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Peroxidase Activity in Murine and Human Hematopoietic Progenitor Cells: Potential Relevance to Benzene-induced Toxicity

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SUMMARY

Peroxidases may be important in the mechanism of toxicity of a number of compounds including benzene, a chemical that has been associated with bone marrow toxicity and leukemia after chronic exposure. The major peroxidase in bone marrow is myeloperoxidase (MPO), which has been previously thought to be expressed at the promyelocytic stage of differentiation. Hematopoietic progenitor cells are important potential cellular targets of bone marrow toxins and leukemogens. We therefore examined peroxidase activity in both murine and human progenitor cells. Murine progenitor populations were purified as lineage-negative cells (>99% enriched) and human progenitor populations were purified as $CD34^+$ cells (>95% enriched). Using conventional biochemical assays for peroxidase activity, murine and human progenitor cells were found to have 30% and 11% of the peroxidase activity of murine and human unpurified marrow, respectively. Peroxidase activity was confirmed in purified murine and human progenitor populations by flow cytometry using a 2,7-dichlorofluorescein assay, adapted to measure peroxidase activity. In addition, two-color flow cytometry of murine whole marrow using phycoerythrin-conjugated antibodies to lineage markers confirmed the peroxidase activity of the murine progenitor cell population. A reverse transcription-polymerase chain reaction assay was developed for MPO mRNA, which was detected in murine progenitor cells. These data show that MPO mRNA is expressed in murine progenitor cells and that both murine and human progenitor cells have marked peroxidase activity. These data may have relevance for studies of hematopoietic cell differentiation and for the examination of mechanisms underlying cell-specific toxicity in bone marrow.

Benzene is an important industrial chemical known to produce hematotoxicity in mice or humans (1, 2). Peroxidase-mediated metabolism of benzene-derived phenolic compounds generates reactive and cytotoxic quinones (3–5), which have been proposed to be responsible for benzene-induced toxicity (6, 7). The bone marrow contains high peroxidase activity (8, 9) and rat, mouse, and human bone marrow can readily bioactivate phenolic metabolites of benzene in a peroxidase-mediated process (10, 11). Additional target organs of benzene, such as Zymbal, harderian, and preputial glands (12), identified in rodent species are also known to be rich in peroxidase activity (13–15), suggesting that peroxidases are important in benzene-induced pathology. It is apparent from these studies that peroxidases play a pivotal role in bioactivation of benzene metabolites and that bone marrow cells containing significant peroxidase activity can be considered as potential targets of benzene toxicity. The bone marrow contains a number of different peroxidases, including MPO, eosinophil peroxidase, and prostaglandin synthase (8, 9, 16–19). Of these enzymes, MPO is known to be present in bone marrow in the highest concentrations and to be synthesized in granulocyte precursor cells at least as early as the promyelocytic stage of differentiation (8).

An important potential target cell population in bone marrow is the hematopoietic progenitor and stem cell compartment. Hematopoietic progenitor cells differentiate into mature blood cell forms, and exposure to benzene *in vivo* is known to depress the number of hematopoietic progenitor cells, measured as colony-forming units-spleen and in other colony-forming assays (20, 21). Recently, it has been shown that alterations in differentiation in myeloid progenitor cells may be of relevance in the pathogenesis of acute myeloid leukemia secondary to benzene exposure (22). These data suggest that hematopoietic progenitor cells are a relevant target cell population of benzene and its metabolites.

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ABBREVIATIONS: MPO, myeloperoxidase; DCFH, 2,7-dichlorodihydrofluorescein; DA, diacetate; TMB, tetramethylbenzidine; bp, base pair(s); PCR, polymerase chain reaction.

The enzymology of hematopoietic progenitor cells has not been investigated due to the difficulties associated with purification of cells in sufficient number and purity. In this study, we have examined the peroxidase activity of murine hematopoietic progenitor cells by two different approaches. Conventional biochemical techniques were used to quantitatively determine peroxidase activity in enriched progenitor cell populations, purified as lineage-negative cells using a cocktail of antibodies to remove cells with lineage-specific markers. Peroxidase activity in enriched progenitor cells and in whole bone marrow was also measured directly using flow cytometry. In addition, we measured the peroxidase activity of human progenitor cells, defined as CD34-enriched populations. Our data show that both murine and human hematopoietic progenitor cells contain peroxidase activity and thus may be potential targets of xenobiotics that are bioactivated by peroxidases.

