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Induction of Apoptosis by Benzene Metabolites in HL60 and CD34$^+$ Human Bone Marrow Progenitor Cells

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SUMMARY

Two cell types, HL60 human promyelocytic leukemia cells and CD34$^+$ human bone marrow progenitor cells, were used as model systems to explore a possible role for apoptosis in the myelotoxicity of the phenolic metabolites of benzene. HL60 cells were treated with either phenol, catechol, hydroquinone, or 1,2,4-benzenetriol and then stained with Hoechst 33342 and propidium iodide and subjected to fluorescent microscopy. Cells with nuclear condensation and fragmentation were scored as apoptotic, and etoposide (40 μM) was used as a positive control. Catechol, 1,2,4-benzenetriol, and hydroquinone induced marked time- (0–24 hr) and concentration- (25–100 μM) dependent apoptosis, whereas phenol (750 μM) did not. Under these conditions, no significant necrosis was observed. The induction of apoptosis was confirmed by internucleosomal cleavage of DNA, assessed by agarose gel electrophoresis. CD34$^+$ cells treated with etoposide (40 μM) or hydroquinone (50 μM) for 18 hr were stained and subjected to fluorescent microscopy as above. The percentage of cells exhibiting nuclear condensation and/or fragmentation as well as high intensity staining significantly increased in both cases. The induction of apoptosis was confirmed using a terminal deoxynucleotidyl transferase assay. These data show that apoptosis can be induced in both HL60 and CD34$^+$ human bone marrow progenitor cells by benzene metabolites. The ability of phenolic metabolites of benzene to induce apoptosis in human bone marrow progenitor cells may contribute to benzene myelotoxicity.

Benzene continues to be of toxicological concern. Current sources of human exposure include industry, gasoline, combustion products, and cigarette smoke (1). The myelotoxic effects of benzene are well established and include various hematopoietic disorders such as pancytopenia, aplastic anemia, myelodysplasia, and AML (1–3).

Once absorbed, benzene is extensively metabolized. Cytochrome P-450 2E1 oxidizes benzene to eventually yield the hydroxylated metabolites phenol, catechol, hydroquinone, and 1,2,4-benzenetriol (4). These phenolic compounds have been shown to accumulate in the bone marrow (5, 6), where they can act as substrates for peroxidases such as MPO (7), which are present at high levels in this tissue. The one-electron oxidation catalyzed by MPO generates the corresponding semiquinones, which in turn can disproportionate to yield benzoquinones (6, 8). Semiquinones and/or benzoquinones may contribute to cytotoxicity by either reacting with cellular nucleophiles, including DNA (9), RNA, and proteins (7), or generating reactive oxygen species, which cause oxidative damage (10). Alternatively, NQO1 can metabolize benzoquinones via a two-electron reduction to generate their hydroquinone derivatives (11); Ganousis *et al.* (12) suggested that this may represent a rate-limiting detoxification reaction in cells with high MPO and low NQO1. The ratio of MPO to NQO1 may therefore represent a bioactivation/detoxification balance that could influence the susceptibility of a cell to toxicity induced by the phenolic metabolites of benzene.

Two major components of bone marrow have been investigated as potential targets of benzene toxicity: stroma and progenitor cells. Previous work with murine stroma (7, 12, 13) has demonstrated that hydroquinone adversely affects macrophage function, including the ability to support hematopoiesis. This is consistent with the high MPO/NQO1 ratio in these cells. Evidence suggesting direct toxicity to progenitor cells after benzene exposure was provided by early inhalation studies in mice in which reductions in colony-forming ability by bone marrow of treated animals was observed (14, 15). Both primitive and more committed progenitor cells have been observed in freshly isolated human CD34$^+$ bone marrow cells, which are capable of parenting virtually all lineages of hematopoietic cells (16–18). The recent demonstration of marked MPO protein and peroxidase activity in these

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ABBREVIATIONS: MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase; AML, acute myeloid leukemia; NQO1, NAD(P)H:quinone oxidoreductase; DMSO, dimethylsulfoxide.

cells (19, 20) provides a metabolic basis for the proposal that $CD34^+$ cells are potential targets of benzene toxicity.

