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Genotoxicity and Identification of Differentially Expressed Genes of Formaldehyde in human Jurkat Cells

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Abstract

Formaldehyde is a common environmental contaminant found in tobacco smoke, paint, garments, diesel and exhaust, and medical and industrial products. Formaldehyde has been considered to be potentially carcinogenic, making it a subject of major environmental concern. However, only a little information on the mechanism of immunological sensitization and asthma by this compound has been known. So, we performed with Jurkat cell line, a human T lymphocyte, to assess the induction of DNA damage and to identify the DEGs related to immune response or toxicity by formaldehyde. In this study, we investigated the induction of DNA single strand breaks by formaldehyde using single cell gel electrophoresis assay (comet assay). And we compared gene expression between control and formaldehyde treatment to identify genes that are specifically or predominantly expressed by employing annealing control primer (ACP)-based GeneFishing™ method. The cytotoxicity (IC_{30}) of formaldehyde was determined above the 0.65 mM in Jurkat cell in 48 h treatment. Based on the IC_{30} value from cytotoxicity test, we performed the comet assay in this concentration. From these results, 0.65 mM of formaldehyde was not revealed significant DNA damages in the absence of S-9 metabolic activation system. And the one differentially expressed gene (DEG) of formaldehyde was identified to zinc finger protein 292 using GeneFishing™ method. Through further investigation, we will identify more meaningful and useful DEGs on formaldehyde, and then can get the information on the associated mechanism and pathway with immune response or other toxicity by formaldehyde exposure.

Keywords: Formaldehyde, genotoxicity, comet assay, GeneFishing, Differentially Expressed Gene (DEG)

Formaldehyde is a common environmental contaminant found in tobacco smoke, paint, garments, medicinal and industrial products, and is a component of diesel and gasoline exhaust[1,2]. Formaldehyde is a typical volatile organic compound (VOC) and has recently received much attention as a chemical irritant responsible for sick building syndrome or sick house syndrome. VOCs are organic solvents that evaporate at room temperature, and diffuse into the rooms from building materials and furnishings. They pollute indoor air, and are thought to cause mucosal irritation of eyes and nose, headaches, and fatigue[3]. Also, Formaldehyde is a ubiquitous compound found in all biological systems liberated by the metabolism of a variety of exogenous and endogenous precursors. Various N-, O- and S-methyl compounds are oxidatively demethylated by demethylases resulting in formaldeyhyde formation[4,5].

Animal studies have demonstrated that high concentrations of formaldehyde can induce tumors in the nasal epithelium of rats and formaldehyde is suspected to be a human carcinogen[6-8]. The specific mechanism by which formaldehyde induces nasal cancer in rodents and upper respiratory tract cancer in humans is largely unknown. However, formaldehyde exposure induces sustained increases in reparative cell proliferation, DNA-protein crosslinks, and has been shown to be mutagenic in nasal respiratory tis-sues lining the rat nose at specific sites[9-13]. Also, ambi-ent formaldehyde, which primarily affects the upper airways and eyes as an irritant, has been shown to cause and exacerbate asthmatic symptoms[14,15]. Formaldehyde probably elicits airway inflammation associated with asthma, such as airway microvascular leakage, secretion into the airway lumen and denudation of airway epithelium[16,17].

As shown by Wantke *et al.*[18], there may be some population susceptible to formaldehyde behaving as an allergen, particularly in children. To date, indoor exposure to formaldehyde has not been proven as an important cause of immunologically mediated respiratory disease in adults.