Experimental Procedures

Materials. DCFH-DA was obtained from Molecular Probes (Eugene, OR). TMB was purchased from Aldrich Chemical Co. (Milwaukee, WI). MPO and Micro-Fast Track mRNA isolation kit version 2.0 were purchased from Calbiochem and Invitrogen (San Diego, CA), respectively. DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Superscript, random primers, and deoxynucleoside triphosphates were obtained from GIBCO BRL (Gaithersburg, MD). RNasin, *Thermus aquaticus* DNA polymerase, and *Sac*I were purchased from Promega (Madison, WI). The Gene Clean II kit was obtained from Bio 101 (La Jolla, CA).

Murine progenitor cell preparation. Lineage-negative cells were prepared as described previously (22). Briefly, bone marrow was extracted from the femurs of male C57BL/6 mice and purified over a discontinuous Lympholyte gradient. Nucleated cells were counted using Turk's solution, and viability was determined using trypan blue exclusion. Marrow was then incubated with lineage-specific antibodies (CD4, CD8, B220, Mac-1, and GR-1) and run over a column containing Sepharose 4B linked to anti-rat κ. The purity of lineage-negative cells was determined using phycoerythrin-conjugated anti-rat κ. A sample of labeled precolumn cells was used as a whole-bone marrow control for peroxidase activity measurements using TMB. Cells that were labeled with lineage-specific antibodies were designated as mature cells. Cells without lineage markers were designated as lineage-negative or progenitor cells. Additionally, labeled precolumn cells (whole bone marrow) were conjugated to phycoerythrin for use in two-color flow cytometry.

TMB assay for peroxidase activity. Peroxidase activity in progenitor cells and whole bone marrow was measured using TMB, as described (16). Briefly, cells were lysed by sonication or freeze thawing four times in liquid nitrogen and were then appropriately diluted in deionized water. A 100-μl sample was incubated at 37° for 4 min in potassium phosphate buffer (50 mM, pH 6) with TMB (1 mM) and hydrogen peroxide (1 mM). The assay was then stopped with 0.1 ml of 5 M phosphoric acid and read at 450 nm with a spectrophotometer. Calibration curves were obtained using MPO in each experiment ($r^2 > 0.99$), and at least two different cell concentrations were used to ensure linearity of response. All measurements made were normalized to cell number, which was determined using Turk's solution.

Flow cytometry. Peroxidase activity was measured using DCFH-DA and flow cytometry (23). Conditions were optimized using HL-60 (peroxidase-rich) and U937 (peroxidase-deficient) cells and then further confirmed in murine whole bone marrow. The final assay conditions were as follows: 1×10^5 to 1×10^6 cells were incubated for 7 min at 37° in the presence of 10 μM DCFH-DA, with or without 10 μM hydrogen peroxide. Flow cytometry was performed on a Coulter Epics 752 flow cytometer equipped with a Coherent 90–5 argon laser operated at 500-mW power, with an excitation wavelength of 488 nm. Emitted light was collected with 525-nm and 575-nm band-pass filters. Data for 70,000 events were collected in list mode for linear forward-angle light scatter, linear 90 degree light scatter, logarithmic orange fluorescence, and logarithmic green fluorescence. The list mode data were analyzed using Coulter Elite software.

Scanning fluorimetry. Human MPO was used to validate the use of DCFH as an assay system for peroxidase activity. DCFH-DA was hydrolyzed according to the method of Ref. 23. Briefly, 10 μl of 10 mM DCFH-DA were added to 40 μl of 0.1 M NaOH. The reaction was incubated for 2 min at 22° and was then stopped with 1.95 ml of 50 mM potassium phosphate buffer, pH 7.8. This reaction mixture was used to test a series of MPO concentrations, with and without peroxide in the reaction mixture. The reactions were run at 37° for 7 min in the dark. The reaction mixture consisted of the following: 10 μM DCFH, with or without 10 μM H_2O_2 and MPO in concentrations from 0.05 to 0.8 unit, in a total volume of 1 ml in 50 mM phosphate buffer, pH 7.4. The reactions were read using an excitation wavelength of 488 nm and an emssion wavelength of 519/520 nm (average value).

