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Use of genetically modified mouse models to assess pathways of benzene-induced bone marrow cytotoxicity and genotoxicity

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Abstract

Benzene induces bone marrow cytotoxicity and chromosomal breaks as a primary mode of action for the induction of bone marrow toxicity. Our research group has used genetically modified mouse models to examine metabolic and genomic response pathways involved in benzene induced cytotoxicity and genotoxicity in bone marrow and in hematopoietic stem cells (HSC). We review our studies using *NQO1*−/− mice and *mEH*−/− mice to examine the roles of these enzymes, NAD(P)H:quinone oxidoreductase-1 (*NQO1*) and microsomal epoxide hydrolase (*mEH*) in mediating benzene-induced toxicity. *NQO1* catalyzes the detoxication of benzene quinone metabolites and *mEH* catalyzes the hydrolysis of benzene oxide. Our studies using gene expression profiling of bone marrow and enriched HSC populations isolated from the bone marrow of benzene-exposed mice demonstrate differential gene expression responses of key genes induced by inhaled benzene. These studies show that benzene toxicity is regulated by a number of genetic pathways that affect the production of reactive metabolites and DNA damage response pathways in a target tissue.

Keywords: Benzene; Hematopoietic stem cell (HSC); Bone marrow; *NQO1*; *mEH*; *p53*

1. Introduction

Benzene is a prototypical hematotoxic and genotoxic carcinogen and a ubiquitous environmental pollutant [1,2]. Oxidation of benzene in the liver by cytochrome *P450 2E1* (*CYP2E1*) to benzene oxide and other reactive intermediates is an initial step in the bioactivation of benzene and is a prerequisite for cellular toxicity [3,4]. Benzene oxide can be hydrolyzed by microsomal epoxide hydrolase (*mEH*) to benzene dihydrodiol that is then converted to a catechol or can undergo ring opening to produce *trans–trans*-muconaldehyde or can spontaneously rearrange to form phenol, which is then hydroxylated in the liver to form hydroquinone (HQ). Once in the bone marrow, it is believed that HQ and catechol are converted by myeloperoxidase to 1,4-benzoquinone (BQ) and 1,2-BQ, respectively, which can be detoxified by reduction via NAD(P)H:quinone oxidoreductase-1 (*NQO1*) [4].

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Benzene biotransformation produces numerous metabolites that can induce cytotoxicity and genotoxicity through diverse mechanisms [1,2,4]. These reactive metabolites include quinones that can bind to cellular macromolecules, including DNA, tubulin, histones and topoisomerase II. Benzoquinones and other benzene metabolites can cause oxidative DNA damage, lipid peroxidation in vivo, formation of hydroxylated deoxyguanosine residues and strand breaks in the DNA of bone marrow cells, implicating a role for reactive oxygen species (ROS) and covalent binding in benzene-induced toxicity. Formation of DNA double-strand breaks (DSB) by ROS and other mechanisms can lead to increased mitotic recombination, chromosomal translocations and aneuploidy [2]. Such genetic consequences may result in protooncogene activation, tumor suppressor gene inactivation, gene fusions and other deleterious changes in stem cells that can ultimately result in leukemic responses.

Enzymes involved in benzene bioactivation and detoxication are key genetic determinants of benzene-induced cytotoxicity and genotoxicity. We and others have used transgenic mouse models with inactivated alleles of genes that code for enzymes involved in the bioactivation and detoxication of benzene to identify critical pathways of biotransformation that lead to bone marrow cytotoxicity and genotoxicity [3,5,6]. These animal models have demonstrated that *CYP2E1* is a key enzyme involved in the initial steps of bioactivation to cytotoxic and genotoxic metabolites [3]. Our group examined the impact of deficiency in two other enzymes involved in key steps of benzene bioactivation and detoxication, *NQO1* and *mEH*, by using the inhalation route of exposure to benzene and genetically modified mouse models deficient in these enzymes [5,6].

