Induction of Granulocytic Differentiation in a Mouse Model by Benzene and Hydroquinone

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Chronic exposure of humans to benzene causes acute myelogenous leukemia (AML). The studies presented here were undertaken to determine whether benzene, or its reactive metabolite, hydroquinone (HQ), affects differentiation of myeloblasts. Benzene or HQ administered to C57Bl/6J mice specifically induced granulocytic differentiation of myeloblasts. The ability of these compounds to induce differentiation of the myeloblast was tested directly using the murine interleukin 3 (IL-3)-dependent 32D.3 (G) myeloblastic cell line, and the human HL-60 promyelocytic leukemia cell line. We have previously shown that benzene treatment of HL-80 myeloblasts activates protein kinase C (PKC) and upregulates the 5-lipoxygenase (LPO) pathway for the production of leukotriene D_4 (LTD_4) and other leukotrienes. We have shown that HQ exposure to HL-80 myeloblasts upregulates LTD_4 production. HQ, in addition to benzene, also induces differentiation of 32D.3(G) myeloblasts. Both compounds interact with cellular signaling pathways normally activated by granulocyte colony stimulating factor (G-CSF) and can replace the requirement for G-CSF. While IL-3 induces a growth response in 32D.3(G) cells, G-CSF has been shown to provide both growth and differentiation signals. Both HQ and LTD_4 induce differentiation and synergize with IL-3 for growth; however, neither supports growth in the absence of IL-3. Benzene, like HQ, also provides a differentiation signal for 32D cells; however, it has no effect on their growth. Unlike G-CSF, benzene, or LTD_4, each of which stimulates terminal differentiation, HQ blocks differentiation at the myelocyte stage, allowing only a small percentage of progenitors to proceed to mature segmented granulocytes. Benzene- and G-CSF-induced differentiation were prevented by the addition of either LPO inhibitors or LTD_4 receptor antagonists, indicating that benzene, like G-CSF, upregulates LTD_4 production. Hydroquinone-induced differentiation was not affected by the LPO inhibitors, but only by the specific receptor antagonists. Thus HQ appears to obviate the requirement for LTD_4 by activating the LTD_4 receptor directly. — Environ Health Perspect 104(Suppl 6):1257–1264 (1996)

Key words: benzene, hydroquinone, granulocytic differentiation, G-CSF, LTD_4, signal transduction

Introduction

Benzene, a widely used industrial chemical and ubiquitous environmental pollutant, is a Class I carcinogen that causes acute myelogenous leukemia (AML) in chronically exposed humans (1–4). Benzene hematotoxicity occurs when its hepatic metabolites (5,6), phenol, catechol, and hydroquinone (HQ), are transported to the bone marrow (7,8) and further oxidized in a peroxidase-mediated (9–11) reaction to biologically reactive intermediates that can potentially affect hematopoiesis. An increased incidence of AML is associated with benzene exposure; therefore, it is important to determine whether benzene or any of its metabolites (such as HQ) directly affects the stem cell or progenitor cells of the myeloid lineage. The ability to alter cytokine-dependent growth and differentiation in hematopoietic progenitor cells appears to be a property of many agents with leukemogenic potential for humans (12). Several studies have reported significant effects of benzene on hematopoietic stem and progenitor cells (13). In one study (14), a dose-dependent depression of all stem cell compartments was observed in BDF1 mice exposed for 16 weeks to airborne concentrations of benzene as high as 99 ppm for 6 hr/day, 5 days/week. The results of this study indicated that the granulocyte/macrophage colony-forming unit (GM-CFU) was much less sensitive than the erythroid CFUs at higher doses of benzene. Another study (15) reported that short-term exposure of mice to benzene induced a shift toward granulocytic differentiation, a growth advantage for granulocytic progenitor cells in the bone marrow and spleen, and to a resultant increase in the total number of granulocytes. These results suggest that benzene or HQ is acting on the myeloid stem or progenitor cells. We report here that the administration of benzene, or HQ, specifically stimulates granulopoiesis in mice and induces granulocytic differentiation in myeloblasts of the human promyelocytic leukemia cell line, HL-60, as well as the normal murine interleukin 3 (IL-3)-dependent myeloblastic cell line, 32D.3 (G). Benzene and HQ do this by replacing the requirement for granulocyte colony-stimulating factor (G-CSF) and leukotriene D_4 (LTD_4), respectively, for induction of differentiation.

Methods

Culture of Cells and Exposure to HQ, Benzene, G-CSF, LTD_4, and Other Agents.

