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High-does nitric oxide induces apoptosis in HL-60 human myeloid leukemia cells

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Abbreviations: NO, nitric oxide; SNP, sodium nitroprusside; PMA, phorbol 12-myristate 13-acetate; HBSS, Hank's balanced salt solution

Abstract

The treatment of human promyeloid leukemia cell line HL-60 cells with nitric oxide (NO) is associated with loss of proliferative capacity and induction of monocytic differentiation. The present results demonstrate that treatment of asynchronous human HL-60 leukemia cells with sodium nitroprusside (SNP), a NO generating agent, is also associated with oligonucleosomal DNA cleavage, including morphological changes, condensation of nuclear chromatin, nuclear fragmentation, and the apoptotic peak of propidium iodide-stained nuclei in flow cytometric analysis. SNP-induced apoptosis was associated with a steep inhibition of the proliferation of the cells in a time- and concentration-dependent manner. Endonucleolytic cleavage was prevented by addition of $ZnSO_4$ or *N*-acetylcysteine, a free radical-scavenging thiol compound. In addition, potassium ferricyanide, which is structurally similar to SNP except for the absence of a nitroso group, did not induce growth inhibition and apoptosis in HL-60 cells. However, the cells which did not undergo apoptosis displayed characteristics of monocytic differentiation, including spreading, vacuolization, expression of monocyte marker CD14, and increased capacity to produce hydrogen peroxide. Since it was of interest to examine whether NO also induce apoptosis in the cells which was undergone differentiation, DNA fragmentation was studied in the cells pretreated with phorbol ester, a monocytic differentiation agent, for 48 h and then monitored for 18 h in the presence of SNP. Flow cytometric analysis revealed no characteristic apoptotic pattern in phorbol 12-myristate 13 acetate-pretreated cells, implying that a more efficient defense mechanism may exist in the differentiating cells. Collectively, these data suggest that NO elaborated in the bone marrow microenvironment might have biphasic regulatory roles in normal and malignant hematopoietic programmed cell death in addition to its already known role as a cell differentiating molecule in HL-60 cells.

Keywords: nitric oxide, differentiation, apoptosis, hematopoietic programmed cell death

Introduction

Programmed cell death, or apoptosis, is a regulated process during which eukaryotic cells die in response to specific stimuli (Kerr *et al.*, 1972). This process provides a mechanism for deletion of specific cell populations in the developing embryo (Hinchliffe, 1981). Apoptosis is also associated with death of lymphocytes treated with glucocorticoids and elimination of autoreactive T-cell clones in the thymus (Wyllie, 1980; Shi *et al.*, 1989; Smith *et al.*, 1989). The death of these cells is preceded by chromatin condensation and cleavage at internucleosomal sites (Wyllie, 1980; Wyllie *et al.*, 1992). Thus, one characteristic of apoptosis is internucleosomal DNA fragmentation. The mechanisms responsible for this endonucleolytic DNA cleavage remain unclear. Nonetheless similar patterns of DNA fragmentation have been observed in lymphocytes deprived of interleukin-2 (IL-2) and in myeloid cells deprived of colony-stimulating factors (Duke and Cohen, 1986; Rodriguez-Tarduchy *et al.*, 1990; Williams *et al.*, 1990). These findings have indicated that certain growth factors may promote survival by suppressing apoptosis (Duke and Cohen, 1986; Rodriguez-Tarduchy *et al.*, 1990; Williams *et al.*, 1990).

