Nitric Oxide Suppression of Human Hematopoiesis In Vitro

Contribution to Inhibitory Action of Interferon-γ and Tumor Necrosis Factor-α

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Abstract

IFN-γ and TNF-α, potent inhibitors of hematopoiesis, induce nitric oxide synthase (NOS) in various cell types. When normal human bone marrow (BM) or CD34$^+$ cells were exposed to NO, inhibition of colony formation was dose dependent and direct. NO induced apoptosis in BM progenitors, as shown by electrophoretic detection of DNA degradation and deoxynucleotidyl transferase assay. Using PCR and immunoprecipitation, we found inducible NOS (iNOS) mRNA and iNOS protein in BM after stimulation with IFN-γ or TNF-α. iNOS mRNA was also detected by PCR in highly purified CD34$^+$ cells; TNF-α or IFN-γ increased iNOS expression. The presence of iNOS in CD34$^+$ cells was confirmed in single cells by immunochemical staining. N^G-Monomethyl-L-arginine (MM-Arg), an NOS inhibitor, partially reversed the effects of TNF-α and, to a lesser extent, IFN-γ in methylcellulose culture of total BM and CD34$^+$ cells, and inhibited apoptosis of BM cells induced by these cytokines. When the effects of competitive iNOS inhibition were tested on more immature progenitors, MM-Arg increased the number of long-term BM culture–initiating cells in control cultures but failed to protect these cells from the inhibitory action of IFN-γ and TNF-α. Our results suggest that NO may be one mediator of cytokine-induced hematopoietic suppression. (*J. Clin. Invest.* 1995. 96:1085–1092.) Key words: bone marrow • cytokines • nitric oxide synthase • CD34$^+$ cells • aplastic anemia

Introduction

Nitric oxide (NO),[1] a radical produced in mammalian cells from arginine in a reaction catalyzed by nitric oxide synthase (NOS), has pleiotropic biologic activities (1–3). NO is produced during inflammatory reactions and has been implicated as a signaling molecule (4–6) as well as a toxic effector (7–10). NO mediates activation or inhibition of various enzyme systems (6, 11), DNA damage (12–15), and oxidative reactions (16–21), with a variety of biologic effects, including killing of microorganisms (22), antiviral activity (23), and cytostasis and cell death (2, 3, 7–10, 24).

Expression of the inducible form of NOS (25, 26) is regulated by cytokines, including IFN-γ and TNF-α. TNF-α and IFN-γ enhance expression of inducible NOS (iNOS) and NO production in a variety of cell types (27–31). The promoter region of iNOS contains several sequences that potentially bind IFN-γ–induced transcription factors, including an interferon regulatory factor-1 (IRF-1) binding element (32, 33). IRF-1 induced by IFN-γ binds to the promoter region of the iNOS gene (34) and enhances iNOS mRNA expression (33, 34). A different activation pathway involving NF-κB/Rel that has been proposed for LPS may also operate for TNF-α–mediated induction of iNOS (35). Because some of the biologic effects of IFN-γ and TNF-α are similar to those described for NO, they may be due to cytokine-mediated induction of NO in the target cells.

A pathophysiologic role of NO has been proposed for several autoimmune diseases (7–9, 36). TNF-α and IFN-γ suppress hematopoietic progenitor cell proliferation (37–40) and have been implicated in the mechanism of bone marrow (BM) failure (41–48). In aplastic anemia, aberrant production of IFN-γ (42, 43) and upregulation of TNF-β (44) in normal human bone marrow likely induce apoptosis of hematopoietic stem cells and progenitor cells. IFN-γ and TNF-α suppress both early and late stages of hematopoiesis and induce programmed cell death (C. Selleri, S. Anderson, N. Young, and J. Maciejewski, manuscript submitted for publication). Involvement of IFN-γ and TNF-α in the regulation of NO production suggested that NO may influence the function of BM and may be relevant for understanding the pathophysiology of hematologic diseases.

Methods

BM cell preparation. BM was obtained from healthy volunteers by aspiration from the posterior iliac crest into syringes containing Iscove's modified Dulbecco's medium (IMDM; GIBCO BRL, Gaithersburg, MD) supplemented 1:10 with preservative-free heparin (O'Neill and Feldman, St. Louis, MO). Informed consent was obtained according to a protocol approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute. Mononuclear BM cells were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC). After washing in HBSS (GIBCO BRL), cells were resuspended in IMDM supplemented with 20% FCS (GIBCO BRL).

Separation of CD34$^+$ cells. CD34$^+$ cells were separated using affinity chromatography (Cellpro, Bothell, WA) and flow cytometry. Briefly, nonadherent BM cells were incubated at room temperature with murine

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1. *Abbreviations used in this paper:* BM, bone marrow; IMDM, Iscove's modified Dulbecco's medium; iNOS, inducible NOS; IRF, interferon regulatory factor; LTBMC, long-term bone marrow culture; LTCIC, long term culture–initiating cells; NO, nitric oxide; NOS, NO synthase; TdT, terminal deoxynucleotidyl transferase.

