Benzo[a]pyrene-Induced DNA-Protein Crosslinks in Cultured Human Lymphocytes and the Role of the GSTM1 and GSTT1 Genotypes

We investigated the influence of glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) polymorphisms upon DNA-protein crosslinks (DPC) induced by benzo[a]pyrene (B[a]P) in cultured human lymphocytes. Lymphocyte samples were collected from 30 healthy nonsmoking hospital administrative workers. DPC was detected with KCl-SDS assay and the distributions of GSTM1 and GSTT1 were determined by polymerase chain reaction. B[a]P was found to induce a significant dose-responsive increase in cytotoxicity and DPC regardless of the genotypes (p<0.05). We did not find statistically significant genetic modification effect of GSTM1 and GSTT1 polymorphisms in the cytotoxicity and DPC formation (p>0.05). In terms of the genes examined, the level of cytotoxicity and DPC formation were found to be highest in the GSTM1-null and GSTT1-null cells. In conclusion, B[a]P induced a significant increase in the cytotoxicity and the level of DPC formation in cultured human lymphocytes. Our findings suggest that DPC could be used as a biomarker of B[a]P exposure.

Key Words: Polymorphism, Genetics; Glutathione Transferase; Benzopyrenes; DNA-Binding Proteins

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are formed due to incomplete combustion of fossil fuels and are ubiquitously present in the environment. Benzo[a]pyrene (B[a]P) is one of a large number of PAH and is frequently used as a representative indicator of total PAH exposure (1). B[a]P is usually inhaled and deposited in the lower lung, where it is readily absorbed. It is then metabolically activated by cytochrome P450 to highly reactive electrophilic forms, including phenol, trans-dihydrodiols, arene oxides, and diol-epoxides, which may be responsible for most of its observed cytotoxic, mutagenic, and carcinogenic effects (1, 2). Biotransformation of chemicals involves metabolic activation and detoxification pathways, which are known to play a primary role in chemical carcinogenesis (3).

Polymorphisms in the genes for metabolism are largely responsible for the different abilities of individuals to activate and detoxify genotoxic agents. Glutathione S-transferase (GST), which forms a multigene family of phase II detoxification enzyme, is involved in the metabolic detoxification of PAH (4-6). This enzyme plays an important role in protecting DNA against damage and adduct formation, by glutathione conjugation to electrophilic substances, particularly those with lipophilic compounds (7). The polymorphism of GSTM1 and GSTT1 gene loci is caused by a gene deletion, which causes a virtual absence of enzyme activity in individuals with the GSTM1- and GSTT1-null genotypes. The GSTM1-null genotype has been known to be associated with an increased risk for various environmentally-induced cancers (8, 9) and similarly, the GSTT1-null genotype with an increased risk for primary brain tumors and myelodysplastic syndromes (10). However, the genotoxic effect of GSTT1 polymorphism is not well understood compared to that of the GSTM1 polymorphism (4, 11). The coordinated activities of polymorphisms of GST may influence cytotoxic, mutagenic, and carcinogenic effects (5, 12). Epidemiologic studies indicate that the coordinated activities of polymorphisms in the GSTM1 and GSTT1 genes are associated with an increased risk for lung and bladder cancers (13, 14).

Although the associations between polymorphisms in genes for metabolism and cancer development has been known, the exact mechanisms of the relationships have not been elucidated. Little has been published on the relationship between these PAH-metabolizing enzymes and the formation of DNA adducts, which reflect DNA damage as a result of environmental exposures to PAH (15). In particular, there are no published data on the relationship between the GSTM1 and GSTT1 polymorphisms and DNA-protein crosslinks (DPC) formation resulting from exposure to B[a]P. DPC is known to be produced by oxygen radicals and other reactive species (16). Exposures to ionizing radiation, formaldehyde, and hexavalent chromium are also known to result in the induction of DPC (17-19). In light of the abundant evidences of DPC...
formation caused by reactive chemicals, it seems quite plausible that DPC is also produced by electrophilic metabolites of B[a]P.

