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Benzene Induces Cytotoxicity without Metabolic Activation

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Abstract: Benzene Induces Cytotoxicity without Metabolic Activation: Takuro NISHIKAWA, *et al*. Departments of Environmental Medicine and Pediatrics, Graduate School of Medical and Dental Sciences, Kagoshima University—Objectives: Benzene has been consistently associated with hematological disorders, including acute myeloid leukemia and aplastic anemia, but the mechanisms causing these disorders are still unclear. Various metabolites of benzene lead to toxicity through the production of reactive oxygen species (ROS), the inhibition of topoisomerase and DNA damage. However, benzene itself is considered to have no mutagenic or cytotoxic activity. In this study, we investigated the effects of benzene itself on a human myeloid cell line with or without benzene metabolizing enzyme inhibitors. **Methods:** A human myeloid cell line, HL-60, was exposed to benzene with or without cytochrome P450 2E1 or myeloperoxidase inhibitor. Cytotoxicity was evaluated in terms of global DNA methylation levels, induction of apoptosis, and ROS production. **Results:** Benzene did not change global DNA methylation levels. However, benzene itself increased the levels of apoptosis and ROS. This cytotoxicity did not change with the addition of benzene metabolizing enzyme inhibitors. Benzene itself increased the mRNA levels of oxidative stress-related genes and transcription factors of activator protein-1. **Conclusions:** Benzene did not influence global DNA methylation in HL-60 cells, but had cytotoxic effects and changed gene expression levels. To elucidate the mechanisms of benzene toxicity, benzene itself as well as benzene metabolites must be investigated.
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Benzene is widely used for manufacturing gasoline and plastics, and is present in cigarette smoke[1)]. More than four million tons of benzene are produced annually in Japan[2)]. However, benzene induces bone marrow toxicity in humans, and causes hematopoietic malignancies and aplastic anemia[3)]. The most frequent human malignancy associated with benzene exposure is acute myeloid leukemia (AML)[1)]. The mechanism by which benzene induces leukemia and aplastic anemia has not been elucidated[1,3)].

It is believed that benzene does not initiate leukemia or aplastic anemia directly. Rather, it acts through a series of benzene metabolites[4–6)]. Benzene is primarily metabolized in the liver by cytochrome P450 2E1 (CYP2E1) to form reactive metabolites such as benzene oxide, catechol and hydroquinone, which can subsequently be metabolized in the bone marrow by myeloperoxidases (MPO) to produce benzoquinone metabolites [4)]. Due to the complexity of benzene's metabolic pathways and the variety of its metabolites, the specific genetic and cellular changes as well as the actual metabolites involved in benzene's toxic and carcinogenic effects have not yet been identified. Since various kinds of CYP, including CYP2E1 and MPO are expressed in the human myeloid cell line HL-60[7)], benzene can be metabolized in HL-60 cells.

Some reactive metabolites of benzene can undergo redox cycling, increasing the production of intracellular reactive oxygen species (ROS), which in turn may damage cellular macromolecules such as DNA, lipids, and proteins, which would lead to disruption of the cellular function[8)]. Benzenetriol, hydroquinone and phenol have been shown to increase levels of 8-oxo-deoxyguanosine[9,10)], a marker of oxidative DNA damage[11)], in HL-60 cells, which are used as a model system for examining the proliferation and differentiation of bone marrow cells. A growing body of evidence suggests that ROS are at least partly involved in mediating the cellular effects of hydroxylated benzene

metabolites[11]. However, it is not yet clear whether benzene itself causes the generation of ROS.

Altered DNA methylation may play an important role in the pathogenesis of AML. Global DNA hypomethylation and promoter hypermethylation of tumor suppressor genes are frequently observed in hematological malignancies [12]. Recent studies have indicated that occupational exposure to low levels of benzene is associated with altered DNA methylation patterns in the peripheral blood leukocytes of gas station attendants and traffic police officers[13]. However, no previous studies have evaluated the epigenetic changes caused by benzene through the use of myeloid cell lines[14].

In this study, we examined the effects of benzene on global DNA methylation in a human myeloid cell line, HL-60. We also determined the cytotoxicity of benzene itself in HL-60 cells with the use of benzene metabolizing enzyme inhibitors, and discuss the mechanism of benzene toxicity.

Materials and Methods

Cell culture and treatments

A human myeloid cell line, HL-60, was kindly supplied by the Japanese Cancer Research Resource Bank. It was maintained in RPMI 1640 medium (SIGMA-Aldrich, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT). The cells were grown in a humidified atmosphere at 37°C in 5% CO_2. Viability was assessed by the trypan blue dye exclusion test.

