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Induction of Centrosome Amplification by Formaldehyde, But Not Hydroquinone, in Human Lymphoblastoid TK6 Cells

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Abstract

Benzene and formaldehyde (FA) are important industrial chemicals and environmental pollutants that cause leukemia by inducing DNA damage and chromosome aberrations in hematopoietic stem cells (HSC), the target cells for leukemia. Our previous studies showed that workers exposed to benzene and FA exhibit increased levels of aneuploidy in their blood cells. As centrosome amplification is a common phenomenon in human cancers, including leukemia, and is associated with aneuploidy in carcinogenesis, we hypothesized that benzene and FA would induce centrosome amplification in vitro. We treated human lymphoblastoid TK6 cells with a range of concentrations of hydroquinone (HQ, a benzene metabolite) or FA for 24 h, allowed the cells to recover in fresh medium for 24 h, and examined centrosome amplification; chromosomal gain, loss, and breakage; and cytotoxicity. We included melphalan and etoposide, chemotherapeutic drugs that cause therapy-related acute myeloid leukemia and that have been shown to induce centrosome amplification as well as chromosomal aneuploidy and breakage, as positive controls. Melphalan and etoposide induced centrosome amplification and chromosome gain and breakage in a dose-dependent manner, at cytotoxic concentrations. HQ, though cytotoxic, did not induce centrosome amplification or any chromosomal aberration. FA-induced centrosome amplification and cytotoxicity, but did not induce chromosomal aberrations. Our data suggest, for the first time, that centrosome amplification is a potential mechanism underlying FA-induced leukemogenesis, but not benzene-induced leukemogenesis, as mediated through HQ. Future studies are needed to delineate the mechanisms of centrosome amplification and its association with DNA damage, chromosomal aneuploidy and carcinogenesis, following exposure to FA.

Keywords

benzene; aneuploidy; chromosomal aberration; carcinogenesis; leukemogen

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AUTHOR CONTRIBUTIONS

Z.J., L.Z., and M.T.S. designed the study; and Z.J., J.B., and J.T. collected the data. Z.J., C.M.M., and L.Z. analyzed the data, prepared draft figures and tables, and had complete access to all of the study data. Z.J. and C.M.M. prepared the manuscript draft with important intellectual input from M.T.S. and L.Z. All authors approved the final manuscript.

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INTRODUCTION

Benzene, a well-established leukemogen, induces myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [Hayes et al., 2001] and probably causes non-Hodgkin's lymphoma [Steinmaus et al., 2008]. Formaldehyde (FA) has been recently classified as a leukemogen by the International Agency for Research on Cancer [IARC, 2012a] and the U.S. National Toxicology Program [NTP, 2011]. The mechanisms by which benzene and FA cause hematopoietic malignancies have not been fully elucidated. Benzene probably causes leukemia by inducing genetic, chromosomal or epigenetic aberrations in hematopoietic stem cells (HSC) [McHale et al., 2012]. FA probably causes leukemia by inducing DNA damage, through the formation of DNA adducts and DNA-protein crosslinks (DPC), and chromosomal aberrations in bone marrow or circulating HSC [Zhang et al., 2009, 2010a].

Previously, we and others reported that aneuploidy, a numerical chromosome aberration and form of genomic instability [Sheltzer et al., 2011; Solomon et al., 2011] that has been associated with leukemogenesis [Schoch and Haferlach, 2002], was induced in the mature blood cells of workers occupationally exposed to benzene [Smith et al., 1998; Zhang et al., 1998; Zhang et al., 2011] and FA [Orsiere et al., 2006]. We also detected aneuploidy in the circulating myeloid progenitor cells of workers exposed to benzene [Zhang et al., 2012] and FA [Zhang et al., 2010b; Lan et al., 2015]. Thus, leukemic stem cells could arise following exposure to these chemicals in vivo through the induction of aneuploidy and genomic instability in circulating hematopoietic stem and progenitor cells.

The mechanisms by which benzene and FA cause aneuploidy remain unclear. Benzene induces cytogenetic changes through its active metabolites, including hydroquinone (HQ) [Zhang et al., 2002]. Previously, we investigated potential mechanisms of aneuploidy induction by HQ in vitro. For comparison, we included etoposide and melphalan, two chemotherapeutic agents that induce therapy-related MDS and AML [IARC, 2000, 2012b; Zhang and Wang, 2014] and are aneugenic [Rowley et al., 1981; Marchetti et al., 2001; Attia et al., 2003; Smith et al., 2003]. We found that both HQ and etoposide induced global methylation—a regulator of genome stability [Robertson, 2005; Cheung et al., 2009] in TK6 cells [Ji et al., 2010]. In a separate study, we found that both HQ and etoposide significantly induced endoreduplication, a process that involves DNA amplification without corresponding cell division that has been proposed to underlie the acquisition of high chromosome numbers by tumor cells [Larizza and Schirrmacher, 1984], in a dose-dependent manner in TK6 cells [Ji et al., 2009]. Additionally, we reported that HQ, melphalan and etoposide each induced DNA/chromosome breakage in TK6 cells [Escobar et al., 2007].

