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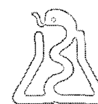

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Ozone Inhalation with 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone and/or Dibutyl Phthalate Induced Cell Cycle Alterations via Wild-type p53 Instability in B6C3F1 Mice

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ABSTRACT. Changes in cell cycle control in the lungs and liver of the B6C3F1 mice (20 males per each group) exposed to ozone (0.5 ppm), 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 1.0 mg/kg), and dibutyl phthalate (DBP, 5,000 ppm) after 52 weeks were examined through Western, Northern blot, and immunohistochemistry based on alterations in protein expression levels of G1/S checkpoints (cyclin D1, cyclin E, and PCNA), G2/M checkpoints (cyclin B1, cyclin G, and cyclin A), negative regulators (p53, p21, GADD45, and p27), and positive regulator (mdm2). Expression levels of cyclins D1, E, G, PCNA, mutant p53, and mdm2 proteins were higher in the lungs and livers treated with combination of toxicants than in those treated with ozone only. Expression levels of the wild-type and mutant p53, p21, GADD45, p27, and mdm2 proteins and mRNAs were higher in toxicant-treated groups than those of the control. Immunohistochemical analysis revealed staining intensities of the PCNA, cyclin D1, c-myc and mdm2 protein- treated lungs and livers were stronger than those of the control group. Our results showed that combined treatment of ozone with NNK/DBP altered the cell cycle control through instability of the wild-type p53 gene. Such pivotal p53-mediated cell cycle alterations may be responsible for the toxicity observed under our experimental condition. These results may be applied to risk assessment of mixture-induced toxicity.

Keywords: Ozone, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, Dibutyl phthalate, Inhalation.

INTRODUCTION

Ozone (O_3) is a common urban air pollutant to which human beings are routinely exposed, and causes, as demonstrated through various laboratory animal and human clinical studies, diverse detrimental effects. Moreover, ozone has a carcinogenic potential in rodents, leading to DNA damage via reactive oxygen species such as hydroxyl radicals, superoxide anions, singlet oxygen, and hydrogen peroxide (Lippmann, 1989). Recent study, ozone induce the free radicals and the inactivation of the antioxidant enzymes, Cu/Zn superoxide dismutase is a major target.

Several nitrosamines derived from tobacco alkaloids are carcinogenic to laboratory animals (Hoffmann and Hecht, 1985; Hecht and Hoffmann, 1989). Among these, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is not only a potent lung carcinogen in rodents, but also is a likely causative factor of human lung carcinogenesis (Hoffmann and Hecht, 1985; Hecht and Hoffmann, 1989).

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A wide range of use has been found for various phthalic acid esters (PAEs), including as plasticizing agents for poly(vinyl chloride) products (Austian, 1973). Dibutyl phthalate (DBP), one of the PAEs, has been used as a plasticizer such as polyvinyl, textile lubricating agents, and resin solvent, and in safety glass, printing inks, paper coatings, and adhesives. It is also used as a perfume solvent and a fixative in cosmetics, a suspension agent for solids in aerosols, a lubricant for aerosol valves, an antifoamer, a skin emollient, and as a plasticizer in nail polish, fingernail elongators, and hairsprays. However, recent studies revealed DBP to be estrogenic in estrogen-responsive human breast cancer cells (Harris et al., 1997; Jobling et al., 1995; Sonnenschein et al., 1995).

Specific molecular events occur at different phases of the mammalian cell cycle. In particular, cell proliferation is controlled at G1/S and G2/M checkpoints. Regulating the cell proliferation by both positive and negative factors ensures that cell cycle phases occur in the correct order and timing. Cell cycle regulators exert their effects by governing the progression of the cell cycle at the checkpoints, and alterations in their expression patterns could contribute to various diseases including tumorigenesis. Thus, failure at the cell cycle checkpoints results in deregulated growth and unrestricted cell cycling, possibly leading to the development of cancer, which is characterized by the loss of cellular growth control. Recent advances in the field of cell cycle regulation have revealed the molecular basis of the restriction point and its role in preventing cells from undergoing unrestricted growth. Although the cell cycle regulatory proteins have been extensively examined *in vitro*, only few studies using *in vivo* animal models are available (Lee and Fukushima, 1998).

In generally, human beings are exposured in various mixed chemical. In spite of the extensive studies on the toxicological actions of ozone, NNK, and DBP, relative little is known of these toxicants in mixed pollutant system. We, thus, hypothesized exposure to various combinations of the above important environmental toxi- cants may cause toxicities through interplay between individual toxicants. Therefore, the aim of this study was to determine the alterations in the cell cycle control in the lungs and livers extracted from B6C3F1 mice after 16, 32, and 52 weeks exposures to the combined treatment of NNK and/or DBP on ozone inhalation. In particular, alterations in the protein and mRNA expression levels of G1/S checkpoints (cyclin D1, E, and PCNA), G2/M checkpoints (cyclin B1, G, and cyclin A), negative regulators (p53, p21, GADD45, and p27), and the positive regulator (mdm2) were examined.

Materials and Methods

Chemicals

NNK (CAS NO. 64091-91-4) was obtained from Chemsyn Science laboratories (Lenexa, KS, USA), with >99% purity as revealed by HPLC analysis (data not shown). Trioctanoin obtained from Wako (Japan) was redistilled prior to use. DBP (CAS NO. 84-74-2) was acquired from Sigma (St. Louis, MO, USA). A diet containing DBP was freshly prepared each week as follows. A predetermined amount of DBP was weighed, added to a small aliquot of ground basal diet, and hand-blended. This premix was then added to a preweighed ground basal diet and blended in a mill for 30 min.

Animals

The male B6C3F1 mice, 4- to 5-weeks-old, were purchased from the Seoul National University (SNU) Laboratory Animal Facility (Seoul, Korea), and acclimated for approximately 7 days prior to initiating chemical exposure and classified each group with 20 mice. When the exposure was beginning, the range of the body weight was Food and water was provided *ad libitum* except during the period of ozone exposure. The rooms were maintained at 23 $\pm$2°C, with a relative humidity of 50 $\pm$ 20% and a 12-h light/dark cycle. All methods used in this study were approved by the Animal Care and Use Committee at SNU and conform to the NIH guidelines (NIH publication No. 86~23, revised 1985).

The experimental groups consisting of 20 male mice each were as follows: (a) unexposed group (control); (b) group exposed to 0.5 ppm ozone (ozone group); (c) group exposed to 1.0 mg NNK/kg body weight (NNK group); (d) group exposed to 5,000 ppm DBP (DBP group); (e) group exposed to 0.5 ppm ozone+1.0 mg/kg NNK (ozone+NNK group); (f) group exposed to 0.5 ppm ozone+5,000 ppm DBP (ozone+DBP group); (g) group exposed to 0.5 ppm ozone+1.0 mg/kg NNK+5,000 ppm DBP (three-combination group).

