p53-dependent Global Genomic Repair of Benzo[a]pyrene-7,8-diol-9,10-epoxide Adducts in Human Cells

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Abstract

The global genomic repair of DNA adducts formed by the human carcinogen (±)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) has been studied by 32P-postlabeling in human fibroblasts in which p53 expression can be regulated. At low BPDE adduct levels (10–50 adducts/10⁸ nucleotides), repair was rapid and essentially complete within 24 h in p53⁺ cells, whereas no repair was detected within 72 h in similarly treated p53⁻ cells. At 10-fold higher BPDE adduct levels, repair under both conditions was rapid up to 8 h, after which a low level of adducts persisted only in p53⁺ cells. These results demonstrate a dependence on p53 for the efficient repair of BPDE adducts at levels that are relevant to human environmental exposure and, thus, have significant implications for human carcinogenesis.

Introduction

The p53 tumor suppressor regulates important cellular responses to DNA damage within human cells. Accumulation of p53 protein in response to DNA damage leads to apoptosis or arrest of the cell cycle, presumably to provide time for repair of DNA damage before cell division or the initiation of a new round of replication. Previous work in this laboratory has established that expression of p53 is required for the efficient global genomic NER ¹ of UV-induced CPDs in human fibroblasts, but is not required for TCR of these lesions (1–3). Skin fibroblasts derived from tumors in patients with LFS, homozygous for mutations in the p53 gene, exhibited a reduced capacity to remove CPDs from the overall genome compared with that of related heterozygous mutants and normal cells (1). Subsequent studies in which the expression of p53 in human fibroblasts could be controlled using a stably integrated tetracycline-regulated p53 gene (2), or in which p53 protein was abrogated by expression of the HPV 16 E6 gene (3), have confirmed these observations. Furthermore, they have established that the global NER of CPDs is much more dependent on p53 activity than is that of the more structure distorting 6-4 photoproducts, repair of which is nearly independent of p53 expression. This suggests that there is some heterogeneity in the requirement of p53 for global NER among different types of DNA lesions.

Whereas UV irradiation is primarily associated with skin cancers, other genotoxins, such as the carcinogenic PAHs, have numerous target organs. Humans are constantly exposed to PAHs; they are widespread contaminants in the environment and are formed as by-products during the inefficient combustion of fossil fuels. They are metabolized in human cells to electrophilic derivatives that form DNA adducts by interacting covalently with purine bases; formation of these adducts can lead to mutation and is thought to be the first stage in PAH-induced carcinogenesis (4). Increased human exposure to PAHs, due to occupation, smoking, or other environmental factors that are associated with enhanced cancer risk, results in the formation of low levels of PAH-DNA adducts in various tissues (5). The efficiency with which human cells are able to process this type of DNA damage is, therefore, an important consideration. However, it remains to be established whether the role of p53 to promote efficient global NER is a general phenomenon, applicable to a variety of classes of DNA damage including PAH-DNA adducts, or whether it is largely restricted to a particular type of UV-induced DNA lesion.

In the present study, we have investigated the repair of adducts formed by BPDE, a reactive metabolite of the potent carcinogen benzo(a)pyrene that binds predominantly to the exocyclic amino position of guanine (6). We have investigated the p53-dependent global NER of BPDE adducts in human fibroblasts in which expression of p53 can be down-regulated by including tetracycline in the culture medium (2). BPDE adducts in DNA were measured using 32P-postlabeling, a very versatile and sensitive technique that allows the detection of DNA adducts at levels much lower than previously reported in DNA repair studies, as low as 1 adduct/10⁹ nucleotides (7). We report that p53 function is required for the efficient global NER in human cells of BPDE adducts, at levels to which certain human populations are exposed. This result has significant implications in terms of human environmental carcinogenesis.

Materials and Methods

Materials. Racemic BPDE was obtained from the NCI 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 previously mentioned sources (8).

Cell Culture and BPDE Treatment. 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 LFS 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 (9). They were grown in the continuous presence of G418 (600 μg/ml) and hygromycin (50 μg/ml) to maintain a selection pressure for transfected cells. Tetracycline (2 μg/ml) was added when suppression of wt p53 was required (2). Experiments were conducted with confluent, contact-inhibited cells to prevent posttreatment replication, or otherwise 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.