This study examined whether formaldehyde has the genotoxic effects and has an influence on the expression of mRNAs, and which mRNAs are responsible for immune response evoked by formaldehyde exposure in Jurkat cells, the human T lymphocyte. The identification of differentially expressed genes (DEGs)

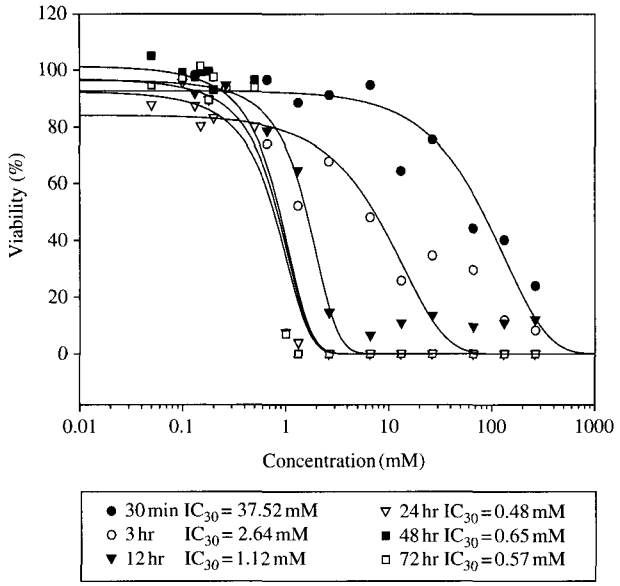

Fig. 1. Effect of formaldehyde on Jurkat cells viability. Dose- and time- response curve assessed at each time points by the trypan blue exclusion assay after treatment. Jurkat cells grown for 1 day in 12-well plates were exposed to formaldehyde in culture medium. IC_{30} value of formaldehyde was calculated to 0.65 mM at 48 h treatment. Values are expressed as percentage of corresponding controls.

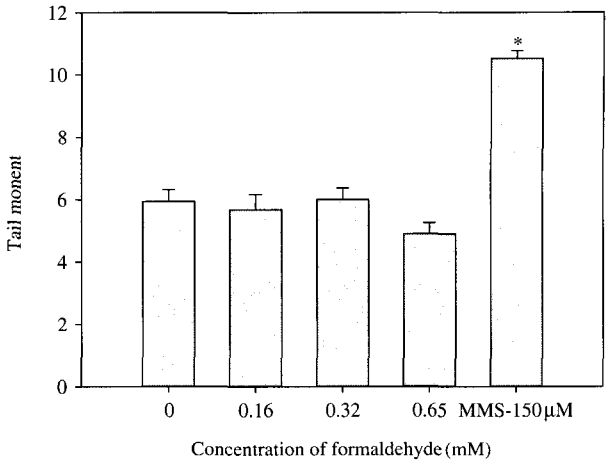

Fig. 2. Detection of formaldehyde -induced DNA strand breaks in Jurkat cells by the Comet assay. Means±SEM of four independent experiments.

may assist in the identification of potential biomarker and may understand molecular toxicological mechanisms of formaldehyde in human lymphocytes. So, we performed the comet assay for detection of genotoxicity and the GeneFishing™ techniques incorporating an annealing control primer, which has specificity to the template and allows only real products to be amplified[19], for identification of DEGs on formaldehyde exposure.

Results

Cytotoxicity of Formaldehyde in human Jurkat cells

Cytotoxicity of Jurkat cells following 30 min, 3 h, 12 h, 24 h, 48 h and 72 h exposure to a range of concentrations of formaldehyde was determined by trypan blue dye exclusion assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of the number of cells survived after treatment. Formaldehyde decreased the viability of the cells studied proportionally to the concentration. And based on results of cytotoxicity assay, 30% inhibitory concentration (IC_{30}) on Jurkat cell growth of formaldehyde was calculated (Fig. 1). As shown in Fig. 1, exposure of 0.65 mM formaldehyde for 48 h resulted in relative survival exceeded 70% compared to solvent control in Jurkat cells. This concentration was considered to be in the acceptable range for conducting the Comet assay[20]. So, we was used this concentration for Comet assay and GeneFishing™ method.

