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Enhancement of Myeloid Cell Growth by Benzene Metabolites Via the Production of Active Oxygen Species

JOSEPH WIEMELS[†] and MARTYN T. SMITH*

Division of Environmental Health Sciences, School of Public Health, 140 Warren Hall, University of California, Berkeley, CA 94720-7360, USA

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In low concentrations, benzene and its metabolite hydroquinone are known to have diverse biological effects on cells, including the synergistic stimulation with GM-CSF of hematopoietic colony formation *in vitro*, stimulation of granulocytic differentiation *in vitro* and *in vivo*, and general suppression of hematopoiesis *in vivo*. These chemicals are also known to be active in the induction of active oxygen species. We used several assays to determine the effects of benzene metabolites (hydroquinone, benzenetriol, benzoquinone) and active oxygen species (xanthine/xanthine oxidase) on cell growth and cell cycle kinetics of the human myeloid cell line HL-60. HL-60 cells treated with these chemicals for 2 h in PBS showed increased growth over untreated controls in a subsequent 18 h growth period in complete media. Incorporation of ^{3}H-thymidine was also increased proportionately by these treatments. Catalase treatment abrogated the increased cell growth of all chemicals, suggesting an oxidative mechanism for the effect of all treatments alike. Cell cycle kinetics assays showed that the growth increase was caused by an increased recruitment of cells from G_0/G_1 to S-phase for both hydroquinone and active oxygen, rather than a decrease in the length of the cell cycle. Benzene metabolite's enhancement of growth of myeloid cells through an active oxygen mechanism may be involved in a number of aspects of benzene toxicity, including enhanced granulocytic growth and differentiation, stimulation of GM-CSF-induced colony formation, apoptosis inhibition, and stimulation of progenitor cell mitogenesis in the bone marrow. These effects in sum may be involved in the benzene-induced "promotion" of a clonal cell population to the fully leukemic state.

Keywords: Hydroquinone, benzoquinone, benzene, active oxygen, cell growth, cell cycle, leukemia

INTRODUCTION

Benzene is an established cause of hematotoxicity and leukemia in man. The mechanisms by which benzene does this remain unclear. Benzene is initially metabolized by cytochrome P4502E1 to benzene oxide and phenol in the liver, and subsequently to hydroquinone, catechol, and

*Corresponding author. Tel.: 510-642-8770. Fax: 510-642-0427. E-mail: martynts@uclink4.berkeley.edu.
[†] Present address: Leukaemia Research Fund Centre, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, England.

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benzenetriol (reviewed in Ref. [1]). These metabolites are fairly stable in the blood, but become oxidized by peroxidases in the bone marrow to highly reactive species. Benzene, via its metabolites, seems to act through several mechanisms – producing DNA strand breaks, topoisomerase II inhibition, and damage to microtubules. These lead to illegitimate recombination, translocations, and aneuploidy (reviewed in Ref. [2]). Epigenetic effects also potentially contribute to the toxicity of benzene, including altered growth and differentiation of progenitor cells,[3–9] and induction or inhibition of apoptosis.[10–12] In this paper, we demonstrate the growth inducing effects of benzene metabolites in HL-60 cells, and discuss the potential involvement of active oxygen species in benzene-induced leukemogenesis.

Evidence is accumulating from several assay systems indicating that active oxygen species contribute to some extent to the toxicological properties of benzene's metabolites.[11,13–18] Among benzene's ultimate metabolites are quinones, which have the capacity to redox cycle.[19] Active oxygen species have been shown to be created following benzene exposure *in vivo* in rodents[13] and in man[18] by using 8-hydroxy-2′-deoxyguanosine (8-oxodG) as a marker. *In vitro*, treatment of HL-60 cells with the metabolites hydroquinone, phenol and benzenetriol, but not catechol, produced 8-oxodG.[13] Benzene metabolites also had extensive and varied effects on the induction of active oxygen species in direct measurements in HL-60 cells.[17] Superoxide was formed in treated cells from three quinonoid metabolites only when the cells were stimulated with 12-O-tetradecanoylphorbol-13-acetate; and H_2O_2 was produced in small amounts from hydroquinone, and in much larger amounts from benzenetriol and benzoquinone. Both hydroquinone and benzenetriol depleted glutathione severely, while benzoquinone had no effect.[17] Active oxygen species have also been determined to be the cause of hydroquinone-induced chromosome breakage (kinetochore-negative micronuclei) based on the ability of antioxidant enzymes to inhibit such breakage.[20] In addition, cellular and specific DNA damage by hydroquinone and benzoquinone were dependent on the addition of the redox facilitators NADH and copper(II).[11]

