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Generation of phosphorylated histone H2AX by benzene metabolites

Mio Ishihama, Tatsushi Toyooka, Yuko Ibuki *

Laboratory of Radiation Biology, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1, Yada, Shizuoka-shi 422-8526, Japan

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ABSTRACT

Benzene is a well known environmental carcinogen which causes myeloid leukemia. DNA damage induced by benzene metabolites such as hydroquinone (HQ) and *p*-benzoquinone (BQ) is one reason for the leukemogenesis. In this study, we showed that treatment with HQ and BQ quickly and clearly generated phosphorylated histone H2AX (γ-H2AX) which has been recently considered an index of the production of double strand breaks (DSBs). HQ and BQ produced discrete foci of γ-H2AX within the nucleus of HL-60 cells in a dose-dependent manner. γ-H2AX appeared after the treatment with HQ and BQ for 2 h, and increased time-dependently up to 4–8 h. HQ and BQ increased intracellular oxidation, and an antioxidant, *N*-acetylcysteine, clearly inhibited the phosphorylation, suggesting that reactive oxygen species produced from HQ and BQ contributed to the generation. γ-H2AX was sensitively detected after treatment with low concentrations of HQ and BQ, compared with the direct detection of DSBs by biased sinusoidal field gel electrophoresis and with the assessment of cytotoxicity based on cell survival. DSBs are the most serious form of DNA damage and are associated with genomic instability leading to myeloid leukemia. γ-H2AX may be a useful tool for judging the genotoxicity of benzene metabolites sensitively.

1. Introduction

Benzene is a ubiquitous environmental chemical that is used mainly as a precursor in the synthesis of numerous products including drugs, dyes, insecticides, and plastics. It is recognized internationally as an hematotoxin and carcinogen, and chronic exposure to it causes acute myeloid leukemia (Golding and Watson, 1999; Huff, 2007). The mechanisms by which benzene exerts its leukemogenic effects are still not well understood, though the results of several studies support the hypothesis that the metabolism of benzene into reactive intermediates is a crucial event in this process (Greenlee et al., 1981; Snyder and Hedli, 1996). The metabolism of benzene produces a number of metabolites, which include benzene oxide, phenol and several hydroxylated compounds (hydroquinone (HQ) and catechol). These metabolites, especially HQ, are thought to be transported to the bone marrow, the site of benzene toxicity, where they can be further metabolized through the actions of peroxidases to the more reactive species *p*-benzoquinone (BQ).

The benzene metabolites, HQ and BQ, have been shown to cause some DNA damage (Snyder and Hedli, 1996; Golding and Watson, 1999; Whysner et al., 2004) including DNA strand breaks (Andreoli et al., 1997; Fabiani et al., 2001), DNA oxidation (Hiraku and Kawanishi, 1996; Oikawa et al., 2001) and DNA adducts (Chenna et al., 1995; Bodell et al., 1996; Gaskell et al., 2004, 2005). These lesions contribute to the leukemogenic effect of benzene. One of the causes of DNA damage is reactive oxygen species (ROS), which are generated from HQ and BQ via both metabolism and auto-oxidation in aqueous solution at physiological pH (Greenlee et al., 1981; Lewis et al., 1988; Snyder and Hedli, 1996). DNA damage caused by ROS includes oxidized bases, abasic sites, DNA strand break and DNA-protein cross links. Oxidized DNA can be repaired by base excision repair and nucleotide excision repair (NER) (Mitra et al., 2001). During replication, areas of single-stranded DNA produced by the repair system can be converted to double strand breaks (DSBs) (Haber, 1999). Therefore, the most serious form of DNA damage, DSBs, can be formed via both direct breaks and replication (Winn, 2003). Other HQ- and BQ-mediated pathways that induce DSBs are also proposed. The formation of DNA adducts is representative of DNA damage by HQ and BQ (Chenna et al., 1995; Bodell et al., 1996; Gaskell et al., 2004, 2005). As DNA alkylators and environmental carcinogens known to cause DNA ad-

Abbreviations: BQ, *p*-benzoquinone; BSFGE, biased sinusoidal field gel electrophoresis; DCFH-DA, 6-carboxy-2,7′-diclorodihydrofluorescein diacetate, di(acetoxy ester); DSBs, double strand breaks; EDTA, ethylenediamine tetra-acetic acid; FDA, fluorescein diacetate; HR, homologous recombination; HQ, hydroquinone; NAC, *N*-acetylcysteine; NER, nucleotide excision repair; NHEJ, non-homologous end-joining; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; γ-H2AX, phosphorylated histone H2AX.

* Corresponding author. Tel./fax: +81 54 264 5799.
E-mail address: ibuki@u-shizuoka-ken.ac.jp (Y. Ibuki).

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ducts generate replication-dependent DSBs (Léonce et al., 2006; Soares et al., 2007; Tanaka et al., 2007), the DNA adducts formed by BQ and HQ are expected to lead to DSBs during the replication process. Furthermore, HQ and BQ generate DSBs by increasing levels of topoisomerase II (Topo II)-mediated DNA cleavage (Frantz et al., 1996; Hutt and Kalf, 1996; Eastmond et al., 2005).