Assessment on DNA Damage of Formaldehyde using the Single Cell Gel Electrophoresis(Comet) Assay

Firstly, we screened using the comet assay in Jurkat cells whether formaldehyde could induce subtle DNA damages, following guideline recommended by IWGTP. The comet assay, as a high throughput toxicity screening tool for detection of DNA damage in mammalian cells, is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells. The intensity and the length of comet images were expressed in terms of the tail moment. Comet assay was carried out at IC_{30} values as maximum concentration. Figure 2 shows the effect of formaldehyde treatment (48 h) in Jurkat cells. While MMS leads to a strongly increased tail moment (10.55±0.25 compared with 5.941±0.36 in controls), formaldehyde (0.16-0.65 mM) did not cause a statistically significant change. At 0.65 mM, DNA migration of Jurkat cells treated to formaldehyde was slightly decreased compared to control (tail moment 4.91±0.34).

Differentially Expressed Amplified cDNA Products (DEGs) in Formaldehyde Treated Cells

To identify genes that are specifically or predominantly expressed in the formaldehyde treated Jurkat

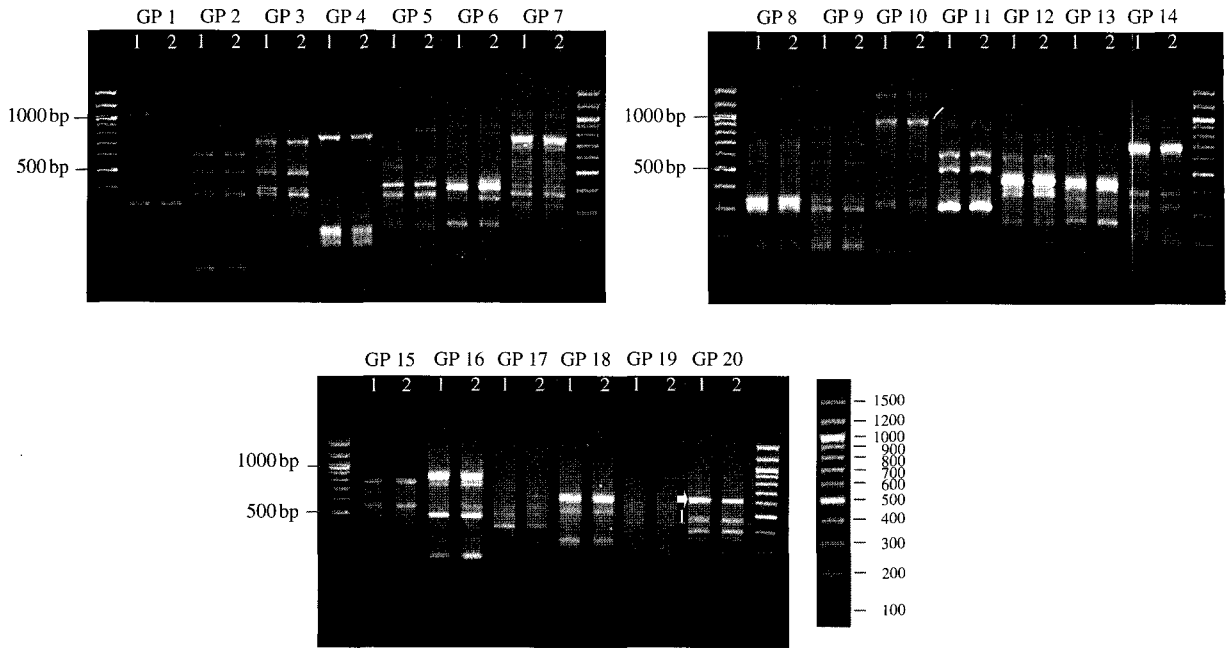

Fig. 3. Differential display of mRNA from Jurkat cells exposed to formaldehyde. 1, Distilled water exposed control; 2, 0.65 mM formaldehyde exposed; GP1-20, arbitrary primer 1-20. The candidate of differentially expressed genes was indicated by box.

