Cell-specific Activation and Detoxification of Benzene Metabolites in Mouse and Human Bone Marrow: Identification of Target Cells and a Potential Role for Modulation of Apoptosis in Benzene Toxicity

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The role of cell-specific metabolism in benzene toxicity was examined in both murine and human bone marrow. Hemopoietic progenitor cells and stromal cells are important control points for regulation of hemopoiesis. We show that the selective toxicity of hydroquinone at the level of the macrophage in murine bone marrow stroma may be explained by a high peroxidase/hicotentamidine adenine dinucleotide phosphate, reduced [NAD(P)H:quinone oxidoreductase (NQO1) ratio. Peroxidases metabolize hydroquinone to the reactive 1,4-benzoquinone, whereas NQO1 reduces the quinones formed, resulting in detoxification. Peroxidase and NQO1 activity in human stromal cultures vary as a function of time in culture, with peroxidase activity decreasing and NQO1 activity increasing with time. Peroxidase activity and, more specifically, myeloperoxidase, which had previously been considered to be expressed at the promyelocyte level, was detected in murine lineage-negative and human CD34+ progenitor cells. This provides a metabolic mechanism whereby phenolic metabolites of benzene can be bioactivated in progenitor cells, which are considered initial target cells for the development of leukemias. Consequences of a high peroxidase/NQO1 ratio in HL-60 cells were shown to include hydroquinone-induced apoptosis. Hydroquinone can also inhibit proteases known to play a role in induction of apoptosis, suggesting that it may be able to inhibit apoptosis induced by other stimuli. Modulation of apoptosis may lead to aberrant hemopoiesis and neoplastic progression. This enzyme-directed approach has identified target cells of the phenolic metabolites of benzene in bone marrow and provided a metabolic basis for benzene-induced toxicity at the level of the progenitor cell in both murine and human bone marrow.—Environ Health Perspect 104(Suppl 6):1177-1182 (1996)

Key words: benzene, cell-specific metabolism, myeloperoxidase, NAD(P)H:quinone oxidoreductase, phenol, catechol, hydroquinone, apoptosis, stromal cells, CD34+ cells

Introduction

Benzene has been extensively used as a solvent in industry and was first identified as a myelotoxic in 1897 (1). Its leukemogenic activity was suggested in 1897 (2) and fully characterized in 1928 (3). The occupational hazards of benzene have been the subject of active debate and continuing regulation (4). Benzene ranks as the 17th chemical in terms of total annual production in 1994 (5) and therefore represents a significant occupational hazard. Although the occupational hazards of benzene are well characterized, the presence of benzene in cigarette smoke (6) and in gasoline (7) renders benzene exposure an environmental as well as an occupational problem. Chronic exposure to benzene results in progressive deterioration in hemopoietic function. Anemia, leukopenia, thrombocytopenia, pancytopenia, and aplastic anemia have all been reported after chronic benzene exposure (8,9). Somewhat paradoxically, benzene also induces marrow hyperplasia (9,10). Acute myelogenous leukemia has been associated most often with benzene exposure, although other forms of leukemia have also been reported (10). It has been noted that leukemia associated with benzene exposure frequently develops following a period of bone marrow depression (10,11).

The mechanisms underlying benzene-induced myelotoxicity and leukemia are unclear, but there is considerable evidence that benzene requires metabolic activation to exert its adverse effects (12). Benzene's major toxicity is myeloid, but the majority of benzene metabolism occurs in the liver (12). Thus, if metabolism is a determinant of toxicity, either activation of benzene must occur in the target tissue or metabolites generated in the liver must be transported to the bone marrow where further metabolism may play a role in the toxic process (Figure 1). The major hepatic metabolites of benzene are phenol, catechol, and hydroquinone (12). Catechol and hydroquinone have been shown to persist in bone marrow after benzene exposure (13). The bone marrow is rich in peroxidase activity (14), and phenolic metabolites of benzene can be activated by peroxidases to reactive quinone derivatives (15-18). Reactive quinones derived from phenolic metabolites can be detoxified by the quinone reductase NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase) (19).

