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Use of OctoChrome fluorescence in situ hybridization to detect specific aneuploidy among all 24 chromosomes in benzene-exposed workers

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Abstract

Benzene is an established human leukemogen. The mechanism of benzene-induced leukemogenesis, however, remains unclear, but chromosomal damage is thought to play a critical role. We previously reported that the loss of chromosomes 5 and 7 (monosomy 5 and 7) and the gain of chromosomes 8 and 21 (trisomy 8 and 21) are significantly increased in benzene-exposed workers in comparison to matched controls. To determine if selective effects of benzene can occur, we employed three-color painting on an 8-square slide to screen numerical changes in all 24 human chromosomes (OctoChrome FISH) in a pilot study of 11 subjects (6 exposed to >5 ppm benzene and 5 age- and sex-matched controls). The effects of benzene on each chromosome were assessed as the incidence rate ratio (IRR) from a Poisson regression model with the strongest effects being reflected by the highest IRR values. Monosomy of chromosomes 5, 6, 7 and 10 had the highest IRRs and statistical significance in this preliminary study (IRR > 2.5, $p < 0.01$). On the other hand, the monosomy levels of six other chromosomes (1, 4, 9, 11, 22 and Y) were unchanged in the exposed workers with IRRs close to 1.0. Similarly, selective effects were also observed on trisomy induction with chromosomes 8, 9, 17, 21 and 22 (IRR > 2.5, $p < 0.01$). These results suggest that benzene has the capability of producing selective effects on certain chromosomes, which is supported by our in vitro findings showing that chromosomes 5 and 7 are more sensitive to loss than other chromosomes following exposure to benzene metabolites. We are currently investigating potential mechanisms for this induction of selective aneuploidy.

Keywords: Benzene; Biomarkers; Chromosomal aberrations; Leukemia

Abbreviations: FISH, fluorescence in situ hybridization; IRR, incidence rate ratio; AML, acute myeloid leukemia; MDS, myelodysplastic syndromes; HQ, hydroquinone; BT, 1,2,4-benzenetriol

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1. Introduction

Benzene is a component of gasoline and an important industrial chemical (>2 billion gal produced annually in the USA) [1]. It is also an established human leukemogen. The mechanism of benzene-induced leukemogenesis remains unclear, but chromosomal damage is thought to play a critical role [2,3].

Leukemias and lymphomas are characterized by clonal chromosomal aberrations that appear to have a central role in tumorigenesis [4,5]. The current thinking as to how these clonal aberrations arise is that random damage occurs to the DNA and/or to the mitotic spindle and that selective advantage causes clones harboring the chromosome abnormalities to grow faster than surrounding cells. The initial damage that leads to the loss or gain of the chromosome or to a structural rearrangement, such as a translocation, is considered to be random rather than selective to the specific chromosomes. An alternate hypothesis for a chemical such as benzene is that its metabolites cause a higher rate of chromosome damage on the chromosomes involved in leukemogenesis and that, as such, are selective in their effects.

Clonal aberrations in chromosomes 5, 7, 8 and 21 are among the most common cytogenetic changes found in acute myeloid leukemia (AML) and precursor myelodysplastic syndromes (MDS). Specifically, monosomy of chromosomes 5 and 7, trisomy of chromosomes 8 and 21, and translocations such as t(8;21) and t(15;17) are found in MDS and AML [4,6]. Earlier work in our laboratory [7] and that of Irons and coworkers [8] established that the benzene metabolites hydroquinone (HQ) and 1,2,4-benzenetriol (BT) produced monosomy of chromosomes 5 and 7 in human lymphocytes and bone marrow cells. Recently, we examined an additional seven chromosomes along with chromosomes 5 and 7 to test the ‘a priori’ hypothesis that chromosomes 5 and 7 are more sensitive to loss induced by HQ and BT than the other seven chromosomes. Chromosomes 5 and 7 were highly sensitive to loss following HQ and BT exposure and significantly more so than the other seven chromosomes, providing significant support for the “a priori” hypothesis [9]. This suggests that benzene metabolites produce selective effects on certain chromosomes at least in vitro.