Phosphorylation of histone H2AX has been recently identified as an early event after the formation of DSBs (Rogakou et al., 1998). In eukaryotes, DNA is packaged into nucleosomes, the core of which is an octameric particle consisting of two each of the class H2A, H2B, H3 and H4 histones. H2AX is a minor component of histone H2A. Within minutes after the introduction of a DSB, several thousand H2AX near the site of the DSB are phosphorylated at serine 139, producing foci within the nucleus that are microscopically visible by immunofluorescence staining (Rogakou et al., 1999). Although the exact role of the phosphorylation of H2AX is still controversial, it appears to be mainly associated with maintaining the genome's integrity by participating in the repair of DSBs (Bassing et al., 2002; Celeste et al., 2002, 2003). The generation of phosphorylated histone H2AX (γ-H2AX) has been well studied using general DSB inducers such as ionizing radiation and anti-cancer drugs, but recently environmental chemicals such as arsenite (Yih et al., 2005; Zykova et al., 2006), methylmethanesulfonate, *N*-ethyl-*N*-nitrosourea, benzo[*a*]pyrene (Zhou et al., 2006), and tobacco smoke (Albino et al., 2004; Tanaka et al., 2007) have been found to generate γ-H2AX.

We also detected γ-H2AX generated by light-irradiated benzo[*a*]pyrene, which was due to the production of ROS (Toyooka et al., 2008). As some researchers reported that H_2O_2 induced γ-H2AX (Li et al., 2006; Tanaka et al., 2007), chemicals which can generate ROS are suspected to induce γ-H2AX. In addition, Topo II inhibitors were reported to generate γ-H2AX (Tanaka et al., 2007; Smart et al., 2008). As described above, benzene metabolites effectively inhibit Topo II and generate DSBs. Therefore, benzene metabolites might generate γ-H2AX via several pathways; production of ROS, inhibition of Topo II, and replication of DNA strands having DNA adducts of benzene metabolites.

We have previously showed that γ-H2AX is a sensitive index of cyto- and genotoxicity. The formation of γ-H2AX foci after a photodynamic reaction is detected at very low doses of chemicals and UVA, compared with estimates of cell survival and direct detection of DSBs (Toyooka and Ibuki, 2006). The toxicity of light-irradiated benzo[*a*]pyrene described above was also detected using γ-H2AX very sensitively at low doses (Toyooka et al., 2008). These studies demonstrated that γ-H2AX is a sensitive indicator of DNA damage and a useful tool for the screening of genetic toxicology. Therefore, γ-H2AX could potentially be used to evaluate the toxicity of benzene.

In this study, we clarified that benzene metabolites generate γ-H2AX, which was related to the production of ROS. γ-H2AX was detectable at low concentrations of the metabolites, suggesting that γ-H2AX is a sensitive indicator of genotoxicity from daily and occupational exposure to benzene. Our goal is to apply γ-H2AX as a sensitive index of the toxicity of several kinds of environmental chemicals.

2. Materials and methods

2.1. Cells and cell culture conditions

HL-60 cells (provided by Japanese Collection of Research Bioresources, Japan) were maintained in RPMI medium supplemented with 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin. The cells were cultured at 37 °C in an atmosphere of 5% CO_2. All experiments were performed with exponentially growing cells.

2.2. Treatment with benzene metabolites

HL-60 cells were treated with various concentrations of HQ and BQ (1–300 μM) for 2 h, washed with phosphate-buffered saline (PBS), and further incubated at 37 °C in an atmosphere of 5% CO_2 for given periods (~24 h). In the experiment on ROS inhibition, *N*-acetylcysteine (NAC) (10 or 25 mM) was added 30 min before the treatment with HQ and BQ.

2.3. Detection of γ-H2AX by Western blotting

HL-60 cells (3×10^6) treated with HQ and BQ were lysed in 40 μl of lysis buffer (50 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulphonyl fluoride) for 4 h on ice. Samples containing 40 μg of whole cell protein were separated on 12.5% polyacrylamide gels (SDS-PAGE), and blotted onto polyvinylidene fluoride (PVDF) transfer membranes. After blocking with 3% non-fat milk, the membrane was incubated with primary antibody against phospho-H2AX (1:1000) (Upstate Biotechnology, UK) overnight at 4 °C, then with secondary antibody conjugated with HRP (Jackson Immuno Research Laboratories, West Grove, PA) for 2 h. Protein expression was visualized with an enhanced chemiluminescence detection kit (GE Healthcare UK Ltd., UK). Actin (Santa Cruz biotechnology, Santa cruz, CA) was used as standard for the equal loading of proteins for SDS-PAGE.

