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Reactive Oxygen Species Mediate Stem Cell Factor Synergy with Granulocyte/Macrophage Colony-Stimulating Factor in a Subpopulation of Primitive Murine Hematopoietic Progenitor Cells

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SUMMARY

Reactive oxygen species (ROS) have been shown to stimulate proliferation and growth responses in a variety of mammalian cell types and to act as important mediators in many cellular processes, including hematolymphopoiesis. We examined the effect on primitive murine hematopoietic progenitor cells (HPC) of ROS generated by xanthine plus xanthine oxidase (xanthine/XO) and various antioxidants. Pretreatment of murine HPC (C57BL/6) with xanthine/XO produced a dose-dependent enhancement of clonogenic response to granulocyte/macrophage colony-stimulating factor (GM-CSF) but not to interleukin-3 or granulocyte colony-stimulating factor. Stem cell factor (SCF), a potent comitogen for many hematopoietic growth factors, also synergized with GM-CSF. However, the synergistic enhancement of GM-CSF with xanthine/XO and SCF was not additive, indicating that xanthine/XO and SCF may target the same subpopulation of HPC. Support for this conclusion came from experiments demonstrating that 1) mutant mice strains constitutively lacking a SCF-responsive population of HPC [White spotted (W/W^v) and Steel (Sl/Sl^d)] are unresponsive to xanthine/XO- and SCF-induced enhancement of GM-CSF and 2) 3,4-epoxybutene, which selectively abrogates SCF synergy with GM-CSF, inhibits xanthine/XO-induced enhancement. As xanthine/XO can mimic SCF in this population of HPC, the possibility exists that ROS also play a role in normal SCF-mediated proliferation of these cells. To test this hypothesis, we used the antioxidants *N-tert*-butyl-α-phenylnitrone, exogenous superoxide dismutase, and catalase. Both *N-tert*-butyl-α-phenylnitrone and superoxide dismutase effectively inhibited SCF and xanthine/XO synergism with GM-CSF, whereas catalase had no effect, indicating that the superoxide anion may be involved. Also, none of these compounds affected SCF synergism with other hematopoietic growth factors, such as interleukin-3 or granulocyte colony-stimulating factor, suggesting a population-specific phenomenon. These findings indicate that xanthine/XO mimics SCF in stimulating a subpopulation of murine HPC to proliferate and that SCF synergy with GM-CSF in this population is sensitive to antioxidant inhibition.

ROS are reactive molecules with the demonstrated ability to damage cellular components and biological systems (1). Oxidative damage includes strand breaks in DNA, inhibition of enzymes due to oxidized amino acids, and membrane disruption via lipid peroxidation (1). The deleterious effects of oxidative stress generally occur after exposure to relatively high concentrations of ROS. However, accumulating evidence suggests that lower concentrations of ROS can also have important physiological roles in cell signaling, signal transduction, and proliferation (2–4). Direct support for this concept is derived from the observation that low-level ROS exposure can increase the growth or growth responses of many types of mammalian cells. Exogenous H_2O_2 at 10^{-8} M results in an increased proliferation of transformed and nontransformed hamster (BHK-21/C13 and BHK-21/PyY) and rat (208F and RFAGT1) fibroblasts (5, 6). Superoxide generated by exogenous xanthine/XO (7) stimulates cultured human and transformed hamster fibroblasts (BHK-21/PyY) (8, 9) and increases the intracellular pH of U937 human histiocytic leukemia cells to the same extent as serum or phorbol ester treatment (7). Many studies have demonstrated the importance of a pro-oxidant state in T lymphocyte development, proliferation, and normal function. In both peripheral T cells and T cell lines, ROS have been implicated in mediating increased proliferation, activation of ornithine decarboxylase, increased gene expression for IL-2 and for the IL-2

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ABBREVIATIONS: ROS, reactive oxygen species; XO, xanthine oxidase; HPC, hematopoietic progenitor cells; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; G-CSF, granulocyte colony-stimulating factor; SCF, stem cell factor; W/W^v, White spotted; Sl/Sl^d, Steel; PBN, *N-tert*-butyl-α-phenylnitrone; SOD, superoxide dismutase; PBS, phosphate-buffered saline; Tempo, 4-hydroxy-2,2,6,6,-tetramethyl piperidinyloxy.