Exposures

The mice were exposed to ozone (0.50$\pm$0.02 ppm) for 6 h per day (between 9:00 AM and 3:00 PM), 5 days per week for 52 weeks in 1.5 m^3 whole-body inhalation exposure chambers (Dusturbo, Seoul, Korea). Ozone (CAS NO. 10028-15-6) was generated from pure oxygen using a silent electric arc discharge ozonator (Model KDA-8, Sam-II Environment Technology, Pusan, Korea) and mixed with the main stream of fil-

tered air before entering the exposure chambers. The ozone concentration in the chambers was monitored using a gas detection system with an O_3 gas sensor (Analytical Technology, USA). The O_3 gas sensor probes were placed within the breathing zone of the mice the middle cage racks. Measurements were taken from 12 locations in each chamber in order to ensure uniformity of ozone distribution, which was enhanced using a recirculation device. Airflow in the chambers was maintained at 15 changes per hour. Wire cages allowed observation of all individually housed animals during exposures. Before and after ozone exposures, the mice were housed five per cage in polycarbonate cages with bottom wire nets. During the test periods, the mice were subcutaneously injected with 1.0 mg NNK per kg body weight in trioctanoin three times per week. They also received diet containing 5,000 ppm DBP for 16, 32, and 52 weeks. The concentration of each test toxicant was determined based on the National Toxicology Program (NTP) carcinogenesis study (National Toxicology Program, 1994, 1995).

Western Blot for Cyclin D1, Cyclin E, PCNA, Cyclin B1, Cyclin G, Cyclin A, Wild-type p53, Mutant p53, p21, GADD45, p27, and mdm2 Protein Levels

Lung and liver tissues of the control and all treated groups were homogenized with a lysis buffer [50 mM Tris at pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonylfluoride (PMSF), 1 μl/ml of aprotinin, 1% igapel 630 (Sigma, St. Louis, MO, USA), and 0.5% deoxycholate] and centrifuged at 14,000 ×g for 30 min. The protein concentration was determined using a Bradford analysis kit (Bio-Rad, Hercules, CA, USA). Equal amount of the proteins were separated on an SDS-12% polyacrylamide gel and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia, Piscataway, NJ, USA). The blots were blocked for 2 h at room temperature with a blocking buffer (10% nonfat milk in TTBS buffer containing 0.1% Tween 20). The membranes were incubated for 1 h at room temperature with specific mouse monoclonal antibodies against cyclins A, B1, D1, E, and G, PCNA, wild-type and mutant p53s, p21, GADD45, p27, mdm2, and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibodies were used at dilutions specified by the manufacturer. After washing with TTBS, the membranes were reincubated with sheep anti-mouse horseradish peroxidase (HRP)-labeled secondary antibodies and visualized using an electrochemiluminescense (ECL) detection kit (Amersham Pharmacia).

Northern Blot for the Cyclin D1, Cyclin E, PCNA, Cyclin B1, Cyclin G, Cyclin A, Wild-type p53, Mutant p53, p21, GADD45, p27, and mdm2 mRNA Levels

Lung and liver tissues of the control and all treated groups were homogenized with TRI reagent (Sigma), and total RNA was isolated according to the manufacturer's instructions. A glyoxal premixture (1.5 μl 5 M glyoxal, 1.5 μl 0.1 M $NaPO_4$, pH 6.5, 7.5 μl DMSO) was added to 4.5 μl (20 μg) total RNA. The RNA denatured with glyoxal and DMSO was incubated at 50°C for 60 min and cooled on ice for 3 min, into which 3 μl of the loading buffer was then added. While gel buffer consisting of 1.0% agarose gel and 10 mM $NaPO_4$, pH 6.5, was prepared, an electrophoresis unit with a circulation system was set up and a gel running buffer (10 mM $NaPO_4$, pH 6.5) prepared. Then twenty microgram of total RNA was separated via electrophoresis and transferred onto nylon membrane. Transfer was initiated immediately after the completion of the glyoxal/RNA gel running. The tank was filled with a transfer buffer (25 mM $NaPO_4$, pH 6.5) and transferred overnight. On the next day, the nylon membranes were dried on a clean bench and wrapped with Saran Wrap, crosslinked for 45 s on a UV-transillumintor (UVP, San Gabriel, CA, U.S.A.), and stored at 4°C. The size standard lane and a duplicate membrane were stained with 0.02% methylene blue in 5% acetic acid to confirm the presence of 28S and 18S rRNAs. Cyclins A, B1, D1, E, and G, PCNA, wild-type and mutant p53s, p21, GADD45, p27, and mdm2 plasmid cDNA were obtained from ATCC. One colony was pitched into 2 ml of the LB medium containing ampicillin and cultured in a 37°C shaking incubator overnight. The plasmid cDNA was then purified using a Minipreps DNA purification kit (Promega). After confirming the size of the plasmid cDNA, and the size and direction of the insert, 1 ml of the cultured transformants was added to 100 ml LB medium containing ampicillin and cultured overnight in a 37°C shaking incubator. The plasmid cDNA was then purified using a Midipreps DNA purification kit (Promega) and stored at -20°C. Cyclin D1, cyclin E, PCNA, cyclin B1, cyclin G, cyclin A, wild-type p53, mutant p53, p21, GADD45, p27, and mdm2 were digested with *Eco*RI/*Xho*I, *Not*I/*Eco*RI, *Hin*dIII, pvu II, *Not*I/*Eco*RI, *Sal*I/*Not*I, *Eco*RI, *Eco*RI/*Xho*I, *Xho*I/*Bgl*II, *Not*I/*Eco*RI, *Nde*I/*Bam*HI, and *Eco*RI restriction enzymes for 3 h at 37°C, respectively, and the linearity was confirmed in 1% agarose gel. After digestion, the products were purified using a Clean up kit (Promega). The antisense cRNA probe was synthesized with Digoxigenin (DIG) RNA probe labeling and a detection kit (BMS, Mannheim, Germany) according to the manufacturer's

protocol. The labeling reaction mixture contained 0.05 μg/μl of the linear plasmid cDNA, 1× *in vitro* transcription buffer, 1×DIG RNA labeling mix (dNTP), 2 units/μl of T7 RNA polymerase, 1 unit/μl of an RNAse inhibitor, at a total volume of 20 μl. The reaction mixture with DNAse and RNAse free water was incubated at 37°C for 2 hour. Finally, 2 μl of 0.5 M EDTA was added to terminate the transcription reaction. The RNA transcript products were analyzed for size confirmation by agarose gel electrophoresis and ethidium bromide staining. Yield of the DIG-RNA probe was detected through a colorimetric detection method using anti-DIG antibodies and stored at -70°C. The RNA membranes were prehybridized in a hybridization buffer (50% formamide, 5× SSC, 0.1% N-lauroyl-sarcosine, 0.02% SDS, and 2% Blocking Reagent) at 68°C for 1~2 h. The prehybridization solution was then discarded, and the hybridization buffer containing the denatured probe (5~10 ng/ml) was added. Hybridization was performed at 68°C overnight. The membranes were rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 1 min at room temperature and incubated in a blocking buffer for 1 h, followed by incubation in an anti-DIG-alkaline phosphatase antibody solution (1 : 10000) for 30 min. The membranes were washed with a maleic acid washing buffer containing 0.3% Tween 20, 6 times for 15 min each with constant shaking at room temperature. Finally, they were incubated in a 25 mM disodium 3-4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo (3.3.1.1)decan]-4-yl phenyl phosphate (CSPD) assay buffer (100 mM Tris-HCl, pH 9.5) for 5 min, then further incubated in a ready-to-use CSPD detection solution for 5 min. Following exposure to film for 15 s to 5 min, they were then incubated at 37°C for 15 min and developed using an auto-developer (AGFA, Belgium).

