MOLECULAR & CELLULAR TOXICOLOGY, Vol. 1, No. 1, 32-39, March 2005

Effects of Polycyclic Aromatic Hydrocarbons on DNA Damage and Plasma Protein Expression in Mouse

Sangnam Oh[1], Eunha Oh[1], Hosub Im[2], Gyu-Chan Jo[3], Donggeun Sul[1,3], Young Whan Kim[4] & Eunil Lee[1,2,5]

[1]Medical Science Research Center, Korea University, Anam-dong 5 Ka, Sungbuk-ku, Seoul 136-705, Korea
[2]School of Public Health, Korea University, Anam-dong 5 Ka, Sungbuk-ku, Seoul 136-705, Korea
[3]Graduate School of Medicine, Korea University, Anam-dong 5 Ka, Sungbuk-ku, Seoul 136-705, Korea
[4]Department of Environmental Health, College of Health Sciences, Korea University
[5]Department of Preventive Medicine, College of Medicine, Korea University, Anam-dong 5 Ka, Sungbuk-ku, Seoul 136-705, Korea
Correspondence and requests for materials should be addressed to E. Lee (eunil@korea.ac.kr)

Accepted March 5, 2005

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are an important class of environmentally prevalent xenobiotics that exert complex effects on the biological system and characterized as probably carcinogenic materials. Single cell gel electrophoresis assays were performed in order to evaluate DNA damage occurring in the T-and B lymphocytes, spleens (T/B-cell), bone marrow, and livers of mouse exposed to mixture of PAHs (Benzo(a)pyrene, Benzo(e)pyrene, Fluoranthene, Pyrene) at dose of 400, 800, or 1600 mg/kg body weight for 2 days. DNA damage of the cells purified from mice was increased in dose dependent manner. In the blood cells and organs, DNA damage was also discovered to vary directly with PAHs. Especially T-cells had been damaged more than B-cell. Plasma proteomes were separated by 2-dimensional electrophoresis with pH 4-7 ranges of IPG Dry strips and many proteins showed significant up-and -down expressions with the dose dependent manner. Of these, significant 4 spots were identified using matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometry. Identified proteins were related to energy metabolism and signal transduction.

Keywords: DNA damage, PAHs, Plasma, Proteomics, MALDI-TOF, Single cell gel electrophoresis assays

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants as a consequence of the incomplete combustion of organic materials and can be found in various compartments of the environment: air, surface water, sediment, soil, food and in lipid tissues of both aquatic and terrestrial organisms. Of the 17 PAHs included in natural or artificial source, only three are produced commercially in the United States in quantities greater than research level: acenaphthene, acenaphthylene, and anthracene. The following compounds are not produced commercially in the United States: benzo[a]anthracene, benzo[b]fluoranthene, benzo[e]pyrene, benzo[j]fluoranthene, benzo[k]fluoranthene, benzo[g, h, i]perylene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1, 2, 3-c,d]pyrene, phenanthrene, and pyrene[1]. These materials metabolically activated in cells by cytochrome P450 (CYP450) enzymes and/or peroxidases to reactive intermediates that damage DNA[2]. Several studies have described two major pathways for metabolic activation. One pathway of activation requires the formation of diolepoxides *via* successive epoxidations catalyzed by CYP450 monooxygenases and an intermediate hydrolysis step catalyzed by microsomal epoxide hydrolase. The other pathway involves the formation of radical cation intermediates *via* enzymatically catalyzed one-electron oxidation[3]. Many studies have demonstrated the malignant potential of PAHs, which is correlated with the expression and activity of the aryl hydrocarbon receptor (AhR)[4,5]. The PAHs-inducible AhR-regulated CYP-4501A1 and CYP-4501B1 enzymes are of particular interest since they oxidize PAHs to reactive intermediates capable of forming DNA adducts that include mutation, and the initiation of malignant transformation[6,7]. Many of metabolites bind covalently to cellular molecules, proteins and DNA in tissues, which is related to mechanism of toxicity (inhibiting cell replication) and carcinogenicity (initiation of leukemia)[8]. PAHs are also supposed to act as a mutagen *via* an indirect mechanism, leading to oxidative DNA damage through the formation of hydroxyl radicals *via* hydrogen peroxide[9].

