Nitric oxide and caspase 3 mediated cytokine induced apoptosis in acute leukemia

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Summary

*Background:* Leukemia is characterized by the uncontrolled accumulation of white blood cells. Recently, cytokines have been used in immunotherapy, which is a new strategy for leukemia treatment.

*Objective:* To investigate the effect of cytokines on induction of apoptosis in acute leukemic cell lines; HL-60, MV4-11, K-562 and Molt-4 and in addition, to study the involvement of nitric oxide (NO) in apoptotic pathways.

*Methods:* Leukemic cell lines were incubated with cytokines; interleukin-1β, tumor necrosis factor-α, and interferon-γ in various concentrations and for variable periods of time. The percent apoptosis and caspase 3 activation were examined by flow cytometry. Moreover, NO production and inducible nitric oxide synthase (iNOS) mRNA were measured by using Griess method and Real-time PCR, respectively.

*Results:* Cytokines caused a time and dose-dependent induction of apoptosis in leukemic cell lines. The highest cell apoptosis was found in K-562 treated with 40 U/ml interferon-γ for 48 hours; this correlated with the result of cell growth inhibition and caspase 3 activation. NO and iNOS mRNA were increased in cytokines treated cells. Moreover, apoptosis was reduced by SMT, an iNOS inhibitor, which confirms the possible involvement of NO in the apoptotic pathway.

*Conclusion:* Cytokines especially interferon-γ induced apoptosis in acute leukemia via NO and caspase 3 pathway. *(Asian Pac J Allergy Immunol 2011;29:102-11)*

*Key words:* Leukemia, apoptosis, cytokine, nitric oxide, caspase

Introduction

Leukemia is a cancer of the white blood cells. Abnormalities in the bone marrow cells cause either the overproduction or underproduction of certain blood cells. Types of leukemia are named after the specific blood cell that becomes cancerous, such as the lymphoid cells or the myeloid cells. The most common four types of leukemia are acute lymphocytic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, and chronic myelogenous leukemia.¹ An estimated 71 new leukemia cases occurred in the Thailand in 2004. Myeloid leukemia has a higher incidence than lymphoid leukemia and acute leukemia is significantly more common than chronic leukemia. The incidence of AML increases with age, and older patients typically have worse treatment outcomes than do younger patients.²

Currently, the preferred treatment of AML involves aggressive induction and consolidation chemotherapy. Chemotherapy is successful at inducing remission of acute myeloid leukemia (AML), but the disease has a high probability of relapse. Strategies to prevent relapse involve consolidation chemotherapy, stem cell transplantation and immunotherapy.³

Immunotherapy is one of the strategies in therapeutic development.⁴ Cytokines which are able to control cell proliferation, differentiation, survival, and apoptosis, have potential as immunotherapeutic agents. Many cytokines such as interferon-alpha have demonstrated the ability to control cancer.⁵ In addition, interleukin-2 (IL-2) and Interferons (IFNs), can be used as an immunotherapeutic in combination with chemotherapy.⁶,⁷ Moreover, the results of an...
increasing number of investigations and greater knowledge about effect of cytokines have increased the opportunities to develop for refined treatment strategies. There are reports of IFN-γ inducing apoptosis by activation of death receptor CD95 in breast tumor cells and in the human myeloid leukemic cell line.\textsuperscript{8} TNF/IFNα combined treatment inhibited the \textit{in vitro} and \textit{in vivo} proliferation of human colon adenocarcinoma cells.\textsuperscript{9} In addition, there is recent evidence that shows that the effect of cytokines on apoptosis may be mediated by the production of nitric oxide as a result of the induction of nitric oxide synthase (iNOS).\textsuperscript{10,11} For instance, IFN-γ and TNF dramatically induced the expression of inducible nitric oxide synthase (iNOS) and apoptosis of lymphoma cells by bone marrow stromal cells in culture.\textsuperscript{12} Interleukin-1β (IL-1β) induces expression of the inducible nitric-oxide synthase (iNOS) with concomitant release of nitric oxide (NO) from cardiac fibroblast cells\textsuperscript{13} and cultured vascular smooth muscle cells from rat.\textsuperscript{14} Therefore, an understanding of the effect and mechanism of cytokine induced apoptosis in leukemia may provide new knowledge which could be applied to therapeutic treatment of leukemic patients.