receptor, and increased binding of nuclear factor-κB (10). These events are all concomitant with T cell activation and development (11, 12). In these examples, ROS-induced activation is inhibited by higher cytotoxic concentrations of ROS or by the addition of ROS scavengers such as catalase or SOD (6, 8, 9). ROS scavengers also suppress normal proliferation of human and rodent fibroblasts and burst-forming units-erythroid formation in an IL-3-dependent murine cell line (9, 13–15). Indirect evidence in support of a role for ROS in cellular proliferation is that cytokines stimulate release of ROS from many cell types, including human fibroblasts, epithelial and endothelial cells, rabbit articular chondrocytes, mesangial cells, leukocytes, and BALB/3T3 cells (2, 6, 16). Interestingly, transformed cells such as HeLa and hamster (BHK-21/PyY) and rat (RFAGT1) fibroblasts, as well as various types of tumor cells (melanoma, colon, pancreatic, ovarian, and breast carcinoma, and neuroblastoma) release ROS constitutively without the requirement for prior stimulation (2). Also, many recent studies have demonstrated a role for ROS as second messenger molecules. ROS are implicated in the signal transduction of tumor necrosis factor-α, transforming growth factor β-1, IL-1, and platelet-derived growth factor (3, 9, 17, 18). ROS scavengers inhibit the responses induced by these cytokines, providing compelling but indirect evidence in support of this newly emerging role for ROS as second messengers. With the use of freshly isolated murine bone marrow, we demonstrated that ROS stimulate selected clonogenic responses in primitive HPC and that SCF synergy with GM-CSF is ROS dependent and sensitive to antioxidant inhibition. Based on the differential effects of antioxidants on SCF synergy with various growth factors, we hypothesized that there are at least two different subpopulations of SCF-responsive cells in murine bone marrow and that ROS involvement in SCF signaling is a population-specific phenomenon.

Materials and Methods

Mice. Male C57BL/6J, (WBB6)F_1/J-W/W^v, and (WCB6)-F_1/J-Sl/Sl^d mice (4–6 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were acclimated for ≥10 days before use and were housed 10 to a cage in sterile chambers with filter tops. Mice were allowed autoclaved food (3000, Agway, Syracuse, NY) and sterilized water *ad libitum*.

Growth factors. Murine recombinant GM-CSF (5×10^7 units/mg), mrSCF (10^5 units/mg), mrIL-3 (5×10^5 units/mg), and human recombinant G-CSF (10^7 units/mg) were generous gifts from Immunex (Seattle, WA).

Bone marrow cell preparation. Lympholyte-M purified nonadherent cells were obtained from bone marrow harvested from mouse femurs as described previously (19). Nucleated cells were enumerated with the use of a hemocytometer with Turk's solution. Cell suspensions were kept on ice unless otherwise noted.

Chemical exposure. Nonadherent bone marrow cells were aliquoted into control and treatment groups, spun at 165 × *g* for 10 min, and resuspended in PBS without bovine serum albumin. Xanthine (Sigma Chemical Co., St. Louis, MO), diluted in PBS, was kept constant at 1.0 mM. XO (Sigma) was diluted in PBS to concentrations ranging from 1.0×10^{-2} to 1.0×10^{-10} units/ml and was always added last to each suspension (1×10^6 cells/ml). PBN (Aldrich Chemical Co., Milwaukee, WI), SOD, and catalase (Sigma) were diluted in PBS and were present during the pretreatment regiment with xanthine/XO. 3,4-Epoxybutene (Aldrich) was also diluted in PBS and was kept in glass test tubes with Teflon caps. Control cells were treated with PBS only. Experimental cells were incubated for 30 min at 37° and 10% CO_2. After a 30-min pretreatment, 1 ml of complete Iscove's medium [(Life Technologies, Grand Island, NY) 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 2 mM L-glutamine, 100 units/mg penicillin/100 mg/ml streptomycin, 50 mM 2-mercaptoethanol] was added to each tube and then spun at 165 × *g* for 10 min. Cells were then resuspended in complete medium and cultured. Cell viabilities were determined through Trypan Blue exclusion after a 30-min pretreatment and were >94% viable. To explore the role of ROS in normal growth factor signaling all antioxidants [including Tempo (Aldrich)] were diluted in complete media and added directly to the methyl cellulose, remaining there for the entire culture period.

Colony-forming unit assay. Pretreated bone marrow cells were plated onto 35-mM culture dishes at concentrations ranging from 0.5 to 1.0×10^4 cells in 1 ml of modified Iscove's medium [1.2% (w/v) methyl cellulose, 10% fetal bovine serum, 100 mg/ml streptomycin/100 units/mg penicillin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol] with recombinant cytokines. Colonies consisting of >50 cells were scored on day 8 with a dissecting microscope. In the absence of growth factor, no colony formation occurred. Correlation of colony morphology with individual cell types was determined through phase-contrast microscopy of methyl cellulose colonies *in situ*.