BPDE was diluted from a freshly prepared 5-nm stock in anhydrous tetrahydrofuran to the required concentration (0.1–1.2 μM) in serum-free medium. 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.
DNA Isolation. Cells were lysed in 10 mM EDTA and 50 mM Tris-HCl (pH 8.0, 1.0 ml) containing 1% SDS and 0.5 mg/ml proteinase K and incubated for 3 h at 37°C. DNA was purified from the lysates using the phenol extraction and RNase treatment method described by Gupta (10), 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 (11). Briefly, 4 µg of DNA were hydrolyzed overnight at 37°C with micrococcal nuclease (0.14 unit) and spleen phosphodiesterase (1.2 µg) in the presence of calcium chloride (10 mM). Enrichment of DNA adducts was achieved by incubation for 1 h at 37°C with nuclease P1 (0.15 unit). 32P-labeling of the DNA digest was for 30 min at 37°C with [γ-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 polyethyleneimine-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, 8.5 M urea (pH 3.5); D3, 0.8 M lithium chloride, and 0.5 M Tris-HCl, 8.5 M urea (pH 8.0). Adducts were detected as radioactive spots on the TLC plate following 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.

Western Blotting. Whole cell extracts were prepared, and protein concentration was determined as described previously (2). Protein (50 µg) was resolved by 12% SDS-PAGE and electroblotted to a nitrocellulose membrane. Membranes were then subjected by immunoblotting with mouse monoclonal antibodies to human p53 (DO-1) or rabbit polyclonal antibodies to human p21 (Ab-1), 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). Proteins were detected by enhanced chemiluminescence (Amersham Life Sciences).

Results

Control of p53 Expression by Tetracycline. The levels of p53 protein in whole-cell extracts in cells were assessed by Western blotting at several times after BPDE treatment. In addition, the levels of p21 were analyzed to evaluate the p53-dependent transcriptional activity in the 041 TR cells. Treatment of p53-suppressed cells with 1.2 µM BPDE resulted in no detectable p53 immediately after treatment, and very little induction of either p53 or p21 was observed up to 72 h after treatment (Fig. 1). In p53- cells, similar treatment resulted in increased p53 levels up to 24 h after treatment and began to return to basal p53 levels by 48 h. p21 levels increased over a similar time period, correlating with increases in p53 levels (Fig. 1). This confirms the efficient suppression of p53 levels and downstream transactivated elements in 041 TR cells by tetracycline.

32P-Postlabeling Analysis of BPDE Adducts. Analysis of DNA isolated from BPDE-treated 041 TR cells revealed the presence of a single radioactive spot as resolved by two-dimensional chromatography and detected by screen-enhanced autoradiography (Fig. 2). This spot corresponds to the major (+)-trans-anti-BPDE adduct formed at the N2-position of guanine that accounts for 80–90% of adducts formed by the parental B(a)P, together with minor products of reactions of the diol epoxide with other positions on both guanine and adenine moieties that have similar chromatographic mobility (12). This radioactive spot was not present in cells treated only with solvent (Fig. 2).

Formation and Repair of BPDE Adducts in 041 TR Cells. 041 TR cells were grown to confluence and then treated with 0.5 µM BPDE. Dilution of adducts by posttreatment replication was minimized by contact-inhibiting the cells, thereby preventing cell division. In the absence of tetracycline (hereafter termed p53-), levels of BPDE adducts increased up to 2 h after treatment, reaching a maximum of about 30 adducts/10⁸ nucleotides. After reaching this maximum, loss of BPDE adducts was rapid, reaching a level of only 8 adducts/10⁸ nucleotides within 8 h; the removal continued to a level of 2 adducts/10⁸ nucleotides by 48 h. In the presence of tetracycline (hereafter termed p53+), adduct levels continued to increase up to 4 h after treatment to a maximum of about 50 adducts/10⁸ nucleotides. Apart from an apparent reduction in BPDE adducts 8 h after treat-
ment, this maximum level persisted up to 72 h after treatment (Fig. 3A).