Immunohistochemistry for PCNA, Cyclin D1, c-myc, and mdm2 Proteins

Lungs and livers of the control and all treated groups were stained through immunohistochemistry [avidin-biotin-complex (ABC) staining kit] (Santa Cruz, Santa Cruz, CA, USA) according to the manufacturer's instructions using anti PCNA, cyclin D1, c-myc, and mdm2 mouse monoclonal antibodies (Calbiochem. La Jolla, CA, U.S.A.). The slides were washed with PBS and deparaffinized in xylenes, 100% ethanol, and 95% ethanol for 5, 10, and 10 min, respectively. They were then incubated in 0.7% hydrogen peroxide for 10 min and washed with PBS. Following incubation with 10% normal serum for 1 h at room temperature, the slides were sequentially incubated with mouse monoclonal anti-PCNA, cyclin D1, c-myc, and mdm2 antibodies (2.5 μg/ml) overnight at 4°C, biotin conjugated anti-mouse secondary antibodies (1 μg/ml) for 1 h at room temperature, and a biotin-avidin complex solution for 30 min at room temperature, with a 5-min washing step, 3 times between each incubation. Finally, upon development of a brown color using a 0.05% diaminobenzidine (DAB) solution containing 0.03% H_2O_2, the cells were counterstained with hematoxilin solution, and a cover slip was then mounted onto a slide glass. Every slide was examined through optical microscopy, and relative intensities of the staining and positive cells in the non-ulcerated area were counted under 200× lens. Three arbitrary fields were chosen in each mouse (total 5 mice per group) for cell counting. Statistical analysis was performed using a Student's *t*-test at the level of $P<0.05$.

RESULTS

Alterations of Cyclin D1, Cyclin E, and PCNA Protein Levels

The expression levels of the G1/S checkpoints such as cyclin D1, cyclin E, and PCNA proteins increased in all toxicant-treated lungs and livers after 16-, 32-, and 52-wk exposures. Generally, the expression level, particularly that of the cyclin D1, was higher in lungs and livers treated with more than one toxicant than those of single exposure. This pattern was more evident in the lungs than in the livers, whereas the opposite was observed with the cyclin E expression, the pattern being more evident in the livers than in the lungs. Interestingly, ozone, on the other hand, induced strong cyclin E expression in the lungs. In all treated groups in this experiment, PCNA was highly expressed in the lungs. In the livers, however, all treated groups excluding DBP group showed induction of PCNA expression (Fig. 1, 16- and 32-wk data not shown).

Alterations of Cyclin B1, Cyclin G, and Cyclin A Protein Levels

The expression levels of the G2/M checkpoints such as the cyclin G protein increased in all toxicant-treated lungs and livers after 16, 32, and 52 wk exposures compared to the control (Fig. 2, 16- and 32-wk data not shown). However, no changes were observed in the protein expression levels of cyclins B1 and A in all treated lungs and livers compared to the control (data not shown).

Alterations of Cyclin D1, Cyclin E, and PCNA mRNA Levels

The mRNA expression levels of cyclin D1, cyclin E, and PCNA were elevated in all toxicant-treated lungs compared to the control. In particular, combination of toxi-

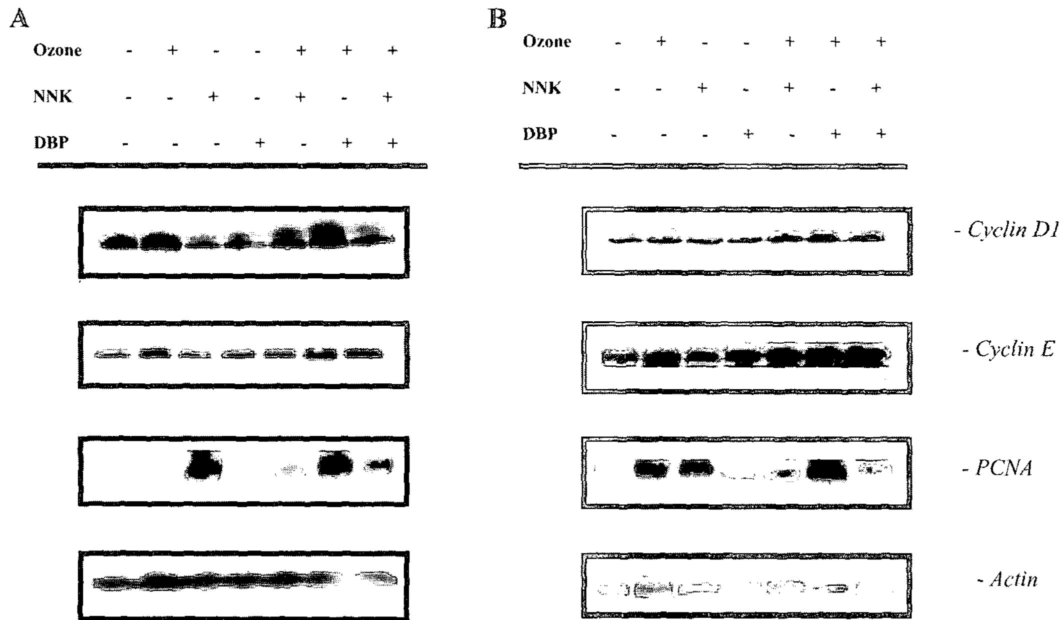

Fig. 1. Expressions of cyclin D1, cyclin E, and PCNA in lungs (A) and livers (B) treated with toxicants for 52 weeks. Total protein was isolated and prepared for Western blotting analysis with appropriate primary antibodies and secondary HRP conjugates, as described in Materials and Methods.