Reverse transcription-PCR. Murine bone marrow was collected as described above. mRNA was isolated from 2×10^5 to 4×10^6 cells using the Micro-FasTrack mRNA isolation kit, version 2.0. mRNA was reverse transcribed using the Superscript RNase H-reverse transcriptase kit and random primers. cDNA was amplified using *T. aquaticus* DNA polymerase and the appropriate primers. The murine MPO cDNA sequence was extracted from GenBank. MPO sense and antisense oligonucleotide sequences used were 5′-AATGTCTTCACCAATGCTTTCC-3′ and 5′-TAGCTTCCTAAACTGAGTGC-3′, respectively. cDNA was amplified for 38 cycles with a Robocycler (Stratagene, San Diego, CA), with 1) an initial denaturing cycle at 93° for 1 min, 2) 38 cycles of annealing at 50° for 30 sec, extension at 72° for 30 sec, and denaturation at 93° for 30 sec, and 3) a final extension cycle of annealing at 50° for 30 sec and extension at 72° for 5 min. Products were visualized on 1% agarose gels containing ethidium bromide. Restriction enzyme digests of the amplified MPO segment were performed using *Sac*I. The MPO segment (543 bp) was isolated after PCR by using the Gene Clean II kit and was digested using *Sac*I, which cut the segment once, into 171- and 372-bp fragments.

Isolation of human progenitor cells. Human bone marrow was obtained, with informed consent, from normal adult volunteers and was enriched for $CD34^+$ cells using the MiniMACS separation system, as directed by the manufacturer (Miltenyi Biotec Inc., Sunnyvale, CA). Briefly, mononuclear cells were isolated using Histopaque-1077 and labeled with anti-CD34 (QBEND/10) and superparamagnetic MACS microbeads. $CD34^+$ cells were obtained by positive selection using a MiniMACS separation unit and column. Separated cells were then labeled for flow cytometric analysis using a fluorescein-conjugated antibody (ICH-3) specific for a different CD34 epitope, compared with QBEND/10.

Results

Murine hematopoietic cells enriched in lineage-negative cells were prepared using a cocktail of lineage-specific antibodies (CD4, CD8, B220, Mac-1, and GR-1), as described in Experimental Procedures. Cells labeled with lineage-specific antibodies were designated as mature cells, and immature (lineage-negative) cells were obtained by negative selection. The purity of lineage-negative or progenitor cells was determined using flow cytometry. Preparations of progenitor cells exhibiting <99% purity were discarded. The average purity of cells used for biochemical assays was 99.6% (Table 1).

The peroxidase content of purified progenitor cells was determined using a conventional biochemical assay with TMB as the substrate. Progenitor cells contained significant peroxidase activity (Table 1). The peroxidase content of each preparation of progenitor cells was also compared with the whole-bone

TABLE 1

Peroxidase activity of murine lineage-negative cells and murine whole-bone marrow cells

In all cases, six matched samples were used. Values are mean ± standard error.

Lineage-negative cell purity[a]	Peroxidase activity[b]		Activity ratio[d]
	Whole bone marrow	Lineage-negative cells	
%	absorbance units/4 min/10^6 cells		%
99.61 ± 0.13	10.76 ± 1.93	3.20 ± 0.86[c]	30 ± 5

[a] As assessed by flow cytometry.
[b] Determined using TMB, as discussed in Experimental Procedures. Conversion to unit equivalents of human MPO activity/10^6 cells can be achieved by multiplying the peroxidase activity by 0.065.
[c] Significantly different from whole bone marrow ($p < 0.05$).
[d] Lineage negative activity as a % of whole bone marrow activity.

marrow sample from which it was purified. The peroxidase content of progenitor cells was approximately 30% that of whole bone marrow, on a per 10^6 cell basis.