Statistical analysis. Five plates were scored for each treatment group, and results are given as the mean ± standard error. Significant differences between pretreated and control groups were determined with the use of Student's *t* test and were calculated using absolute values. All statistics were calculated with Excel 4.0 (Microsoft Corp., Redmond, WA).

Results and Discussion

We evaluated the effects of ROS and ROS scavengers on the clonogenic response of murine HPC induced by IL-3, GM-CSF, and SCF. Xanthine/XO is a well-characterized system that yields a flux of both superoxide radicals and hydrogen peroxide (20–22). Pretreatment of murine HPC with xanthine/XO *in vitro* resulted in a selective dose-dependent enhancement of colony formation in response to GM-CSF (Fig. 1A). Individually, neither xanthine nor XO had an effect on colony formation. Also, heat-inactivated XO in combination with xanthine was equally ineffective (data not shown). These results indicate that the ROS generated by this system and not the compounds themselves were responsible for increased clonogenic response. The biphasic proliferative response seen in Fig. 1A was consistent with xanthine/XO-induced proliferation reported for other *in vitro* cultures in which low doses of ROS stimulate but increased concentrations inhibit proliferation (9). Xanthine/XO-induced clonogenic enhancement appeared to be growth factor specific as there was no increase in colonies formed in response to IL-3 (Fig. 1B).

SCF predictably synergized with GM-CSF to increase colony number but was not capable of supporting colony formation alone (23). Murine HPC pretreated with xanthine/XO exhibited an increase in GM-CSF response that was comparable to that observed with SCF. However, pretreatment with xanthine/XO was not additive with SCF in recruiting HPC to respond to GM-CSF (Fig. 1B). One possible explanation for this finding is that xanthine/XO and SCF were acting on the same population of HPC. Evidence in support of this hypothesis was derived from a series of experiments with bone marrow from Sl/Sl^d and W/W^v mice. Sl/Sl^d and W/W^v mice are defective in SCF and its receptor, c-*kit*, respectively (24, 25). Consequently, SCF synergism with other cytokines

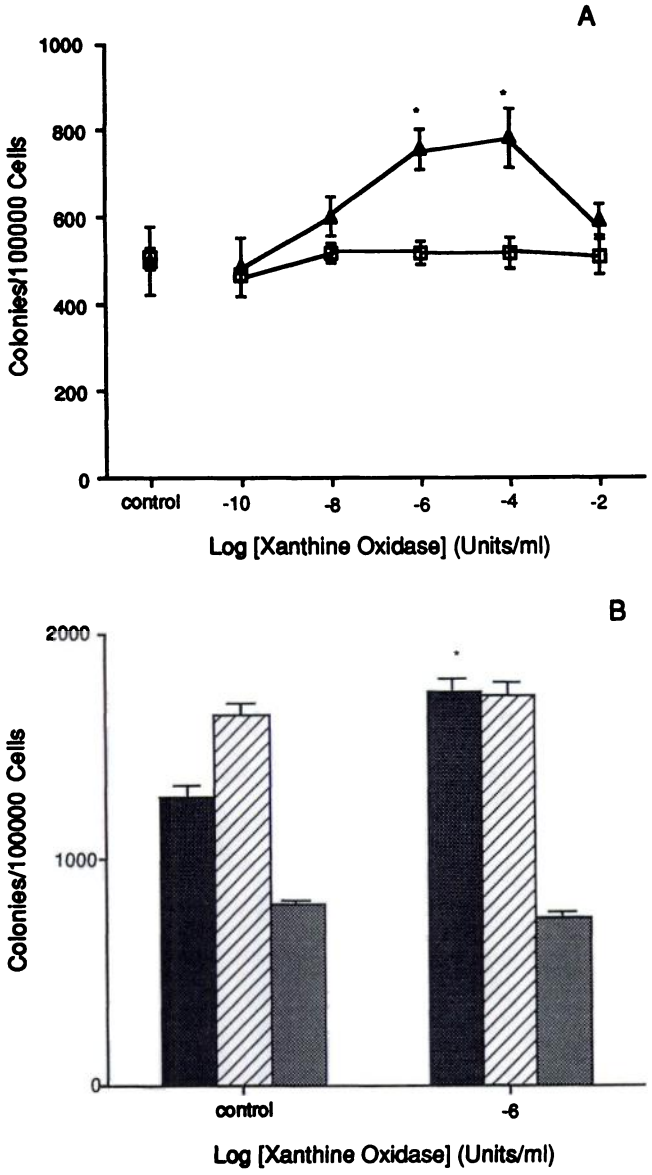

Fig. 1. A, Effects on GM-CSF (5 ng/ml)-stimulated colony formation of C57BL/6 bone marrow cells of *in vitro* pretreatment with various concentrations of XO with (▲) and without (□) 10^{-4} M xanthine. B, Effect of 10^{-4} M xanthine (+) and 10^{-6} units/ml XO (−) on C57BL/6 bone marrow cells treated with GM-CSF (5 ng/ml) (■), GM-CSF (5 ng/ml) plus SCF (10 ng/ml) (*striped bars*), and IL-3 (2 ng/ml) (*shaded bars*). *Error bars*, 1 SE for five cultures. *, Significant increase compared with control pretreated with vehicle (PBS) ($p < 0.05$).