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anti-human CD34 IgM, washed in PBS, and incubated with streptavidin-conjugated goat $F(ab')_2$ anti-mouse IgM. After washing with PBS supplemented with 2% human albumin, cells were applied to an affinity column containing biotin-coated beads, and the $CD34^+$ cell fraction was eluted with PBS. An aliquot of the eluted cells was stained with phycoerythrin-conjugated anti-CD34 HPCA-2 monoclonal antibody (Becton Dickinson, Mountain View, CA) to assess the purity of the eluted cells. Usually, 70–90% of separated cells were $CD34^+$. For higher purity preparations, cells were further fractionated; column-purified cells were stained with FITC-labeled anti-$CD34^+$ mAb (Becton Dickinson), washed with PBS, and sorted by microfluorometry (Epics V; Coulter, Hialeah, FL). The purity of cells obtained by combining affinity chromatography and flow cytometry was 97–99%.

Hematopoietic cell culture. For short-term suspension cultures, $CD34^+$ BM cells were cultured in 96 round-bottom well plates at a density of 5×10^4 cells/ml (1×10^4 cells/well) in medium consisting of IMDM, 20% FCS, 50 ng/ml IL-3 (Genzyme, Boston, MA), 50 ng/ml stem cell factor (Amgen, Thousand Oaks, CA), and 2 U/ml erythropoietin (Ortho, Raritan, NJ). TNF-α and IFN-γ were obtained from Boehringer Mannheim, Indianapolis, IN. Diethylenetriamine (DETA) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Its adduct with NO (DETA/NO) was prepared as previously described (49). Its structure is $H_2NCH_2CH_2N[N(O)NO]^-CH_2CH_2NH_3^+$, and its Chemical Abstracts registry number is 146724-94-9. The half-life of DETA/NO in pH 7.4 buffer at 37°C is 20 h (50), and 2 mol of NO are released for each mol of DETA/NO that hydrolyzes (49). N^G-Monomethyl-L-arginine (MM-Arg) was purchased from Calbiochem (San Diego, CA), and 3-aminobenzamide was obtained from Sigma Chemical Co. (St. Louis, MO).

Long-term BM cultures (LTBMCs) were performed according to a previously described method (51, 52). 1×10^7 mononuclear BM cells were used to initiate LTBMC. Culture medium consisted of stem cell medium (Terry Fox Laboratory, Vancouver, Canada) supplemented with 1×10^{-6} M hydrocortisone hemisuccinate (Sigma Chemical Co.). After 1 wk of culture at 37°C, the incubator temperature was changed to 33°C. Medium changes were performed weekly. Nonadherent cells were removed from the dishes, washed, and counted; half the cells were returned to the original dishes, and the other half were used in re-plating experiments to measure the output of clonogenic progenitors. TNF-α and IFN-γ were added weekly at appropriate concentrations. After 5 wk of culture, nonadherent cells were harvested using trypsin (GIBCO BRL), washed, and replated in methylcellulose to estimate the numbers of long term culture-initiating cells (LTCICs). The number of clonogenic cells was converted to the absolute number of LTCIC by dividing by 4 (52, 53).

Hematopoietic progenitors were measured in methylcellulose cultures. Freshly isolated or previously cultured BM cells were placed in methylcellulose in the presence of 50 ng/ml IL-3 (Genzyme), 20 ng/ml GM-CSF (Boehringer Mannheim), 50 ng/ml stem cell factor, and 2 U/ml erythropoietin (Amgen). Total BM cells were plated at a density of 1×10^5 cells in 1-ml culture medium in 35-mm dishes. $CD34^+$ cells were cultured at a density of 1×10^3 cells/0.5 ml methylcellulose in 48-well plates. TNF-α and IFN-γ were added to the culture at appropriate concentrations. All cultures were performed in duplicate. All experimental procedures were performed in endotoxin-free plasticware; according to the manufacturers' information, the levels of endotoxin contamination in the cytokine preparations and mAb were 3 endotoxin U/mg by the limulus assay, and antibodies used in all experiments contained < 1 ng/ml of endotoxin. As a negative control for flow cytometry experiments, isotype-matched mAb were employed (Dako, Carpinteria, CA).

Apoptosis assays. DNA fragmentation was measured after extraction of low molecular weight DNA. 2×10^6 cells were resuspended in 900 μl 1 × Tris-EDTA buffer (Quality Biological, Inc., Gaithersburg, MD) and lysed with 25 μl 20% SDS (54). High molecular weight DNA was precipitated for 6 h in the presence of 5 M NaCl. The high molecular weight fraction was pelleted by high-speed centrifugation, and the fragmented DNA was extracted from the aqueous phase with phenol and chloroform and then precipitated with ethanol. After resuspension in water, DNA was electrophoresed using 1.5% agarose gel and visualized by ethidium bromide staining.

To quantitate the number of cells undergoing apoptosis, cells were harvested, fixed with 4% neutral buffered formalin, and cytocentrifuged onto siliconized slides. Apoptotic cells were stained using the terminal deoxynucleotidyl transferase (TdT) method (Apotag; Oncor, Gaithersburg, MD). Endogenous peroxidase was first quenched with 0.5% hydrogen peroxide, and the cells were permeabilized using company-supplied equilibration buffer. The 3′ OH ends of degraded DNA were reacted with TdT and digoxygenin-labeled ATP for 30 min. After washing with PBS, slides were reacted with an anti-digoxygenin mAb conjugated to peroxidase, washed, and developed with 3,3′-diaminobenzidine tetrahydrochloride (Pierce, Rockford, IL). Stained cells were counted using a light microscope.

