

p53 Controls Global Nucleotide Excision Repair of Low Levels of Structurally Diverse Benzo(g)chrysene-DNA Adducts in Human Fibroblasts¹

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ABSTRACT

Benzo(g)chrysene is a widespread environmental contaminant and potent carcinogen. We have measured the formation and nucleotide excision repair of covalent DNA adducts formed by the DNA-reactive metabolite of this compound in human fibroblasts, in which expression of the p53 tumor suppressor gene could be controlled by a tetracycline-inducible promoter. Cells were exposed for 1 h to 0.01, 0.1, or 1.2 μM (\pm)-anti-benzo(g)chrysene diol-epoxide, and DNA adducts were assessed at various post-treatment times by subjecting isolated DNA to ³²P-postlabeling analysis. Four major DNA adducts were detected, corresponding to the reaction of either the (+)- or (–)-anti-benzo(g)chrysene diol-epoxide stereoisomer with adenine or guanine. Treatment with 1.2 μM resulted in a level of 1100 total adducts/10⁸ nucleotides for both p53-proficient and -deficient cells; removal of adducts was not observed in either case. In cells treated with 0.1 μM , the maximum level of total adducts at 24 h was 150/10⁸ nucleotides in p53-proficient cells and 210 adducts/10⁸ nucleotides in p53-deficient cells. A concentration of 0.01 μM resulted in a maximum of 20 adducts/10⁸ nucleotides in p53-proficient cells at 4 h, but 40 adducts/10⁸ nucleotides persisted in p53-deficient cells at 24 h. Whereas there were clear differences in the time course of adduct levels in p53-proficient compared with p53-deficient cells treated with 0.1 μM or 0.01 μM , these levels did not decrease extensively over 3 days. This is likely because of the stabilization of the diol-epoxide in cells, and consequent exposure and formation of adducts for many hours after the initial treatment. Furthermore, despite minor quantitative differences, all 4 of the adducts behaved similarly with respect to the effect of p53 expression on their removal. p53 appears to minimize the appearance of benzo(g)chrysene adducts in human cells by up-regulating global nucleotide excision repair and reducing the maximum adduct levels achieved. The fact that this p53-dependent effect is noted at levels of DNA adducts that are commonly found in human tissues (*i.e.*, <100 adducts/10⁸ nucleotides) because of environmental factors such as smoking is particularly significant with respect to human carcinogenesis related to environmental exposure.

INTRODUCTION

Humans are periodically exposed to environmental agents that damage DNA. Physical agents such as UV or ionizing radiation, and some chemical carcinogens, can interact with DNA and elicit a change in its structure (1). The damage that results from exposure to these agents can interfere with normal DNA transactions, including replication (2) and transcription (3), and can be an initiating factor in tumorigenesis. Among the wide variety of environmental agents that interact with DNA, of particular concern in human carcinogenesis is the class of chemicals known as PAHs.³ PAHs are formed as a by-product of inefficient combustion of fossil fuels, and are wide-

spread environmental pollutants present in cigarette smoke, cooked meat, industrial effluents, and vehicle exhaust fumes (4). These highly aromatic, hydrophobic molecules are processed in human cells by the cytochrome P450 and epoxide hydrolase enzyme systems (5). In an attempt to oxygenate and render them water-soluble to facilitate excretion, PAHs are metabolized to form highly reactive diol-epoxide derivatives that interact with purine bases to form covalent DNA adducts. These PAH-DNA adducts represent substantial blocks to replication (2) and transcription (6), they are highly mutagenic (7), and their formation represents the first stage in PAH-induced carcinogenesis (4).

Complex mechanisms have evolved to minimize the deleterious effects of agents such as PAHs by repairing the resulting DNA damage shortly after it is formed. The most versatile mechanism for the removal of bulky DNA lesions is NER, a process that involves recognition of the lesion, unwinding of the DNA strands around the lesion site, excision of a 24–32 nucleotide oligomer containing the lesion, repair replication using the undamaged strand as a template, and ligation of the resulting patch to the contiguous DNA (8). This multistage mechanism in human cells requires the participation of at least 30 proteins. Accumulating evidence has demonstrated that, in addition to its well-established roles in cell cycle arrest and apoptosis (9), the p53 tumor suppressor protein plays an important role in regulating the efficiency of this DNA repair mechanism. Studies from our laboratory (10–12) and others (13–16) have determined that NER of UV-induced CPDs but not 6–4 photoproducts is dependent on p53. Several investigators have also documented a role for p53 in base excision repair, which deals primarily with small DNA modifications such as oxidative and alkylation-induced DNA lesions (17–19). The involvement of p53 appears to be restricted to global repair, *i.e.*, the removal of lesions throughout the genome; TCR of CPDs, a mechanism targeted to the removal of lesions from the transcribed strands of expressed genes, was shown in our laboratory to be p53-independent (10–12). The p53-dependent repair of CPDs has since been found to be mediated in large part by p48, a component of the UV DNA damage binding protein complex that is absent in cells derived from xeroderma pigmentosum (complementation group E) patients and rodents, both of which lack efficient global NER of CPDs but retain proficient global NER of 6–4 photoproducts (20, 21). These studies combined have provided compelling evidence that p53 is involved in the efficient repair of some but not all of the classes of DNA damage. Therefore, it was important to determine the other types of environmental DNA damage, in addition to CPDs, that require p53 for efficient global NER, as well as the implications of this requirement for carcinogenesis and human health.