HL-60 human promyelocytic leukemia cells (ATCC #240-CCL, Rockville, MD) were cultured at 37°C, 5% CO_2 in RPMI 1640 medium (Mediatech, Washington, DC) supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS). Cells (5 x 10^6/ml) from passages 18 to 42, were treated with or without benzene in RPMI...
1640/2 mM glutamine/10% FBS for 7 days at 37°C, after which differentiation to granulocytes was assessed. Because of benzene’s volatility, cells were treated with benzene in microscopes with teflon-lined screw caps and very small head space. LTD₄ was added directly to the culture medium (see Table 1 and figure legends for concentrations used). The 5-lipoxygenase (LPO) inhibitors and LTD₄ receptor antagonists were also added directly to the culture medium, or concentrations used, but 20 min prior to the addition of the inducing agent.

The IL-3-dependent myeloblastic cell line, 32D.3, derived from normal bone marrow of C3H/HeJ mice, has a normal karyotype and is nonleukemic (16). The clone was previously characterized for IL-3-dependence (17) and for G-CSF induction of differentiation [32D.3 (G)] (18). 32D.3 (G) cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM) with 2 mM glutamine/10% FBS and 3 U/ml recombinant murine IL-3 (rMuIL-3) at 37°C, 5% CO₂, 32D cells (2.5 x 10⁵/ml) in IMDM/2 mM glutamine/10% FBS were treated with or without benzene and with or without 3 U/ml rMuIL-3. When HQ was used, the cells were pretreated with 2 μM HQ in Dulbecco’s phosphate-buffered saline minus Ca²⁺ and Mg²⁺ (PBS)/2 mM glucose [PBS-A] for 30 min at 37°C, after which the cells were collected, washed twice with PBS, and placed into culture in IMDM/2 mM glutamine/10% FBS with or without 3 U/ml rMuIL-3. LTD₄ (as well as recombinant human G-CSF [rHuG-CSF]), the LPO inhibitors, and the LTD₄ receptor antagonists were added as for HL-60 cells, but in the presence or absence of IL-3. Unless otherwise indicated (in figure or table legends), a sample of each culture was removed for cell counting after 3 days of incubation, and the remainder of the culture diluted to 2.5 x 10⁵ cells/ml to ensure optimal growth conditions. Incubation was continued for 4 days, after which the cells were analyzed for the granulocytic phenotype.

**Table 1. Morphological examination of benzene-induced granulocytic differentiation in HL-60 cells.**

| System      | Myeloblast | Promyelocyte | Myelocyte | Metamyelocyte | Mature granulocyte | Total granulocytes |
|-------------|------------|--------------|-----------|---------------|--------------------|--------------------|
| HL-60       | 36         | 45           | 2         | 15            | 2                  | 19                 |
| HL-60 + 5 mM benzene | 15        | 29           | 16        | 30            | 10                 | 56                 |

Cells were treated with benzene as described for Figure 4. Morphological examination was performed by Cytospin preparation and May-Grunwald/Giemsa staining. Percentages were based on the average obtained by counting 200 cells on each of triplicate slides in six experiments.

**Assessment of Differentiation.** Granulocytic differentiation was assessed by four factors: the acquisition of granulocytic morphology—specifically promyelocytes, myelocytes, metamyelocytes, and mature segmented cells; the development of superoxide production—as measured by the reduction of nitroblue tetrazolium (NBT); the development of chloroacetate esterase activity; and, in the case of HL-60 cells, the appearance of the L-12-2 granulocyte-specific surface antigen. Morphological assessment of differentiation was performed by Cytospin preparation (Shandon Scientific Ltd, Pittsburgh, PA) and May-Grunwald/Giemsa staining (Sigma Chemical Co, St. Louis, MO). Percentages were based on the average obtained by counting 200 cells on each of triplicate slides. For NBT reduction, cells were incubated for 1 hr with 0.125 nmol/liter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 0.05% NBT. Cells were fixed on a slide, counterstained with safranin, and the percentage of cells containing black granules of reduced NBT was determined. Chloroacetate esterase activity was determined by incubating fixed cells with a diazonium salt, AS-D chloroacetate, and Red Violet at pH 6.3 for 5 min at 37°C, after which the percentage of cells containing red granules was determined. The presence of L-12-2 surface antigen was detected by fluorescence microscopy using rhodamine-labeled antimouse immunoglobulin against the monoclonal antibody.

**Treatment of Animals with Benzene and HQ.** Inbred C57BL/6J male mice (22–25 g) received benzene (600 mg/kg bw) in corn oil or HQ (25 or 50 mg/kg bw) in PBS ip, twice per day, 7 hr apart, for 2 days. Controls received corn oil or PBS. Eighteen hours after the final injection, the animals were killed by cervical dislocation, their femurs removed, and the bone marrow cells obtained (as described below).