Reverse transcription PCR for human iNOS. Total RNA was extracted from constant numbers of mononuclear marrow cells and $CD34^+$ cells using RNAsol (Cinna/Biotecx, Friendswood, TX). Contaminating DNA was digested using RNase-free DNaseI (Boehringer Mannheim). RNA was reextracted with phenol and chloroform, precipitated with ethanol, and diluted in RNase-free water. After reverse transcription using $oligo(dT)_{16}$ primer, iNOS cDNA was amplified using primers 5′ CGGTGCTGTATTTCCTTACGAGGCGAAGAAGG 3′ and 5′ GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC 3′ specific for iNOS and primers specific for human β-actin. The PCR products were electrophoresed on 1.2% agarose gels and transferred onto nytran filters (Schleicher & Schuell, Keene, NH). The blots were then hybridized with an internal oligonucleotide 5′ labeled with ^{32}P using T4-kinase (Boehringer Mannheim). After washing under stringent conditions (0.1 × SSC, 0.5% SDS), membranes were exposed to x-ray film and developed.

Immunocytochemical staining and immunoprecipitation. BM cells were starved for 2 h in methionine- and cysteine-deficient medium containing 2% dialysed FCS, washed, and resuspended in the medium containing 2 μCi/ml of [^{35}S]methionine and cysteine (Trans-label; ICN Biomedicals, Irvine, CA). Total BM cells were cultured for 24 h in the presence of TNF-α or IFN-γ, lysed in radioimmunoprecipitation buffer, and, after addition of mouse serum, cleared for 2 h with protein-A agarose (Boehringer Mannheim). After overnight incubation with 2 μg of mouse anti-mouse iNOS peptide 961–1144 monoclonal IgG with cross-reactivity to human iNOS (Transduction Laboratories, Lexington, KY), cell lysates were incubated with rabbit anti-mouse IgG (Pierce) for 2 h and reacted with 30 μl protein-A agarose suspension for 2 h. After multiple washing steps, agarose-bound complexes were denatured by boiling in loading buffer containing 1% mercaptoethanol, electrophoresed on 8% SDS-polyacrylamide, and either transferred onto nitrocellulose membranes (Novex, San Diego, CA) or vacuum-dried and exposed to x-ray film. After blocking for 2 h (Superblock; Pierce), blots were reacted with 2 μg of mouse anti-iNOS mAb (Transduction Laboratories) over night and developed with alkaline-phosphatase-conjugated rabbit anti-mouse IgG (Pierce) for 2 h. Bands of 130 kD corresponded to the human iNOS.

For immunocytochemistry, cultured purified $CD34^+$ cells were washed in PBS and cytocentrifuged onto siliconized slides. After fixation with ice-cold 50:50 methanol/acetone for 10 min, slides were blocked with PBS-containing 10% normal goat serum, reacted with rabbit anti-human iNOS peptide IgG purified from antiserum (kindly provided by Merck Research Laboratories, West Point, PA) and developed with Texas red-conjugated goat anti-rabbit IgG (Vector Lab, Burlingame, CA). Immunizing peptide was used to block anti-human iNOS polypeptide IgG. As positive controls, 293 cells transfected with human iNOS cDNA were used (25).

Results

Effects of NO on colony formation of marrow cells in methylcellulose cultures. To test the effects of NO on the growth of marrow cells in vitro, we used DETA/NO, a compound which

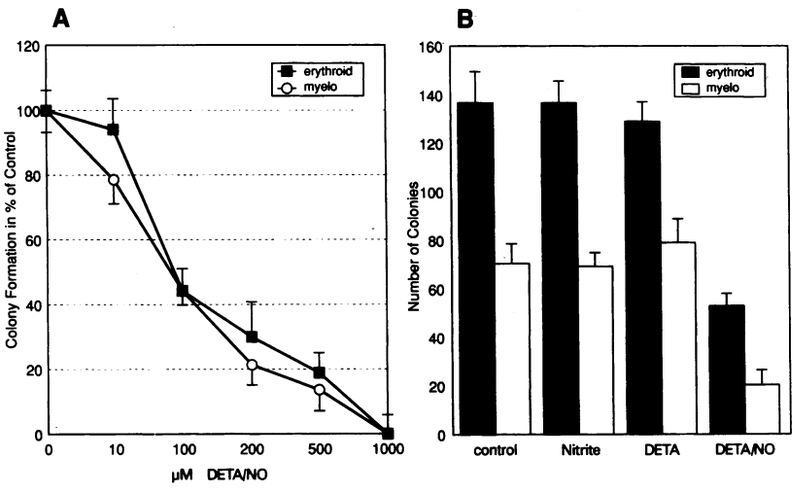

Figure 1. NO inhibits colony formation by human total and CD34$^+$ purified BM cells. (*A*) Dose-dependent inhibition of colony formation by DETA/NO in CD34$^+$ cells. Values represent the percentage of colony formation relative to DETA as control. The graphs represent results from two independent experiments. (*B*) NO released during the decomposition of DETA/NO inhibits colony formation by total BM cells; DETA and nitrite do not affect colony formation. Bars represent mean colony formation in a representative experiment (duplicate measurements). Concentrations used were 100 μM DETA/NO, 100 μM DETA, and 200 μM sodium nitrite.