To assess whether this p53-dependent repair of BPDE adducts occurred at differing maximum levels of DNA adducts, and in cells that were not growth-inhibited, additional experiments were carried out with actively growing cells subjected to both lower and higher concentrations of BPDE. Before these experiments, cells were radiolabeled with [3H]thymidine, then trypsinized and mixed together to ensure even distribution of the label, and replated in the required number of dishes. Calculated adduct levels were corrected to account for dilution of parental DNA by posttreatment DNA synthesis. Treatment with 0.1 μM BPDE resulted in an increase up to 1 h after treatment in p53− cells to a maximum of 7 adducts/108 nucleotides, followed by complete and rapid repair between 8 and 24 h. In contrast, BPDE adducts increased up to 4 h after treatment to 9 adducts/108 nucleotides in p53− cells. Some repair was observed after 24 h, but a detectable level of 3 adducts/108 nucleotides persisted up to 72 h after treatment (Fig. 3B). This confirmed that BPDE adducts were refractory to repair at these levels in p53− cells and that this effect was independent of the growth state of the cells. After treatment of both p53+ and p53− cells with 1.2 μM BPDE, the level of adducts increased to a level of about 250 adducts/108 nucleotides after 1 and 4 h, respectively. In both cases, proficient and rapid repair was observed up to 8 h after treatment, when the level of adducts was reduced to around 20 adducts/108 nucleotides in both cases. Removal of adducts continued to less than 2 adducts/108 nucleotides by 72 h in p53+ cells; however, in p53− cells a low level of around 20 adducts/108 nucleotides persisted up to 72 h after treatment (Fig. 3C). Adduct levels reported here are at least one order of magnitude higher than the 1 adduct/108 nucleotides detection limit of the 32P-postlabeling assay.

Analysis of Replication after BPDE Treatment. In growing cells that were radiolabeled with [3H]thymidine, the ability to replicate DNA over a period of 72 h after BPDE treatment was assessed by density labeling with BrdUrd and FuUrd and separation of hybrid density-replicated DNA from parental density DNA by centrifugation in cesium chloride gradients. In 1.2 μM-treated p53+ cells, DNA replication is almost completely inhibited over the 72-h posttreatment period, whereas in p53− cells replication of DNA proceeds through nearly one round (Fig. 4).

Discussion

Evidence that p53 is specifically involved in global NER was obtained from studies in human fibroblasts derived from patients with LFS, a cancer-prone genetic disorder. LFS cells are heterozygous for mutations in one allele of p53. Homozygous derivatives of these cells were deficient in the global genomic NER of UV-induced CPDs, but proficient in TCR of these lesions (1). A coincident report demonstrated similar disruption of NER efficiency in human cells in which the wild-type activity of p53 was defective (13), and these results have been largely confirmed in later studies. The global NER of CPDs was subsequently shown to be much more p53-dependent than that of 6-4 photoproducts in Escherichia coli, in which the induction of the SOS response is required for the efficient global repair of CPDs but not for TCR. Interestingly, as with p53 regulation of repair, the repair of 6-4 photoproducts is much less dependent on SOS induction (14).

We have now demonstrated that the efficient global NER of BPDE adducts in human cells is also p53 dependent. The results from the present study demonstrate that, unlike the qualitative requirement of p53 for the repair of CPDs, the requirement for p53 in global NER of BPDE adducts is quantitative (i.e., dependent on the level of adducts being repaired). The results also indicate that the increase in repair is related to the initial basal levels of p53 protein rather than a damage-dependent increase, because, in repair-proficient p53− cells, the removal of BPDE adducts was almost complete before p53 stabilization.
(Fig. 1). The observed persistence of BPDE adducts in p53- cells cannot be due to toxicity of the compound because these cells are able to replicate over the 72-h period immediately following treatment (Fig. 4) and must, therefore, be related to a defect in the action or regulation of the DNA repair machinery. A recent study demonstrated activation of p53-enhanced repair of BPDE adducts in a transfected reporter plasmid following treatment of host cells with high levels of thymidine dinucleotides (15); it is likely that the enhanced repair in this specialized system was due to the same pathway characterized in this study.

It is also noteworthy that the maximum level of BPDE adducts was never attained immediately after treatment. The adduct level always increased, irrespective of p53 status, and reached a maximum at 2–4 h after treatment before any repair was observed. This is consistent with similar treatment of other cell types^ and treatment of MCF-7 breast carcinoma cells with diol epoxide metabolites of another chemical carcinogen, dibenzo(a,l)pyrene (16). It is possible that the lipophilic diol epoxide molecules are sequestered and stabilized in lipids within cells and released slowly, thereby extending the half-life of these labile compounds beyond the initial 1-h incubation period (17). It is, therefore, important to carefully monitor the levels of chemically induced DNA adducts during the first 8 h after treatment; failure to do so would most likely lead to underestimation of the maximum level of BPDE adducts formed and, consequently, the rate at which adducts are removed from the genome.