Apoptosis or programmed cell death plays an important role in hematopoietic regulation, mediating clonal deletion of thymocytes as well as the programmed senescence of other lymphoid and myeloid cells (21, 22). Nonphysiological induction of apoptosis in the progenitor compartment could deplete the stem cell population of the marrow, potentially resulting in defective hematopoiesis. Two experimental systems with elevated MPO/NQO1 ratios were used to determine the potential of phenolic benzene metabolites to induce apoptosis. The human promyelocytic leukemia cell line HL60 represents a promyelocyte capable of differentiation to monocytic or granulocytic cells. Closer to the *in vivo* situation, freshly isolated human $CD34^+$ bone marrow cells include more primitive as well as partially committed progenitor cells. In this article, we demonstrate the ability of phenolic metabolites of benzene to induce apoptosis in both HL60 and $CD34^+$ human progenitor cells.

Materials and Methods

Chemicals. Hydroquinone, catechol, phenol, etoposide, Hoechst 33342, propidium iodide, Proteinase K, Ribonuclease A, and Histopaque 1077 were obtained from Sigma Chemical (St. Louis, MO). 1,2,4-Benzenetriol was obtained from Aldrich Chemical (Milwaukee, WI). All other reagents used were of analytical grade or better.

HL60 cells. The human promyelocytic cell line HL60 (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% 200 mM L-glutamine, and 1% penicillin/streptomycin. Cell culture medium and supplements were obtained from GIBCO (Grand Island, NY). Cells were kept at 37° in a humidified atmosphere containing 5% CO_2.

HL60 cells that had reached a density of ~0.4-0.8 × 10^6 cells/ml were resuspended in fresh media to yield a density of ~1 × 10^6 cells/ml and treated as follows. Cells were incubated for 0, 3, 6, 12, or 24 hr after the addition of either 40 μM etoposide; 10–100 μM hydroquinone, catechol, or 1,2,4-benzenetriol; or 750 μM phenol. Stock solutions were made in DMSO so that the treatment volume added was ≤0.1% of the total cell suspension volume. DMSO was used as the vehicle control.

Fluorescent microscopy. After the above exposures, 0.5–1.0 × 10^6 cells/sample were collected and stained with 1 μg/ml Hoechst 33342 at 37° for 10 min. Cells were then pelleted and resuspended in 5 μg/ml propidium iodide in phosphate-buffered saline and visualized at 200× under UV illumination via fluorescent microscopy. Cells with condensed, and fragmented chromatin and high-intensity staining were scored as apoptotic. Necrotic cells were identified as those that failed to both exclude propidium iodide and demonstrate condensed and fragmented chromatin.

Gel electrophoresis. Approximately 1.5 × 10^6 cells/sample were collected and pelleted. Cells were lysed with ddH_2O and incubated at room temperature for 20 min with 10 mg/ml RNase A. The gel above the wells of a 1.8% agarose gel was removed and replaced with 0.8% agarose gel containing 1% sodium dodecyl sulfate and 1 mg/ml Proteinase K. Once loaded, samples were run at 20 V for 1 hr followed by 4–6 hr at 100 V. Gel was then stained in a solution of ethidium bromide, destained as necessary, visualized with UV, and photographed.

$CD34^+$ cells. Human bone marrow was obtained with informed consent from healthy adult volunteers (three white women, two white men, one black man, and one Asian woman). No obvious variation in the ability of phenolic metabolites to induce apoptosis was evident among ethnic groups and genders. $CD34^+$ cells were isolated using the VarioMacs separation system as directed by the manufacturer (Miltenyi Biotec, Sunnyvale, CA). Briefly, mononuclear cells were isolated using Histopaque 1077, labeled with anti-CD34 QBEND10 and superparamagnetic MACS microbeads, and run through VarioMacs RS+ magnetic columns. Separated cells were then labeled for flow cytometric analysis using phycoerythrin-conjugated anti-HPCA2 antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) specific for a CD34 epitope other than QBEND10. Flow cytometric analysis was then performed to determine purity. Isolated $CD34^+$ cells ≥95% pure were resuspended in MyeloCult H5100 media (Stem Cell Technologies, Vancouver, British Columbia, Canada) plus 1 μM hydrocortisone and 1% penicillin/gentamicin to yield a density of ~0.6-1 × 10^5 cells/ml.

