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Original Article

Interleukin-1 beta and tumor necrosis factor-alpha induced apoptosis *via* caspase in leukemic K 562 and HL-60 cell lines

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Abstract

Recently, some cytokines were used as immunotherapy for leukemic patients, which improved cancer therapy by apoptosis induction. This study investigated the effects of interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) on induction of cell apoptosis in leukemic cell lines, K562 and HL60. Both of leukemic cell lines were treated with IL-1β and TNF-α in various concentrations and incubation times. Cell viability and growth inhibition were analyzed by using trypan blue staining and MTT assay, respectively. Apoptotic cells were stained with Annexin-V-FITC and analyzed by flow cytometry. In addition, the involvement of caspase activation in the apoptotic pathway was investigated. The results indicated that percentage of cell viability was decreased while cell apoptosis increased after treatment with 2 ng/ml IL-1β or 20 ng/ml TNF-α in both leukemic cell lines. The increased sub G1 population from cell cycle analysis and cell morphology indicated increasing apoptosis in cytokines treated cells. Caspase 3 and caspase 8 activity were increased after IL-1β and TNF-α treatment. These results suggest that IL-1β and TNF-α could induce apoptosis *via* caspase cascade pathway in K562 and HL60 cell lines.

Keywords: interleukin-1 beta, tumor necrosis factor-alpha, apoptosis, caspase, leukemia

1. Introduction

Leukemia is cancer that affects the blood-forming stem cells. The abnormal blood cells grow in an uncontrolled way. They develop in bone marrow and spread into the blood circulation and other organs, such as spleen, lymph nodes, liver, and central nervous system. There are several types of leukemia, which can be grouped by how rapidly the cancer develops, acute or chronic leukemia, and by the types of white blood cell, which are affected, myelocytic and lymphocytic leukemia (Pizzo *et al.*, 2006). In Thailand, leukemia is ranked as the eighth most common cancer in males and the tenth in females (Wiangnon, 2007). Acute myeloid leukemia and adult-onset acute lymphocytic leukemia are aggressive diseases and poor prognosis (Lagadinou *et al.*, 2010). Acute leukemia is a neoplastic disease characterized by rapid accumulation of primitive hematopoietic cells. Acute leukemia is subclassified as myeloid or lymphoid depending on the origin of the malignant cell. Acute lymphocytic leukemia (ALL) is predominantly a disease of childhood with 75% of all cases occurring in patients younger than 15 years of age. In contrast, acute myeloid leukemia (AML) is more common in adults with an incidence that increases with age. Most cases of chronic myelogenous leukemia (CML) occur in adults, but children may develop the disease. CML accounts for most cases of myeloproliferative diseases and for 20% of all

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leukemias. The development of a malignant cell clone is due to the dysregulation of the balance between cell proliferation and cell apoptosis (Schulera and Szendeb, 2004).

Apoptosis or programmed cell death is a process for self-killing of cells through the activation of an intracellular pathway leading to cellular changes. This process is essential in the homeostasis of normal tissues of the body. Abnormalities in cell death control lead to a variety of diseases, including cancer, autoimmunity, and degenerative disorders. There are increasing evidences that the processes of neoplastic transformation, progression, and metastasis concern changes in apoptotic pathways (Cotter, 2009). Apoptosis can be induced by several exogenous injuries such as heat shock, cytokine defects, ionizing radiation, and drugs (Fulda *et al.*, 2010). The pathway is executed by the activated caspases. However, there are some studies in cell line system showed that a caspase-independent pathway of cell apoptosis exists when induced by a mitochondrial apoptosis-inducing factor (Zhang and Bhavnani, 2006). Some cytokines have the anti-proliferation effect and were used as immunotherapy in cancer such as interferon gamma (IFN-γ), interferon alpha (IFN-α), tumor necrosis factor alpha (TNF-α) and some interleukins (Kujawski and Talpaz, 2007). Interleukin-1 beta (IL-1β) and TNF-α are key inflammatory cytokines in many diseases (Kufe *et al.*, 2003). These cytokines are involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The objective of this study was to investigate whether cytokines, IL-1β and TNF-α, could induce apoptosis and is involved in caspase dependent pathways of leukemic cell lines, HL60 (human acute promyelogenous leukemia) and K562 (human chronic myelogenous leukemia).