at neutral pH spontaneously releases NO into the solution with a half-life of 20 h at 37°C. When DETA/NO was added to methylcellulose cultures of CD34$^+$ cells, both myeloid and erythroid cells decreased in colony-forming ability (Fig. 1 *A*). To exclude the possibility that the inhibition of colony formation was due to the carrier molecule, DETA, or to nitrite generated in the medium from released NO, both substances were added to the culture as controls. No effect on colony formation was observed with DETA and nitrite used at appropriate concentrations (Fig. 1 *B*). The inhibitory effect of NO was dose dependent. The presence of accessory cells was not required for DETA/NO suppression. The CD34$^+$ cell population used in this experiment includes committed and primitive progenitor cells (55, 56).

NO-mediated hematopoietic inhibition is associated with the induction of apoptosis of BM cells. The observed suppression of colony formation by NO could be due to a reversible inhibition of progenitor cell cycling without loss of viability or to the induction of cell death. Analysis of low molecular weight DNA extracted from total BM cultured in suspension in the presence of the NO-releasing compound DETA/NO showed a nucleosomal DNA degradation pattern on agarose gels stained with ethidium bromide characteristic of apoptosis (Fig. 2). Low molecular weight DNA extracted from the same number of cells cultured in the presence of DETA as a control reagent did not show this pattern. Furthermore, 3-aminobenzamide, a poly-ADP–ribose synthetase inhibitor, suppressed the apoptotic degradation of DNA in cells cultured with DETA/NO (Fig. 2). NO-induced programmed cell death was detectable within the first 24 h of culture. To test whether hematopoietic progenitors were affected by the action of NO, we added DETA/NO to the cultures of highly purified CD34$^+$ BM cells. Using the in situ TdT method, we demonstrated that NO induced apoptosis of CD34$^+$ cells in culture (Fig. 3).

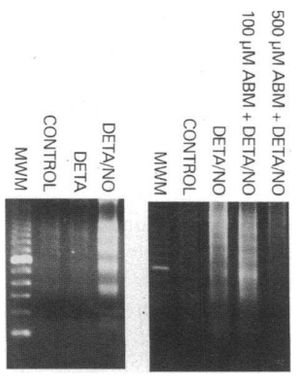

Figure 2. NO induces apoptosis of total BM cells in cultures. Results of agarose gels stained with ethidium bromide after electrophoresis of low molecular weight DNA extracted from constant numbers of cells grown in the presence of DETA/NO or DETA as control. ABM, 3-aminobenzamide.

Expression of iNOS in marrow cells stimulated by IFN-γ or TNF-α. Because NO inhibited BM cell proliferation, and iNOS, a key enzyme in the synthesis of NO, can be induced in a variety of cell types with TNF-α or IFN-γ, we tested whether these cytokines regulated the expression of iNOS in BM cells. Using PCR, we demonstrated the presence of iNOS mRNA in cultured unstimulated total BM cells. A very strong signal was obtained from cells cultured in the presence of TNF-α and IFN-γ (data not shown). Immunoprecipitation and immunoblot of cell lysates demonstrated that BM cells expressed low levels of iNOS, and the expression of this enzyme was enhanced by IFN-γ and TNF-α (Fig. 4 *B*). Because the iNOS signal in total BM cells might be due to the presence of accessory cells, we tested whether more immature cells contained in the CD34$^+$ cell population expressed iNOS. To minimize the possibility of contamination with mature cells potentially expressing iNOS, we used preparations containing 98% CD34$^+$ cells. Simultaneous staining with CD14 mAb, specific for monocytes and macrophages, and anti-CD2 mAb for lymphocytes, showed less than 3% of CD14$^+$ and 2% of CD2$^+$ cells in CD34$^+$ cell preparations used for PCR. iNOS mRNA was detectable in the CD34$^+$ cells cultured for 24 h in medium alone, and IFN-γ– or TNF-α–stimulated cells showed a stronger amplification signal (Fig. 4 *A*). Immunocytochemical staining with human iNOS-specific antiserum of CD34$^+$ cells confirmed the presence of iNOS protein at the single cell level in freshly purified CD34$^+$ cells from some donors. The presence of iNOS protein in a proportion of cultured CD34$^+$ cells was consistent with the PCR results, with low untreated and cytokine-enhanced expression of iNOS in CD34$^+$ cells (Fig. 4, *C* and *D*). To determine if more immature progenitors, defined by the expression of CD38 antigen, contained iNOS mRNA, we sorted CD34$^+$CD38^{high} (Fig. 5 *A*) and CD34$^+$CD38^{dim} (Fig. 5 *B*) cells and performed RT-PCR on both cell fractions. Human iNOS mRNA was detectable both in less mature CD34$^+$CD38^{dim} and in mature CD34$^+$CD38^{high} BM cells (Fig. 5 *C*).

