

Carcinogenic Potential of Benzene and Toluene When Evaluated Using Cyclin-dependent Kinase Activation and p53–DNA Binding

Craig Dees,¹ Minoo Askari,² and Don Henley²

¹Risk Analysis Section, Health Sciences Research Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee; ²University of Tennessee, Knoxville, Tennessee

Benzene is carcinogenic, whereas toluene is thought to have little carcinogenic potential. Benzene and toluene were found to activate cyclin-dependent kinase 2 in rat liver epithelial (RLE) and HL60 cells. pRb105 was hyperphosphorylated in RLE cells treated with either solvent. Kinase activation and subsequent hyperphosphorylation of pRb105 and p53 by benzene or toluene may be responsible for their growth promotional effects, but it does not account for increased potential of benzene to induce cancer. Therefore, we examined the ability of these solvents to increase p53-DNA site-specific binding in RLE cells. Benzene increased p53–DNA site-specific DNA binding in RLE cells compared to control levels or the effects of toluene. Increased p53–DNA site-specific binding by benzene may be caused by damage to cellular DNA. If so, although both solvents appear to have promotional activity, the increased potential of benzene to damage DNA may be responsible for the difference in the ability of benzene to cause cancer. — Environ Health Perspect 104(Suppl 6):1289–1292 (1996)

Key words: p53, pRb105, tumor promoter, phosphorylation, DNA damage, cyclin-dependent kinase

Introduction

Recently, we showed that benzene, toluene, and chloroform induce hyperphosphorylation of the tumor-suppressor gene product p53 (1). Hyperphosphorylation of p53 and pRb105 by promoters like 12-*O*-tetradecanoylphorbol-13-acetate (TPA), benzene, toluene and chloroform is probably due to their effects via protein kinase C (PKC) or by inducing a cascade effect in kinases responsible for signal transduction (1–4). Stimulation of kinase activity and the subsequent hyperphosphorylation of tumor-suppressor gene products, may partially account for the promotional effects exhibited by benzene, toluene, and chloroform. Promoter attenuation of p53's ability to induce cell cycle arrest may also keep p53 from preventing the replication of damaged

DNA (5). Therefore, posttranslational modification of p53 or other tumor-suppressor gene products induced by benzene or toluene may also be a factor in determining the relative carcinogenicity of the two solvents.

Since benzene and toluene both appear to have adverse effects on molecular mechanisms that control cell growth and prevent the duplication of genetic errors, these effects do not explain the apparent differences in the ability of the two solvents to cause cancer. It is clear that benzene is a carcinogen, but toluene is thought to be either noncarcinogenic or to have little potential to induce cancer in hematopoietic cells.

To resolve the difference in the carcinogenic potential of benzene and toluene, we

examined the ability of these two solvents to induce cyclin-dependent kinase 2 (Cdk2) activity and to hyperphosphorylate pRb105. Cdk2 has been previously associated with the promotional effects of estrogen and phosphorylates the tumor-suppressor gene product pRb105 (6). We hypothesized that differences in kinase induction and post translation modification of proteins like pRb105 might account for the apparent differences in carcinogenicity shown by benzene and toluene. Hyperphosphorylation of pRb105 was examined in rat liver epithelial (RLE) and HL60 cells that had been treated with benzene or toluene. Cdk2 kinase activity in cells treated with benzene and toluene was also determined.

Previous studies have also suggested that p53 DNA site-specific DNA binding is increased in cells treated with DNA-damaging agents and chemotherapeutic agents (7). We hypothesized that the ability to damage DNA might be responsible for the apparent differences in carcinogenic potential of benzene and toluene. Therefore, we examined p53–DNA binding in RLE cells treated with toluene or benzene.

Materials and Methods

Cell lines examined in this study included WB-F344 RLE cells, which were a gift from Dr. James Trosko of Michigan State University (1). Because RLE cells appear to produce a wild-type p53 and pRb105 (8), they were used for immunoprecipitation of pRb105, Cdk2, and p53–DNA sequence-specific binding studies. Because the HL60 cells, obtained from the American Type Culture Collection, produce mutant p53 and the pRb105 status is unknown, they were only examined for Cdk2 activation.

RLE cells were maintained in Richter's medium, and HL60 cells were maintained in RPMI 1640. Both cell lines were incubated at 37°C in a 5% CO₂ atmosphere. RLE cells were maintained with 5% (v/v) calf serum and HL60 cells in 20% fetal bovine serum. Prior to studies on Cdk2 activation, the respective serum concentrations were reduced to 0.5% (v/v) for 48 hr.