$CD34^+$ cells were treated immediately after isolation with either 40 μM etoposide or 50 μM hydroquinone and incubated at 37° in a humidified atmosphere containing 5% CO_2 for 18 hr. Eighteen hours after treatment, flow cytometric analysis of cells indicated retention of CD34 antigen. Cells were then collected and stained as above with Hoechst 33342 and propidium iodide and visualized at 200× under UV illumination via fluorescent microscopy. Cells demonstrating condensed and/or fragmented chromatin with high-intensity staining were scored as apoptotic. Necrotic cells were identified as cells that failed to both exclude propidium iodide and show condensed and/or fragmented, intensely stained chromatin.

TdT assay. DNA fragmentation was detected in $CD34^+$ cells by the TdT assay as modified from Gorczyca *et al.* (23). Briefly, 2–3 × 10^5 cells/treatment were collected and fixed in ice-cold 1% formaldehyde for 15 min and then permeablized with −20° 70% ethanol for ≥1 hr. Cleaved DNA was labeled for 1 hr at 37° with 2 μM dig-11UTP (Becton Dickinson Immunocytometry Systems) by 10 units of TdT (GIBCO), after which cells were incubated with a fluorescein isothiocyanate-labeled antidigoxigenin antibody (Becton Dickinson Immunocytometry Systems) for 30 min at room temperature in the dark. Finally, cells were resuspended in 0.5 ml of 0.25 μg/ml propidium iodide in phosphate-buffered saline and subjected to flow cytometry. Cells demonstrating green fluorescence were scored as positive and considered to be apoptotic.

Statistical analysis. Data were analyzed by one-way analysis of variance and Dunnett's *t* test for comparisons of multiple samples with a single control.

Results

HL60 cells were treated with ≤750 μM phenol, 100 μM catechol, hydroquinone, 1,2,4-benzenetriol, or 40 μM etoposide (positive control) and assessed for induction of apoptosis 12 hr after treatment (Fig. 1). Cells demonstrating condensed and/or fragmented chromatin were scored as apoptotic. The positive control, 40 μM etoposide, produced a significant increase in apoptotic morphology beginning 3 hr after treatment, whereas apoptosis in vehicle control-treated HL60 cells never exceeded 7.5%. Of the phenolic metabolites administered, hydroquinone and 1,2,4-benzenetriol were more effective at inducing apoptosis than catechol (Fig. 1), whereas phenol at concentrations as high as 750 μM failed to produce significant increases in apoptosis (Fig. 1). None of the treatments resulted in significant necrosis. Internucleosomal fragmentation assessed by agarose gel electrophoresis was consistent with fluorescent microscopy counts (Fig. 2), with the exception of the degree of fragmentation after 1,2,4-benzenetriol treatment. The significant but relatively faint laddering observed with 1,2,4-benzenetriol in the presence of clear apoptotic morphology may indicate an accumulation of DNA cleavage products at the level of large fragments of DNA (300 kb, 50 kb). Large fragments are the precursors to the internucleosomal fragments observed in conventional laddering gels. Apoptotic morphology and the appearance of