Oxygen radicals have been implicated in carcinogenesis given the observations that hyberbaric oxygen tension, radiation, quinones, and inhibitors of antioxidant defense are tumor promoters.[21] Some recent studies have focused on the effect of specific oxidants on cell growth. Active oxygen species were shown to be mitogenic in a murine epidermal cell line[22] and in human fibroblasts.[23] In these studies the production of exogenous active oxygen species by xanthine/xanthine oxidase and various chemicals (H_2O_2, potassium superoxide, etc.) was found to stimulate cell growth, and this stimulation could be abrogated with the addition of antioxidants or antioxidant enzymes. Using a variety of treatment conditions with kidney fibroblasts, Burdon and colleagues have suggested that the intracellular balance between H_2O_2 and glutathione may be of significance in both proliferative and senescent states.[24–26] Other researchers have implicated thioredoxin as a central redox regulating molecule, with such activities as regulation of protein nucelotide interactions, signal transduction, and cytokine-like effects.[27]

A recent study demonstrated that active oxygen produced by xanthine/xanthine oxidase was able to potentiate the clonogenic effects of GM-CSF in $CD34^+$ cells.[28] This activity was previously demonstrated with hydroquinone[8] as well as for a variety of chemotherapeutic agents which cause secondary leukemia.[29] In these studies, $CD34^+$ cells were treated in a glutathione-and antioxidant-free environment, i.e., phosphate-buffered saline. Similar treatment conditions were used in studies of altered myeloid diferentiation and inhibition of apoptosis by hydroquinone in cell lines by Kalf and coworkers.[7,12] Production of reactive oxygen species is an appealing common mechanism for all of the biological activities observed in these assays. In the present study we consider growth

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stimulation by various benzene metabolites and xanthine/ xanthine oxidase in HL-60 cells treated in a fashion similar to those used in the studies described above. Benzene metabolites and active oxygen species were found to stimulate cell growth at sub-toxic concentrations, and this stimulation was prevented by catalase. In addition, the enhanced growth was found to be related to the increased recruitment of cells into cycle.

MATERIALS AND METHODS

HL-60 cells, obtained from ATCC, were grown in complete media: RPMI with 10% FBS and 1% penicillin/streptomycin supplement (all from Gibco BRL). Hydroquinone, benzoquinone, and benzenetriol were all from Aldrich and of greater than 99% purity. Xanthine and xanthine oxidase were from Sigma. Chemicals were immediately used after dissolution in sterile water.

Cells in log phase growth were washed in PBS, and resuspended in PBS at a concentration of 10^6 cells per ml in 10 ml in TI-25 tissue culture flasks (Corning). Cells were treated for 2 h with various concentrations of chemicals or vehicle control, washed again in PBS, and returned to complete media, again at a concentration of 10^6 cells per ml in fresh flasks. Catalase (Sigma, 41,000 units/mg, 140 mg/ml) or superoxide dismutase (3170 units/ mg protein) were added to some cultures, just prior to the other chemicals. Catalase was added at a level of 2000 units/ml, and superoxide dismutase at 100 units/ml. For cell growth experiments, cell number and viability were measured 18 h after treatments by using trypan blue dye and a hemocytometer. For ^{3}H-thymidine-labeling experiments, 5 μCi ^{3}H-thymidine (Amersham) was added to the cultures after returning to complete media. Cells were harvested 18 h later. Cells were washed in PBS, resuspended in 2 ml PBS, and 200 μl trichloroacetic acid (TCA) was added and vortexed. The TCA precipitatable material was collected on glass microfiber filters (GF-A, Whatman) under vacuum. The filters were washed twice in succession with each 2 ml 5% TCA in water, methanol/water 1 : 1, and finally ethanol/ether 1 : 1. The filters were then placed in a scintillation counter vial, and air dried. The filters were shaken with 10 ml scintillation solvent, and counts measured on a scintillation counter (Beckman).