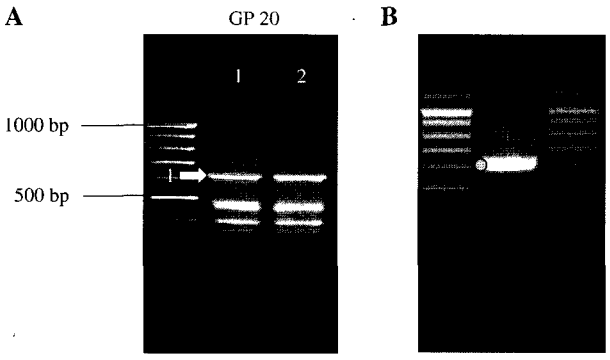

Fig. 4. (A) ACP RT-PCR using the arbitrary a ACP primer (#20) was performed with mRNA from Jurkat cells exposed to formaldehyde. 1, Distilled water exposed control; 2, 0.65 mM formaldehyde exposed; arrow, differentially expressed gene (DEG). (B) Reamplified product of DEG gel band extracted.

cells, we compared the mRNA expression profiles of vehicle treated cells and 0.65 mM formaldehyde treated cells. To do this, the mRNAs from both types of cells were extracted and subjected to ACP RT-PCR analysis using a combination of 20 arbitrary primers and two anchored oligo (dT) primers (dT-ACP 1 and dT-ACP 2). This method is depicted in the Materials and Methods. The analysis generated about 100 amplicons, 1 of which was markedly down-regulated in formaldehyde treated cells compared to control cells. Example of the identification of this DEG by this analysis is shown in Figure 3. For annotation of this DEG, we sequenced directly with extracted band of this PCR product. As result, this DEG was matched with zinc finger protein 292 (ZNF292) (Fig. 4).

Discussion

In this study, we demonstrated that formaldehyde at non-lethal concentrations exerts non-genotoxic effects with comet assay and effects on mRNA expression in human T lymphocyte, Jurkat cell line.

The living human body is constantly exposed to low concentrations of formaldehyde derived from various exogenous and endogenous sources potentially leading to endogenous and immediate alteration of cellular functions. Therefore, concerns on this chemical were evoked in the many research fields. Numerous studies on formaldehyde have primarily performed to elucidate a mechanism of carcinogenesis and toxicity in nasal derived model or respiratory related model[9-13,21]. Also, Inhaled chemicals as like formaldehyde are classified as air toxicants when they have the potential to produce adverse health effects in humans after inhalation exposure. These effects include carcinogenic as

well as noncarcinogenic responses such as asthma[22]. There are a few reports on the mechanism of immunological sensitization and asthma that may be induced by formaldehyde[23-27]. So, we performed with Jurkat cell line, a human T lymphocyte, to assess the induction of DNA damage and to identify the DEGs related to immune response or toxicity by formaldehyde.

In Jurkat cells, formaldehyde was not induced a significant DNA damage in the IC_{30} for 48 h. Especially, it was observed the reduced DNA migration after formaldehyde exposure of maximum concentration (0.65 mM) compared to control, in spite of not having a significance. It has also been reported by others. In the mouse lymphoma L5178Y cells (AstraZeneca, unpublished data), formaldehyde was also found to reduce the tail moment after 3 h of exposure to 0.1 mM, and the same effect was observed in freshly isolated human leukocytes after 4 h of exposure to 0.8 mM[28]. It is not known why the tail moment was significantly increased both in the vehicle control. As one hypothesis, Merk and Speit[29] noticed that the DNA/protein-crosslinking (DPC) agent formaldehyde reduced γ-ray induced DNA migration in V79 Chinese hamster cells. However, the biological significance of DPC for mutagenesis and carcinogenesis is at present poorly understood. It is still a matter of debate whether DPC are directly involved in the formation of mutations, whether specific types of mutations are induced and whether these are responsible for formaldehyde-induced carcinogenesis.

