Hydroquinone Stimulates Granulocyte–Macrophage Progenitor Cells in Vitro and in Vivo

R. Henschler,¹ H.R. Glätt,² and C.M. Heyworth³

¹Department of Hematology/Oncology, Freiburg University Medical Center, Freiburg, Federal Republic of Germany; ²Department of Toxicology, Deutsches Institut für Ernährung-ungsforschung, Bergholz-Rehbrücke, Germany; ³Department of Experimental Haematology, Christie Hospital, Manchester, United Kingdom

To investigate whether hydroxylated metabolites of benzene may be responsible for the amplification of granulocyte–macrophage progenitor cells (GM–CFC) observed in mice that inhale benzene, groups of six C57BL6 mice were injected with hydroquinone (HQ) (75 mg/kg) or HQ (50 mg/kg) plus phenol (PHE) (50 mg/kg) twice daily for 11 days. Deviations in blood leucocyte and erythrocyte levels by up to one-third were noted in the treated groups; however, the peripheral blood differential counts were unchanged. Although no changes in bone marrow cellularity were observed in mice treated with HQ, cellularity was decreased by a factor of two in the mice that had received HQ plus PHE. The number of GM–CFC per femur was doubled in both treated groups. In vitro experiments using the murine multipotent hematopoietic progenitor cells FDCP mix also showed a duplication of GM–CFC formation in the presence of HQ at concentrations between 10⁻⁶ M and 10⁻¹⁰ M. When HQ and PHE were present at equimolar concentrations, significantly increased colony formation was still observed with 10⁻¹² M of metabolites. The effect was independent of the concentration of GM–colony-stimulating factor used. We suggest that HQ is a major mediator of the stimulatory effect of benzene on GM–CFC in mice. In addition, the in vitro data indicate that a direct effect on GM–CFC is involved. — Environ Health Perspect 104(Suppl 6):1271–1274 (1996)

Key words: bone marrow, benzene, hydroquinone, phenol, granulocyte–macrophage colony-forming cells, granulocyte–macrophage colony-stimulating factor

Introduction

Benzene has been well recognized as a hazardous industrial chemical for many years (1). Exposure to benzene has been associated with increased incidence of blood dyscrasias, including cytopenias, aplastic anemias, myelodysplastic syndromes, and leukemias (2–4). Damage to the hematopoietic system by benzene has been predominantly linked to the formation and accumulation in bone marrow of hydroxylated derivatives such as hydroquinone (HQ) and catechol (5,6). This was shown to be due to the conversion of phenol (PHE), the major in vivo metabolite of benzene, by myeloid cell-derived myeloperoxidase in the bone marrow (7). Oxidation of HQ to p-benzoquinone (BQ) via a semi-quinone intermediate is considered a key step towards the generation of toxic intermediates in bone marrow (8); however, open-ring products such as trans-trans-muconaldehyde may also be responsible for cytotoxic damage following benzene exposure (9,10).

Types of permanent DNA damage caused by benzene or its metabolites include structural chromosome aberrations, aneuploidy, sister chromatid exchange, micronuclei (11), and gene mutations (12). Hydroquinone and BQ have been shown to result in the formation of DNA adducts, whose structures have been characterized in part (13). Other studies have looked at short-term effects of hydroxylated benzene metabolites on different cellular compartments of bone marrow, i.e., different types of stroma cells, hematopoietic stem cells, progenitor cells, and maturing cells of different blood cell lineages. Mainly, marrow-adherent cells, including mononuclear phagocytes, have been defined as targets of hematopoietic damage by benzene (14–17).

Benzene application augments precursor cells of granulocyte–macrophage (GM) lineage in bone marrow of mice during and after chronic inhalation (18). Work by Irons and colleagues (19) demonstrated a synergistic effect of hydroquinone (HQ) on granulocyte–macrophage progenitor cells (GM–CFC) in vitro. Analysis of cytofluorometrically sorted bone marrow cells and plating at low cell numbers demonstrated that this is a direct—i.e., not stroma cell-mediated—effect on GM–CFC.

We investigated whether HQ administered in vivo could reproduce the increase in GM–CFC seen in mice that inhaled benzene chronically (18). We used the protocol that had been reported by Eastmond et al. (20) to reproduce, after ip injection of HQ and PHE, the decrease in bone marrow cellularity associated with chronic inhalation of benzene. To determine if this is a direct effect on the progenitor cells, we conducted analogous in vitro experiments.

