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Benzo[a]pyrene-induced Modification on p53 and Related Proteins

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벤조피렌에 의한 p53 및 관련 단백질 변화

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요 약

PAH 위해성 평가의 생체지표 개발을 위하여, benzo[a]pyrene을 인체 간암 세포주인 HepG2 세포에 처리 하여 암 억제 단백질인 p53 및 관련 단백질의 발현 양상에 대하여 연구하였다. HepG2 세포의 생존력은 benzo[a]pyrene을 노출 시킨 군에서 농도가 증가 할수록 감소하였다. p53과 인산화 p53의 발현 양상은 benzo[a]pyrene 농도 의존적으로 증가하는 경향을 보였으며, 반면에 아세틸화 p53은 benzo[a]pyrene의 농도가 증가할수록 감소하는 경향을 나타내었다. 세포 주기 조절에 관련된 p21 단백질은 화학 물질 처리에 의해서 p53과 마찬 가지로 증가하였으나, CdK4와 Rb 단백질의 발현에는 변화가 없었다. 상관분석 결과 Benzo[a]pyrene 노출, 세포 생존력, p53, 인산화 p53, p21이 서로 높은 상관성을 보였다. 본 연구의 결과는 p53 단백질의 축적이 benzo[a]pyrene 독성에 있어 매우 중요한 현상이며, 이는 선택적인 지표와 함께 p53이 benzo[a]pyrene과 같은 PAH계열의 물질의 위해성 평가를 위한 민감한 생체 지표로써 개발될 수 있음을 시사한다.

Key words : benzo[a]pyrene, p53, p21, biomarker, risk assessment

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH), of which Benzo[a]pyrene (BaP) is the most commonly studied and measured, are ubiquitous environmental agents and they commonly believed to significantly contribute to human cancers. BaP is not manufactured and has no industrial use. It is ubiquitously distributed throughout the environment as a consequence of its formation during the combustion of organic matter. Carcinogenic and mutagenic effects of BaP have been well investigated in animals and other mammalian cell systems (Harvey, 1991; Pei *et al.*, 1998). PAHs are themselves chemically inert and hydrophobic. However, they undergo metabolic activation in mammalian cells to diol-epoxides that bind

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covalently to cellular macromolecules, including DNA, thereby causing errors in DNA replication and mutation that initiate the carcinogenic process (Phillips, 1999).

The tumor suppressor p53 is the most frequent target genetic alterations in human cancers, with mutations occurring in almost 50% of all human tumors (Cariello *et al*., 1994). It has been suggested that p53 may play an important role in DNA repair, cell cycle arrest, apoptosis upon environmetal stresses (Oren, 1999). The activated p53 induces several mechanism including protein stabilization and modification of the protein by phosphorylation and acetylation. The p53 can be acetylated in vivo in response to a variety of cellular stress signals (Sakaguchi *et al*., 1998; Pearson *et al*., 2000; Prives *et al*., 2001) and p53 is a transcription factor that recognizes specific binding sites within numerous target gene including mdm2, cyclin G, bax, and p21/WAF1/CIP1, which mediate p53-dependent cell cycle arrest and/or apoptosis (Sakaguchi *et al*., 1998; el-Deiry 1998; Bargonetti *et al*., 2002; Lagger *et al*., 2003). The cyclin dependent kinase inhibitor p21/WAF1/CIP1 is an important regulator of cell cycle progression, sensence, and differentiation (Appella *et al*., 2001). In this study, Bezo[a]pyrene toxicity was investigated on human hepatoma HepG2 cells, focusing on its effect on tumor suppressor protein, p53, to identify biomarkers for PAH risk assessment.

MATERIALS AND METHODS

Cell culture and cell treatment

The human hepatoma HepG2 cells were maintained in DMEM (GIBCO BRL Life Technologies) supplemented with 10%(v/v) fetal bovine serum and 1% antibiotics at 37°C in CO_2 atmosphere. BaP was dissolved in dimethyl sulfoxide (DMSO) as a 1000x stock. For BaP treatment, cells were 70～80% confluent, and the medium was exchanged with fresh medium(serum free) containing various concentrations of BaP in DMSO. Treated and control cells were incubated for 24 hours and harvested for the analysis.