Further support for a selective effect of benzene on certain chromosomes comes from studies of exposed workers. Forni and coworkers using classical cytogenetic methods reported that benzene exposure caused significantly more aberrations in certain chromosomes than in others [10–12]. Following up on this work we examined the loss of chromosomes 1, 5, 7, 8 and 21 in the peripheral blood cells of highly exposed workers and found significant effects only for chromosomes 5, 7, and 8 but not chromosomes 1 and 21 [3,13,14]. Recently, we have examined an additional seven chromosomes and some selectivity was observed, especially for loss and gain of chromosome 6 [15]. However, to determine if selectivity truly occurs it is necessary to examine all 24 chromosomes simultaneously on a single slide. This is now possible using multicolor fluorescence in situ hybridization (FISH). We have devised a 3-color chromosome painting method that allows for the simultaneous detection of all 24 chromosomes and leukemia-specific structural aberrations in a single slide using multicolor (OctoChrome) FISH. Here, the OctoChrome FISH method has been applied in a pilot study to determine its usefulness for studying the potentially selective effects of benzene on aneuploidy induction in exposed workers.

2. Methods

2.1. Study population

Subjects ($n = 11$) for this pilot OctoChrome FISH analyses were selected from a large molecular epidemiology study aimed at evaluating hematologic, cytogenetic and molecular endpoints in workers exposed to benzene in Tianjin, China [16]. The six exposed workers came from one of the shoe factories studied and the five unexposed controls were from a clothing manufacturing plant located in the same general geographical area as the shoe factory. The two groups of workers were frequency matched by age, sex, alcohol consumption and current smoking status. The detailed characteristics of the study subjects are summarized in Table 1. Institutional Review Boards at the U.S. National Cancer Institute, and the National Institute of Occupational Health and Poison Control, China CDC, Beijing approved the study. Participation was voluntary, written informed consent was obtained. The peripheral blood samples were collected in June 2000.

2.2. Exposure assessment

Prior to phlebotomy, individual benzene and toluene exposure was monitored by wearing an organic vapor passive monitor badge as previously described [17]. Personal full shift air monitoring took place about every month over a 3-month period, resulting in about 3–4 personal air measurements per person. Average individual benzene exposure was calculated for the whole observation period and separately for the last month before biological sample collection. Benzene and toluene were not detected in air samples from the control factories [16]. The benzene exposure levels (1 month prior to phlebotomy) in exposed workers were 44.50 ± 25.57 ppm (mean $\pm$ S.D., 142.4 ± 81.82 mg/m^3) and <0.04 ppm (<0.13 mg/m^3) in unexposed controls (Table 1). High levels of benzene were also present in the urine of the exposed workers (Table 1).

2.3. OctoChrome FISH

The OctoChrome device was originally conceived by Dr. Zhang in our group and is currently manufactured by CytoCell (Banbury, UK). Using this method one can detect most specific chromosomal rearrangements related to human leukemia and lymphoma on a single slide. The OctoChrome FISH device is based on the preloading of three-color painting probes (FITC as green, Texas Red as red, and Aqua as blue) onto an eight-squared device. All the 24 whole chromosome paints are directly labeled in one of the three fluorophores and reversibly bound to one of the eight squares. Thus, each square contains three different chromosome paints, each in a different color as shown on the CytoCell web site (http://www.cytocell.com/files/OctoChrome_Results_Sheets.pdf). The chromosome probes are organized on the slide in a way that allows for easy detection of leukemia- and lymphoma-related chromosomal translocations, such as t(15;17), t(8;21), t(14;18), etc. The fixed lymphocyte metaphases prepared from the blood of benzene-exposed workers and controls were dropped onto the eight-square slides matched to the OctoChrome device. The simultaneous denaturation of the probes and target DNA, and the use of rapid formamide-free stringency wash after overnight hybridization simplifies the FISH procedure. The detailed protocol provided by CytoCell will be followed; this can be located online at http://www.cytocell.com/files/CMS_Octochrome.pdf.

Table 1
Demographics of study subjects

	Control	Exposed
Sex		
Male	2 (40)[a]	3 (50)
Female	3 (60)	3 (50)
Alcohol		
Yes	2 (40)	2 (33)
No	3 (60)	4 (67)
Currently smoking		
Yes	1 (20)	2 (33)
No	4 (80)	4 (67)
Age at interview (years)	35.00 ± 11.60[b]	34.50 ± 5.68
Body mass index	21.58 ± 3.26	21.91 ± 3.06
Benzene air level (ppm)	<0.04	44.50 ± 25.57
Benzene urine level (μg/L)	0.53 ± 0.73	720.67 ± 1287.36

[a] Subject number: *N* (%).
[b] Mean $\pm$ standard deviation.

2.4. Image analysis and scoring procedure

After the hybridization, post-washing and DAPI staining steps, metaphase spreads on each square of the eight-square slide were scanned and localized automatically using Metafer software (MetaSystems, Germany) and then evaluated on the computer screen. A total of 35,852 metaphase cells (averaging > 3000 per subject, and >400 per chromosome) were selected and scored according to the criteria listed in our previous publications [7,14]. The data were recorded on a specially designed scoring sheet. All cells with structural changes were labeled and images of these cells captured on the image analysis system for further review.