As an independent confirmation of peroxidase activity in progenitor cells, enzyme activity was measured directly using flow cytometry. The fluorescent probe DCFH-DA has been used previously as a probe for flow cytometric measurement of peroxidase activity (23). DCFH-DA is taken up into cells by diffusion and intracellularly hydrolyzed to DCFH by esterases. DCFH can then be oxidized to its fluorescent product (green) via peroxidase activity. DCFH can also be oxidized to its fluorescent product, however, by reactive oxygen species (23). To ensure that DCFH-DA could be used as a valid assay of peroxidase activity, DCFH-DA was chemically hydrolyzed to DCFH and fluorescence calibration curves for MPO in the presence and absence of hydrogen peroxide were obtained under a variety of conditions. Optimal conditions were determined where hydrogen peroxide did not induce fluorescence and the assay was quantitative for peroxidase activity. A MPO calibration line was determined using optimal assay conditions and is shown in Fig. 1. Identical conditions were then used for flow cytometric measurement of peroxidase activity.

Flow cytometric analysis of peroxidase activity in peroxidase-rich HL-60 and peroxidase-deficient U937 cells confirmed that this assay could be used as a measure of peroxidase activity in cells (data not shown). The technique was then applied to both purified progenitor cells and whole bone marrow, and marked hydrogen peroxide-stimulated fluorescence could be detected in this cell fraction (Fig. 2). Flow cytometric assay of peroxidase activity in progenitor cells was also verified by two-color fluorescence analysis of Ficoll-purified bone marrow. Antibodies to lineage markers were used to label the more mature cells in unpurified whole marrow, and these were then detected by a secondary antibody conjugated to phycoerythrin. Peroxidase

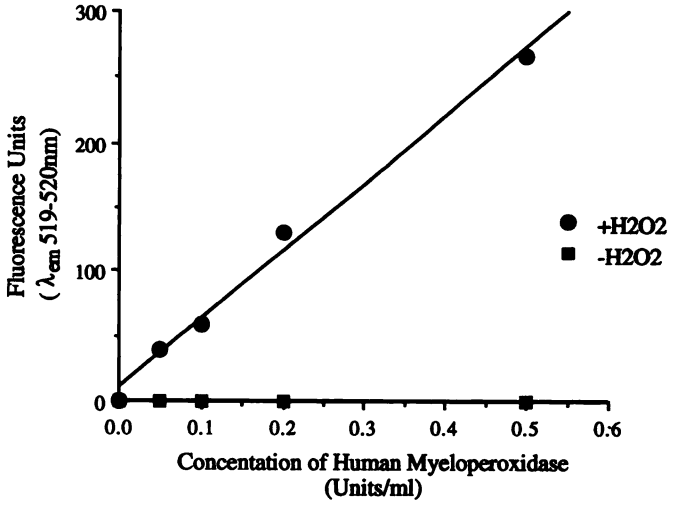

Fig. 1. Concentration and peroxide dependence of DCFH fluorescence with human MPO ($r^2 > 0.99$).

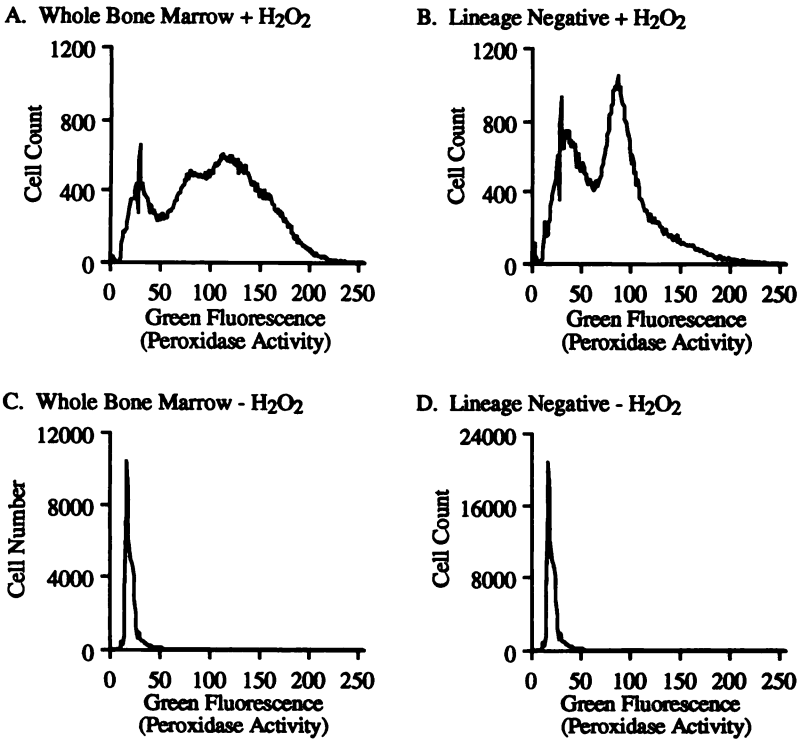

Fig. 2. Peroxidase activity in murine whole bone marrow (A and C) and purified progenitor (lineage-negative) cells (B and D). Data shown are from flow cytometric plots in the presence (A and B) or absence (C and D) of hydrogen peroxide.