MTT Assay

Cell viability was measured using MTT assay. Cells at the exponential phase were collected and transferred into each well (about 10^4～10^5 cells in 180 μL/well). The cells were incubated for various concentration and then 50 μL of 2 mg MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide) solution was added to each well (0.1 mg/well). After incubating for 4 h, the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL of DMSO and the A_{540} was read on a scanning multi-well spectrophotometer (Molecular Device Co., Sunnyvale, CA). All experiments were performed in triplicate.

Western blotting

Cells (2×10^6cells/mL) were cultured with BaP and harvested at the indicated times. Cells were washed with ice-cold phosphate-buffered saline (PBS, pH 7.4). Cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 50 mM NaF, 50 μg/mL leupeptin and 50 μg/mL aprotinin and incubated on ice for 30 min. After centrifugation (15,000 g for 10 min at 4°C), the supernatant was collected and protein contents in lysates were determined by Bradford analysis (Bio-Rad). Equal amounts of protein per lane were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibody for 1 hour and then extensively washed with TTBS buffer. And the membranes were incubated for 1 hour with secondary antibody. After extensive washing with TTBS buffer, the immune complexes were detected by enhanced chemiluminescence detection system (Amersham) and exposed to X-ray film.

Table 1. List of Antibidies

Antibodies	Ca. No.
p53	SantaCruz, sc−126
p−p53 (ser15)	Cell Signaling, 9286
a−p53 (Lys320)	Upstate, 06−915
p21	Upstate, 05−345
CdK4	SantaCruz, sc−601
Rb	SantaCruz, sc−102

Chemicals

Benzo[a]pyrene was purchased from Sigma(B1760) and antibodies were purchased from SantaCruz, Cell Signaling and Upstate (Table 1).

Data analysis

After band intensity analysis (Kodak 1D 3.6), all analyses were performed using SigmaPlot 8.0 and SigmaStat 5.0.

RESULTS AND DISCUSSIONS

The effect of BaP on cell viability was studied in HepG2 cells. 10 μM of BaP treatment decreased in the cell viability to almost 25% of that of control by 24 h (Fig. 1). The cell death induced by BaP might be due to apoptosis, however, to prove this hypothesis, further analysis are needed.

In order to find out whether p53 protein is involved in the cell death induced by BaP, cellular p53 levels was measured using western blot analysis. The p53 protein level was augmented in BaP treatments (Fig. 2). Because the phospholyration of the serine15 residue of p53 is known as a very early step in the activation of p53, we assessed the amount of p53 phospholated at serine 15 after BaP treatment, using an antibody that specifically recognizes the phosphorylated serine 15 residue of p53. The phosphorylation of p53 at serine 15 was induced by BaP treatment, in a concentration depenant manner (Fig. 2). Chemical response of phosphorylated p53 could be more sensitive than that of p53. However, the level of acetylated p53 at lysine 320, which is also an activated form of p53, decreased by BaP exposure (Fig. 2A.). The meaning of p53 acetylation and the way by which this process is regulated have not yet fully understood (8), therefore, further researches are needed to explain the result observed in this study. The β−actin protein level remained unchanged following BaP exposure (data not shown).

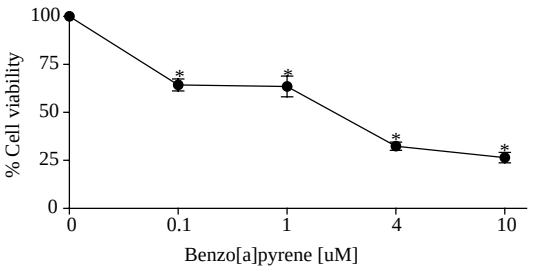

Fig. 1. Efffect of BaP on cell viability. HepG2 cells were treated 0, 0.1, 1, 4, 10 μM BaP for 24 h. Cell viability was determined by MTT assay. The results are shown as the mean ± S.D. of triplicate culture. Asteriks denotes significant difference from control ($p < 0.05$)