\textbf{Methods}

\textbf{Leukemic cell lines}

A K-562 leukemic cell line (Human Erythroleukemia leukemia; AML-M6) was obtained from Prof. Dr. Watchara Kasinrerk, Faculty of Associated Medical Sciences, Chiangmai University. HL-60 leukemic cell line (Human acute promyelogenous leukemia; APL), MV4-11 (Human acute monocytic leukemia; AML-M5), and Molt-4 (Human acute lymphocytic leukemia; ALL) were purchased from Cell Lines Services.

\textbf{Leukemic cell culture}

HL-60, K-562 and Molt-4 cells were cultured in RPMI-1640 medium and MV4-11 was cultured in Iscove Modified Dulbecco’s Medium (IMDM) supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin in humidified incubators at 37°C, 5% CO\textsubscript{2} \cite{11}. Cells were treated with IL-1β, TNF-α and IFN-γ (Chemical international) in triplicate to obtain a final concentration of 4, 40 and 400 U/ml, then incubated for 24 and 48 hours. In addition, normal myeloid progenitor cell was used as a normal cell control. Briefly, CD34 positive cells were enriched from mononuclear cells, which were collected from from the peripheral blood of healthy subjects and cultured, following an established protocol.\textsuperscript{15}

\textbf{Cell growth inhibition assay}

The percentage of cell growth inhibition following culture for 24 and 48 hours was determined by the MTT assay kit, based on the enzymatic reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to form formazan crystals within mitochondria. Untreated cells were used as controls. A 10 μl aliquot of MTT labeling reagent was added to 100 μl of cell suspension in each well, and incubated for 4 hours. 100 μl of solubilization solution was added and mixed well. The absorbance was then measured with an ELISA plate reader at the wavelength of 570 nm. The percent cell growth inhibition was calculated.\textsuperscript{16}

\textbf{Detection of apoptosis}

The percentage of cell apoptosis was determined by staining with Annexin-V-FITC and Propidium iodide (PI) (BD Biosciences). The 1x10\textsuperscript{5} cells were washed with PBS and centrifuged at 12,000 rpm for 5 min to get the pellets. After that, the pellets were added to 1X binding buffer and stained with Annexin-V-FITC and Propidium iodide (PI), then left at room temperature for 15 min in the dark and analysed by flow cytometry. In addition, cells were stained with Wright-Giemsa to observe the apoptotic features under light microscopy. The percentage of apoptosis in the results section is shown as percentage apoptosis of cytokine treated cell subtract and the percentage of apoptosis of untreated cell.

\textbf{Caspase-3 activation determination}

The caspase-3 activation was analysed by staining with 1 μl of FITC specific for caspase-3 (Calbiochem) and incubated for 1 hr in a 37°C incubator with 5% CO\textsubscript{2}. Cells were centrifuged at 3000 rpm for 5 min and the supernatant was removed. Then, the cell pellet was washed twice and re-suspended in 300 μl of washing buffer and analysed by flow cytometry. The percentage of FITC intensity was measured and calculated for caspases-3 activation. The untreated cell at 24 hrs acted as controls. The results for controls were considered to be equal to a one fold increase.
Quantification of NO production
To determine the NO concentrations, the cells were centrifuged at 12,000 rpm for 5 min and the supernatant was used. Assessment of NO production was performed by using the Griess reagent that measures nitrite (NO$_2^-$), which is the major NO metabolite in the cell culture, 100 μl of Griess reagent was added to 100 μl of the sample in 96 well plates. After incubation at room temperature for 15 min, samples were measured with an ELISA plate reader at the wavelength of 570 nm. The amount of nitrite was calculated from a standard curve using NaNO$_2$ at concentrations of 0.1-100 μM.

Real-time RT-PCR for iNOS mRNA expression
Real-time RT-PCR was used to determine iNOS mRNA expression. The iNOS primers used for the reaction were (5’→3’): sense GCT GTA TTT CCT TAC GAG GCG AAG AA and anti-sense CTT GTT AGG AGG TCA AGT AAA GGGC. The primer for the β2-microglobulin gene was used as the internal control using the following primer sequences (5’→3’): sense CAT CCA GCG TAC TCC AAA GA and anti-sense GAC AAG TCT GAA TGC TCC AC. These primers were synthesized by Invitrogen Ltd. The leukemic cells were harvested and total RNA was extracted using the TRIzol reagent according to the manufacturer’s protocol (Invitrogen). The synthesis of cDNA was according to the manufacturer’s instructions with the reverse transcriptase kit (Invitrogen). The real-time PCR was processed with a total volume of 50 µl containing 25 µl SYBR Green permix, 1 µl sense primers, 1 µl anti-sense primers, and 2 µl cDNA templates. The protocol for real-time PCR consisted of 35 cycles and the cycling parameters were as follows: denaturation at 94 °C for 50 seconds, annealing at 60 °C for 50 seconds and elongation at 72 °C for 10 seconds. A real-time PCR instrument was used (iCycleriQ® Multicolor Real-time PCR Detection system; Bio-Rad, USA). The measurements for each sample were performed in triplicate. iNOS expression (fold increase) was calculated by the iCycleriQ® software. The level of iNOS mRNA were normalized relative to β2-microglobulin mRNA.