Materials and Methods

Chemicals

Hydroquinone, CAT, and PHE (research grade) were purchased from Sigma Chemical Co. (Munich, Germany). Substances were dissolved fresh in phosphate-buffered saline (PBS) and added to the cell cultures at 1 or 0.33% w/v. For treatment of mice, chemicals were dissolved in PBS, buffered to a pH of 7.4 with 100 mM Tris–phosphate, sterile filtered, aliquoted, and kept frozen at –70°C until use. The concentration was adjusted for a constant injection volume of 0.2 ml per mouse.

Mice

Male B6D2F1 mice, 6 to 10 weeks old, were obtained from the Deutsche Tiererversuchsanstalt (Hannover, Germany). They
were allowed standard rodent food and water ad libitum throughout the studies. Mice were treated and kept in groups of six. Injections (50–75 mg/kg/day in 0.2 ml solvent, ip) were administered during the day twice daily, with at least a 6-hr interval between injections, following the schedule of Eastmond et al. (20). On day 12 of the trials, blood smears were obtained from the tail vein, animals were killed by cervical dislocation, and bone marrow cells from femora were prepared according to standard methods (21).

Cytology
Blood and bone marrow smears were performed on glass slides, stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany) and evaluated microscopically. Cell counts were performed in 0.3% acetic acid for white blood cells or in 1% methylene blue in PBS for red cells.

FDCP-mix Cells
Factor-dependent cells Paterson (FDCP)-mix cells (22) were kept in Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO, Paisley, UK) supplemented with 20% pre-selected horse serum (Sigma) and 2% mIL-3 conditioned medium (23). Cells were incubated in a humidified atmosphere at 37°C and 5% CO₂. Blast morphology was controlled using cytopsin preparations at regular intervals. For agar plating, cells were washed twice in IMDM and resuspended at 5000 cells/ml in IMDM containing 20% fetal calf serum (FCS) (GIBCO), 10% (v/v) of 100 mg/ml bovine serum albumin ([BSA]; Boehringer Mannheim) and cytokines dissolved in PBS/1% BSA. Recombinant mouse GM–CSF and interleukin-3 (IL-3) were also from Boehringer Mannheim. A mixture of cells, growth factors, and cytokines was preincubated for 30 min at 37°C, after which 10% v/v of 3.3% agar in H₂O was added; the resulting plating mix was allowed to gel in 1-ml culture dishes (NUNC, Wiesbaden, Germany) before incubation in a humidified atmosphere at 37°C and 5% CO₂ in air. Colonies were evaluated under a light microscope on day 7. Colonies containing more than 30 cells were scored positive.

Results
Effects in Mice Injected with Hydroquinone and Phenol
Groups of six mice were injected ip twice daily with PBS, HQ, or equimolar HQ and PHE. Animal weights remained constant throughout the treatment period (25 g). No abnormal behavior was registered, except for some minutes of behavioral irritation after the first dose in animals that received combined HQ and PHE. Blood leukocyte and erythrocyte counts were relatively unchanged between the treated and control groups, except for an anemia in the HQ + PHE group, and a slight leukocytosis in the HQ-treated group, which was not statistically significant (Table 1). Bone marrow cellularity was reduced upon treatment with HQ + PHE, but not in HQ-treated animals (Table 1). No changes in the blood differential cell count were observed (data not shown). When femur bone marrow cells were assayed for GM–CFC, all mice in the treated groups displayed elevated numbers of GM–CFC per femur in comparison with the levels of the control animals (Figure 1). No GM–CFC could be grown from spleen cells of the animals.

Table 1. Blood and bone marrow cellular parameters following exposure of mice to hydroquinone or hydroquinone plus phenol.

| Parameter                      | Control     | Hydroquinone | Hydroquinone + phenol |
|--------------------------------|-------------|--------------|-----------------------|
| Blood leukocytes, x10⁶/ml      | 49.4 ± 8.0  | 69.5 ± 18.6  | 51.8 ± 27.8           |
| Blood erythrocytes, x10¹²/ml   | 33.0 ± 5.8  | 36.3 ± 13.4  | 24.7 ± 8.3            |
| Bone marrow cellularity, x10⁵/femur | 64.0 ± 14.1 | 59.5 ± 12.0  | 36.8 ± 9.8            |

*Values are means ± SD of six mice. *Statistically different from control using Student’s t-test (p<0.01).