Human myeloid leukemia cell lines, such as HL-60 and U-937, proliferate autonomously (Sundstrom and Nilsson, 1976; Collins, 1987). However, these cells have retained the capacity to respond to inducers of differentiation with cessation of growth and appearance of a more mature phenotype (Koeffler, 1983). Previous

reports demonstrated that nitric oxide (NO), derived as purified gas or released from the pharmacologic NO donors, caused monocytic differentiation of HL-60 cells and altered gene expression (Boss, 1989; Magrinat *et al.*, 1992). The treated cells stopped proliferating, became spread and vacuolated, and increased expression of nonspecific esterase and the monocytic marker CD14. NO-induced monocytic differentiation of these cell line was also associated with the increased steady-state expression of mRNA for tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), but decreased expression of mRNA for the proto-oncogenes c-*myc* and c-*myb* (Magrinat *et al.*, 1992).

Despite the evidence showing that NO induces monocytic differentiation in HL-60 cells, it has been known that NO is also involved in the apoptosis of human hematopoietic cells, mouse splenocytes, macrophages, and thymocytes (Maciejewski *et al.*, 1995; Cui *et al.*, 1994; Albina *et al.*, 1993; Fehsel *et al.*, 1995). Since NO can do regulatory function in the cells at a low concentration while toxic effect at higher concentration, the biological functions of NO may be partially dependent on the concentration of NO. The results demonstrate that DNA of NO-induced HL-60 cells is cleaved into multiples of nucleosome-sized fragments in a dose- and time-dependent manner. On the other hand, the cells which did not undergo apoptosis displayed characteristics of monocytic differentiation. In addition, phorbol-ester-induced monocytic like cells revealed no characteristic patters of internucleosomal DNA cleavage. These findings suggest that NO has biphasic regulatory roles in normal and malignant hematopoietic programmed cell death and differentiation.

Materials and Methods

Reagents

Human interferon-γ (1 x 10^6 U/mg) was purchased from Genzyme (Munich, Germeny). Anti-CD14 monoclonal antibody was obtained from Becton-Dickinson (San Jose, CA). Sodium nitroprusside (SNP), potassium ferricyanide, *N*-acetylcysteine, phorbol 12-myristate 13-acetate (PMA), propidium iodide, 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), and $ZnSO_4$ were purchased from Sigma (St, Louis, MO). The 123-bp DNA ladder was purchased from Bethesda Research Laboratories (Bethesda, MD). Genomic DNA purification kit was obtained from Promega (Madison, WI). Thirty five-mm and 100-mm tissue culture dishes were purchased from Nunc (Naperville, IL). RPMI 1640, Hanks' balanced salt soultion (HBSS), phosphate buffered saline (PBS), and fetal bovine serum (FBS) and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Cell cultures

The human promyelocytic leukemia HL-60 cell line was maintained at 5 x 10^5 cells/ml in RPMI1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM), and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were seeded at 1 x 10^6 per 35-mm culture dish. On the following day, exponentially growing cells were exposed to various agents indicated in the results section.

To obtain PMA-induced adherent cells, exponentially growing HL-60 cells were treated with 100 nM PMA. After 48 h incubation, non-adherent cells were washed twice with warm HBSS and further incubated with SNP as indicated in the result section.

Cell viability and morphological assessment

Cell viability was determined by a trypan blue exclusion test. Cells which permitted trypan blue uptake, interpreted as non-viable (necrosis), were expressed as a percentage of the total cell number. Cells after agents treatment were cytospinned, fixed in methanol, and stained with Wright-Giemsa for the determination of morphological and quantitative analysis of apoptosis.

DNA extraction and electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described (Maciejewski *et al.*, 1995). Briefly, genomic DNA was purified by Wizard Genomic DNA purification Kit (Promega). After ethanol precipitation, samples of 5 mg in each lane were subjected to electrophoresis on a 1.4% agarose at 50 V for 3 h. DNA was stained with ethidium bromide.