**Determination of Bone Marrow Cellularity and Morphologic Hematology.** The epiphasial plate on each end of the femur was removed and the femur was flushed with 1 ml of 100% FBS using a syringe with a 25-gauge needle. The marrow was collected in a tissue culture tube and a single cell suspension prepared by passing the marrow through the syringe two additional times. A sample (100 μl) of the cell suspension was used to prepare slides, which were then stained (as described below) and used for differential cell counts. The remainder of the cell suspension was diluted in cold lysing buffer (10 mM Tris HCl, pH 7.4, 155 mmol/liter NH₄Cl) to remove red blood cells, and the number of nucleated cells was determined by hemocytometer. Viability was tested by Trypan Blue exclusion and was always >98%. Staining was carried out as follows: the slides were dipped in Wright stain (Hema Quik II) (Curtin Matheson, Houston, TX) for 3 min, washed in tap water for 3 min, and allowed to air dry for at least 30 min. Counterstaining was carried out for 7 min in Giemsa (Azure B Type) prepared fresh as a 1:50 dilution in Hydron buffer, pH 6.8. The slides were then washed for 30 sec under running tap water and allowed to air dry. Differential cell counts were carried out under oil immersion at 100 times. Five hundred cells per slide were counted and the percentage of cells at each stage of differentiation was determined.

**Statistical Analysis.** Data between groups were analyzed using the Student’s t-test, or ANOVA followed by Dunnett’s t-test. Results are expressed as mean values ± SD. A p<0.01 was considered significant.

**Results**

**Stimulation of Granulocytic Differentiation in Mice by Benzene and Hydroquinone**

As can be seen in Figure 1, ip treatment of mice with benzene results in an overall depression of bone marrow cellularity selective for the lymphoid and erythroid lineages, while the granulocytic lineages actually show an increase in cell number and a shift toward granulocytic differentiation. These results directly support those previously obtained by Seidel (14), and Dempster and Snyder (15). A more in-depth examination of the effects of benzene (and its metabolite HQ) on the induction of granulocytic differentiation in mice (Figure 2) indicated that benzene was able to stimulate the differentiation of myeloblasts, as measured by an increase in the percent of promyelocytes and intermediate granulocytic progenitors in the bone. 

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mature marrow one day after the last benzene injection (3 days after the initiation of treatment), but had no effect on the number of myeloblasts. Neither was the number of mature granulocytes stimulated by day 3. However, in experiments carried out for 7 days, benzene significantly increased the number of mature granulocytes compared to control values (Figure 3). The experiments presented here are representative of three such experiments each, all of which gave similar results. Identical results were obtained when the data were expressed as percentage of total cells counted. Hydroquinone administered to mice for 3 days, at a dose of 50 mg/kg bw, also stimulated granulocytic differentiation as indicated by an increased percentage of promyelocytes and intermediate granulocytic progenitors (Figure 2). In contrast to benzene, HQ doubled the number of myeloblasts and stimulated differentiation to GB forms to a greater extent than benzene, yet the terminal differentiation of GB to mature granulocytes was limited. HQ administered at 25 mg/kg body weight caused a lesser, but significant, stimulation of granulopoiesis (data not shown). At these doses of benzene or HQ, there was no loss of animals, nor any overt signs of toxicity, and the viability of the cells flushed from the femurs was always >98%.

Granulocytic Differentiation of Myeloblasts by Benzene and Hydroquinone

These in vivo results suggested that benzene per se, or by its metabolism to HQ, is capable of inducing granulocytic differentiation. However, these results do not indicate whether either compound causes induction directly or indirectly in vivo. To answer this question and to develop a system for use in further mechanistic studies involving the induction of granulocytic differentiation, we turned to the use of myeloid cell lines. The well-studied human promyelocytic leukemia cell line, HL-60, which consists of approximately 35 to 45% myeloblasts and 55 to 65% promyelocytes, was selected because it has been used in many studies as a surrogate for the GM-CFU and because a number of agents have been shown to induce terminal granulocytic differentiation in these cells (19). The normal IL-3-dependent mouse myeloblastic 32D.3 (G) cell line was chosen because it has been adapted to terminally differentiate to granulocytes in response to G-CSF (18) and because any data obtained using this cell line can be