Reagents

Benzene was purchased from SIGMA-Aldrich (CHROMASOLV® Plus, for HPLC, purity ≥ 99.9%) and Wako Pure Chemical (purity; 99.8%, Osaka, Japan). Chlormethiazole-HCl (CMZ) and dimethyl sulfoxide (DMSO) were purchased from SIGMA-Aldrich. 4-Aminobenzoic acid hydrazide (ABAH) was purchased from Tokyo Chemical Industry (Tokyo, Japan). H_2O_2 was purchased from Santoku Chemical Industries (Tokyo, Japan). Catalase was purchased from Boehringer-Mannheim (Mannheim, Germany) and Worthington Biochemical (Lakewood, NJ). 5-Aza-2'-deoxycytidine was purchased from Wako Pure Chemical.

Determination of 5-methyl-2'-deoxycytidine (mdC) by HPLC

HL-60 cells were suspended in RPMI-1640 containing 10% FCS at 4×10^5/m*l*, and benzene diluted in DMSO was then added. The final concentration of DMSO was less than 0.1%. The cells were incubated at 37°C in 5% CO_2. After being exposed to benzene for 72 h, the cells were immediately chilled in an ice-water bath, washed with ice-cold Dulbecco's phosphate buffered saline (DPBS) and then stored as cell pellets at –80°C until analysis. The cells were incubated with ribonuclease A (10 μg/sample, Nacalai Tesque, Kyoto, Japan) at 37°C for 30 min, and DNA was then extracted from the cells with a QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan) and enzymatically digested to nucleosides, as described elsewhere[15]. The nucleosides were separated with ODS-80TS (TOSOH, Tokyo, Japan), as described elsewhere[15]. 2'-Deoxycytidine (dC), 5-methyl-2'-deoxycytidine (mdC) and 2'-deoxyguanosine (dG) were visualized by UV/Visible detection at 280 nm. Reference standards of dC (Wako Pure Chemical), mdC (Tokyo Chemical Industry), and dG (SIGMA-Aldrich) were used. %mdC was calculated as 100 x (moles of mdC/moles of dG).

Evaluation of apoptosis by flow cytometry

HL-60 cells suspended in RPMI-1640 at 4×10^5/m*l* were incubated with or without benzene at 37°C in 5% CO_2 for 1 h. FCS was added to the cells at 10%, and the cells were incubated further. Benzene concentrations in the medium were reported to decrease rapidly at 37ºC[16]. FCS contains proteins and lipid, and may adsorb benzene. The presence of FCS generates bubbles during the preparation of benzene solutions. To avoid any effects of FCS on apoptosis, we prepared benzene solution without FCS, incubated HL-60 cells for 1 h in the absence of FCS, and then added FCS to reduce cell damage due to the serum-free conditions[17]. After incubation, the cells were harvested by centrifugation, washed with ice-cold DPBS and fixed with 70% ethanol overnight at –20°C. The fixed cells were suspended with DPBS containing propidium iodide (PI; SIGMA-Aldrich) (15 μg/m*l*) and RNase A (50 μg/m*l*) and incubated for 30 min at 37°C in the dark. Apoptotic cells were measured using a FACScan cytometer (Becton Dickinson, Mountain View, CA) and the data were analyzed using WinMDI software version 2.9. Apoptotic cells were determined as the percentage of signals to the left of the G1 peak (sub G0/G1 population, see Fig. 2a)[18].

For the detection of phosphatidylserine as a marker of apoptosis, an Annexin V-fluorescein isothiocyanate (FITC) and PI double-labeling kit purchased from Trevigen (Gaithersburg, MD) were used, and apoptotic cells were stained according to the manufacturer's instructions. Briefly, after 8 h of exposure to benzene with or without 250 U/m*l* of catalase as described above, samples were harvested and washed, and then stained with Annexin V-FITC and PI. The cells were evaluated using FACScan, and the data were analyzed with WinMDI software version 2.9. Apoptosis was determined by evaluating the percentage of events that accumulated in the Annexin V-FITC-positive and PI-negative position (see Fig. 2c). For experiments with CMZ and ABAH, HL-60 cells were pre-incubated with RPMI1640-10% FCS containing 50 μM CMZ, 100 μM ABAH, or both CMZ (50 μM) and ABAH (100 μM) for 20 h. Cells were then suspended in RPMI-1640 containing the same concentrations of enzyme

inhibitors and exposed to 5 mM benzene. After 1 h of incubation, FCS was added to the cells to a final concentration of 10%, and the cells were incubated for 7 h. The double-staining procedures were the same as those described above.

Measurement of the intracellular generation of ROS

HL-60 cells suspended in phenol red-free RPMI-1640 at 4×10^5/m*l* were incubated with dichlorofluorescin-diacetate (DCFH-DA; Molecular Probes), as described elsewhere[19], and then exposed to 5 mM benzene with or without catalase (250 U/m*l*) for 60 min. The oxidative conversion of DCFH to dichlorofluorescein (DCF) was measured using FACScan. For experiments with CMZ, the cells were pre-treated as described above in "Evaluation of apoptosis by flow cytometry", and then suspended in phenol red-free RPMI-1640 at 4×10^5/m*l*. After the addition of the same concentration of CMZ, loaded with probes, the cells were exposed to 5 mM benzene for 60 min at 37°C. ROS levels were measured as described above.