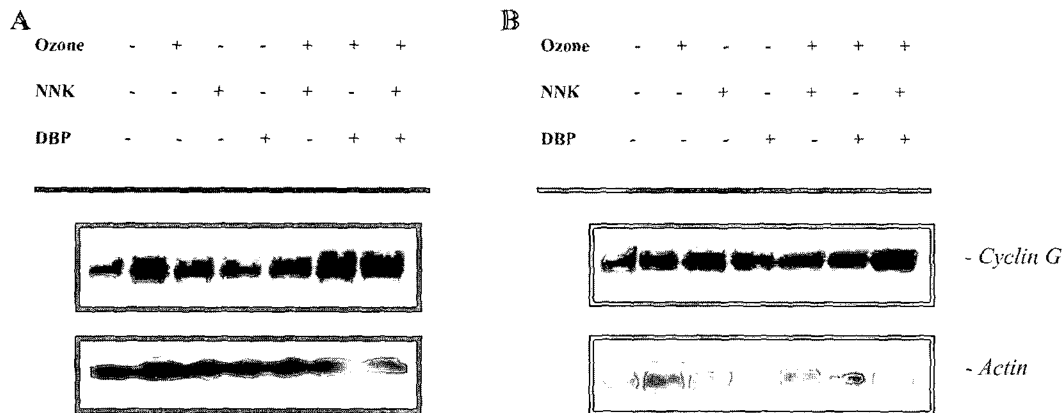

Fig. 2. Expressions of cyclin G in lungs (A) and livers (B) treated with toxicants for 52 weeks. Total protein was isolated and prepared for Western blotting analysis with appropriate primary antibodies and secondary HRP conjugates, as described in Materials and Methods.

cants induced higher expression levels than those of single exposures. However, a similar pattern was not clearly observed in the livers; instead, cyclin D1 mRNA expression level in liver was high in the ozone+NNK and ozone+DBP groups (Fig. 3, 16- and 32-wk data not shown).

Alterations of Cyclin B1, Cyclin G and Cyclin A mRNA Levels

The cyclin G expression levels were higher in combination of two or three toxicant-treated lungs and livers compared to the control and single exposure group in lung and liver. The expression levels of cyclin B1 and cyclinA in lung and liver have no critical exchange (Fig. 4, 16- and 32-wk data not shown).

Alterations of p53, p21, GADD45, and p27 Protein Levels

Expression levels of wild-type p53, mutant p53, p21, GADD45, and p27 proteins were higher in all toxicant-treated lungs and livers compared to the control. In particular, all proteins were expressed in higher amounts combinations of two and three toxicants expressed higher amounts

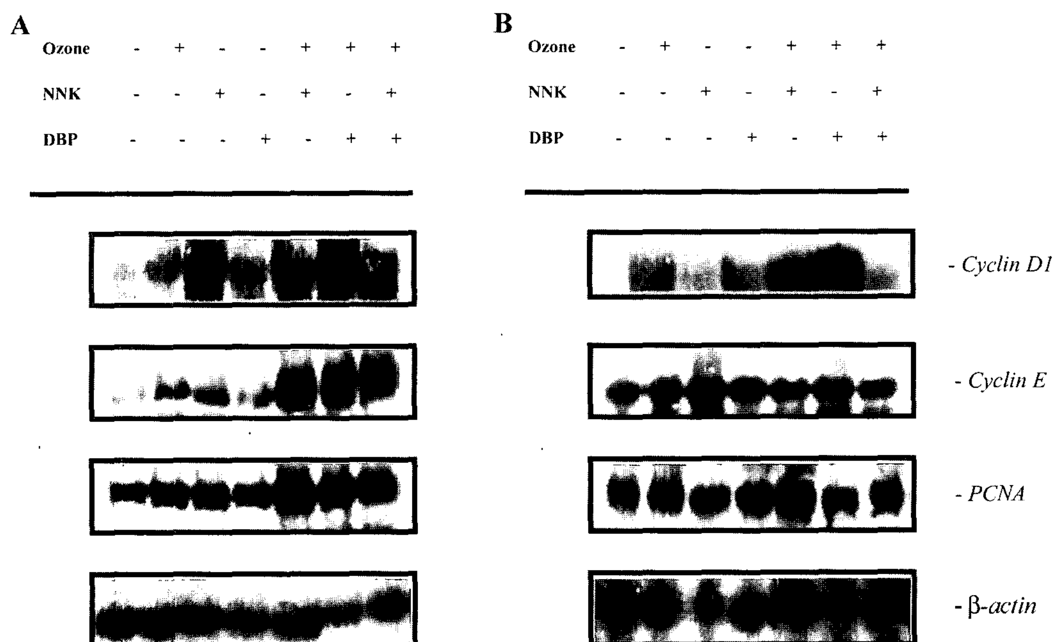

Fig. 3. Expression levels of cyclin D1, cyclin E, and PCNA mRNA determined through Northern blot analysis. mRNA was extracted from lungs (A) and livers (B) of mice exposed to toxicants for 52 weeks, and Northern blot was performed as described in Materials and Methods.

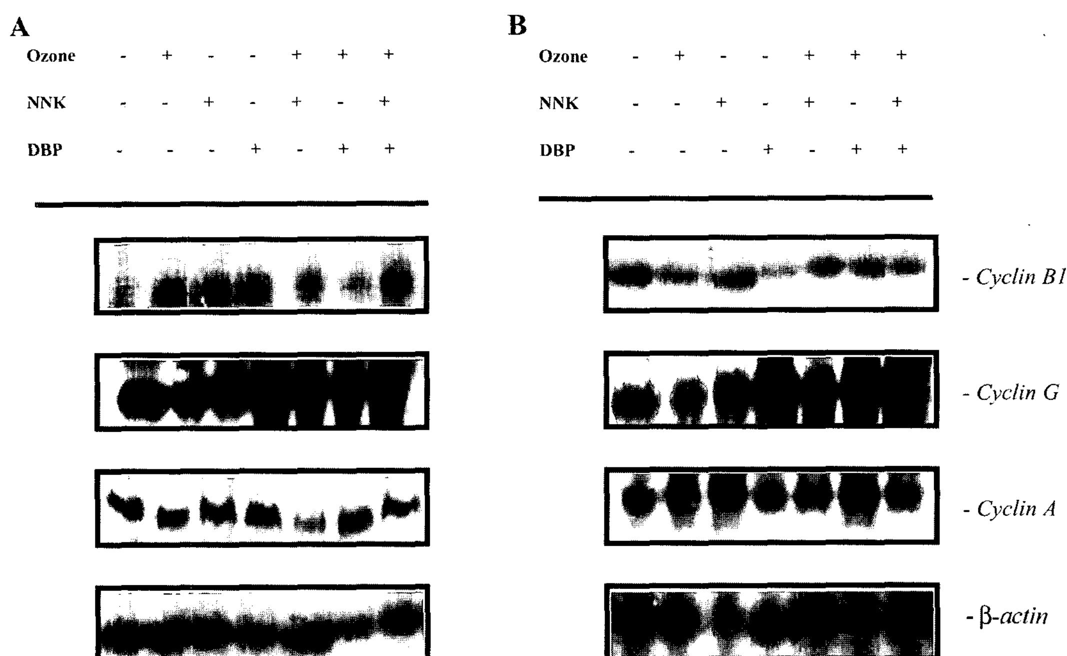

Fig. 4. Expression levels of cyclin B, cyclin G, and cyclin A mRNA determined through Northern blot analysis. mRNA was extracted from lungs (A) and livers (B) of mice exposed to toxicants for 52 weeks, and Northern blot was performed as described in Materials and Methods.

of each protein (Fig. 5, 16- and 32-wk data not shown).