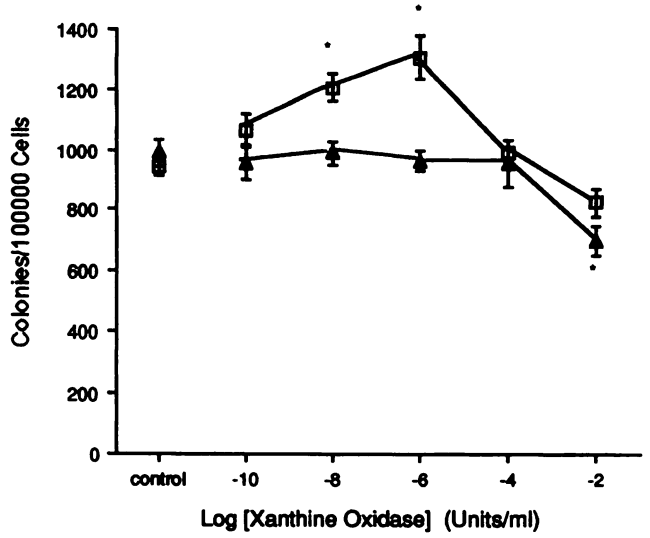

Fig. 2. Effect of xanthine/XO (10^{-4} M xanthine with various concentrations of XO) pretreatment on GM-CSF (5 ng/ml) stimulated colony formation of W/W^v (□) and Sl/Sl^d (▲) bone marrow cells. *Error bars*, 1 SE for five cultures. *, Significant increase compared with control pretreated with vehicle (PBS) ($p < 0.05$).

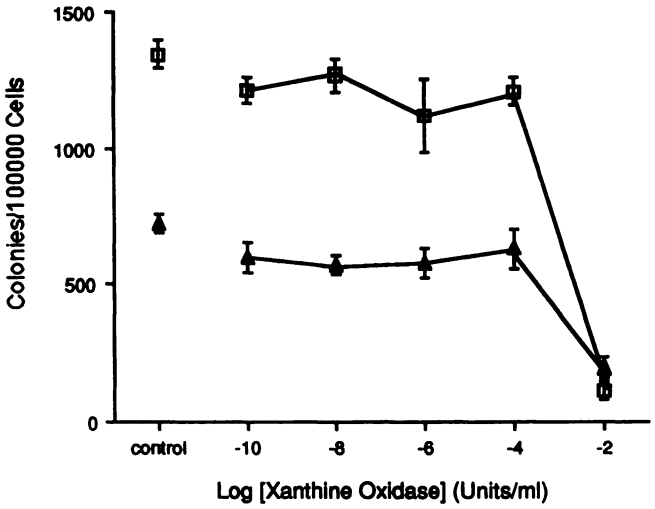

Fig. 3. Effects of 3,4-epoxybutene on xanthine/XO (10^{-4} M xanthine with various concentrations of xanthine oxidase) induced colony formation in GM-CSF (5 ng/ml) stimulated C57BL/6 bone marrow cells; □, xanthine/XO without EB; ▲, xanthine/XO with 10^{-8} M EB. *Error bars*, 1 SE for five cultures. *, Significant increase compared with control pretreated with vehicle (PBS) ($p < 0.05$).

cannot be demonstrated in bone marrow from these mice (26). Consistent with a common target population, xanthine/XO did not enhance GM-CSF response in bone marrow cells from either W/W^v or Sl/Sl^d mice (Fig. 2). In contrast, bone marrow from Sl/Sl^d and W/W^v littermate control mice exhibited an xanthine/XO-enhanced GM-CSF clonogenic response comparable to that observed in C57BL/6 mice (data not shown). Previous studies in our laboratory have demonstrated that pretreatment of C57BL/6 bone marrow cells with 3,4-epoxybutene, a metabolite of butadiene, selectively suppresses SCF synergism with GM-CSF but not with other cytokines (26). Pretreatment of C57BL/6 HPC with epoxybutene completely eliminated xanthine/XO synergism with GM-CSF (Fig. 3). This finding provides further support that xanthine/XO was targeting the same population of HPC that are recruited by SCF to respond to GM-CSF. It is unlikely that xanthine/XO was inducing the formation of SCF in this assay as SCF strongly synergizes with IL-3 (27) and xanthine/XO pretreatment has no effect on IL-3 response. For the same reason, we believe the production of other comitogenic factors, such as G-SCF and IL-11, is an unlikely explanation for these observations. Moreover, the production of proliferative factors, such as IL-3 and GM-CSF, or of differentiation factors, such as macrophage colony-stimulating factor, is also unlikely because xanthine/XO pretreatment of HPC did not result in colony formation in the absence of growth factors (data not shown).