Determination of mRNA levels by real-time PCR

Real-time PCR was used to measure the mRNA levels of epigenetic-related enzymes, and cell cycle regulatory, apoptosis-related, oxidative stress-related and transcription factor-related genes (Table 1). HL-60 cells were exposed to 5 mM benzene as described above in the section entitled "Evaluation of apoptosis by flow cytometry". Total RNA was then isolated from the cells by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Complementary DNA was synthesized by reverse transcription from total RNA using reverse transcriptase and oligo-dT20 (Toyobo, Osaka, Japan). The resulting cDNA was amplified using the FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Mannheim, Germany) under the following conditions: 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Real-time PCR was performed using a Thermal Cycler Dice® Real Time System (Takara Bio Inc., Otsu, Japan). The details of the primers used in this experiment are shown in Table 1. The relative level of mRNA was calculated using cycle time (*Ct*) values, which were normalized against the value of *GAPDH*. Relative quantification (fold change) between different samples was calculated according to the $2^{-\Delta\Delta Ct}$ method[20].

Statistical analysis

Values are shown as means ± standard error (SE). Statistical analysis was performed using StatView version 5.0 for Windows (SAS, Institute Inc., Cary, NC). Differences between groups were tested by non-parametric Wilcoxon tests. A probability value of less than 0.05 was considered to be statistically significant.

Results

Levels of global DNA methylation

In HL-60 cells exposed to 5 mM benzene, the cell number was less than in the control from 24 to 48 h (Fig. 1a). However, the levels of %mdC did not change in the presence of benzene in concentrations up to 5 mM (Fig. 1b). The levels of %mdC from 4 to 96 h of incubation also did not change (Table 2). Although the %mdC levels in HL-60 cells incubated with 1.3% DMSO did not change relative to the control, the %mdC levels with 100 μM 5-aza-2-deoxycytidine (AzaC) decreased significantly to around 1.4% compared to 4% in the control (Fig. 1c).

Levels of apoptotic cells

Figure 2b shows that the proportion of sub G0/G1 cells (corresponding to apoptotic cells, see Fig. 2a) increased among cells exposed to benzene after incubation for 4, 8 and 24 h. The induction of apoptosis by benzene was confirmed by another method. Annexin V-FITC-positive and PI-negative cells, which are considered to be apoptotic, also increased in HL-60 cells exposed to benzene alone (Fig. 2c). This increase was dependent on the benzene concentration (Fig. 2d). Since the increase at 0.05 mM and 0.5 mM was slight, albeit significant, we investigated the effects of benzene at 5 mM in later experiments. Neither CYP2E1 inhibitor (CMZ), MPO inhibitor (ABAH) nor catalase altered the levels of apoptosis induced by benzene (Fig. 2e, f).

Levels of intracellular ROS

Intracellular ROS were quantified by a flow-cytometric analysis of the ROS-sensitive fluorescent probe DCFH-DA. Fluorescence intensity was significantly increased in HL-60 cells exposed to 5 mM benzene for 60 min, and was decreased by co-incubation with catalase (Fig. 3a, b). We also investigated the effects of CMZ on ROS generation. Exposure to benzene consistently increased the fluorescence intensity (2.34 ± 0.40, mean ± SE, n=17). Exposure to benzene also increased the fluorescence intensity (1.56 ± 0.41) in cells that had been pretreated with CMZ, however, this increase was significantly less than that found in cells that were not treated with CMZ. To determine the levels of ROS by flow cytometry, we selected a presumably intact cell population by a gate (polygon in Fig. 3C). Exposure to benzene decreased the population from 80.67 ± 0.87% to 76.06 ± 1.25%. Exposure to benzene also decreased the population that had been pretreated with CMZ from 80.13 ± 1.02% to 75.55 ± 1.40%. In contrast to the generation of ROS, CMZ did not affect the decrease of the cell population induced by exposure to benzene.