Characterization of cell morphology and CD14 expression

HL-60 cells (5 x 10^5 /ml) were treated with 0.5 mM SNP indicated in the results section for 3 days. Morphological changes were observed under a reverse microscope and the cells were photographed under an Olympus microscope. Cell size and granulity were evaluated using a FACStar (Becton Dickinson, Rutherford, NJ). The optical specifications: Argon-ion laser, 488 nm emission wavelength, and 200 mW operating power. Analysis was performed from 10,000 cells per tube. Data acquired in the dual mode and processed using consort 30 software with Hewlett-Packard computer. CD14 expression was determined by flow cytometric analysis of phycoerythrin-conjugated anti-CD14 monoclonal antibody (Becton-Dickinson).

Measurement of hydrogen peroxide

Hydrogen peroxide generation was measured by the method of chemiluminescence assay as described previously (Porter *et al.*, 1992). HL-60 cells (5 x10^5 /ml)

were washed in HBSS and resuspended in 1 ml prewarmed veronal buffered saline (VBS) containing 300 mM luminol. Chemiluminescence was monitored in a luminescence analyzer (Biolumat LB9505, Berthold, Germany). Six samples were assayed simultaneously and maintained at 37°C : readings were taken at 1 min intervals. Cells were monitored for 10 min prior to addition of stimulus and then for a further 1 h.

Flow cytometric analysis

Flow cytometric analysis was also performed to identify apoptotic cells as described (Krishan, 1975). Approx. 10^6 cells per experimental condition were harvested, washed with HBSS and sequentially resuspended in 0.1 % Nonidet P-40 and 50 μg/ml propidium iodide solution, final concentrations. The content of DNA per cell was estimated by flow cytometry.

Results

The treatment of human HL-60 promyeloid leukemia cells with NO is associated with the induction of monocytic differentiation (Boss, 1989; Magrinat *et al.*, 1992). However, it was also reported that NO is involved in the apoptosis of human hematopoietic cells, mouse splenocytes, macrophages, and thymocytes (Albina *et al.*, 1993; Cui *et al.*, 1994; Fehsel *et al.*, 1995; Maciejewski *et al.*, 1995). To determine whether NO has a capacity to induce DNA fragmentation in HL-60 cells, exponentially growing cells were exposed to various concentrations of SNP, a NO generating agent, and, at various intervals, morphological change patterns for apoptotic characteristics were analysed. Exposure of exponentially growing HL-60 cells to various concentrations of SNP (0.5-2 mM) resulted in morphological changes, including cell shrinkage and blebbing, condensation of nuclear chromatin, and nuclear fragmentation (Figure 1). However, there was no detectable morphological changes in untreated cells. Morphological changes were present at low but