Another potential mechanism of aneuploidy induction is through centrosome amplification, first proposed by Theodore Boveri in 1914 [Boveri, 1914]. The centrosome is an organelle that serves as the main microtubule organizing center as well as a regulator of cell-cycle progression. The centrosome duplicates once during each cell cycle and the two centrosomes form spindle poles and direct the formation of the bipolar spindles, which is essential for accurate chromosome segregation into daughter cells [Bornens, 2012]. The presence of more than two centrosomes (centrosome amplification) severely disturbs the mitotic process and results in chromosome segregation errors. Centrosome amplification is a common

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phenomenon in human cancers and is thought to play an important role in carcinogenesis [D'Assoro et al., 2002; Fukasawa, 2005; Basto et al., 2008; Anderhub et al., 2012; Godinho et al., 2014]. Centrosome aberrations have also been associated with leukemia [Kearns et al., 2004; Neben et al., 2004; Xu et al., 2005; Nitta et al., 2006].

The goal of the current study was to determine whether HQ and FA induce centrosome amplification in the human lymphoblastic cell line TK6. To the best of our knowledge, centrosome amplification associated with benzene or its metabolites has not been examined previously. Kumari et al. previously reported that high levels of FA (300 μM) induced aneuploidy, polyploidy and abnormal centrosome defects in Chinese hamster ovary (CHO) cells [Kumari et al., 2012]. We included melphalan and etoposide as positive controls in the study as they were previously reported to induce centrosome amplification in human lymphocytes [Efthimiou et al., 2013] and human U2OS and mouse NIH3T3 cells [Shimada et al., 2011] in vitro, respectively. We investigated centrosome amplification, chromosome aberrations (aneuploidy and breakage) and cytotoxicity induced by treatment with several concentrations of each chemical for 24 h, followed by a 24-h recovery period.

MATERIALS AND METHODS

Cells and Chemicals

The human lymphoblastoid cell line, TK6, was maintained in RPMI 1640 medium (GIBCO, San Diego, CA) containing 10% FBS (Omega Scientific, Tarzana, CA) and 1% penicillin and streptomycin (Omega Scientific) at 37°C in a 5% CO_2 moist atmosphere, at a density of between 2×10^5 and 1×10^6 cells/ml. Immediately before treatment for all experiments, FA (37% solution) was diluted in PBS (1×) and HQ (≥99% purity) was dissolved in PBS (1×). Melphalan (≥95% purity) and etoposide (≥98% purity) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20° C. The final concentration of DMSO present in cell cultures was 0.1%. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Treatment and Cytotoxicity

Prior to each treatment, TK6 cells were counted with trypan blue solution. The viabilities for all experiments were higher than 90%. Cells were prepared in complete medium at a viable density of 0.4×10^6/ml for the chemical treatments. The chemicals were added to the cells at a ratio of 1/100 (v/v). The FA concentrations were 50, 75, 100, 125, 150, 175, and 200 μM with PBS (1×) as the vehicle control. This FA dose range was chosen based on the reported endogenous concentration of FA in the blood of rats, monkeys, and humans is approximately 2 to 3 μg/g (66.6–100 μM) [Heck et al., 1985; Casanova et al., 1988]. Similar to our previous studies in TK6 cells, the HQ concentrations selected were 0, 5, 10, and 15 μM with PBS (1×) as the vehicle control [Escobar et al., 2007; Ji et al., 2010]. The melphalan concentrations were 0.5, 1, and 2 μM and the etoposide concentrations were 0.1, 0.2, and 0.4 μM, with DMSO as vehicle control.

TK6 cells were treated for 24 h after which the cells were counted with trypan blue solution and pelleted by centrifugation. The medium was discarded and the cells were washed with 5 ml fresh medium. The cells were pelleted by centrifugation and the medium was discarded

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again. The cell pellet was fully suspended in fresh medium at a viable density of 0.4×10^6/ml and the cells were allowed to recover for 24 h (about 1.5 cell cycles) to determine the effects of treatment on centrosomes and chromosomes. Three separate treatment experiments were conducted for HQ, melphalan and etoposide, and six separate experiments were conducted for FA.

Cytotoxicity was evaluated by relative increase in cell count (RICC) [Galloway et al., 2011; Honma 2011]. RICC was calculated as:

[Final cell density (treated) – initial recovery cell density (treated)]/[final cell density (control) – initial recovery cell density (control)] × 100%.

Centrosome Amplification

After culturing the treated cells in fresh medium for 24 h, the cells were pelleted by centrifugation and re-suspended in PBS (1×). The slides were prepared by cyto-spin and immediately immersed in ice-cold methanol for 10 min to fix the cells. The slides were dried in air at room temperature and stored at −20° C under a nitrogen atmosphere until use.

Prior to staining, the slides were warmed to room temperature and immersed in 0.5% Triton X-100 in PBS (1×) at 37°C for 5 min to permeabilize the cells. The slides were rinsed once with PBS (1×), blocked with 3% BSA in PBS at 37°C for 30 min, and rinsed with PBS (1×) again. Mouse monoclonal anti-γ-tubulin (Sigma-Aldrich, cat# T6557, 1:100 diluted with 1% BSA in PBS (1×)) was then added to the slides, followed by incubation at 37°C for 60 min. The slides were rinsed in PBS (1×) twice, 5 min each, and subsequently incubated with FITC-conjugated secondary antibody (Jackson IR, cat# 115-095-003, 1:100 diluted with 1% BSA in PBS (1×)) at 37°C for 60 min. The slides were rinsed in PBS (1×) twice, 5 min each and then dried in air at room temperature.