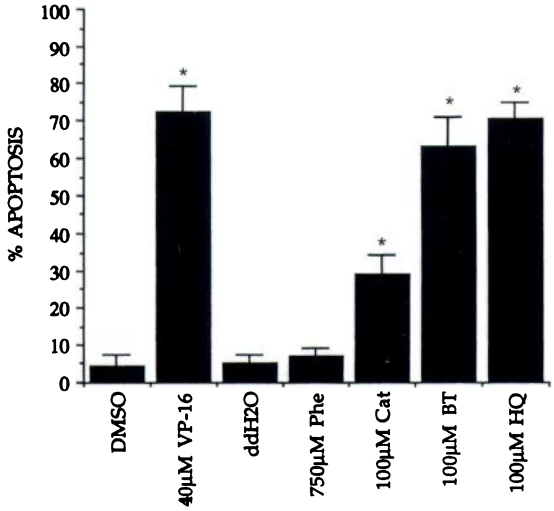

Fig. 1. Percentage of apoptosis in HL60 cells 12 hr after treatment with DMSO (vehicle control), 40 μM etoposide (*VP-16*, positive control), ddH_2O (*ddH20*, vehicle control), 750 μM phenol (*Phe*), 100 μM catechol (*Cat*), 100 μM 1,2,4-benzenetriol (*BT*), or 100 μM hydroquinone (*HQ*). Cells were collected 12 hr after treatment and scored based on nuclear morphology as described in Materials and Methods. Data are mean ± standard deviation ($n \geq 3$ separate experiments). *, Percentage of apoptosis is significantly greater than that of respective controls ($p \leq 0.05$).

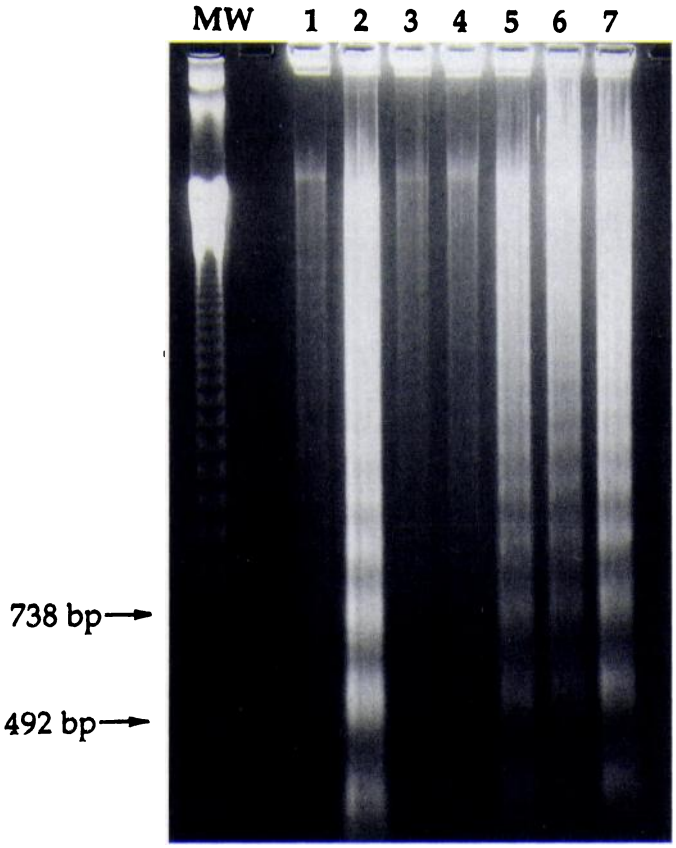

Fig. 2. Internucleosomal fragmentation in HL60 cells 6 hr after treatment with vehicle control DMSO (*lane 1*), 40 μM etoposide (*lane 2*), vehicle control ddH_2O (*lane 3*), 750 μM phenol (*lane 4*), 100 μM catechol (*lane 5*), 100 μM 1,2,4-benzenetriol (*lane 6*), or 100 μM hydroquinone (*lane 7*). A similar laddering pattern is observed 12 hr after treatment. Gel is representative of ≥3 experiments.

large fragments of DNA in the absence of DNA laddering have been described previously (24). HL60 cells demonstrating condensed and/or fragmented chromatin after treatment with hydroquinone are shown in Fig. 3 (*left*).

Catechol, 1,2,4-benzenetriol, and hydroquinone induced apoptotic nuclear morphology in a time- (0–24 hr) and concentration- (10–100 μM) dependent manner (Fig. 4). Hydroquinone-induced apoptosis was significantly higher than control at concentrations of >10 μM and increased with time (Fig. 5). Internucleosomal fragmentation increased in intensity, consistent with the increase in cells showing nuclear apoptotic morphology (Fig. 6).