activity (green fluorescence) in progenitor cells (orange-negative signal) of whole bone marrow was measured using two-color fluorescence (Fig. 3A). The signal from purified lineage-negative cells gated on orange-negative is shown in Fig. 3B for comparison. When fluorescence was measured by gating on the well characterized progenitor region (24–26) of the flow cytometric light-scatter plots of Fig. 3, A and B, green fluorescence could also be detected (Fig. 3, C and D). These data confirmed the peroxidase activity of progenitor cells.

The peroxidase activity measured using either TMB or DCFH-DA could reflect the presence of a number of different peroxidases. To determine whether MPO was expresed in progenitor cells, a PCR assay was developed for MPO mRNA and applied to progenitor cells. Fig. 4 shows that MPO mRNA could be detected in progenitor cells and to a lesser extent in whole bone marrow. The resultant PCR product was cleaved using the restriction enzyme *Sac*I and the expected 171- and 372-bp cleavage products were obtained (data not shown). The relatively small amount of MPO mRNA in whole bone marrow is not unexpected, because MPO mRNA is known to become negligible with myeloid differentiation from the promyelocytic stage (18). In addition, the presence of marked enzyme activity in the absence of MPO mRNA has been well characterized in HL-60 cells (18).

The peroxidase activity of human progenitor cells was also measured using both biochemical and flow cytometric techniques. Human progenitor cells were purified as $CD34^+$ cells, and CD34 enrichment resulted in a cell population where >95% of cells were positive for this marker. Peroxidase activity was then measured using TMB and was compared with that of human bone marrow before purification. Human CD34-enriched cells contained 10.8 ± 4.5% (mean ± standard error, three experiments) of the peroxidase activity of whole bone marrow. This result was also verified using flow cytometry and the results are shown in Fig. 5. Both precolumn (Fig. 5A) and postcolumn (CD34-enriched) (Fig. 5B) cells demonstrated marked stimulation of peroxidase activity (green fluorescence) in the presence of hydrogen peroxide.

Discussion

Our data show the presence of marked peroxidase activity in both murine and human hematopoietic progenitor cells. Murine progenitor cells were enriched by using antibodies to the lineage markers CD4, CD8, B220, Mac-1, and GR-1. Human progenitor cells were defined on the basis of $CD34^+$ cells, because CD34 is expressed on immature blast cells of various lineages, including virtually all hematopoietic cells capable of forming colonies *in vitro* (27–29). Both murine and human progenitor populations represent heterogeneous cell populations. The human CD34-enriched population is known to include progenitor cells of different lineages and different stages of differentiation and can be further subdivided by the use of additional markers (30). The murine population obtained is less well characterized but, based on the antibodies used for purification, would also be expected to include progenitor cells of different lineages.

This work demonstrating peroxidase activity in $CD34^+$ cells is in agreement with a recent study by Strobl *et al.* (30), who examined the expression of MPO in human hematopoietic progenitor cells using a fluorescein-labeled antibody to MPO and flow cytometry. The latter technique, however, measures the amount of enzyme rather than functional enzyme activity. In contrast to our work, which measured total peroxidase activity in both mouse and human progenitor cells, Strobl *et al.* (30) measured the amount of MPO protein in human cells.