Induction of DNA damage by a variety of agents, such as BaP, is known to activate the tumor suppressor p53, which in turn acts as transcriptional regulator of several target genes. One of the main targets is the gene encoding the Cdk inhibitor, p21. Treatment of the human hepatoma HepG2 cells with BaP led to strongly increased p21 protein levels (Fig. 2). Increase of this protein might be related with cell cycle arrest, however the levels of Cdk4 and Rb proteins were not modified by BaP exposure (Fig. 2). The meaning of increase of p21, with no modification of its downstream signaling proteins, Cdk4 and Rb, remains uncertain. Further studies are needed to illucidate whether benzo[a]pyren−induced p53 accumulation lead to cell cycle arrest through p21 signaling pathway.

Time dependent effects of BaP on p53, acetylated p53 and p21 were shown in Fig. 4. The p53 protein level increased as early as 3 h after BaP treatment and this augmentation remained until 24 h. Whereas, the peak of the p21 protein level occurred at 24 h after treatment. The results of time−course study

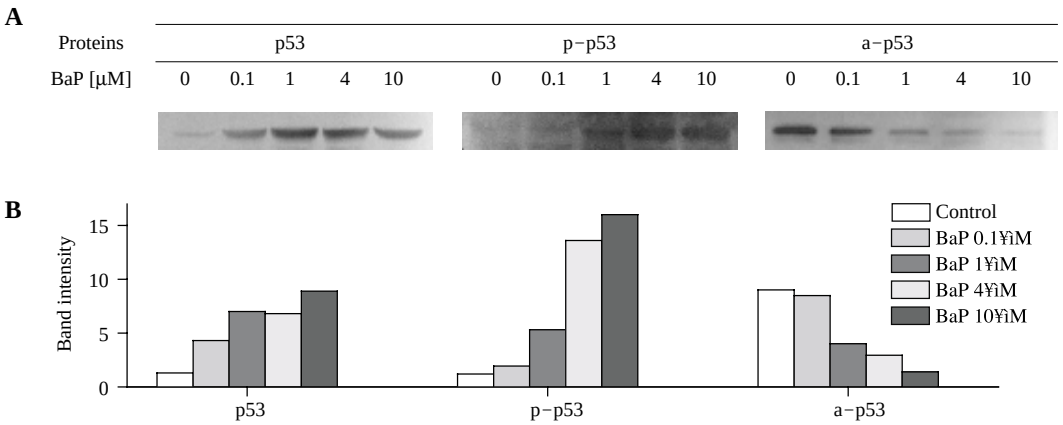

Fig. 2. Concentration-dependent effect of BaP on p53, phosphorylated p53 and acetylated p53. Expression of p53, phosphorylated p53 and acetylated p53 in different concentrations of BaP (0～10 μM, 24 h) treated HepG2 cells by Western blot analysis (A); densitometric analysis (B).

Table 2. Correlation between the different parameters

	Viability	p53	p-p53	a-p53	p21	Cdk4	Rb
[BaP]	**−1.000 (0.017)**	0.900 (0.083)	**1.000 (0.017)**	−0.825 (0.086)	**1.000 (0.017)**	−0.700 (0.233)	−0.783 (0.133)
Viability		−0.90 (0.083)	**−1.000 (0.017)**	**1.000 (0.017)**	−1.000 (0.017)	0.700 (0.233)	0.783 (0.133)
p53			0.900 (0.083)	−0.900 (0.083)	0.900 (0.083)	−0.600 (0.350)	−0.783 (0.133)
p-p53				**−1.000 (0.017)**	**1.000 (0.017)**	−0.700 (0.233)	−0.783 (0.133)
a-p53					−1	0.700 (0.233)	0.783 (0.133)
p21						−0.700 (0.233)	−0.783 (0.133)
Cdk4							0.783 (0.133)

The values in bold face indicate significant correlations at $p < 0.05$ (Spearman correlation test). Numbers in paranthesis indicate p value.

suggest that BaP-induced p53 protein accumulation could lead increase in the p21 protein level. Acetylated p53 level decreased by BaP treatment in time-course study (Fig. 3), as in exposure level study, shown in a 1. Given the as yet incomplete understanding of the meanings of p53 acetylation, further researches are needed to explain BaP-induced decrease of acetylated p53 observed in this study.