The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml) and scored under a fluorescence microscope at a magnification of 1,000×. All slides were coded to prevent observer bias before scoring. Five thousand cells were evaluated for each treatment concentration in each experiment. As shown in Figure 1, normal cells have one or two centrosomes, and centrosome amplification is defined as a cell bearing three or more centrosomes.

Chromosome Aberrations

Two hours before harvesting the TK6 cells, colcemid (0.1 μg/ml, Invitrogen, Carlsbad, CA) was added to each culture to obtain a sufficient number of metaphase spreads. Twenty-four hours after culturing the treated cells in fresh medium, the cells were pelleted by centrifugation and suspended in hypotonic solution (0.075 M KCl) for 30 min at 37°C. The cells were fixed three times with freshly prepared Carnoy's fixative (methanol : glacial acetic acid = 3:1). The fixed cells were dropped onto glass slides, which were air dried and stored at −20°C under a nitrogen atmosphere.

Prior to staining, the slides were warmed up to room temperature and then stained with Giemsa. The metaphase spreads were scanned and localized automatically using Metafer

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software (MetaSystems, Altlussheim, Germany). All slides were coded to prevent observer bias before scoring. Metaphases were scored at 1,000× magnification to detect chromosomal aberrations. Only metaphase spreads in which the cells appeared intact with the chromosomes condensed and well spread, and the centromeres and chromatids were readily visible, were scored. Two hundred cells were targeted for each treatment concentration in each experiment. Chromosome numbers were counted for each metaphase spread scored. Chromosomal gain and chromosomal loss were defined as 48 to 55 chromosomes and 40 to 46 chromosomes, respectively, since a normal TK6 cell has 47 chromosomes. Chromosomal breakage (chromatid breaks and chromosome breaks) were scored according to *An International System for Human Cytogenetic Nomenclature* (2005) [Schaffer and Tommerup, 2005]. Chromatid gaps and chromosome gaps were recorded but not included in the chromosomal breakage calculation.

Statistical Analysis

Negative binomial regression was used to test for differences in centrosome amplifications and chromosome aberrations between the cells treated with each concentration of FA, HQ, melphalan, and etoposide and their respective controls [Zhang et al., 2005, 2011], because (1) it can naturally adjust for differences in the total number of cells counted; (2) it is commonly used when the outcome variable is a count, such as the number of cells with centrosome amplification or chromosome aberration; (3) it allows for overdispersion. Fisher's exact test was applied to test for differences in centrosome amplification between the cells treated with each concentration of FA and the control cells within individual experiment. Data are presented as mean ± SE (standard error).

RESULTS

Centrosome Amplification in TK6 Cells Treated With FA, HQ, Melphalan, and Etoposide

Centrosome amplification was examined by fluorescent microscopy at 1,000× magnification in TK6 cells. The median number of cells scored and interquartile range was 5,000 ± 0 for each of the three to six experiments conducted per chemical treatment concentration. Representative cells with the normal complement of centrosomes (1–2) and with centrosome amplification (≥3) are presented in Figure 1.

The proportion of cells with centrosome amplification was determined after 24-h treatment with each concentration of each leukemogen, followed by 24-h recovery, and are presented in Figure 2, along with the cytotoxicity data. Data are expressed as •, the number of cells per 1,000 scored. As shown in Figure 2A, FA significantly induced centrosome amplification in a dose-dependent manner ($P_{trend} < 0.0001$). At the three highest treatment concentration of FA, the average frequency of cells with centrosome amplification at 150, 175, and 200 μM FA was 2.42 ± 0.73 (mean ± SE), 4.60 ± 1.81, and 6.73 ± 3.37•, respectively, each significantly higher than that of the untreated control cells (1.15 ± 0.31•), across six independent experiments. The RICC at these concentrations were 54.6 ± 7.0, 12.6 ± 7.7, and −3.0 ± 5.0% (listed by increasing concentration).

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HQ did not significantly induce centrosome amplification at any concentration tested (5, 10, and 15 μM), across three independent experiments (Fig. 2B). HQ did induce cytotoxicity with increasing concentration, however, with RICCs of 75.1 ± 3.2%, 59.7 ± 4.1%, and 39.5 ± 9.1% at 5, 10, and 15 μM, respectively.

The potent human leukemogens melphalan and etoposide each significantly induced centrosome amplification in a dose-dependent manner, across three independent experiments (P_{trend} < 0.0001, Figs. 2C and 2D, respectively). The average frequency of cells with centrosome amplification at the top two concentration of 1 μM and 2 μM melphalan were 6.87 ± 2.17• and 25.13 ± 4.44•, respectively, each significantly (P < 0.0001) higher than that of the control cells (1.60 ± 0.35•), as shown in Figure 2C. The corresponding RICCs were 70.7 ± 11.9 and 17.3 ± 5.5%. Etoposide was the most potent inducer of centrosome amplification, resulting in significantly (P < 0.0001) higher average frequencies of positive cells at each concentration tested: 14.07 ± 1.38 (0.1 μM), 48.80 ± 10.81 (0.2 μM), and 96.47 ± 10.49• (0.4 μM), compared with the control cells (1.87 ± 1.01•, Fig. 2D). The corresponding RICCs were 56.9 ± 7.6, 15.2 ± 7.5, and −7.9 ± 4.6%.