Recently, single cell gel electrophoresis, also known as "the comet assay" has been used extensively to determine the extent and character of DNA damage after low level exposure to toxicants, including strand breaks, alkali-labile sites, DNA crosslink-

Table 1. Up and down Regulated Proteins in Plasma Among the Exposed Mice

Spot areas	PI	MW (kDa)	Protein name	Function
A	5.3	83	Calpastatin	Protease inhibitor/protein metabolism
B	4.8	50	Interleukin receptor-associated kinase	Protein serine/threonine kinase activity Signal transduction/cell communication
D	5.2	38	Ubiqnitin-like 1	Ubiquitin-specific protease activity /Protein metabolism
C	5.2	50	Interleukin receptor-associated kinase	Protein serine/threonine kinase activity Signal transduction/cell communication

ing, and incomplete excision repair sites[10,11]. In animal studies, this assay has been carried out in lymphocytes and multiple organ including liver, lung, spleen, kidney, and bone marrow to determine DNA damage and to characterize mutagens[12-14]. Proteome analysis offered a means of conveniently analyzing differential gene expressions at the protein level by comparing the 2-DE patterns of proteomes under different conditions after exposure to compounds of toxicological relevance. Moreover, a large range of immobilized pH gradient (IPG) strips and more advanced 2-DE analysis have made it possible to identify a number of proteins whose level significantly increased or decreased after treatment with toxic compounds in cells and animals.

The concentrations of individual compounds used in our study were guided by Health Assessment Document for Diesel Emissions [US EPA, 1998] and we mixed with four compounds to make the PAHs and applied to mouse for 2 days but with some modifications.The magnetic cell sorting (MACs) method had been used to isolate the different cell types, i.e. T-lymphocytes, B-lymphocytes. By using the comet assay, DNA damage in each cell type in mouse exposed to PAHs mixture was evaluated as genotoxic effects. Analysis of differential expressed plasma proteins was performed using proteomics tools including 2D-PAGE.

DNA Damage in Blood Cells and Organs

Single cell gel electrophoresis (SCGE, comet assay) has been performed in lymphocytes and multiple organ including spleen, bone marrow, liver to determine DNA damaged by exposure to PAHs.

The results of the comet assay using the mice peripheral T- and B-lymphocytes are shown in Fig. 1A and B The mean value of the Olive tail moment of the control T- and B-lymphocytes was 1.39 ± 0.24 and 1.37 ± 0.26, respectively. In control B-lymphocytes and T-lymphocytes, DNA damages were mea-

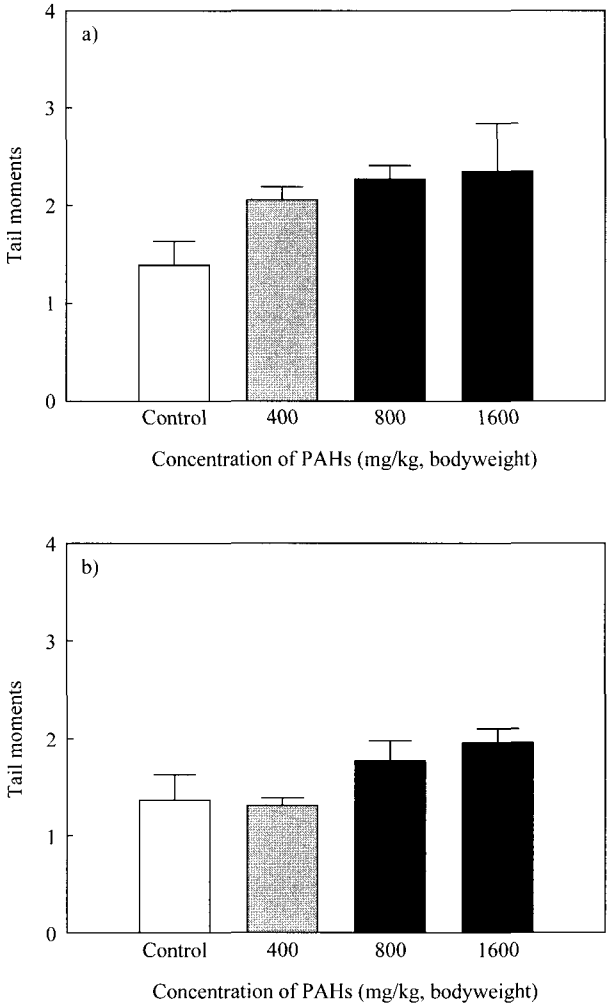

Fig. 1. Diagrams showing the DNA damage in lymphocytes exposed to PAHs. A significant effect was seen between control and all exposures, indicating a higher level of genotoxicity for PAHs. Above the data in comet assay, the mean tail moment was demonstrated elevated DNA damage with increasing concentration. A. T-lymphocytes, B. B-lymphocytes.

