BIOLOGICAL EFFECTS OF SIX CHLORINATED POLYCYCLIC AROMATIC HYDROCARBONS

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Introduction

Many kinds of polycyclic aromatic hydrocarbons (PAHs) are discharged from various emission source in air environment and in aqueous environment[1]. PAHs discharged in the atmosphere are flowed into aqueous environment through natural falling or rain water[2,3]. It seems PAHs are received the changes (oxidation, reduction, decomposition, substitution, conjugation etc.) by chemical reaction in the aqueous environment, photoreaction, metabolism of the aquatic organism and various treatment processes. Especially, in the case of raw water for drinking water source, these compounds are exposed chlorine in the process for the disinfection in drinking water treatment plant and then are reacted oxidation and/or chlorine displacement. Among disinfection by products, there are many compounds causing the adverse effect to the human[4]. It is guessed that PAHs are also similarly changed to various reaction products by chlorination in the process for the disinfection. Additionally, it is afraid the reaction products affect on human health by drinking[5,6]. However, there is not much information on the reaction products of PAHs by chlorination in drinking water treatment plant.
The chlorinated forms of PAHs were identified by gas chromatography with mass spectrometric detector in selected ion monitoring (SIM) mode after the extraction using solid phase cartridge from water sample.
We have examined the cytotoxicity using cultured cells, the mutagenicity by umu test and effect on the process of differentiation to the primitive heart cells or the nervous cells by the expression level of the specific genes.

Materials and Methods

Chemicals

Fluoranthene (FL),benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a] pyrene (B[a]P), benzo[ghi]perylene (B[ghi]P), indeno[1,2,3-cd]pyrene (IP), dichloromethane and acetonitrile (pesticide residue PCB analysis grade), methanol (HPLC grade), sodium hypochlorite solution, and L(+)-ascorbic acid sodium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Laboratory water was purified by a Milli-Q gradient A10 Elix system with an EDS polisher (Millipore, Bedford, MA, USA). Individual stock solutions were prepared to 1000 mg/l in acetonitrile. All stock solutions were stored at -20˚C, whereas working solutions were prepared fresh for each use by dilution of the standard stock solution.

Gas Chromatography With Mass Spectrometric Detection (GC/MS)

GC was carried out using an HP6890 Series Gas Chromatograph system (Hewlett Packard, Wilmington, DE, USA) with an HP6890 Series auto-sampler and split/splitless injector. The analytical column was a DB-5 fused silica capillary column, 30 m × 0.25 mm ID, 0.25 mm film thickness (J&W Scientific, Folsom, CA, USA). The oven temperature program was as follows: 100˚C initial temperature for 1 min, ramped at 10˚C/min to 220˚C, at 3˚C/min to 280˚C, holding for 5 or 10 min at 280˚C. The carrier gas (helium) flow was set to 1.2 mL/min. Pulsed splitless injection of a 2 μL volume was carried out at 250˚C. The MS was carried out using a 5973 Mass Selective Detector (Hewlett Packard, Wilmington, DE, USA) in electron ionization mode with an ionization voltage of 70 V and ion source temperature of 280˚C. The instrument was operated in selected ion monitoring (SIM) mode. Two selected ions for each compound were monitored for identification by following mass number (*m/z*); FL:202, mono-chlorinated FL:236, di-chlorinated FL:270, B[b]F;252, mono-chlorinated B[b]F;286, di-chlorinated B[b]F;320, B[k]F;252, mono-chlorinated B[k]F;286, di-chlorinated B[k]F;320, B[a]P;252, mono-chlorinated B[a]P;286, di-chlorinated B[a]P;320, B[ghi]P;276, mono-chlorinated B[ghi]P;310, di-chlorinated B[ghi]P;344, B[ghi]P;276, IP;276, mono-chlorinated IP;310, di-chlorinated IP;344.

Analytical Procedures

The working solutions were prepared from the stock solution with acetonitrile before use. The 6mg/L working solution was dropped into purified water with 10mM phosphate buffer (pH 7.0) as mimic tap water. The final

concentration of PAH was 3μg/L and that of acetonitrile was 0.05% (v/v). After stirring 10 min at room temperature, five hundreds mL was divided as the original water sample, the reaction time at 0 hour. Then, the sodium hypochlorite solution was added to the solution so that the concentration of free chlorine became 1mg/L. With stirring at 20°C, five hundreds mL of the solution was taken at the reaction time of 1, 2, 4, 6 and 24 hours, respectively. One mL of 10g/L sodium ascorbic acid solution was added into five hundreds mL of the sample water solution in order to remove chlorine. The sample water adjusted to pH3.5 by 9% (v/v) nitric acid.