The *p53* DNA damage response is activated in the bone marrow in response to inhaled benzene [7–9]. Relative to wild-type (*p53*+/+) mice, *p53*+/− mice and *p53*−/− mice show a greatly attenuated expression of *p53* transcriptional target genes involved in cell cycle control and apoptosis in the bone marrow, two important biological processes that regulate the response of this target tissue to benzene. In wild-type mice, benzene exposure induces the expression in mouse bone marrow of *p21*, *gadd45*, *cyclins* and *bax*, all hallmark genes of the *p53* DNA damage response that is greatly reduced in *p53* deficient mice [7–9]. We have extended these studies by using gene expression profiling by microarrays and RT-PCR in enriched HSC cell populations isolated from the bone marrow of benzene-exposed mice [10].

In this manuscript, we review our studies using transgenic mouse models with inactivated alleles of genes that code for enzymes involved in the bioactivation and detoxication of benzene to identify critical pathways of biotransformation that lead to bone marrow cytotoxicity and genotoxicity [5,6]. We also review our studies using gene expression profiling to examine mRNA levels of several key genes involved in apoptosis, cell cycle control and DNA repair [7,10]. Detailed methods and results from these studies are presented in published literature [5–7,10].

2. *NQO1*−/− mice and benzene-induced genotoxicity and cytotoxicity

In these studies, we examined the frequency of micronucleated reticulocytes (MN-RET) in blood as a measure of genotoxicity and assessed white blood cell (WBC) count and bone marrow cellularity as measures of bone marrow cytotoxicity in male and female mice (wild-type and *NQO1*−/− mice) exposed to 0, 10, 50 or 100 ppm benzene 6 h/day, 5 days/week for 2 weeks [5]. Using these experimental conditions, benzene induced a concentration-dependent increase in the frequency of MN-RET in male wild-type mice and *NQO1*−/− mice; there were no significant differences between the two genotypes at any exposure level (Table 1). In female wild-type mice, there was no concentration-dependent increase in the frequency of MN-RET. However, in female *NQO1*−/− mice benzene induced a clear exposure-dependent increase in the frequency of MN-RET. These results indicate that *NQO1* is critical in detoxifying benzene metabolites leading to genotoxic activity in female mice.

Benzene-induced hematotoxicity results in a decrease in WBC counts and bone marrow cellularity [1]. In this same study, differences in hematotoxicity among the male mice were concentration dependent. Male *NQO1*−/− mice exhibited significant hematotoxicity at a benzene concentration of 50 and 100 ppm while a significant effect in male wild-type mice only occurred at a benzene concentration of 100 ppm. This difference indicates that male *NQO1*−/− mice are more sensitive than male wild-type mice to benzene-induced

Table 1
Summary of genotoxic and cytotoxic effects of inhaled benzene in genetically modified mouse models that are deficient in enzymes involved in benzene bioactivation and detoxication

Transgenic/mutant mouse model	Effects on genotoxicity in bone marrow	Effects on cytotoxicity in bone marrow	Reference
CYP2E1−/−	Reduced (males)	Reduced (males)	[3]
NQO1−/−	No effects (males), increased sensitivity (females)	Increased sensitivity (males and females)	[5]
mEH−/−	Reduced (males), no effects (females)	Reduced (males), no effects (females)	[6]

hematotoxicity. In female mice, the *NQO1*−/− mice were also more sensitive to benzene-induced hematotoxicity at the 50 and 100 ppm exposure concentrations compared to the wild-type mice.

The difference in responses between the male *NQO1*−/− and wild-type mice with respect to genotoxicity and hematotoxicity suggests that different metabolites are responsible for DNA damage and hematotoxicity.

3. *mEH*−/− mice and benzene-induced genotoxicity and cytotoxicity

These studies used the same experimental design as described above and we again examined the frequency of MN-RET in blood as a measure of genotoxicity and assessed bone marrow cytotoxicity in male and female mice (wild-type and *mEH*−/− mice) [6] (Table 1). In male wild-type mice, benzene induced a concentration dependent increase in the frequency of MN-RET with up to a 14-fold increase at 100 ppm compared to 0 ppm. This genotoxic response was greatly reduced at all exposure levels in the male *mEH*−/− mice. There was a significant increase in MN-RET only at the 100 ppm exposure level in the female wild-type and *mEH*−/− mice. The magnitude of the increase was not different between the female wild-type and *mEH*−/− mice.