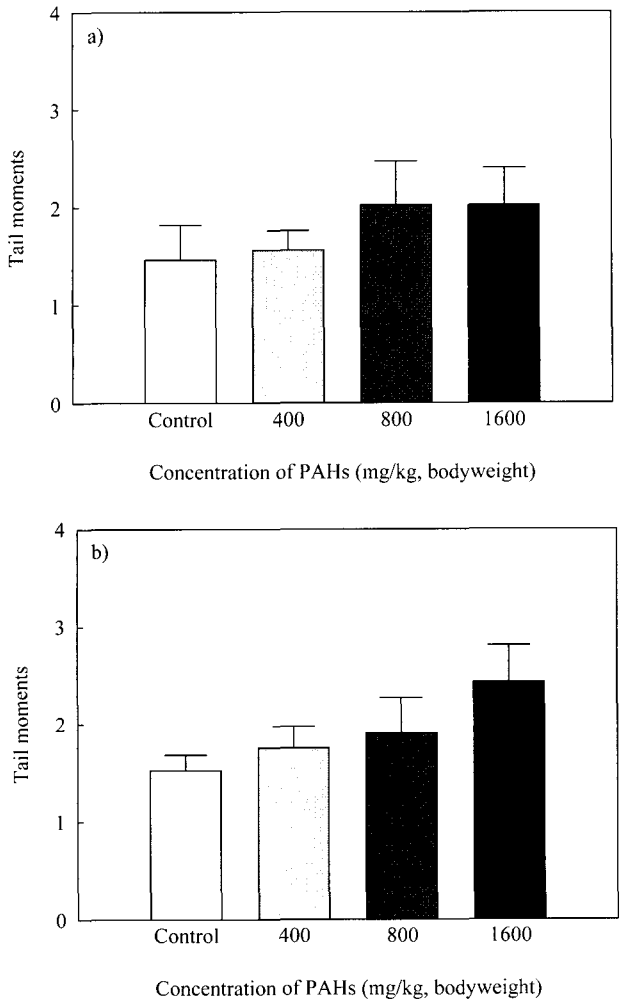

Fig. 2. Diagrams showing the DNA damage in T-cells and B-cell from the spleen exposed to PAHs. A significant effect was seen between control and all exposures, indicating a higher level of genotoxicity for PAHs. Above the data in comet assay, the mean tail moment was demonstrated elevated DNA damage with increasing concentration. A. T-cell (spleen), B. B-cell (spleen).

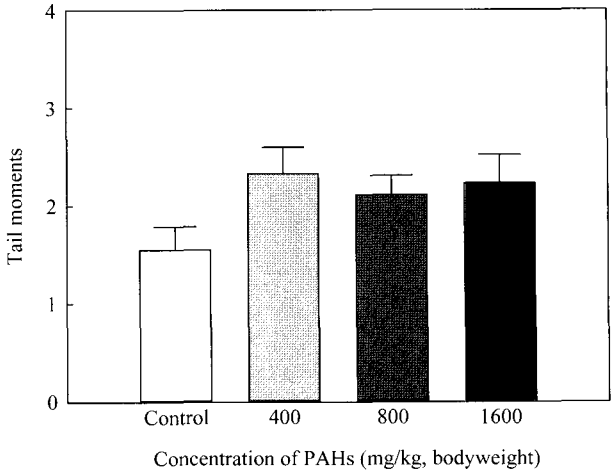

Fig. 3. Diagrams showing the DNA damage in cells from the bone marrow exposed to PAHs. A significant effect was seen between control and all exposures, indicating a higher level of genotoxicity for PAHs. Above the data in comet assay, the mean tail moment was demonstrated elevated DNA damage with increasing concentration.

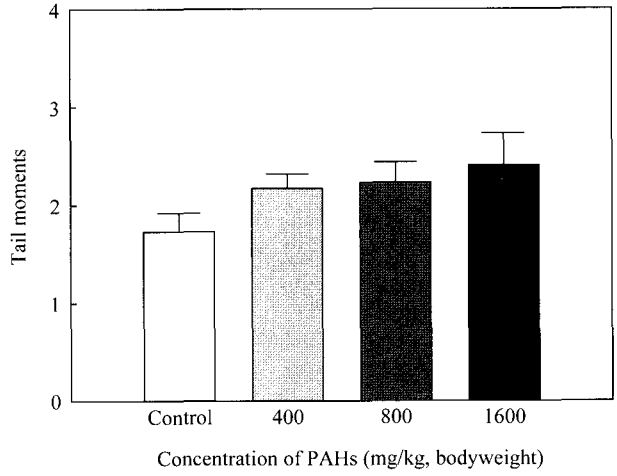

Fig. 4. Diagrams showing the DNA damage in cells from the liver exposed to PAHs. A significant effect was seen between control and all exposures, indicating a higher level of genotoxicity for PAHs. Above the data in comet assay, the mean tail moment was demonstrated elevated DNA damage with increasing concentration.

with no statistically significant differences. After 2 days of PAHs exposure, the mean values of the Olive tail moments of T-lymphocytes and B-lymphocytes of mice exposed to 800 mg/kg PAHs were 2.27 ± 0.14 and 1.77 ± 0.21, respectively. DNA damage of the both lymphocytes in mice exposed to PAHs increased with increasing concentration. Especially, T-lymphocytes had been damaged more than B-lymphocytes against exposure to PAHs, and a significant increase in DNA damage occurred after 2 days at all PAHs exposure levels ($p < 0.05$).