The use of in vitro lymphocyte culture assay offers a well-controlled approach to the understanding of the metabolizing effects on genotoxicity (20). Since human lymphocytes are known to adequately express xenobiotic-metabolizing enzymes, lymphocyte cultures can be an excellent means of evaluating the genotoxicity of a variety of chemicals (3).

In the present study, we investigated the formation of DPC induced by B[a]P in cultured human lymphocytes, and in particular, the influence of GSTM1 and GSTT1 polymorphisms on cytotoxicity and genotoxicity.

**MATERIALS AND METHODS**

**Cell preparation and exposure to B[a]P**

Samples of peripheral blood (10 mL) were collected in EDTA tubes from 30 volunteers composed of healthy hospital administrative workers in Incheon, Korea. The individuals were males (age 25-35 yr) and current nonsmokers. None of the volunteers had been occupationally exposed to PAH or cytotoxic chemotherapeutic agents. All samples were collected in the morning and used for lymphocyte separation and cultures in the afternoon. Lymphocytes were isolated using the Ficoll-Histopaque procedure, and cultured in RPMI 1640 medium with 15% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.) in the presence of CO2 at 37°C for 24 hr. Cultured lymphocytes were then treated with either 5 μg/mL B[a]P (Sigma Chemical Co., St. Louis, MO, U.S.A.) or 10 μg/mL B[a]P after dissolved with DMSO (Sigma). Control cells were treated with DMSO only.

**DNA-protein crosslinks**

DPCs were measured as described by Zhitkovich and Costa (21) with minor modification. In brief, the cells were subjected to lysis with 0.5 mL of 2% SDS and 1 mM PMSF (Sigma) in 20 mM Tris-HCl (pH 7.5) in a total volume of 1.5 mL. After a further addition of 0.2 mM Tris-HCl (pH 7.5), the mixture was vigorously vortexed for 10 sec and heated at 65°C for 10 min. Precipitate was formed by cooling the sample for 5 min and collected by centrifugation at 5,000 × g for 5 min at 4°C. The pellet was resuspended in 1 mL of 0.2 M KCl in 20 mM Tris-HCl, heated at 65°C for 10 min, cooled, and centrifuged at 5,000 g for 6 min at 4°C. This washed precipitate was then incubated with 0.2 mg/mL proteinase K (Sigma) at 50°C for 3 hr in 1 mL of a reaction mixture containing 0.1 M KCl and 10 mM EDTA in 20 mM Tris-HCl (pH 7.5). Released SDS was removed by cooling the sample in the presence of 100 μg of bovine serum albumin (Sigma). The amount of DNA in the supernatant was determined using a fluorescent dye (Hoechst 33258) in a TKO 100 DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) with excitation and emission wavelengths at 365 and 460 nm, respectively. The total DNA was determined by measuring the free DNA in the supernatants during the several washing steps. The DPC level was determined as the percentage of DNA cross-linked to proteins.

**Genotyping**

The analysis of genetic polymorphism was performed by PCR amplification in a Techne progene thermal cycler (Techne Limited, Cambridge, U.K.). PCR reactions were carried out in a total volume of 50 μL in the presence of 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.2 mM of each dNTP (Takara Shuzo Co., Shiga, Japan); 2.0 mM MgCl2; 1.25 units Taq polymerase Takara); 20 pmol of each primer; and 100 ng genomic DNA as template. The primers and the PCR method used to detect GSTM1 and GSTT1 were as described in a previous study (22). A 268-bp fragment of the β-globin gene was co-amplified as an internal positive control. The presence of the GSTM1 and GSTT1 genes was indicated by amplified 215-bp and 480-bp products, respectively.

**Statistical analysis**

Results were analyzed statistically using the repeated measures ANOVA for individual data and the ANOVA for grouped data to determine the differences in cytotoxicity and DPC formation among the experimental groups. The statistical analysis was used to examine genotypes singly and in combination. A p-value of less than 0.05 was considered statistically significant.

**RESULTS**

Among the 30 study subjects, 53.3% (n=16) had a homozygous deletion of the GSTM1 gene and 50.0% (n=15) a homozygous deletion of the GSTT1 gene.