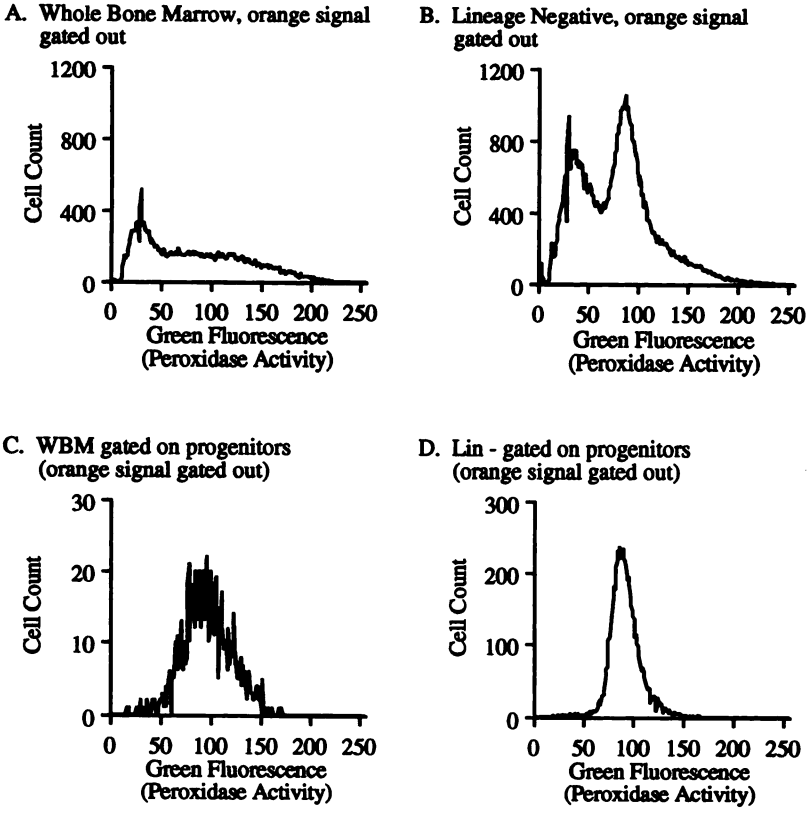

Fig. 3. Peroxidase activity in murine whole bone marrow (*WBM*) (A and C) and purified progenitor (lineage-negative) cells (*Lin* −) (B and D). Data shown are from two-color flow cytometric plots using a variety of gating protocols. A and B, Gated on the orange-negative signal; C and D, gated on the orange-negative signal and the well characterized progenitor region (24–26) of the flow cytometric plot.

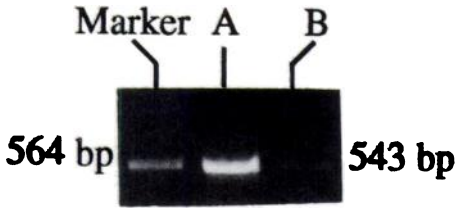

Fig. 4. PCR of murine progenitor cell MPO mRNA. *Marker*, *HindIII* digest of λ DNA (564 bp); *lane A*, lineage-negative cell mRNA (543 bp); *lane B*, whole-bone marrow mRNA (543 bp).

Those authors also utilized CD34 as a marker of progenitor cells but, in addition, subfractionated this population further by use of the marker CD33 and other antigens. CD33 is expressed by a subpopulation of $CD34^+$ cells, and the $CD34^+$ $CD33^{low}$ population is thought to represent a cell population at an earlier stage of differentiation than the $CD34^+$ $CD33^{high}$ population (28, 31). Strobl *et al.* (30) showed that $CD34^+$ $CD33^{high}$ cells, which correspond to myeloid progenitor cells, contained MPO, whereas the more primitive $CD34^+$ $CD33^{low}$ cells did not. These data, taken together with our work, show clearly that the human myeloid progenitor cell population contains peroxidase activity and, more specifically, MPO.

An important question involves the role that cell-specific metabolism plays in conferring susceptibility to potential hematopoietic target cell populations. Leukemias are monoclonal malignancies originating in hematopoietic stem and progenitor cells in bone marrow. Previous studies suggested that, in the vast majority of cases, acute myelogenous leukemia arises at the level of multilineage or single-lineage myeloid progenitor cells (32–34). MPO comprises up to 5% of the dry weight of granulocytes and has been shown to be specific to cells of myeloid lineage (9). Although very small amounts of MPO message can be detected in a small fraction of leukemias of lymphoid origin (35), MPO expression has been used as a diagnostic indicator of myeloid leukemia (36). MPO expression has previously been considered to occur in promyelocytes (8), which are at a later stage of cell differentiation than are the target cells for neoplastic transformation in leukemogenesis. Our data, however, showed that both MPO mRNA and functional peroxidase enzyme activity are first expressed in murine hematopoietic progenitor cells. In addition, our work demonstrated functional peroxidase activity in $CD34^+$ human cells, which, when taken together with the recent data of Strobl *et al.* (30), suggests that expression of MPO also occurs in the human hematopoietic progenitor cell compartment.