The fortified water samples were concentrated according to the Solid Phase Extraction method as described below with Oasis HLB Plus Extraction Cartridge (Waters, Milford, MA, USA) prepacked with *N*-vinyl-pyrrolidone polymer resin[7]. The cartridges were equilibrated with 5 mL dichloromethane, 5 mL methanol and 5 mL water, respectively. Extraction of water samples was carried out at a flow rate of approximately 10 mL/min. Air was then passed though the cartridges for 5 min, and nitrogen gas for 10 min. The analytes were eluted from the cartridges with 5 mL dichloromethane. After evaporating the samples to less than 1 mL under a gentle nitrogen steam, the eluate was adjusted to a final volume of 1.0 mL with dichloromethane for GC/MS analysis. The automatic concentrator used was a Sep-Pak Concentrator Plus (Waters, Milford, MA, USA).

Evaluation of Cytotoxicity

HepG2 cells were cultured using the Essential modified Eagle's medium containing 10% (v/v) fetal calf serum, 1/100 volume of the 100-fold concentration non-essential amino acid solution, 1mM sodium pyruvate. Two hundred cells were seeded in the 96-well plate using the Essential modified Eagle's medium containing 0.5% (v/v) fetal calf serum, 1/100 volume of the 100-fold concentration non-essential amino acid solution, 1mM sodium pyruvate and with the each chlorinated PAHs extract. After one or two days, the viability was measured by Cell-Titer-Glo Luciferase Cell Viability Assay kit (Promega Corp.) or by lactose-dehydrogenase activity assay (Wako Chem. Corp.; Osaka, Japan) following the manufactural manual. In the case of mouse ES cells, the cytotoxicity was evaluated under the same condition aforesaid. The growth medium for maintenance of the mouse ES cells was used Dulbecco's Modified Eagle's Medium containing high concentration of glucose, added 20% (v/v) Fetal calf serum, 1/100 volume of the 100-fold concentration non-essential amino acid solution, 1mM sodium pyruvate, 0.1mM β-mercaptoethanol and 10^3 units of leukemia inhibitory factor (LIF). The serum was heated at 55 °C for 30min before using. The primary mouse fibroblast cells was cultured with Dulbecco's Modified Eagle's Medium containing high concentration of glucose, added 10% (v/v) fetal calf serum, 1/100 volume of the 100-fold concentration non-essential amino acid solution, 1mM sodium pyruvate and 0.1mM β-mercaptoethanol. The mouse ES cells were cultured on the feeder cells that were seeded on the tissue culture's plastic dish covered gelatin. The feeder cells were seeded 4-8 x 10^4 cells/cm^2. The growth medium was changed every day and the mouse ES cells were transferred to the new dish attached feeder cells every three days. The dish covered gelatin was prepared by following procedures. The 0.1% gelatin solution was added into the tissue culture's plastic dish and discarded after keeping for 2 hrs in the CO_2 incubator at least and then the dish was washed twice with PBS. The feeder cells were prepared from the primary mouse fibroblast cells by treatment with 10μg/mL mytomicin C for 2hrs at 37 °C for 2 hrs in the CO_2 incubator.

Evaluation of Mutagenicity

The mutagenicity was evaluated by umu test system using the chlorinated PAHs extract using the ume test kit (UMURACK-AT; JIMRO, Tokyo, Japan).

Effect on The Process of Differentiation by The Expression Level of The Specific Genes

We established the differentiation conversion system to transfer from embryonic stem cells to the specific organs' preliminary cells. A single cell suspension of 3.75 x 10^4 cells/mL with the growth medium was made from the monolayer-cultured mouse ES cells and seeded 20 μL on the cover of bacterial plastic dish and then incubated to make embryoid body for three days in the CO_2 incubator upside down for three days. Then, embryoid body was transferred into the bacterial 96 well plate with 200 μL of the Dulbecco's Modified Eagle's Medium containing high concentration of glucose, 1/100 volume of the 100-fold concentration non-essential amino acid solution, 1mM sodium pyruvate, 0.1mM β-mercaptoethanol and 10% fetal calf serum in the CO_2 incubator. After two days, the embryoid body was transferred into the non-coated tissue culture dish. A part of cells of embryoid body was differentiated to heart cells.