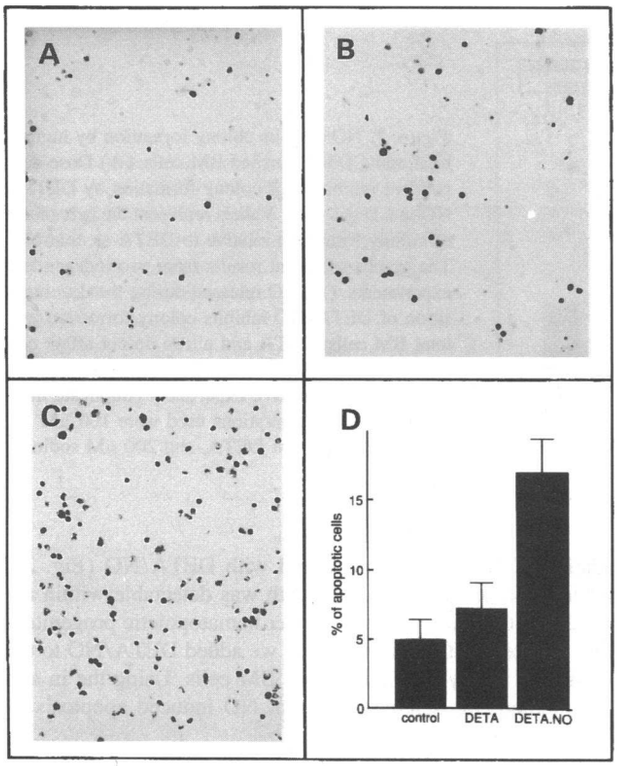

Figure 3. NO induces apoptosis of CD34$^+$ cells in culture. Purified CD34$^+$ cells were grown in suspension culture in the presence of DETA/NO. (*A*) Photomicrograph of control cells after in situ TdT-apoptosis assay. (*B*) Cells grown in the presence of DETA as control. (*C*) Cells cultured in the presence of DETA/NO. Dark-stained cells are apoptotic. (*D*) Bars represent percentages of apoptotic cells in culture using the in situ TdT-apoptosis assay.

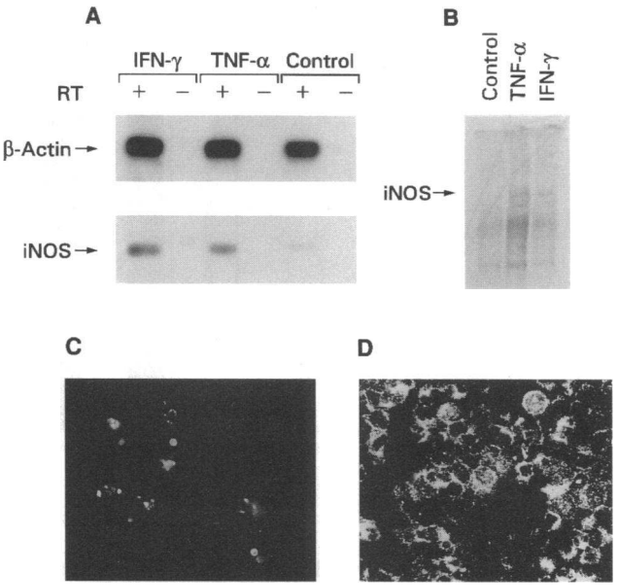

Figure 4. Expression of iNOS in CD34$^+$ cells after stimulation with IFN-γ or TNF-α. (*A*) Bands represent products of the RT-PCR reaction for iNOS and actin mRNA from highly purified CD34$^+$ cells stimulated with TNF-α or IFN-γ. *Upper gel,* actin; *lower gel,* iNOS. (*B*) Immunoprecipitation of human iNOS in total BM cells stimulated with IFN-γ or TNF-α. Bands of 130 kD correspond to human iNOS. (*C*) and (*D*) Photomicrographs of CD34$^+$ cells cultured in the presence of IFN-γ. (*C*) Control staining with preimmune serum. (*D*) Staining with anti-human iNOS peptide polyclonal IgG; reactivity of immune IgG was completely blocked by immunizing peptide (not shown).

Effects of inhibition of iNOS on the proliferation of BM progenitors. Because NO inhibited hematopoietic colony formation and IFN-γ and/or TNF-α increased the expression of iNOS in BM progenitors, we tested whether the inhibitory effects of these cytokines were related to NO production. Addition of MM-Arg, a competitive inhibitor of iNOS, to methylcellulose cultures of total BM cells partially reversed the suppressive effects of IFN-γ and/or TNF-α on colony growth (Table I). MM-Arg showed more pronounced effects on cultures that included TNF-α. Parallel results were obtained in colony cultures from CD34$^+$ cells (Table I). Similar degrees of inhibition by IFN-γ and TNF-α were observed for both erythroid and myeloid series. Within myeloid subsets combining granulocytic, macrophage, and granulocytic-monocytic colonies, preferential survival of macrophage colonies after IFN-γ and TNF-α was seen in both total and CD34$^+$ cell cultures. In cultures supplemented with MM-Arg, granulocytic, macrophage, and granulocytic-monocytic colonies were represented at similar proportions as in control cultures (data not shown).