Exogenous xanthine/XO is known to result in ROS production (28); it has been previously suggested that ROS are

involved as second messengers in signal transduction (3, 29). To determine whether xanthine/XO-induced enhancement of clonogenic response is due to the generation of ROS, we tested three ROS scavengers: PBN, SOD, and catalase. SOD is a ubiquitous oxioreductase that has been shown to scavenge superoxide (30). PBN is a stable and extremely permeable lipophilic radical spin trap and an effective free radical scavenger (31, 32). Catalase is a heme protein that catalyzes the reduction of hydrogen peroxide to water (33). The addition of SOD or PBN completely abrogated xanthine/XO synergy with GM-CSF (Fig. 4, A and B). The oxidation of xanthine by XO enzymatically formed superoxide, which subsequently underwent either spontaneous or SOD-catalyzed dismutation to H_2O_2. We believe that the inhibitory effects of SOD provide presumptive evidence that the superoxide anion is the ROS involved in mediating xanthine/XO-induced synergy with GM-CSF. If the H_2O_2 formed by the dismutation of superoxide is involved in mediating xanthine/XO proliferation, then SOD (which actually makes H_2O_2) would be unable to completely inhibit this effect. This interpretation is further supported by the lack of inhibition seen with catalase, an enzyme that is very specific for H_2O_2 (Fig. 4C). Unequivocal identification of the specific ROS involved in this proliferative response was beyond the scope of this present investigation. In any case, these results indicate that a pro-oxidant signal is involved in mediating proliferation in these cells.

We conducted additional experiments to explore the hypothesis that ROS are involved in normal SCF signaling in murine bone marrow cells. The addition of either PBN or SOD directly to cell cultures completely inhibited SCF synergism with GM-CSF (Fig. 5, A and B). Independently, we used another low molecular weight ROS scavenger and SOD mimic, Tempo, which was equally effective in inhibiting SCF synergy with GM-CSF-induced proliferation (Fig. 5D). Antioxidant sensitivity seemed to be population specific as neither SOD nor PBN altered G-CSF response or SCF synergism with G-CSF (Fig. 6, A and B). Consistent with our earlier findings, catalase had no inhibitory effect on any growth factor or combination of growth factor tested (Figs. 5C and 6C). These findings provide evidence that ROS are required for SCF synergy with GM-CSF and that there are at least two separate SCF-responsive populations in murine bone marrow.

The concentrations of SOD that are effective in inhibiting xanthine/XO-induced proliferation in this assay system are 2–3 orders of magnitude lower than would be predicted based on the known kinetics of this enzyme. Trivial explanations include the possibility that less superoxide was generated than we assumed in our kinetic analysis. For example, if the