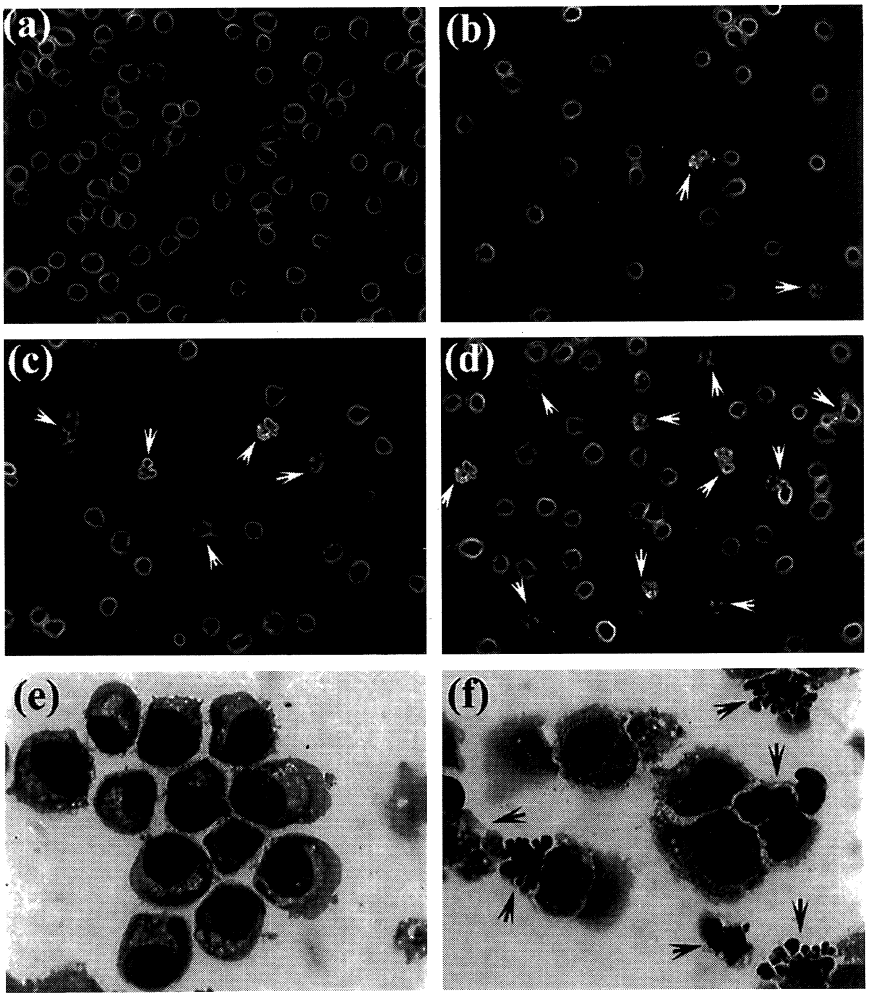
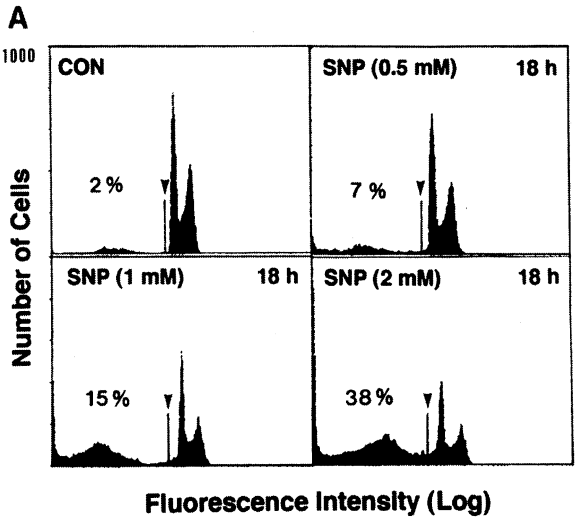


Figure 1. Morphological examination of SNP-treated cells. HL-60 cells (5 x 10^5 cells/dish) were incubated for 18 h with medium alone (a and e) or medium containing various concentrations of SNP (0.5-2 mM) (b, c, d, and f). Then, the cells were photographed by microscope (a-d: x400), or cytospinned, fixed in methanol, stained with Wright-Giemsa for the determination of morphological and quantitative analysis of apoptosis, and photographed by microscope (e and f: x1000). Note the apoptotic cells with highly condensed chromatin and apoptotic bodies (arrows).

detectable levels in cells treated for 6 h with 2 mM SNP (data not shown); this pattern was more apparent with 2 mM SNP at 18 h. This pattern of morphological changes (blebbing and shrinkage) was in concert with flow cytometric analysis by propidium iodide staining (Figure 2 A). Apoptosis of HL-60 cells was also recognized by the appearance of DNA fragments equivalent to approx. 200 base pairs and multiples thereof. A ladder pattern of the DNA fragmentation, as detected by electropho-resis, was not observed under basal conditions. However, such fragmentations were appeared as early as 6 h after cells were exposed to 2 mM SNP and was time-dependent (Figure 2B). Figure 2C shows a dose response pattern of cells treated with SNP for 18 h. The apoptosis-inducing effect of SNP was dose-dependent in the range of 0.5 - 2 mM. However, higher concentrations of SNP (> 3 mM) caused necrosis and less typical apoptotic change (data not shown) and corrsponded with the decrease of cell viability (Figure 3B). Figure 3 illustrates the time-course of growth and viability of HL-60 cells treated with various concentrations of SNP (0.5-3 mM). Compare to untreated cells, treatment of HL-60 cells with SNP caused a progressive inhibition of growth in a concentration-dependent fashion. Time dependent decrease in cell growth was clearly distinct at 12 h. Cell viability was the same in the control and SNP-treated groups up to 6 h, while decreased gradually to about 5-15 % by 24 h in the 2-3 mM SNP-treated cells as determined by trypan blue exclusion.