![Figure 1. Influence of HQ and PHE on bone marrow GM–CFC content in mice. Total femur GM–CFC content is shown in control mice, HQ-treated mice and HQ + PHE-treated mice. Each dot represents the mean value of three measurements from the combined contents of both femurs of one mouse. The horizontal line indicates the means of all animals within one group. Wilcoxon’s rank test showed statistical significance in the differences between control and HQ-treated, and controls and HQ- and PHE-treated groups (p<0.005).](image1)

![Figure 2. Influence of HQ and PHE at constant concentrations of growth factors on FDCP-mix colony formation in vitro. Colony formation by FDCP-mix cells was determined in the presence of (A) various concentrations of hydroquinone and a constant concentration of GM–CSF (50 ng/ml) and IL-3 (0.3 ng/ml); (B) various concentrations of HQ and a constant concentration of IL-3 (2% mL-3-conditioned medium yielding a final concentration >150 U/ml IL-3); and (C) various equimolar concentrations of HQ + PHE and constant GM–CSF (50 ng/ml) and IL-3 (0.3 ng/ml). Values are means ± SD from three independent cultures. Statistically significant differences from controls using Student’s t-test are indicated, *p<0.01; **p<0.05.](image2)
Effect of Hydroquinone on Formation of Granulocyte–Macrophage Colonies in Vitro

When HQ was present at noncytotoxic concentrations, the number of GM colonies formed by FDCP-mix cells was enhanced about 2-fold, if either GM–CSF or IL-3 was used as the cytokine driving colony growth (Figure 2A,B). When PHE was present at equimolar concentrations in addition to HQ, a left shift of the dose–response curve of HQ by 2 to 3 logs was observed (Figure 2C). Addition of PHE to HQ-containing cultures did not result in toxicity at concentrations as high as 10^{-5}M. Variation of GM–colony stimulating factor (CSF) concentrations at constant levels of HQ did not affect the ability of HQ to elicit enhanced colony formation at both plateau and also non-saturating cytokine concentrations (Figure 3). This suggests synergistic interaction of HQ and PHE with either GM-CSF or IL-3.

Discussion

We observed an increase in total GM–CFC in the bone marrow of mice treated with HQ or an equimolar mixture of HQ and PHE. Snyder et al (18) reported the presence of increased numbers of GM–CFC in animals that inhaled benzene chronically. The results of the present study indicate that this effect may be mediated by the metabolite HQ. It cannot be determined, however, if HQ itself, or metabolic products of HQ, such as BQ or trans-trans-muconaldehyde, are the moieties that ultimately act on the GM–CFC. A bias also remains with regard to the comparability of the concentrations of the substances reached in vivo between the benzene inhalation protocol and the HQ + PHE injection protocol; this is because direct measurements of bone marrow levels of PHE, HQ, and their metabolites have not been determined for the latter protocol. A relative comparability may be deduced from the fact that the doses of HQ and PHE injected are able to reproduce the bone marrow toxicity that is achieved by inhalation of benzene for the same interval (20).

In previous experiments (R. Henschler and CM Heyworth, unpublished data), instead of an expected change of differentiation pattern upon exposure to HQ, we noted an increase in cell numbers in liquid cultures of murine progenitor cell FDCP-mix that had been induced to differentiate into the GM lineage. When FCDP-mix cells were subsequently analyzed in colony assays, a profound increase in colony numbers was observed in the presence of HQ. Because colony assays of FDCP-mix cells used as few as 5000 cells/ml, the resulting increase in colony formation in these assays is most likely a direct effect of HQ and GM–CSF on the colony-forming cells and not an indirect effect via bystander cells. These studies demonstrate that FDCP-mix cells retain the capability of primary bone marrow cells to reproduce the synergistic effect of GM–CSF and HQ, or HQ and PHE, on myeloid colony formation, as reported by Irons et al. (19) for unfractionated bone marrow or bone marrow cells enriched for primitive populations. As FDCP-mix cells are multipotential cells, however, and as the primary populations used by Irons et al. (19) also contain substantial numbers of multipotential precursors in addition to GM–CFC, it is possible that HQ and GM–CSF act on a multipotential precursor of GM–CFCs rather than on GM–CFCs themselves. They may induce these “pre–GM–CFCs” to undergo extra cell divisions to yield increased numbers of GM–CFC. Another possibility is that HQ and GM–CSF counteract, for example, an apoptotic mechanism that normally results in cell death of a proportion of potential colony-forming cells. Additional work will be necessary to elucidate the mechanisms underlying the synergism between hematopoietic growth factors and benzene metabolites.