In suspension cultures of total BM and purified CD34$^+$ cells, MM-Arg also partially prevented IFN-γ– and TNF-α–induced apoptosis (Fig. 6).

To determine whether cytokine-mediated NO production showed differential inhibitory effects on less mature CD34$^+$CD38^{dim} and more committed CD34$^+$CD38^{high} cells, we cultured these two fractions in suspension in the presence of IFN-γ and TNF-α, with or without MM-Arg. After 7 d of culture, cells were counted to estimate the effects of MM-Arg on overall proliferation. Although the proliferative capacity of CD34$^+$CD38^{dim} cells is lower than that of CD34$^+$CD38^{high} cells, IFN-γ and TNF-α showed inhibitory effects on both cell populations. In the presence of 500 μM MM-Arg, the inhibitory effects of the cytokines were less pronounced (for IFN-γ, 48 vs 26% inhibition in cultures of CD34$^+$CD38^{high} and 15 vs 5% in cultures of CD34$^+$CD38^{dim} cells, without or with MM-Arg, respectively; for TNF-α, 36 vs 0% inhibition in cultures of CD34$^+$CD38^{high} and 39 vs 0% in cultures of CD34$^+$CD38^{dim} cells without or with MM-Arg, respectively). To determine the effects of MM-Arg on the numbers of colony-forming progenitors in these cultures after stimulation with IFN-γ and TNF-α, cells derived from primary suspension cultures of CD34$^+$CD38^{dim} and CD34$^+$CD38^{high} cells were placed in methylcellulose, and after 14 d the colony numbers were counted (pre-CFU-assay). MM-Arg failed to protect against the IFN-γ–induced decrease in the numbers of colony forming cells, and the protective effect of MM-Arg on the number of colony-forming cells in cultures with TNF-α was less pronounced than the effects seen in the primary methylcellulose assays (data not shown).

Because clonogenic assays measure the function of committed progenitors, we used LTBMC to study the protective effects of MM-Arg on more immature progenitors (Table II). When the output of CFU in LTBMC was measured in sequentially performed replating experiments, MM-Arg showed effects similar to those obtained with primary methylcellulose culture. MM-

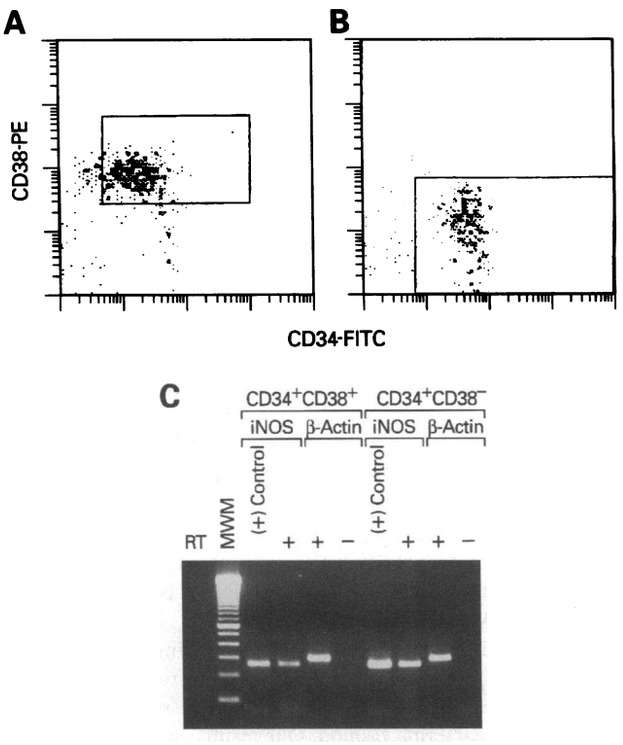

Figure 5. Expression of human iNOS mRNA in $CD38^{high}$ and $CD38^{dim}$ $CD34^+$ cells. Purified $CD34^+$ cells were sorted according to the expression of CD38 antigen. $CD34^+CD38^{high}$ (*A*) and $CD34^+CD38^{dim}$ (*B*) cell fractions were used for mRNA extraction and subjected to RT-PCR. Human iNOS and β-actin amplification products were electrophoresed in agarose gels and stained with ethidium bromide (*C*).

Arg increased the numbers of CFU in control cultures by 40% in week 1, 36% at week 2, and 30% at week 3, in comparison with treated control cultures. A protective effect on inhibition of CFU production by IFN-γ and TNF-α was seen; the numbers of CFU measured in the nonadherent cell fraction after week 1 were completely restored. A lesser effect of MM-Arg on CFU output was seen in week 2 (19 and 39% higher CFU numbers with IFN-γ and TNF-α, respectively) and week 3 (32 and 46% higher CFU numbers for IFN-γ and TNF-α) of LTBMC. Although the presence of MM-Arg in LTBMC increased the total output of CFU in cultures not treated with IFN-γ and TNF-α, cytokine-suppression of LTCIC survival was unaffected (Table II). Similar to the primary methylcellulose assay, surviving myeloid colony–forming cells in IFN-γ and TNF-α were predominantly of the macrophage type (data not shown).

