Role of TP53 in repair of N-(deoxyguanosin-8-yl)-4-aminobiphenyl adducts in human transitional cell carcinoma of the urinary bladder

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The global genomic repair of DNA adducts was examined in human papillary transitional cell carcinoma (TCC) cell lines after exposure to N-hydroxy-4-acetylaminobiphenyl (N-OH-ABP), the proximate carcinogenic metabolite of the human bladder carcinogen 4-aminobiphenyl (ABP). ³²P-post-labeling analysis of TCC cultures exposed to N-OH-ABP revealed a major adduct, identified as the 3′,5′-bisphosphate derivative of N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP). The amount of adduct formation in TCC10 was dependent upon the dose and the duration of exposure and ranged between 1 and 5 adducts/10⁷ nucleotides. To test if p53 regulates repair of the dG-C8-ABP adduct in genomic DNA, an isogeneic set of cell lines was obtained by infection of the TCC10 cultures with a retroviral construct expressing a transdominant mutant of p53, namely a Val→Ala mutation at codon 143. The TDM143-TCC10 line expressing the mutant form of p53 was selected. The rate of repair of dG-C8-ABP was compared between TCC10 and TDM143-TCC10 cultures after treatment with 15 µM N-OH-ABP. The rate of disappearance of the adduct was monitored over a period of time after chemical treatment. ³²P-post-labeling analysis of dG-C8-ABP in parental TCC10 showed its rapid removal, the majority of adducts disappearing within 48 h. In contrast to TCC10, TDM143-TCC10 was relatively slower in repair of dG-C8-ABP. After 24 h DNA repair TDM143-TCC10 showed an ~3-fold greater amount of dG-C8-ABP compared with TCC10. These results imply that p53 plays a role in the repair of ABP adducts and that in p53 null cells the unrepaired DNA damage could cause accumulation of mutations, which might contribute to increased genomic instability and neoplastic progression.

Introduction

Arylamines are among the most potent known human carcinogens. Occupational and environmental exposure to arylamines and related carcinogens is the primary cause of urinary bladder cancer in humans (1,2). Occupational exposure to arylamines is prevalent in the rubber, coal, textile, printing, cable and leather processing industries (3–5). Environmental exposure to arylamines is rampant because of their occurrence in cigarette smoke, fossil fuels and other sources (6–8). Arylamine exposure could also result from inhalation and reduction in vivo of nitroaromatic compounds that are widely prevalent in auto exhausts. Several agents of this class of chemicals that exhibit carcinogenic activity in humans have been identified, including 4-aminobiphenyl (ABP), 2-naphthylamine and benzidine (4,7–9). ABP is a constituent of cigarette smoke and the DNA adduct derived from it has been detected in the blood and also in the DNA of target bladder tissues of smokers (11–13). Metabolic conversion of ABP to a reactive electrophile and its binding to DNA is thought to be essential for manifestation of the genotoxic and carcinogenic effects of ABP.

Utilizing ABP and its actual metabolite of the human bladder carcinogen 4-aminobiphenyl (ABP), 2-naphthylamine and benzidine (4,7–9). ABP is a constituent of cigarette smoke and the DNA adduct derived from it has been detected in the blood and also in the DNA of target bladder tissues of smokers (11–13). Metabolic conversion of ABP to a reactive electrophile and its binding to DNA is thought to be essential for manifestation of the genotoxic and carcinogenic effects of ABP.
Materials and methods

Materials

Spleen phosphodiesterase, micrococcal nuclease, nuclease P1, potato apyrase and fetal bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO). T4 polynucleotide kinase was purchased from US Biochemical Corp. (Cleveland, OH). Antibodies for α-tubulin and p53 were purchased from Oncogene Research Products (Boston, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The reagents for chemiluminescent detection and quantitation of proteins in western blots were purchased from New England Nuclear Life Science Products (Boston, MA). EMerck PEI-cellulose TLC plates were purchased from VWR (Pleasantville, NJ). Ham’s F12 nutrient mixture was purchased from Gibco (Gaithersburg, MD). N-OH-ABP was prepared by acetylation of N-OH-ABP with acetyl chloride using methods described earlier (38,39). The dG-C8-ABP and N-deoxyadenosin-8-yl)-4-aminobiphenyl (dA-C8-ABP) standards were synthesized as described previously (22,39,40). The purity of the standard adducts and N-OH-ABP was determined to be >99% based on chromatographic analyses.