$CD34^+$ cells were freshly isolated from normal human bone marrow aspirates and treated with 40 μM etoposide (positive control) or 50 μM hydroquinone and, 18 hr after treatment, were assessed for induction of apoptosis. Cells demonstrating condensed and/or fragmented chromatin as well as high-intensity staining were scored as apoptotic (Fig. 3, *right*). Treatment with 40 μM etoposide induced 73.83 ± 10.85% apoptosis compared with the 9.57 ± 1.67% background apoptosis in control. Treatment with 50 μM hydroquinone induced 26.33 ± 2.56% apoptosis, which was significantly greater ($p \leq 0.05$) than background levels (Fig. 7). TdT assay revealed significantly increased DNA fragmentation in both etoposide- and hydroquinone-treated $CD34^+$ cells, consistent with the induction of apoptosis observed by fluorescent microscopy (Fig. 8).

Discussion

These data clearly demonstrate that certain phenolic metabolites of benzene induce apoptosis in HL60 cells and human $CD34^+$ bone marrow cells. Hydroquinone and 1,2,4-benzenetriol were most effective, catechol was much less so, and phenol failed to induce apoptosis above control levels.

There are two probable mechanisms by which phenolic metabolites of benzene cause cellular damage, either of which may result in the induction of apoptosis by these compounds. The first involves the oxidation of phenolic metabolites by peroxidases to yield reactive semiquinones and quinones (6–8). Both semiquinones and benzoquinones are capable of disrupting cellular function by either binding to cellular nucleophiles, including DNA (9), RNA, and protein thiol groups (7), or generating free radicals. HL60 and $CD34^+$ cells have marked MPO activity and low levels of NQO1, a two-electron reductase that can detoxify reactive quinones and protects against hydroquinone-induced cytotoxicity in murine bone marrow stromal cells (7, 11, 12, 25, 26). Hydroquinone is more readily oxidized by MPO relative to catechol and phenol due to the higher specificity (V_{max}/K_m) of the enzyme for hydroquinone, which Subrahmanyam *et al.* (27) showed to be 5-fold higher than that of catechol and 16-fold higher than that of phenol. The enzyme content in target cells and the differential bioactivation of phenolic metabolites by MPO are consistent with the potential of these compounds to induce apoptosis in our study. A second possible mechanism by which phenolic metabolites of benzene may induce apoptosis involves the generation of reactive oxygen species via autoxidation. 1,2,4-Benzenetriol and hydroquinone autoxidize more readily than either catechol or phenol. Hydroquinone and 1,2,4-benzenetriol (but not catechol) have been demonstrated to produce oxidative DNA damage in HL60 cells *in vitro*. Oxidative DNA damage was also observed in murine bone marrow *in vivo* after benzene or