The results of DNA damages in spleen cells are summarized in Fig. 2A (T-cell), B (B-cell) and cells from the bone marrow and liver in Fig. 3 and 4, respectively. A similar trend with regard to DNA damage was also observed in the bone marrow spleens and livers of mice exposed from 0 to 1600 mg/kg PAHs for 2 days. In the T-lymphocytes and bone marrow cells, the plateau in the mean values was

showed, implying that no additional DNA damage occurred with increasing exposure. The statistical analyses of mean ranked data (not shown) revealed significant differences within cell type. With cells from the liver, a significant effect was seen between control and all exposures, indicating a higher level of genotoxicity for PAHs. Above the data in comet assay, the mean tail moment for most cells mixture demonstrated elevated DNA damage with increasing concentration.

Proteomic Analysis

Analysis of differential expressed proteins was carried out using 2D-PAGE. Four concentrations of PAHs, 0 (control), 400, 800, or 1600 mg/kg were used to identify the biological markers of PAHs in the mice plasma. Figure 2 shows the 2-DE pattern of the plasma exposed to PAHs using pI 4-7 range and total 560 spots were detected in the gel. Many spots were found to be down regulated in the exposed mouse plasma. The down regulated spots with 300 percentages were identified by MALDI-TOF/MS. Four protein spots, calpastatin, interleukin receptor-associated kinase, ubiqnitin-like 1, interleukin receptor-associated kinase, were identified, including the proteins that were involved in signaling and energy metabolism mainly.

PAHs are environmentally prevalent xenobiotics that exert complex effects on the biological system and characterized as probably potent tumor initiators and mutate the cellular DNA. It has been reported that PAHs exposure causes DNA single-strand breakage[12,15] and their metabolites bind covalently to cellular molecules, proteins and DNA in several tissues. The binding of PAHs metabolites to DNA provides a potential mechanism by which cell replication can be inhibited, or leukemia can be initiated[8]. However, nucleophilic xenobiotics such as phenol and hydroquinones are also prone to lose an electron, thereby forming free radicals in peroxidase-catalyzed reactions. These radicals attack the sugars, purines and pyrimidines in DNA, resulting in strand breakage[22].

The concentrations of individual compounds used in our study were guided by Health Assessment Document for Diesel Emissions [US EPA, 1998] and we mixed with four compounds to make the PAHs and applied to mouse for 2 days but with some modifications. In this experiment, we evaluated the effects of PAHs with regard to DNA damage in various cells and organs, including lymphocytes, bone marrow, the spleen, and the liver. In the case of the blood cells, we used the magnetic cell sorting (MACs) method to separate the different cell types, i.e. T-lymphocytes and B-lymphocytes and assessed the differences in DNA damage for each cell type in mice exposed to PAHs. This study suggests that the toxicities capable of causing DNA damage can cause changes in protein expression in mice exposed to PAH concentrations containing B(a)P as the major toxic components. DNA damage was evaluated by the alkaline version of the comet assay in various cell type exposed to 0 (control), 400, 800, or 1600 mg/kg of PAHs for 2 days (fig. 1-4). Increasing concentrations had caused significant genotoxicity with some differences (eg. the plateau patterns of damaged cell were various to each other slightly). This study reports very significant genotoxic effects occurring in the blood cells and all organs after 2 days of exposure. Especially, T-

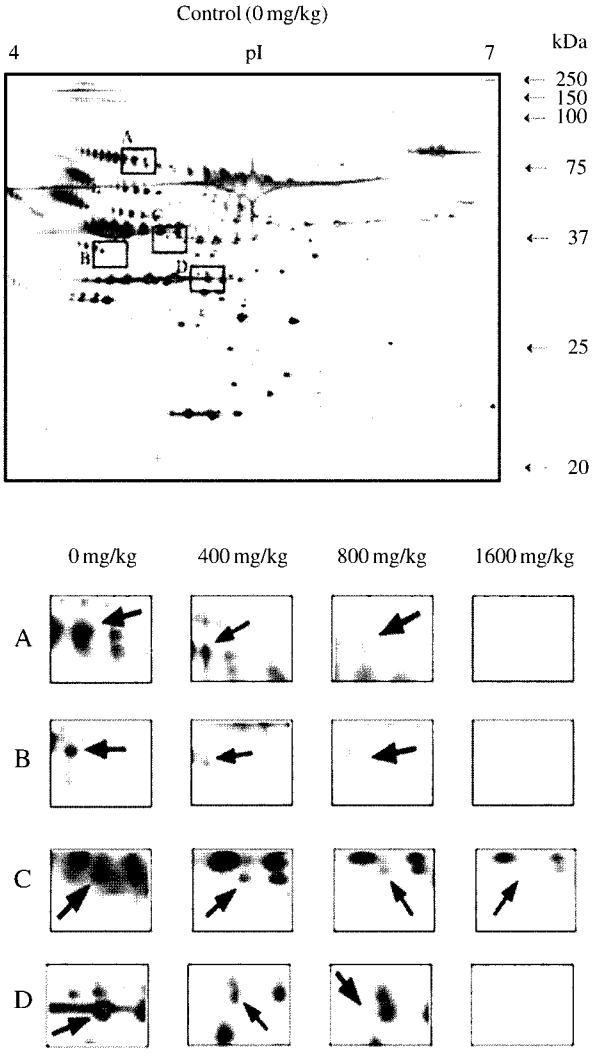

Fig. 5. The 2-DE pattern of the plasma in mice exposed to PAHs using pI 4-7 range. The down regulated each spot with 300 percentages was shown to small squares (A, B, C, and D) with increasing concentrations.