Inhibitory effect of iNOS inhibitor
To confirm that NO mediates cytokine induced apoptosis in leukemic cells, a highly selective iNOS inhibitor, S-Methylisothiourea Sulfate (SMT) was used. Cells treated with cytokine were incubated with and without 1 ng/ml of SMT (iNOS inhibitor) for 24 and 48 hrs at 37°C in a
humidified atmosphere of 5%CO$_2$, and then investigated for NO production, iNOS mRNA expression, cell apoptosis and caspase 3 activation.

**Statistical analysis**

The experiments were performed in triplicate to compare between the control (untreated cell) and the cytokine treated cells and the results were expressed as mean ± S.D. The statistical analysis was performed by t-test. Difference is considered statistically significant at $p$-value < 0.05.

**Results**

**Effect of cytokines on cell growth inhibition of leukemic cell lines**

We evaluated the effect of cytokines on cell growth inhibition of leukemic cell lines. Leukemic cell lines were treated with various concentrations (4, 40 and 400 U/ml) of IL-1β, TNF-α and IFN-γ. The change in growth inhibition was measured by MTT assay after treatment for 24 and 48 hrs. Treatment of leukemic cells with cytokine, IL-1β, TNF-α, and IFN-γ showed a higher percentage of cell growth inhibition than untreated cells. Interestingly, the highest percentage of cell growth inhibition of HL-60, MV4-11, K-562 and Molt-4 was found in cells treated with 40 U/ml of IFN-γ at 48 hrs (Figure 1).

**Effect of cytokines on cell apoptosis of leukemic cell lines**

We examined key features of apoptosis, including PS externalization and morphological features to determine whether the increase in cell growth inhibition induced by cytokines was due to apoptotic cell death. As an early marker of apoptosis, PS externalization was detected by AnnexinV and PI staining. From the results for cell apoptosis, we found that 40 U/ml of IFN-γ at 48 hrs produced significantly increased apoptosis in leukemic cell lines, K-562, HL-60, MV 4-11 and Molt-4. In addition, 400 U/ml of TNF-α at 48 hrs induced significantly increased apoptosis of K-562 and MV4-11 but 400 U/ml IL-1β induced apoptosis only in the K-562 cell line. To investigate the synergistic effect of these cytokines, suitable concentrations of each cytokine were used to examine the effect of a combination of cytokines on cell apoptosis. The combination of IL-1β, TNF-α and IFN-γ had less effect on cell apoptosis than a single cytokine treated cell (Figure 2). Moreover, morphological changes of apoptotic features, including reduction in the volume and nuclear chromatin condensation of HL60, MV4-11, K562 and Molt4 cells, were found after treatment with 40 U/ml of IFN-γ for 48hr. The results indicated that cytokine treatment, especially IFN-γ, increased the percentage

**Figure 2.** Percentage of cell apoptosis of HL-60 (a), MV4-11 (b), K-562 (c), Molt4 (d) cell line. Cells were treated with various concentrations of IL-1β, TNF-α and IFN-γ for 24 and 48 hrs. AnnexinV-FITC intensity was analyzed by flow cytometry and calculated for the percentage of cell apoptosis. *, $p < 0.05$ compared with control group, Mix = 400 U/ml of IL-1β, 400 U/ml of TNF-α and 40 U/ml of IFN-γ.
of cell apoptosis in leukemic cells. However, the cytokines could not induce apoptosis in normal cells (data not shown).

**Effect of cytokines on caspase-3 activation**

To investigate the apoptotic signaling pathway involved in caspase-3 activation after cytokine treatment, caspase-3 activation was determined using specific recognition sequence for caspase-3 and determined by flow cytometry. Increased caspase-3 activation was observed after treatment of leukemic cells with IL-1β, TNF-α and IFN-γ. 40 U/ml of IFN-γ treated K-562 at 48 hrs showed the highest caspase-3 activation. These results suggest that cytokines could induce caspase-3 activation in leukemic cells (Figure 3). IL-1β could activate caspase 3 only in HL-60 cells but the caspase 3 activation showed not correlate with percentage of apoptosis in IL-1β treated cells.