Figure 1. Benzene metabolism and production of reactive metabolites in bone marrow.

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Abbreviations used: NQO1, NAD(P)H:quinone oxidoreductase; MPO, myeloperoxidase; DCFH-DA, 2,7-dichlorofluorescein diacetate; MDS, myelodysplastic syndrome; MEM, minimal essential medium; ICE, interleukin-1,β-converting enzyme.
Metabolism of benzene is complex, however, and other metabolites such as benzene oxide, trans-trans-muconaldehyde, and 1,2,4-benzene triol have also been proposed to play a role in benzene toxicity (12,20).

Within the bone marrow, both hematopoietic progenitor cells and bone marrow stromal cells are potential targets of benzene toxicity (10,21,22) (Figure 2). The progenitor compartment includes hemopoietic stem cells and the CD34+ compartment contains all clonogenic cells in human bone marrow, including both short- and long-term repopulating cells (23). Progenitor cells are thought to be the cells of origin for leukemias (24) and are attractive as potential targets of toxins such as benzene, whose toxic effects are not restricted to a single hemopoietic lineage. The stroma, on the other hand, provides a supporting framework within the medullary cavity of the bone marrow for the process of blood cell development (25,26).

Stromal cells are critical in regulation of normal hemopoiesis (26) and have been considered as important targets of benzene toxicity (21,22,27–29). Stromal cells are intimately associated with developing blood cells and regulate hemopoiesis via direct cell-to-cell interactions, the production of extracellular matrix components, and the secretion of soluble mediators such as cytokines and eicosanoids (26,28,30).

Although metabolism is central to benzene toxicity, studies of the metabolic capability of human bone marrow are scarce and we are not aware of any studies in critical cellular subpopulations such as human stroma and CD34+ cells. In this paper we examine the enzymology and potential consequences of cell specific bioactivation of phenolic metabolites of benzene in both murine and human bone marrow.

**Methods**

**Purified Bone Marrow-derived Macrophages.** These are purified as described by Thomas et al. (31). Cultures typically result in >95% macrophages.

**Stromal Fibroblasts.** The fibroblastoid cell line, designated LTF, was obtained from single-cell clones of long-term bone marrow cultures and cultured as described by Thomas et al. (32).

**HL-60 (Promyelocytic Human Leukemia).** These were obtained from the American Type Culture Collection (ATCC) and were cultured as described by ATCC (33).

**Human Bone Marrow, Stromal, and CD34+ Preparations.** Human bone marrow was obtained from healthy volunteers by aspiration.

For human stroma, the method of Kojima et al. (34) was used. Mononuclear cells from bone marrow specimens were separated on Ficoll/Hypaque and plated at 20×10⁶/10 ml of α-minimal essential medium (MEM) supplemented with 12.5% horse serum, 12.5% fetal calf serum, and 10⁻⁶ M hydrocortisone in 100×20 mm tissue culture dishes sometimes containing 12-mm round glass cover slips to facilitate microscopy. Cultures were incubated at 37°C in 5% CO₂/95% air. Cells are refed weekly by exchanging half the medium for fresh medium. Adherent stromal cultures were composed mainly of fibroblastoïd cells with some adipocytes, macrophages, and endothelial cells.

CD34+ cells were purified using magnetic bead technology and a Miltenyi (Miltenyi Biotec, Sunnyvale, CA) CD34+ cell purification kit (QBEND/10, mouse IgG1). Briefly, cells were labeled with a modified CD34+ antibody and then purified using colloidal magnetic beads that recognize the modified CD34+ antibody. Cells were purified by two passes through a magnetized column in the Vario-MACS system. From a single isolation (50–200×10⁶) cell, we obtain 0.5 to 1×10⁶ cells of greater than 96% purity, as assessed by flow cytometry (35).

**Peroxidase and NQO1 Activity Measurements.** In the case of NQO1, dicoumarol-inhibitable reduction of dichlorophenolindophenol was followed at 600 nm (36), whereas for peroxidase a spectrophotometric assay based on the oxidation of tetramethylbenzidine was used (35). Peroxidase activity was expressed as absorbance U/4 min/mg protein as described by Schattenberg et al. (35). Flow cytometric assays for peroxidase activity were performed using 2,7-dichlorofluorescein diacetate (DCFH-DA) as previously described (35).