The TD_{50} level is defined as the dose at which 50% of chemically-treated cells remain viable after a 24 h incubation in complete media. Cells treated as above for 2 h in various concentrations of chemical were washed and returned to complete media. Live cells were counted after 24 h. At this point, necrotic and apoptotic cells were degraded – i.e., viability was high (> 93%) but debris from dead cells was apparent (data not shown). The dose at which 50% of the cells were recovered (average of four experiments) is defined as the TD_{50}.

Cell cycle kinetics were measured using bromodeoxyuridine labeling and flow cytometry. In the first experiment, it was tested whether hydroquinone treatment affected the time of cell cycle. Cell cycle time was measured by the speed at which the labeled S-phase cells continued through the cell cycle. Cells were labeled for 30 min, then treated with hydroquinone. The labeled cells were washed in PBS and divided in two groups, one receiving 20 μM hydroquinone treatment (a dose which does not induce cell death) and one control. Cells were treated for 2 h, returned to media, and an aliquot removed for fixing and staining as described below. Cells were then grown for an additional 24 h, with samples removed after 10 h and at 24 h. These cells were immediately fixed and stored. In the second experiment it was determined if hydroquinone affected recruitment of cells into cycle by measuring BrdUrd incorporation pre- and post-hydroquinone treatment. Cells were washed and placed in PBS. BrdUrd was added with hydroquinone and control PBS, and left in the culture for the two hour treatment period. These cells were washed, fixed and stained. Parallel cultures without BrdUrd were labeled with BrdUrd for 30 min,

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1 h following treatment, and 12 h following treatment. All processed samples were analyzed simultaneously on a FACScan flow cytometer using Lysis II software. This enabled cells to be analyzed using the same set of regions (areas on 2-dimensional plots to measure frequency and X and Y mean values) since the FACScan detectors and gain settings are close to constant during a single session. All experiments were performed in duplicate, and repeated once on a different day with a different batch of HL-60 cells, with similar results.

For flow cytometry, cells were pelleted and washed twice in PBS, resuspended in 200 µl PBS, and then fixed in 5 ml 70% ice-cold ethanol while vortexing. After 30 min on ice, cells were pelleted and placed in 2 N hydrochloric acid/0.5% Triton X-100 for 30 min to denature the DNA. Cells were pelleted and the cell pellet neutralized with 1 ml 0.1 M sodium tetraborate. Cells were then spun down and stored in 5 ml 70% ethanol at −20°C overnight. The fixed cells were spun down and rehydrated in 1% BSA, 0.5% Tween-20, and then counted by microscopy. One million cells were placed in a microcentrifuge tube and 20 µl FITC-labeled Anti-BrdUrd (Becton Dickenson) was added, and cells incubated at room temperature for 30 min. Cells were washed once in the incubation medium, and then resuspended in 1 ml PBS with 50 µg/ml propidium iodide and analyzed by FACScan using Lysis II software (Becton Dickenson).

RESULTS

Benzene Metabolites and Active Oxygen Stimulate Cell Growth

Cell growth was significantly increased after hydroquinone treatment compared to PBS-treated controls, at doses between 10 and 40 µM (Figure 1). The chemically treated cells which exhibited growth stimulation continued to outgrow controls in the 1–2 days following analysis, resulting in confluence and media exhaustion far sooner than controls (data not shown). Addition of catalase just prior to hydroquinone abrogated the increased cell growth at doses lower than 50 µM. At higher doses catalase treatment protected the cells from hydroquinone toxicity (Figure 1). Similar results were also demonstrated

