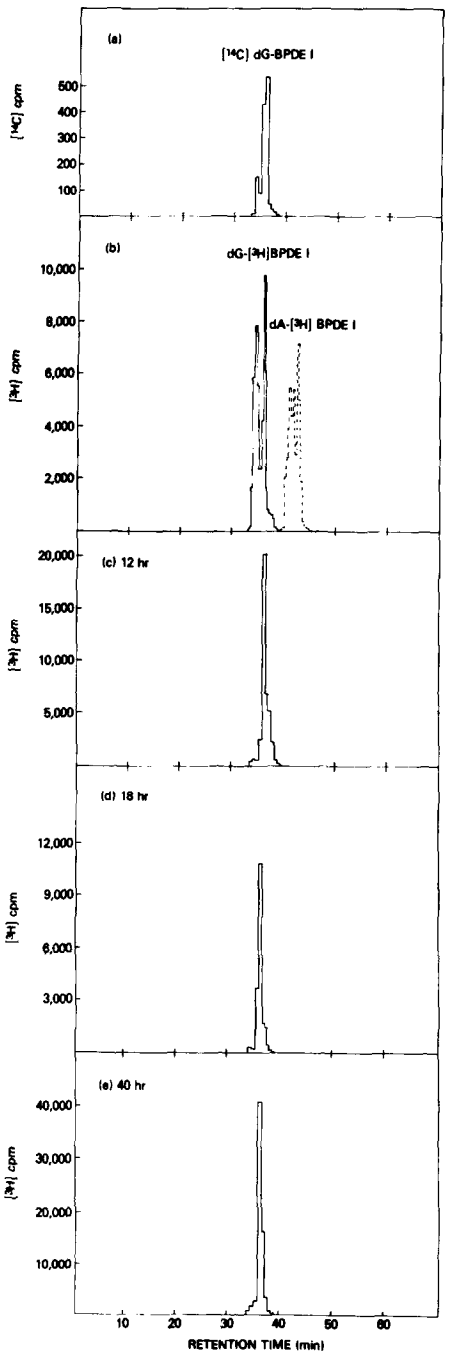

Figure 2. HPLC profiles of DNA adducts formed in BALB/3T3-A31 clone 1-13 cultures incubated with [6-^{3}H]BP. (a) *in vitro* marker from digest of BPDE I modified [^{14}C] purine DNA. (b) *in vitro* marker formed by reaction of [^{3}H] BPDE I with poly d(G) and poly (dA) respectively. (c)-(e) DNA adducts isolated from 1-13 cells after 12 hr (c), 18 hr (d), and 40 hr (e) exposure to 2.0μM of [6-^{3}H] BP. DNA isolated from cells exposed to 0.5μM of [6-^{3}H]BP exhibits an identical elution pattern.

and in secondary mouse embryo cells (MEF,11). The deoxycytidine adduct was also detected in 10T1/2 cells (18). Both BPDE I- and BPDE II-dGua at comparative level were found in baby hamster kidney cells (BHK,II) and A549 human alveolar tumor cells (17). The 7S enantiomers of BPDE I-dGua and BPDE II-dGua were produced in mouse skin (19). In HEC (13), besides BPDE I- and BPDE II-dGua adducts, small amounts of both deoxyadenosine and deoxycytidine adducts were also found. Cells of some of these systems, like human cells, are resistant to transformation induced by BP. However, HEC (28-31) and 10T1/2 cells (32) are readily transformed by this carcinogen. It is not known which types of these BP-DNA adducts are correlated most strongly with the carcinogenic process.

Minor adducts (33) and differential rate of excision of multiple adducts have been implicated in mutagenic and transformation processes in some systems (11,13,17). Our results clearly indicate that this is not the case with the highly transformable 1-13 cells, since only one type of BP-DNA adduct (BPDE I-dGua) was observed in all the DNA samples examined. Our assays are able to detect minor adducts at quantities as little as one thousandth of that of the total adducts produced. Should minor adducts be formed in 1-13 cells they probably represent less than 0.1% of the total adducts formed. Unless these minor adducts are a thousand fold more efficient in inducing transformation than deoxyguanosine adduct, it is impossible for them to be the major cause of transformation. Our data also show that BPDE I-dGua is persistent in BP-treated cells over extented periods. Therefore, formation of this adduct appears responsible for the induction of transformation by BP in 1-13 cells, provided that the covalent binding of carcinogen to DNA is a necessary step in chemical carcinogenesis.

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