Cell lines and culture

TCC cultures were initiated and established using published methods (34). The TCC10 immortalized cell line was initiated from a biopsy of a non-invasive tumor of a patient at the University of Wisconsin Hospitals and Clinics. The TCC10 cell line overcame senescence due to a defect in the G1 checkpoint resulting from a mutation in p16 and hence emerged from growth crisis without treatment with any chemical or viral agent (35). This cell line showed a stable karyotype upon analysis at passage 35. The TDM143-TCC10 cell line was generated by transformation of the immortal TCC10 line using a retroviral vector carrying a TDM at codon 143. The cell line was established. The cultures were maintained in selection medium for ~10 days prior to shifting them to a normal growth medium containing the neomycin resistance gene. Following successful infection of TCC10 in vitro, the rate of repair of ABP–DNA adducts was compared after treatment of these cultures with N-OH-ABP. 32P-post-labeling analysis of TCC cultures exposed to N-OH-ABP showed a major adduct which was identified as N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) based on its chromatographic properties compared with a synthetic standard. The result presented in this study shows that p53 protein modulates the rate of removal of dG-C8-ABP adducts from genomic DNA. This implies that p53 regulates repair of DNA adducts in TCC10 and that a p53 mutation, which is commonly observed in human bladder cancers, might enhance overall mutability to endogenous and exogenous agents. Increased levels of unrepaired DNA damage could accelerate the progression of bladder neoplasia to an advanced stage. Thus, the above findings have important implications in environmental carcinogenesis.

Results

The genotypic status of TP53 in parental TCC10 was determined to be wild-type based on nucleotide sequencing analysis of PCR samples of the cDNA (data not shown). Additionally, sequencing analysis of exons 5–9 (performed by OncorMed, Gaithersburg, MD) of TCC10 genomic DNA revealed no sequence alterations in the above coding region of TP53, indicating a normal p53 genotype. Furthermore, the functional
status of p53 in the TCC10 isotype was assessed by western blot analysis of the cell lysate using a pan-tropic antibody that recognizes all forms of p53. The protein product was detected by enhanced chemiluminescence after hybridization with anti-mouse IgG. Figure 1 shows western blot analyses of parental line TCC10 along with its isogenic set, in which the wild-type function of p53 was nullified due to the expression of a human papilloma virus 16-E6 oncoprotein (E6-TCC10) or as a result of expression of a TDM p53 (TDM143-TCC10). The TCC10 line shows a band for p53 of marginal intensity, suggestive of the wild-type allele in TCC10. In contrast, the cell lysate from TDM143-TCC10 shows an intense p53 band (>20-fold compared with TCC10) consistent with reports of stabilization of p53 as a result of the mutation (41–43). This was further confirmed by nucleotide sequencing of the cDNA derived from TDM143-TCC10 (data not shown). The sequencing data revealed a base transition mutation at codon 143 (G→T) in conformity with the published reports (43). In contrast to TDM143-TCC10, E6-TCC10 cells failed to show a band in the region of p53. The lack of a p53 band in E6-TCC10 is due to sequestering of p53 by the E6 oncoprotein and rapid degradation of the complex (44). Furthermore, the functional status of wild-type p53 was evidenced by measurement of p53 levels 24 h after irradiation of TCC10 (Figure 1). There was a 4-fold induction of p53 in TCC10 following irradiation with 18 Gy. Based on the nucleotide sequencing and the above western analyses, the functional status of p53 was confirmed to be normal in TCC10 while it was defective in TDM143-TCC10.