2. Materials and Methods

2.1 Leukemic cell lines

K562 leukemic cell line (Human chronic myelogenous leukemia; CML) was obtained from the Faculty of Associated Medical Sciences, Chiang Mai University and HL60 leukemic cell line (Human acute promyelogenous leukemia; APL) was purchased from Cell Lines Services.

2.2 Leukemic cell culture

Cells were maintained in RPMI 1640 medium (GIBCO™, Invitrogen) supplemented with 10% fetal bovine serum along with 1% penicillin-streptomycin (GIBCO™, Invitrogen) and cultured at 37°C in a humidified atmosphere of 5% CO_2. Cells were treated with IL-1β and TNF-α (Chemical international) in triplicate to obtain the final concentration of 0.2, 2 and 20 ng/ml, and then incubated for 12, 24, and 48 hrs.

2.3 Cell viability count

Twenty microliters of cells were mixed with equal volume of 0.4% trypan blue (Sigma-Aldrich, St. Louis, U.S.A.). Cell viability count was performed using hemocytometer under a light microscope.

2.4 Detection of cell apoptosis

Cell apoptosis percentage of cytokine treated cells was determined by flow cytometry. The cells were washed twice with PBS and centrifuged at 12,000 rpm for 5 min to get the cell pellets. After that, the cell pellets were added with 1X binding buffer and stained with Anexin-V-FITC and Propidium iodine (PI), then leaved at room temperature for 15 min in dark and analyzed by flow cytometry (Miller, 2004). In addition, cells were stained with Wright-Giemsa to observe the apoptotic feature under a light microscope.

2.5 Cell cycle analysis

SubG1 population, which represents cell apoptosis was investigated in cell cycle analysis (Cycle TEST™ plus DNA Reagent kit; Beckton Dickinson). Approximately 10^6 cells were collected by centrifugation at 1,500 rpm for 5 min and washed three times with 1 ml buffer solution. The cell pellet was gently resuspended in 250 µl solution A (trypsin buffer) and kept at room temperature for 10 min, after which 200 µl solution B (trypsin inhibitor and RNase buffer) was added, and the system was kept at room temperature for another 10 min. Then, 200 µl cold solution C (Propidium iodide) was added, gently mixed, and incubated in the dark at 2–8°C for 10 min. The cell suspension was then subjected to flow cytometry analysis (Cao *et al.*, 2006).

2.6 Caspase 3, caspase 8 and caspase 9 activity determination

Treated cells ($1.0x10^6$ cells/ml) were stained with 1 µl of FITC specific for caspase 3 (Calbiochem) in separated tubes and incubated for 1 hr at 37°C with 5% CO_2. Cells were centrifuged at 3,000 rpm for 5 min and supernatant was discarded. The cells pellets were then washed twice and resuspended in 300 µl of wash buffer and analysed by flow cytometry. The percentage of FITC intensity was measured and calculated for caspases 3, caspase 8 and caspase 9 activity (Contini *et al.*, 2007).

2.7 Statistical analysis

Data were analysed by SPSS version 17.0 for window. A difference is considered statistically significant at *p-value* <0.05.

3. Results

3.1 Effect of cytokines on cell viability of leukemic cell lines

Percentage of cell viability of K562 and HL-60 cells decreased after treatment with IL-1β and TNF-α treatment. IL-1β was reduced cell viability in both leukemic cell lines. The lowest percentage of cell viability for K562 was 54.6± 3.6% after treatment with 20 ng/ml TNF-α at 48 hrs (Figure 1A, B); whereas the lowest percentage of cell viability for HL-60 was 55.2±2.8% after treatment with 20 ng/ml TNF-α at 48 hrs (Figure 2 A, B).

3.2 Effect of cytokines on apoptosis of leukemic cell lines

The percentage of apoptotic cells was analyzed by flow cytometry. In K562, the highest percentage of apoptosis was 11.1±4.3% after 20 ng/ml TNF-α treatment at 48 hrs (Figure 3A). In HL-60, the highest percentage of apoptosis was 5.5±0.4% after 2 ng/ml of IL-1β treatment at 24 hrs (Figure 3B). The combination of IL-1β and TNF-α showed less effects on cell apoptosis than single treatment of each cytokine. The morphological changes to apoptotic feature included reduction in the volume and nuclear chromatin condensation of K562 and HL-60 cells after treated with 2 ng/ml IL-1β for 24 hrs and 20 ng/ml TNF-α for 24 hrs (data not shown). The results indicated that IL-1β and TNF-α treatment increased cell apoptosis in both K562 and HL-60 cells.