**Determination of Apoptosis in HL-60 Cells by Fluorescent Microscopy.** For assessment of apoptosis by fluorescent microscopy, 10 μl of Hoechst 33342 (50 μg/ml) (Sigma, St. Louis, MO) was added to 0.5 ml of treated cell suspension and incubated for 10 min at 37°C. Cells were then centrifuged and the supernatant removed. Propidium iodide was then added (50 μl 5 μg/ml) and samples kept on ice until fluorescence microscopy. Hoechst 33342 is a nuclear dye and normal cells have a blue homogenous nucleus. Apoptotic cells have condensed and fragmented chromatin and frequently show the presence of apoptotic bodies. Apoptotic cells also show an elevated fluorescence intensity relative to control cells (37,38). Cells that appear pink (i.e., do not exclude propidium iodide) and do not have condensed chromatin are scored as necrotic. For scoring, at least 200 cells were counted.

**Results and Discussion**

Stroma is a heterogeneous system containing different cell types such as fibroblasts, macrophages, endothelial cells, and adipocytes. Within murine bone marrow stroma, the stromal macrophase is selectively sensitive to the toxic effects of the benzene metabolite hydroquinone (32). We have shown that this can be explained on the basis of cell-specific metabolism within bone marrow stroma (31,39).

Specifically, the bone marrow-derived macrophase had an increased capability to activate hydroquinone to the reactive 1,4-benzoquinone via peroxidatic oxidation and a decreased ability to detoxify 1,4-benzoquinone via NQO1 (Figure 3). The concept of the cytotoxicity of phenols being influenced by a balance between peroxidase-mediated oxidation and NQO1-mediated reduction of any reactive quinones generated was originally developed by Smart and Zannoni (40). Since that time, the critical role of both peroxidases and NQO1 in activation and detoxification respectively in bone marrow has been supported by many studies (15–17,41–44). The murine bone marrow-derived macrophase also had a decreased glutathione S-transferase activity relative to the stromal macrophase and whole marrow (45). Glutathione content did not differ between macrophages and stromal fibroblasts (45), suggesting it did not play a role in the selective toxicity of hydroquinone in murine stroma.
In purified murine bone marrow-derived macrophage cultures, hydroquinone reduced detectable interleukin-1 (IL-1) activity, suggesting that hydroquinone interfered with macrophage function by depressing macrophage IL-1 secretion (32). Hydroquinone inhibits IL1α processing from its precursor form (pre-IL1α) to the mature cytokine (IL1α) in murine bone marrow-derived macrophages and in the P388D1 macrophage monocyte cell line (46). The protease responsible for IL1α processing is the enzyme calpain (47,48) and we have shown that hydroquinone decreases calpain activity and content in bone marrow macrophages (Figure 4). Calpain exists in two isozymic forms—calpain I and calpain II. The effects of hydroquinone were specific to calpain II using immunoblot analysis (49). This work provides a potential mechanism for the effects of hydroquinone on IL1α secretion at the level of the macrophage in bone marrow stroma (Figure 4). It also demonstrates the utility of examining cell-specific bioactivation in heterogeneous systems and shows how the data can be used to identify potential target cells.

Studies of metabolism in human bone marrow are scarce and we are not aware of any studies in critical subpopulations such as human stroma or CD34+ cells. Metabolism of the aromatic hydrocarbon 7,12-dimethylbenz[a]anthracene by human marrow mononuclear cells (50) and benzo[a]pyrene by human lymphocytes (51) has been observed, so metabolism of benzene in situ in human marrow cannot be discounted. In preliminary data we have been unable to detect any metabolism of benzene to phenolics or open-ringed metabolites in murine bone marrow or in human marrow mononuclear cells using high performance liquid chromatography (HPLC) with conventional UV/visible detection. Clearly, metabolism studies need to be performed using much more sensitive methods of detection such as employing 14C-benzene.