To verify the involvement of NO on SNP-induced apoptosis, we evaluated the effect of *N*-acetylcysteine, a free radical-scavenging thiol compound (Chang *et al.*, 1992) and potassium ferricyanide, which is structually similar to SNP except for the absence of a nitroso group. As can be seen in a representative experiment (Figure 4), SNP-induced internucleosomal DNA cleavage was greatly inhibited by *N*-acetylcysteine, while potassium ferricyanide alone had no evidence of fragmentation after exposure for 18 h. The effect of SNP was also protected by addition $ZnSO_4$ (0.5 mM).

We next asked whether the cells which did not undergo apoptosis can display characteristics of monocytic differentiation. Thus, HL-60 cells were treated for 72 h with 0.5 mM SNP and analyzed the spreading capacity, vacuolization, cell size, CD14 expression, and PMA-induced peroxide production. As shown in Figure 5, cells exposed to SNP showed increased size, vacuolization, and polarization as determined by flow cytometry (but not adherence). Similar morphologic changes have been reported after treatment with agents (such as PMA or 1,25 dihydroxy-



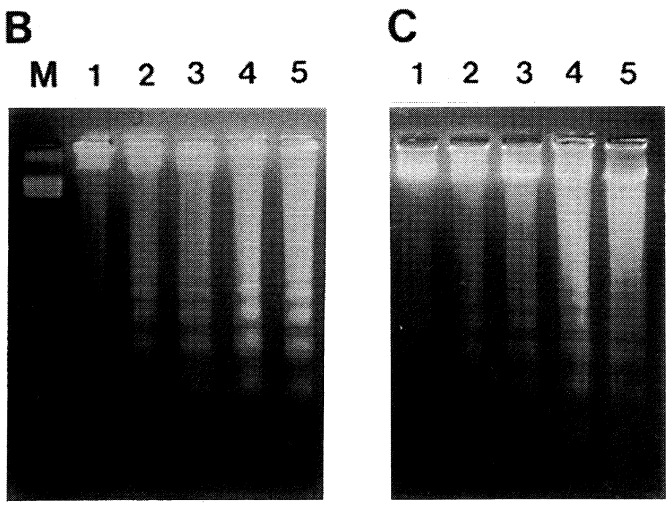


Figure 2. Analysis of endonucleosomal DNA cleavage by flow cytometry and agarose gel electrophoresis from HL-60 cells. **(A)** HL-60 cells were treated for 18 h with various concentrations of SNP (0.5-2 mM). Then, the cells were harvested, stained with propidium iodide solution, and their DNA content was analyzed by flow cytometer as described in Materials and Methods. The experiment was repeated four times and representative histograms are presented. **(B)** Time-dependent effects of SNP on DNA fragmentation. HL-60 cells were incubated for various times with 2 mM SNP. Then, genomic DNA was purified and subjected for electrophoresis. Lane M, 123-bp DNA ladder; 1, untreated control cells; 2, 6 h; 3, 12 h; 4, 18 h; 5, 24 h. **(C)** Dose-dependent effects of SNP on DNA fragmentation. HL-60 cells were incubated for 18 h with various concentrations of SNP. Then, genomic DNA was purified and subjected for electrophoresis. Lane 1, untreated control cells; 2, 0.5 mM SNP; 3, 1 mM SNP; 4, 2 mM SNP; 5, 3 mM SNP.