Hyperphosphorylation of pRb105 in RLE cells was examined by adding 0 to 2% (v/v) benzene or toluene to the medium of the adherent cells similar to procedures described previously (1). Briefly, pRb105 was radiolabeled using [³²P]-*ortho*-phosphoric acid and the cells were lysed (1). pRb105 was immunoprecipitated (1). Total protein of all extracts was determined using

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Address correspondence to Dr. C. Dees, Research and Development, PhotoGen L.L.C., #1805, 790 N. Cedar Bluff Road, Knoxville, TN 37923. Telephone: (423) 531-3192. Fax: (423) 458-2879. E-mail: GENASE@aol.com

Abbreviations used: Cdk2, cyclin-dependent kinase 2; HSP, heat shock protein; PBS, phosphate-buffered saline; PKC, protein kinase C; RLE, rat liver epithelial (cells); TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

a commercial BCA protein assay (Pierce Biochemicals, Rockford, IL). Samples were equalized on total protein before immunoprecipitations were performed. Anti-pRb105 monoclonal antibody was obtained from Oncogene Sciences (Manhasset, NY). Radiolabeled cell extracts were incubated with the antibody and protein A/G agarose overnight. The agarose immune complexes were precipitated using microcentrifugation, and then the supernatants were removed and discarded. The agarose beads were washed with lysis buffer and then centrifuged. Standard denaturing gel sample buffer was added, and the immunoprecipitates examined using denaturing gel electrophoresis followed by autoradiography. Polyacrylamide gels used were 8.0% from a commercial source (Novex Inc., San Diego, CA). Immunoprecipitation studies were performed similar to the kinase assay protocols except [³²S]-methionine labeled extracts from RLE cells were used (1). Anti-p53 (ab-1) and anti-heat shock protein (HSP) 72/73 were obtained from Oncogene Sciences.

RLE or HL60 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by the addition of cold lysis buffer (Tris 20 mM pH 7.5, NaCl 250 mM, 0.1% NP-40, NaF 10 mM, sodium vanadate [NaVO] 1 mM, phenylmethylsulfonyl fluoride [PMSF] 1 mM). After 15 min on ice, the lysates were centrifuged at 20,000g for 15 min (4°C). Cdk2 was precipitated from equal amounts of cell extracts using purified rabbit anti-Cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G agarose. Cdk2 immunoprecipitates were washed (3×) with the lysis buffer and twice with kinase buffer (Tris 40 mM, pH 7.5, MgCl₂ 10 mM). The immunoprecipitates were suspended in 30 μl of kinase buffer supplemented with 400 μg/ml histones (type II-SS, Sigma chemical, St. Louis, MO), 5 μM ATP, 0.5 μM dithiothreitol, 0.5 mM EGTA, and 5 μCi γ-[³²P]-ATP for 20 min at room temperature. The reaction was stopped using gel electrophoresis sample buffer, and the reaction products were separated on a 14% polyacrylamide gel (Novex).

Cells were cultured in 175-cm² flasks in Richter's medium supplemented with 0.5% calf serum. The medium was replaced before adding compounds for test with fresh medium without serum. Cells were then incubated for 2 hr. Untreated control cells were also examined. Nuclear extracts from the cells were prepared as described (5). Briefly, the medium was removed

from the cells, and the monolayers were washed with PBS, pH 7.4. Cells were lysed by the addition of 2.5 ml buffer (20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, and 0.1% Triton X-100 in 20 mM HEPES buffer, pH 7.6). The lysate was centrifuged at 800g for 4 min and the resulting pellet was diluted with 3 vol of 500 mM NaCl in buffer (see above) and then incubated at 4°C for 30 min with agitation. The mixture was centrifuged at 35,000g for 10 min and the supernatants containing p53 were removed for immediate analysis. The total protein content of the extracts was determined using BCA protein assays (Pierce Biochemicals). Protein content for all samples was equalized before performing the binding assay. The consensus p53 binding sequence determined by Funk (GGACATGCCCGGGCATGTCC) (9) was synthesized, prepared in double-stranded form, and end-labeled with [³²P]-ATP. Binding reactions consisted of 20 μg nuclear protein, 0.5 ng ³²P-labeled oligonucleotide, 0.5 μg salmon sperm DNA (Sigma) with buffer (without Triton) in a final volume of 25 μl. Binding reactions were incubated at room temperature for 20 min and 8 μl of the reaction mixtures were separated on 6% nondenaturing polyacrylamide gels (Novex) and visualized by autoradiography.