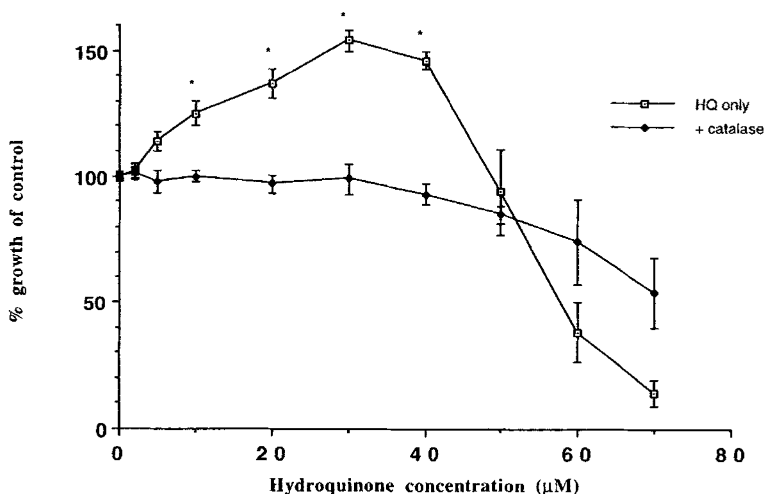

FIGURE 1 Effect of hydroquinone on HL-60 cell growth with and without catalase. Cells were treated with hydroquinone or PBS in the presence (◆) or absence (□) of catalase for 2 h, returned to complete media, and counted by microscope 18 h later. Growth is expressed as the percentage difference between hydroquinone treated cells and control cells. $^{*}p < 0.05$ for the difference, two-tailed t-test. Standard deviations are indicated by error bars.

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for benzoquinone, benzenetriol, and xanthine/xanthine oxidase (data not shown).

Benzene Metabolites and Active Oxygen Stimulate Cell Proliferation

Both hydroquinone and benzoquinone significantly increased cell proliferation in the 18 h following treatment, to levels 1.5–2 times that in PBS-treated controls (Figure 2). Benzoquinone showed a greater effect than hydroquinone at doses that were an order of magnitude lower (Figure 2B), suggesting that it may be the reactive species involved in causing the effect of hydroquinone. This effect was greatest at doses lower than but close to the TD_{50} of these chemicals: the TD_{50} for hydroquinone was 45 µM, and benzoquinone, 3.0 µM. Enhanced growth, at

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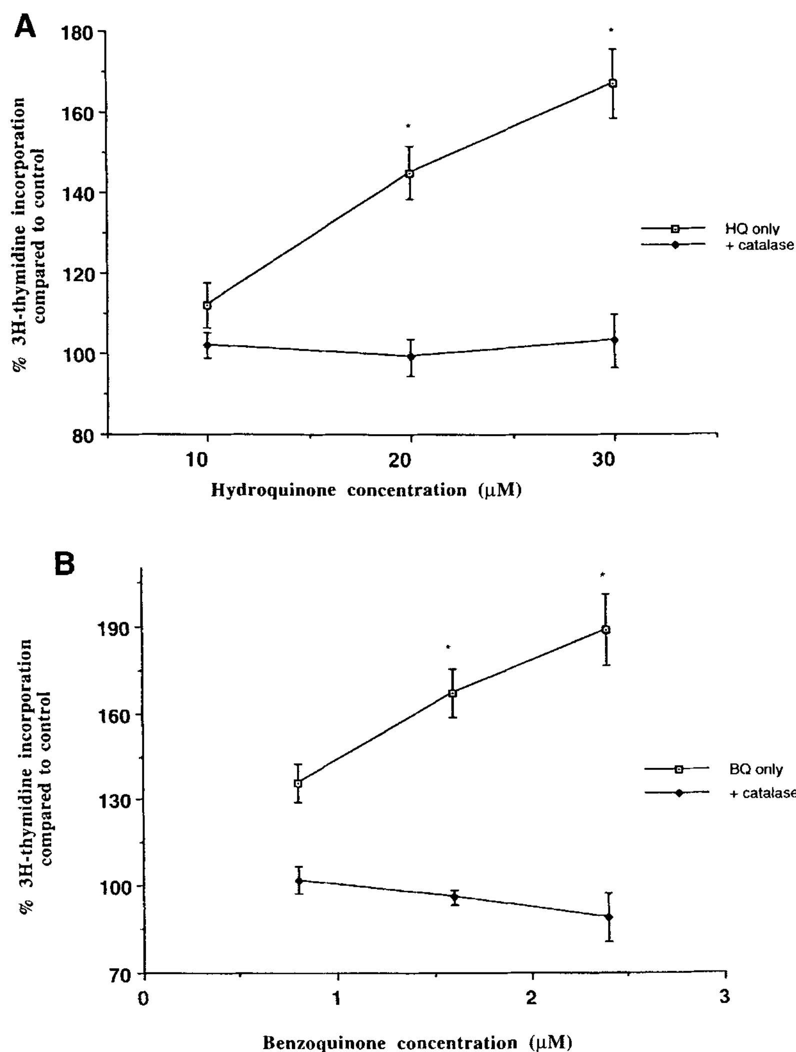

FIGURE 2 HL-60 cell proliferation (^{3}H-thymidine labeling) induced by hydroquinone and benzoquinone and the effects of catalase. Values are expressed as percentage difference compared to controls. A, hydroquinone only (□), with catalase (◆). B, benzoquinone only (□), with catalase (◆). *$p < 0.05$ for the difference, two-tailed t-test.