Utilizing TCC10, we first characterized the DNA adducts generated in culture after exposure to N-OH-AABP. An autoradiogram of the 2-dimensional TLC of post-labeled products was also obtained from TCC10 cultures exposed to N-OH-AABP (Figure 2D) and N-OH-ABP chemically bound DNA (Figure 2E). As shown in Figure 2, there is complete disappearance of adduct 1 following nuclease P1 digestion in these samples. The synthetic dGp-ABP standard was similarly labeled after nuclease P1 digestion under the same conditions and was also found to be sensitive to nuclease P1 digestion (Figure 2F), an observation which is in accordance with published reports (19). In the case of calf thymus DNA chemically modified with N-OH-ABP the disappearance of adduct 1 was followed by intensification of another spot, adduct 2 (Figure 2E). Spot 2 corresponds to the 3′,5′-bisphospho-8-yl)-4-aminobiphenyl (dGp-C8-ABP). As demonstrated in Figure 2A–C, the TLC spots corresponding to 1 were extracted three times with 3.7 M ammonium hydroxide in isopropyl alcohol and analyzed on a HPLC interfaced to a radioactivity detector using conditions described in an earlier study (19). The product from TCC10 exhibited the same retention time (10 min) as synthetic samples (data not shown), thereby further affirming its structural identity. Based on the chromatographic properties the above adduct 1 has been identified as dG-C8-ABP. This conclusion was further strengthened by the finding of its sensitivity to hydrolysis by nuclease P1.

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Prior to quantification of dG-C8-ABP DNA adducts from TCC cultures, optimum conditions were determined for DNA hydrolysis, butanol extraction and the kinase reaction as described (45–47). Using synthetic deoxyguanosine phosphate and dG-C8-ABP standards, the efficiency of the kinase reaction was determined to be ~55%. Similarly, the efficiency of the butanol extraction step was found to be >95%. Based on the amount of label incorporated into normal nucleotides, the concentrations of enzymes (micrococcal nuclease and spleen phosphodiesterase) and the time of incubation for DNA hydrolysis were optimized such that >95% of the DNA was completely hydrolyzed to 3’-mononucleotides.

Utilizing [3H]N-OH-ABP bound to DNA (sp. act. ~1 pmol/ nmol DNA), the dynamic range of response was tested using a 10 µg quantity of DNA, the amount that is normally used for quantification of adducts in TCC cultures. Normal calf thymus DNA was spiked with varying amounts of [3H]N-OH-ABP chemically bound DNA (in the range 100–1250 pmol equiv. total nucleotides) to a final amount of 10 µg. The mixtures were hydrolyzed to 3’-mononucleotides, butanol extracted and labeled with T4 kinase in the presence of excess amounts of [γ-32P]ATP. The label associated with adduct 1 was extracted from TLC plates as described in Materials and methods and the radioactivity measured using a scintillation counter. Figure 3 shows the relationship between amount of dG-C8-ABP adduct recovered from the TLC spots versus the quantity of N-OH-ABP bound DNA (expressed as nmol total nucleotides) with which the reaction mixture was spiked. A linear relationship was observed between the radioactivity in adducts and the amount of N-OH-ABP bound DNA.

To test if adduct formation is dependent upon dose, TCC10 cultures were exposed to varying concentrations of N-OH-ABP, ranging between 5 and 25 µM. Following exposure to the chemical for 12 h the cells were harvested, the DNA purified and adduct analyses were carried out and quantified as described in Materials and methods. As shown in Figure 4, the amount of adduct formation was dependent upon the concentration of N-OH-ABP. In order to estimate relative labeling index, a known aliquot of the same DNA hydrolysate sample was labeled for normal nucleotides prior to butanol extraction. The radioactivity associated with the total nucleotides was determined following separation on TLC plates (12,13). Based on the ratios of the radioactivity in ABP–DNA adducts versus the radioactivity in total nucleotides in TCC10 cultures exposed to 5–25 µM N-OH-ABP, the dG-C8-ABP adduct levels were estimated to be in the range 1–5 adducts/10^7 nucleotides.

Cell viability measurements after 12 h chemical treatment revealed no cytotoxic effect up to a concentration of 15 µM. At higher doses there was a dose-dependent cytotoxic response and the effect on cell viability was significant. For this reason, the DNA repair studies with N-OH-ABP were conducted at a dose of 15 µM. At this 15 µM dose the effects of various durations of exposure to the chemical were tested. Figure 5 shows the relationship between the amount of dG-C8-ABP formation versus the period of exposure to the chemical. Up to 12 h there were significant increases in the amount of dG-C8-ABP formation, after which the level appears to plateau.