Figure 1. Benzene-induced selective depression of bone marrow cellularity in C57BL/6J mice. Groups (n=4) of male C57BL/6J mice were established. One group served as the control and received only corn oil. The other groups were injected ip with benzene (600 mg/kg bw) in corn oil twice per day, 7 hr apart, for 2 days. Eighteen hours after the final injection, the animals were sacrificed by cervical dislocation, their femurs removed, and the nucleated bone marrow cells obtained and counted as described under methods. An analysis of the total numbers of cells of the various lineages in the bone marrow was carried out. A sample of the bone marrow cell suspension was used to prepare a slide for differential count. The slide was stained and analyzed as described under methods. For each slide 500 cells were counted and the percentage of each cell type determined. This percentage was multiplied by the total number of cells per femur to determine the number of a given cell type, and that was normalized to the number of cells in the control group. Data are expressed as the mean ± SD (n=4). Where there were differences between groups, they were significant at the p≤0.01 level. Lymph, lymphocytes; NEC, nucleated erythroid cells; PM, promyelocytes; Neut, neutrophils; Eos, eosinophils.

Figure 2. The induction of granulocytic differentiation in C57BL/6J mice by benzene or HQ. Groups of mice (n=4) were injected with benzene (600 mg/kg bw) in corn oil or HQ (50 mg/kg bw) in PBS-A, twice daily for 2 days. The control animals received corn oil or PBS-A only. Eighteen hours after the final injection, the mice were killed and their bone marrow obtained. A morphological analysis of the bone marrow was carried out as described under methods. Data are presented as absolute numbers of myeloid cells per femur represented by blast cells and total granulocytic cells of 500 cells analyzed. Qualitatively similar results were obtained when the data were expressed as the percent of nucleated bone marrow cells. The results are expressed as the mean ± SD (n=4); *p≤0.001 as compared with the control group. The data for various cell types from the individual marrows are extremely tight (they range from 30±0.001 to ±0.03) and too small to be plotted by the printer/plotter; therefore, no error bars are visible. PM, promyelocytes; GB, myelocytes, metamyelocytes, and band (ring) forms; GS, segmented mature granulocytes.

Figure 3. The induction of granulocytic differentiation in C57BL/6J mice by benzene over time. Groups of mice (n=4) were treated with benzene as described for Figures 1 and 2. Several groups were sacrificed, and their nucleated bone marrow cells harvested, 18 hr after the last benzene injection (3 days after the initiation of treatment) as before. However, several other groups were maintained for an additional 2 days and then sacrificed (5 days after treatment initiation), and still other groups were maintained for 2 more days before being sacrificed (7 days after treatment initiation). All total and differential counts were determined as for Figures 1 and 2. PM, promyelocytes; GB, myelocytes, metamyelocytes, and band (ring) forms; GS, mature segmented granulocytes.
directly compared with that obtained from the mouse.

**Benzene-induced Granulocytic Differentiation in HL-60 Cells**

As can be seen from the data presented in Table 1 and Figure 4, exposure of HL-60 myeloblasts to benzene, followed by incubation for 7 days, resulted in terminal granulocytic differentiation as measured by morphology (determination of the percent of cells classified as myelocytes or higher granulocytic forms) and the development of several characteristics of the granulocytic phenotype. No significant induction of nonspecific esterase activity (Figure 4), indicative of monocytes, was observed, nor was monocyte morphology observed (Table 1). The ability of benzene to induce granulocytic morphology, as well as the appearance of other markers of the granulocytic phenotype, was a function of the concentration of benzene used over the range of 0.1 mM to 5 mM (data not shown). As can be seen in Table 1, benzene treatment of HL-60 cells caused a significant decrease in the number of normally occurring myeloblasts and promyelocytes with a corresponding shift toward more intermediate progenitors and mature granulocytes. The majority of the differentiated progenitors appeared to be metamyelocytes and myelocytes, but a significant number of terminally differentiated granulocytes was observed (Table 1).

**Involvement of Protein Kinase C and the 5-LPO Pathway in Benzene-induced Granulocytic Differentiation of HL-60 Cells**

Sustained activation of protein kinase C (PKC) is essential to the induction of differentiation in HL-60 cells (20). Both retinoic acid and dimethylsulfoxide (DMSO) induce HL-60 cells to differentiate to granulocytes by activation of PKC (21). Benzene activates PKC in intact rabbit platelets (22) and in the macrophagelike cell lines, P388D1 and BAC1.2F5CSF-1 (Carlson and Kalf, unpublished data). An indication that benzene also activates PKC in HL-60 cells is provided by the benzene-induced phosphorylation of cellular proteins in these cells, as well as the ability of benzene-treated HL-60 cell membrane preparations to carry out the phosphorylation of histone III-S, a PKC substrate, in an *in vitro* assay (23,24). Sphinganine, a potent inhibitor of PKC in *in vitro* and in cell systems (25), prevents not only benzene-induced phosphorylation of cellular proteins, but also differentiation to granulocytes (23,24), implicating the phosphorylation of these proteins by PKC in benzene-induced granulocytic differentiation of HL-60 cells.