Expression levels of mRNAs of various genes

We investigated the expression levels of mRNA of 30

Table 1. Primer sets used in this study

Category	Gene name	Product size (bp)	Forward primer	Reverse primer	Reference
Epigenetic systems	*DNMT1*	142	5'-GAAGAATTATCCGAGGAGGGCTA-3'	5'-GGGCTTCACTTCTTGCTTGGTT-3'	-
	DNMT3A	111	5'-ACGCCAAAGGACCCTGCGGT-3'	5'-TGGCTGGGGCTCACTCCGCT-3'	-
	DNMT3B	156	5'-TTCCCCACGTTCCACCCGAG-3'	5'-GTGAGGTCGATGGTAAGGTAAGAGC-3'	-
	HDAC1	102	5'-CAAGCTCCACATCAGTCCTTCC-3'	5'-TGCGGCAGCATTCTAAGGTT-3'	(31)
	SUVH1	115	5'-AAGAAGATCCGCGAACAGGAA-3'	5'-GGAACTGCTTGAGGATACGCAC-3'	(31)
	SUVH2	102	5'-ATCCCACCTGGTACTCCCATCT-3'	5'-GCAAAGCGAATACTGTGTGCC-3'	(31)
	EZH2	144	5'-GCGACTGAGACAGCTCAAGAGGT-3'	5'-GTCAGGATGTGCACAGGCTGT-3'	(31)
	EP300	126	5'-ATGGCCGAGAATGTGGTGGAAC-3'	5'-GTCGTGCTCCAAGTCAAATAGAG-3'	(31)
Enzyme	*TOP2A*	75	5'-AGTCGCTTTCAGGGTTCTTGAG-3'	5'-TTTCATTTACAGGCTGCAATGG-3'	(32)
	MPO	123	5'-AAGCTGCTTCTGGCCCTAGCAG-3'	5'-CTCCTCCATGGAGCTCAGCAC-3'	-
Apoptosis	*BCL2*	135	5'-GCAGAAGTCTGGGAATCGATCTG-3'	5'-TGCATAAGGCAACGATCCCATC-3'	-
	BAX	92	5'-CCTGTGCACCAAGGTGCCGG-3'	5'-GGTCTTGGATCCAGCCCAACAG-3'	-
	NOXA	153	5'-CGCGCAAGAACGCTCAACCGA-3'	5'-GCAGTCAGGTTCCTGAGCAGAAG-3'	-
	SOCS1	178	5'-GGAGCGGATGGGTGTAGGGG-3'	5'-GAGGTAGGAGGTGCGAGTTCAG-3'	(33)
Oxidative stress	*HMOX1*	119	5'-GAGGAACTTTCAGAAGGGCCAG-3'	5'-AGACTGGGCTCTCCTTGTTGCG-3'	(34)
	NQO1	119	5'-GCTCCAAGCAGCCTCTTTGAC-3'	5'-GACTTGCCCAAGTGATGGCC-3'	-
	KEAP1	121	5'-ACGGCTGCATCCACCACAACA -3'	5'-AGGAGACGATTGAGGACAGCCA-3'	-
	NFKB1	134	5'- AATGACAGAGGCGTGTATAAGG -3'	5'-GAGCTGCTTGGCGGATTAG-3'	-
	RELA	130	5'-GTTCACAGACCTGGCATCC-3'	5'-TGTCACTAGGCGAGTTATAGC-3'	(35)
	SOD1	236	5'-CATCATCAATTTCGAGCAGA -3'	5'-GCCACACCATCTTTGTCAGCAG-3'	(36)
	SOD2	181	5'-ACTGCAAGGAACAACAGGCC -3'	5'-CAGCATAACGATCGTGGTTTAC-3'	-
	CAT	188	5'-GCGGTCAAGAACTTCACTGA-3'	5'-GCTAAGCTTCGCTGCACAGGT-3'	(34)
	OGG1	145	5'-CCCCAGACCAACAAGGAACT-3'	5'-TGGAACCCTTTCTGCGCTTT-3'	(37)
	GPX1	174	5'-CCTCCCCTTACAGTGCTTGT-3'	5'-GTACCTTGCCCCGCAGGG-3'	-
AP-1	*FOS*	159	5'-CTCCGTGCCAGACATGGACCTATCT-3'	5'-GAAGACGTGTAAGCAGTGCAGCTG-3'	-
	JUN	142	5'-AAGAACTCGGACCTCCTCACCT-3'	5'-CGTTCTTGGGGCACAGGAACTG-3'	-
Cell cycle	*CDKN2A(ARF)*	87	5'-GAGAACATGGTGCGCAGGT-3'	5'-GATGTGAACCACGAAAACCCTC-3'	(38)
	CDKN2A (p16)	150	5'-ACTCTCACCCGACCCGTGCA-3'	5'-GACATCGCGATGGCCCAGCT-3'	-
	CDKN1A (p21)	146	5'-GGAAGACCATGTGGACCTGT-3'	5'-GGCGTTTGGAGTGGTAGAAA-3'	(39)
	CDKN2B (p27)	134	5'-GGAGAACAAGGGCATGCCCAG-3'	5'-TCCCGAAACGGTTGACTCCGTTG-3'	-
Internal Control	*GAPDH*	120	5'-CCATGGCACCGTCAAGGCTGA-3'	5'-ACGACGTACTCAGCGCCAGCA-3'	(40)