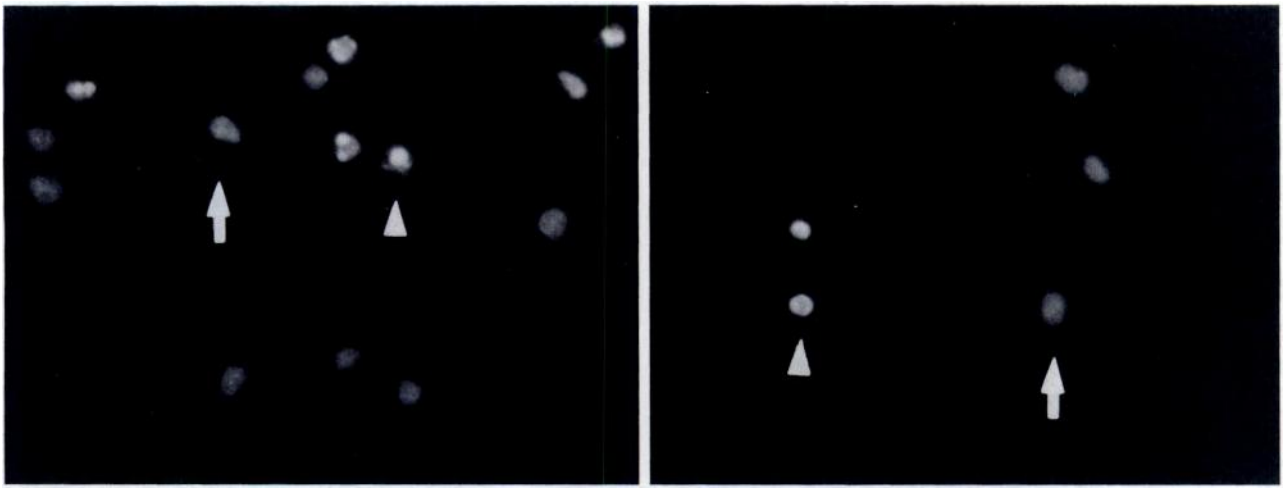

Fig. 3. Nuclear morphology of normal (*arrow*) and apoptotic (*arrowhead*) HL60 (*left*) and CD34+ (*right*) cells. Cells were treated with 50 (HL60) or 100 (CD34+) μM hydroquinone for 6 (HL60) or 18 (CD34+) hr, stained with Hoechst 33342 and propidium iodide, and photographed under UV illumination at 400× magnification.

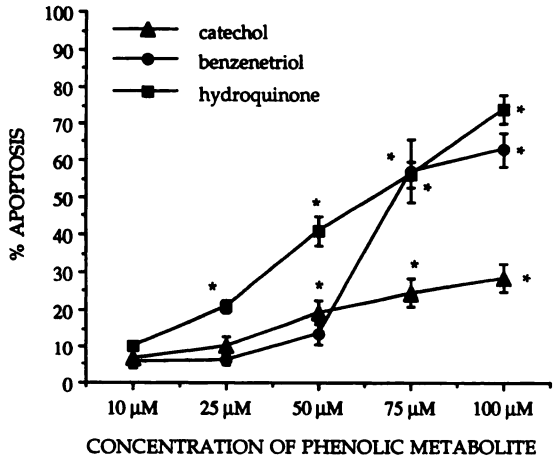

Fig. 4. Percentage of apoptosis in HL60 cells 12 hr after treatment with 10–100 μM hydroquinone, 1,2,4-benzenetriol, or catechol. Cells were collected 12 hr after treatment and scored based on nuclear morphology as described in Materials and Methods. Treatment with 750 μM phenol induced 7.42 ± 0.79% apoptosis, which was not significantly greater than the 5.25 ± 0.99% for vehicle control. Treatment with the positive control 40 μM etoposide produced 72.78 ± 3.22% at 12 hr. Data are mean ± standard error ($n \geq 3$ separate experiments). *, Percentage of apoptosis is significantly greater than control ($p \leq 0.05$).

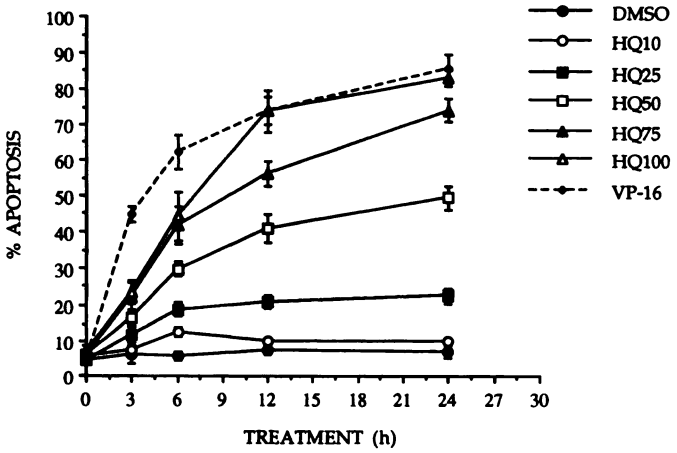

Fig. 5. Percentage of apoptosis in HL60 cells treated with vehicle control (*DMSO*); 40 μM etoposide (*VP-16*); or 10, 25, 50, 75, or 100 μM hydroquinone (*HQ*). Cells were collected 0, 3, 6, 12, or 24 hr after treatment and scored based on nuclear morphology as described in Materials and Methods. Data are mean ± standard error ($n = 4$ separate experiments). All data points are significantly greater than control at time points of ≥3 hr except 10 μM hydroquinone (at all times) and 25 μM hydroquinone (at 3 hr) ($p \leq 0.05$).