3.3 Effect of cytokines on sub G1 population in leukemic cell lines

The sub G1 population was represented as hypodiploid peak, probably due to the presence of apoptotic cells with DNA content was less than 2n. The percentages of sub G1 population in K562 treated with 2 ng/ml IL-1β and 20 ng/ml TNF-α at 24 hrs were 11.4±0.4% and 17.5±0.5%, respectively (Figure 4A) and at 48 hrs were 8.5±0.4% and 11.5± 0.5%, respectively (Figure 4B). Whereas the percentages of sub G1 population in HL-60 treated with 2 ng/ml IL-1β and 20 ng/ml TNF-α at 24 hrs were 4.3±0.1% and 4.0±0.1%, respectively (Figure 5A) and at 48 hrs were 3.2±0.4% and 3.3± 0.2%, respectively (Figure 5B).

3.4 Effect of IL-1β and TNF-α on caspase activation of apoptotic signaling pathway

The caspase 3 activity in K562 was increased to 4.8± 0.8% after IL-1β treatment for 24 hrs, whereas it was increased from 3.1±0.4% to 7.3±0.1% after TNF-α treatment for 24 to 48 hrs, respectively. The caspase 3 activation in HL-60 was increased to 4.5±1.2% and 3.9±0.4% after IL-1β treatment for 24 and 48 hrs respectively, whereas it was increased to 3.5± 0.3% and 6.8±0.8% after TNF-α treatment for 24 and 48 hrs.

In addition, the caspase 8 activity was increased after IL-1β and TNF-α treatment while no statistical significantly

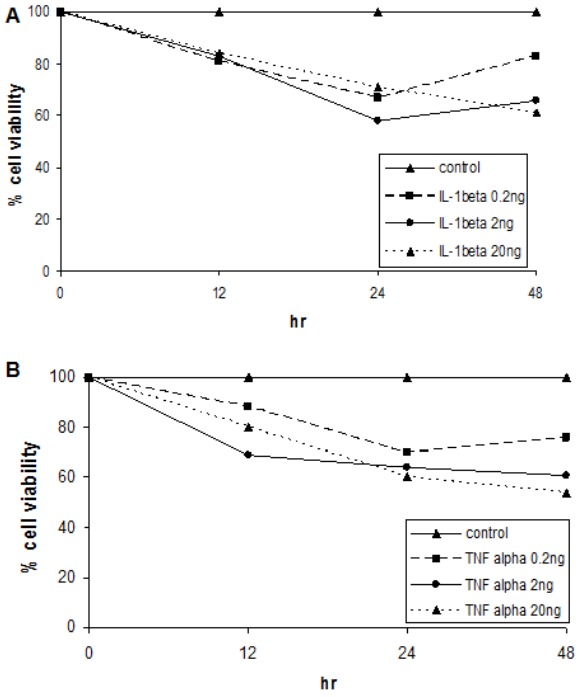

Figure 1. Percentage of cell viability of K562 cell line after IL-1β (A) and TNF-α (B) treatment with various concentrations and times. Cells were stained with trypan blue and the number of viable cells were counted and calculated in percentage of cell viability.

