Differential Effects of Nitric Oxide on Erythroid and Myeloid Colony Growth From CD34+ Human Bone Marrow Cells

By Paul J. Shami and J. Brice Weinberg

Nitric oxide (NO) is a reactive molecule with numerous physiologic and pathophysiologic roles affecting the nervous, cardiovascular, and immune systems. In previous work, we have demonstrated that NO inhibits the growth and induces the monocytic differentiation of cells of the HL-60 cell line. We have also demonstrated that NO inhibits the growth of acute nonlymphocytic leukemia cells freshly isolated from untreated patients and increases monocytic differentiation antigens in some. In the present work, we studied the effect of NO on the growth and differentiation of normal human bone marrow cells in vitro. Mononuclear cells isolated from human bone marrow were cultured in semisolid media and treated with the NO-donating agents sodium nitroprusside (SNP) or S-nitroso-acetyl penicillamine (SNAP) (0.25 to 1 mmol/L). Both agents decreased colony-forming unit–erythroid (CFU-E) and colony-forming unit–granulocyte macrophage (CFU-GM) formation by 34% to 100%. When CD34+ cells were examined, we noted that these cells responded to SNP and SNAP differently than did the mononuclear cells. At a concentration range of 0.25 to 1 mmol/L, SNP inhibited the growth of CFU-E by 30% to 75%. However, at the same concentration range, SNP increased the number of CFU-GM by up to 94%. At concentrations of 0.25 to 1 mmol/L, SNAP inhibited the growth of CFU-E by 33% to 100%. At a concentration of 0.25 mmol/L, SNAP did not affect CFU-GM. At higher concentrations, SNAP inhibited the growth of CFU-GM. Although SNP increased intracellular levels of cGMP in bone marrow cells, increasing cGMP in cells by addition of 8-Br-cGMP (a membrane permeable cGMP analogue) did not reproduce the observed NO effects on bone marrow colonies. These results demonstrate that NO can influence the growth and differentiation of normal human bone marrow cells. NO (generated in the bone marrow microenvironment) may play an important role modulating the growth and differentiation of bone marrow cells in vivo.

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MATERIALS AND METHODS

Cell source. After obtaining informed consent, iliac crest bone marrow samples were aspirated from patients undergoing bone marrow transplantation according to a protocol approved by the Duke and VA Institutional Review Boards. Mononuclear cells were isolated using a ficoll-Hypaque density gradient and were resuspended in RPMI-1640 before plating in the clonogenic assays. CD34+ cells were then incubated for 1 hour at room temperature in an AIS MicroCELLector T-25 flask coated with anti-CD34 antibodies. The nonadherent cells were discarded, 4 mL of PBS were added, and the side of the flask was tapped several times to release the CD34+ cells from the antibody coat. The cells were washed in PBS and resuspended in RPMI-1640 before plating in the clonogenic assays. The cell fraction obtained was >85% CD34+ by flow cytometry and constituted 0.8% to 1% of the original cell number. The viability of the cells after isolation was 100% as determined by Trypan blue exclusion. In some experiments, the CD34+ population was further enriched by flow cytometry and sorting. In brief, the CD34+ cell fraction obtained using the AIS flasks was incubated at 4°C for 30 minutes with fluorescein isothiocyanate-labeled mouse antihuman CD34+ antibody (Biosource International, Camarillo, CA). The CD34+ cells were then sorted under sterile conditions using a cell sorter. The sorted cells were 100% CD34+ and were used for culture in methylcellulose media described in the following section.

Culture conditions. Mononuclear bone marrow cells and CD34+ cells were cultured in semisolid media purchased from Stem Cell Technologies (Vancouver, BC, Canada). The medium contained 30% fetal bovine serum, 10% agar leukocyte conditioned medium, 1% bovine serum albumin, 0.9% methylcellulose, 0.1 mmol/L 2-mercaptoethanol, 2 mmol/L glutamine, and 3 μmol/L purified human urinary erythropoietin. Bone marrow mononuclear cells were cultured at a density of 50,000/mL. Fifteen thousand cells per well were plated in 16 mm tissue culture plates. CD34+ cells were cultured at a density of 10,000 cells/mL. One thousand six hundred cells per well were plated in 12 mm tissue culture plates. Samples were cultured in duplicate at 37°C in a humidified room air 5% CO2 atmosphere.