An active LPO pathway of metabolism that converts arachidonic acid (AA) to the peptidoleukotriene, LTD₄, is essential for normal (26,27) as well as leukemic (28,29) myeloid progenitor cell differentiation. LTD₄ is an essential intermediate in CSF-induced clonal growth of GM-CFU (26,27). It has been demonstrated that HL-60 cells express a granulocytic phenotype if, during induction, PKC-activated release of AA from plasma membranes, and lipooxygenation of AA to LTD₄, occurs (26). Benzene-treatment of HL-60 cells results in a release of AA from membrane lipids and a subsequent increase in LTD₄, both of which are inhibited by sphinganine (23,24).

Several inhibitors of 5-LPO were tested for their ability to prevent benzene-induced granulocytic differentiation of HL-60 cells as measured by morphology and a functional parameter, superoxide production (NBT reduction). From Table 2 it is apparent that the highly specific 5-LPO inhibitor, AA-861 (30), as well as caffeic acid, prevented benzene-induced granulocytic differentiation and that this inhibition could be overcome by the concomitant addition of LTD₄ to the reaction. To further implicate LTD₄ in the differentiation process, we tested the ability of LTD₄ receptor antagonists to inhibit benzene-induced differentiation. HL-60 cells were incubated with benzene in the presence or absence of MK-571, a highly specific inhibitor of LTD₄ binding to its high affinity receptor (31). Benzene-induced

![Figure 4. Benzene-induced granulocytic differentiation of HL-60 myeloblasts. Cells (5 x 10⁶/ml) were cultured with and without 5 mM benzene, as described in the methods section, for up to 7 days. The development of the granulocytic phenotype was determined by measuring those characteristics presented in the figure. Each analysis was carried out as described in the methods section; each set of values represents the mean ± SD of three experiments, where each sample was carried out in triplicate. * Significantly different from the control cells at p < 0.001. Reprinted from Hazel et al. (24) with permission of AlphaMed Press.](image-url)

| System         | Morphology | NBT reduction |
|----------------|------------|---------------|
| Control        | 12.3 ± 5.5 | 16.7 ± 3.5    |
| + Benzene      | 37.5 ± 3.8 | 45.7 ± 3.3    |
| + Benzene/AA-861 | 17.5 ± 3.9 | 20.8 ± 3.7    |
| + Benzene/AA-861/100 mM AA-861 | 37.7 ± 3.7** | 42.8 ± 3.3** |
| LTD₄           | 23.7 ± 3.3 | 18.3 ± 1.7    |
| + Benzene/LTD₄ | 54.7 ± 10.5** | 28.7 ± 4.1** |

*Significantly different from control and benzene-treated cells pretreated with inhibitors. **Significantly different from cells treated with benzene and inhibitors, p < 0.01. Cells (5 x 10⁶/ml) were preincubated in culture with the 5-LPO inhibitors, caffeic acid (100 μM) or AA-861 (1 μM), and with LTD₄ (1 μM) in RPMI 1640/10% FBS for 10 min at 37°C, 5% CO₂. Benzene (5 mM) was added directly to the culture and NBT reduction and granulocytic differentiation were determined after incubation for 7 days. Values represent the mean ± SD of at least two experiments carried out in triplicate.
granulocytic differentiation of HL-60 cells was completely inhibited by the LTD₄ receptor antagonist and differentiation was restored by the addition of excess LTD₄ (Figure 5). Another receptor antagonist, LY163443 (32), gave similar results (data not shown). Taken together, these results indicate that both production of LTD₄ and its interaction with, and activation of, its receptor are essential for benzene-induced granulocytic differentiation.

Granulocytic Differentiation of the Myeloblastic Cell Line 32D.3 (G) by Benzene and Hydroquinone
To compare the induction of granulocytic differentiation by benzene and HQ with that of the normal physiological inducer, G-CSF, we turned to the IL-3 dependent diploid myeloblastic cell line, 32D.3 (G), which was derived from normal mouse bone marrow and adapted to differentiate in the presence of G-CSF (18). Table 3 presents data from one representative of four experiments that gave similar results. Few differentiated myeloid cells were detected in the presence of IL-3 alone, which, although obligatory for proliferation and survival of the myeloblast, does not induce differentiation. rHuG-CSF, which is fully capable of binding to the mouse G-CSF receptor, induced terminal differentiation to granulocytes. Substitution of noncytotoxic concentrations of benzene or HQ in place of G-CSF also induced differentiation in 32D myeloblasts (Table 3). While both benzene and HQ replaced the necessity of G-CSF for differentiation to granulocytes, neither was able to obviate the dependence of the cells on IL-3 for survival and growth (data not shown).