Cytotoxicity was studied in cells by determining the inhibition of cell proliferation at different concentrations of B[a]P (0, 5, and 10 μg/mL) (Table 1). Cell numbers decreased in a dose-dependent manner upon B[a]P addition regardless of the genotype (p<0.05). Mean number of cells expressed as a percentage of the control cells was 94.5% at 5 μg/mL B[a]P and 88.4% at 10 μg/mL B[a]P. When the collected data were analyzed by the GSTM1 genotype, the percentage of the cells decreased with the GSTM1-null genotype. However, this decrease did not reach a statistical significance (p<0.05). We did not find a statistically significant interaction between GSTM1 polymorphism and B[a]P concentrations in the decrease of cell numbers (p>0.05). When the data was analyzed for the GSTT1 genotype, there was no statistically significant dif-
ference of the cell numbers by GSTT1 polymorphism (p > 0.05). Interaction term of GSTT1 polymorphism and B[a]P concentrations also did not reach a statistical significance in the analysis (p > 0.05).

Results were further analyzed on the basis of the GSTM1 and GSTT1 combinations. The highest level of inhibition of cell proliferation was observed with the GSTM1-null/GSTT1-null genotype at both B[a]P concentrations (Fig. 1). However, the differences among the genotype subgroups were not statistically significant (p > 0.05).

B[a]P also caused a significant elevation in the DPC level of cells in a dose-dependent manner regardless of the genotype (p < 0.01) (Fig. 2, 3). The mean levels of DPC were 2.34 at 0 μg/mL, 7.29 at 5 μg/mL B[a]P, and 13.49 at 10 μg/mL B[a]P.

Table 1. Inhibition of cell proliferation with respect to the GSTM1 and the GSTT1 polymorphisms in cultured human lymphocytes

| Genotype   | No. of subjects | Cell number (% of control) 5 μg/mL B[a]P | 10 μg/mL B[a]P | p for trend* |
|------------|-----------------|------------------------------------------|----------------|-------------|
| GSTM1 genotype |                 |                                          |                |             |
| Positive (+) | 14              | 95.86 ± 8.30                            | 91.59 ± 12.04  | <0.05       |
| Null (-)     | 16              | 93.36 ± 8.58                            | 85.64 ± 9.22   | <0.01       |
| p for interaction’ | > 0.05 |                                          |                |             |
| GSTT1 genotype |                 |                                          |                |             |
| Positive (+) | 15              | 98.28 ± 7.65                            | 89.27 ± 9.46   | <0.01       |
| Null (-)     | 15              | 90.78 ± 7.58                            | 87.56 ± 12.39  | <0.01       |
| p for interaction’ | > 0.05 |                                          |                |             |
| All donors  | 30              | 94.53 ± 8.40                            | 88.41 ± 10.86  | <0.01       |

*, Test for trend of cell proliferation at different concentrations of B[a]P using repeated measures ANOVA. ’, Test for interaction of trend of cell proliferation at different concentrations of B[a]P by genotype of GSTM1 or GSTT1 using repeated measures ANOVA.

With respect to the GSTM1 genotype, the level of DPC increased in the case of the GSTM1-null genotype without statistical significance (p > 0.05). We did not find a statistically significant interaction between the GSTM1 polymorphism and B[a]P concentrations in the formation of DPC (p > 0.05). When the data were analyzed for the GSTT1 genotype, there was no statistically significant difference of the level of DPC by the GSTT1 polymorphism (p > 0.05). There was no statistical significance of the interaction between the GSTT1 polymorphism and B[a]P concentrations in the formation of DPC (p > 0.05).