Following optimization of the conditions for dG-C8-ABP formation, the kinetics of DNA repair were monitored by measuring the rate of disappearance of adducts. To monitor the kinetics of repair, TCC10 cultures were exposed to 10 µM N-OH-ABP for 12 h, after which they were allowed to grow for up to 72 h in F12 supplemented medium with 3% serum devoid of the chemical agent. Figure 6A and B shows the amounts of dG-C8-ABP adducts that remained in the DNA at varying time periods after treatment of TCC10 and TDM143–TCC10 cultures with the chemical. After about 24 h there was a rapid removal of these adducts from DNA and a majority of them disappeared within 48 h post-treatment, suggesting...
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Fig. 5. Effect of duration of exposure to N-OH-AABP on the formation of dG-C8-ABP in TCC10. TCC10 cultures were exposed to 15 µM N-OH-AABP for varying periods of time as indicated above. Following chemical treatment cells were harvested and adduct analyses carried out and the results are presented as described in the legend to Figure 4.

Fig. 6. Kinetics of disappearance of the dG-C8-ABP adduct in TCC10. TCC10 (A) and TDM143-TCC10 (B) cultures exposed to 10 µM N-OH-AABP for 12 h. After exposure to the chemical they were allowed to recover for varying periods of time after which the cells were harvested and the dG-C8-ABP adducts were estimated per unit amount of DNA as described in Materials and methods.

Fig. 7. Comparison of dG-C8-ABP levels in TCC10 and TDM143-TCC10 cells at 0 and 25 h post-treatment of TCC cultures with N-OH-AABP. TCC10 and TDM143-TCC10 were exposed to 15 µM N-OH-AABP for 8 h. The total amount of dG-C8-ABP adducts in 5 µg DNA were determined as elaborated earlier. The data shown here are representative of the findings from two independent experiments.

that TCC10 has the necessary DNA repair system for expedient removal of the above DNA damage. The drop in adduct levels is not because of cell proliferation since measurement of the total cell counts at the time of harvest following post-treatment with chemical showed insignificant differences.

To test the effect of p53 function on repair, both the TCC10 and TDM143-TCC10 cell lines were cultured under the same conditions and simultaneously exposed to 15 µM N-OH-AABP for 8 h. Following completion of the chemical treatment they were either harvested immediately or after 25 h recovery time to repair the DNA damage caused by the chemical agent. Figure 7 shows a comparison of the dG-C8-ABP adduct levels at 0 and 25 h post-treatment with N-OH-AABP. There were no major differences in the amounts of dG-C8-ABP adducts between TCC10 and TDM143-TCC10 immediately after completion of treatment with the chemical. However, 25 h following chemical treatment the adduct levels were significantly different between the TCC10 isotypes. In the parental TCC10 containing wild-type p53 a majority (>75%) of the adducts were removed from DNA within 25 h. In contrast, the p53-defective TDM143-TCC10 line was relatively inefficient in removal of the dG-C8-ABP adduct from the genomic DNA (~30%). Nonetheless, TDM143-TCC10 was still capable of repairing the damage resulting from the DNA adduction process, although the rate of removal was much slower when compared with the parental TCC10 line.

Discussion

The results presented here show that human TCC lines form dG-C8-ABP as the main adduct when exposed to the carcinogenic proximate metabolite of the human bladder carcinogen ABP. Detection of these adducts was based on chromatographic analysis of the post-labeled products of the DNA hydrolysate containing 3′-mononucleotides. The DNA hydrolysate from TCC10 yielded a product with the same chromatographic properties as the product obtained by post-labeling analysis of authentic synthetic dGp-C8-ABP. The detection of dG-C8-ABP in TCC10 is in conformity with earlier reports by us wherein we have demonstrated that normal human uroepithelial cells contain the enzyme system(s) needed for biochemical activation of N-OH-AABP to reactive electrophilic intermediates that bind to DNA (22,23). These activating enzymes were shown to be distributed in the microsomes and their activities were blocked by paraoxon, a metabolic inhibitor of acetyltransferases (23). Incubation of [3H]N-OH-AABP with calf thymus DNA in vitro in the presence of TCC10 microsomes revealed binding of label to nucleic acids which amounted to 31 ± 3 pmol/mg DNA/mg microsomal protein. These activities were in the same range (25.2 pmol/mg DNA/mg protein) as those reported for normal human uroepithelial cells (23). Thus TCC10 have retained their metabolic capability to activate these arylhydroxamic acid derivatives of ABP.