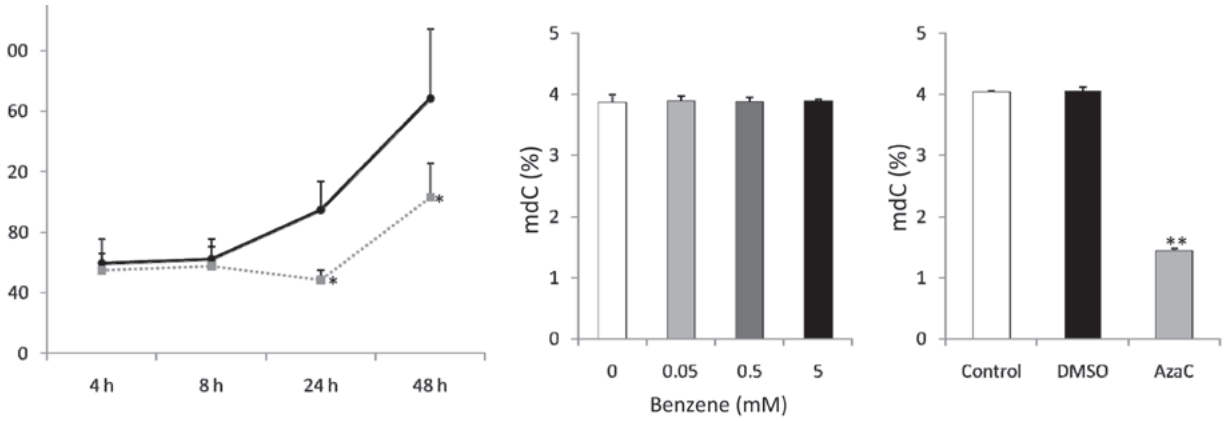

Fig. 1. Cell numbers and %mdC levels of HL-60 cells exposed to benzene and other reagents. (a) Growth of HL-60 cells and HL-60 cells exposed to benzene for up to 48 h. HL-60 cells were exposed to 5 mM benzene diluted with DMSO (■, gray line). Closed circles (●) indicate results for HL-60 cells incubated with 0.1% DMSO. (b) %mdC levels in HL-60 cells exposed to the indicated concentrations of benzene (up to 5 mM) for 72 h. (c) %mdC levels in HL-60 cells incubated for 72 h with no supplement (Control), 1.3% DMSO or 100 μM AzaC. The data are presented as means + SE values from four to eight independent experiments, except for (c). For (c), data are obtained from two independent experiments conducted in quadruplicate. %mdC was determined as described in Materials and Methods. *p<0.05, **p< 0.01 vs. Control.

Table 2. Changes in %mdC as a function of incubation time with 5 mM benzene

	4 h	8 h	24 h	48 h	96 h
DMSO	4.04 ± 0.03	4.07 ± 0.02	4.04 ± 0.03	4.07 ± 0.05	4.05 ± 0.03
Benzene	4.05 ± 0.04	4.08 ± 0.03	4.05 ± 0.06	3.99 ± 0.05	4.01 ± 0.03

Benzene was diluted with DMSO. Data are means ± S.E. from four independent experiments.

genes (Table 1) in HL-60 cells that had/had not been exposed to benzene. Among genes related to epigenetic systems such as DNA methyltransferases (Dnmts) and histone modification enzymes, only the mRNA levels of *DNMT3A* were increased 2-fold after 4 h of exposure to 5 mM benzene (Fig. 4a). The mRNA level of the genes related to cell cycle regulation and apoptosis, such as *p15, p16* and the *BCL2* protein family, were not changed by exposure to benzene (data not shown). On the other hand, mRNA levels of oxidative stress-related genes, such as *NQO1* and *HMOX1*, and transcription factor-related genes, such as *FOS* and *JUN*, increased after exposure to benzene (Fig. 4b–e).

Discussion

Benzene, an important industrial chemical and a ubiquitous environmental pollutant, is an established carcinogen and myelotoxicant. Epidemiological studies have found that, exposure to benzene is associated with the development of AML and aplastic anemia in humans. However, the mechanism by which benzene induces hematological disorders has not been elucidated[1, 3].

Recently, altered DNA methylation has been suggested in epidemiological studies to be one of the potential mechanisms underlying the leukemogenesis associated with benzene[13]. Global DNA hypomethylation may play an important role in human leukemogenesis contributing to carcinogenesis through the generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting[21]. Ji *et al*. reported that hydroquinone, a metabolite of benzene, induced global DNA hypomethylation in TK6 lymphoid cells[14] . However, in our study, benzene did not induce global DNA hypomethylation at all (Fig. 1b). In addition, after cells were exposed to benzene for 4, 8, 24, 48, and 96 h, %mdC levels were not changed (Table 2). The difference in the cell lines used for the experiments may explain these conflicting results. Other possible explanations are the differences in the hydroquinone concentrations used in the experiments or the co-existence of other metabolites in our experiment. Since we tried to elucidate the carcinogenic mechanism of benzene in AML, we investigated the DNA methylation status after exposure to benzene in a myeloid cell line, HL-60 . The mRNA level of *DNMT3A*, which causes *de novo* DNA methylation, increased about 2-fold after 4 h of exposure to benzene

(Fig. 4a). This finding suggests that benzene may affect the epigenetic profile, such as promoter hypermethylation of tumor suppressor genes, but not global DNA methylation. The %mdC levels in HL-60 cells in our experiments are similar to those reported earlier by Aarbakke, *et al*.[22] and decreased when cells were incubated with AzaC.