Results

To determine if pRb105 was hyperphosphorylated in RLE cells treated with benzene or toluene, we treated RLE cells with concentrations of solvent ranging from 0 to 2% (v/v). Phosphorylation of p53 in RLE cells increases with the dose of benzene (Figure 1a) or toluene (Figure 1b) applied to the cells. Therefore, RLE cells hyperphosphorylate pRb105 in a dose-dependent response to the solvents.

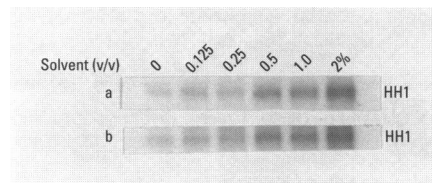


Figure 1. (a) Phosphorylation of pRb105 in rat liver epithelial (RLE) cells increases with the applied dose of benzene. (b) Phosphorylation of pRb105 RLE cells also increases with the applied dose of toluene. Benzene and toluene were added to the medium at a concentration of 2% (v/v) and incubated with the cells for 2 hr.

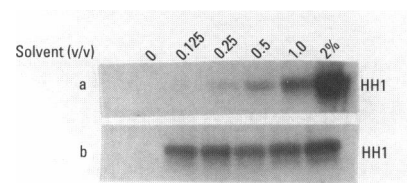


Figure 2. (a) Activation of cyclin-dependent kinase 2 (Cdk2) occurs in rat liver epithelial (RLE) cells treated with benzene in relation to the applied dose. (b) Cdk2 activation also occurs when the HL60 cells are treated with benzene. Lanes 1 contain histone incubated with immunoprecipitated Cdk2 from cell extracts from control cells (no solvent); lanes 2, cells treated with 0.125%; lanes 3, 0.25%; lanes 4, 0.5%; lanes 5, 1.0%; lanes 6, 2% (v/v). RLE cells were incubated in medium containing benzene for 4 and 2 hr for HL60 cells.

Cdk2 activity increases in relation to the applied dose of benzene (Figure 2a) added to the medium of RLE or to HL60 cells (Figure 2b). Similar dose-dependent increases in Cdk2 activity are obtained when using toluene (data not shown). Differences in the amount of Cdk2 activation may be caused by different susceptibilities of the cells to the two solvents. However, it cannot be determined if this variation is caused by some feature of the experimental methods used. Since the solvents are not fully miscible in the medium, the applied dose under these conditions is difficult to control. In addition, the solvents, especially at high doses, may also be highly toxic to the cells. Longer application of the solvents at 1 or 2% (v/v) resulted in toxic death of RLE cells.

DNA-damaging and chemotherapeutic agents increase p53-DNA site-specific binding in RLE cells (Figure 3A). Benzene treatment of RLE cells was found to increase p53-DNA binding when compared to control cells (Figure 3B). p53-DNA binding was also increased over control levels in cells treated with toluene (Figure 3B), but the effect was markedly less than that produced by benzene.

Increased amounts of HSP 72/73 is precipitated from benzene- and toluene-treated RLE cells using the anti-HSP72/73 antibody (Figure 4). However, it cannot be determined if anti-HSP coprecipitates p53 or anti-p53 coprecipitates HSP.

Discussion

Since benzene and toluene have been previously shown to be potent PKC-activating promoters, it is likely that the hyperphosphorylation of p53 is mediated through effects on PKC as has been previously

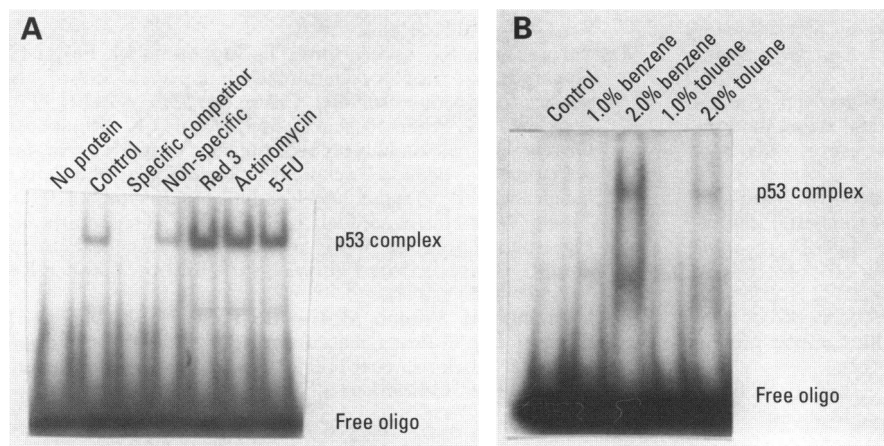


Figure 3. (A) DNA-damaging chemicals and chemotherapeutic agents increase p53-DNA site-specific DNA binding in rat liver epithelial (RLE) cells. (B) Increased p53-DNA site-specific binding occurs when RLE cells are treated with 2% benzene or toluene. Little or no increase in p53-DNA binding occurs when 1% benzene or toluene is added to the medium.