Numerous studies have shown that formaldehyde is genotoxic and mutagenic to mammalian cells[6,30,31]. Formaldehyde significantly induced DPC, sister chromatid exchanges and micronuclei with V79 and L5178Y cells, in parallel with the induction of cytotoxicity (relative cloning efficiency). In contrast, treatment with formaldehyde did not significantly induce gene mutations in the HPRT test with V79 cells[29,32]. Speit and Merk suggest that the mutagenic effect of formaldehyde is based on a clastogenic or recombinogenic mechanism and are in line with the observed strong effects of formaldehyde in cytogenetic tests[32,33].

To detect DEGs that are transcribed at low levels, highly specific polymerase chain reaction (PCR) amplification is required. Several reverse transcription-polymerase chain reaction (RT-PCR) methods have been used to date to identify novel genes or expressed sequences. Differential display methods, which are based on PCR using short arbitrary primers, are simple and fast but suffer from high rates of false positives. They are also biased toward detecting more abundant transcripts. Recently, suppression subtractive hybridization has been used to identify genes[34,35] but it is labor-intensive and also prone to false positives. The annealing control primer (ACP)-based RT-PCR method involves an ACP that has a unique tripartite structure in that its distinct 30- and 50-end portions are separated by a regulator. This ACP-based RT-PCR system is easy and accurate and lacks false positives[19,36].

In this study, GeneFishing™ method was applied to gene expression analysis of formaldehyde in Jurkat cells because of its effectiveness and convenience. We identified one gene down-regulated by formaldehyde, which was presumed to be the zinc finger protein 292. Considering that this data was a preliminary data using 20 arbitrary primers, further investigation have to be progress with more primer set and many meaningful and useful DEGs on formaldehyde may be identified.

Through these results, we can get the information on the associated mechanism and pathway with immune response or other toxicity by formaldehyde exposure.

Methods

Materials

37% Formaldehyde solution was purchased from Sigma (USA). RPMI-1640, trypan blue and fetal bovine serum (FBS) were the products of GIBCO® (California, USA). Low melting agarose was a product of Amresco (Solon, OH, USA). All other chemicals used were of analytical grade or the highest grade available.

Cell lines and Culture

Jurkat cell line was purchased from Korean Cell Line Bank and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture medium was 90% RPMI-1640 with 1 mM sodium pyruvate, 0.1% pluronic supplemented with 10% fetal bovine serum (FBS; Gibco, USA) plus penicillin, streptomycin. The cells had been maintained by 3-4 day passages.

Cytotoxicity (Cell Growth Inhibition)

Cytotoxicity of cells was checked by the trypan blue exclusion assay. For the determination of cell cytotoxicity, 1×10^6 Jurkat cells were treated to various concentrations of formaldehyde in 12-well plate for 30 min, 3 h, 12 h, 24 h, 48 h and 72 h, respectively. After the staining of 0.4% trypan blue (Life Technologies, MD, U.S.A), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer, and then average number of cells per section was calcul-

ated. Cell viability of treated chemical was related to controls that were treated with the solvent. The 30% inhibitory concentration (IC_{30}) of a particular agent was defined as that concentration that causes a 30% reduction in the cell number versus the untreated control. The IC_{30} values were directly determined from the semi-logarithmic dose-response curves. All experiments were repeated twice in an independent experiment.