From the Department of Medicine, Division of Hematology-Oncology, VA and Duke University Medical Centers, Durham, NC. Submitted April 15, 1995; accepted September 12, 1995.

Address reprint requests to J. Brice Weinberg, MD, VA and Duke University Medical Centers, 508 Fulton St, Durham, NC 27705.

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cGMP solutions were made immediately before use and added to correspond to the amount of NO delivered to the bone marrow cells added directly to the cell cultures at the indicated final concentrations. The cultures that were treated with SNP were exposed to light important to note that the concentrations of the NO donors do not likely much less because of the very short half-life of NO in solution. 8-Bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP) was purchased from Chem Biochem Research Inc (Salt Lake City, UT). SNP and SNAP solutions were made immediately before use and added directly to the cell cultures at a final concentration of 30 or more cells. Under our culture conditions, the majority of the bone marrow cells when tested by trypan blue exclusion. Cell extracts were then suspended in assay buffer supplied by the manufacturer. One hundred microliters of cell extract were mixed with 100 µL of 1-21 labeled cGMP solution. One hundred microliters of rabbit anti-cGMP antiserum were added followed by 100 µL of donkey antirabbit IgG antibody coupled to fluomicrospheres (all solutions and reagents supplied by the manufacturer). The suspension was incubated at room temperature for 15 to 20 hours. The samples' luminescences were measured in a scintillation counter. Measurements were made in duplicate. The cGMP concentration was deduced from a standard curve and expressed as picograms/million cells.

NO donors and chemicals. Sodium nitroprusside (SNP), and S-nitrosoacetyl penicillamine (SNAP) were used as NO sources. SNP was purchased from Elkins-Sinn (Cherry Hill, NJ), and SNAP was purchased from Chem Biochem Research Inc (Salt Lake City, UT). SNP and SNAP solutions were made immediately before use and added directly to the cell cultures at the indicated final concentrations. The cultures that were treated with SNP were exposed to light for 30 seconds to enhance NO release by a photolytic effect. It is important to note that the concentrations of the NO donors do not correspond to the amount of NO delivered to the bone marrow cells in solution. The actual NO concentration delivered to the cells is likely much less because of the very short half-life of NO in solution. 8-Bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP) was purchased from Sigma Chemical Company (St Louis, MO). 8-Br-cGMP solutions were made immediately before use and added to the cell cultures at a final concentration of 3 mmol/L.

Analysis. Colonies were scored 10 to 18 days after plating using an inverted light microscope. A colony was defined as a group of 30 or more cells. Under our culture conditions, the majority of the erythroid colonies observed at the time of scoring were colony-forming unit-erythroid (CFU-E) colonies and did not display a typical burst configuration. Therefore, we used only these colonies to analyze the effect of NO on erythroid precursors. Statistical analysis was done using the Student's t-test. Differences were considered statistically significant for P < .05.

RESULTS

Effect of SNP on the mononuclear fraction of bone marrow cells. We first studied the effect of SNP on colony formation using the bone marrow mononuclear fraction as a source of progenitor cells. SNP did not affect the viability of the bone marrow cells when tested by trypan blue exclusion. SNP was added to bone marrow cells cultured in semisolid media as described in Materials and Methods. Colonies were assayed at days 10 to 18. At concentrations of SNP as described in the Materials and Methods section. Colonies were assayed at days 10 to 18. At concentrations of 0.25 to 1 mmol/L, SNP inhibited the growth of CFU-E by 66% to 100% (P < .05 for all treatments) (Fig 2). At the same concentrations, it inhibited the growth of CFU-GM by 53% to 100% (P < .05 for all treatments) of 0.5, 0.75, and 1 mmol/L) (Fig 2). As noted with SNP treatments, the colonies obtained from cells treated with SNAP were smaller than colonies obtained from untreated cells. There was no significant difference between the extent of CFU-E inhibition and CFU-GM inhibition at all the SNAP concentrations used.