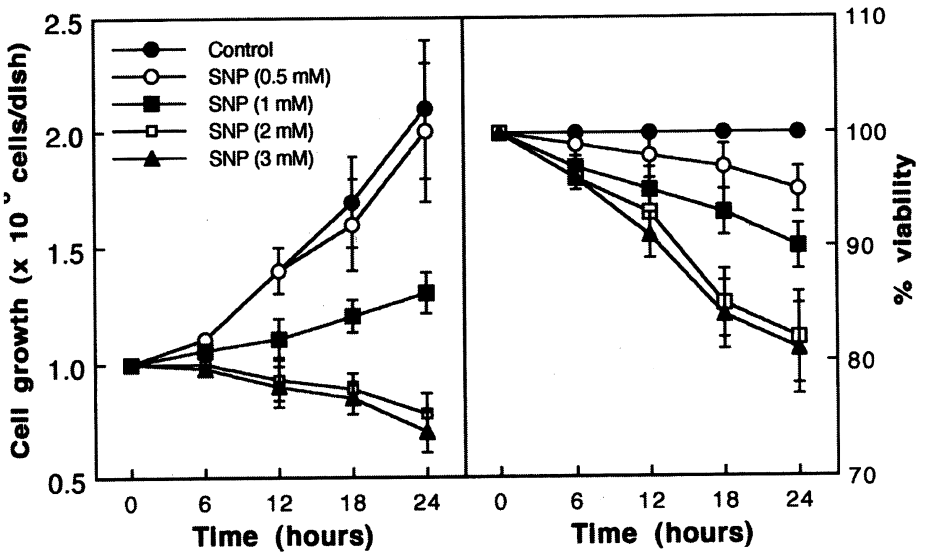


Figure 3. Dose-dependent effects of SNP on the growth and viability of HL-60 cells. HL-60 cells (1 x 10^6 cells/dish) were cultured for the indicated times in the absence (●) or presence of 0.5 mM (○), 1 mM (■), 2 mM (□), and 3 mM (▲) SNP. Growth **(A)** and viability **(B)** were determined as described under Materials and Methods. Data represent mean ± SE of three independent experiments.

Figure 4. Effects of $ZnSO_4$, N-acetylcysteine, or potassium ferricyanide on DNA fragmentation. HL-60 cells (1 x 10^7 cells/dish) were incubated for 18 h with SNP (2 mM) + $ZnSO_4$ (0.5 mM), SNP + *N*-acetylcysteine (5 mM), or potassium ferricyanide (2 mM). Then, genomic DNA was purified and subjected for electrophoresis. Lane M, 123-bp DNA ladder; 2, untreated control cells; 3, SNP; 4, SNP + $ZnSO_4$; 5, SNP + *N*-acetylcysteine; 6, potassium ferricyanide.

vitamin D_3) known to induce monocytic differentiation in these cells (Weinberg, 1981). Functionally, as neutrophilic and monocytic cells differentiate, they acquire an increased capacity to generate hydrogen peroxide. HL-60 cells treated with SNP showed enhanced PMA-stimulated peroxide production (Table 1), indicating that functional as well as morphologic differentiation had taken place. Moreover, as shown in Table 1, cells treated with SNP had large increases in the expression of CD14 monocytic marker (the endotoxin/endotoxin binding-protein receptor).

Although the cells which did not undergo apoptosis display characteristics of monocytic differentiation, it does not indicate whether maturation of the cells to monocyte is more resistant to NO than that of undifferentiated or transformed cells. To address this question, we examined the effect of SNP to induce apoptosis in PMA-induced monocytic cells. HL-60 cells were treated for 48 h with PMA and then washed twice with warm HBSS. Then we performed the treatment under identical conditions to PMA-induced adherent cells. No characteristic apoptotic morphology or cytometric histogram were observed after 18 h of SNP treatment (up to 2 mM) (Figure 6). In addition, under the same conditions, SNP was also ineffective at inducing apoptosis in normal peripheral blood mononuclear cells (data not shown). This result suggests that preferential survival of PMA-induced differentiated cells among NO-treated HL-60 cells may be related to their lower sensitivity to NO toxicity.