Granulocytic Differentiation of 32D Myeloblasts by LTD₄
Since LTD₄ is a downstream effector of G-CSF-induced signal transduction and benzene has been shown to produce LTD₄ via activation of PKC with subsequent release of AA (23,24) and upregulation of the 5-LPO pathway (Table 2), we tested the ability of LTD₄ to replace either G-CSF or benzene (as well as HQ) in the induction of granulocytic differentiation in 32D myeloblasts. As can be seen in Figure 6, LTD₄ is capable of replacing these inducing agents in the concentration-dependent induction of terminal differentiation in 32D myeloblasts.

Effects of 5-LPO Inhibitors and LTD₄ Receptor Antagonists on the Ability of Hydroquinone to Induce Granulocytic Differentiation in 32D Myeloblasts
The induction of differentiation by G-CSF (Table 4A) and benzene (data not shown) is inhibited by a specific 5-LPO inhibitor, as expected, since both G-CSF (33) and benzene induce the release of AA (the substrate for 5-LPO) from plasma membranes (23,24). However, the ability of HQ to induce differentiation is not prevented by the 5-LPO inhibitor, suggesting that HQ, in contrast to G-CSF and benzene, does not induce the formation of LTD₄, but rather functions in some other way, perhaps by interacting with the LTD₄ receptor.

Table 3. Induction of granulocytic differentiation in 32D cells by benzene and HQ.

| System                  | Percentage of cells counted |
|-------------------------|-----------------------------|
|                         | Morphology | NBT reduction |
| IL-3                    | 75 ± 3.2    | 4.3 ± 0.6     |
| + G-CSF                 | 30.8 ± 2.5  | 36.7 ± 1.7    |
| + Benzene               | 53.0 ± 1.0  | 55.0 ± 0.7    |
| + HQ                    | 67.8 ± 3.3  | 66.8 ± 1.4    |

Cells (2.5×10⁵/ml) were pretreated with HQ (2 μM in PBS-A) or PBS-A for 30 min at 37°C. The cells were harvested by centrifugation, suspended in IMDM (2.5×10⁵/ml) containing 3 U/ml rMulL-3 and 10% FBS. rHuG-CSF (0.15 ng/ml) or benzene (5 mM) was added to the PBS-A-pretreated cells. Controls and HQ-pretreated cells received only PBS-A. After 3 days of culture at 37°C, 5% CO₂, the medium was changed and the cells diluted to the original concentration to insure optimal growth conditions. The incubation was continued for 4 more days, at which time parameters indicative of granulocytic differentiation were assessed. The values listed represent the mean ± SD of the results of triplicate wells. Data are presented as the percentage of cells showing granulocytic differentiation (promyelocytes to mature segmented granulocytes) of a total of 200 cells counted.

| Table 4. Effects of AA-861 and MK-571 on G-CSF- and HQ-induced differentiation of 32D cells. |
|---------------------------------------------------------------|
| System                  | Percentage of cells counted |
|-------------------------|-----------------------------|
|                         | Morphology | NBT reduction |
| A.                      |              |               |
| Control                 | 17.5 ± 2.6  | 12.5 ± 3.6    |
| +G-CSF                  | 70.8 ± 3.5  | 48.2 ± 10.6   |
| +G-CSF/A-861            | 31.9 ± 0.9  | 12.6 ± 2.4    |
| +HQ                     | 81.5 ± 1.3  | 48.6 ± 5.7    |
| +AA-861/A-861           | 66.1 ± 1.7  | 38.6 ± 2.0    |
| B.                      |              |               |
| Control                 | 17.5 ± 2.6  | 12.5 ± 3.6    |
| +G-CSF                  | 70.8 ± 3.5  | 48.2 ± 16.6   |
| +G-CSF/MK-571           | 32.8 ± 0.8  | 26.8 ± 6.2    |
| +HQ                     | 81.5 ± 1.3  | 48.6 ± 5.7    |
| +MK-571/A-861           | 24.1 ± 1.8  | 15.2 ± 2.5    |