2.5. Statistical methods to identify which chromosomes and types of abnormalities are most sensitive to the effects of benzene

Poisson regression was applied in this study because: (1) it is commonly used when the outcome variable is a count; (2) it can naturally adjust for differences in the denominator (total number of cells tested); (3) it provides interpretable associations between two measures, called incidence rate ratios (IRRs) [18], that describe how the aneuploidy frequency increases as the benzene exposure level increases. For all models, we also tested for over-dispersion by comparing the rel-

Table 2
Distribution of aneuploidy among all 24 chromosomes in benzene-exposed workers and their matched controls

Chromosome	Monosomy				Trisomy			
	Control	Exposed	IRR[a]	*p*-Value[a]	Control	Exposed	IRR	*p*-Value
1	1.59 ± 0.29[b]	1.84 ± 0.25	1.2	0.5	0.26 ± 0.11	0.6 ± 0.14	2.4	0.1
2	2.18 ± 0.36	0.61 ± 0.15	0.3	<0.0001	0.42 ± 0.16	0.22 ± 0.09	0.5	0.2
3	1.7 ± 0.27	2.66 ± 0.35	1.6	0.02	0.38 ± 0.13	0.6 ± 0.15	1.6	0.3
4	2.32 ± 0.35	2.74 ± 0.33	1.2	0.4	0.37 ± 0.14	0.67 ± 0.16	1.8	0.2
5	0.79 ± 0.22	2.07 ± 0.2	2.6	0.003	0.07 ± 0.07	0.36 ± 0.12	5.4	0.1
6	0.88 ± 0.22	3.22 ± 0.35	3.6	0.0001	0.06 ± 0.06	0.43 ± 0.13	7.7	0.05
7	0.59 ± 0.2	2.19 ± 0.2	3.7	0.0003	0.07 ± 0.07	0.48 ± 0.14	7.3	0.06
8	2.38 ± 0.33	4.15 ± 0.46	1.7	0.002	0.37 ± 0.13	1.16 ± 0.24	3.1	0.006
9	2.85 ± 0.36	2.79 ± 0.36	1.0	0.9	0.28 ± 0.11	0.93 ± 0.21	3.4	0.009
10	0.72 ± 0.22	2.23 ± 0.3	3.1	0.0007	0 ± 0	0.32 ± 0.11	n/a	n/a
11	2.62 ± 0.35	2.79 ± 0.36	1.1	0.7	0.09 ± 0.06	0.56 ± 0.16	6.1	0.02
12	2.1 ± 0.31	4.15 ± 0.46	2.0	0.0003	0.23 ± 0.1	0.86 ± 0.21	3.7	0.01
13	2.72 ± 0.41	1.77 ± 0.25	0.7	0.04	0.48 ± 0.17	0.32 ± 0.11	0.7	0.4
14	1.64 ± 0.29	3.02 ± 0.34	1.9	0.004	0.21 ± 0.11	0.78 ± 0.18	3.7	0.02
15	1.92 ± 0.29	2.48 ± 0.3	1.3	0.2	0.38 ± 0.13	0.43 ± 0.12	1.1	0.87
16	1.69 ± 0.29	3.37 ± 0.34	2.0	0.0006	0.15 ± 0.09	0.6 ± 0.14	3.9	0.03
17	2.6 ± 0.33	3.51 ± 0.35	1.4	0.06	0.3 ± 0.11	0.92 ± 0.18	3.1	0.008
18	2.74 ± 0.38	3.84 ± 0.39	1.4	0.05	0.26 ± 0.12	0.55 ± 0.15	2.1	0.2
19	1.69 ± 0.29	3.64 ± 0.35	2.2	<0.0001	0.15 ± 0.09	0.67 ± 0.15	4.4	0.02
20	3.27 ± 0.44	1.88 ± 0.26	0.6	0.004	0.61 ± 0.19	0.43 ± 0.13	0.7	0.4
21	2.19 ± 0.32	4.35 ± 0.47	2.0	0.0002	0.33 ± 0.12	1.57 ± 0.28	4.8	0.0002
22	2.66 ± 0.35	2.93 ± 0.37	1.1	0.6	0.18 ± 0.09	1.07 ± 0.22	5.8	0.001
X	1.94 ± 0.33	4.00 ± 0.39	2.1	0.0002	0.72 ± 0.2	1.44 ± 0.24	2.0	0.03
Y	0.69 ± 0.35	0.53 ± 0.22	1.1	0.9	1.39 ± 0.49	1.67 ± 0.38	1.7	0.2

[a] IRRs (incidence rate ratios) and *p*-values calculated from Poisson regression models as described in Section 2.
[b] Mean ± standard error.

ative fit of the Poisson model to an equivalent negative binomial model. Of interest is ranking the chromosomes by their "sensitivity" to benzene exposure. We ranked the chromosomes based on the estimated strength of associations (using IRR and *p* value) from the 24 regressions.