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comparable levels, was also demonstrated with benzenetriol (data not shown). Catalase again inhibited the cell growth increase. Catalase-treated cells concurrently treated with hydroquinone (Figure 2A), benzoquinone (Figure 2B), or benzenetriol (data not shown) did not show any increase in proliferation over controls. In contrast to the situation with catalase, addition of superoxide dismutase to cells exposed to 2 μM benzoquinone had negligible effects on the stimulation of cell proliferation induced by benzoquinone (data not shown).

Given our results with catalase, we postulated that active oxygen species may play the mediating role in the enhanced cell proliferation. To test this idea, the same assays were applied to a xanthine/xanthine oxidase system used for the generation of active oxygen. Treatment with xanthine/xanthine oxidase caused a dose-dependent increase in cell growth up to toxicity levels (Figure 3). Catalase again prevented the cell growth-enhancing effects of the treatment, as well as partially inhibiting toxicity at the highest dose used. This suggests that hydrogen peroxide is involved in producing these effects.

Benzene Metabolites and Active Oxygen Induce Cells to Enter Cell Cycle, But Do Not Decrease Cell Cycle Time

In the first FACS experiment, cells were prelabeled with BrdUrd, then treated in hydroquinone/PBS or PBS alone. For analysis of data, regions were drawn around the BrdUrd-labeled, and the unlabeled, non-cycling cells. A control run omitting BrdUrd was first analyzed, and a line was drawn to contain > 99% of the cells beneath it. Below this line are the non-cycling cells, above cells in the S-phase of the cell cycle. Fifty-six percent of HL-60 cells labeled with BrdUrd (i.e., fell in the higher region) in the 30 min BrdUrd-labeling period prior to hydroquinone treatment (Figure 4A). At 10 h after exposure to hydroquinone or PBS control, a proportion of the S-phase labeled cells moved into G_2/M and G_0/G_1 phases (not shown). At the 24 h time point the BrdUrd-labeled cells mostly returned to G_0/G_1 phase (Figure 4B and C). These data are in concordance with the cell doubling time of HL-60, which is 30 h (data not shown). An additional region was drawn to delineate BrdUrd-labeled cells in G_0/G_1 (Figure 4B and C). There

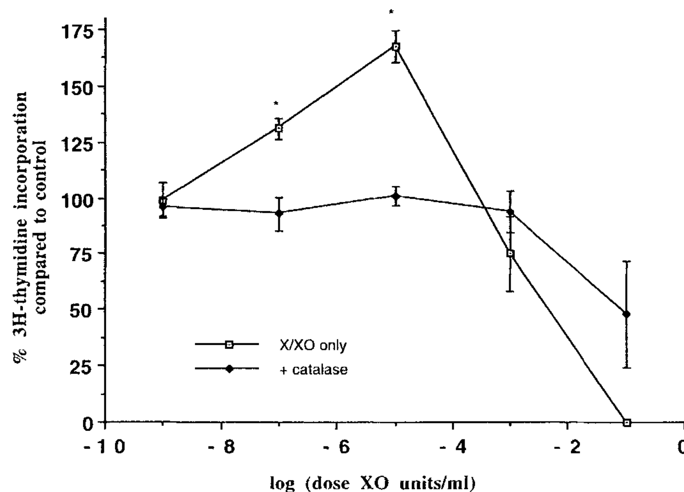

FIGURE 3 Effect of xanthine (1 mM) and xanthine oxidase (XO, 10^{-9}–0.1 units/ml) on HL-60 cell proliferation and the effects of catalase. Xanthine was present in constant concentration in all flasks. Values are expressed as percentage difference compared to controls. Xanthine/xanthine oxidase only (□), with catalase (◆). *$p < 0.05$ for the difference, two-tailed t-test.