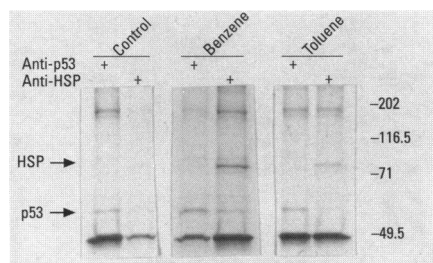


Figure 4. Increased amounts of heat-shock protein (HSP) are precipitated from rat liver epithelial (RLE) cells treated with 2% benzene and to a lesser extent from toluene-treated cells by anti-HSP antibody. No evidence of coprecipitation of HSP was found when anti-p53 antibody was used. Cells were incubated in medium containing solvent for 2 hr before detergent lysis.

demonstrated for phorbol 12-myristate 13-acetate (PMA) (1-4). Results presented here suggest that the growth promotional effects of benzene and toluene may in part be caused by hyperphosphorylating another cell cycle and transcription controlling protein pRb105. This result is in agreement with studies that show PMA treatment of cells results in the hyperphosphorylation of p53 and pRb105 (1,10). Results presented here suggest that Cdk2 activation is

responsible for the hyperphosphorylation of pRb105. It remains to be determined if benzene or toluene directly affect Cdk2 or this is a result of a cascade effect on signal transduction enzymes.

Other growth-stimulating promoters also have been shown to increase Cdk2 activity. Estradiol stimulates Cdk2 in cultured human breast cells and increases the phosphorylation of pRb105 (6). The xenoestrogen DDT also increases Cdk2 activity (10). Hyperphosphorylation of pRb105 and p53 that occurs as a result of the effects of chemical promoters on kinase activity may be an essential component of their growth-stimulating properties. Hyperphosphorylation of p53, for example, has been shown to decrease the ability of p53 to activate transcription (11).

No outstanding differences between the ability of benzene or toluene to phosphorylate pRb105 or induce Cdk2 activation were found that might explain the difference in the carcinogenic potential of these two solvents. However, p53-sequence-specific DNA binding was found to be elevated in cells treated with 2% benzene when compared to cells treated with toluene or control cells. Increased p53 DNA site-specific DNA binding has been demon-

strated in cells treated with DNA-damaging or chemotherapeutic agents that affect DNA-manipulating enzymes (5). DNA-damaging chemicals and chemotherapeutic agents increase p53-DNA binding in RLE cells (Figure 3A), as do high concentrations (2% v/v) of benzene (Figure 3B), and to a lesser extent, toluene (Figure 3B). Lower concentrations of toluene or benzene (1% v/v) fail to markedly increase p53-DNA binding in RLE cells (Figure 3B). If benzene-induced increase in p53 DNA site-specific DNA binding can be attributed to a molecular mechanism occurring in response to DNA damage (5), benzene may be more capable of damaging genetic material. Therefore, this may account for the carcinogenicity demonstrated by benzene in relation to toluene. However, other cellular stresses including hypoxia have also been shown to increase p53-DNA binding, but the increased p53 levels are thought to occur by a different mechanism than radiation-induced DNA damage (12). The tumor promoter okadaic acid, an inhibitor of protein phosphatases, has been shown to also increase the steady-state level of p53 without altering p53-DNA binding (13). Heat shock has been shown to increase the intracellular levels of p53, which is bound by heat shock protein (14). Therefore, cellular effects by benzene that result in stress may be responsible for the increase we see in p53-DNA binding. Increased levels of HSP were precipitated from benzene- and toluene-treated cells (Figure 4). However, no evidence of coprecipitation of p53 by anti-HSP or precipitation of HSP by anti-p53 could be found. Therefore, we cannot attribute the change in p53-DNA binding in solvent-treated cells to HSP, nor can we rule it out. Whatever cellular stress is indicated by increased p53-DNA binding after treatment of benzene that is not evident with toluene, it still may represent a central difference between the two solvents that is directly related to carcinogenicity. Further studies will be required to determine if benzene-induced p53-DNA binding is directly related to DNA damage or is caused by some other form of cellular stress.

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