3. Results

The data from this pilot study of six highly exposed workers and five controls is shown in Table 2. Selective effects of benzene exposure on certain chromosomes were observed. For example, the monosomy frequencies of chromosomes 1, 4, 9, 11, 22 and Y were essentially identical in the exposed and control workers (IRRs = 1.0–1.2, $p > 0.4$). In contrast, the monosomy frequencies of chromosomes 5, 6, 7 and 10 were very significantly elevated in the benzene-exposed workers (IRR > 2.5, $p < 0.01$).

Similar to the selective effects seen for monosomy frequencies above, the IRRs for trisomy of the 24 chromosomes were often very different (Table 2). Benzene exposure had essentially no effect on trisomy of chromosome 15 [IRR = 1.11 (95% confidence interval: 0.47–2.63)], but produced highly significant increases in trisomy of chromosomes 8, 9, 17, 21 and 22 (IRR > 2.5, $p < 0.01$). The highest IRRs were obtained for trisomy of chromosomes 6 and 7 but the *p* values only approached significance. A larger sample size may therefore produce somewhat different rankings but the data clearly indicate that selective effects are likely to be observed in a larger series of study subjects.

4. Discussion

OctoChrome FISH enables one to simultaneously examine all 24 chromosomes and detect most spe-

cific chromosomal rearrangements related to human leukemia and lymphoma on a single slide. Using this method we have investigated the effects of benzene exposure on all 24 chromosomes simultaneously in a pilot study of 6 exposed workers and 5 matched controls and looked for selective effects. The pilot data suggest that this technology will be very useful for examining whether benzene produces selective effects on certain chromosomes of importance in leukemia development. The major advantages of the OctoChrome FISH device in comparison with other FISH assays are: (1) simultaneous hybridization and examination of all 24 chromosomes on the same slide; (2) detection of specific chromosomal rearrangements related with human leukemia and lymphoma; (3) examination of many more metaphase spreads than with other assays such as SKY and/or G-banding; (4) greater cost-effectiveness and efficiency than SKY and/or G-banding.

The results of this pilot study indicate that benzene significantly alters the aneuploidy status of some chromosomes more than others. Monosomy of chromosomes 5, 6, 7 and 10 was significantly affected by benzene exposure but it had essentially no effect on monosomy of chromosomes 1, 4, 9, 11, 22 and Y. This will require confirmation in larger studies, which are ongoing. We are currently using OctoChrome FISH on more than 80 subjects, which should give us sufficient power to detect selective effects if they are present. Studies in cell culture are also ongoing with the OctoChrome FISH assay, the results of which should complement the data from exposed workers and matched controls.

Overall, results from the pilot study presented here suggest that benzene has the capability of producing selective effects on certain chromosomes, which is supported by our in vitro findings showing that chromosomes 5 and 7 are more sensitive to loss than other chromosomes following exposure to benzene metabolites. These preliminary findings are in agreement with previous work on the alkylating chemotherapy drug, melphalan that produces secondary leukemias with aberrations of chromosomes 5 and 7. In a series of studies, Mamuris et al. showed that melphalan caused selective damage to chromosomes 5, 7, 11, and 17 both in vitro [19] and in patients treated with this alkylating agent [20,21].

Quite how benzene metabolites would produce selective aneuploidy is an open question that we are investigating. Such selective loss would probably not be caused by inhibition of microtubule assembly, a known effect of HQ and BT [22,23], because effects on the mitotic spindle should be non-selective. For example, chloral hydrate, a well-established aneuploidy-inducing agent that inhibits spindle formation but does not damage DNA directly [24], did not cause selective aneuploidy in earlier studies performed in our laboratory [25]. However, there are a number of ways that HQ and BT could be acting as the process of chromosomal segregation is dependent on many different cellular components and is controlled by multiple signaling pathways. We are currently investigating several possibilities.

In conclusion, the pilot study presented here shows that a new multicolor FISH cytogenetic technique, called OctoChrome FISH, is very useful for studying the potential selective aneuploidy-inducing effects of benzene and for examining most specific chromosomal rearrangements related to human leukemia and lymphoma in exposed workers. The pilot study presented here suggests that certain chromosomes may be more affected by benzene exposure than others, but confirmation of these findings is required in a larger study that is currently underway.

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