Alteration of mdm2 Protein Level

In the lung, the expression level of mdm2 was increased in all toxicant-treated lungs and livers compared to the control (Fig. 6, 16- and 32-wk data not shown). In lung and liver, especially, the effect to the over expression of mdm2 was more clear in the groups

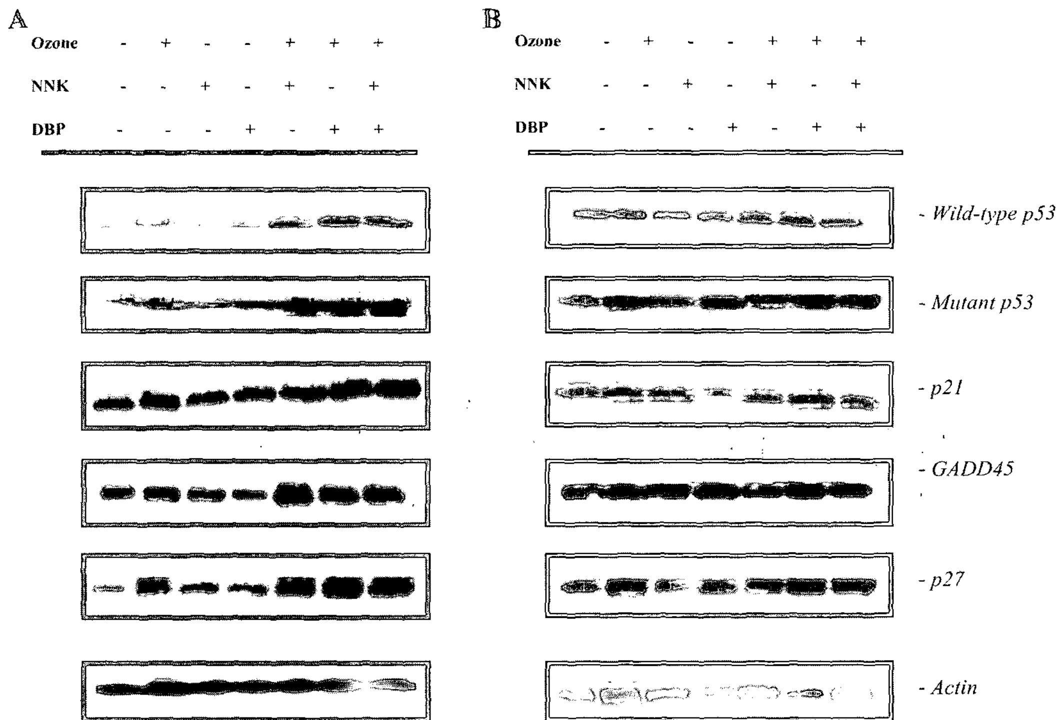

Fig. 5. Expressions of wild-type p53, mutant p53, p21, GADD45, and p27 in lungs (A) and livers (B) of mice exposed to toxicants for 52 weeks. total protein was isolated and prepared for Western blotting analysis with appropriate primary antibodies and secondary HRP conjugates, as described in Materials and Methods.

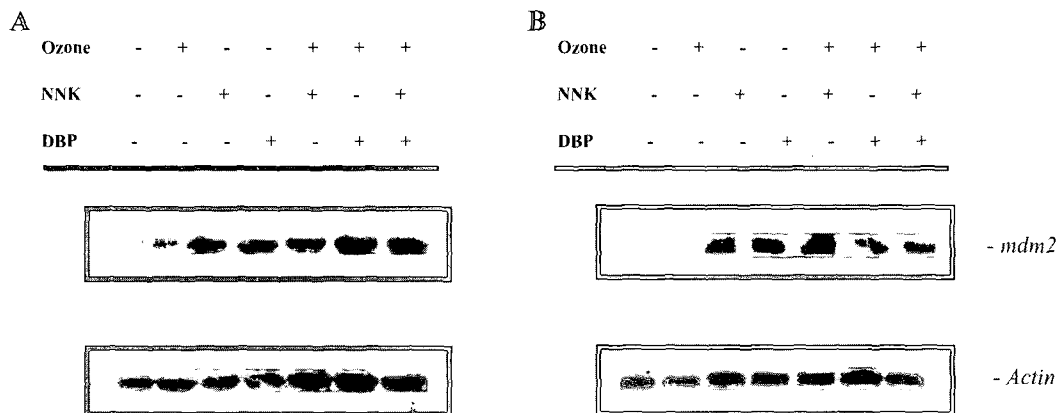

Fig. 6. *Expression of mdm2 in lungs (A) and livers (B) of mice treated with toxicants for 52 weeks. Total protein was isolated and prepared for Western blotting analysis using appropriate primary antibodies and secondary HRP conjugates, as described in Materials and Methods.*

included both NNK and DBP not ozone.

Alterations of p53, p21, GADD45, and p27 mRNA Levels

In the lung, the protein level of p53 both wild type and mutant were increased in two or three combination treated groups compared to control and single treated group. And this pattern was observed in p27 and GADD45, however, this pattern was observed only wild type p53 and p27 in liver and the protein level of mutant form of p53 and GADD45 had no critical exchange.

Alteration of mdm2 mRNA Level

The In the lung, mdm2 expression levels were elevated in all toxicant-treated compared to those of the control after 52-wk exposure (Fig. 8, 16- and 32-wk data not shown). The expression level of oncogenes such as mdm2 protein was higher in the lungs and liv-