Table 2 shows Spearman correlation analysis between different parameters tested in this study. Statistical study reveals that BaP exposure levels are negatively correlated with cell viability, and positively with phosphorylated p53 and p21 protein levels. Among 6 proteins tested, phosphorylated p53 and p21 are negatively correlated with cell viability, whereas positive correlation was observed between acetylated p21 protein and cell viability. These overall results suggest that exposure to BaP lead to cell death and the accumulation of the phosphorylated p53 and p21 proteins levels may be deeply involved in this process.

It is well known that metabolite of BaP are capable of binding to cellular macromolecules including DNA. Therefore, p53 activation and phosphorylation of serine 15 seems to be a downstream response to BaP-induced DNA damage and these results suggest that p53 plays important roles in defense against BaP induced genotoxicity. Taken into account overall results, p53 and p21 seems pertinent biomarkers for BaP exposure and after calibration and validation, these proteins could be used for PAH risk assessment. However, further work is mecessary to eluci-

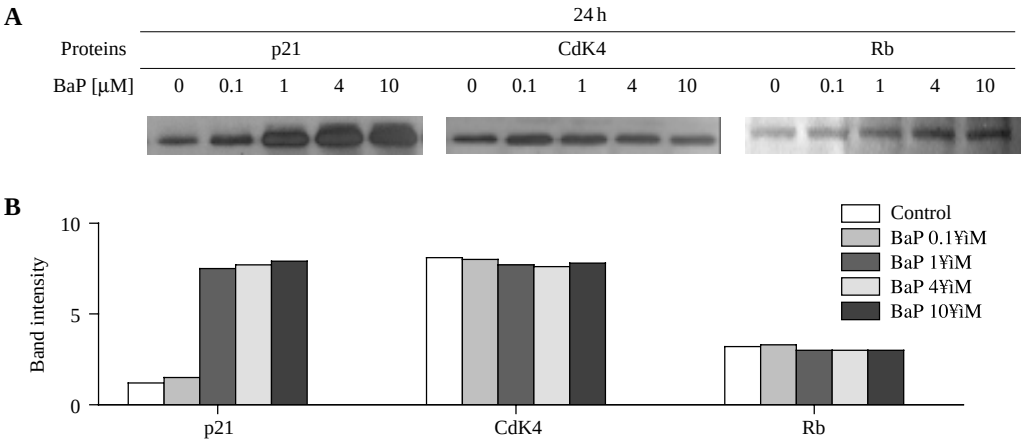

Fig. 3. Concentration-dependent effect of BaP on p21, Cdk4 and Rb. Expression of p21, Cdk and Rb in different concentrations of BaP (0～10 μM, 24 h) treated HepG2 cells by Western blot analysis (A); densito-metric analysis (B).