Discussion

We studied cytokine pathways leading to the induction of NO production and NO effects on human hematopoiesis in vitro. NO decreased colony formation by total human BM and purified $CD34^+$ cell preparations, demonstrating that the effects of NO were direct with regard to a cell population containing hematopoietic progenitor cells (55, 56). The detection of apoptosis in these experiments, in both total BM and $CD34^+$ cells, suggests that the suppressive effects of NO may be at least partially attributed to irreversible cytotoxicity. In the presence of IFN-γ and/or TNF-α, iNOS expression appeared to be enhanced not only in total BM containing mature accessory cells but also in the immature cells defined by the CD34 surface antigen. Although in isolated $CD34^+$ cells the expression of iNOS showed some variability, there was a concordance between iNOS expression and the presence of cytokines in culture in both iNOS-specific RT-PCR and immunocytochemistry performed on

Table I. Effect of NOS Inhibition on Colony Formation

	MM-Arg concentration					
	0 μM		250 μM		500 μM	
Treatment	Erythroid	Myeloid	Erythroid	Myeloid	Erythroid	Myeloid
BM cells						
TNF-α	62±9	66±9	82±12*	79±14*	114±10‡	100±11‡
IFN-γ	47±8	47±9	53±13	43±8	88±15‡	67±13*
IFN-γ + TNF-α	35±9	33±6	79±12‡	63±11‡	44±11*	55±15*
$CD34^+$ cells						
TNF-α	43±7	50±6	62±8*	72±11*	63±8*	67±12*
IFN-γ	52±6	47±8	89±2‡	78±9‡	74±12*	74±13*
IFN-γ + TNF-α	27±6	27±9	36±8*	53±10*	48±13‡	51±12*

N^G-Monomethyl-L-arginine partially abrogates the inhibitory effects of IFN-γ and TNF-α on colony formation by total BM and $CD34^+$ cells in vitro. The values (mean±SD) represent colony formation as a percentage of control and reflect combined data from the five experiments performed. The mean total numbers of colonies in control total BM cultures were 186 ± 44 erythroid and 84±17 myeloid colonies and 66±8 erythroid and 65±9 myeloid colonies for $CD34^+$ cells. The number of colonies in $CD34^+$ cell cultures at 250 μM MM-Arg was 91±12% (erythroid) and 107±10% (myeloid) of control, and, at 500 μM MM-Arg, 107±12% (erythroid) and 105±7% (myeloid) of control. Since addition of MM-Arg influenced colony formation, the percentage of inhibition of colony formation in the samples with cytokines and MM-Arg was calculated using MM-Arg alone as control. For comparison, colony formation by total BM cells at 250 μM MM-Arg was 74±3% (erythroid) and 81±5% (myeloid) of control and, in the presence of 500 μM MM-Arg, 91±16% (erythroid) and 88±8% (myeloid) of control. Statistical analysis (paired *t* test), comparing cytokine vs. cytokine + MM-Arg: $^*P < 0.05$, $^‡P < 0.01$.

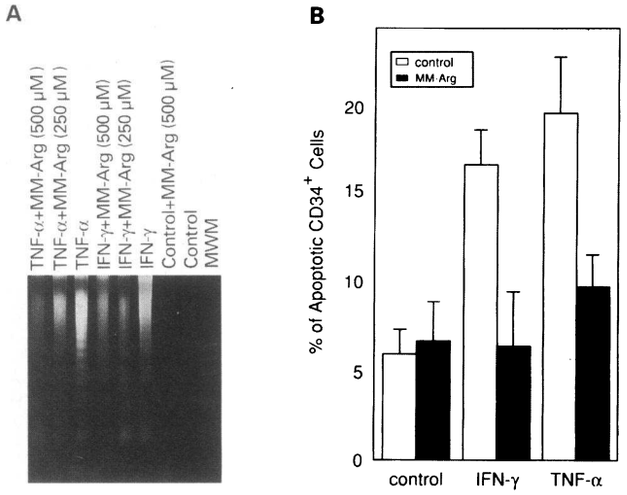

Figure 6. N^G-Monomethyl-L-arginine (MM-Arg) partially prevents IFN-γ– and TNF-α–induced programmed cell death. (*A*) Agarose gels were stained with ethidium bromide after electrophoresis of low molecular weight DNA extracted from constant numbers of total BM cells grown in suspension culture in the presence of IFN-γ and TNF-α. (*B*) Bars represent percentages of apoptotic cells in cultures of $CD34^+$ cells enumerated using the in situ terminal deoxynucleotidyl transferase–apoptosis assay. Statistical analysis (paired *t* test): $n = 5$; control vs. IFN-γ, $P < 0.01$; control vs. TNF-α, $P < 0.01$; IFN-γ vs. IFN-γ + MM-Arg, $P < 0.05$; TNF-α + MM-Arg vs. TNF-α, $P < 0.05$.

highly purified $CD34^+$ cells. Competitive inhibition of iNOS partially reversed the inhibitory effects of IFN-γ and/or TNF-α on colony formation by total BM and $CD34^+$ cells and inhibited cytokine-induced apoptosis. Thus, TNF-α and/or IFN-γ mediated hematopoietic suppression, in part, through an NO pathway. The less protective activity of MM-Arg in the pre-CFU assay and the lack of comparable effects of iNOS inhibition on the generation and maintenance of LTCIC in the presence of IFN-γ and/or TNF-α suggest that NO may not be the principal mediator of LTCIC inhibition under these circumstances. Although iNOS mRNA was detectable in the immature $CD34^+CD38^{dim}$ cell population enriched for LTCIC, very primitive hematopoietic cells may be incapable of iNOS expression and/or the mechanism of cytokine action on these cells may be more complex. The differences in the ability of MM-Arg to reverse the inhibitory effects of TNF-α and IFN-γ in primary methylcellulose cultures, pre-CFU assays, and LTBMC may also be related to the experimental conditions. At present, the question of whether more or less mature progenitor cells show differential susceptibility to NO-mediated inhibition cannot be conclusively resolved.