The activity of critical enzymes in human bone marrow stroma and progenitor populations has not been examined. In Figure 5 we show preliminary data demonstrating that the peroxidase and NQO1 activity in human stromal cultures varies as a function of time in culture. Peroxidase activity of human stroma decreases with time in culture whereas the NQO1 activity increases. Since freshly aspirated human marrow has high peroxidase and low NQO1 activity, this suggests that the metabolic phenotype of mature stoma may be unrepresentative of human bone marrow and influenced by culture conditions or cell differentiation. Clearly, whether human stromal cultures, which are used for toxicological studies, actually bear any resemblance in a metabolic sense to cells in marrow is an important question. An alternative explanation for this observation is that stromal microenvironments exist in situ in bone marrow with low peroxidase and high NQO1 activity that are not removed by aspiration.

The role that cell-specific metabolism plays in conferring susceptibility to potential
hematopoietic target cell populations is unclear. Peroxidase, and specifically myeloperoxidase (MPO), has been considered to be expressed at the level of the promyelocyte (14). The promyelocyte is at a much later stage of differentiation than myeloid progenitor cells, which are considered the target cells for neoplastic transformation in leukemogenesis. Using both biochemical and flow cytometric measurements of peroxidase activity (Figure 6), we showed that both murine lineage-negative progenitors and human CD34+ progenitors expressed marked peroxidase activity (35). MPO mRNA was also detected in murine progenitors (35). Strobl et al. have also subdivided the MPO-positive phenotype further using the CD33+ marker (52). These authors showed that CD34+, CD33high cells contained MPO, but that the more primitive CD34+ CD33low cells did not.

Some potential consequences of an elevated MPO/NQO1 ratio are shown in Figure 7. Cells with this metabolic phenotype are potential targets of phenolic metabolites of benzene as a result of bioactivation and subsequent covalent binding, altered production of regulators of hemopoiesis, or apoptosis. It is difficult to overestimate the role of imbalanced apoptosis in hemopoietic disease. Hemopoietic cells are likely to undergo apoptosis as a result of growth factor deprivation, toxins, or as an integral part of hemopoiesis (53–56). Inappropriate apoptosis of progenitor cells would be expected to exert a profound effect on normal hemopoiesis (55–57). We have found that hydroquinone is a potent inducer of apoptosis in the human promyelocytic HL-60 cell line (Figure 8), which has a high MPO/NQO1 ratio. In preliminary experiments we have also found that hydroquinone can induce apoptosis in CD 34+ human progenitor cells (not shown). This is a potentially important finding that needs to be pursued.

The data suggest that benzene may induce its hemopoietic toxicity via induction of apoptosis. Hydroquinone, however, can also inhibit the IL-1 processing enzyme calpain, which is known to play a central role in induction of apoptosis (58). This raises the fascinating possibility that in addition to directly inducing apoptosis in critical cells such as progenitor cells, benzene and its metabolites may inhibit apoptosis induced by other stimuli via inhibition of critical proteases in the apoptotic cascade such as calpain and interleukin 1β converting enzyme (ICE). Inhibited apoptosis has been associated with neoplastic progression (55,59,60) and may therefore contribute to benzene leukemogenesis. Myelodysplastic syndrome (MDS or preleukemia) and acute myeloid leukemia are both characterized by a high degree of proliferation, but fully developed leukemias have a much lower rate of apoptosis than MDS, resulting in a more proliferative and lethal condition (57). Inhibition of apoptosis may therefore play a role in the progression of MDS to acute myeloid leukemia (57).

In summary, we have characterized, using cell-specific metabolic studies, potential target cells of benzene toxicity in the bone marrow. We propose a critical role for the MPO/NQO1 balance in determining cellular sensitivity to phenolic metabolites of benzene. These studies have provided a metabolic basis for the toxic effects of phenolic metabolites of benzene at the level of the human progenitor or CD34+ population, which are considered the initial target cells for development of leukemia. Consequences of a high MPO/NQO1 ratio include increased covalent binding, altered production of stromal-derived regulators, and apoptosis. Our data suggest that modulation of apoptosis in target cells may play an important role in aberrant hemopoiesis and neoplastic progression.

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