1,2,4-benzenetriol administration (10). A role for reactive oxygen species in apoptosis has been suggested (28), and the different abilities of the phenolic metabolites of benzene to undergo autoxidation correspond approximately to the extent of apoptosis we observed after treatment of HL60 cells with these compounds. It is also possible that a combination of both mechanisms described above may result in apoptosis. Autoxidation of hydroquinone and 1,2,4-benzenetriol would be expected to generate H_2O_2, which could then fuel turnover of MPO, producing reactive quinones. Further work is in progress to distinguish among these possibilities.

The results of studies in animals have indicated that phenolic metabolites of benzene concentrate and persist in bone marrow after benzene exposure (5, 29). The degree to which the concentrations of benzene metabolites used in our study correspond to those likely to result from occupational or environmental exposure to benzene is unclear. Although human exposure to benzene would be expected to occur at considerably lower levels and over longer periods of time than previously used in acute animal disposition studies (5, 6, 29, 30), the actual concentrations of phenolic metabolites in human bone marrow after acute or chronic exposure to benzene are not known. In our study, we investigated the adverse effects of acute exposure of primary human CD34+ bone marrow cells to benzene metabolites, and the effects of chronic low-dose exposures are unknown.

The pattern of myelotoxicity that often develops after chronic exposure to benzene suggests a perturbation of normal hematopoiesis. Aplastic anemia, myelodysplasia, and AML have all been associated with benzene exposure (2). The bone marrow progenitor compartment (CD34+ cells) is responsible for self-renewal and generation of virtually all lineages of hematopoietic cells. Pancytopenia and aplastic anemia represent degrees of hematopoietic failure in which significant reductions in circulating blood cells are observed.

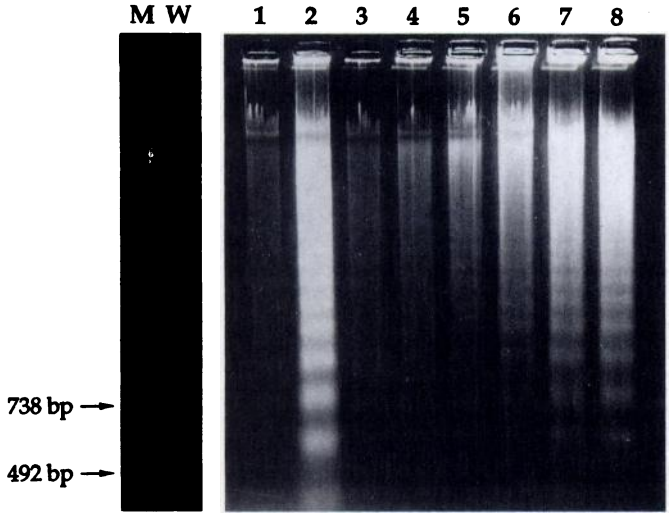

Fig. 6. Internucleosomal fragmentation in HL60 cells 6 hr after treatment with increasing concentrations of hydroquinone. Cells were collected 12 hr after treatment with DMSO vehicle control (*lane 1*); 40 μM etoposide (*lane 2*); ddH_20 vehicle control (*lane 3*); or 10 μM (*lane 4*), 25 μM (*lane 5*), 50 μM (*lane 6*), 75 μM (*lane 7*), or 100 μM (*lane 8*) hydroquinone and analyzed by laddering gel electrophoresis as described in Materials and Methods. Gel is representative of ≥ 3 experiments.