We have used recently the ³²P-postlabeling technique to assess the formation and removal of different classes of DNA damage, including adducts formed by PAHs. ³²P-postlabeling is a highly sensitive technique commonly used for the detection of PAH-DNA adducts in human tissues from exposed individuals such as cigarette smokers (22, 23). Using this approach, we demonstrated that the efficient

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³ The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; NER, nucleotide excision repair; TCR, transcription-coupled repair; CPD, cyclobutane pyrimidine dimer;

BPDE, benzo(a)pyrene diol-epoxide; B(g)CDE, benzo(g)chrysene diol-epoxide; wt, wild-type.

global NER of the major *N*2-guanine adduct formed by BPDE is p53-dependent (24). This p53 dependence was most pronounced at the low levels of adducts that are significant in terms of human exposure to PAHs through smoking or other environmental exposures, and it is therefore particularly significant in terms of human environmental carcinogenesis (24). The involvement of p53 in the global NER of BPDE adducts has since been confirmed by Wani *et al.* (25), who additionally demonstrated that TCR of these adducts was not affected by p53 status. However, among PAHs, benzo(*a*)pyrene is rather unusual in that it forms one DNA adduct with guanine almost exclusively (26). Complex environmental agents such as cigarette smoke include many different PAHs, most of which form several covalent DNA adducts with both guanine and adenine (27). We are interested in the possibility that some of these adducts might require p53 for their efficient repair, whereas others would be relatively independent with respect to repair. Therefore, it is of interest to investigate the repair of related compounds that form a broader spectrum of adducts, not only in terms of investigating the repair of structurally diverse DNA lesions, but also to represent more closely the wide spectrum of environmental DNA damage to which humans are commonly exposed.

Among the best characterized of the PAHs is benzo(*g*)chrysene, for which the arrangement of the five-ring structure compared with those of benzo(*a*)pyrene (Fig. 1) results in considerable differences in its reactivity toward DNA. Whereas benzo(*a*)pyrene is metabolized to 4 optically active diol-epoxide stereoisomers, the primary DNA-reactive metabolite is (+)-*anti*-BPDE, which reacts almost exclusively with the *N*2 position of guanine, in the *trans*-orientation; this results in the formation of one major adduct that accounts for 80–90% of total adducts formed by the parental compound (26). Metabolism of benzo(*g*)chrysene, on the other hand, results in the formation of 4 “fjord” region stereoisomers, all of which have significant reactivity toward DNA (28). The steric hindrance on formation of a fjord-region diol-epoxide moiety, because of its proximity to adjacent aromatic rings, results in reduced planarity. Consequently, these metabolites are less ionic than BPDE, more selective in their reaction with the highly nucleophilic DNA centers, and less susceptible to aqueous hydrolysis (29). Significantly, they react with both adenine and guanine bases to form several structurally distinct covalent DNA adducts (28).

In the present study, we have used ³²P-postlabeling to assess the global NER of the four principal DNA adducts formed by (±)-*anti*-B(*g*)CDE. ³²P-postlabeling facilitates detection and resolution of several discrete DNA adducts formed on reaction of this species with both adenine and guanine bases in DNA. We demonstrate p53-dependence for the global NER of each of these B(*g*)CDE adducts,

most notable at the lower, biologically significant levels of DNA adducts found in human tissues. The results are consistent with our previous studies (24), which documented p53-dependent repair of BPDE adducts. These studies demonstrate that p53 has a role in the repair of structurally diverse DNA adducts and are particularly significant in terms of carcinogenesis related to human environmental exposures.

MATERIALS AND METHODS

Materials. (±)-*anti*-B(*g*)CDE was obtained from the National Cancer Institute Chemicals Repository, Midwestern Research Institute, Kansas City, MO. Antibodies used in Western blotting procedures were from Santa Cruz Biotechnologies (Santa Cruz, CA). Enzymes and materials for the ³²P-postlabeling assay were obtained from sources mentioned previously (28).

Cell Culture and B(*g*)CDE Treatment. Human 041 TR cells were grown as monolayers in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. These cells, originally obtained from Dr. G. Stark (Cleveland Clinic Foundation, Cleveland, OH), were derived from spontaneously immortalized Li-Fraumeni syndrome skin fibroblasts (termed 041 mut) that are homozygous for mutant p53. 041 mut cells were stably transfected with a tetracycline-regulated system for expression of wt p53 (30). They were grown in the continuous presence of G418 (600 μg/ml) and hygromycin (50 μg/ml) to maintain selection pressure for transfected cells. Tetracycline (2 μg/ml) was added when suppression of wt p53 expression was required. Experiments were conducted with growing cells that had been radiolabeled with [³H]thymidine (0.2 μCi/ml) to allow quantitation of parental, adduct-containing DNA in each sample.