It is generally accepted that benzene toxicity is mediated through the metabolic formation of reactive metabolites [3–6]. Various metabolites of benzene are considered to lead to toxicity through the production of ROS, inhibition of topoisomerase and induction of DNA damage[3–6]. We investigated whether benzene itself is actually cytotoxic. Through the use of two different methods, we confirmed that benzene induced apoptosis in HL-60 cells. Benzene increased the sub G0/G1 fraction and Annexin V-FITC-positive/PI-negative cells after 8 h of exposure (Fig. 2). We then investigated whether this action of benzene was due to its metabolites. We pretreated HL-60 cells with CMZ, ABAH or both CMZ and ABAH, and then exposed the treated cells to benzene in the presence of inhibitors. These inhibitors did not change the levels of apoptosis induced by benzene. This finding suggests that benzene itself induced apoptosis in HL-60 cells. To exclude the possibility that contaminants in the benzene preparation increased the levels of apoptosis, we used two benzene preparations from two different manufacturers with different purities (99.8 and 99.9%). At the same benzene concentration, the benzene preparations showed similar results, indicating that benzene itself, rather than any contaminants, induced apoptosis. Figure 2b shows that the proportion of sub G0/G1 cells decreased as incubation proceeded. Slight apoptosis may occur in HL-60 cells at the time of exposure to benzene due to incubation without FCS and mechanical stirring. Such apoptotic cells may be lost or degraded further during incubation.

Benzene metabolites have been reported to generate ROS in HL-60 cells[10], and ROS induce apoptosis in various cell lines[8,9]. Therefore, we determined intracellular ROS levels by flow cytometry with DCFH . Non-fluorescent DCFH has been reported to change to fluorescent DCF upon reaction with H_2O_2 and peroxidases[23]. Exposure to benzene increased the fluorescence of HL-60 cells and this fluorescence was decreased by the addition of catalase. Even though HL-60 cells were pretreated with CMZ, the fluorescence intensity increased in HL-60 cells exposed to 5 mM benzene. This finding indicates that benzene itself generated ROS in HL-60 cells. Although CMZ partially inhibited the generation of ROS caused by exposure to benzene, CMZ did not inhibit apoptosis or the decrease of a presumably intact cell population. While benzene metabolites might be involved in generation of ROS, we consider the cytotoxicity seen in our experiments to be caused by benzene itself rather than by any benzene metabolites. Catalase, which decreased the levels of ROS induced by benzene, did not suppress the level of apoptosis induced by benzene. Therefore, ROS might not be a trigger of apoptosis in HL-60 cells exposed to benzene. Although frequently used solvents, including DMSO and ethanol, did not induce apoptosis in HL-60 cells (data not shown), lipophilic solvents such as benzene might cause apoptosis due to a direct effect on the cellular membrane. We are planning to explore whether other lipophilic solvents induce apoptosis in human cell lines.

Since benzene itself increased the levels of ROS in HL-60 cells, we studied the expression levels of genes of a redox-sensitive signaling pathway by real-time PCR. Exposure of HL-60 cells to benzene resulted in a remarkable increase in oxidative stress-related genes (*HMOX1* and *NQO1*) (Fig. 4b, c) and transcription factor (*FOS* and *JUN*) mRNA levels (Fig. 4d, e). *HMOX1* and *NQO1* act as intracellular antioxidant molecules regulated by Nrf-2 (NF-E2 related factor-2)[24]. *FOS* and *JUN* belong to the AP-1 (activator protein) family, which regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, and stress. AP-1 in turn controls several cellular processes including differentiation, proliferation, and apoptosis[25] . Activation of the *FOS* gene has been especially associated with the promotion of neoplastic transformation[25]. In our study, mRNA levels of NFκB signaling-related genes did not increase. This may have been because the oxidative stress that is induced by benzene is only weak, or specifically activates Nrf-2 and AP-1 signaling. Depending on the level of ROS, different redox-sensitive transcription factors are activated and coordinate distinct biological responses. The expression levels of genes change due to mechanical stress[26, 27], and may be modulated by changes in the CO_2 concentration or pH under exposure to benzene. Such effects may remain and induce large variations, especially in experiments with 4 h of incubation. We did not investigate the changes in gene expression levels in CMZ-pretreated cells because CMZ itself may affect gene expression levels[28].

The concentrations of benzene used in this study were relatively high. However, 5 mM benzene corresponds to 0.045% (v/v) and 395 ppm. Aksoy reported that benzene concentrations in workplaces were 210 to 650 ppm and those in adhesives and thinners were between 9 and 88%[29]. In a C57B1/6 mouse experiment, the incidence of leukemia was increased after exposure to benzene at 300 ppm[30]. Therefore, our benzene concentrations were similar to those that have been shown to lead to the development of leukemia after long-term exposure.