Biochemical activation of N-OH-AABP and DNA binding could occur through two different pathways, involving either the arylnitrenium or arylamidonium ion intermediates (21,23,48). Arylnitrenium ions could be formed from N-OH-
AABP by transacetylation to form the N-acetoxy derivative followed by deacetylation. In contrast, arylnitrenium ions are formed from the N-acetoxyacetyl derivative of ABP, which could conceivably arise from N-OH-AABP by the action of certain peroxidases (21,48,49). Interaction of the arylnitrenium ion with DNA generates the acetyl derivatives of the adduct whereas the arylnitrenium intermediates yield adducts without the acetyl group. The formation of dG-C8-ABP in urothelial DNA, which is devoid of the acetyl group, suggests that the ultimate reactive species responsible for binding to DNA is the arylnitrenium ion. This is further evidenced by the finding that N-OH-ABP generates the arylnitrenium ion as an intermediate and yields dG-C8-ABP (Figure 2B) as the main product when it binds to DNA under acidic conditions. The formation of dG-C8-ABP as the primary product in TCC10 and the absence of its acetylated adducts in urothelial DNA strongly suggests acetyltransferase-mediated activation of N-OH-AABP to be primarily responsible for DNA binding. This finding is also in accordance with our earlier report wherein in normal human uroepithelial cells we reported acetyltransferase-mediated activation of N-OH-AABP to be a major pathway for bioactivation of N-OH-AABP (21).

Calf thymus DNA that was chemically interacted with N-OH-ABP yielded dA-C8-ABP as a minor product (Figure 2E) which could be detected by 32P-post-labeling methods following nuclease P1 enrichment. Nuclease P1-treated DNA hydrolysates of TCC10 cells exposed to N-OH-AABP failed to show a spot in the region of dA-C8-ABP, although both TCC10 and TDM143-TCC10 yielded the same reactive intermediate, the arylnitrenium ion. The reason for the failure to detect dA-C8-ABP in TCC cultures remains unknown and could possibly be due to variations in its stability or reactivity of the reactive intermediate in the intracellular environment.

The results presented in Figures 3–6 show quantitative analyses of formation of dG-C8-ABP adducts in TCC10 cultures exposed to N-OH-AABP. Using synthetic dGp-C8-ABP standards and [3H]N-OH-ABP bound DNA, conditions were optimized for quantitative analysis of dG-C8-ABP in TCC10. The limit for quantification of dG-C8-ABP was ~1 adduct/5×109 nucleotides. To ascertain the quantitative response at the highest DNA concentration (10 µg) that was used for analyses of TCC cultures, ABP bound DNA of known specific activity (1 pmol/nmol, prepared by chemical reaction with [3H]N-OH-ABP) was spiked with calf thymus DNA. Subsequently they were subjected to post-labeling analysis. Based on the amount of label recovered in the TLC spots associated with dG-C8-ABP and the specific activity of [γ-32P]ATP, the adduct was estimated to be ~0.38 pmol/nmol nucleotides (Figure 3). The efficiency of the kinase reaction under our experimental conditions was found to be 55% and, when corrected for the above, the amount of dG-C8-ABP adduct amounts to 0.7 pmol/nmol DNA. Based on the amount of tritium label bound to unit weight of DNA and the specific activity of [3H]N-OH-ABP, the total adducts in chemically bound DNA was estimated to be 1 pmol/nmol nucleotides. Of the total adducts in N-OH-ABP chemically bound DNA, ~80% are thought to comprise dG-C8-ABP (50). Thus the theoretical estimate of dG-C8-ABP adducts in the spiked samples is ~0.8 pmol/nmol. The value of 0.7 pmol/nmol DNA, determined by post-labeling methods of analysis, is in close approximation to the above theoretical estimates. This is further confirmed by recovery studies using synthetic dGp-C8-ABP, wherein ~85% of the products were finally recovered during post-labeling analyses of DNA hydrolysates. Thus the quantitative methods of analysis of adducts in TCC10 were validated. As expected, the amount of dG-C8-ABP formation in TCC10 was found to be dependent upon the dose (Figure 4) and the duration of exposure (Figure 5). Under the above conditions the amount of dG-C8-ABP in TCC10 varied between 1 and 5 adducts/107 nucleotides. This amount is about two orders of magnitude higher than the limit of quantification. Hence, we adopted this method to monitor the kinetics of repair of adducts.