It is significant that p53 was required for repair only at low levels of BPDE adducts. The sensitivity of the 32P-postlabeling assay has been integral in establishing this quantitative nature of p53-dependent NER. Previous studies have used a variety of methods to monitor the repair of BPDE adducts, however, few of those methods achieve the sensitivity of the 32P-postlabeling assay for the detection of PAH-DNA adducts, and, consequently, few studies have focused on the repair of BPDE adducts, or other DNA lesions, at the low levels reported here. The lowest levels reported in this study (around 1 adduct/107 nucleotides) are comparable with those found in vivo. 32P-postlabeling has been used in human biomonitoring studies to assess the environmental exposure to carcinogens, especially PAHs, in humans with occupations, physical environments, or lifestyles that have enhanced cancer risk (7). For example, the total level of PAH-DNA adducts, including BPDE adducts, present in DNA isolated from lung biopsy tissues derived from human smokers were as high as 33.4 adducts/107 nucleotides in one study (18). Similar levels of DNA adducts are found in populations that are exposed to PAHs through occupation or physical environment (reviewed in Ref. 5). Because the reported levels of PAH-DNA adducts present in human tissues are comparable with the levels of BPDE adducts that remained unrepaired in cultures of p53- human cells in the present study, these adducts may persist in tissues in which the function of p53 has been compromised. In view of the fact that p53 is defective in a large percentage of human tumors, the p53-dependent NER of low level BPDE adducts in human cells is a significant observation.

Recent studies have shed light on the mechanisms by which p53 might regulate the efficiency of global NER. p53 protein has no effect on NER in vitro, suggesting that the up-regulation of global repair is due to transactivation (19, 20). Because the extent to which p53 is involved in DNA repair is dependent on the type of DNA lesion, it seemed likely that it might regulate the initial recognition stages of NER. Recently, p53 has been found to regulate the expression of the p48 gene (21); the p48 protein, in turn, regulates the activity of the UV-DDB, an activity absent from cells derived from a subset of XP-E patients (22). XPE cells that lack UV-DDB activity exhibit the same DNA repair phenotype as p53- cells in that they are deficient in global NER but proficient in TCR of CPDs, and proficient in repair of 6-4 photoproducts (21). It will be of interest to establish whether the repair of BPDE adducts in XPE cells is comparable with that in p53- cells. It will also be of interest to study the repair of BPDE adducts in Chinese hamster ovary cells; these are also deficient in p48 (22) and exhibit deficient global NER of CPDs (23). The fact that Chinese hamster ovary cells are deficient in this p53-regulated repair pathway and that p53 is required for efficient NER of certain DNA adducts, including those formed by BPDE, may raise important implications and concerns for the use of rodent models for risk assessment in genetic toxicology. If some lesions are repaired in a p53- and p48-dependent manner while others are not, the deficiency in this NER pathway could lead to misleading results in genotoxicity assays. Caution should, therefore, be exercised in the use of genotoxicity testing protocols in which p53 and/or p48 are deficient or abrogated.

In addition to the UV-DDB complex, there are other protein complexes that may be involved in the initial recognition stages of NER, and the nature of the primary recognition element is currently the matter of some debate. Using purified protein complexes and affinity chromatography, Sugasawa et al. (24) demonstrated that the XPC-HR23B complex recognizes 6-4 photoproducts as a likely first step in NER. However, a complex involving XPA and RPA proteins is also a possible candidate for the initial recognition of DNA lesions (25).

Repair assays using whole-cell extracts derived from p53- and p53- cells have also demonstrated a p53-dependent repair of abasic sites, suggesting that p53 may also be involved in base excision repair (26). It seems likely that different DNA lesions may be recognized by different elements on the basis of the structure of these lesions in a manner that is either p53-dependent or p53-independent, and that a hierarchy of lesion recognition exists. This would certainly explain the homogeneous and quantitative nature of p53-dependent DNA repair among the types of DNA lesions investigated thus far (2, 3). It would also explain why, at high BPDE adduct levels, efficient repair was observed in p53- cells, indicative of a p53-independent repair pathway.

In summary, we report that DNA adducts formed by a potent chemical and environmental carcinogen, BPDE, are repaired in a p53-dependent manner. This p53-dependent DNA repair is observed at low adduct levels, comparable with those found in certain human populations. The results from this study have implications in terms of both human chemical carcinogenesis and the basis by which NER proceeds in human cells. Studies are continuing in an attempt to resolve the nature of DNA lesions, the repair of which is dependent on p53, the recognition processes that initiate NER of these lesions, and the broader implications for human cancer risk assessment.

Acknowledgments

We thank James Ford for advice and for providing the 041 TR cells carrying the tet-regulated p53. We are also grateful to Ann Ganesan and Allen Smith for helpful discussions and critical reading of the manuscript.

References


[^4]: Unpublished data.


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