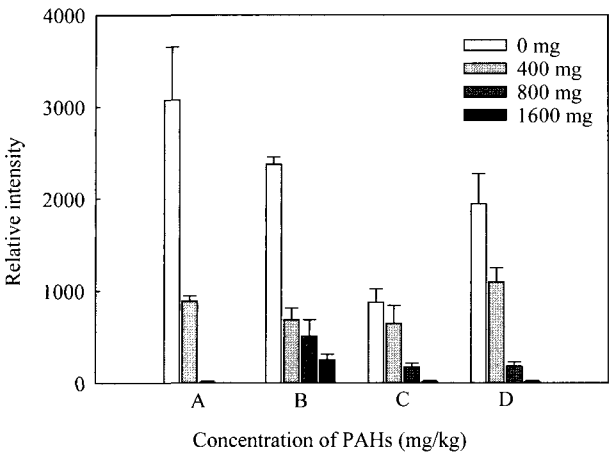

Fig. 6. Relative intensity of changed plasma proteins in mice exposed to PAHs. The spot volumes were analyzed by total spot normalization and each spot quantity. Each bar represents the mean of relative intensity of each spot.

lymphocytes were more sensitive to exposure of PAHs than B-lymphocytes and immuno-toxicological effects might be differing to each other. But the detail mechanism on this difference was unknown yet.

2D-PAGE allowed the analysis of a number of proteins in plasma whose level was significantly changed after the exposure of PAHs compared with the oil control. Many of spots were found to be down regulated in the exposed mouse plasma with significance and relative to the immunosuppressive effects of PAHs that impair the body's natural defense mechanisms have been documented in mammals, and PAHs have been shown to alter the immune system of mammals[9]. In mice, B(a)P suppresses antibody production to both T-dependent and T-independent antigens[24], which appear to exert their immunosuppressive activity by altering the B-cell maturation or function and suppress the cytotoxic T-cell activity. One of the mechanisms for the immunosuppressive effects of B(a)P might be the induction of aryl hydrocarbon hydroxylase[25], which would lead to generation of the reactive diol epoxides by inducing the CYP450 isoenzymes[26]. Since the proteins including serine/threonine kinase activity, signal transduction, and cell communication were obviously down-regulated, physiological and biological processes might be slowed or arrested, then cells started to die so called apoptosis. By Down-regulating protease inhibitors, enhanced protease activity induced proteins not to carry out proper function, rather than to degrade fragments.

Through the comet assay and proteomics tool including 2D-PAGE, biological effects in mouse treated with PAHs mixture could be estimated and compared with untreated mouse in the level of DNA and protein.

Methods

Animals

Specific pathogen free male, Sprague-Dawley rats were obtained from the Samtaco Animal Breeding Company (Osan, Korea) and housed under standard laboratory conditions (Tm; 24±2°C, humidity; 50± 10% and 12-hour day and night cycles). Animals were allowed to acclimatize to the facility for 1-2 weeks, and were then observed for abnormal behavior. They were given free access to a standard chow diet and drinking water, and were 6-8 weeks old on the first day of exposure.

Experimental Design

We used mixture of four PAHs (Benzo(a)pyrene, Benzo(e)pyrene, Fluoranthene, Pyrene) exposure groups: dose of 400, 800, or 1600 mg/kg and an unexposed control for period of 2 days. 4 mice were inoculated with PAHs mixture in each experiment (Total: 16 mice). Mice were finally sacrificed and bloods and tissues were collected in order to determine the extent of DNA damage. Plasma in each sample had been storage at −70°C until sample preparation for proteomic analysis was performed.