Conclusion

While benzene had no influence on global DNA methylation levels in HL-60 cells, benzene itself induced cytotoxic effects and changes in gene expression levels. To better understand the mechanisms that underlie the

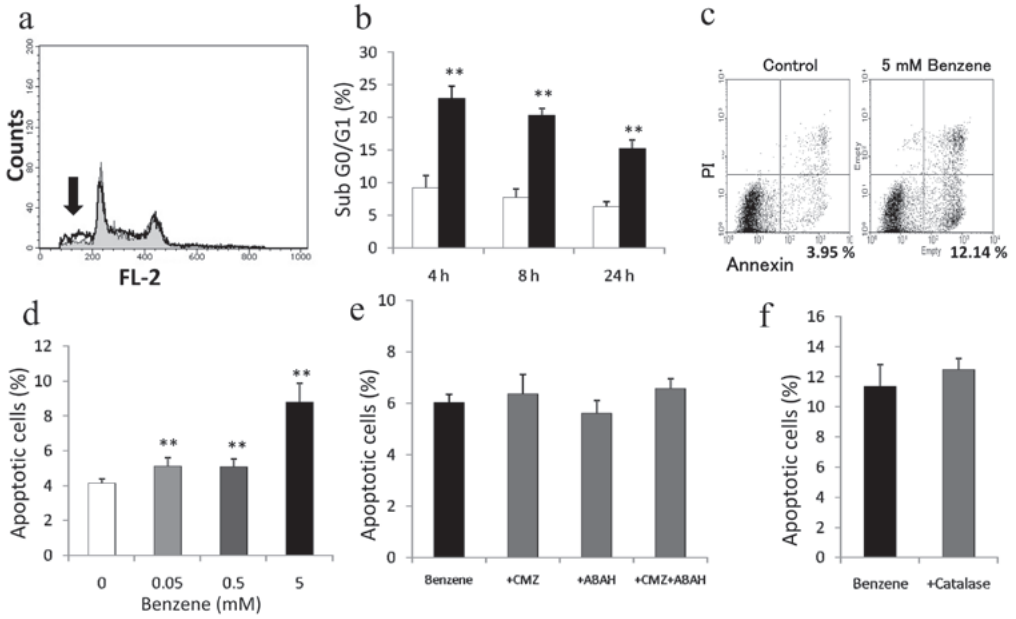

Fig. 2. Proportion of apoptotic cells in HL-60 cells exposed to benzene and other reagents. (a) Representative histograms of ethanol-fixed and PI-stained HL-60 cells exposed to 0 (filled gray line) or 5 mM benzene (black line) for 8 h. Benzene was diluted directly with RPMI-1640, and HL-60 cells were exposed to 0 or 5 mM benzene in RPMI-1640 for 1 h. Next, FCS was added to the HL-60 cells at 10%. PI staining was carried out as described in Materials and Methods. The arrow shows the sub G0/G1 phase. (b) The percentage of cells in the sub G0/G1 phase was measured in HL-60 cells (□) and HL-60 cells exposed to 5 mM benzene (■) for 4, 8 and 24 h. The exposure conditions were the same as those described above. (c) Representative dot grams of HL-60 cells (left) and HL-60 cells exposed to 5 mM benzene (right) for 8 h. HL-60 cells were double-stained with Annexin V-FITC and PI as described in Material and Methods. Cells in the lower right quadrant, which were stained with Annexin V-FITC but not PI, were considered to be apoptotic. The percentages of cells in the lower right quadrant are listed. The exposure conditions were the same as those described above. (d) Percentages of apoptotic cells in HL-60 cells and HL-60 cells exposed to benzene (up to 5 mM) for 8 h. The exposure conditions and double-staining procedures were the same as those described above. (e) Percentages of apoptotic cells in HL-60 cells exposed to 5 mM benzene for 8 h in the presence of enzyme inhibitors. HL-60 cells were incubated with RPMI1640-10% FCS containing 50 μM CMZ (CYP2E1 inhibitor), 100 μM ABAH (MPO inhibitor), or both CMZ (50 μM) and ABAH (100 μM) for 20 h. Cells were then suspended in RPMI-1640 containing the same concentrations of enzyme inhibitors and exposed to 5 mM benzene. After 1 h of incubation, FCS was added to the cells at 10%, and the cells were incubated for 7 h. The double-staining procedures were the same as those described above. (f) Percentages of apoptotic cells in HL-60 cells exposed to 5 mM benzene for 8 h with or without 250 U/m*l* of catalase (H_2O_2 scavenger). The exposure conditions and double-staining procedures were the same as those described above. The data are presented as means + SE from three to eight independent experiments. $^{**}p<0.01$ vs. HL-60 cells that were not exposed to benzene in the corresponding condition.