The finding that murine and human hematopoietic cells contain significant peroxidase activity may be of considerable importance in explaining the pathogenesis of blood dyscrasias and the differential susceptibility of myeloid cells to a variety of hematotoxic agents. In the case of benzene, the production of micronuclei, nondysjunctional events, and aneuploidy (37–39) by benzene-derived quinones generated *in situ* in hematopoietic progenitor cells via peroxidase-mediated metabolism represents a possible mechanism underlying benzene-induced acute myelogenous leukemia. The toxicological relevance of peroxidase activity in progenitor cells may also extend to other drugs and toxins. For example, clozapine and carbamazepine, drugs known to induce agranulocytosis, have been shown to be bioactivated by peroxidases (40–42), and the hematopoietic toxicity of 1,3-butadiene may also be a function of bioactivation to reactive epoxides by peroxidases (43, 44). There has been little work examining the cell-specific nature of bioactivation mechanisms in bone marrow. Hematopoietic progenitor cells,

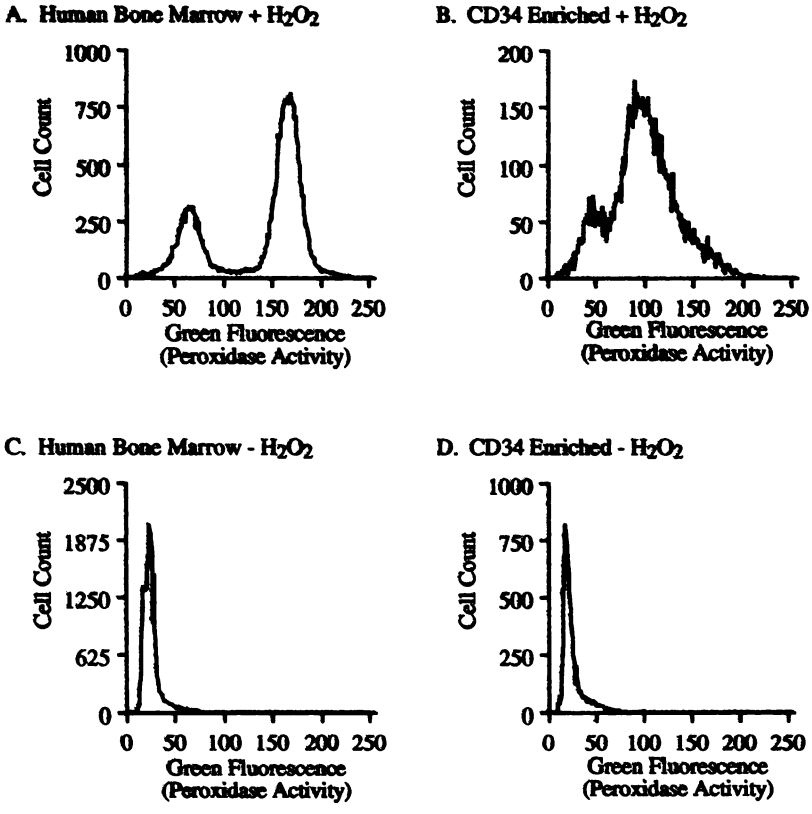

Fig. 5. Peroxidase activity in human bone marrow before (A and C) and after (B and D) CD34 enrichment. Data are shown for activity in the presence (A and B) and absence (C and D) of hydrogen peroxide. Enriched cells were 95% $CD34^+$.

because of their marked peroxidase activity, may be target cells for compounds that are activated via this enzyme.

In summary, we have demonstrated that MPO mRNA is expressed in murine progenitor cells and that both murine and human progenitor cells have marked peroxidase enzyme activity. These findings may have relevance for studies of cell differentiation and the characterization of mechanisms underlying cell-specific toxicity in bone marrow.

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