Effect of SNAP on the mononuclear fraction of bone marrow cells. To ascertain that the growth inhibitory effects observed with SNP treatment were due to NO and not to the NO-carrying molecule, we treated cells obtained from the mononuclear fraction of the bone marrow with SNAP. SNP is a compound that liberates NO in solution. Bone marrow cells were cultured in semisolid media with increasing concentrations of SNAP as described in the Materials and Methods section. Colonies were assayed at days 10 to 18. At concentrations of 0.25 to 1 mmol/L, SNAP inhibited the growth of CFU-E by 66% to 100% (P < .05 for all treatments) (Fig 2). At the same concentrations, it inhibited the growth of CFU-GM by 53% to 100% (P < .05 for all treatments) of 0.5, 0.75, and 1 mmol/L) (Fig 2). As noted with SNP treatments, the colonies obtained from cells treated with SNAP were smaller than colonies obtained from untreated cells. There was no significant difference between the extent of CFU-E inhibition and CFU-GM inhibition at all the SNAP concentrations used.

Effect of SNP on CD34+ cells. The growth inhibitory effects of NO described above could be due to one or more of the following effects: a direct effect on bone marrow progenitors, induction of growth inhibitory factors in accessory cells, and inactivation of growth factors or their receptors. To determine whether NO acted directly on progenitor cells, we enriched the progenitor cell population by positively selecting CD34+ cells from the bone marrow using a panning technique with anti-CD34 antibody. CD34+ enriched cells constitute a population of enriched progenitors.

![Fig 1. Effect of SNP on the growth of colonies from the mononuclear fraction of the bone marrow.](image-url)
with very low numbers of mature or accessory cells. CD34<sup>+</sup> cells were cultured in semisolid media in the presence of increasing concentrations of SNP. Colonies were scored at days 10 to 18. At a concentration of 0.25 to 1 mmol/L, SNP decreased the number of CFU-E obtained from CD34<sup>+</sup> cells by 30% to 75% (with \( P < .05 \) for all the treatments) (Fig 3). However, at the same concentrations, it increased the growth of CFU-GM by up to 94% (\( P < .05 \) for the 0.5, 0.75, and 1 mmol/L treatments) (Fig 3). To confirm these results, we repeated the experiments using a population of 100% CD34<sup>+</sup> cells obtained by cell sorting as described in the Materials and Methods section. When such cells were used, SNP (0.25 to 1 mmol/L) inhibited the growth of CFU-E by 29% to 95%, but did not affect the growth of CFU-GM (data not shown).

**Effect of SNAP on CD34<sup>+</sup> cells.** To provide further evidence that the observed effects on CD34<sup>+</sup> cells were due to NO, we did experiments in which CD34<sup>+</sup>-enriched cells were cultured in semisolid media and treated with increasing concentrations of SNAP. At concentrations of 0.25 and 0.5 mmol/L, SNP inhibited the growth of CFU-E by 33% and 81%, respectively (\( P < .05 \) for both treatments) (Fig 4). At a concentration of 0.25 mmol/L, it did not inhibit CFU-GM, but at a concentration of 0.5 mmol/L it inhibited CFU-GM by 30% (Fig 4). However, the latter change did not reach statistical significance. At concentrations of 0.75 and 1 mmol/L, SNP totally inhibited the growth of CFU-E and CFU-GM (\( P < .05 \)) (Fig 4).

**Effect of SNP on cGMP levels in bone marrow cells.** One of the main intracellular targets of NO is the soluble guanylate cyclase enzyme. For example, NO induces the relaxation of vascular smooth muscle cells by increasing intracellular levels of cGMP through the activation of guanylate cyclase. Consequently, we determined the effect of NO on cGMP levels in bone marrow cells. Mononuclear cells from the bone marrow were isolated by density centrifugation and cultured in RPMI-1640 at a density of \( 1 \times 10^6 \) cells/mL. The cells were treated with 1 mmol/L SNP and harvested after 15, 30, 45, and 60 minutes. SNP increased the intracellular levels of cGMP in a time dependent manner with almost a sixfold increase at 60 minutes as compared with controls (the differences reached statistical significance with \( P < .05 \) for the 30-, 45-, and 60-minute time points) (Fig 5).