2.4. Detection of γ-H2AX by immunofluorescence staining

HL-60 cells (1×10^6) were treated with HQ and BQ for 2 h. After incubation for the indicated period (~8 h), they were fixed in 2% paraformaldehyde for 15 min on ice and then in 70% ethanol for 20 min at −20 °C. The fixed cells were immersed in buffer containing 100 mM Tris–HCl, 50 mM ethylenediamine tetra-acetic acid (EDTA), and 0.5% Triton X-100 for 15 min at room temperature for better permealization, and blocked with 1% bovine serum albumin (BSA) for 1 h at 37 °C. After being washed with PBS, they were incubated with primary antibody against phospho-H2AX (1:200) at 4 °C overnight, then with secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson Immuno Research Laboratories) for 3 h at room temperature. To confirm the distribution of foci, the nucleus was stained with propidium iodide (PI) (20 μg/ml). Images were acquired on a fluorescence microscope (IX70; Olympus, Japan). Cells were judged as "positive" for γ-H2AX foci if they displayed five or more discrete dots of brightness. At least 300 cells were counted for each experimental condition.

2.5. Viability assay (FDA assay)

Cell viability was estimated by the fluorescein diacetate (FDA) assay. FDA is hydrolyzed by cytoplasmic esterases into fluorescent fluorescein in living cells. Cells treated with HQ and BQ were suspended in PBS containing FDA (0.1 mg/ml) and incubated for 15 min at 37 °C. The viability of cells was determined by measuring the fluorescence intensity of FDA hydrolyzed inside the cells using a flowcytometer (FCM) (Epics XL; Coulter, Billerica, MA).

2.6. Detection of DSBs by BSFGE

DSBs were detected with a biased sinusoidal field gel electrophoresis (BSFGE) system (Atto, Japan) as described previously (Toyooka and Ibuki, 2005). In brief, the cells treated with HQ and BQ were solidified in 1% low-melting agarose. The agarose plugs were treated with proteinase K (0.5 mg/ml) and ribonuclease A (1 mg/ml), and electrophoresed in a 0.8% agarose gel. The gel was visualized by staining with ethidium bromide.

2.7. Flow cytometric detection of intracellular ROS

The intracellular generation of ROS in HQ and BQ-treated cells was investigated using the 6-carboxy-2,7′-dichlorodihydrofluorescein diacetate, di (acetoxymethyl ester) (DCFH-DA) (Molecular Probes, Eugene, OR). Cells were incubated in the presence of 10 μM of DCFH-DA for 1 h. The medium was then changed and they were treated with HQ and BQ for 1 h. The fluorescence intensity of DCFH-DA inside the cells was determined using a FCM.