Results and Discussion

Condition of Chlorination

If PAHs remain in the treated water without removing under the water treatment process, PAHs might react with

chlorine at 1mg/L free chlorine or less. The final concentration of PAHs in the experimental water was decided considering the solubility at 3.0μg/L. In this condition, non-chlorinated PAHs dissolved in water were recovered from 80% to 120% by SPE in Materials and Methods. The concentration of free chlorine was decreased with time-dependent and reached about 0.8mg/L after 24 hours in all case of six kinds of PAH under this condition. This means that the quantity of chlorine for the reaction had sufficiently remained after 24 hours and that the reaction had almost perfectly advanced.

Identification of Chlorinated PAHs

The GC/MS system in selected ion monitoring mode was used for the analysis of PAHs and chlorinated PAHs. PAHs decreased with time-dependent in the case of all six kinds of PAH. FL, B[b]F and B[ghi]P were slowly decomposed in comparison with B[k]F, B[a]P and IP. The time in which the initial concentration decreased to the half was 0.6 hours in B[a]P, 1.5 hours in B[k]F and IP, 3.2 hours in B[b]F, 3.8 hours in B[ghi]P and 4.1 hours in FL. However, FL, B[ghi]P and IP were detected the original compounds after 24 hours. From these results, the reactivity for the chlorine was sensitive in order of B[a]P>B[k]F>B[b]F=IP>B[ghi]P>FL. If the concentration of the chlorine in reaction solution might be higher, the original compounds decompose more quickly. The mole ratio of the target compound and free chlorine concentration may be an important element. Mono-chlorinated PAHs were formed in the case of all six kinds of PAH. Di-chlorinated PAHs were detected in the case of B[k]F and B[a]P. However, Tri-chlorinated forms were not detected in this condition.

Cytotoxicity of Chlorinated PAHs

The extracted compounds from chlorine solution dissolved PAHs, crude chlorinated PAHs, were exposed in both HepG2 cells and mouse undifferentiated ES cells for 24hrs. Chlorinated B[a]Ps showed the significant cytotoxicity highest in comparison with original compound in mouse undifferentiated ES cells and three chlorinated PAHs showed higher by Cell-Titer-Glo Luciferase Cell Viability Assay kit. However, chlorinated B[k]F and B[ghi]P did not show the significant cytotoxicity higher in comparison with the original compounds (Fig.1). In the case of B[a]P, the concentration that did not show the lethal effect was 0.2mg/L or less (Fig.2).

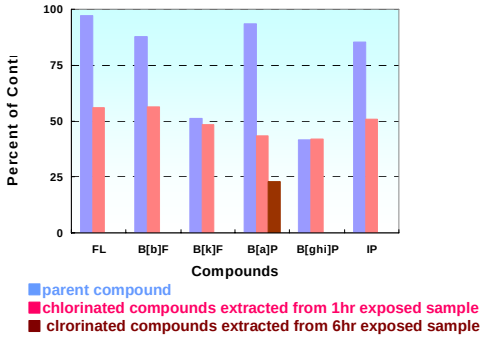

Fig.1 Cytotoxicity of PAHs and Chlorinated PAHs in Mouse Undifferntiated Cells.

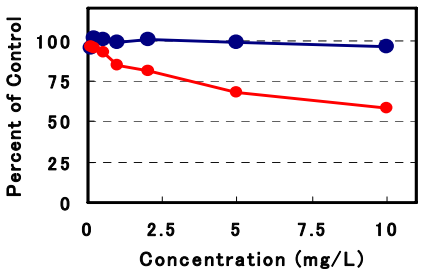

Fig. 2 Cytotoxicity of B[a]P and chlorinated B[a]P by LDH assay
●; mono-chlorinated B[a]P
▲; di-chlorinated B[a]P