Discussion

Apoptosis is a highly regulated process of cell death and appears to be an essential and critical mechanism used by biological organisms to maintain homeostasis. The loss of the ability by the cell to undergo spontaneous apoptosis rather than increasing cell proliferation rates has been suggested to be one of the mechanisms involved in some tumor malignancies

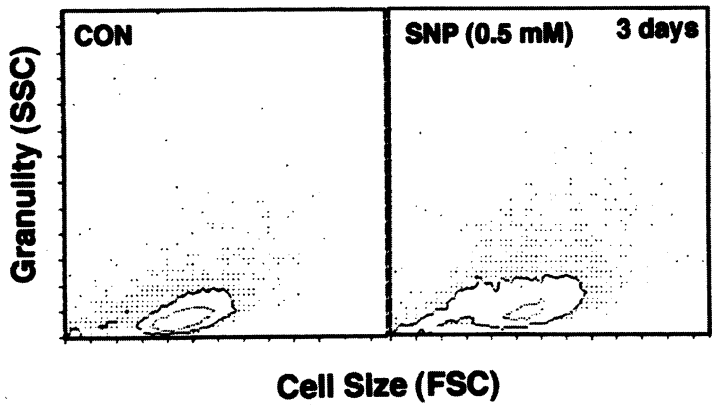


Figure 5. Increment of cell size and granulity in SNP-treated HL-60 cells. HL-60 cells (5×10^5 cells/dish) were incubated for 72 h with medium alone or medium containing 0.5 mM SNP (addition of days 0 and 1). Then, the cell size and granulity were analysed by Flow cytometer. Contour graphs represent one of the five similar experiments.

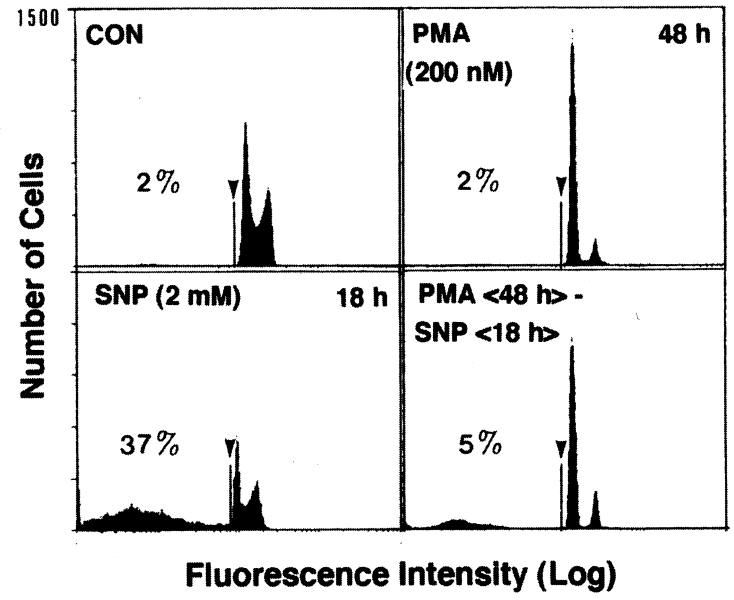


Figure 6. Effect of SNP to induce apoptosis in PMA-induced monocytic cells. HL-60 cells were pretreated for 48 h with PMA and the cells were washed twice with warm HBSS. Then, PMA-induced adherent cells were treated with 2 mM SNP. After 18 h, the cells were harvested, stained with propidium iodide soultion, and their DNA content was analyzed by flow cytometer. The position of the apoptotic peak is indicated as arrows. The experiment was repeated four times and representative histograms are presented.