Cells (2.5×10⁵/ml) were pretreated with a final concentration of 2 μM HQ or treated with rHuG-CSF (0.15 ng/ml) as described in Table 3. The 5-LPO inhibitor AA-861 or the LTD₄ receptor antagonist MK-571 was added to each sample at 1 μM final concentrations. Morphological analysis was performed as described for Table 3.
Therefore, we carried out an experiment to ascertain whether the ability of HQ to induce terminal granulocytic differentiation in 32D myeloblasts could be prevented by an LTD₄ receptor antagonist. The addition of the specific antagonist MK-571 completely blocked HQ-induced terminal granulocytic differentiation (Table 4B) indicating that HQ may interact with the ligand-binding domain of the receptor to activate its signal and initiate the cascade of events that result in granulocytic differentiation. Hydroquinone can be oxidized in the cell to p-benzoquinone (BQ), a bifunctional alkylating agent that can interact indiscriminately with available sulphydryl groups on membrane-bound proteins (4,34). Therefore, the possibility exists that BQ may bind covalently to sulphydryl groups located at, or very near, the ligand-binding domain of the LTD₄ receptor, obviating the need for LTD₄ and activating the receptor in a constitutive, rather than signal-driven manner (as is the case with LTD₄).

**Effects of LTD₄ versus HQ on 32D Myeloblast Proliferation and Granulocytic Differentiation**

The indication from LTD₄ receptor antagonist studies that HQ, like LTD₄, induces granulocytic differentiation through direct interaction with, and activation of, the LTD₄ receptor was an interesting one. To further study this phenomenon, we examined the effects of both LTD₄ and HQ on 32D cell proliferation and differentiation, in the presence and absence of IL-3, over time.

An analysis of the kinetics of stage-specific granulocytic differentiation was performed on 32D myeloblasts induced by LTD₄ or HQ in the absence of IL-3. This allowed for a determination of the inductive effects of LTD₄ or HQ, in the absence of any competitive proliferative signal from IL-3. Figure 7 demonstrates that LTD₄ was incapable of providing a proliferative signal, as evidenced by the low number of promyelocytes detected over the 6-day period, but was capable of producing a significant number of terminally differentiated granulocytes, as well as an increased number of intermediate progenitors. At each time point examined, the predominance of cell type (differentiation stage) present was the mature segmented granulocyte. Absence of a proliferative burst was also detected upon HQ treatment; but, unlike LTD₄, HQ induced predominately myelocytes at any given time point (Figure 8). HQ appears unable to induce significant differentiation beyond this point and, therefore, results in an incomplete differentiation program in these cells.

**Discussion**

Exposure of C57BL/6J mice to a dose of benzene (35) known to depress other hematopoietic cell lineages (Figure 1) significantly stimulated granulocytic differentiation as well as the total number of granulocytes, indicating that granulopoiesis was also occurring (Figure 2). While benzene could not provide a growth signal for myeloblasts, it did stimulate their differentiation to promyelocytes and intermediate progenitors, but did not stimulate the production of mature granulocytes by day 3. However, when measured at day 7, benzene had increased the number of segmented mature granulocytes (Figure 3). Our results show a general increase in granulocytes after benzene exposure and are consistent with those that show an increase in the number of granulocytes in bone marrow of DBA/2J mice after short-term exposure to benzene (15).
a major metabolite of benzene found in the bone marrow, also stimulated proliferation and differentiation of granulocytic progenitor cells by day 3 when administered to C57BL/6J mice (Figure 2) as well as in mouse 32D myeloblasts in culture (Figures 7, 8). In contrast to benzene, HQ provided both proliferative and differentiative signals for myeloblasts in vivo that increased the numbers of all progenitor forms, but appeared incapable of inducing terminal differentiation (Figure 2) for reasons that are not yet clear.

To study the mechanism(s) whereby benzene and HQ stimulate granulopoiesis, we investigated whether the inductive effect of benzene or HQ on granulocytic differentiation could be reproduced in myeloblasts in culture. Benzene at noncytotoxic concentrations caused a dose-dependent specific induction of terminal granulocytic differentiation in HL-60 myeloblasts (Table 1; Figure 4), as measured by morphological and functional parameters (superoxide production, chloroacetate esterase activity, and the appearance of the specific surface antigen L-12-2) that are specific for granulocytic differentiation. Benzene induction produced a majority of intermediate progenitors (myelocytes, metamyelocytes and band forms); however, a significant number of mature granulocytes were also observed (Table 1). These results correspond to earlier observations that the granulocytic differentiation of HL-60 cells induced by certain agents is somewhat incomplete and defective (19).