Blood Cell Preparation

Blood samples, comprising 1-2 ml of heparinized whole blood, were collected by cardiac puncture from each mouse, and comet assays were carried out within 3 hours. T-lymphocytes and B-lymphocytes were positively selected with magnetic beads (Magnetic cell sorting (MACS CD4 or CD45R isolation kit; Miltenyi Biotec) according to the manufacturer's instructions. Unfractionated leukocytes were prepared by removal of red blood cells from whole blood, *via* centrifugation with Ficoll-Paque solution. Leukocytes were incubated with each type of monoclonal antibody (20 μl of MACS microbeads per 10^7 total cells) for 15 minutes at 6-12°C. After washing cells by adding 10-20X the labeling volume of PBS buffer, the cells were centrifuged at 300 xg for 10 minutes. The supernatant was then removed completely and resuspended in an appropriate amount of buffer (500 μl of buffer per 10^8 total cells). The supernatant was again removed completely, and the cells were applied onto a prepared MS column (Miltenyi Biotec), which was placed in the magnetic field of a MACS separator and washed with 500 μl buffer. After the elimina-

tion of negative cells, the column was removed from the MACS separator, placed on a suitable collection tube, and each type of cells was collected and washed with PBS buffer, in preparation for the comet assay.

Tissue Sample Preparation

The bone marrow, spleen, and liver tissue samples were prepared for comet assay according to the methods described by Farris, with minor modifications[15]. Femurs were removed and marrow was flushed with cold RPMI 1640 medium containing 100 IU/ml penicillin, 100 UI/ml streptomycin, and 10 % Fetal bovine serum using a syringe fitted with a 23-gauge needle. Spleens and livers were minced with a scalpel blade, and the tissue was mashed with a syringe plunger. The cell suspensions of marrow, spleen, and liver were filtered through a 25-μm metal mesh. The filtered suspension was moved in 15 ml conical tubes, allowed to stand for five minutes and produced 2-3 ml of upper single cell suspension. The collected bone, spleen, and liver cells were washed with PBS buffer in preparation for the comet assay.

Comet Assay (SCGE, Single cell gel electrophoresis)

Single cell gel (SCG) electrophoresis or 'Comet assay' is a rapid and very sensitive fluorescent microscopic method to examine DNA damage and repair at individual cell level. Migration length is generally believed to be related directly to fragment size and would be expected to be proportional to the level of single strand break and alkali-labile sites. The comet assay was performed according to Singh with minor modification [Singh, N.P. *et al.*, 1988]. Normal melting point agarose (Amresco, NMA) and low melting point agarose (Amresco, LMA) were dissolved in PBS (Gibco BRL) using microwave. In brief, 100 μl of 1% NMA was added onto a fully frosted slides precoated with 50 μl of 1% NMA for a firm attachment and the slides were allowed to solidify with cover slips in the refrigerator for 5 min. After solidification of the gel, the cover slips were removed and lymphocytes in 50 μl mixed with 50 μl of 1% LMA were added. The cover slips were added on the layer and the slides were allowed to solidify in the refrigerator for 5 min. After removing cover slips, 100 μl of 0.5% LMA was added on the third layer and the slides were placed with cover slips again in the refrigerator for 5 min. The slides were submersed in the lysis solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris-HCl, pH 10; 1% Triton ×-100, pH 10 were added fresh) for 1 h. The slides were then placed in unwinding buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 20 min and electrophoresis was carried out using the same solution for 20 min at 25 V and 300 mA (0.8 V/cm). After electrophoresis, the slides were neutralized by washing three times with neutralization buffer (400 mM Tris-HCl, pH 7.4) for 5 min each and were stained with 50 μl of 10 μg/ml ethidium bromide. The slides were examined using a Komet 4.0 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with an excitation filter of 515-560 nm and a barrier filter 590 nm. For each treatment group, two slides were prepared and each 50 randomly chosen cells (total 100 cells) were scored manually. The parameter of Olive tail moment (% DNA x distance of center of gravity of DNA) was calculated automatically using the Komet 4.0 image analysis system.

Two-dimensional Gel Electrophoresis (2DE)

Sample preparation

The sample preparation of plasma for 2DE was performed as previously described[16]. Lipids and salts were removed from the sample using molecular cut off column (3 kDa; Amicon, Millipore, Bradford, MA). For this step, plasma and sample buffer containing of 7 M urea, 2 M thiourea, 40 mM Tris (0.5 M, pH 8.5), 4% CHAPS, 65 mM DTT, 1% IPG buffer (pH 4-7 L) and 1% protease inhibitors were mixed with equal volume in the column. After centrifugation, at 3500 rpm 12°C for 4 times of 1 h each the supernatant was aliquoted and stored at −70°C. The concentration was measured by modified Bradford assay method[17].

First Dimension-Isoelectric Focusing (IEF)

Immobiline Dry Strips (4-7 L, 24 cm) were used for IEF. For the comparison of running kits (vertical SDS -PAGE running systems), prepared protein sample solution (50 μg) was mixed with rehydration buffer (containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, 65 mM DTT, a trace of bromophenol blue) up to 450 μl at total volume per one sample. The IPG strips were rehydrated in the presence of the sample for 12 h and focused using the IPGphor™ IEF system (Amesham Pharmacia Biotech, UK) for 81.5 kVh. After the first dimension run, the IPG gel strips were equilibrated twice for 15 min, under gentle shaking at room temperature, first in solutions (equilibration buffer: 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% DTT and, next in equilibration buffer containing 2.5% iodoacetamide. After equilibration, the proteins were separated in the second dimension (SDS-PAGE) on 9-16% gradient polyacrylamide gels made by Ettan Dalt gradient

maker (Amesham Pharmacia Biotech, UK). Running conditions were 1 w/gel for 30 min and 10 w/gel for 8 h at 10°C until the tracking dye reached the end of the gel.