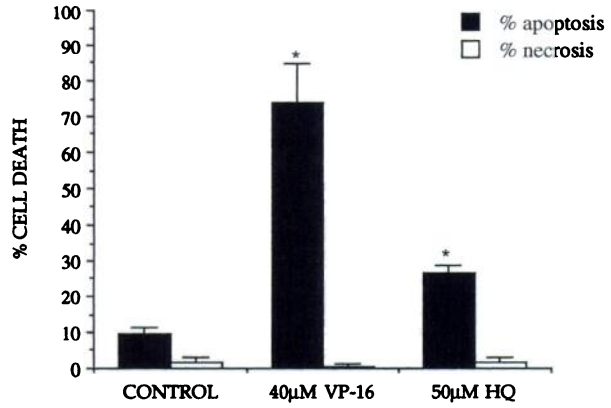

Fig. 7. Percentage of cell death in $CD34^+$ cells in control and after treatment with 40 μM etoposide (*VP-16*) or 50 μM hydroquinone (*HQ*). Cells were collected 18 hr after treatment and scored based on nuclear morphology as described in Materials and Methods. Data are mean ± standard error ($n \geq 6$ separate experiments). *, Percentage of apoptosis is significantly greater than that of control ($p \leq 0.05$).

It has been suggested (2, 31, 32) that acquired aplastic anemia may be attributed to disruption of the ability of stem cells to undergo self-renewal and/or differentiation to more committed progenitor cells, due in part to depletion of this population. There are fewer $CD34^+$ cells (particularly fewer long term culture-initiating cells) in bone marrow from patients with aplastic anemia relative to normal control samples, and the $CD34^+$ cells that are isolated demonstrate reduced clonogenic potential (33, 34). Therefore, depletion of $CD34^+$ cells via apoptosis induced by phenolic metabolites of benzene represents a potential mechanism underlying benzene-induced aplastic anemia. In a recent study of several patients with myelodysplastic syndrome, Raza *et al.* (35) found that ineffective hematopoiesis was most likely due to excessive apoptosis in the bone marrow of these patients. Thus, pathological induction of apoptosis may also play an important role in the development of myelodysplasia.

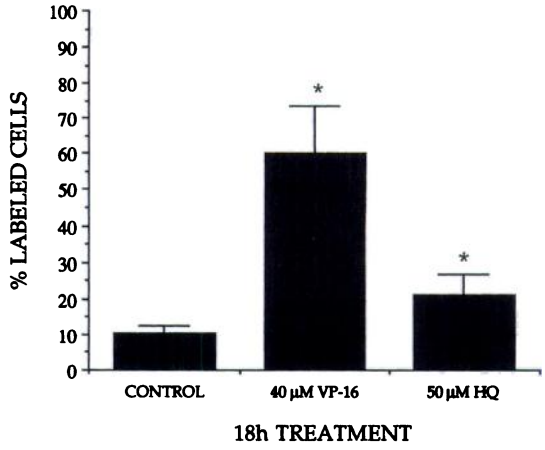

Fig. 8. Percentage of $CD34^+$ cells demonstrating DNA cleavage as indicated by TdT assay. Cells were collected 18 hr after treatment, labeled for the presence of DNA cleavage, and analyzed by flow cytometry as described in Materials and Methods. Data are mean ± standard error ($n \geq 4$ separate experiments). *, Percentage of TdT+ cells is significantly greater than that of control ($p \leq 0.05$). *VP-16*, etoposide; *HQ*, hydroquinone.

AML, which develops after chronic exposure to benzene, is often preceded by pancytopenia or aplastic anemia (32). A recent follow-up study of recovered aplastic anemics found an increased risk of AML, particularly after immunosuppressive therapy, in these patients compared with the general European population (36). Of further interest is the development of AML as a secondary cancer after chemotherapy with various DNA-damaging agents, including the topoisomerase II inhibitor etoposide, which was used in our study as a positive control because of its potent ability to induce apoptosis. Marsh and Geary (37) reviewed a possible relationship among the clonal disorders aplastic anemia, myelodysplastic syndrome, and AML in which aplastic anemia, resulting from a stem cell defect, progresses to myelodysplastic syndrome and then AML, perhaps due to additional damage in the progenitor compartment. The apparent contribution of apoptosis to aplastic anemia and myelodysplasia may therefore also be relevant to the development of AML secondary to benzene exposure.

The data that we presented demonstrate that the bone marrow progenitor compartment represents a direct target for phenolic metabolites of benzene. Induction of apoptosis by metabolites of benzene and consequent depletion of the bone marrow progenitor compartment may therefore represent an important mechanism contributing to the hematopoietic toxicity of benzene.

Acknowledgments

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