When the combinations of polymorphisms of GSTM1 and GSTT1 were analyzed, the highest induction of DPC coefficient was observed with the GSTM1-null/GSTT1-null genotype at 10 μg/mL B[a]P (Fig. 4). At a lower B[a]P concentration of 5 μg/mL, the DPC coefficient was highest with the GSTM1-null/GSTT1-positive combination. However, no significant difference (p > 0.05) was observed among the genotypic combinations at either B[a]P concentration.
Our results indicate that B[a]P, without external metabolic activation, induced a significant dose-dependent increase in both cytotoxicity and DPC formation in cultured human lymphocytes (p<0.01). However, both GST M1 and GST T1 polymorphisms did not play significant roles in the modification of B[a]P-induced genetic damage. We could not observe any significant interactions of B[a]P concentrations and the GST M1 or the GST T1 for the effect of cytotoxicity and DPC formation.

Human lymphocytes used in this study were obtained from healthy, non-smoking hospital administrative workers who had not been exposed to PAH or other DNA damaging agents by their occupations. Therefore, the experiments were relatively well-controlled in terms of the subject environment for genotoxicity assay. In vitro lymphocyte culture has a recognized validity as an assay tool because the blood lymphocytes represent an integrated DNA adduct burden from exposure to genotoxins via diverse routes, such as ingestion, inhalation, and absorption (23). In addition, human lymphocytes are known to directly metabolize a variety of chemicals, which induce genotoxic responses (3).

Genetic polymorphisms in enzymes involved in carcinogen metabolism influence cancer susceptibility, and glutathione S-transferases are involved in the detoxification of metabolic electrophilic compounds by binding to them directly or catalyzing their conjugation (24). GST M1 and GST T1 polymorphisms are associated with the metabolism of PAH, which is a recognized potential carcinogen (25-27). Deletion of the GST M1 gene has also been shown to be associated with an increase in the level of DNA-adduct (25) or sister chromatid exchange (26). It has also been associated with various cancers, including lung cancer (27), malignant mesothelioma (28), and bladder cancer (29) in epidemiologic studies. Although GST M1 is involved in the detoxification of a wide range of electrophilic compounds, such as active metabolites of PAH, our study did not show statistically significant modification of DPC formation after benzo[a]pyrene treatment by the GST M1 polymorphism.

It is not known whether the GST T1 deletion can modify the risks associated with exposure to toxic and carcinogenic chemicals. The GST T1 deficiency has been shown to potentiate the induction of chromosomal damage, and the carriers of the null genotype may be at increased cancer risk (30). In contrast, it was also reported that the GST T1 deficiency could be protective under some circumstances (31). Our study shows that there is no significant difference between the GST T1 genotypes in terms of cytotoxicity and DPC formation. The GST T1 polymorphism did not change the effect of benzo[a]pyrene on the cytotoxicity and DPC formation, either. This negative result may be partly due to either the relatively minor role GST T1 plays in the detoxification of B[a]P or to the low expression of GST T1 in human lymphocytes (32).

The coordinated activities of GST M1 and GST T1 may further modulate the susceptibility to cancer. When both GST M1 and GST T1 genes were investigated for a possible association with lung cancer, Kelsey et al. found this gene interaction carried the risk for lung cancer (13). Our study also showed that the genotoxicity of B[a]P was the highest in the absence of both GST M1 and GST T1 genes. However, this association was not statistically significant and the small number of blood donors in our study limited the interpretation of the interactive effects of the genetic polymorphisms.

In this study, we evaluated the genotoxicity by DPC formation, since many established or suspected carcinogens, such as ionizing radiation, ultraviolet, and formaldehyde, and nickel and chromium compounds are known to induce covalent DPC (17-19, 33, 34). It is a significant DNA defect that impairs the gene expression and chromatin structure, and can also lead to a deletion of DNA sequences during DNA replication because of the high probability of resisting repair (35). In contrast to a previous result, which did not show the DPC formation after B[a]P treatment by alkaline elution analysis (36), our results showed a significant DPC formation by B[a]P.

Our present study did not find the genetic modification effect of the GST M1 and GST T1 polymorphisms in the DPC formation. However, the DPC formation, which represents the DNA damage, was found to be increased in the case of the GST M1-null and GST T1-null genotypes at a high B[a]P concentration. This work suggests a possibility that DPC could be used as a biomarker of benzo[a]pyrene exposure, and in vitro lymphocyte culture assay could be a useful tool for evaluating genotoxic susceptibility to environmental cancers.
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