Visualization

Proteins were visualized for image analysis using the silver staining method performed according to Blum *et al.* with some modifications[18]. Briefly, the gels were fixed in 50% methanol and 12% acetic acid, followed by washing three times for 20 min in 50% ethanol. The gels were then sensitized by incubating in 0.02% sodium thiosulfate followed by washing three times for 20 sec in double distilled water. The gels were immersed in 0.1% silver nitrate for 20 min, then rinsed two times for 20 sec in double distilled water. The development stage was carried out in 6% sodium carbonate and 0.05% formaldehyde (37%). Finally, the reaction was terminated with 50 % methanol and 12 % acetic acid.

Image Analysis

The silver-stained gels were scanned using a 800 × 1600 dpi instrument (UMAX, UTA 2100XL) and the image files were transformed into TIF files with linear gray scale values. The computer analysis of 2D-image was carried out using the ImageMaster 2D Elite Software (Version 4.01) (Amersham Pharmacia Biotech, Amersham, UK)

MALDI-TOF/MS Analysis and Protein Identification

Each spot was in-gel digested with some modifications[19,20]. The gel spots were excised with a scalpel, crushed, and destained by washing with 25 mM ammonium bicarbonate, 50% acetonitrile. The gels were dehydrated by addition of acetonitrile, rehydrated in ice by adding 10-20 μl of 25 mM ammonium bicarbonate with 10 μg/ml of sequencing grade trypsin (Promega), and incubated at 37°C for 12-15 h. Peptides were extracted by adding 25 μl of solution containing 50% acetonitrile, 0.1% trifluoroacetic acid. The extraction was repeated three times and completed by adding 20 μl of acetonitrile. The extracted solutions were pooled and evaporated to dryness in a Speed Vac centrifuge. Samples were reconstituted in 10 μl of 0.1% trifluoroacetic acid and treated with ZipTips™ containing C18 resin (Millipore Co. Bedford, MA, USA) according to the manufacturer's instructions. The washed peptides were eluted with saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 0.1% trifluoroacetic acid). Monoisotopic masses (M+1) of tryptic fragments were measured in a Perspective Biosystem MALDI-TOF-MS voyager DE-STR Mass Spectrometer (Framingham, MA, USA). The spectra obtained were internally calibrated by trypsin peaks. Proteins were identified by searches of a homo sapience subset of NCBInr (NCBInr.10.21.2003) database using the MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit) in order to identify the proteins. Known keratin masses and trypsin autodigest products were excluded from the searches. The parameters were set as one of missed cleavage and acrylamide modification. Protein identities were assigned if at least five peptides masses were matching within a maximum of 50 ppm error and the candidate agreed with the estimated pI and Mw from the 2D gel.

Statistical Analysis

Statistic analyses were performed using the SAS version 6.12 software, and we used the analysis of variance (ANOVA) method to determine the differences in olive tail moments between exposure and control groups.

Acknowledgements

This work was supported by the Ministry of Environment and the Medical Research Center for Environmental Toxico-Genomics & Proteomics of Korea University

References

1. The International Agency for Research on Cancer (IARC), IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans; polynuclear aromatic compounds, part 1, chemical, environmental and experimental data, **Vol 32** (1986).
2. Melendez-Colon, V.J., Luch, A., Seidel, A. & Baird, W.M. Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites. *Carcinogen.* **20**, 1885 -1891 (1999).
3. Melendez-Colon, V.J., Luch, A., Seidel, A. & Baird, W.M. Formation of stable DNA adducts and apurinic sites upon metabolic activation of bay and fjord region polycyclic aromatic hydrocarbons in human cell cultures. *Chem. Res. Toxicol.* **13**, 10-17 (2000).
4. Rigdon, R. & Neal, J. Relationship of leukemia to lung and stomach tumors in mice fed benzo(a) pyrene. *Proc. Soc. Exp. Biol.* **130**, 146-148 (1969).
5. Mann, K.K., Matulka, R.A., Hahn, M.E., Trombino, A.F., Lawrence, B.P., Kerkvliet, N.I. & Sherr, D.H. The role of polycyclic aromatic hydrocarbon metabolism in dimethylbenz[a]anthracene-induced pre-B lymphocyte apoptosis. *Toxicol. Appl. Phamacol.* **161**, 10-22 (1999).