B(*g*)CDE was diluted to the desired concentration (0.01–1.2 μM) in serum-free DMEM from a freshly prepared 5 mM stock in anhydrous tetrahydrofuran. Treatment was for 1 h at 37°C, after which cells were washed twice in PBS, and lysed immediately or incubated in appropriate medium for up to 72 h and lysed.

Preparation of Whole-Cell Extracts and Western Blotting. Whole-cell extracts were prepared, and protein concentration was determined as described previously (12). Fifty μg of protein was resolved by 12% SDS-PAGE and electroblotted to a nitrocellulose membrane. Membranes were then immunoblotted with mouse monoclonal antibodies to human p53 (DO-1) or rabbit polyclonal antibodies to human p21 (Ab-1). These primary antibodies were diluted 1/1000 in 1% nonfat milk in PBS followed by incubation with horseradish-peroxidase-conjugated secondary antibody (1/5000 in 1% nonfat milk/PBS). The secondary antibody was detected by enhanced chemiluminescence (Amersham Life Sciences).

DNA Isolation. Cells were lysed in 10 mM EDTA and 50 mM Tris-HCl (pH 8.0; 0.5 ml) containing 1% SDS and 0.5 mg/ml proteinase K (Life Technologies, Inc.), and incubated for 3 h at 37°C. DNA was purified from the lysates by phenol extraction and RNase treatment (31), resuspended in 1/100 SSC (1.5 mM sodium chloride and 0.15 mM sodium citrate), and stored at –20°C before analysis.

³²P-Postlabeling. DNA samples were subjected to the nuclease P1 enrichment method of postlabeling analysis (32). Briefly, 4 μg DNA was hydrolyzed overnight at 37°C with micrococcal nuclease (0.14 units) and spleen phosphodiesterase (1.2 μg) in the presence of calcium chloride (10 mM). After treatment with nuclease P1 (0.15 units for 1 h at 37°C), DNA digests were labeled for 30 min. at 37°C with [³²P]ATP (50 μCi) and T4 polynucleotide kinase (6 units). Separation of radiolabeled DNA adducts was achieved by multidirectional chromatography of radiolabeled DNA digests on 10 × 10 cm anion-exchange polyethylenimine-cellulose TLC plates using the following solvents: D1, 1.0 M sodium phosphate (pH 6.0) on to a paper wick; D2, 3.5 M lithium formate and 8.5 M urea (pH 3.5); and D3, 0.8 M lithium chloride, 0.5 M Tris-HCl, and 8.5 M urea (pH 8.0). Adducts were detected as radioactive spots on the TLC plate after screen-enhanced autoradiography for up to 18 h at room temperature. Quantitation of DNA adducts was achieved by excising these radioactive spots and subjecting them to Cerenkov counting in a Beckman Scintillation counter.

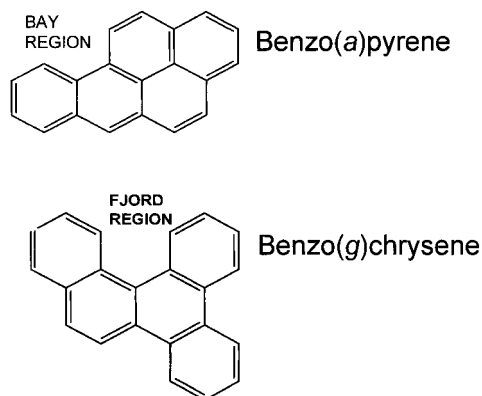


Fig. 1. Chemical structures of benzo(*a*)pyrene and benzo(*g*)chrysene, two structurally related PAHs.

RESULTS

p53 Levels in Whole-Cell Extracts from B(g)CDE-treated 041 TR Cells. The levels of p53 protein in whole-cell extracts were assessed by Western blotting at several times after B(g)CDE treatment. In addition, the level of p21 was analyzed in the same samples to evaluate the p53-dependent transcriptional activity in the 041 TR cells. Cells grown in the presence of tetracycline to repress p53 and treated with 1.2 μM B(g)CDE contained little or no detectable p53 immediately after treatment, and showed no induction of p53 up to 72 h after treatment (Fig. 2). However, some p21 expression was observed, suggesting p53-independent accumulation of this protein in response to B(g)CDE. Cells grown in the absence of tetracycline to allow p53 production, when treated with 1.2 μM B(g)CDE, showed increasing levels of p53 up to 24 h post-treatment, after which the level remained constant up to 72 h. p21 levels increased over a similar time period, correlating with increases in p53 levels (Fig. 2). This confirms the efficient suppression of p53 levels by tetracycline in 041 TR cells after B(g)CDE treatment, as observed in our previous studies with both UV (12) and BPDE (24); however, the p53-independent accumulation of p21 was not observed on treatment of tetracycline-suppressed 041 TR cells with these other DNA-damaging agents.