Benzene caused dose-dependent hematotoxicity in male wild-type mice. In contrast, male *mEH*−/− mice had no apparent hematotoxicity. Female *mEH*−/− mice did not differ in response to benzene compared to the wild-type female mice; no significant hematotoxicty occurred in female wild-type or *mEH*−/− mice.

These studies show that benzene did not induce genotoxicity and hematotoxicity in male mice deficient in *mEH* enzyme activity. Female wild-type mice exhibited very little response to benzene with no significant differences in hematotoxicity and genotoxicity between the female wild-type and *mEH*−/− mice.

4. Toxicogenomic responses in bone marrow and hematopoietic stem cells

We extended our previous studies examining gene expression changes induced by benzene in the bone marrow by examining cell populations enriched for HSC [7,10]. HSC enriched cell populations were prepared from bone marrow of benzene-exposed or unexposed mice by magnetic and flow cytometry based cell sorting for *Lin*-, *Sca*-1$^+$, CD117 (c-*kit*)$^+$ cells. Total RNA from total bone marrow and enriched HSC were converted to cDNA for use in gene expression analysis by microarray and quantitative RT-PCR. The microarray data are published elsewhere and will only be briefly mentioned [10]. Transcription of *p21* and several other genes involved in G_1/S and G_2/M cell cycle control is upregulated in response to *p53* activation by DNA damage in association with cell cycle arrest [11]. Our laboratory and others have demonstrated that *p21*, a cyclin-dependent kinase inhibitor, is elevated in bone marrow in response to benzene [7–10]. We therefore quantified the mRNA levels of *p21*, *p53* and *Mdm*-2 in bone marrow and HSC samples of benzene-exposed mice compared to unexposed controls (100 ppm benzene for 6 h, 5 days/week for 2 weeks) to assess the *p53*-dependent DNA damage response (Table 2). The level of *p53* mRNA was not altered in benzene-exposed total bone marrow or HSC by RT-PCR. The *p21* mRNA level was increased significantly in total bone marrow by RT-PCR but was not significantly altered in HSC. Likewise, the *Mdm*-2 mRNA level was significantly induced in total bone marrow by RT-PCR but was not significantly altered in HSC.

Table 2
Fold increase of mRNA levels measured by RT-PCR for specific genes in total bone marrow and in hematopoietic stem cell (HSC)-enriched cell populations in benzene exposed mice (100 ppm) compared to unexposed mice [10]

Function	Gene	Total bone marrow mRNA	HSC-enriched population mRNA
Apoptosis	*Bcl2*	NC[a]	NC
	Bax	↑ 2.3	↑ 2.3
Cell cycle control/cell proliferation	*p21*	↑ 15.4	NC
	Gadd45a	↓ 1.3	↓ 2.0
	Cyclin G	↑ 6.4	↑ 3.4
	MDM2	↑ 1.5	NC
	Wig1	↑ 5.4	↑ 12.7
	p53	NC	NC

↑, increase ↓, decrease.
[a] NC, no change compared to unexposed mice.

Compared to unexposed mice, *gadd45a* mRNA level was down-regulated in benzene-exposed HSC and in total bone marrow (Table 2). The *p53* inducible gene *wig1* was significantly induced in both HSC and total bone marrow from benzene-exposed mice but was induced to a greater extent in HSC. The level of *Bcl-2* mRNA was investigated since the ratio of *Bax:Bcl-2* protein is an important regulator of apoptosis. *Bax* was induced to similar levels in both HSC and total bone marrow and *Bcl-2* expression was shown to be unchanged in bone marrow and HSC samples.

