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

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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 promotion 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 to the difference in the ability of benzene to cause cancer.

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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 phosphorlates 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
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 [35S]-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 [NaVO3] 1 mM, phenylmethylsulfonylfluoride [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 (3x) 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 (GGACATGCC-GGCGCATGTCC) (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% non-denaturing 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.

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 chemotherapy 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
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