³²P-Postlabeling Analysis of B(g)CDE Adducts. Analysis of DNA isolated from (\pm)-anti-B(g)CDE-treated 041 TR cells revealed the presence of four discrete radioactive spots, as resolved by two-dimensional chromatography and detected by screen-enhanced autoradiography (Fig. 3). To establish the identity of these individual adducts, calf thymus DNA samples treated with optically pure B(g)CDE, obtained from Prof. David H. Phillips (Institute of Cancer Research, Sutton, United Kingdom), were analyzed. Cochromatography experiments, combined with the chromatographic evidence described in Giles *et al.* (28) demonstrated that the adducts generated by treatment of 041 TR cells with (\pm)-anti-B(g)CDE were formed by reaction of (-)-anti-B(g)CDE with dG (adduct 1); (+)-anti-B(g)CDE with dG (adduct 2); (-)-anti-B(g)CDE with dA (adduct 3); and another product of (-)-anti-B(g)CDE with dA (adduct 4). Less intense radioactive spots, observed only at the higher dose of (\pm)-anti-B(g)CDE, were not identified or quantitated in the subsequent *in vivo* experiments; these are probably minor products of the reaction of (+) or (-)-anti-B(g)CDE with DNA, because each of these optically active diol-epoxide derivatives are known to form several adducts with DNA *in vitro* (Ref. 28; Fig. 3).

Formation and Repair of B(g)CDE Adducts in 041 TR Cells. In our previous studies (24), we established that the p53-dependent repair of BPDE adducts was most notable when those adducts were present at low levels. Therefore, we treated cells with B(g)CDE in a manner that would induce similarly low levels of adducts to assess whether they were subject to enhanced global NER in the presence of functional p53. 041 TR cells were treated with 0.01 μM B(g)CDE either in the presence of tetracycline (to suppress p53 expression, and

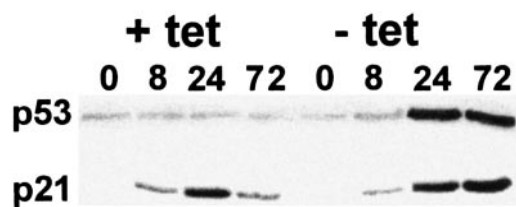


Fig. 2. Western blot analyses of p53 and p21 protein levels in whole-cell extracts from 041 TR fibroblasts treated with 1.2 μM B(g)CDE, as described in "Materials and Methods." Extracts were prepared at the various times indicated after B(g)CDE treatment of cells that were grown and then incubated after treatment in the presence or absence of tetracycline.

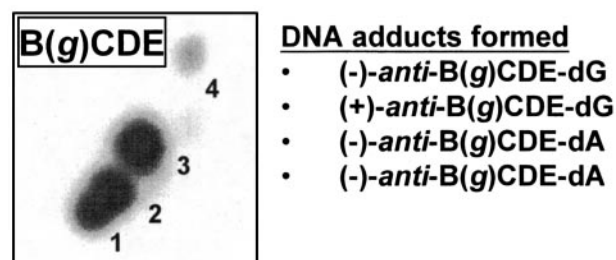


Fig. 3. Representative autoradiogram of TLC separation of ³²P-postlabeled DNA isolated from 041 TR fibroblasts treated with B(g)CDE. Screen-enhanced autoradiography was for 24 h at room temperature.

hereafter termed p53⁻) or in its absence (to permit p53 expression, and hereafter termed p53⁺). This treatment induced levels of adducts similar to those found in human tissues as a result of environmental exposure to PAHs. In the absence of p53, maximum levels of 16, 15, and <1 per 10⁸ nucleotides were achieved after 24 h for adducts 2, 3, and 4 respectively; adduct 1 reached a maximum of 6 per 10⁸ nucleotides after 72 h (Fig. 4A). The levels of each adduct remained constant between 24 and 72 h, with no removal being observed during this period. In the presence of p53, maximum (or close to maximum) levels of DNA adducts were again observed at earlier time points (between 4 and 8 h), reaching 5, 9, 8, and <1 adduct per 10⁸ nucleotides for adducts 1–4. These maxima were lower than those observed in the corresponding p53-deficient cells, and this level of adducts remained constant up to 72 h (with the exception of adduct 1, which continued to increase slightly up to this last time point).