Proposed mechanisms for NO toxicity include binding to iron in the prosthetic groups of a variety of enzymes (2, 5, 7, 18), *S*-nitrosation of proteins (17–21), and mono- or poly-ADP-ribosylation leading to post-translational modification of proteins and also to NAD depletion (11, 57, 58). The inhibitory effects of NO on BM proliferation could be related to all of these mechanisms and more. However, DNA toxicity with single-strand DNA breaks and base deamination may be also responsible for NO-mediated suppression (12–15). Our experiments support this theory. NO-mediated DNA damage in BM cells might be responsible for poly-ADP–ribosylation and subsequent apoptosis, as suggested in other studies (59). Blocking of poly-ADP–ribosylation inhibited NO-mediated nucleosomal degradation. Because nuclear poly-ADP–ribosylation is involved in the cellular response to single-strand DNA breaks (60, 61), inhibition of this reaction may prevent access of either nucleases or DNA repair machinery to DNA, both resulting in apoptotic DNA degradation.

Table II. Effect of NOS Inhibition on LTCIC

	Control	IFN-γ	TNF-α	INF-γ + TNF-α
Control	131±20	19±8	54±15	8±5
MM-Arg	166±14	31±9	58±12	11±6

Inhibition of iNOS increases the output of LTCIC but does not protect them from the inhibitory effects of IFN-γ and/or TNF-α on the generation and maintenance of LTCIC in long-term cultures. Values represent the numbers of LTCIC±SD generated in the cultures initiated with 1×10^7 total BM cells. The results summarize data from all five experiments performed.

NO production may be toxic to surrounding cells or autotoxic (7–9, 36). Expression of iNOS has been well documented in macrophages (27, 28). Thus, in BM, accessory cells could release NO in paracrine fashion. Our results suggest, however, that immature hematopoietic cells contained in the $CD34^+$ cell population themselves express iNOS and produce NO. The ability to express iNOS is not restricted to more mature, committed $CD34^+$ cells which are phenotypically defined by high levels of CD38 expression, because more primitive $CD34^+$ cells sorted on the basis of low or absent expression of CD38 marker also contained detectable levels of iNOS mRNA. Although we cannot rigorously exclude that the ability to detect iNOS in $CD34^+$ cells was related to contamination by the more mature cells, very low numbers of contaminating cells in our preparations suggest that $CD34^+$ cells are capable of expressing iNOS. In any event, induction of NO release in BM accessory cells in vivo may have pathophysiologic implications on the regulation of BM function that are of equal importance to endogenous NO production in BM progenitors.

Although the ability to express iNOS has been most frequently associated with terminally differentiated cells such as macrophages and neutrophils, cytokine-inducible NO production may also be physiologically important in early cells, including hematopoietic progenitors. It can only be speculated whether iNOS expression is a function of maturity, a lineage-specific marker, or a general physiologic reaction in response to cytokine-mediated signals. Preferential survival of macrophage colonies in cultures stimulated with IFN-γ and TNF-α may be related to their lower sensitivity to these cytokines and/or to NO toxicity rather than to induction of macrophage differentiation of the committed progenitors.

The results of our studies demonstrate that cytokines such as TNF-α and IFN-γ may contribute to the regulation of NO production in BM and explain their suppressive activities. TNF-α is present in normal BM, but IFN-γ can only be detected in BM under pathologic conditions, and the induction of iNOS expression by each of these cytokines suggests possibly different functional roles for NO in normal hematopoiesis and in disease. Since the protective effect of iNOS inhibition on TNF-α– and IFN-γ–mediated suppression of BM proliferation was only

partial, other intracellular mechanisms triggered by IFN-γ or TNF-α may contribute to their antiproliferative actions on BM progenitors. Such mechanisms include inhibition of cell cycling and induction of apoptosis by not only nitrogen radicals but also reactive oxygen species (62, 63) and by other signaling pathways.

Overexpression of IFN-γ and TNF-α in BM has been described in some BM failure disorders (42–48). Increased production of NO due to the action of these cytokines during disease may acutely result in induction of apoptosis of progenitor cells as demonstrated by our in vitro experiments. Programmed cell death may reflect a physiologic reaction protective against accumulated DNA damage. Although the mutagenicity of NO is still a matter of controversy, chronically elevated NO production could overwhelm normal DNA repair mechanisms and result in somatic mutations. Similar mechanisms may also act in connection with oxygen radicals generated in response to inflammatory stimuli. Further experimentation is needed to test whether production of reactive oxygen and nitrogen species can lead to mutations associated with malignancies.

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