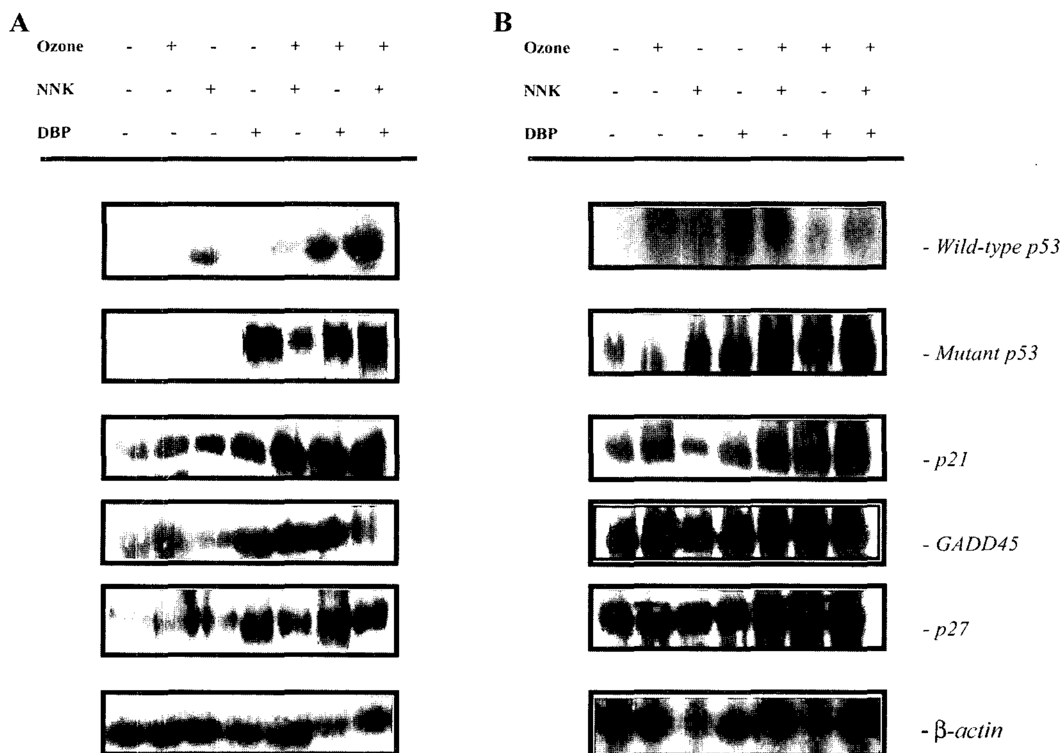

Fig. 7. Expression levels of wild-type p53, mutant p53, p21, GADD45, and p27 mRNA determined through Northern blot analysis. mRNA was extracted from lungs (A) and livers (B) of mice exposed to toxicants for 52 wk, and Northern blot was performed as described in Materials and Methods.

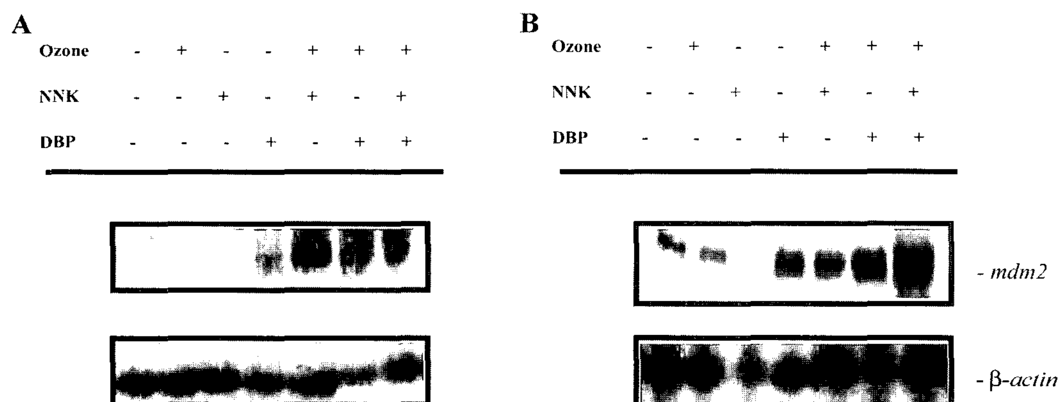

Fig. 8. Expression levels of mdm2 mRNA determined through Northern blot analysis. mRNA was extracted from lungs (A) and livers (B) of mice exposed to toxicants for 52 wk, and Northern blot was performed as described in Materials and Methods.

ers exposed to combinations of two and three toxicants than in the ozone-treated ones, similar to the protein expression level.

Alterations of PCNA, Cyclin D1, c-myc, and mdm2 Protein Expressions

Lungs and livers of the control and all treated groups were stained for PCNA, cyclin D1, c-myc, and mdm2 proteins through immunohistochemistry (Figs. 9, 10). Positive cells of PCNA, cyclin D1, c-myc, and mdm2 in the control group were found scattered. On the other hand, most cells of the treated groups were positively stained, and their staining intensity was stronger than that of the control group. The number of PCNA-positive cells in arbitary area were higest in the three-combination (178 $\pm$13) and ozone+DBP (168 $\pm$21) groups was higher than control group (88 $\pm$11, 91 $\pm$18). In addition, the number of cyclin D1-positive cells was more

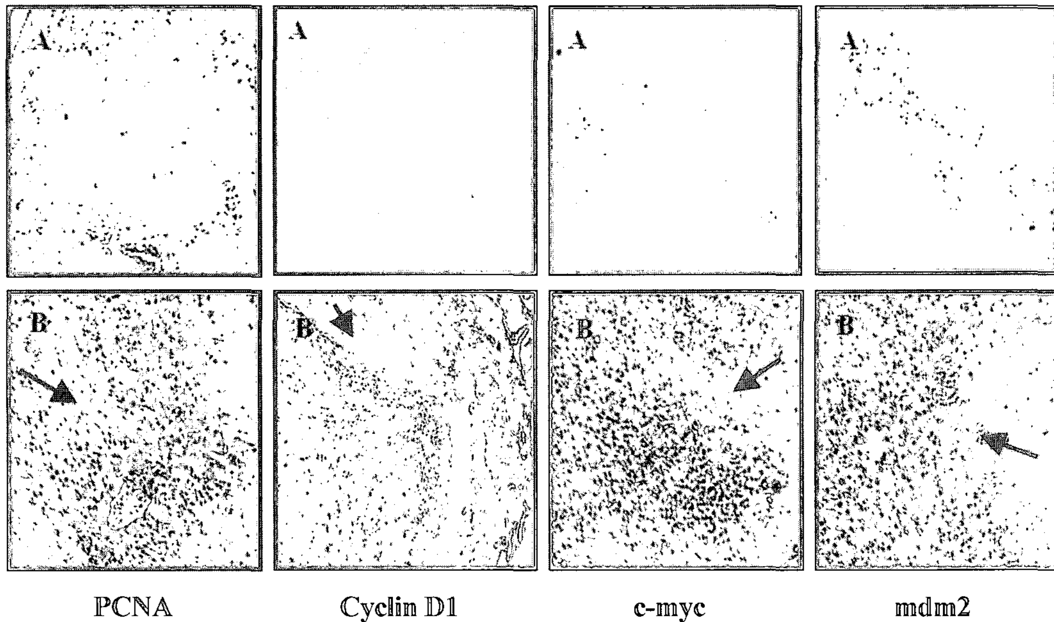

Fig. 9. Immunohistochemical detections of PCNA, cyclin D1, c-myc, and mdm2 in mouse livers: (A) unexposed control, (B) treated group. Original magnification: 100 ×.