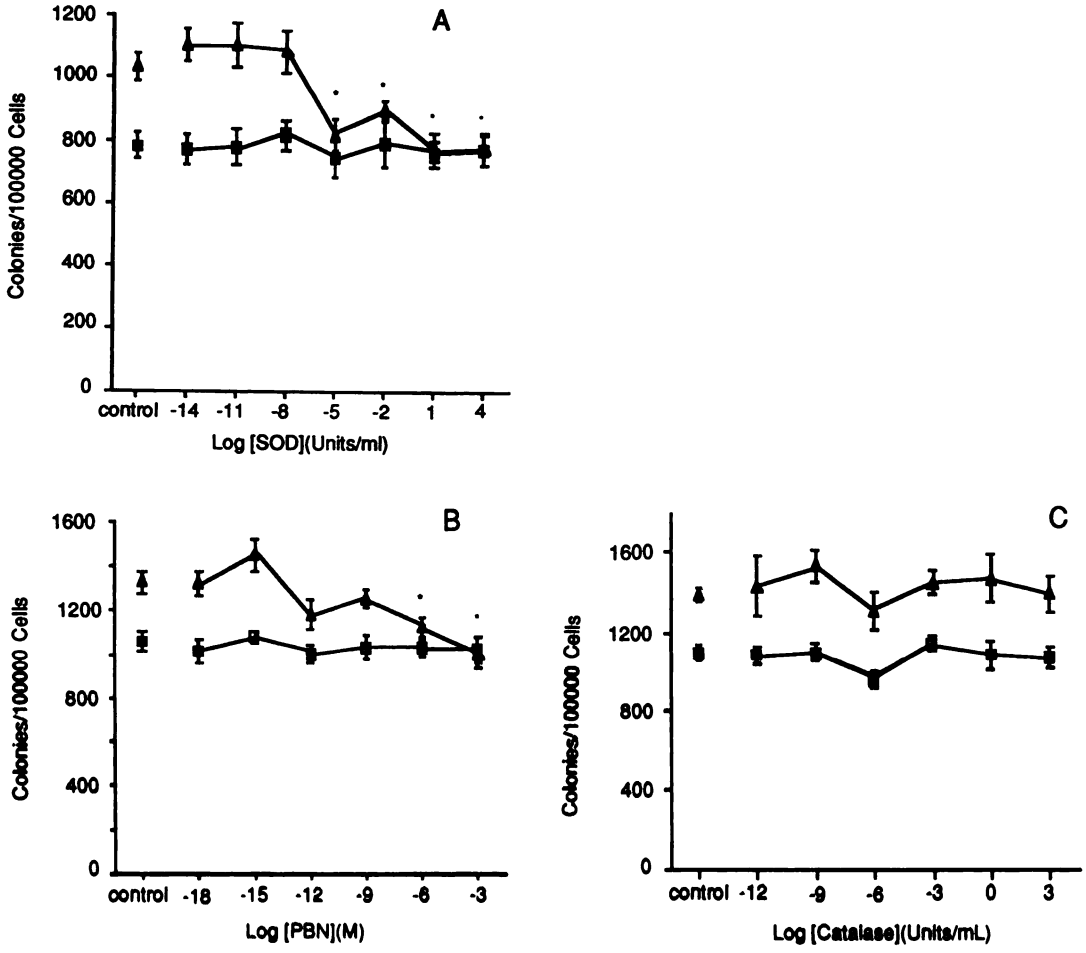

Fig. 4. Effects of SOD (A), PBN (B), and catalase (C) on xanthine/XO synergy with GM-CSF stimulated colony formation in C57BL/6 bone marrow cells. For each graph: □, GM-CSF (5 ng/ml); ▲, GM-CSF (5 ng/ml) + xanthine/XO (10^{-4} M xanthine + 10^{-6} units/ml xanthine oxidase). *Error bars*, 1 SE for five cultures. *, Significant increase compared to control pretreated with vehicle (PBS) ($p < 0.05$).