Table 1 Cytotoxicity of PAHs and Its Chlorinated PAHs in HepG2 Cells

Concentration	FL	FL-Cl	B[b]F	B[b]F-Cl	B[k]F	B[k]F-Cl	B[ghi]P	B[ghi]P-Cl	B[a]P	B[a]P-Cl(1h)	B[a]P-Cl(6h)	IP	IP-Cl
0.05	100	100	100	100	100	100	100	100	100	100	100	100	100
0.1	100	100	100	100	100	100	100	100	100	100	100	100	100
0.25	100	100	100	100	100	100	100	100	100	100	100	100	100
0.5	100	100	100	100	100	100	100	100	100	100	100	100	100
1	100	100	100	100	100	100	100	99.1	100	100	68.3	100	100
2.5	100	100	100	100	100	100	100	99.6	100	100	54.1	100	100
5	100	96.8	100	100	100	95.4	100	95.2	100	51	37.4	100	98.5

In the case of HepG2 cells, chlorinated B[a]Ps showed the significant cytotoxicity higher in comparison with original compound by lactose-dehydrogenase activity assay after exposure of 24hrs (Table 1) and 48hrs (data not shown), especially the chlorinated B[a]P extracted from the 6hr reaction solution. However, the other five

chlorinated PAHs did not show the significant cytotoxicity comparing the original compounds under this condition. From these results, chlorinated PAHs shows the cytotoxicity, but the sensitivity is different with the cell species. Among six PAHs, chlorinated B[a]P causes strongest effect.

Mutagenicity of Chlorinated PAHs

The chlorinated B[a]P showed the mutagenicity at 0.01mg/L concentration without metabolic activation and 0.003mg/L with metabolic activation by the umu test. B[a]P showed the mutagenicity at 0.04mg/L with metabolic activation and did not show at 1mg/L or less without metabolic activation.

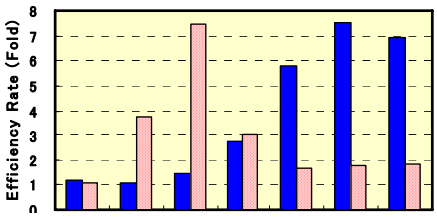

Fig.3 Mutagenicity of Clroinated B[a]P
■without metabolic activation
□with metabolic activation

Effect on the expression level of the specific genes in the process of differentiation

First, we identified the specific marker genes for the differentiated stage, preliminary endoderm cells and primary heart cells. Among several candidate genes, Hepatic Nuclear Factor-4 (HNF-4), transcription factor working at early developmental stage transferring from stem cells to endoderm cells, was selected as specific marker gene. GATA-4 gene was also selected the specific marker gene for primary heart cells. The cassette plasmid, that was able to induce luciferase by the interaction between the regulatory region of luciferase gene and cellular endogenous factors, has constructed (pGL3Neo). The HNF-4 pomoter region (-1837 ～-1) and GATA-4 promoter region (-2000nt～-1) were from transcription initiation site in the upstream of HNF-4 and GATA-4 were isolated by PCR and inserted into the pGL3Neo plasmid at the regulatory position of luciferase gene, respectively . For the confirmation of the functional efficiency of the promoter region, the expression screening systems of the fluorescent protein have constructed. These promoters were inserted pDsREed-Express-1 (Clontech) for the red fluorescent protein and pZsGreen1-1 (Clontech) for green fluorescent protein. The constructed plasmid was transfected into mouse ES cells with TransFast Reagent (Promega) by following the manufactual manual. The transformed cells were selected by Neomycine (G418) and kept to proliferate. After induction of the differentiation, the green and red fluorescence proteins were detected on 7th day, respectively. These results indicated the isolated promoter regions were effective for transcriptional control of the gene located in the downstream. The maximum non-toxicity concentration was examined using undifferentiated ES cells. Cells were exposed with compounds and then ATP concentration was measured after 24 hours as index of living cells. The chlorinated B[a]P was exposed at 0.005mg/L for 12days from the start day of the differentiation process. In this condition, there were no remarkable effects on the expression level of GATA-4 and HNF-4, but the morphological change was observed.

Conclusion

In this paper, we show PAHs react with chlorine and change to chlorinated forms. The chlorination mechanism of PAHs is individually different. Chlorinated PAHs shows the cytotoxicity, but the sensitivity is different with the cell species. Among six PAHs, chlorinated B[a]P causes strongest effect. The chlorinated B[a]P showed the mutagenicity without and with metabolic activation by the umu test. There were no remarkable effects on differentiation process under the established differentiation conversion system.

Acknowledgements

This study was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology-Japan and the Ministry of Health, Labour and Welfare of Japan.

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