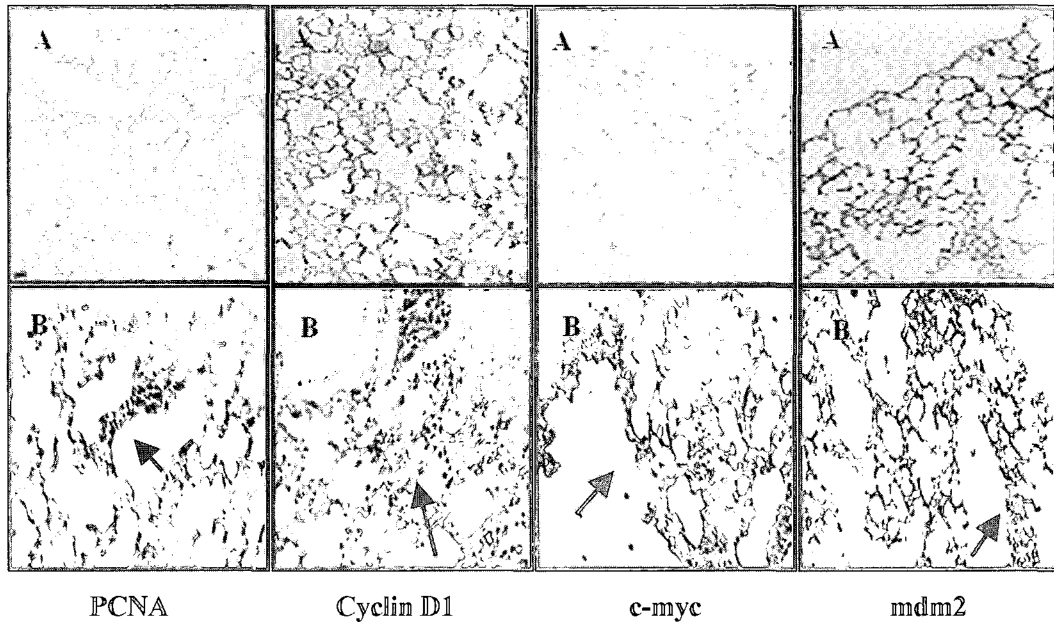

Fig. 10. Immunohistochemical detections of PCNA, cyclin D1, c-myc, and mdm2 in mouse lungs: (A) unexposed control, (B) treated group. Original magnification: 100 ×.

higher in the ozone+NNK (162 ±21) and three-combination (164 ±19) groups than control group (78 ±14, 101 ±21) (Table 1). Distribution patterns of mdm2 and c-myc positive cells were similar to those of PCNA and cyclin D1 proteins.

In the tree-combination group, the numbers of mdm2 in lungs (166 ±14) and liver (175 ±22) was highest and the c-myc in lungs (178 ±19) and liver (184 ±23) was higher than control groups , respectively (Table 1). In addition, the expression levels of PCNA, cyclin D1, c-myc, and mdm2 proteins were higher in the lungs and livers exposed to combinations of two or three toxicants than those of the ozone-treated ones (Table 1). The staining intensity patterns observed during the 16-

Table 1. Number of PCNA, CyclinD1, mdm2 and c-myc-positive cells was counted in the test material treated mice and the control mice for 52 wk

Group	n	PCNA		Cyclin D1		mdm2		c-myc	
		lung	liver	lung	liver	lung	liver	lung	liver
Unexposed control	5	88 ± 11	91 ± 18	78 ± 14	101 ± 21	81 ± 13	102 ± 17	98 ± 12	103 ± 14
Ozone	5	$144 \pm 12^{*}$	$141 \pm 14^{*}$	$143 \pm 16^{*}$	$157 \pm 19^{*}$	$145 \pm 17^{*}$	$146 \pm 21^{*}$	$152 \pm 25^{*}$	$144 \pm 19^{*}$
NNK	5	$141 \pm 16^{*}$	$161 \pm 12^{*}$	$152 \pm 19^{*}$	$164 \pm 12^{*}$	$134 \pm 22^{*}$	$152 \pm 13^{*}$	$149 \pm 19^{*}$	$159 \pm 22^{*}$
DBP	5	$158 \pm 14^{*}$	$151 \pm 18^{*}$	$154 \pm 17^{*}$	$152 \pm 17^{*}$	$141 \pm 24^{*}$	$144 \pm 16^{*}$	$151 \pm 12^{*}$	$162 \pm 14^{*}$
Ozone+NNK	5	$141 \pm 20^{*}$	$159 \pm 16^{*}$	$162 \pm 21^{*,**}$	$161 \pm 23^{*}$	$163 \pm 19^{*}$	$152 \pm 11^{*}$	$166 \pm 16^{*}$	$151 \pm 14^{*}$
Ozone+DBP	5	$163 \pm 15^{*}$	$168 \pm 21^{*,**}$	$154 \pm 11^{*}$	$157 \pm 16^{*}$	$155 \pm 12^{*}$	$164 \pm 13^{*}$	$174 \pm 22^{*,**}$	$168 \pm 19^{*,**}$
Ozone+NNK+DBP	5	$178 \pm 13^{*,**}$	$165 \pm 19^{*,**}$	$158 \pm 14^{*}$	$164 \pm 19^{*}$	$166 \pm 14^{*,**}$	$175 \pm 22^{*,**}$	$178 \pm 19^{*,**}$	$184 \pm 23^{*,**}$

The number of PCNA, cyclin D1, mdm2, and c-myc-positive cells were counted under the ×200 field. In each mouse, 3 arbitrary fields in the non-ulcerated area were chosen for cell counting. The results are expressed as mean±standard deviation (n=5). *$P<0.05$ in comparison with the control group. **$P<0.05$ in comparison with the ozone group.

and 32-wk studies were similar to those of 52-wk study, showing a time-dependent increase (Data not shown).

DISCUSSION

Carcinogenesis is a multistage process driven by carcinogen-induced and spontaneous genetic, and/or epigenetic damages to susceptible cells. Such cells are believed to achieve selective growth advantage and undergo clonal expansion as a consequence of the altered gene expression, including activation of the proto-oncogenes and/or inactivation of the tumor suppressor gene. In this mechanism, the cell cycle was affected by oncogenes, tumor suppressor genes and related proteins, for instance, cyclin, CDK, mdm2, GADD45 and other related proteins. In normal state, in these proteins were regulated and interact with closely. When the cell was exposured with carcinogens, mutagens or any other chemicals, these interactions were confused and the cell cycle checkpoint was operated. In this reason, screening of the relationship between chemicals and proteins related cell cycle and carcinogenesis were important to understand the cancer. Although, the screening of the effects to the humans or animals was difficult for the variety and complexity of the mechnism of organs, so the detection and screening of the effects among the two or more combination of the chemicals were difficult. However, in general, human beings were exposured with various chemicals in life, so the relationship between the chemicals was important. In environment, many chemicals were exist and in these chemicals, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), Dibutyl phthalate (DBP) and ozone were toxic and recent studies show that each was induced the mutation and cancer in vitro and *in vivo*. These chemicals were potent carcinogens in our environment and may be responsible for the increasing incidence of human cancer, characterization of its effect of combination in human remains incomplete. The principal aim of the present study was to provide a more thorough examination of interaction with NNK, DBP and ozone to animal model and provide the clue to understand the mechanism of the cancer and to this, we were performed to determine the alterations of cell cycle control in the lungs and livers of B6C3F1 mice after 16, 32, and 52-wk exposures to combined treatments of NNK and/or DBP with ozone inhalation.