**Effect of cGMP on bone marrow and CD34<sup>+</sup> cells.** Since NO increased intracellular levels of cGMP in bone marrow cells, we sought to determine whether cGMP would reproduce the NO-induced changes in the bone marrow. We, therefore, treated mononuclear cells obtained from the bone marrow with the membrane permeable cGMP analog 8-Br-cGMP. When added to bone marrow mononuclear cells at a concentration of 3 mmol/L, 8-Br-cGMP had no effect on CFU-E, but it inhibited CFU-GM by 53% (\( P < .05 \)) (Fig 6A). When added to CD34<sup>+</sup>-enriched cells at the same concentrations, 8-Br-cGMP had no effect on CFU-E, but it inhibited CFU-GM by 34% (\( P < .05 \)) (Fig 6B).

**DISCUSSION**

In the present work, we have used clonogenic assays to study the effect of NO on human bone marrow growth. When...
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CFU-GM

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0 0.25 0.5 0.75 1

SNAP (mM)

Fig 4. Effect of SNAP on the growth of colonies from CD34' cells. SNAP was added directly to the semisolid methylcellulose cell suspension at the indicated concentrations. Colonies were scored at days 10 to 18. There was an average of 82 CFU-E and 34 CFU-GM per well in the controls. SNAP inhibited the growth of CFU-E at all the concentrations used. SNAP did not affect CFU-GM at 0.25 mmol/L but inhibited their growth at higher concentrations. The data points represent means ± one standard error of the mean. Asterisks indicate statistically significant differences (P < .05) between treatments and controls. Results shown are the averages of three separate experiments.

the whole mononuclear fraction of the bone marrow was used as a source of progenitor cells, NO inhibited the growth of both CFU-E and CFU-GM. NO increased intracellular levels of cGMP in these cells, and exogenously added 8-Br-cGMP partially reproduced the NO effects. However, when the progenitor cell population was enriched and treated with NO, the erythroid and myeloid lineages were affected differently. SNP inhibited erythroid colony growth, but enhanced myeloid colony growth. When SNAP was used as a source of NO, erythroid colonies were inhibited at concentrations of 0.25 and 0.5 mmol/L, while myeloid colony growth was not significantly inhibited at the same concentrations. At higher concentrations, SNAP inhibited both erythroid and myeloid colony growth totally. While exogenously added 8-Br-cGMP partially reproduced the NO effects on the mononuclear fraction of the bone marrow, it did not have the same effects on CD34' cells.

Our results suggest that NO may affect the growth of bone marrow colonies from precursor cells both directly and indirectly. In the presence of accessory and mature mononuclear cells, it inhibits the growth of both CFU-E and CFU-GM. It could, therefore, induce the production of cytokines or factors in the accessory cells, which in turn, would inhibit progenitor cell growth. In fact, we have previously reported that NO increases the mRNA levels of tumor necrosis factor-α and interleukin 1-β in cells of the HL-60 human myeloid leukemia cell line. NO has also been shown to enhance the production of tumor necrosis factor in interleukin-1-α stimulated human mononuclear cells. A similar effect could be operating here. On the other hand, NO had different effects when added to CD34' cells. When added to this population of enriched progenitor cells, it inhibited the growth of CFU-E, but enhanced the growth of CFU-GM. This growth enhancing effect was seen when SNP was used as a source of NO. When SNAP was used at 0.25 mmol/L, it had no effect on CFU-GM, even though it inhibited CFU-E.