**Effect of cytokines induced iNOS mRNA expression and NO production in leukemic cell lines**

We next examined whether NO production by iNOS was involved in leukemic cell apoptosis after cytokines treatment. The expression of iNOS mRNA of cytokine treated cells was examined by real-time PCR. The results showed that 40 U/ml of IFN-γ treated cells for 48 hrs could induce iNOS expression in leukemic cells (Figure 4). In addition, accumulation of nitrite, the end product of nitric oxide metabolism, in the culture medium was also quantified using Griess method. Results were compared against a NaNO₂ standard curve and nitric oxide concentrations were calculated. The levels of nitrite induced were statistically significant after treatment of HL-60, MV4-11 and K-562 with 40 U/ml of IFNγ for 48 hrs. However, the highest level of nitrite was found in HL-60 cells treated with 40 U/ml of IFNγ (Figure 5). The data shows that the effect of cytokine on induction of iNOS expression and subsequent nitric oxide production correlates with the apoptosis results.

**Effect of iNOS inhibitor on cytokines induced leukemic cell apoptosis and caspase 3 activation**

Since nitric oxide production by iNOS was sufficient to induce leukemic cell apoptosis and it might occur upstream of caspase activation, we examined whether nitric oxide production is a requisite step for caspase activation and apoptosis. Leukemic cells were treated with IL-1β, TNF-α and IFN-γ in the presence and absence of SMT, a specific iNOS inhibitor, after which iNOS expression, NO production, caspase-3 activation and apoptosis were examined. We found that treatment with SMT, the iNOS inhibitor, was able to block cytokine-induced iNOS expression and nitric oxide production. Treatment with SMT also

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**Figure 3.** Percentage of caspase-3 in HL-60 (a), MV4-11 (b), K-562 (c), Molt4 (d) cell line. Cells were treated with IL-1β, TNF-α and IFN-γ for 24 and 48 hrs. Caspase3 labeled FITC, as the fluorescent marker, was stained and measured by flow cytometry. * , p <0.05 compared with control group.
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Figure 4. iNOS mRNA expression of HL-60 (a), MV4-11 (b), K-562 (c), Molt4 (d) cell line. Cells were treated with IL-1β, TNF-α and IFN-γ for 24 and 48 hrs. iNOS mRNA expression was normalized to the beta-2-microglobulin (β2M) housekeeping gene and analyzed by Real-time PCR. *, p <0.05 compared with control group

Figure 5. Nitrite concentrations for the HL-60 (a), MV4-11 (b), K-562 (c), Molt4 (d) cell line. Cells were treated with various concentration of IL-1β, TNF-α and IFN-γ for 24 and 48 hrs. Cell culture supernatants were assayed for relative levels of nitrite (NO2-) by Griess assay. *, p <0.05 compared with control group
reduced the AnnexinV-FITC signal, indicating decreased apoptosis (Figure 6A). Interestingly, it could also prevent cytokine induced caspase-3 activation (Figure 6B). Taken together, these data suggest that cytokine-mediated nitric oxide production is necessary for activation of caspase and execution of apoptosis.

**Discussion**

Apoptosis, or programmed cell death, plays a critical role in the regulation of tissue homeostasis, especially in cell systems with a high turnover rate such as hematopoietic cells. Imbalances between proliferation and cell death may lead to impaired development, degenerative diseases and tumor formation. Also, cytotoxic therapy and immunotherapy of leukemia and lymphoma predominantly mediates cell death through induction of apoptosis. Understanding the molecular events by which apoptosis is induced by cytotoxic therapies provides a paradigm to link normal growth control, malignant transformation and the response to therapy.

There is evidence that cytokines IL-1β, TNF-α, and IFN-γ- and their signaling pathway induce apoptosis in many cells. IL-1β signaling, which is mediated through MAPK activation of JNK and p38, can also lead to induced apoptosis in pancreatic RINm5F cells. TNF-α, along with its numerous effector functions, is a potent inducer of apoptosis. One study reported that TNF-α induces apoptosis in endothelial cells via phosphorylation and down-regulation of Bcl-xl. TNF-α inhibited both *in vitro* and *in vivo* proliferation of human
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colon adenocarcinoma cells. It also suppressed erythropoiesis by inhibiting the generation of Glycophorin A positive cells derived from CD34+ cells. IFN-γ signaling is involved in activation of receptor-associated JAK and the subsequent phosphorylation of STAT-1. IFN-γ sensitizes human myeloid leukemic cells to death receptor-mediated apoptosis. Although, these cytokines have not been demonstrated to exert any direct cellular cytotoxicity, they are known to act as a sensitizing agent to other stimuli.