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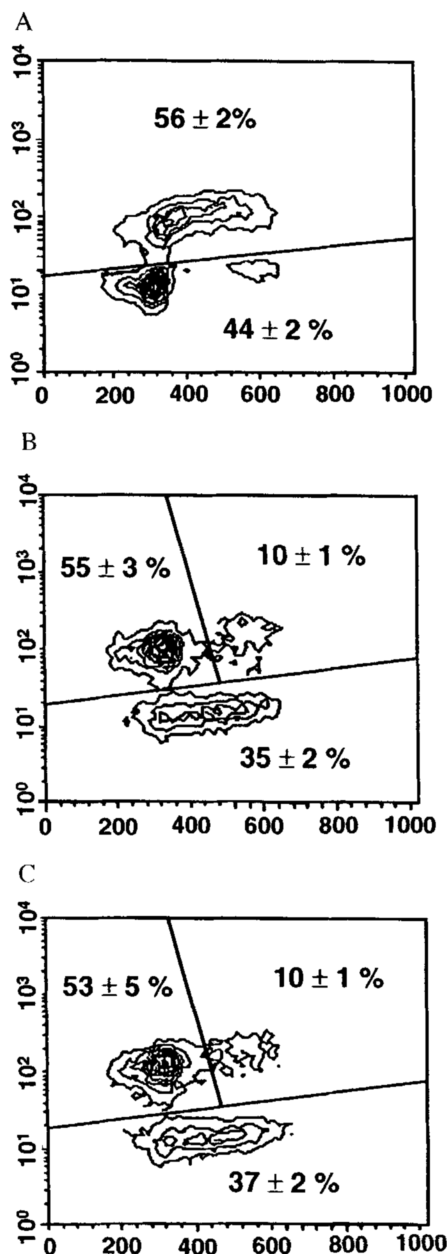

FIGURE 4 Effect of hydroquinone treatment on HL-60 cell cycle time. *X*-axis is DNA content (propidium iodide staining), and *Y*-axis is cell proliferation (BrdUrd content as indicated by anti-BrdUrd, FITC-labeled antibody). A, HL-60 cells labeled for 30 min with BrdUrd. The percentage of cells falling within the regions are indicated. Standard errors were calculated based on duplicate samples. B, same sample as A following 2 h treatment in PBS and 24 h incubation in complete media. C, same sample as A following 2 h treatment in 20 μM hydroquinone in PBS and 24 h incubation in complete media. There were no statistical differences in the cell populations outlined in the three regions in B and C.

were no differences between the speed at which control and hydroquinone-treated cells traversed the cell cycle, indicated by the lack of a difference between the amounts of cells in the upper two regions of Figure 4B and C. Hydroquinone treatment therefore had no effect on reducing cell cycle time.

In the second experiment, unlabeled cells were subjected to hydroquinone treatment while simultaneously being labeled with BrdUrd, or labeled with BrdUrd after the treatment time to determine if hydroquinone effects on cell cycle were delayed. Not only did cells label well with BrdUrd while in PBS, but hydroquinone significantly induced a greater number of cells to label with BrdUrd and to a higher level (Figure 5A and B). Sixty-one percent of the hydroquinone-treated cells labeled with BrdUrd, while 51% of the untreated cells labeled with BrdUrd ($p < 0.001$, χ^2 test). The extent of labeling, that is, the average *Y* value for the S-phase cells, was also greater: 83 to 59 (Figure 5A and B). The increased recruitment of cells into the S-phase persisted after the treatment was finished. There were significant differences between untreated and hydroquinone-treated cells 1 h following treatment (18% of cells cycling in untreated control, 29% in hydroquinone-treated, $p < 0.001$), and 12 h following treatment (38% of control, and 49% in hydroquinone-treated, $p < 0.001$).

Experiments similar to those outlined in Figures 4 and 5 were performed with benzoquinone and the xanthine/xanthine oxidase system in the presence or absence of catalase. Benzoquinone and xanthine/xanthine oxidase produced a similar profile to hydroquinone, and catalase prevented the enhancing effects of hydroquinone, benzoquinone, and xanthine/xanthine oxidase treatments (data not shown).