Due to the potentially broad nature of DNA lesions induced by benzene that may be repaired by diverse pathways, we analysed expression of key genes involved in the primary DNA repair pathways. We examined the mRNA levels of 11 DNA repair genes: *rad51*, *rad54*, *Rpa*, *Xpa*, *Xpc*, *Xpg*, *Apx*, *Pcna*, *DNApolβ*, *pkrdc*, *Ku80*. Although certain of these genes showed increased levels of mRNA in HSC only (*rad51* = 1.47-fold increase, *Ku80* = 1.33-fold) none of these genes was significantly altered (> 1.50-fold) in total bone marrow or in HSC following in vivo benzene exposure. It is of importance to note that by microarray analysis of mRNA isolated from HSC, two genes known to be involved in DNA double strand break repair, *Nbn* (*nibrin*) and *H2ax*, were significantly elevated (> 1.50-fold) suggesting the induction of double strand breaks in this target cell population [10].

5. Conclusion

The mechanism that leads to leukemia in some individuals following benzene exposure is unclear but several aspects of benzene toxicity are known. Benzene must undergo biotransformation to exert its toxic effect. While the exact metabolites responsible for the carcinogenic, hematotoxic and genotoxic effects of benzene are uncertain, several reports discussing benzene toxicity have demonstrated interactions between combinations of phenol and HQ, phenol and catechol, and HQ and muconaldehyde leading to a brad spectrum of DNA damage [1,2,4]. Key determinants of genetic susceptibility and risk in response to the toxic effects of benzene may likely be the enzyme systems involved in the bioactivation and detoxication reactions of benzene metabolism and the DNA repair enzymes required to restore genomic integrity following DNA damage.

In mice, gender and enzymes involved in the bioactivation and detoxication of benzene are significant factors affecting benzene-induced bone marrow cytotoxicity and genotoxicity. *NQO1* deficiency results in increased benzene-induced genotoxicity, hematotoxicity and DNA damage response in female mice whereas male *NQO1*−/− mice showed an increase in sensitivity for hematotoxicity but not genotoxicity relative to wild-type mice [5]. *mEH* deficiency results in elimination of benzene-induced bone marrow genotoxicity and cytotoxicity [6]. Since humans vary in their expression of these enzymes, these enzymes are likely determinants of benzene genetic susceptibility.

In an effort to further identify genes involved in benzene toxicity and carcinogenesis, we have utilized a toxicogenomic approach to identify gene expression profiles in the bone marrow and in the relevant target cell population, HSC, following in vivo exposure to benzene. Toxicogenomic analysis of HSC

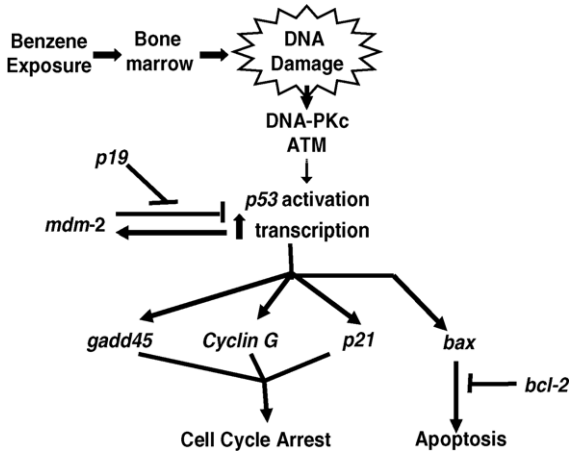

Fig. 1. Genetic network examined in bone marrow and in hematopoietic stem cells for alterations in gene expression due to inhaled benzene (modified from [7]).

revealed numerous genes with altered mRNA levels following in vivo exposure to benzene (Fig. 1). The pattern of gene expression suggests that benzene-exposed HSC undergo genomic responses involved in regulating critical steps in the cellular genotoxic stress response. The HSC and bone marrow genotoxic stress response to inhaled benzene included genes involved in apoptosis, growth control of damaged HSC and HSC growth arrest. These data are consistent with cellular studies showing suppression of hematopoietic cell growth [12,13]. The pattern of gene expression suggests that HSC isolated immediately following a 2-week exposure to 100 ppm benzene are not actively proliferating. This study also highlights the use of enriched target cell populations for gene expression profiling. Determination of mRNA levels by RT-PCR revealed several key differences between HSC and total bone marrow in response to benzene. Understanding the pathways affected by benzene exposure in the appropriate target cell population may better define the mechanism of benzene-induced toxicity and leukemogenesis.

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