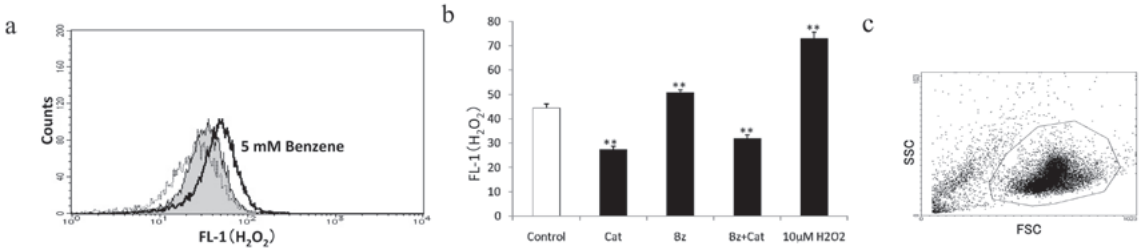

Fig. 3. Levels of intracellular ROS after exposure to benzene.(a) Representative histograms of HL-60 cells exposed to nothing (gray, filled), 5 mM benzene (black bold line) or 5 mM benzene with 250 U/m*l* catalase (dotted line) . Intracellular ROS levels were quantified using flow-cytometry analysis with the ROS-sensitive fluorescent probe DCFH-DA as described in Materials and Methods. (b) Fluorescence intensities of HL-60 cells exposed to nothing (Cont), catalase (Cat), 5 mM benzene (Bz), 5 mM benzene and catalase (Bz+Cat) or 10 μM H_2O_2 (■) for 60 min. ROS levels were measured as described above. (c) A representative dot gram of HL-60 cells. To analyze ROS in cells, we selected presumably intact cells with a polygon. The data are presented as means + SE of three independent experiments conducted in duplicate. $^{**}p<0.01$ vs. Control.

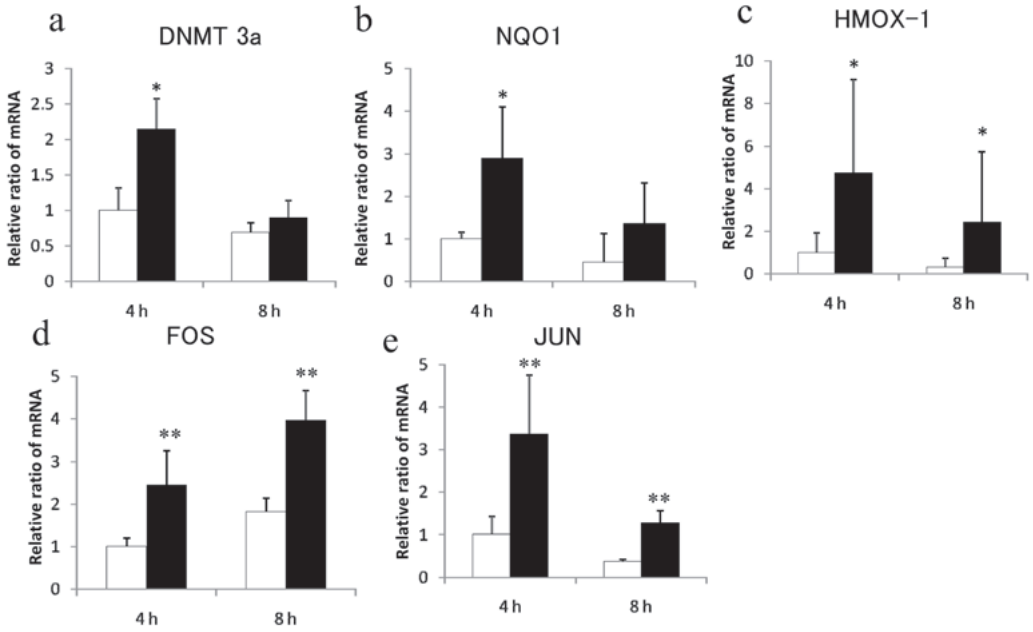

Fig. 4. Expression levels of mRNA of various genes in HL-60 cells exposed to benzene. HL-60 cells were exposed to nothing (□) or 5 mM benzene (■) for 4 or 8 h as described in the text and the legend for Fig. 2(a). The mRNA levels of an epigenetic systems-related gene (*DNMT3A*), oxidative stress-related genes (*NQO1, HMOX1*), and transcription factor-related genes of the AP-1 family (*FOS, JUN*) were determined as described in Materials and Methods. The relative values for the respective mRNA in HL-60 cells without exposure at 4 h were set to 1.0. The data are presented as means + SE of five independent experiments. *p<0.05, **p<0.01 vs. HL-60 cells that were not exposed to benzene in the corresponding condition.

toxic and carcinogenic effects of benzene, we should investigate the effects of benzene itself as well as those of benzene metabolites.

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