Chromosome Aberrations in TK6 Cells Treated With FA, HQ, Melphalan, and Etoposide

We examined chromosomal aberrations (gain, loss, and breakage) by Giemsa staining in TK6 metaphases from cells treated with all three concentrations of HQ, etoposide and melphalan and with the top four concentrations of FA, following 24-h treatment and recovery. Two hundred cells were scored for each of the three to six experiments conducted per chemical concentration with the exception of 175 μM FA (200 ± 24, median ± interquartile range) and 0.4 μM etoposide (200 ± 55.5).

Aneuploidy

Data (% positive cells) and dose–response (P_{trend}) for chromosomal gain and loss are presented in Figure 3. Neither FA (Fig. 3A) nor HQ (Fig. 3B) showed a significant increase in chromosome gain. In contrast, both melphalan and etoposide induced significant dose-dependent increases in chromosome gain (P_{trend} <0.001 and <0.0001, respectively) as shown in Figures 3C and 3D. Melphalan significantly increased the percentage of cells with chromosome gain at the top concentration (2 μM) to 18.17 ± 3.00, compared with 7.50 ± 2.93 in the control cells. Etoposide significantly increased the percentage of cells with chromosome gain at the top two concentrations (0.2 μM and 0.4 μM) to 13.33 ± 3.32 and 21.20 ± 1.20, respectively, compared with 8.33 ± 0.60 in the control cells. None of the four leukemogens significantly induced chromosome loss at any concentration, however, a noticeable increase in chromosomal loss at the highest HQ concentration (15 μM) may drive the borderline significant P_{trend} (0.0493).

Chromosomal Breakage

Neither FA nor HQ significantly induced chromosomal breakage at any concentration (Figs. 4A and 4B). Both melphalan and etoposide significantly induced chromosomal breakage in a dose-dependent manner (P_{trend} <0.0001, Figs. 4C and 4D). Compared with the percentage of vehicle control cells (0.50 ± 0.29), melphalan significantly increased levels to 2.00 ± 0.29, and 3.67 ± 1.30, at 1 and 2 μM respectively (Fig. 4C), and etoposide significantly increased

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levels to 1.83 ± 0.17, 2.50 ± 0.76, and 6.96 ± 0.46, at 0.1, 0.2, and 0.4 μM, respectively (Fig. 4D).

DISCUSSION

Here, we sought to determine whether FA and HQ, which have been shown to induce aneuploidy in human blood cells [Zhang et al., 2005, 2010b], could induce centrosome amplification in TK6 cells. We included melphalan and etoposide as positive controls for centrosome amplification [Efthimiou et al., 2013]. We examined centrosome amplification, chromosomal gain, loss, and breakage in TK6 cells that were treated with several concentrations doses of each chemical for 24 h, washed and allowed to recover for 24 h.

In our study, melphalan and etoposide induced chromosome gain and breakage in a dose-dependent manner, at concentrations that produced cytotoxicity, confirming previous findings of aneuploidy and chromosome breakage by melphalan and etoposide in patients with therapy-related leukemia [Rowley et al., 1981; Smith et al., 2003; Pedersen-Bjergaard et al., 2006; Pedersen-Bjergaard et al., 2008]. Similarly, in our study, centrosome amplification was induced in a dose-dependent manner by melphalan and etoposide, confirming it as a potential mechanism underlying leukemogenesis associated with these leukemogens [Shimada et al., 2011; Efthimiou et al., 2013].

Though both HQ and FA produced cytotoxicity in the current study, only FA-induced centrosome amplification and neither chemical induced chromosomal aberrations. Thus, we have shown for the first time that centrosome amplification is a potential mechanism of FA-induced leukemogenesis but not benzene-induced leukemogenesis, at least as mediated through HQ. Previously, we reported that altered global methylation and endoreduplication were potential mechanisms of aneuploidy induction by the benzene metabolite HQ in TK6 cells. Thus, it is possible that benzene and FA cause aneuploidy induction through different mechanisms.

In our study, FA significantly elevated the centrosome amplification frequency at moderate to highly cytotoxic concentrations (150–200 μM) rather than at concentrations close to the normal background range (66.6–100 μM) of human blood [Heck et al., 1985; Casanova et al., 1988]. To the best of our knowledge, this is the first report of such an effect of FA in human cells. Kumari et al. recently reported the induction of increased centrosome number and size in CHO cells treated with a very high concentration of FA (300 μM) for 4 h followed by a 48-h recovery. They also reported aneuploidy in wild-type CHO cells and CHO cells deficient in the nucleotide excision repair (NER) gene XPF, at this very high treatment concentration [Kumari et al., 2012]. FA did not increase aneuploidy in TK6 cells in the current study, in keeping with previous negative findings for monosomy 7 and trisomy 8 in vitro in expanded human erythroid progenitor cells by our group [Ji et al., 2014] and myeloid progenitor cells (10–50 μM FA) by Kuehner et al. [2012]. Aneuploidy findings in vitro contrast with our positive findings in myeloid progenitor cells in vivo, for monosomy 7 and trisomy 8 in a small number of exposed (n = 10) versus unexposed control workers (n = 12) [Zhang et al., 2010b] and more recently for monosomy and trisomy of multiple chromosomes in larger numbers of exposed (n = 29), and control workers (n = 23), using a

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chromosome-wide aneuploidy study (CWAS) approach [Lan et al., 2015]. Previously, we suggested that this discrepancy between in vitro and in vivo effects may be explained by the interaction of FA with a physiological agent to mediate its effects in vivo; this requires further investigation [Lan et al., 2015]. The lack of chromosomal aberrations in vitro could also be caused by a cell cycle delay and should be examined in future studies given the novel and important finding of centrosome amplification induced by FA.