Similar treatment of cells with 0.1 μM B(g)CDE resulted in levels of adducts that are comparable, within an order of magnitude, to levels documented in human tissues in exposed individuals. Cells treated with 0.1 μM B(g)CDE in the absence of p53 reached a maximum level of adducts between 8 and 24 h after treatment (31, 82, 94, and 10 per 10⁸ nucleotides for adducts 1–4; Fig. 4B). By 72 h, the levels of these adducts had been reduced slightly to 26, 41, 67, and 8 per 10⁸ nucleotides. In the presence of p53, maximum levels of adducts were observed at earlier time points, with maxima of 23, 66, 59, and 8 per 10⁸ nucleotides being achieved between 4 and 8 h after treatment. These maxima were notably lower than those observed in similarly treated p53⁻ cells, and were additionally reduced to 14, 28, 34, and 4 adducts per 10⁸ nucleotides by 72 h.

In p53⁻ cells treated with 1.2 μM B(g)CDE, levels of B(g)CDE adducts reached maxima of 200, 390, 412, and 33 per 10⁸ nucleotides, for adducts 1–4 respectively, between 8 and 72 h after treatment with the chemical (Fig. 4C). Adducts 2 and 4, which reached their maximum levels at 8 and 24 h, respectively, decreased slightly in frequency to 228 and 29 per 10⁸ nucleotides after 72 h, whereas adducts 1 and 3 continued to increase and achieved their maximum levels at this time point. Adducts 1–3 were each present at approximately 6–12 times the quantity of adduct 4. In p53⁺ cells, maximum adduct levels were achieved at 8 h (363 and 21 per 10⁸ nucleotides for adducts 2 and 4, respectively) or 24 h (300 and 478 per 10⁸ nucleotides for adducts 1 and 3, respectively). Some reduction of these levels was observed at 72 h (218, 264, 395, and 19 per 10⁸ nucleotides for adducts 1–4, respectively). (The adduct levels reported here are at least 1 order of magnitude higher than the 1 adduct per 10¹⁰ nucleotides detection limit of the ³²P-postlabeling assay.)

DISCUSSION

It is becoming increasingly clear that structurally diverse DNA lesions are processed by the NER machinery with variable efficiency.