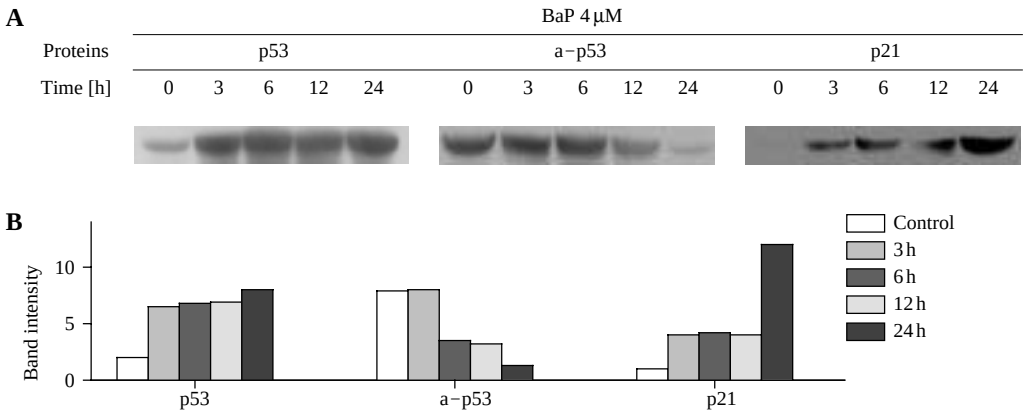

Fig. 4. Time-dependent effects of BaP on p53, acetylated p53 and p21. Expression of p53, acetylated p53 and p21 at different time points (0～24 h) in BaP (4 μM) treated HepG2 cells by Western blot analysis (A); densitometric analysis (B).

date the molecular mechanism leading to modification of the p53 and p21 proteins levels after BaP treatment.

CONCLUSIONS

BaP toxicity was investigated on HepG2 cells, focusing on its involvement in tumor suppressor protein, p53. HepG2 cell viability decreased upon BaP exposure. Concentration-dependant increase of protein induction was observed on p53, as well as on posphorylated p53, whereas acetylated p53 level decreased by BaP treatment. P21 level increased by chemical treatment, in a concentration-dependant manner, whereas its downstream signaling proteins, such as, Cdk and Rb, did not changed by BaP exposure. These results suggest that p53 and p21 accumulation is an important phenonmenon for BaP toxicity, and thus, concomitantly with chemical specific biomarker, these proteins could be identified as sensitive biomarkers for BaP-like PAH risk assessment.

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REFERENCES

Appella E and Anderson CW. Post-translational modifications and activation of p53 by genotoxic stress, Eur J Biochem 2001; 268(10): 2764-2772.

Bargonetti J and Manfredi JJ. Multiple roles of the tumor suppressor p53, Curr. Opin. Oncol. 2002; 14(1): 86-91.

Cariello NF, Beroud C and Soussi T. Database and software for the analysis of mutation at the human p53 gene, Nucleic acids REs 1994; 22(17): 3549-3550.

el-Deiry WS. Regulation of p53 downstream genes, Semin Cancer Biol 1998; 8(5): 345-357.

Harvey RG. Polycyclic Aromatic Hydrocarbons: Chemistry and Carcinogenicity, Cambridge University Press. Cambridge. UK. 1991.

Lagger G, Doetzlhofer A, Schuettengruber B, Haidweger E, Simboeck E, Tischler J, Chiocca S, Suske G, Rothen-eder H, Wintersberger E and Seiser C. The tumor suppressor p53 and histone deacetylase 1 are antagonis-tic regulators of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 gene, Mol Cell Biol 2003; 23(8): 2669-2679.

Oren M. Regulation of the p53 tumor suppressor protein, J Bio Chem. 1999; 274(51): 36031-36034.

Pearson M, Carbone R, Sebastiani C, Cioce M, Fagioli M, Saito S, Higashimoto Y, Appella E, Minucci S, Pandolfi PP and Pelicci PG. PML regulates p53 acetylation and premature senescence induced by oncogenic Ras, Nature 2000; 406(6792): 207-210.

Pei XH, Nakanishi Y, Takayama K, Bai F and Hara N. Benzo[a]pyrene Acivates the Human p53 Gene through Induction of Nuclear Factor kB Activity, The journal of Biological Chemistry 1999; 274(49): 35240-35246.

Phillips DH. Polycyclic aromatic hydrocarbon in the diet, Mutation Research 1999; 443(1-2): 139-147.

Prives C and Manley JL. Why is p53 acetylated, Cell 2001; 107(7): 815-818.

Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW and Appella E. DNA damage activates p53 through a phosphorylation-acetylation cascade, Genes Dev 1998; 12(18): 2831-2841.