The discrepancies between the observed effects of SNP and SNAP on CD34' cells are most likely due to the different amounts of NO liberated in solution by the two compounds. At all the concentrations used, SNAP had a more marked growth inhibitory effect than did SNP. The effect of NO on bone marrow growth and differentiation therefore appear to be concentration-dependent. At high concentrations, it potently inhibits the growth of both the erythroid and myeloid pathways. At lower concentrations, it seems to act as a signal that preferentially inhibits the growth of erythroid cells and enhances the growth of myeloid cells. The latter effect may be due to a direct effect on the bone marrow progenitors because it was observed only when an enriched population of CD34' cells was used as a cell source. Even though it increased the intracellular levels of cGMP in bone marrow cells, the NO effects appear to be cGMP-independent because raising intracellular cGMP levels with the membrane permeable cGMP analogue 8-Br-cGMP did not reproduce the NO effects.

NO has been reported to affect cell growth in different settings. It inhibits tumor cell growth and is cytostatic or

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Fig 5. Effect of SNP on cGMP levels in bone marrow mononuclear cells. SNP was added to bone marrow mononuclear cells cultured in RPMI-1640. The cells were lysed at the indicated time points and intracellular cGMP levels were measured. The data points represent means ± one standard error of the mean. Asterisks indicate statistically significant differences (P < .05) between treatments and controls. Results shown are the averages of two separate experiments.
cytocidal for certain microorganisms.\textsuperscript{1,2} NO has also been shown to inhibit lymphocyte proliferation.\textsuperscript{11} NO also affects cellular progression through the cell cycle of murine macrophage-like cells.\textsuperscript{12} While Pipili-Synetos et al\textsuperscript{13} demonstrated that NO could inhibit angiogenesis, Ziche et al\textsuperscript{14} have shown that NO mediates angiogenesis and promotes endothelial cell growth and migration. NO has also been shown to amplify fibroblast growth factor-2–induced mitogenesis of rat aortic smooth muscle cells.\textsuperscript{15} Punjabi et al\textsuperscript{16} have shown that interferon-γ and lipopolysaccharide inhibited murine bone marrow colony growth through a NO-dependent mechanism. Finally, Maciejewski et al\textsuperscript{17} reported that NO inhibits the growth of human bone marrow cells in vitro. They did not observe a selective effect on either the erythroid or the myeloid lineage.

NO can alter cell growth and differentiation by affecting different intracellular targets. Although the guanylate cyclase-cGMP signal transduction pathway is a major target of NO in the vascular system,\textsuperscript{2,3,9} it is not clear from the results we report here that NO affects the hematopoietic system solely through cGMP. Other potential mechanisms of action and intracellular targets include protein sulphydryl group nitrosylation,\textsuperscript{7} mitochondrial respiration,\textsuperscript{18} G protein activation,\textsuperscript{19} activation of nuclear factor-κB,\textsuperscript{20} inhibition of ribonucleotide reductase,\textsuperscript{21,22} protein adenosine diphosphate ribosylation,\textsuperscript{23,24} and DNA damage and mutation.\textsuperscript{25} NO has also been shown to induce apoptosis in murine peritoneal macrophages and in human bone marrow cells.\textsuperscript{17,26} It is also remarkable that NO at very low concentrations prevents apoptosis in B cells through the maintenance of bcl-2 levels.\textsuperscript{27}

It is clear from our observations and from those reported by other investigators that NO can play multiple physiologic and pathophysiologic roles affecting bone marrow cell growth and differentiation. It affects bone marrow physiology at different levels. It can mediate increased blood flow to the marrow under hematopoietic stress.\textsuperscript{28} Our work and the work of others\textsuperscript{15,17} show that it can also have a significant effect on the growth of cells of the erythroid and myeloid pathways. This is especially significant because the bone marrow contains numerous cells that could be potential sources of NO. At low to moderate levels, NO could selectively switch bone marrow cell production from the erythroid to the myeloid pathway. At high levels of production, it could potently inhibit the growth of both erythroid and myeloid cells. NO could, therefore, be an important mediator in the generation of the anemia associated with chronic inflammatory states (such as rheumatoid arthritis and cancer).

Therapeutic modulation of NO production and/or NO effects in vivo might significantly affect hematopoiesis.

REFERENCES


Differential effects of nitric oxide on erythroid and myeloid colony growth from CD34+ human bone marrow cells

PJ Shami and JB Weinberg