Table 1. Induction of differentiation by NO in HL-60 cells. HL-60 cells were cultured with or without interferon-γ (IFN-γ, 100 U/ml) on day 0 or SNP (0.5 mM) on day 0 or day 0 and 1. Cells were harvested on day 3, and CD14 expression was determined by flow cytometric analysis of PE-conjugated Leu-M3 (anti-CD14) antibody. The capacity of hydrogen peroxide generation was measured after stimulation with 100 nM PMA by chemiluminometer. Results presented (mean ± SD) were calculated from three duplicated experiments.

Conditions	CD14 (% cells)	Peroxide (x 10^6) (photons/60 min)
Control	5 ± 1.6	8.7 ± 1.9
IFN-γ (0 day)	69 ± 4.9	27.8 ± 4.4
SNP (0 day)	38 ± 3.6	11.9 ± 2.7
SNP (0, 1 day)	56 ± 4.3	23.3 ± 3.1

(Kerr and Lamb, 1984; Allan *et al.,* 1992). The present findings demonstrate that NO induces apoptosis in human myeloid leukemia cell line HL-60. NO inhibited the growth of HL-60 cells, and induced morphological changes, including condensation of nuclear chromatin, degradation of nuclear DNA in these cells into integer multiples of approximately 200 base pairs, and the apoptotic peak of propidium iodide-stained nuclei in flow cytometric analysis. In the present work, the kinetics of DNA clevage were dependent on the concentration of NO.

Although NO had the capacity to induce oligonucleosomal DNA cleavage in HL-60 cells, the cells which did not undergo apoptosis displayed characteristics of monocytic differentiation, including spreading,

vacuolization, expression of monocyte marker CD14, and increased capacity to produce hydrogen peroxide. These results indicate that NO may play biphasic roles in controlling the fates of human hematopoietic cells between cell death and differentiation. In addition, our results further suggest that the concentration of NO derived from several sources is crucial in controlling the induction of either apoptosis or differentiation of HL-60 cells, because internucleosomal DNA cleavage highly depends on the concentration of NO used in our experiments.

NO-induced growth inhibition, morphological changes (blebbing and shrinkage), and DNA fragmentation are evidences of apoptosis in HL-60 cells, but do not indicate whether maturation of the cells to monocyte has the capacity to resist NO toxicity. Previous reports domonstrate that the prominent effect of NO is a cytotoxicity on tumor target cells and microorganisms, but do not on normal cells (termed as terminally differentiated cells such as macrophages and neutrophils) (Fehsel *et al.,* 1995; Maciejewski *et al.,* 1995). Thus, we assessed further the effect of NO in phorbol ester-induced differentiating HL-60 cells. We found that phorbol-ester-induced monocyte-like cells revealed no characteristic patterns of internucleosomal DNA cleavage by NO. Moreover, SNP was also ineffective at inducing apoptosis in normal peripheral blood mononuclear cells (data not shown). This result suggests that a more efficient defense mechanism to NO toxicity may exist in the differentiating cells.

In conclusion, our results indicate that NO may play biphasic roles in controlling human hematopoietic cell death and differentiation. Hematopoietic cell death, growth, and differentiation depend on a complex interplay between progenitor cells and other cells present in the bone marrow microenvironment (e.g., endothelial cells, macrophages, fibroblasts, and fat cells). Although many soluble factors secreted by these cells are involved in normal and malignant cell fates, the concentration of NO derived from several sources and the extent of cellular differentiation might play key roles in normal and malignant hematopoietic cell death and differentiation. Although the precise mechanisms of apoptosis in HL-60 cells are not fully understood, the results presented in this study might have implications not only for the study of hematopoietic programmed cell death and differentiation, but also for the understanding of the possible complications of the NOS induction and of the clinical use of NO donors or antagonists.

Acknowledgement

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