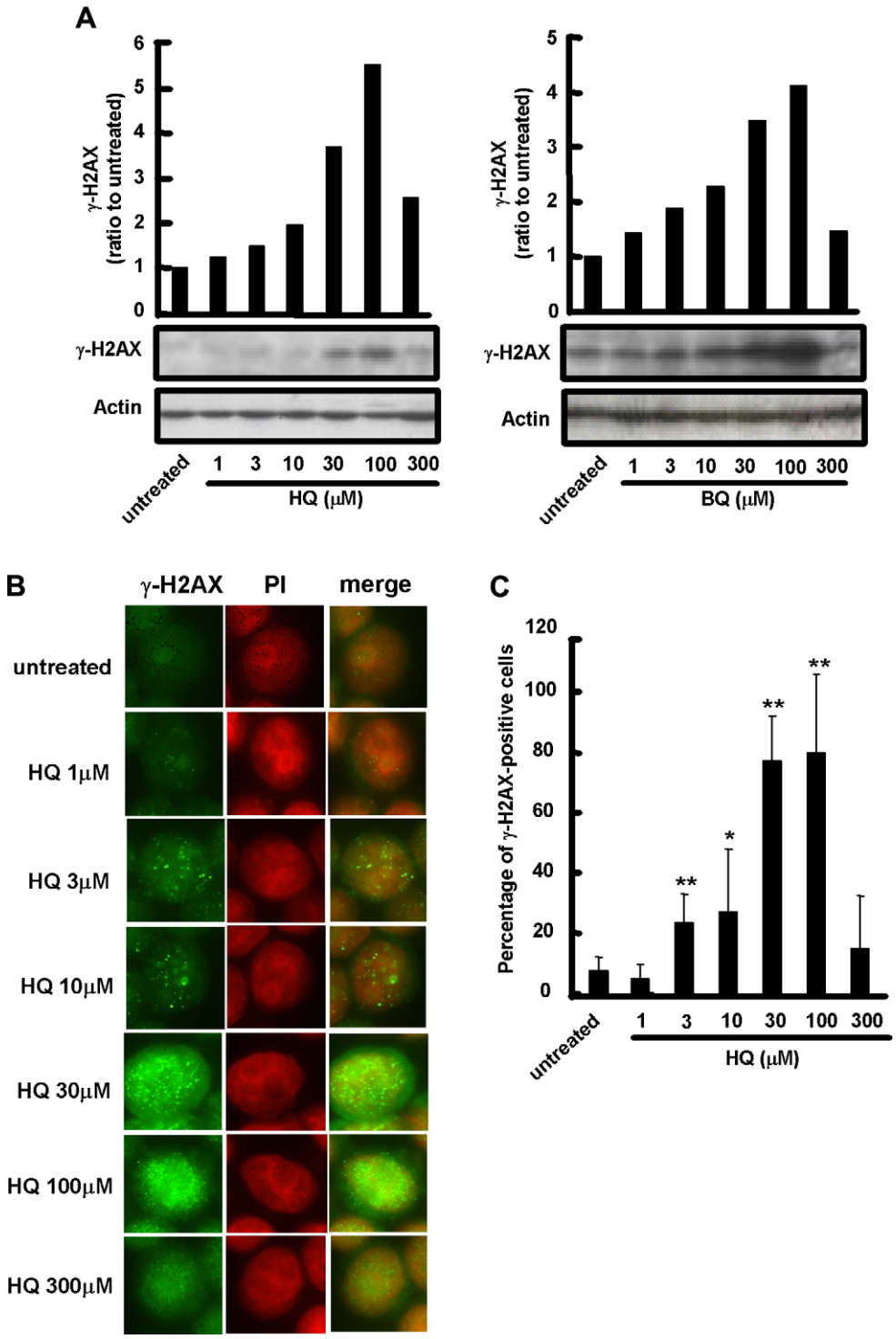

Fig. 1. Generation of γ-H2AX after treatment with HQ and BQ. HL-60 cells were treated with HQ and BQ for 2 h and further cultured for 2 h. (A) Western blot analysis of γ-H2AX generated after treatment with HQ and BQ. Actin is a standard for the equal loading of proteins for SDS-PAGE and the values (γ-H2AX/Actin) in graphs are expressed as a ratio to the untreated control. (B) Images of γ-H2AX foci generated after treatment with several doses of HQ for 2 h. Left panel: γ-H2AX detected by immunofluorescence staining, center panel: nuclei detected by PI staining, right panel: merged images. (C) Percentages of γ-H2AX-positive cells. Cells having more than 5 foci within the nucleus were counted as γ-H2AX-positive.

2.8. Statistics

All experiments were repeated two or three times. Data are represented as the mean ± S.D. (n = 3–5). Data were analyzed by a one-way ANOVA followed by Dunnett's t test for comparisons between groups. Statistical significance is represented when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Generation of γ-H2AX after treatment with benzene metabolites

The treatment with HQ and BQ generated γ-H2AX dose-dependently (Fig. 1). Fig. 1A shows the appearance of γ-H2AX 2 h after treatment with HQ and BQ for 2 h. The generation increased in a dose-dependent manner up to 100 μM and decreased at 300 μM. γ-H2AX is known to produce discrete foci within the nucleus that are microscopically visible by immunofluorescence staining (Rogakou et al., 1999). Images of γ-H2AX foci generated by treatment with HQ are presented in Fig. 1B. PI staining shows the location of the nucleus. HQ produced discrete dots of γ-H2AX in the nucleus. The cells containing over five foci in the nucleus were judged as "positive" for γ-H2AX. A significant number of cells positive for γ-H2AX foci were observed from 3 μM of HQ (Fig. 1C). A similar result of immunofluorescence staining was obtained after treatment with BQ (data not shown).

γ-H2AX was maintained for at least 8 h after the treatment with HQ and BQ for 2 h. This was confirmed by both Western blotting (Fig. 2A) and immunofluorescence staining (Fig. 2B). The generation increased up to 1–2 h after the treatment and plateaued at 4–8 h.

3.2. Induction of DSBs and survival after treatment with HQ and BQ

The generation of γ-H2AX has been attributed to the induction of DSBs (Rogakou et al., 1998). The detection of DSBs was carried out using BSFGE. Induction of DSBs immediately after treatment with HQ and BQ for 2 h was observed from more than 30 μM of HQ and BQ (Fig. 3A). It was difficult to detect DSBs on the treatment with less than 10 μM of HQ and BQ.

DSBs are the worst form of DNA damage, leading to cell death. Fig. 3B shows the survival 24 h after treatment with several concentrations of HQ and BQ. HQ induced cell death in a dose-dependent manner, which was significant from 30 μM, consistent with the appearance of DSBs. More than 8 h is required for the detection of cell death after treatment with 30 μM of HQ (Fig. 3C). BQ caused no cell death at 30 μM.