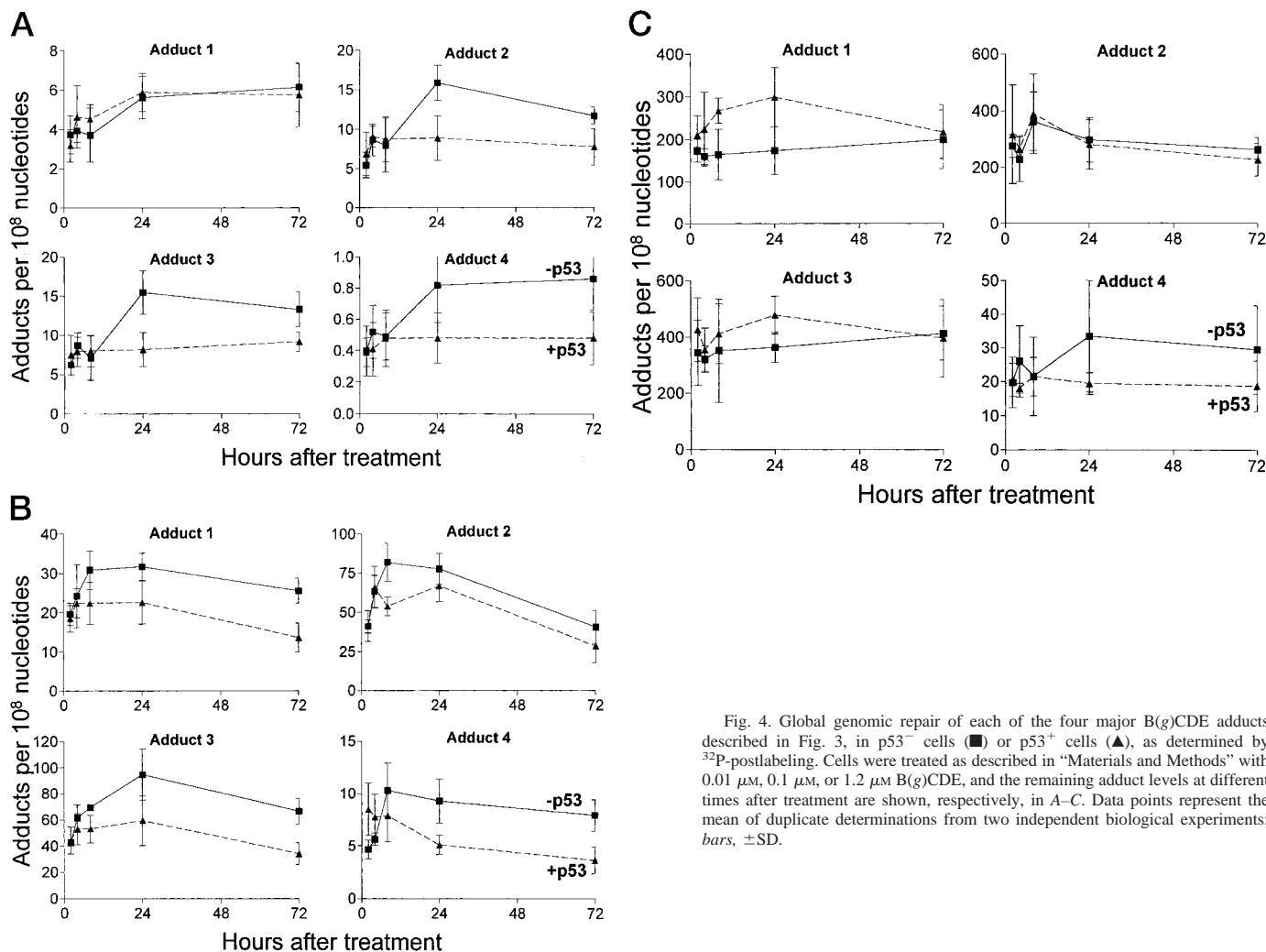


Fig. 4. Global genomic repair of each of the four major B(g)CDE adducts described in Fig. 3, in $p53^-$ cells (■) or $p53^+$ cells (▲), as determined by ^{32}P -postlabeling. Cells were treated as described in "Materials and Methods" with 0.01 μM , 0.1 μM , or 1.2 μM B(g)CDE, and the remaining adduct levels at different times after treatment are shown, respectively, in A–C. Data points represent the mean of duplicate determinations from two independent biological experiments; bars, \pm SD.

Studies that have documented the repair of the two major classes of UV-induced DNA damage, CPDs and 6–4 photoproducts, have demonstrated that the latter are removed much more efficiently than the former in repair-proficient mammalian cells (33) and in *Escherichia coli* (34, 35). Furthermore, 6–4 photoproducts, unlike CPDs, undergo global NER in a $p53$ -independent manner in mammalian cells (12), and recent evidence has suggested that there may be differences in the role of xeroderma pigmentosum (complementation group C) in the repair of CPDs versus 6–4 photoproducts (36). Given the emerging evidence that there may be different protein requirements for efficient NER among structurally diverse DNA lesions, it is important, and timely, to consider the repair of various classes of DNA damage to which humans are exposed to gain a broader appreciation of the versatility of the NER process, and in particular its role in the prevention of carcinogenesis in humans.

Our studies that demonstrated the $p53$ -dependent global NER of DNA adducts formed by a widespread chemical carcinogen, benzo(a)pyrene, made significant progress in this direction (24). They also demonstrated the feasibility of using the ^{32}P -postlabeling assay, a supremely sensitive method that has been used for the detection of DNA adducts in human biopsy samples (22, 23), to study the repair of DNA adducts at levels found in human tissues as a result of environmental exposure. This is an important development, because similar studies that investigate the repair of UV-induced photoproducts require the induction of significantly higher levels of DNA damage than are routinely encountered in the environment. However, a disadvan-

tage associated with studies of the repair of chemically induced DNA adducts is that the unreacted DNA-damaging agent persists in cells after the initial exposure period. Other factors, including solubility, diffusion across membranes, and reaction of the chemical with cellular components other than DNA, can impose additional degrees of experimental variability. Whereas these issues were resolved and experimental variation was minimized in the experiments described here, it remained impossible to establish a reliable quantitation of DNA damage immediately after treatment (0 h), because of the presence of B(g)CDE in the cells after harvesting, and continued exposure of DNA to the damaging agent during cell lysis and processing of DNA samples. Therefore, adducts continued to be formed, and the 0 h time point was considered extremely variable and unreliable. For this reason, the time courses for the experiments reported here are arbitrarily set to begin at 2 h after treatment, a more reliable indicator of "initial" levels of DNA damage than that in the compromised 0 h samples.

In the present study, we used a derivative of human Li-Fraumeni fibroblasts, spontaneously immortalized and homozygous for a mutation in the $p53$ gene. These cells, termed 041 TR (30), had been engineered to express wt $p53$ under the control of a tetracycline-inducible promoter, allowing studies of the effect of $p53$ status on DNA repair efficiency. Studies conducted previously with 041 TR cells in this laboratory demonstrated that $p53$ is required for the global NER of CPDs, but not 6–4 photoproducts, and that $p53$ is not required for TCR of CPDs (12); these observations have been con-

firmed in other p53-deficient cell systems, including those that have undergone transformation with SV40 (37) and the human papilloma virus E6 epitope (13) to abrogate p53 function. Experiments conducted here with 041 TR cells have revealed that the efficient global NER of DNA adducts formed by benzo(g)chrysene, a potent carcinogen that reacts with both adenine and guanine, also depends on expression of the p53 protein. These results demonstrate the generality of the p53 requirement for the efficient repair of DNA adducts formed by PAHs. They are also consistent with our recent observations that the requirement for p53 in the repair of PAH-DNA adducts is most pronounced when these adducts are present at low, biologically significant levels (24). It is especially interesting that removal of DNA adducts, at levels that are known to be present in human tissues, is significantly impaired in the absence of a protein of which the gene is mutated in >50% of human tumors.