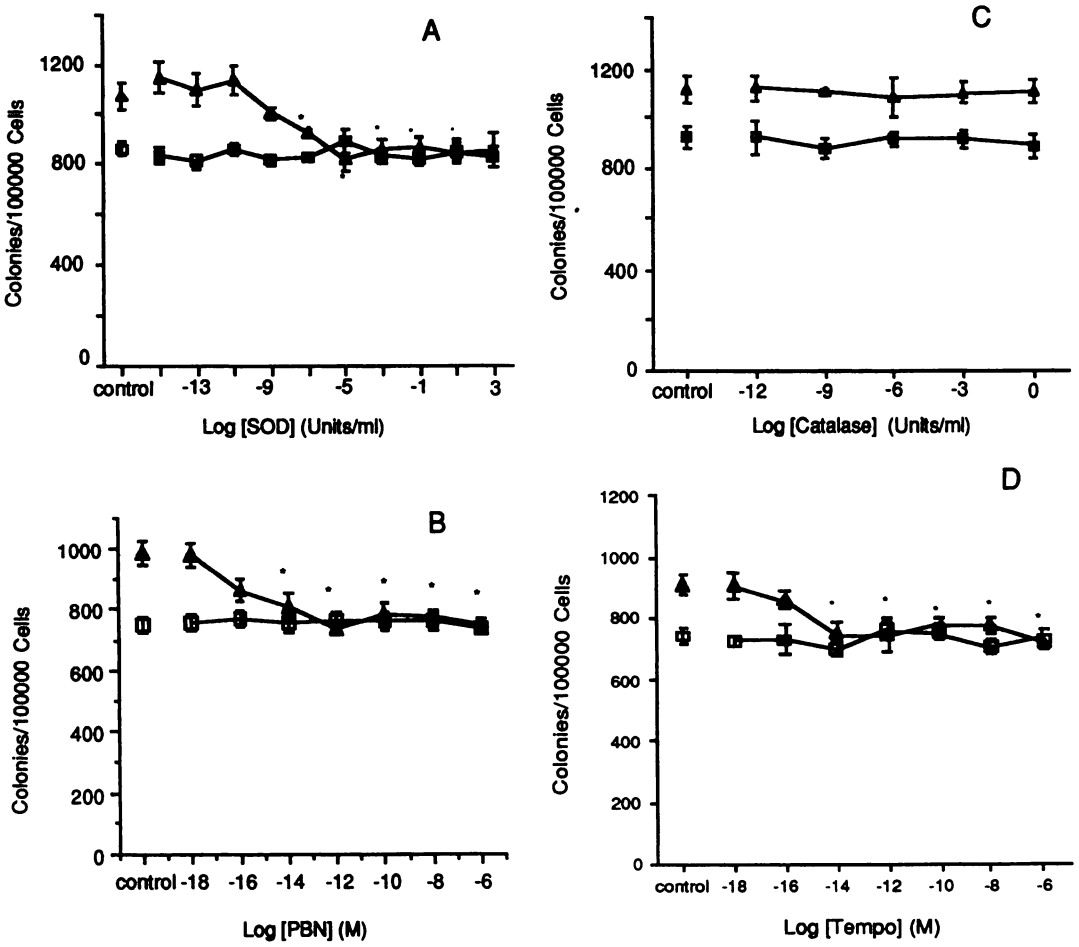

Fig. 5. Effects of SOD (A), PBN (B), catalase (C), and Tempo (D) on SCF synergy with GM-CSF stimulated colony formation in C57BL/6 bone marrow cells. For each graph: □, GM-CSF (5 ng/ml); ▲, GM-CSF (5 ng/ml) + SCF (10 ng/ml). *Error bars*, 1 SE for 5 cultures (omitted when smaller than the symbol). *, Significant increase compared with control pretreated with vehicle (PBS) ($p < 0.05$).

XO preparations were less active (or the SOD was more active) than assumed, then less superoxide would be present and. consequently, less SOD would be required. Albeit, a 2- or 3-order-of-magnitude discrepancy in the activity of these commercial enzyme preparations seems extremely unlikely. Also, the levels of SOD that inhibit SCF synergy with GM-CSF are remarkably low and are consistent with those needed to inhibit xanthine/XO-induced proliferation. The potential for SOD to play a nonenzymatic role (e.g., receptor mediated) in this system was also considered. Despite some support for this concept in the literature, it is difficult to reconcile with the inhibitory effect seen with PBN or Tempo, both of which are low molecular weight radical scavengers and are structurally very different from SOD (31). Therefore, we believe that the most plausible explanation involves structure-specific compartmentalization of SOD, most likely occurring at a receptor or near the surface of the responder cells. Highly specific localization could result in concentrations of SOD substantially higher than those found in solution.

The data presented in this report are consistent with superoxide as the primary ROS mediating xanthine/XO- and SCF-induced proliferation. However, other experiments revealed that exogenous H_2O_2 induced a similar proliferative effect to xanthine/XO.[1] Our results demonstrated that catalase, a known H_2O_2 scavenger, has no effect on xanthine/XO-induced proliferation (Fig. 4C). However, catalase was able to abrogate H_2O_2-induced enhancement, providing evidence that the catalase was active and that H_2O_2 was susceptible to scavenging (data not shown). Additional experiments demonstrated that SOD inhibited xanthine/XO-induced proliferation (Fig. 4A) Therefore, we believe our results argue against a direct role for H_2O_2 in this proliferative signal. One possible explanation is that exogenous H_2O_2 results in an increase in the superoxide level in these cells. Additional support for this hypothesis comes from experiments demonstrating that SOD and SOD mimics are effective in inhibiting H_2O_2-mediated proliferation in this same population of cells (data not shown). Exogenous H_2O_2 has been shown to increase the levels of superoxide in endothelial cells (34). Also, exogenous SOD protects against H_2O_2-induced oxidative damage to hepatocytes, which could indicate increased superoxide generation after H_2O_2 exposure (35). Therefore, we believe it is feasible that H_2O_2 stimulation involves the intracellular formation of superoxide and that this radical mediates H_2O_2 as well as xanthine/XO- and SCF-induced pro-

[1] D. W. Pyatt, W. S. Stillman, and R. D. Irons, unpublished observations.