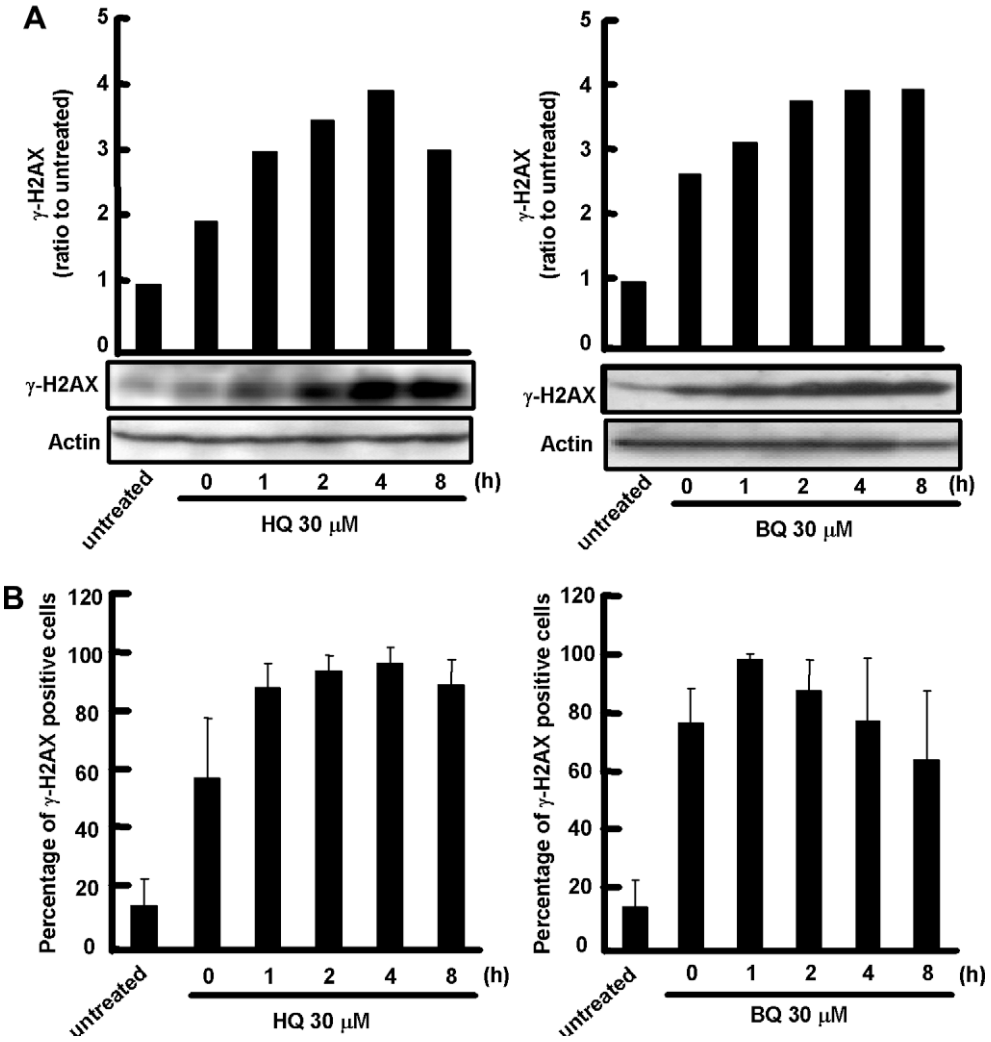

Fig. 2. Time-dependent increase of appearance of γ-H2AX. HL-60 cells were treated with HQ and BQ (30 μM) for 2 h and further cultured for the period indicated. The appearance of γ-H2AX was detected by Western blotting (A) and immunofluorescence staining (B).