It was of additional interest in these experiments that the Western blot analysis of protein extracts, despite efficient suppression of p53 in 041 TR by tetracycline after B(g)CDE treatment, revealed significant p21 expression. Such expression was not observed in the same cells after treatment with either UV (12) or BPDE (24), in which expression of both p53 and p21 was efficiently suppressed, and suggests that this chemical can induce p21 in a p53-independent manner. This is in contrast to studies that have suggested that B(g)CDE (38) and other PAH-diol-epoxide metabolites including BPDE (39) induce p53 efficiently in human mammary carcinoma MCF-7 cells, but that this induction is followed by poor induction of p21 and G₁ arrest, and suggests the expression of a p53 protein that is unable to carry out normal transactivation. Our studies are in better agreement with those of Mahadevan *et al.* (40), who found that treatment with a related PAH, dibenz(a,l)pyrene, induced p53 and p21 efficiently in normal human diploid fibroblasts (although they observed no p53-independent expression of p21). These apparently contrasting results may be because of differences between the cell systems used in the experiments.

Despite the structural similarity of B(g)CDE with BPDE, we found considerable differences in the profile and kinetics of formation and removal of the corresponding DNA adducts. After treatment of 041 TR cells with BPDE, we reported previously that maximum levels of adducts were observed 2–4 h after treatment (24). In contrast, treatment of the same cells with B(g)CDE not only resulted in higher levels of DNA adducts, but the maximum adduct levels were achieved at much later time points, generally after 24 h. Clonogenic survival assays using VA13 cells, selected as a repair-proficient cell line with wt p53 function, demonstrated comparable viability in untreated cells and those treated with 0.1 μM or 0.01 μM B(g)CDE (data not shown). Therefore, the apparent absence of significant DNA repair after treatment at these low doses is highly unlikely to be a result of B(g)CDE toxicity and suggests that NER of these adducts proceeds very slowly in comparison with those formed by BPDE. The increase in adduct levels up to late time points is consistent with the higher stability of the fjord-region diol-epoxides such as B(g)CDE in aqueous solution, compared with the more hydrolytic bay-region BPDE (41). It has been demonstrated that, once they enter the cell, diol-epoxide chemicals are stabilized by associating with lipids, after which they are slowly released, thereby extending the period of exposure to the DNA-damaging agent for several hours after its removal from the culture medium (42, 43). Indeed, it is clear from our previous studies (24) and that of others (44) that PAH-DNA adducts continue to be formed many hours after the diol-epoxide has been removed from the culture medium. Our own observations with other repair-proficient and deficient cell lines have also revealed increases in B(g)CDE adduct levels beyond 24 h after treatment.⁴

Therefore, it appears that there is a balance between formation and repair of DNA adducts; in the first few hours after treatment the rate of adduct formation exceeds the rate of adduct removal; at the maximum adduct level the rates of formation and repair are equal, after which the rate of repair can exceed the rate of adduct formation. Because B(g)CDE is more stable than BPDE, it is likely that the cellular DNA continues to be exposed to damage for a longer period after treatment; this would explain why maximum adduct levels were observed 24 h after treatment in most cases. Additionally, it would explain what appears to be a slower loss of these adducts compared with those formed by BPDE, because continued formation of DNA adducts would obscure the effect of adduct removal by the NER machinery. It may also be the case that B(g)CDE adducts are relatively poor substrates for NER. Very few studies have addressed the repair in cells of PAH-DNA adducts other than those formed by BPDE, and none have investigated cellular excision of B(g)CDE adducts. However, *in vitro* studies have demonstrated significant variability in the repair of different PAH-DNA adducts, with benz(a)anthracene adducts being repaired particularly slowly by human cell extracts (45) and benzo(c)phenanthrene adducts being refractory to removal by cell extracts at a mutational hotspot of the *ras* oncogene (46) compared with BPDE adducts. Therefore, it might be that DNA adducts formed by fjord region diol-epoxides, such as the reactive metabolites of benz(a)anthracene, benzo(c)phenanthrene, and benzo(g)chrysene, are relatively poor substrates for NER compared with adducts of bay region diol-epoxides such as BPDE.