DISCUSSION

Pretreatment of HL-60 cells with subtoxic concentrations of hydroquinone had remarkable

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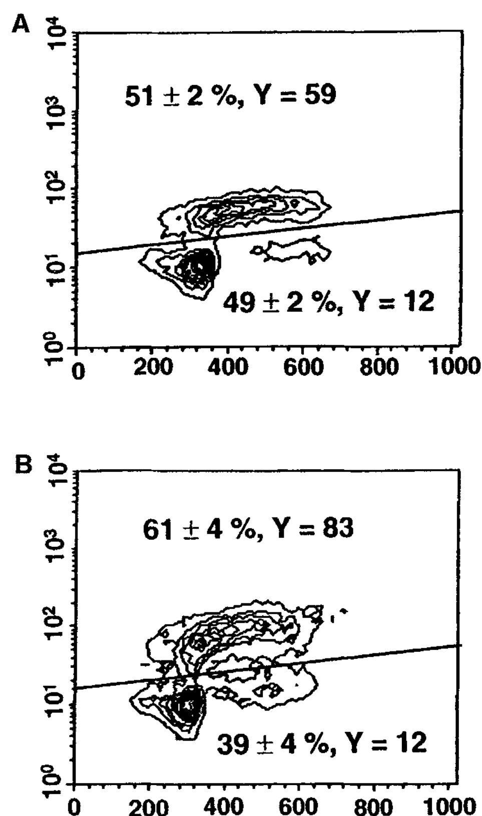

FIGURE 5 Effect of hydroquinone on the recruitment of HL-60 cells into cell cycle. A, cells labeled with BrdUrd for 2 h in PBS. B, cells labeled with BrdUrd in the presence of 20 μM hydroquinone. Percentages of cells falling within the two regions are noted, as well as the average *Y* value (BrdUrd labeling) for all of the cells falling within the region. The difference in percentages of cells was statistically significant ($p < 0.05$).

enhancing effects on cell growth (Figure 1). This enhancement was also apparent in a measure of cell proliferation, ^{3}H-thymidine incorporation into DNA (Figure 2A). Hydroquinone's ultimate metabolite, 1,4-benzoquinone, also caused this proliferative increase, and the effect of both compounds was inhibited by catalase (Figure 2A and B). This suggests that their inductive effects on cell proliferation were mediated by active oxygen species. We therefore tested the effects of a widely used active oxygen generating system, xanthine combined with xanthine oxidase, in the same assays. Xanthine/xanthine oxidase produced an enhancing effect on HL-60 cell growth, which was prevented by the addition of catalase (Figure 3).

The proliferative enhancement caused by the benzene metabolites hydroquinone and benzoquinone followed a linear dose–response curve over a small range of treatments (Figure 2). It should be noted that toxicity to HL-60 cells, i.e., induction of apoptosis, occurred only at doses greater than 30 μM hydroquinone and 2.5 μM benzoquinone (TUNEL assay in flow cytometry, data not shown). Beyond this point the proliferative enhancement effects of the chemicals were balanced by their toxic effects. At treatments at or below those stated above, proliferation occurs in the absence of cell death.

In order to further delineate the specific effects of hydroquinone on cell cycle behavior, cell cycle kinetics were measured by a flow cytometry assay. The question asked was whether hydroquinone acts to increase the rate of the cell cycle by decreasing its length, or whether hydroquinone acts to recruit a higher than normal proportion of G_0/G_1 cells to enter into S phase. Our data support the theory that hydroquinone acts to recruit cells from G_0/G_1 into cycling (Figure 5), and that this recruitment is responsible for the increased cell growth in the 24 h period following exposure to chemical. Hydroquinone does not have any effect on the cell cycle time of labeled S-phase cells (Figure 4) thus showing that the growth increase is not a result of decreased cell cycle length. Our data also suggest that the mechanism by which hydroquinone induces the effects on cell cycle is similar to that induced by active oxygen, as both exposures have very similar effects in these assays. This is in accord with a growing literature on the growth promoting effects of active oxygen in various cell types. Active oxygen has been associated with the enhancement of cell growth in several other studies,[22–24,26,30] and specific gene activation of growth-regulating genes have been shown to follow oxygen radical production.[31–33]

Benzoquinone was found to be the most active metabolite in enhancing cell proliferation, an

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effect which was completely abrogated by the removal of H_2O_2 (via catalase, Figure 2). Interestingly, benzoquinone was the most active metabolite among several tested in producing H_2O_2 in HL-60 cells,[17] further suggesting that H_2O_2 is the active species in cell growth induction. Further, it was observed that superoxide dismutase did not alter the stimulation of cell proliferation induced by benzoquinone (data not shown). We cannot rule out superoxide as playing a role in the growth stimulation, but we can conclude that extracellular superoxide is most likely not the cause. It could be argued that contaminants in the catalase preparation, including other enzymes, were actually causing the effects on growth ascribed to catalase in the present study. The catalase preparation used was suspended in a solution of 0.1% thymol, but this was diluted 2870 times for a final thymol concentration in media of 24 nM. The test chemicals were added at concentrations 2–5 orders of magnitude higher concentration, and it is unlikely that the small amount of thymol could inhibit an effect from a wide variety of chemicals and treatment conditions. We also conclude that SOD contamination of the catalase preparation could not cause the effects ascribed to catalase, since SOD itself had none.