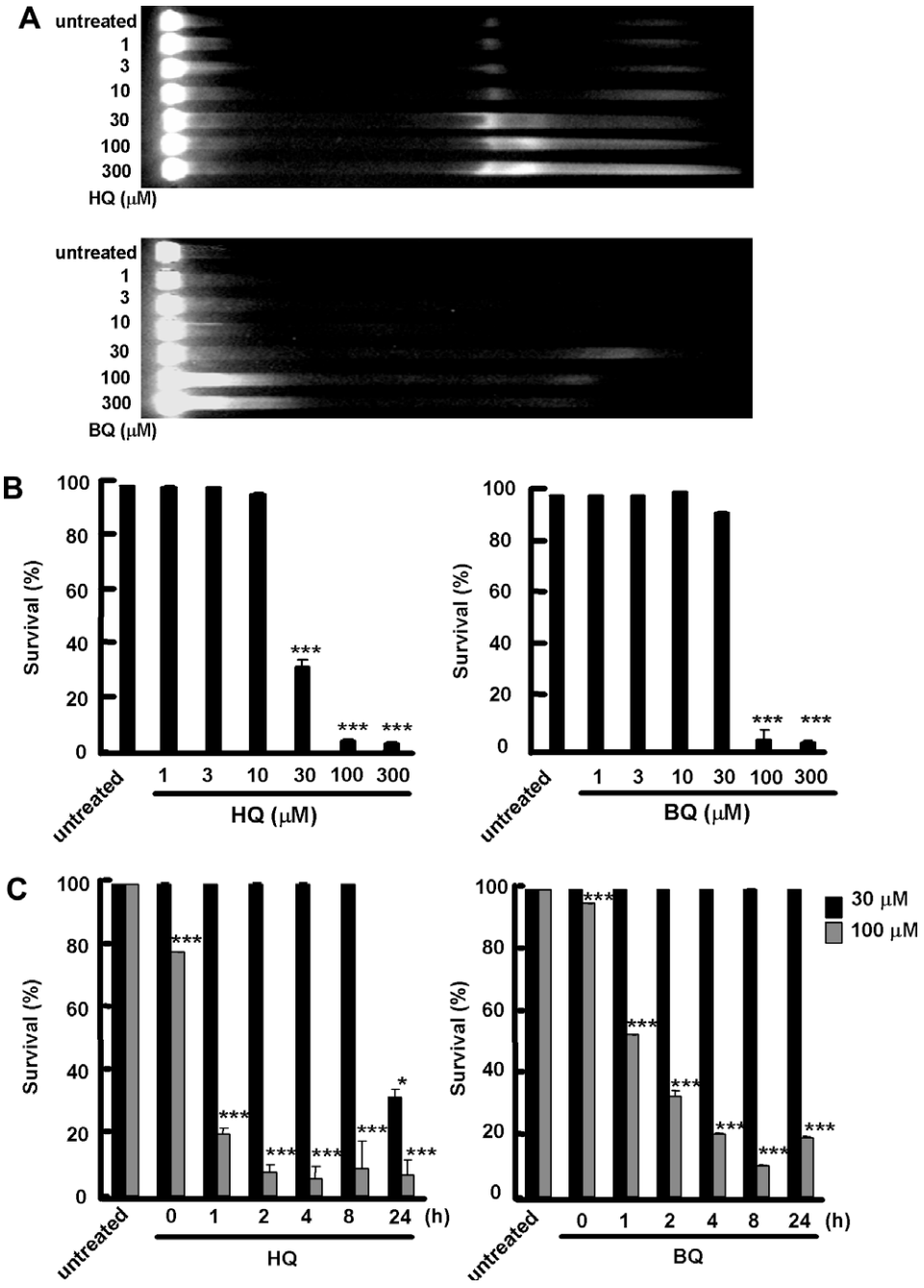

Fig. 3. Formation of DSBs and cell death after treatment with HQ and BQ. (A) Induction of DSBs. HL-60 cells were treated with several doses of HQ and BQ for 2 h. They were solidified in 1% low-melting agarose and treated as described in Section 2. The gel stacks containing the cells were loaded onto a 0.8% agarose gel, and BSFGE was carried out. (B) Dose-dependent cell death. HL-60 cells were treated with several doses of HQ and BQ for 2 h. The cells were further cultured for 24 h and suspended in PBS containing FDA. They were incubated for 10 min at 37 °C. Viability was determined using a FCM. (C) Time-dependent cell death. Black column: 30 μM, gray column: 100 μM.

3.3. Generation of γ-H2AX caused by ROS from HQ and BQ

Some reports attributed the toxicity of benzene metabolites to the production of ROS (Lewis et al., 1988; Snyder and Hedli, 1996; Whysner et al., 2004). Treatment with HQ and BQ induced intracellular peroxidation, showing the production of ROS from HQ and BQ (Fig. 4A). In addition, the antioxidant NAC completely inhibited the cell death and DSBs caused by HQ or BQ (Fig. 4B and C). γ-H2AX generated by HQ and BQ was also suppressed by treatment with NAC (Fig. 4D). One of the ROS produced by HQ and BQ was reported to be H_2O_2 (Lewis et al., 1988; Hiraku and Kawanishi, 1996). Fig. 4E shows the generation of γ-H2AX after treatment with H_2O_2. H_2O_2 clearly increased the number of γ-H2AX-positive cells.

4. Discussion

In this study, we clarified that benzene metabolites effectively generate γ-H2AX, which has been identified as an early event after the formation of DSBs (Rogakou et al., 1998). On partial irradiation using a pulsed microbeam laser, γ-H2AX was generated in the exposed area, where DSBs were formed and some repair proteins like Rad50, BRCA1, etc. were colocalized (Rogakou et al., 1999; Paull et al., 2000), indicating that γ-H2AX is a sign of DSBs and recruits