We considered whether centrosome amplification associated with FA could in fact be explained by increases in polyploidy, as such cells would have multiple centrosomes. Although we did not score polyploidy specifically in the study, the percentage increase in cells with polyploidy induced by melphalan and etoposide in TK6 cells in our previous (unpublished) data was much lower than that of centrosome amplification in the current study, suggesting that the observed multiple centrosomes in the present study were unlikely to be caused solely by polyploidy. Previous findings from our group and others suggest that FA does induce polyploidy in vitro and in vivo. Though Kuehner et al. did not see significant changes in aneuploidy in myeloid progenitor cells in vitro, they did see a dose-dependent increase in tetraploidy at 10 to 50 μM FA [Kuehner et al., 2012]. In our CWAS data in myeloid progenitor cells of exposed workers, we also reported significant increases in tetrasomy, indicative of tetraploidy [Lan et al., 2015]. Kumari et al. reported polyploidy (ranging from triploid to octaploid) in wild-type CHO cells and XPF-deficient CHO cells at 300 μM FA [Kumari et al., 2012]. In light of the in vitro versus in vivo discrepancy in aneuploidy and given the potential for polyploidy to manifest as centrosome amplification, in future studies, we will examine centrosome amplification in interphase cells of FA-exposed and unexposed workers in which we have extensively examined chromosome aberrations.

The lack of effect of HQ on both centrosome amplification and chromosomal aberrations was apparent up to 15 μM, at which cells were 40% viable; higher, more cytotoxic concentrations were not examined in this study. Previously, we did report the induction of chromosome breakage by HQ in studies comparing HQ, etoposide and melphalan in TK6 cells. In one study, HQ, melphalan and etoposide each induced DNA breaks at both 5q31 and 11q23 chromosome regions in a dose-dependent manner in TK6 cells, with HQ and melphalan having a stronger effect on 5q31 and etoposide having a stronger effect on 11q23, as measured by COMET-FISH [Escobar et al., 2007]. In another study, we found that HQ induced specific cytogenetic alterations characteristic of both melphalan and etoposide, though at a lower frequency [Ji et al., 2010]. In a third study, both HQ and etoposide significantly induced endoreduplication in a dose-dependent manner and etoposide potently induced translocations of chromosomes 11 and 21 and SCA while HQ induced the latter two in TK6 cells [Ji et al., 2009]. The lack of effect of HQ in the current study compared with previous studies may be explained by the different experimental conditions used. Here, we used lower HQ concentrations, up to 15 μM, compared with 20 and 25 μM in the previous studies [Escobar et al., 2007; Ji et al., 2010]. In the present study, we treated the cells for 24 h and allowed them to recover for 24 h before assessing chromosome aberrations, while the previous studies treated the cells for 1 h [Escobar et al., 2007] and 48 h [Ji et al., 2010], respectively, with no recovery period . We hypothesize that the lack of chromosome

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aberrations induced by HQ in the present study may be due to repair of DNA damage during the 24-h recovery period.

Potential mechanisms of centrosome amplification include multiple centrosome duplication cycles in one cell cycle; failure of cytokinesis; centriole splitting; and acentriolar centrosome formation, as reviewed [D'Assoro et al., 2002]. The most common and well-studied mechanism of centrosome amplification occurs as a result of failure of the normally tight coordination between centrosome and DNA replication, such as during stalling of the cell cycle in response to DNA damage which can lead to multiple centrosome duplication cycles. A number of chemical agents and physical factors which produce DNA damage have been demonstrated to induce centrosome amplification, e.g. 6-thioguanine in chicken DT40 B-lymphoma cells [Robinson et al., 2007], irradiation in human KK-47 bladder cancer cells [Kawamura et al., 2004], ultra-violet light and cisplatin, a DNA cross linker, in human U2OS and mouse NIH3T3 cells [Shimada et al., 2011], hydrogen peroxide in human Chang liver cells [Chae et al., 2005] and doxorubicin, a topoisomerase II poison, in human retinal epithelial RPE1 cells [Douthwright and Sluder 2014].

The chemicals that were positively associated with centrosome amplification in our study cause different kinds of DNA damage. Melphalan is an alkylating agent, etoposide is a topoisomerase II inhibitor and FA, a classic cross linker, is a strong inducer of DPC, inadequate repair of which was proposed to lead to further DNA damage, and consequently to chromosomal aberrations and carcinogenesis [Merk and Speit 1998; Speit et al., 2000]. Increased DPC were observed in the blood cells of workers occupationally exposed to FA [Shaham et al., 1997; Shaham et al., 2003] and more recently by us in the bone marrow of mice exposed to FA by nose-only inhalation [Ye et al., 2013; Zhang et al., 2013].