The cell cycle is composed of many related proteins, among these proteins, the p53 gene is known to suppress the tumor growth via various mechanisms. First, p53 induces G1 arrest and apoptosis through the accumulation of p21 and GADD45 (Lewin, 1997). However, several recent reports suggest that p53 may actually accelerate G2/M progression under DNA damage, and that this accelerated exit from G2/M may be associated with the induction of apoptosis by p53 (Lee and Fukushima, 1998). Rocha *et al.* (2003) reported that p53 specifically represses the cyclin D1 promoter activity via Bcl-3 downregulation. In our results, similarities were ovserved in in the lungs of mice exposed to NNK/DBP with ozone inhalation (Figs. 1, 3, and 5). Therefore, the combined treatment of ozone with NNK/DBP may affect p53-mediated apoptosis, and such deregulated apoptosis may be one of the important underlying causes of toxicity. Previous results obtained by our group indicating that the combined exposure to ozone, NNK and/or DBP synergistically enhances genotoxicity, thus, also support current findings (Kim *et al.*, 2002). Among the genes known, the p53-responsive elements are p21, GADD45, and mdm2. The p53 protein causes G1 arrest in the cell cycle, and induces differentiation, DNA repair or apoptosis via transcriptions of p21 and GADD45 (Guillouf *et al.*, 1995; Skladanowski and Larsen, 1997). Our results also showed that G2/M

arrest caused by the test toxicants was accompanied by increases in the expression levels of wild-type p53 protein, and cyclin G mRNA and protein (Figs. 2, 4, and 5). Cyclin G is a transcriptional target gene of p53. Recent reports suggest that cyclin G is transcriptionally activated by p53, and cyclin G, in turn, negatively regulates the stabilization of p53 family of proteins (Ohtsuka *et al.*, 2003). Therefore, ozone inhalation in combination with NNK and/or DBP exposure appears to induce G2/M arrest through cyclin G accumulation, and such accumulation might have negatively affected the stability of wild-type p53 in this study. The increase in the expression level of mutant p53 protein supports the role of cyclin G in the negative feedback regulation of p53 (Fig. 5). Moreover, the accumulated cyclin G might be associated with mdm2 protein expression (Fig. 6). Overall, cyclin G accumulation caused by ozone inhalation combined with NNK/DBP treatment may play a key role in the p53-mdm2 autoregulated module.

This study showed that the p27 mRNA and protein expression levels also have positive correlation with cyclin G, and negative correlation with the protein levels of cyclins B1 and A at G2/M arrest (Figs. 2, 4, 5, and 7). These results suggest that the p27 mRNA and its protein may also be associated with G2/M arrest in a manner similar to that of p53 through the up-regulated cyclin G protein and down-regulated cyclins B1 and A mRNAs along with their proteins. Beniston and Campo (Beniston and Campo, 2003), upon examining the effects of quercetin, a potent mutagen found in high levels in bracken fern, found that quercetin arrested cells at G1/S as well as G2/M in correlation with the wild-type p53 activation. However, the expression of bovine papillomavirus type 4 (BPV-4) E7 overcame this arrest, leading to the development of tumorigenic cell lines. Such arrest was linked to the elevation of the cyclin-dependent kinase inhibitor (cdki) p27 with a concomitant reduction of cyclin E-associated kinase activity. This elevation of p27 may be due not only to the increased protein half-life, but also to the increased mRNA transcription. These results support our findings that exposure to combination of toxicants increased level of the p27 mRNA as well as proteins. Therefore, in this incresement of the p27 was response against combined exposure to a certain degree as long as the wild type p53. In our study, the expression of oncogenes such as the mutant p53 and mdm2 protein and mRNA was also higher in lungs and livers treated with combination of toxicants compared to those treated with ozone only and control (Figs. 5, 6, 7, 8, 9 and 10 and Table 1). Therefore, elevated oncogens with decreased normal p50 activity could be one of the underlying reasons of the multiple toxicities examined in our study. The p21 is a cyclin-dependent kinase inhibitor, whose abundance increased in cells exposed to radiation or other DNA-damaging agents. Such an increase activates a G1 checkpoint, allowing time for DNA repair prior to entering the S phase. Overall, due to the disturbance of the cell cycle progression by cyclin D1 and GADD45, the role of p21 in providing protection against toxicity induced by combination of toxicants was not successfully accomplished in this study, even though the p21 expression level was increased. This is why the combined treatment resulted in high expression levels of PCNA and cyclin D1, which should have been suppressed effectively by p21. Moreover, the unstable wild-type p53 as well as the increased inhibitory functions of mdm2 and c-myc on p53 might have also disturbed the function of normal p21, an indication that the damaged tissue did not have sufficient time to repair. Amplification or over-expression of the proto-oncogene mdm2 was observed in many sarcomas, glioblastomas, and breast carcinomas (Leach *et al.*, 1998; Innocente *et al.*, 1999). The c-myc expression level is tightly linked to the cell growth (Stewart et al., 1995), almost undetectable in growth-arrested cells and transiently elevated in response to mitogens (Masramon et al., 1998), which are indications that c-myc promotes cell proliferation and contributes to the cellular transformation. The immunohistochemical results of this study revealed that the combined treatment of ozone and NNK/DBP induced abnormal cell proliferation (Figs. 9 and 10, Table 1). Recently, Hildesheim *et al.* (2002) reported that c-myc protein suppressed the expression of GADD45 induced by p53 protein, a novel pathway through which myc promotes the cell cycle entry and prevents the growth arrest of the transformed cells. The amplification of c-myc was frequent in during the advanced stages of tumor invasion and was found to be associated with mutations in the p53 tumor-suppressor gene (Masramon *et al.*, 1998). These immunihistochemical results also confirmed the above results. The lungs and livers of the control and all treated groups were stained for PCNA, cyclin D1, c-myc, and mdm2 proteins through immunohistochemistry. In addition, the expression levels of the PCNA, cyclinD1, c-myc, and mdm2 proteins were higher in the lungs and livers of all combination-treated groups than in the ozone-treated ones and control group, which is revealed that combination of NNK and/or DBP with ozone, induced much more decrease of the p53 stability. In the c-myc proteins, the crucial relationship with other proteins was not found, however, the expression level of the mdm2 expressed in DNA damaged was increased in two or three combina-

tion trated groups (Figs. 9 and 10, and Table 1).

In conclusion, the expression levels of the proteins in cyclinD1, cyclinE, PCNA, cyclinG with increase of the mutant p53 were increased in two or three combined groups compared to the single treated groups and control. In other cell cycle related proteins, the effect of the change of the expression level was observed more clear in two or three combined treated groups than single treated groups. Based on these results, we are able to find out that increased mutant p53 by two or three combined treatment with NNK, DBP and ozone might be related closely with other cell cycle regulated proteins.

These results may be applied to the risk assessment of toxicity induced by treatment with combination of toxicants.

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