As was previously shown, activation of PKC appears to be involved in the induction of granulocytic differentiation in benzene-treated HL-60 myeloblasts. This is because the phosphorylation of cellular proteins, as well as the induced differentiation, was prevented by the concomitant presence of benzene and the specific PKC inhibitor sphinganine (23,24). Benzene-induced differentiation of HL-60 myeloblasts to granulocytes also appears to involve the induction of a functioning 5-LPO pathway for production of LTD₄, since differentiation is inhibited by the 5-LPO inhibitors caffeic acid and AA-861 (Table 2) and by the specific LTD₄ receptor antagonist, MK-571 (Figure 5). Inhibition by each of these agents was prevented by the concomitant addition of LTD₄. These results together support the view that a role for benzene in the induction of granulocytic differentiation in HL-60 myeloblasts—like that of G-CSF—is to produce LTD₄, which is a necessary signal for granulocytic differentiation. Although G-CSF has not yet been demonstrated to directly cause the production of LTD₄, it has been shown to cause the activation of phospholipase A₂ and the subsequent release of AA from cell membranes (33).

Benzene is also capable of inducing granulocytic differentiation in the diploid IL-3-dependent murine myeloblastic cell line, 32D.3 (G), whereas IL-3 induces only a growth response (Table 3). rHuG-CSF provides not only a differentiation signal (Table 3), but, in the absence of IL-3, a growth signal for 32D myeloblasts as well (24). Benzene, unlike G-CSF, is incapable of inducing growth in the absence of IL-3, but does provide the differentiation signal, and neither G-CSF nor benzene can synergize with IL-3 to stimulate growth (24). LTD₄ and HQ are also incapable of supporting growth of 32D cells in the absence of IL-3, but can synergize with IL-3 to enhance growth (Figure 7). Both LTD₄ and HQ induce granulocytic differentiation in 32D myeloblasts (Figure 8). Either benzene or HQ (Table 3), as well as LTD₄ (Figure 6), can replace G-CSF for induction of differentiation. Note that, in the experiment reported in Table 3, no effort was made to optimize concentrations, so that the magnitude of benzene- or HQ-induced differentiation cannot be quantitatively compared with that of G-CSF. These results, in total, indicate that benzene, with its metabolite HQ, can induce granulopoiesis in 32D mouse myeloblasts since both the number of progenitor cells and terminally differentiated granulocytes increased; and they support our in vivo results concerning the induction of granulopoiesis by benzene and HQ in mice (Figure 2). The ability of HQ to synergize with other cytokines/growth factors (as seen in Figure 7 for IL-3) has been previously documented. Irons et al. (36), showed that pretreatment of nonadherent murine bone marrow cells, or lineage-restricted hematopoietic cells, with HQ, in vitro, followed by culture in complete medium for 8 days, significantly enhanced the number of granulocyte–macrophage colonies induced by recombinant GM-CSF. Optimal enhancement was observed with 1 μM HQ, was largely independent of the concentration of GM-CSF, and was not observed with other marrow metabolites such as phenol or catechol.

The fact that benzene-induced granulocytic differentiation of myeloblasts in culture is prevented by inhibitors of PKC and 5-LPO supports the view that benzene-induced differentiation might also be due to benzene per se, not only the result of its metabolism to HQ in the myeloblast. Consequently, not all of the granulopoietic activity of benzene in vivo can be attributed to its metabolism to HQ. During chronic benzene exposure, benzene and HQ are both present in the bone marrow; therefore both can contribute to granulocytic differentiation. On the basis of results presented here and those previously obtained in our laboratory, benzene, like G-CSF, appears to activate the AA cascade and upregulate the 5-LPO pathway for the production of LTD₄. Hydroquinone appears to obviate the requirement for LTD₄ by activating the LTD₄ receptor directly since specific LTD₄ receptor antagonists, but not 5-LPO inhibitors, prevent HQ-induced granulocytic differentiation in myeloblasts.

One can speculate about the roles of benzene and HQ in benzene-induced AML. As a known clastogen, HQ may cause a leukemogenic initiating event in the myeloblast [i.e., one of the translocations or deletions characteristic of AML (4)], and at the same time covalently bind to the LTD₄ receptor to constitutively activate this signal-driven process and induce granulocytic differentiation, which is incomplete and arrested at the myelocyte stage. Blocks in the developmental program of terminal hematopoietic cell differentiation appear to be a major step in tumor progression (37). The ability of HQ to stimulate an increase in the number of myeloblasts, both in vivo (Figure 2) and in vitro (Figure 7) by an as yet undetermined mechanism, may then result in the classic picture of AML: an overproliferation of incompletely differentiated myeloid cells. Concomitantly, benzene may provide a promotal effect on the initiated myeloblast, via constitutive activation of PKC, and overexpression of its activity, with resultant pleotropic effects on morphology and growth control.

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