The role of cell growth enhancement and active oxygen in benzene toxicity has been a continuing interest of several laboratories including our own.[13,34] Irons and colleagues have shown that hydroquinone pretreatment induces a higher number of bone marrow cells to become responsive to GM-CSF in a colony-forming assay.[8] Recently they extended this assay to include reactive oxygen species, which also induced this recruitment.[28] A number of other chemicals have varied results in this assay, and it has been proposed that the assay is peculiarly sensitive to leukemogenic chemicals and not sensitive to chemicals that may be myelotoxic but not leukemogenic.[29] The treatment conditions, however (30 min in PBS), would favor the production of active oxygen species and it cannot be ruled out that the effect is active oxygen species-induced, at least in part. An effect that is shared by a variety of chemicals capable of altering redox state in cells, and reactive oxygen species themselves, most probably relates to an altering of redox sensitive signal transduction pathways in cells. Indeed the discovery that *ras/rac* mediates mitogenic signaling in fibroblasts by active oxygen species[30] underscores the notion that active oxygen species are an important component of normal cell signaling and that a chemical's effect on cell growth or differentiation cannot be explored without an examination of its effects on active oxygen production or the redox state of the cells.

Irons and colleagues' work has recently been extended by Henschler *et al.* to test whether cells from animals treated *in vivo* also demonstrated elevated Granulocyte-Macrophage Colony Forming Units in Culture (GM-CFC).[3] Indeed this relationship existed; GM-CFC per mouse femur were more than doubled in treated animals. Henschler and coworkers also tested CFC formation from factor-dependent cells following hydroquinone and phenol treatment. CFC were increased with hydroquinone treatment, and the addition of phenol caused a further increase in the order of 2–3 orders of magnitude.[3] The authors suggested that hydroquione may induce "pre-GM-CFC" to undergo extra cell divisions, a mechanism supported by the data in the present study. In other work, Farris and colleagues demonstrated that benzene-induced hematosuppression is followed by an increased percentage of progenitor cells in S-phase.[35] Upon cessation of the benzene exposure, the granulocytic lineage overwhelmed the bone marrow as a result of the need to repopulate the hematopoietic system. This hyperplastic state is reminiscent of the data presented here and that performed by other researchers, who have seen enhanced granulocytic proliferation and differentiation both *in vivo* and *in vitro*.

In bone marrow progenitor cells, differentiation is inextricably linked to proliferation, that is, cells divide a defined number of times at each stage and need growth factors to maintain

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both viability and growth. Specific stages of growth are linked to high proliferative capacity; these are the "blast" stages. But even in these stages, cells are irreversibly committed to apoptosis if removed from the ideal conditions for proliferation.[36] The cell cycle stage by which the commitment to grow or enter apoptosis is the G_1/S transition, it is at this stage where most cell cycle "checkpoints" are in place.[37] We have shown that hydroquinone and active oxygen species are able to induce cells to cross this checkpoint at a greater rate than normal. Bypassing this checkpoint could explain hydroquinone's inhibition of staurosporine-induced apoptosis seen by Kalf and coworkers.[12]

Several genes are known to be activated by redox mechanisms, including *egr-1*,[32] NF-kappa B,[33] and *fos*, *jun*, and *myc*[31,38] (reviewed in Ref. [39]). Indeed such gene activation or suppression has recently been shown to occur in response to chemicals such as the chemotherapeutic daunorubicin,[40] and the benzene metabolites hydroquinone[41] and trans, trans-muconaldehyde.[42] Both of these chemicals are known to be inducers of active oxygen species. It is likely that the cell growth enhancement in the present study was a direct effect on transcription of redox sensitive genes or the cellular signal transduction pathways which control them. In either case, active oxygen effects on cell cycle related genes – their expression and phosphorylation status following exposure to chemicals – is a fertile research area.

Acknowledgements

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