We hypothesize that FA may induce centrosome amplification through DNA damage triggered by DPC. Fanconi anemia pathway components, such as FANCD2 and BRCA2, play important roles in the DNA damage response to FA-induced genotoxicity [Jacquemont and Taniguchi, 2007; Ridpath et al., 2007; Zhang et al., 2010a; Ren et al., 2013]. Though we did not measure DPC directly in TK6 cells in the current study, previously we reported greater induction of DPC, micronuclei and chromosomal aberrations, and increased toxicity, by FA in the FANCD2-deficient human lymphoblast cell line PD20 [Timmers et al., 2001], compared with its FANCD2-sufficient counterpart PD20-D2 [Ren et al., 2013]. Disruption of Fanconi anemia pathway signaling also causes chromosome instability by disrupting centrosome maintenance, among other mechanisms, as reviewed by Nalepa and Clapp, [2014]. Fanconi anemia pathway-deficient cells exhibit abnormal centrosome numbers under normal conditions [Lee et al., 1999; Tutt et al., 1999; Nakanishi et al., 2007; Kim et al., 2013; Nalepa et al., 2013] and upon exposure to DNA crosslinking agents [Kim et al., 2013; Zou et al., 2013].

Future studies are necessary to delineate the role of DNA damage and repair in the induction of centrosome amplification induced by FA. Repair-deficient human cell lines are useful models in which to test hypotheses regarding the roles of specific repair genes or pathways such as FANCD2 in the PD20 and PD20-D2 cell lines. In XPF-deficient CHO cells, Kumari et al. showed delayed double-strand break repair following treatment with FA in compared

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with wild-type cells, but they examined centrosome amplification only in the wild-type cells. Future studies with human DNA damage response cell models should measure centrosome amplification and markers of DNA damage such as DPC and micronuclei, as well as chromosome aberrations, in these cell models. FA-induced DNA damage, DPC, and MN have been reported in many cell types in vitro, as we summarized previously [Zhang et al., 2009; IARC, 2012a].

CONCLUSIONS

Centrosome amplification was induced by FA, but not by HQ, in a concentration-dependent manner in TK6 cells. Thus, centrosome amplification is a potential mechanism underlying FA-induced leukemogenesis but not benzene-induced leukemogenesis as mediated by HQ; additional studies are required to determine the propensity of other benzene metabolites to induce centrosome amplification. Neither FA nor HQ was associated with significant increases in chromosome gain, loss and breakage in TK6 cells. Future studies are needed to delineate the mechanisms of centrosome amplification and its association with DNA damage, chromosomal aneuploidy and carcinogenesis, following exposure to FA.

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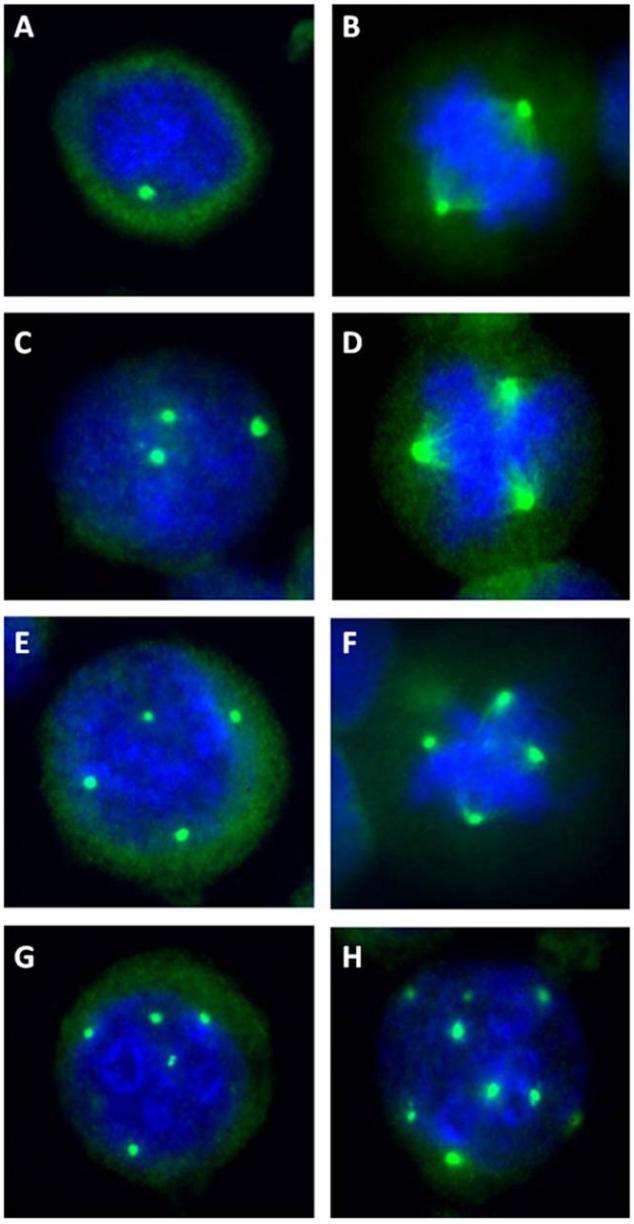

Fig. 1.

Examples of centrosome amplification observed under the microscope. The centrosome and nucleus are stained green (FITC) and blue (DAPI), respectively. Normal cells have one or two centrosomes (**A** and **B**). Centrosome amplification is defined as three or more centrosomes in one cell (**C–H**).