6. Thirman, M., Albrecht, J., Krueger, M., Erickson, R., Cherwitz, D., Park S., Gelboin, H. & Holzman. J. Induction of cytochrome CYPIA1 and formation of toxic metabolites of benzo[a]pyrene by rat aorta: a possible role in atherogenesis. *Proc. Natl. Acad. Sci. USA.* **91**, 5397-5401 (1994).
7. Kress, S. & Greenlee, W. Cell-specific regulation of human CYP1A1 and CYP1B1 genes. *Cancer Res.* **57**, 1264-1269 (1997).
8. Andrews, L.S., Snyder, R Amdnr, M.O., Doull, J. & Klaassen, C.D. (Eds.), Toxic effects of solvents and vapors. Toxicology : *The basic science of poisons.* 681-722 (1992).
9. Andreoli, C., Leopardi, P. & Crebelli, R. Detection of DNA damage in human lymphocyte by alkaline single cell gel electrophoresis after exposure to benzene or benzene metabolites. *Mutat. Res.* **377**, 95-104 (1997).
10. Kassie, F., Pazefall, W. & Knasmüler, S. Single cell gel electrophoresis assay : a new technique for human biomonitoring studies. *Mutat. Res.* **463**, 13-31 (2000).
11. Meller, P., Knudsen, L.E. & Wallin, H. The comet assay, a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer Epidemiology, Biomarker & Preventions* **9**, 1005-1015 (2000).
12. Sasaki, E., Nishidate, F. Izumiyama, N. Matsusaka. & S. Tsuda. Simple detection of chemical mutagens by the alkaline single-cell gel electrophoresis (Comet) assay in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow). *Mutat. Res.* **391**, 215-231 (1997).
13. Miyamae, K. Zaizen, K. Ohara, Y. Mine. & Y.F. Sasaki. Detection of DNA lesions induced by chemical mutagens by the single cell gel electrophoresis (Comet) assay. 1. Relationship between the onset of DNA damage and the cgaracteristics of mutagens. *Mutat. Res.* **393**, 99-106 (1997).
14. McNamee, J.R.N. McLean, C.L. Ferrarotto. & P.V. Bellier. Comet assay: rapid processing of multiple samples. *Mutat. Res.* **466**, 63-69 (2000).
15. Farris, G.M., Robinson, S.N., Wong, B.A., Wong, V.A., Hahn, W.P. & Shah, R. Effects of benzene on splenic, thymic and femoral lymphocytes in mice. *Toxicology.* **118**, 137-148 (1997).
16. Möller, A., Soldan, M., Völker, U. & Maser, E. Monitoring daunorubicin-induced alterations in protein expression in pancreas carcinoma cells by two-dimensional gel electrophoresis. *Toxicology.* **160**, 129-138 (2001).
17. Ramagli, L.S. in: Link, A.J. (Ed.) Quantifying Protein in 2-D PAGE Solubilization Buffers in 2-D Proteome Analysis Protocols Humana Totowa, *NJ, USA*, pp. 99-103 (1999).
18. Blum, H., Beier, H. & Gross, H.J. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis.* **8**, 93-99 (1987).
19. Sechi, S. & Chait, B.T. Modification of Cysteine Residues by Alkylation. A Tool in Peptide Mapping and Protein Identification. *Anal. Chem.* **70**, 5150-5158 (1998).
20. Quadroni, M. & James, P. Proteomics and automation. *Electrophoresis.* **20**, 664-677 (1999).
21. Wu, C-H., Hsieh, C-L., Song, T-Y. & Yen, G-C. Inhibitory effects of Cassia tora L. on benzo[a]pyrene-mediated DNA damage toward HepG2 cells. *J. Agric. Food Chem*, **49**, 2579-2586 (2001).
22. Sul, D., Oh, E., Im, H., Yang. M. & Lee, E. DNA damage in T- and B-lymphocytes and granulocytes in emission inspection and incineration workers exposed to polycyclic aromatic hydrocarbons. *Mutat. Res.* **538**, 109-19 (2003).
23. Halliwell, B. & Gutteridge, M.C. Free radicals in biology and medicine: B. Halliwell, M.C. Gutteridge (Ed.), Oxford Science publications (1999).
24. Marczynski, H.P. Rihs, B. Rossbach, J. Holzer, J. Angerer, M. Scherenberg, G. Hoffmann, T. Bruning & M. Wilhelm, Analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine and DNA strand breaks in white blood cells of occupationally exposed workers: comparison with ambient monitoring, urinary metabolites and enzyme polymorphisms. *Carcinogenesis.* **23**, 273-281 (2002).
25. Binkova, J. *et al.* Biomarker studies in northern Bohemia. *Environ. Health Perspect.* **104**, 591-597 (1996).
26. Sram, K. Podrazilova, J. Dejmek, G. Mrackova & T. Pilcik. Single cell gel electrophoresis assay: sensitivity of peripheral white blood cells in human population studies. *Mutagenesis.* **13**, 99-103 (1998).