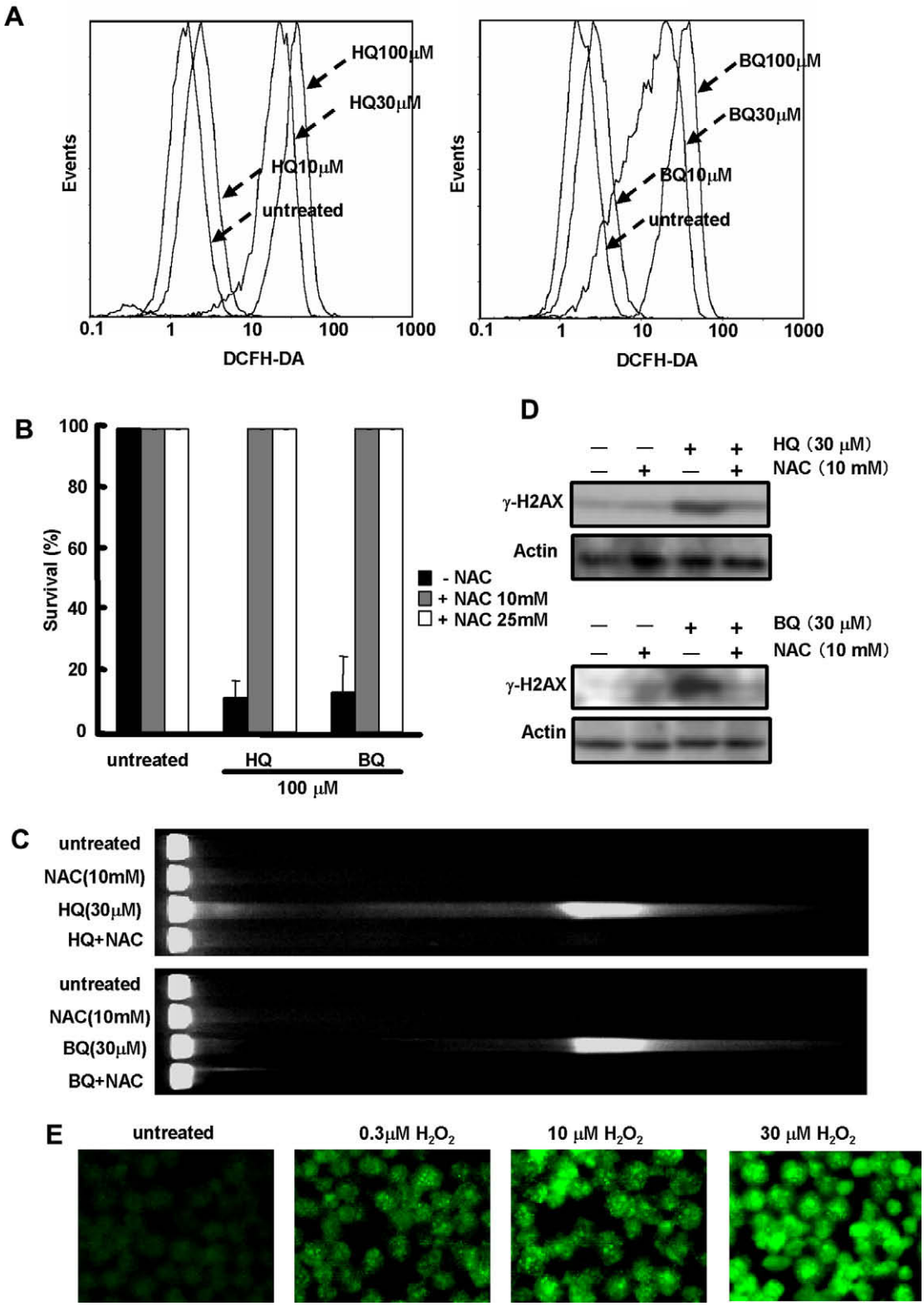

Fig. 4. Relationship between generation of γ-H2AX and formation of ROS by HQ and BQ. (A) Generation of intracellular ROS by treatment with HQ and BQ. HL-60 cells were incubated in the presence of 10 μM of DCFH-DA for 1 h. The medium was changed and the cells were treated with HQ and BQ for 2 h. The fluorescence intensity was analyzed with FCM. (B) Effect of NAC on survival rates following treatment with HQ and BQ. HL-60 cells were treated with HQ and BQ (100 μM) for 2 h in the absence or presence of NAC. Survival was determined by FDA assay. Black column: 0 mM NAC, gray column: 10 mM NAC, white column: 25 mM NAC. (C) Effect of NAC on the formation of DSBs following treatment with HQ and BQ. HL-60 cells were treated with HQ and BQ (30 μM) for 2 h in the absence or presence of NAC (10 mM). BSFGE was carried out as in Fig. 3A. (D) Effect of NAC on generation of γ-H2AX following treatment with HQ and BQ. HL-60 cells were treated with HQ and BQ (30 μM) for 2 h in the absence or presence of NAC (10 mM). The generation of γ-H2AX was examined by Western blotting. (E) Generation of γ-H2AX after treatment with H_2O_2. HL-60 cells were treated with several doses of H_2O_2 for 2 h. The presence of γ-H2AX was examined by immunofluorescence staining.

some of the molecules needed for their repair. Radiation-induced γ-H2AX foci/cell were reported to be linearly correlated with the number of DSBs/cell (Rothkamm and Löbrich, 2003). In immunofluorescence staining in Fig. 1, clear foci were observed from low doses ($\geqslant$3 μM) of HQ and BQ, suggesting that low doses of HQ and BQ could generate DSBs. DSBs are the most critical form of DNA damage because they affect both strands and no intact template is available for the repair of one strand leading to the erroneous rejoining of broken DNA. At least two mechanisms, homologous recombination (HR) and non-homologous end-joining (NHEJ), are known for the repair of DSBs. Although HR is an error-free repair pathway, NHEJ is an error-prone one. Mistakes in the repair of DSBs may be an important factor in the development of genomic instability (Rassool et al., 2007; Sallmyr et al., 2008). Increased NHEJ misrepair in response to excess DSBs formed by benzene metabolites could lead to increased genomic instability, resulting in the increased possibility of myeloid leukemia. Some researchers demonstrated that myeloid disease involves increased ROS production and DSBs, which add further mutations leading to disease progression (Rassool et al., 2007; Sallmyr et al., 2008). Daily exposure to benzene might promote the progress.