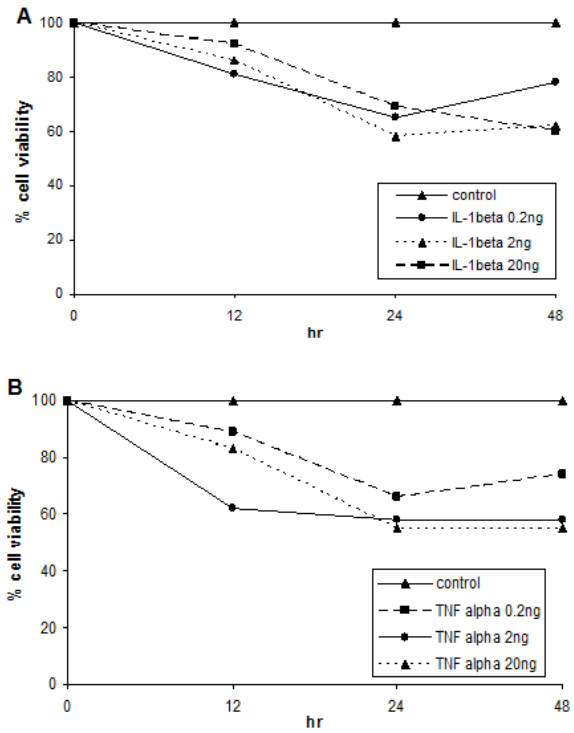

Figure 2. Percentage of cell viability of HL-60 after IL-1β (A) and TNF-α (B) treatment with different concentrations and times. Cells were stained with trypan blue and the number of viable cells were counted and calculated in percentage of cell viability.

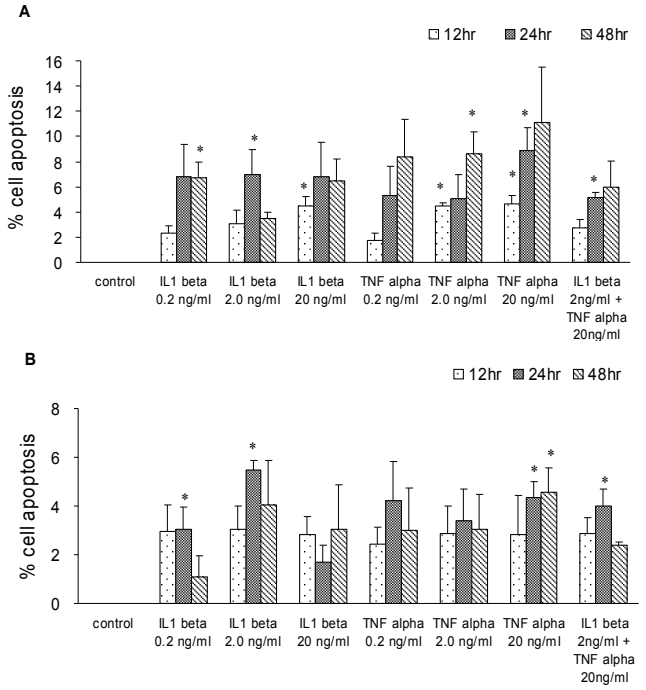

Figure 3. Percentage of cell apoptosis of K562 (A) and HL-60 (B) cell after IL-1β or TNF-α treatment with different concentrations and times. The percentage of cell apoptosis was calculated from Annexin-V-FITC intensity (*, p <0.05 compared to control group).

increase of caspase 9 activity after cytokines treatment. (Figure 6 A, B). These results indicated that IL-1β and TNF-α could induce caspase 3 and caspase 8 activation in K562 and HL-60 cells.

4. Discussion

Immunotherapy has been used for leukemic patients treatment by cell apoptotic induction. Apoptosis is mediated through three different pathways: extrinsic death receptor pathway, intrinsic or mitochondrial pathway, and stress-induced pathway (Ersvaer *et al.*, 2007). Factors recognized as apoptotic inducers include heat shock, free radicals, and some pro-inflammatory cytokines. Some report showed that reactive oxygen species-induced cell death of rat primary astrocyte through mitochondria-mediated mechanism (Wang *et al.*, 2009). In recent reports, some cytokines such as IFN-γ and IFN-α were used as immunotherapy (Smit*h et al.*, 2004). IFN-γ increased stress-induced apoptosis in AML patients and affected cell proliferation and regulation of apoptosis (Green, 2003), whereas IFN-α was widely used to treat CML patients (Kujawski and Talpaz, 2007).

In this study, IL-1β and TNF-α were chosen for studying the ability to induce cell apoptosis in human chronic myelogenous leukemia and acute promyelogenous leukemia. Although presently, there is still no clue concerning the effects of these two cytokines on apoptosis in leukemia, our