Our previous studies demonstrated that the p53-dependent repair of BPDE adducts was observed at low adduct levels, comparable with the level of carcinogen-DNA adducts found in human tissues from environmentally exposed individuals (24). The effect of p53 in reducing the level of B(g)CDE adducts was also observed only at the lower concentrations (0.1 μM and 0.01 μM) that generate B(g)CDE adducts at levels found in exposed populations. Whereas a clear difference between p53-proficient and deficient cells was observed after these treatments, this was, for the most part, a difference in the maximum levels of B(g)CDE adducts generated, with more adducts appearing in the p53-deficient cells. Therefore, p53 appears to be most active in minimizing the levels of DNA adducts formed through the continued exposure of DNA to B(g)CDE in cells and, therefore, the continued formation of DNA adducts. In cells with wt p53 activity, the maximum level of DNA adducts was lower and this occurred at earlier time points (4–8 h), whereas in p53-deficient cells the level of adducts continued to increase to a higher level, reaching a maximum at a later time point (24–72 h). Therefore, it is likely that a difference observed in the repair of B(g)CDE adducts in p53-proficient and deficient cells is determined by the point at which the rate of DNA adduct repair exceeds that of formation; this occurs sooner after treatment in cells that have wt p53 activity and results in a lower maximum level of B(g)CDE adducts. No such effect of p53 on global NER of B(g)CDE adducts was observed when cells were treated with 1.2 μM , the highest concentration used in these experiments. Indeed, adducts 1 and 3 appeared to be formed at a higher frequency in p53⁺ cells at intermediate time points, although significance of this observation is unclear because of the increased experimental error at high doses of B(g)CDE. An exception was adduct 4 (Fig. 4C), which was present at approximately 6–12-fold lower levels than adducts 1–3 (33 adducts per 10⁸ nucleotides) and which appeared to be removed more efficiently in p53-proficient cells (although the experimental error renders this observation statistically insignificant). Our previous study also demonstrated no difference in the kinetics of BPDE adduct removal when these adducts were present at levels of >300 per 10⁸ nucleotides; p53 was only involved in the repair of the last remaining 5–10%

⁴ D. Lloyd and P. Hanawalt, unpublished observations.

of adducts, corresponding to biologically relevant levels of DNA damage found in human tissues (24).

The reason for the p53-dependent repair of carcinogen-DNA adducts being specific to low levels of DNA damage is unclear. It may be that p53 is involved in the repair of a fraction of the total adducts, that this fraction is less accessible to DNA repair machinery and possibly buried in chromatin, and that p53 regulates their accessibility. This is consistent with the demonstration that p48, a p53-regulated protein and component of the UV DNA damage binding protein activity that is absent in xeroderma pigmentosum (complementation group E) patients and in rodents, has considerable sequence homology to proteins involved in remodeling chromatin (47). Several lines of evidence support this hypothesis; for example, it has been demonstrated that p48 is required for the repair of UV-induced CPDs, in a mechanism that is regulated by p53, in both human (21) and rodent cells (48). p48-deficient XP-E cells (21) and rodent cells (49, 50) exhibit the same repair phenotype as p53-deficient cells in that they lack global NER of CPDs; stable transfection of rodent cells with functional human p48 reverses this DNA repair defect (49). However, the role of p48 in the repair of PAH-DNA adducts, and particularly at the low levels reported here, remains to be ascertained. An alternative explanation is that the biologically significant levels of BPDE adducts generated in these experiments represent, by themselves, an insufficient signal to recruit lesion recognition proteins; therefore, p53 might be required to up-regulate the recognition process. In any event, it is important to consider the repair of these low levels of DNA damage, not only because they represent a level of DNA damage that is more relevant to the levels formed in human tissues, but also because the proteins involved in the removal of DNA adducts are clearly dependent on adduct frequency.

In addition to the sensitivity of the assay and the analysis of DNA repair at low levels of DNA damage, we were able to assess the repair of the four major structurally distinct adducts formed by the same DNA-damaging agent, and establish whether there are differences in the rate and mechanism by which they are removed from the genome. The major difference among the adducts formed by B(g)CDE is the base with which the diol-epoxide reacts, either guanine or adenine, and corresponding differences in reaction sites on the base and adduct orientation (51); one might expect differences in the manner in which these structurally distinct adducts are removed. Whereas all of the adducts, when present at low levels, behaved similarly with respect to the effect of p53 expression, there was a general, but quantitatively minor, trend that the global NER of B(g)CDE-adenine adducts was more affected by p53 expression than that of corresponding guanine adducts. This is noteworthy because PAH-adenine adducts are considered more harmful than corresponding guanine adducts; they are more mutagenic (52, 53) and potentially those PAHs that form a greater proportion of adenine adducts are more carcinogenic.

In summary, we have used a highly sensitive experimental system to assess the repair of biologically significant levels of DNA adducts formed by the PAH and potent human carcinogen, benzo(g)chrysene. We find that the levels of adducts depend on the tumor suppressor protein p53; taken together with our previous study (24) they establish that this p53 requirement for the repair of biologically significant levels of adducts is a general phenomenon among the PAHs, an important and widespread class of environmental carcinogens. There are considerable clinical implications to these studies because they demonstrate the requirement of a gene product that is commonly mutated in human tumors for the repair of levels of DNA damage that are found frequently in human tissues. Therefore, they are highly significant in terms of human carcinogenesis related to environmental exposure. Studies are continuing to exploit these experimental approaches to study the repair in human cells of structurally diverse,

environmentally significant levels of DNA damage, and the importance of these repair processes in the prevention of cancer.

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