REFERENCES

1. Collegium Ramazzini. International Conference on Benzene. Am J Ind Med 7:361 (1985).
2. Laskin S, Goldstein BD. Benzene toxicity: a critical review. J Toxicol Environ Health (Supp 2):23–36 (1977).
3. Goldstein BD. Clinical hematotoxicity of benzene. Adv Mod Environ Toxicol 4:51 (1983).
4. Aksoy M. Benzene as a leukemogenic and carcinogenic agent. Am J Ind Med 4:589 (1985).
5. Andrews LS, Sasse HA, Gillette JR. 1,4-H-Benzene metabolism in rabbit bone marrow. Life Sci 25:567 (1979).
6. Rickert DE, Baker TS, Bus JS, Barrow CS, Irons RD. Benzene disposition in the rat after exposure by inhalation. Toxicol Appl Pharmacol 49:417 (1979).
7. Sawahata T, Rickert DE, Greenlee WF. Metabolism of benzene and its metabolites in bone marrow. In: Toxicology of the Blood and Bone Marrow (Irons RD, ed). New York:Raven Press, 1985:141–158.
8. Greenlee WF, Sun JD, Bus JS. A proposed mechanism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites. Toxicol Appl Pharmacol 59:187 (1981).
9. Witz G, Rao GS, Goldstein BD. Short-term toxicity of trans-trans-muconaldehyde. Toxicol Appl Pharmacol 80:511 (1985).
10. Latriano L, Goldstein BD, Witz G. Formation of muconaldehyde, an open-ring metabolite of benzene, in mouse liver microsomes: an additional pathway for toxic metabolites. Proc Natl Acad Sci USA 83:8356 (1986).
11. Dean BJ. Recent findings on the genetic toxicity of benzene, toluene, xylenes and phenols. Mutat Res 154:153 (1985).
12. Glatt HR, Padykula R, Berchtold G, Ludewig G, Platt KL, Klein J, Oesch F. Multiple activation pathways of benzene leading to products with varying genotoxic characteristics. Environ Health Perspect 82:81 (1989).
13. Jowa L, Winkle S, Kalf G, Snyder R. Deoxyguanosine adducts formed from benzoquinone and hydroquinone. In: Biological Reactive Intermediates. III: Mechanism of Action in Animal Models and Human Disease (Kocsis J, Jollow DJ, Wittmer CM, Nelson JO, Snyder R, eds). New York:Plenum Press, 1985:825.
14. Garnett H, Cronkhite EP, Drew RT. Effect of in vivo exposure
to benzene on the characteristics of bone marrow adherent cells. Leuk Res 7:803 (1983).

15. Gaido K, Wierda D. Modulation of stromal cell function in DBA/2 and B6D2F1 mice exposed to benzene or phenol. Toxicol Appl Pharmacol 81:469 (1985).

16. Lewis JG, Odom B, Adams DO. Toxic effects of benzene and benzene metabolites on mononuclear phagocytes. Toxicol Appl Pharmacol 92:246 (1988).

17. Laskin DL, MacEachern L, Snyder R. Activation of bone marrow phagocytes following benzene treatment of mice. Environ Health Perspect 82:75 (1989).

18. King AG, Landreth KS, Wierda D. Hydroquinone inhibits bone marrow pre-B cell maturation in vitro. Mol Pharmacol 32:807 (1987).

19. Irons RD, Stillman WS, Colagiovanni DE, Henry VA. Synergistic interaction of the benzene metabolite hydroquinone on myelopoietic stimulating activity of granulocyte-macrophage colony-stimulating factor in vitro. Proc Natl Acad Sci USA 89:3691 (1992).

20. Eastmond DA, Smith MT, Irons RD. An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. Toxicol Appl Pharmacol 91:85 (1987).

21. Spooncer E, Heyworth CM. Colony assays of murine haemopoietic cells. In: Haemopoiesis—A Practical Approach (Molineux G, Tests NG, eds). New York: Oxford University Press, 1992.

22. Spooncer E, Heyworth CM, Dunn A, Dexter TM. Self-renewal and differentiation of interleukin-3 dependent multipotent stem cells are modulated by stromal cells and serum factors. Differentiation 31:111 (1986).

23. Karasuyama H, Melchers F. Establishment of mouse cell lines which secrete large quantities of interleukin 2,3,4 or 5, using modified cDNA expression vectors. Eur J Immunol 18:97 (1988).