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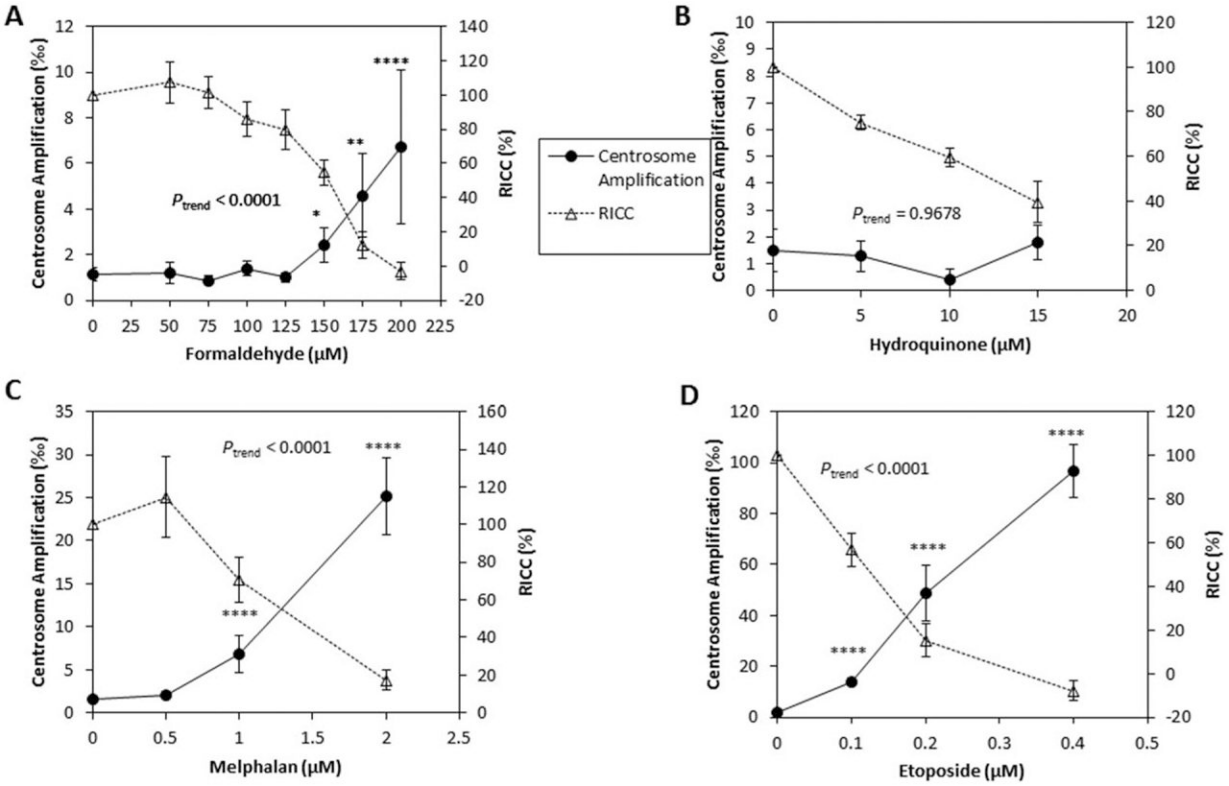

Fig. 2.

Centrosome amplification and RICC in TK6 cells treated with FA, HQ, melphalan, or etoposide. Centrosome amplification (• of cells with ≥3 centrosomes) and RICC (%) are shown for each treatment concentration of: (**A**) FA, (**B**) HQ, (**C**) Melphalan, and (**D**) Etoposide. Data are presented as mean ± SE of three to six experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, relative to the control. P_{trend} is shown for centrosome amplification. RICC, relative increase in cell count.

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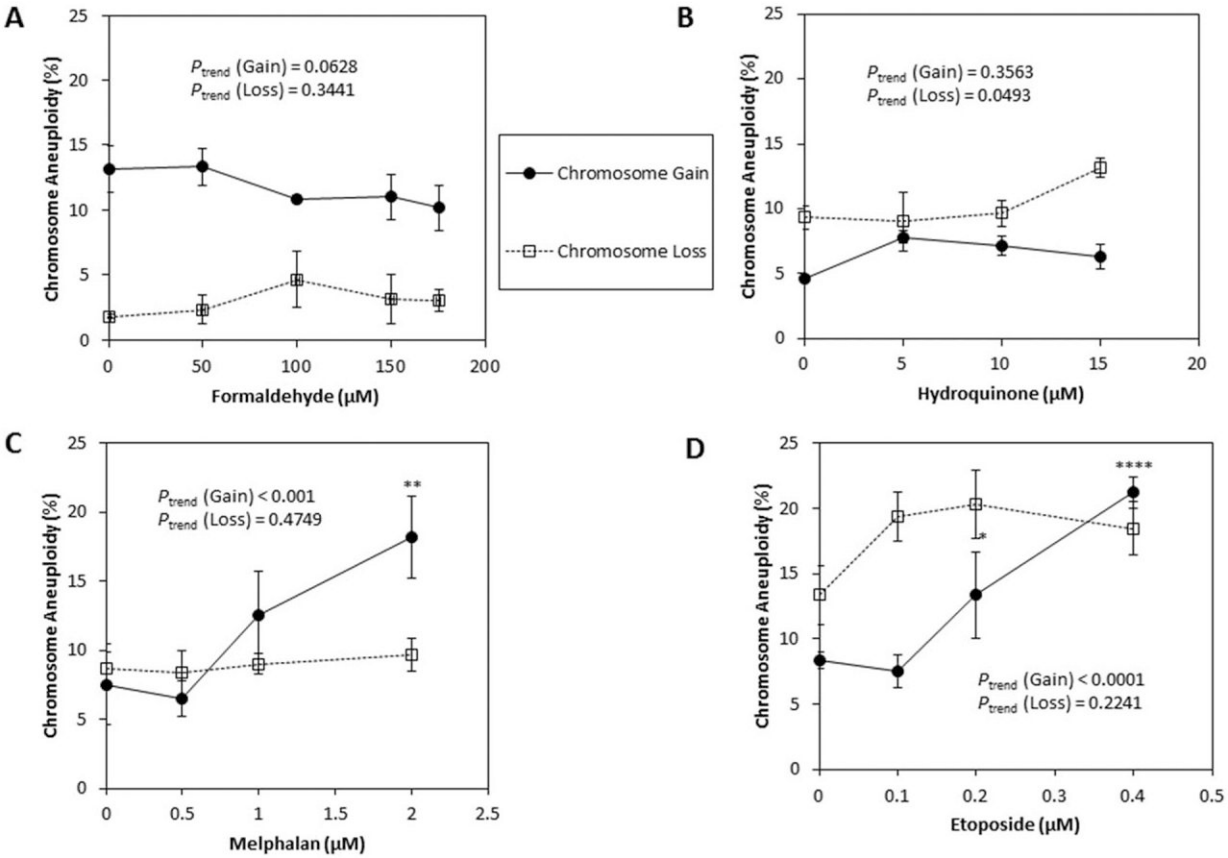