Although γ-H2AX is generated through production of DSBs, several different pathways for the formation of DSBs after treatment with HQ and BQ are suspected. (1) ROS produced from HQ and BQ directly form DSBs, leading to γ-H2AX. Some researchers reported that H_2O_2 generated γ-H2AX (Li et al., 2006; Tanaka et al., 2007). The formation in proximity of two single strand breaks by ROS might contribute to the process. (2) ROS produced from HQ and BQ form oxidized DNA like 8-oxo-7,8-dihydro-2′-deoxyguanosine and DSBs are generated by the repair system (Haber, 1999; Mitra et al., 2001; Winn, 2003). Oxidative damage of DNA bases is subjected to processing by base excision repair or NER. If two lesions are located more than three nucleotides apart on opposite strands, the oxidative DNA glycosylases are capable of recognizing and cleaving at the sites of both lesions, giving rise to a DSB. In addition, the processing of clustered damage by oxidative DNA glycosylases converts such lesions to DSBs (Yang et al., 2004, 2006). (3) DNA adducts of HQ and BQ form and DSBs are produced in the replication stage. HQ and BQ form some DNA adducts. Gaskell et al. (2005) have demonstrated that the reaction of DNA with BQ yielded four adducts, the major product being a deoxycytidine adduct and that HQ formed a deoxyguanosine adduct. When the advancing DNA replication fork runs into the DNA adduct, the formation of DSBs would occur. This is not restricted to benzene metabolites, but applies to a number of DNA cross-linking agents (Niedernhofer et al., 2004) and DNA alkylators (Léonce et al., 2006; Soares et al., 2007). (4) DSBs are induced by inhibition of Topo II. Topo II is an essential sulfhydryl (SH)-dependent endonuclease required for replication, recombination, chromosomal segregation, and maintenance of chromosomal structure. HQ and BQ bind to an essential SH group, leading to inhibition of the normal functioning of Topo II, resulting in the formation of DSBs (Frantz et al., 1996; Hutt and Kalf, 1996; Eastmond et al., 2005). Notably, BQ increased TopoIIα-mediated DNA cleavage primarily by enhancing the forward rate of scission (Lindsey et al., 2004). In this study, an antioxidant, NAC, which was present during treatment with HQ and BQ for 2 h, completely suppressed the formation of γ-H2AX. Considering that DSBs were not observed after treatment with HQ and BQ in the presence of NAC, the generation of γ-H2AX in the early stage (~2 h) might be due to the direct formation of DSBs by ROS. With time, the generation of γ-H2AX increased up to 4–8 h after the treatment with HQ and BQ. This might be due to the repair of modified DNA, replication of DNA having adducts or Topo II-mediated scission.

Fig. 5 shows a comparison of the sensitivity of detection for benzene metabolite-induced toxicity in terms of survival, DSBs by BSFGE and γ-H2AX. For the detection of cell death caused by benzene metabolites, a definite time for culture is required (24 h in this study), whereas, DSBs and γ-H2AX were detectable immediately after the treatment. The sensitivity of detection for γ-H2AX was about 10-fold higher than that for DSBs by BSFGE. This high sensitivity was similar to previous results in the evaluation of other chemicals. Phototoxicity of environmental chemicals and toxicity of photo-oxidized chemicals were detectable at low concentrations, compared with survival and DSBs (Toyooka and Ibuki, 2006; Toyooka et al., 2008). Yu et al. (2006) reported that γ-H2AX foci formed very soon after chemical treatment and that the damage was not detectable by a neutral comet assay, suggesting the value of γ-H2AX as a sensitive and rapid indicator of DNA damage. The risk of exposure to benzene might be effectively assessed by γ-H2AX. To make this practical, further study of chronic exposure in lower doses would be needed. While, we should keep in mind that this might be a very unspecific marker, as many other exposures including ROS-generating and DNA-reactive chemicals may also generate the same biological response.

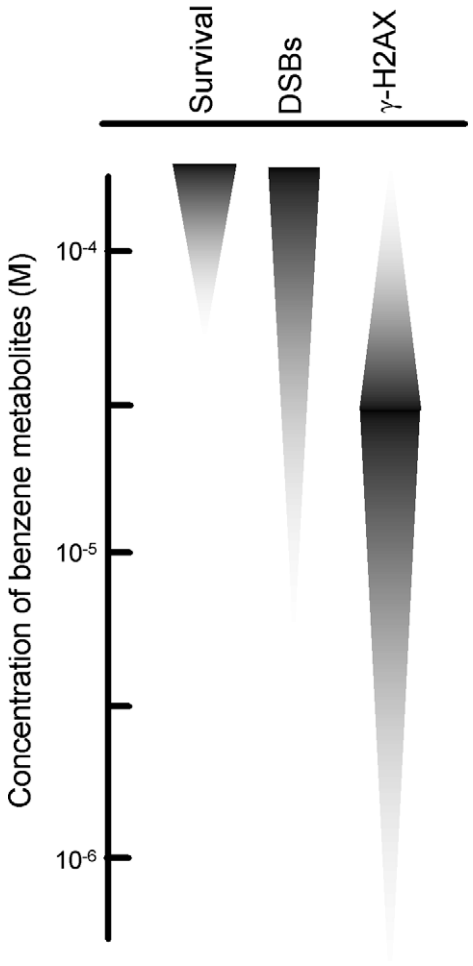

Fig. 5. Comparison of sensitivity for detection of toxicity of benzene metabolites.

In summary, we clarified that benzene metabolites, BQ and HQ, clearly generated γ-H2AX. The γ-H2AX was detectable at low concentrations, compared with the detection of DSBs by electrophoresis. Considering reports that some environmental chemicals having mutagenic and carcinogenic activities generate γ-H2AX, the detection of γ-H2AX might become a sensitive index of the genotoxity of environmental chemicals involving benzene metabolites.

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