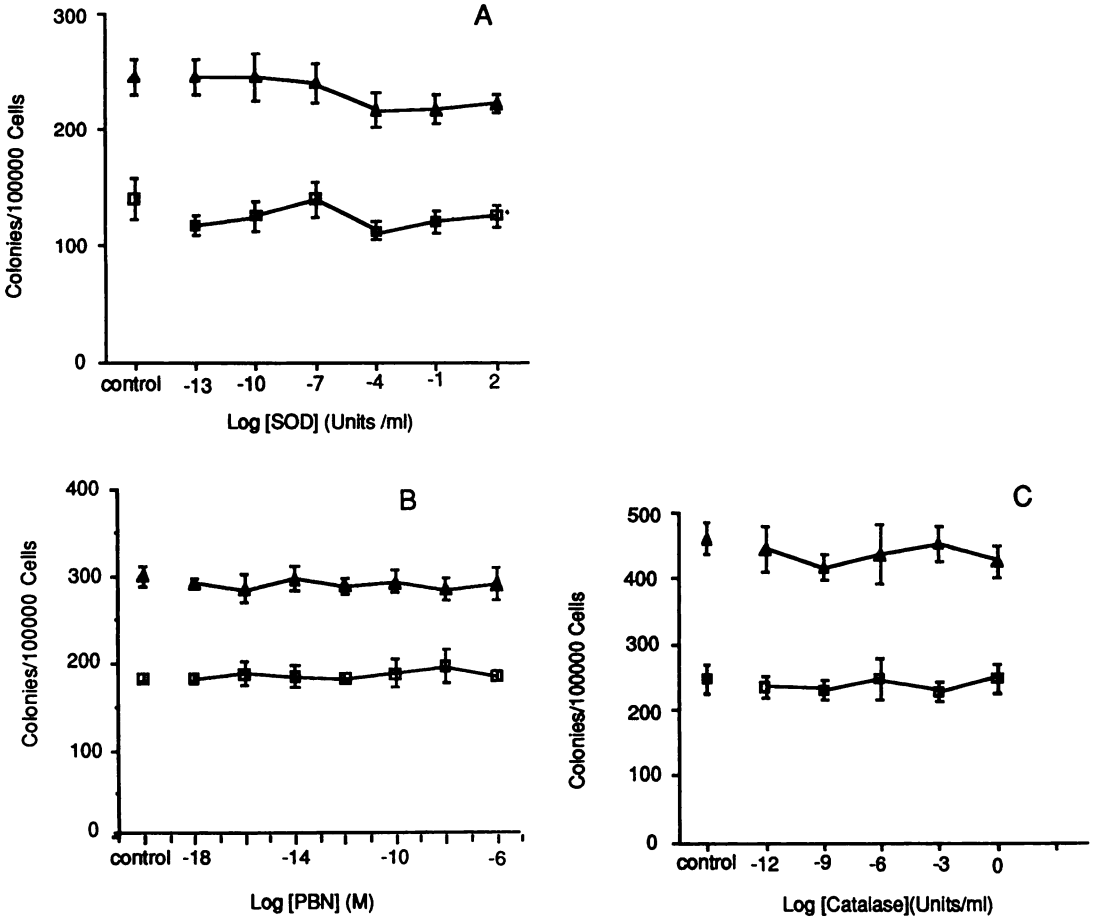

Fig. 6. Effects of SOD (A), PBN (B), and catalase (C) on SCF synergy with G-CSF stimulated colony formation in C57BL/6 bone marrow cells. For each graph: □, G-CSF (10 ng/ml); ▲, G-CSF (10 ng/ml) + SCF (10 ng/ml). *Error bars*, 1 SE for five cultures. *, Significant increase compared with control pretreated with vehicle (PBS) ($p < 0.05$).

liferative signals within this population of murine bone marrow cells.

Evidence presented in this study is consistent with an emerging role of ROS as potential mediators in cellular proliferation and describes a ROS-dependent population of HPC in primary murine bone marrow cells. The precise identity of this subpopulation remains obscure. However, based on previous studies and the available literature, we believe the most likely candidate is the pre-T cell. This population of cells is found in murine bone marrow (36) and is responsive to SCF (37). Several studies have shown that Jurket and Esb-L T cell lines can be stimulated by low-level ROS exposure to proliferate (12, 38). In addition, both human and murine pre-T lymphocytes (39, 40) as well as T cell lines (11, 12) are extremely sensitive to inhibition by antioxidants. T cell activation by reactive oxygen appears to proceed via activated nuclear factor-κB, which results in increased expression of IL-2 and the IL-2 receptor (10, 12). Accordingly, we are exploring the role of nuclear factor-κB in ROS-mediated activation of HPC.

ROS, such as superoxide, fulfill several important prerequisites for second messengers: they are small, diffusable molecules; cells seem to be exquisitely sensitive to slight changes in intracellular concentrations; and ubiquitous cellular enzymes can rapidly synthesize and destroy them, permitting tight molecular regulation (1, 30). Regardless of the precise mechanism, these results provide evidence of the involvement of ROS in SCF synergism with GM-CSF within a select subpopulation of murine bone marrow cells and suggest that at least two population-specific signaling pathways for SCF exist in murine bone marrow, one of which is dependent on ROS and one that is not.

Acknowledgments

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