This study shows that cytokine could induce apoptosis in leukemic cell lines. The percentage of cell growth inhibition and cell apoptosis depended on dose and time incubation of cytokine treated cells. Moreover, this study also investigated if the apoptosis signaling pathway induced by cytokine could be involved in caspase pathway and NO.

Since NO is enzymatically synthesized from L-arginine in macrophages, the immunological function of NO revealed the induction of cytotoxicity against tumor cells and surrounding tissues. High concentrations of NO induce cell death in several cell types. These include macrophages, thymocytes, pancreatic islets, certain neurons, and tumor cells. Although the precise mechanism that determines the cellular sensitivity against NO induced apoptosis is not clearly elucidated, the pro-apoptotic effects of NO on these cells seem to be activation of the apoptotic signaling cascade (such as caspases),

**Figure 6B.** Effect of iNOS inhibitor on percentage of caspase-3 (fold increase) of HL-60, MV4-11, K-562, and Molt4 cell line. Cells were treated IL-1β, TNF-α and IFN-γ and incubated with iNOS inhibitor for 24 hrs (a-d) and 48 hrs (e-h). AnnexinV-FITC intensity was analyzed by flow cytometry and calculated for the percentage of cell apoptosis. *, p <0.05 compared with without iNOS inhibitor.
mitochondrial cytochrome c release, or regulation of cell survival and apoptotic gene expression. Furthermore, the threshold of the NO level triggering apoptosis is different from one cell to the other. NO is a multifunctional molecule, functioning as either a pro-apoptotic or an anti-apoptotic effector, depending on the levels of NO generated. There is a report of NO donor-induced cell growth arrest and apoptosis contributing to an inhibitory effect on CFU-GM formation in the HL-60 cell line model.

Expression of iNOS in response to cytokine stimulation was originally observed in the macrophage, where it is involved in NO-mediated cell damage and apoptosis. Subsequent data have shown that other cell types are capable of expressing iNOS on stimulation with cytokines. For example, TNF-α stimulates NF-κB activation of cornal endothelial cells (CECs), which is known to upregulate iNOS. IFN-γ also induces iNOS expression through activation of JAK, STAT-1 and IFNγ response factor (IRF-1) proteins.

From the results, IL-1β could activate caspase 3 only in HL-60 cells but the caspase 3 activation did not correlate with the percentage of apoptosis in IL-1β treated cells. In addition, iNOS expression was correlated with caspase 3 activity and the percentage of apoptosis only in TNF-α and IFN-γ treated acute leukemia cell lines for 48 hr but was not correlated in IL-1β treated some leukemic cells. It is possible that IL-1β could also induce NO mediated apoptosis via other caspases such as caspase-1 or a caspase-independent pathways could be involved in the process. The results of our study suggest that IL-1β is not a good candidate for induction apoptosis in leukemic cell compared with other cytokines.

The results show IFN-γ to be highly effective in the induction of iNOS expression and generation of high levels of NO production that induce apoptosis in both acute myeloid and lymphoid leukemia cell lines. The highest induction of apoptosis was found in IFN-γ treated K-562 (AML-M6) concomitant with high production levels of NO. IL-1β and TNF-α could induce apoptosis and produce NO at lower levels than IFN-γ. In addition, the level of apoptosis in ALL appeared lower than AML-M3 and AML-M6 leukemic cell line. Therefore, it is suggested that the apoptosis was cell type specific and related to NO production. Moreover, an iNOS inhibitor was used to confirm the occurrence of NO mediated cytokine induced apoptosis in this study. The results showed that NO might provide upstream signaling of cytokine-3 in the apoptotic signaling pathway induced by cytokines in leukemic cell lines.

Interestingly, cytokines could not induce apoptosis in normal cells. It may due to the susceptibility and levels of receptor of cytokine are different between leukemic and normal cells. In addition, it was found that many normal cells are not killed by TNF-α and this may be related to NF-κB trans-activation by blockade of NF-κB, which sensitizes the cell to TNF-α and augments induced apoptosis cell death. In summary, in the present study we have shown that cytokines, especially IFN-γ, induce apoptosis mediated by NO and caspase 3 pathways in acute leukemic cell lines. Therefore, cytokines could be used as immunotherapy in leukemic patients.

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