Single Cell Gel Electrophoresis (Comet) Assay

DNA damages were detected by the alkaline version of standard comet assay described by Singh *et al.*[37,38] with minor modifications[20,39]. For the comet assay, 8×10^5 cells were seeded into 12 wells plate (Falcon 3043) and then treated with formaldehyde. At all doses of formaldehyde used in the experiment, the cell viability exceeded 70%. In the experiments, parallel cultures were performed and methyl methane sulfonate (MMS) were used as a positive control. After treatment with formaldehyde for 48 h, cells were centrifuged for 3 min at × 100 g (about 1,200 rpm), and gently resuspended with PBS and 100 μL of the cell suspension was immediately used for the test. Cells were mixed with 0.1 mL of 1% low melting point agarose (LMPA, Gibco BRL, Life Technologies, Inc., MD, USA) and added to fully frosted slide (Fisher Scientific, PA, USA), had been covered with a bottom layer of 100 μL of 1% normal melting agarose (Amresco, OH, USA). The cell suspension was immediately covered with cover glass and the slides were then kept at 4°C for 5 min to allow solidification. After removing the cover glass gently, the slides were covered with a third layer of 100 μL of 0.5% LMPA by using a cover glass and then the slide were kept again at 4°C for 5 min. The cells embedded in the agarose on slides were lysed for 1.5 h in reaction mixture of 2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris-HCl (pH 10), 1% Triton X-100 at 4°C. Slides were then placed in 0.3 M NaOH containing 1 mM Na_2-EDTA (approximately pH 13) for 20 min to unwind DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage. After the electrophoresis, the slides were washed gently to remove alkali and detergents that would interfere with ethidium bromide staining, by placing the slides vertically in glass jar containing 0.4 M Tris (pH 7.5) three times for 10 min. The slides were stained by 50 μL of ethidium bromide in distilled water solution on each slide, and then covering the slide with a cover glass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed each sample. All experiments were repeated in an independent test. Measurement was made by image analysis with Komet 3.1 (Kinetic Imaging Limited, Liverpool, UK) system, determining the mean tail moment (percentage of DNA in the tail times tail length) of the 50 cells. Differences between the control and the other values were tested for significance using one way of analysis of variance (ANOVA).

GeneFishing™ Reverse Transcription-Polymerase Chain Reaction

Total RNAs from Jurkat cells treated to formaldehyde and vehicle were isolated using Trizol (Gibco-BRL, Grand Island, NY, USA) and purified with RNeasy mini kit (Qiagen). Reverse transcription (RT) was conducted using the GeneFishing™ DEG kits (Seegene, Seoul, South Korea) as follows; 3 μg of the extracted RNAs were put into a tube containing RNase-free water and 10 μM dTACP1 with a final volume of 9.5 μL using a DNA Thermal Cycler 9600 (Perkin Elmer, Foster City, CA, USA). Equal amounts of RNA were compared with identify differentially expressed bands between the samples. The mixture was incubated at 80°C for 3 min and spun briefly after chilling on ice. Twenty μL of reaction solution, consisting of 5X RT buffer, 2 mM dNTP, RNase inhibitor, and M-MLV RT (Promega, Madison, WI, USA), was added to the mixture. The tube was incubated at 42°C for 90 min, heated at 94°C for 2 min, chilled on ice and spun briefly. The synthesized first-strand cDNA was diluted by adding 80 μL of RNase-free water. The cDNA samples were stored at 20°C until use. Polymerase chain reaction (PCR) amplification was conducted using the same GeneFishing™ DEG kits (Seegene Co. Korea) in 50 μL of reaction solution, consisting of 10X buffer without $MgCl_2$, 25 mM $MgCl_2$, 5 μM arbitrary ACPs, 10 μM dTACP2, 2 mM dNTP, 2.5 U Taq DNA polymerase (Genecraft, Ludinghausen, Germany), and 50 ng of first-strand cDNA, using a DNA Thermal Cycler 9600 (Perkin Elmer). This kit comprises 20 different arbitrary annealing control primers. The thermal cycler was preheated to 94°C before the tubes were placed in it. The program of PCR amplification was as follows; 1 cycle at 94°C for 5 min, 50°C for 3 min, and 72°C for 1 min, 40 cycles at 94°C for 40 s, 65°C for 40 s, and 72°C for 40 s, and 72°C for 5 min. The DNA fragments produced by PCR were separated by electrophoresis on a 2% agarose gel. The bands were photographed using polaroid film under ultraviolet light after ethidium bromide staining and analyzed by a

densitometry (Pharmacia, Uppsala, Sweden).

Direct Sequencing

The differentially expressed band was re-amplified and extracted from the gel by using the GENCLEAN® (II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly sequenced with ABI PRISM® (3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

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