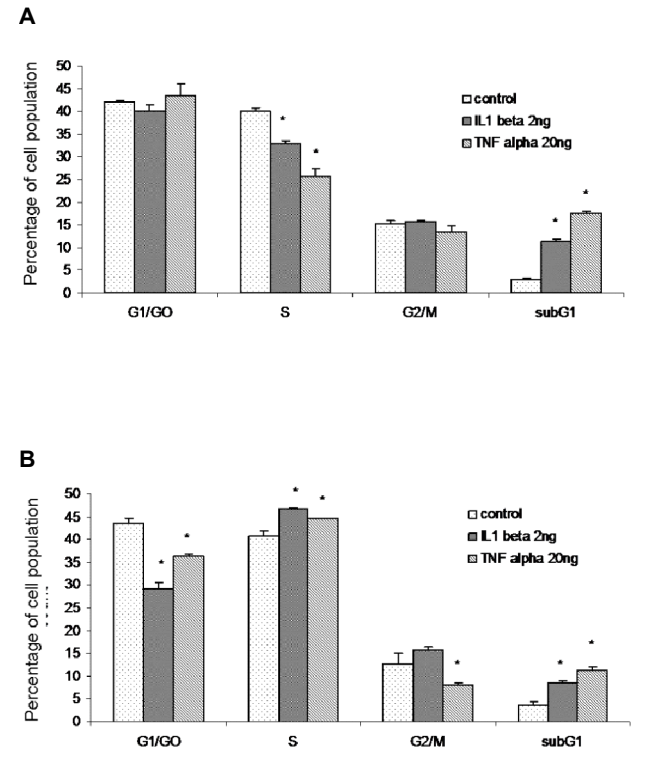

Figure 4. Percentage of cell population in cycle phase fractions of K562 cell line. Cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24 (A) and 48 hrs (B). Cell cycle phase was analyzed by flow cytometry.

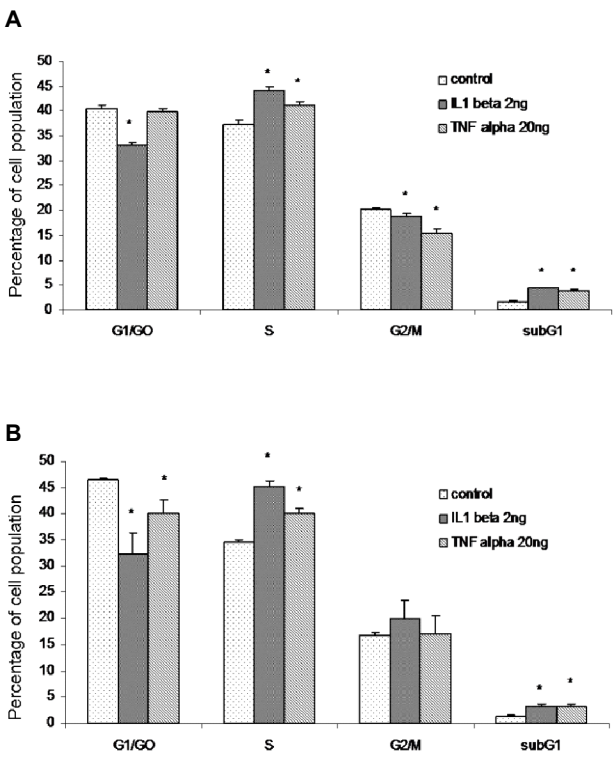

Figure 5. Percentage of cell population in cycle phase fractions of HL-60 cell line. Cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24 (A) and 48 hrs (B). Cell cycle phase was analyzed by flow cytometry.