Fig. 3.

Chromosomal gain and loss in TK6 cells treated with FA, HQ, melphalan, or etoposide. Chromosome gain and loss (% of cells with ≥48 and ≤46 chromosomes, respectively) are shown for each treatment: (**A**) FA, (**B**) HQ, (**C**) Melphalan, and (**D**) Etoposide. Data are presented as mean ± SE of three to six experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, relative to the control. P_{trend} is shown for centrosome amplification.

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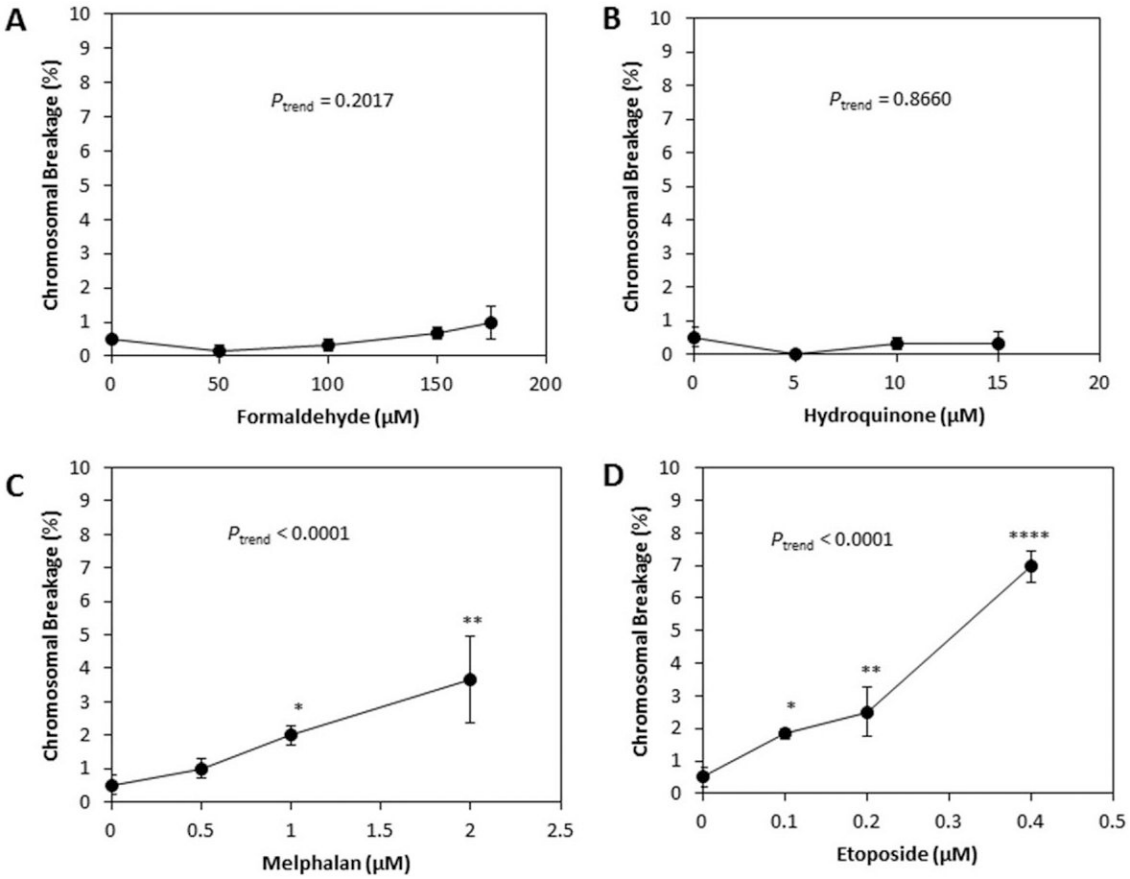

Fig. 4.

Chromosomal breakage in TK6 cells treated with FA, HQ, melphalan, or etoposide. Chromosome breakage (% of cells with breaks among the total of cells scored) is shown for each treatment: (**A**) FA, (**B**) HQ, (**C**) Melphalan, and (**D**) Etoposide. Data are presented as mean ± SE of three to six experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P <$ 0.0001, relative to the control. P_{trend} is shown for chromosomal breakage.