The rate of disappearance of dG-C8-ABP was used as an indicator of repair efficiency. The results presented in Figure 6 show that dG-C8-ABP adducts persist in TCC10 for the first 24 h, after which there is rapid removal. The majority of the adducts are removed from TCC10 cultures within 48 h after treatment with the chemical, suggesting that TCC10 have the repair machinery to correct DNA damage resulting from bulky adducts. The decrease in adduct amounts are not due to dilution of adduct DNA by replication or cell proliferation since total cell counts at the last time point were no higher than those of samples collected immediately after chemical treatment. In fact, the total cell counts at the 48 h recovery time were 90%, when compared with control cultures that did not undergo treatment with N-OH-ABP. This is in part due to cytotoxic effects or arrest of the cell cycle following DNA damage caused by N-OH-ABP treatment. Cell cycle analysis revealed that this effect could last up to 72 h (data not shown). Lastly, the generation time of these TCC cultures in enriched medium containing 3% serum was found to be 48 h. Hence, a 5-fold reduction in adduct levels within 24 h could not be accounted for by cell proliferation effects.

Comparison of the amounts of dG-C8-ABP that remained 24 h post-treatment with N-OH-ABP between the isogeneic set of TCC10, wherein wild-type p53 function was either expressed normally (TCC10) or nullified by a mutation (TDM143-TCC10), reveals that p53 is involved in the repair of these adducts. Although similar amounts of dG-C8-ABP adducts were initially detected in TCC10 versus TDM143-TCC10, a significantly larger amount (3-fold excess relative to TCC10) remained in the DNA of TDM143-TCC10 after a 25 h recovery period. The marginal difference observed in the rate of repair in TDM143-TCC10 presented in Figures 6B and 7 might, in part, be attributed to differences in the treatment regimen between the two sets of experiments (see legends to Figures 6B and 7). Nonetheless, the results suggest that p53 is involved in the repair of DNA damage caused by N-OH-ABP.

Earlier evidence for the involvement of p53 in global DNA repair comes from studies in human fibroblasts derived from patients with Li–Fraumeni syndrome, a disorder that results from a germline defect in one of the TP53 alleles. The cell lines from these subjects show reduced repair of UV-induced pyrimidine dimers compared with normal cells (33). Similarly, abrogation of p53 by introduction of the HPV16 E6 oncoprotein or a dominant-negative p53 in human colon cell lines resulted in a 3- to 5-fold reduction in nucleotide excision repair and host–cell reactivation of UV-irradiated reporter plasmid DNA (30,51). Recently, wild-type p53 was shown to be required for efficient global genomic repair of benzo[a]pyrene diol epoxide-induced DNA adducts from the overall genome (31,32). The present study demonstrates that p53 is also involved in the repair of bulky adducts generated from the environmental carcinogen ABP and thus are in accordance with recent reports (30–32). In these studies we have used a temperature-sensitive
mutants of p53 which manifests trans-dominant function at 37°C, the temperature that was used for the growth and propagation of these TCC lines. TDM143-TCC10 was not completely defective in DNA repair since it was partially effective in the removal of dG-C8-ABP. At present it is unclear whether inefficient removal of adducts is due to the specific codon position of the mutation or due to a temperature-dependent alteration in conformation of p53. This work was supported by NCI grant CA-82035. We wish to acknowledge the support of the Environmental Health Sciences Center of the University of Wisconsin for use of the Cell Marker Research Core Facility funded by NIHES grant ES09990.

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