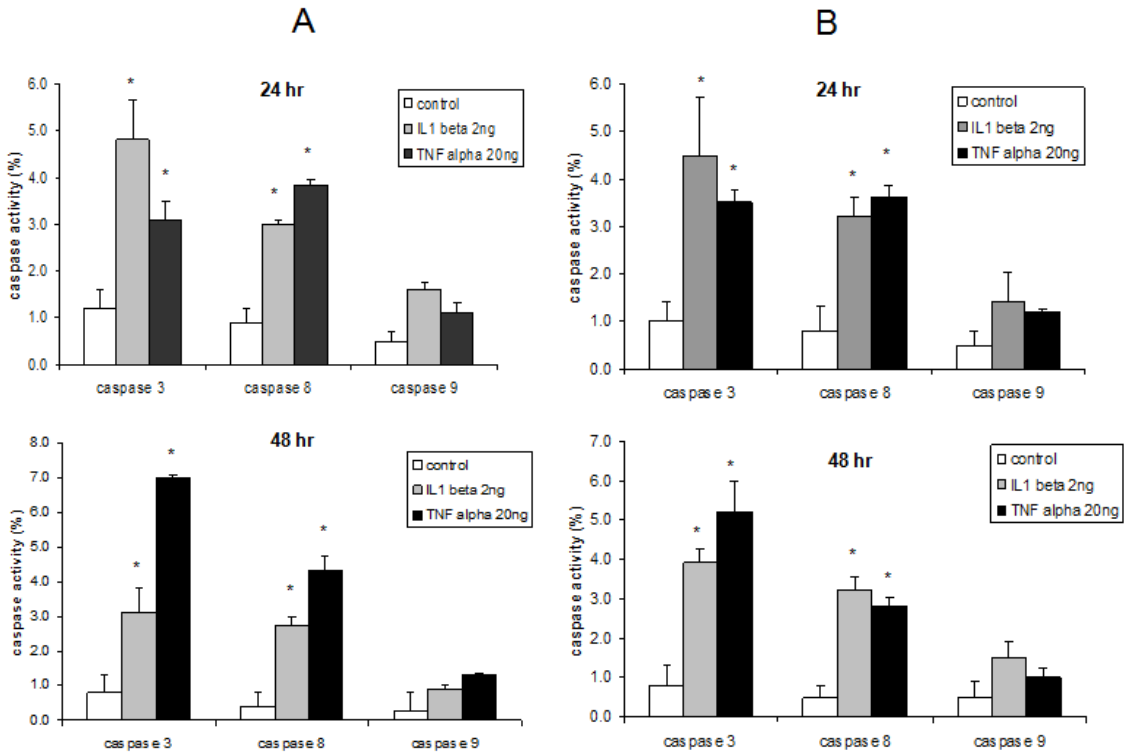

Figure 6. Percentage of FITC intensity of caspase 3, caspase 8 and caspase 9 in K562 (A) and HL-60 (B) cell lines. Cells were treated with IL-1β and TNF-α for 24 hrs and 48 hrs. FITC as the fluorescent marker was stained and measured the intensity by flow cytometry.

results show that both IL-1β and TNF-α are able to reduce cell viability and induce cell apoptosis in both leukemic cell lines. The sub G1 peak in cell cycle analysis also confirms increased apoptosis in cytokines-treated leukemic cells. Sub G1 cell analysis resulted in free nuclei, was stained with propodium iodide for relative DNA content measurement. Cells undergoing apoptosis will lose part of their DNA due to the DNA fragmentation in later apoptosis leading to increased Sub G1 fraction. Moreover, apoptotic feature in leukemic cell lines treated with two cytokines was observed under light microscope, apoptosis was not seen in normal cells.

The combination of IL-1β and TNF-α at the suitable concentrations could not enhance apoptotic effect than single cytokine. Therefore, these cytokines were not shown synergistic effect on induction of apoptosis. Recently, there is evidence showed that mitochondrial DNA damage is involved in apoptosis caused by pro-inflammatory cytokines in human osteoarthritis (OA) Chondrocyte (Kim, 2009). El Btaouri *et* al. (2006) revealed that IL-1β induced cell apoptosis through adenylyl cyclase and ERK1/2 inhibition in primary cultured of thyroid cells. It suggested that these cytokines could induce apoptosis in different signaling pathways and depend on cell types.

In addition, the caspase pathway was found to be involved in cytokine-induced cell apoptosis in both leukemic cell lines. Apoptotic pathways lead to a cascade of events that ultimately converge to the activation of caspase-3, which is an effector enzyme. Caspase-3 is a cysteine protease with aspartic specificity and it is a well-characterized effector of apoptosis. The pro-caspase-3 is sequestered as a zymogen, where upon proteolysis at a conserved DEVD sequence, is converted to the active enzyme capable of disassembling the cell. Cell death can become disregulated under various conditions and multiple disease states. Sensitive and reproducible detection of active caspase-3 is critical to advance the understanding of cellular functions and multiple pathologies of various etiologies (Fox and Aubert, 2008). In addition, caspase 8 was also shown to be involved in extrinsic apoptotic signaling pathways induced by these cytokines.

From results of this study can be concluded that IL-1β and TNF-α could induce cell apoptosis in K562 and HL60 cells *via* caspase dependent pathway. However, elucidation of the precise mechanisms and other signaling pathways should be done in further study.

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