EFFECT OF CYTOKINES ON APOPTOSIS OF LEUKEMIC CELL LINES

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ABSTRACT

Leukemia has a high incidence rate in children and also occurs in adults. Recently, some cytokines were used as immunotherapy for leukemic patients, which improved cancer therapy by induction of apoptosis. This study investigated the effect of cytokines, interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), on induction apoptosis in leukemic cell lines, K562 and HL60, via caspase cascade pathway. Leukemic cell lines were treated with cytokines in various concentrations and for various incubation times. Cell viability and growth inhibition were analysed by using trypan blue staining and MTT assay, respectively. Apoptotic cells were then stained with Annexin-V-FITC and analysed 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 growth inhibition and cell apoptosis were increased after treatment with 2 ng/ml of IL-1β or 20 ng/ml of TNF-α in both cell lines. The increased subG1 populations from cell cycle analysis indicated increased apoptotic cells. Caspase 3 activation was increased after IL-1β and TNF-α treatment. TNF-α could induced caspase 8 activation in both leukemic cell lines. Whereas, IL-1β treatment induced caspase 8 and 9 activation in HL60 cell. From the data it is suggested that IL-1β and TNF-α could induce cell apoptosis in both leukemic cell lines, via caspase cascade pathway.

KEY WORDS : CYTOKINE / APOPTOSIS / LEUKEMIA

110 pp.

ผลของไซโตคายน์ต่อการเกิดอะพอพโตซีสในเซลล์มะเร็งเม็ดเลือดขาว
(EFFECT OF CYTOKINES ON APOPTOSIS OF LEUKEMIC CELL LINES)

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บทคัดย่อ

มะเร็งเม็ดเลือดขาวพบได้ทั้งในเด็กและในผู้ใหญ่ ปัจจุบันมีการนำสารไซโตคายน์บางชนิดมาใช้ในการรักษาผู้ป่วยมะเร็งเม็ดเลือดขาวโดยทำให้เกิดการกระตุ้นการตายแบบอะพอพโตซีส ในการวิจัยครั้งนี้ได้ศึกษาผลของสารไซโตคายน์ชนิด Interleukin-1 beta (IL-1β) และ Tumor necrosis factor alpha (TNF-α) ในการกระตุ้นการตายแบบอะพอพโตซีสในเซลล์มะเร็งเม็ดเลือดขาวชนิด K562 และ HL60 ผ่านทาง caspase pathway โดยทำการเพาะเลี้ยงเซลล์มะเร็งทั้งสองชนิดและทดสอบกับไซโตคายน์ที่ความเข้มข้นและเวลาต่าง ๆ กัน จากนั้นวัดปริมาณเซลล์ที่มีชีวิตอยู่ การยับยั้งการเจริญเติบโตของเซลล์ ลักษณะและปริมาณเซลล์ที่ตายแบบอะพอพโตซีส โดยย้อมด้วย Annexin-V-FITC และยังศึกษาปริมาณ subG1 ในวงจรของเซลล์ด้วยเครื่อง flow cytometer ผลการศึกษาพบว่าทั้ง IL-1β และ TNF-α สามารถลดจำนวนของเซลล์ที่มีชีวิตและเพิ่มการยั้งยั้งการเจริญของเซลล์มะเร็งเม็ดเลือดขาวได้ ซึ่งสัมพันธ์กับการเพิ่มจำนวนเซลล์ตายแบบอะพอพโตซีสและปริมาณเซลล์ในระยะ subG1 มีค่าเพิ่มขึ้นด้วย ผลจากการศึกษาการตายแบบอะพอพโตซีส ผ่าน caspase pathway พบว่า IL-1β และ TNF-α สามารถกระตุ้นให้เกิด activated caspase 3 เพิ่มขึ้นในมะเร็งเม็ดเลือดขาวทั้งสองชนิด นอกจากนี้ IL-1β สามารถกระตุ้น activated caspase 8 และ 9 ในเซลล์มะเร็งชนิด HL60 ส่วน TNF-α สามารถกระตุ้น activated caspase 8 แต่ไม่สามารถกระตุ้นการทำงานของ activated caspase 9 ได้ในเซลล์มะเร็งทั้งสองชนิด จากการศึกษาสามารถสรุปได้ว่า IL-1β และ TNF-α สามารถกระตุ้นการตายแบบอะพอพโตซีสในมะเร็งเม็ดเลือดขาวทั้งสองชนิด ผ่านทาง caspase pathway

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LIST OF ABBREVIATIONS

Abbreviations	Terms
α	alpha
β	beta
γ	gamma
º C	degree celsius
AIF	Apoptosis-inducing factor
ALL	Acute lymphocytic leukemia
AML	Acute myelogenous leukemia
Apaf-1	Apoptosis protease activating factor-1
APL	Acute promyelogenous leukemia
Bax	Bcl-2 associated x protein
Bcl	B cell lymphoma
Caspase	Cysteine asparted-specific proteinase
CD95	Fas receptor
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
DISC	Death inducing-signaling complex
DNA	Deoxyribonucleic acid
FADD	Fas associated death domain
FBS	Fetal bovine serum
FITC	Fluorescence isothiocynate
hr	hour
IAP	Inhibitor of apoptosis protein
IFN	interferon
IL	interleukin
kDa	kilo Dalton

LIST OF ABBREVIATIONS (continued)

Abbreviations	Terms
mg	milligram
min	minute
ml	milliliter
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
ng	nanogram
O.D	Optical Density
PBS	Phosphate Buffer Saline
PI	Propidium iodide
SEM	Standard error of mean
TNF	Tumor necrosis factor
µg	microgram
µl	microliter

CHAPTER I
INTRODUCTION

Leukemia is cancer that affects the blood-forming stem cells. The abnormal blood cells grow in an uncontrolled way. It develops in the bone marrow and spreads into the blood circulation and to other organs, such as spleen, lymph nodes, liver, and central nervous system. There are several types of leukemia which can group by how rapidly the cancer develops, acute or chronic leukemia, and grouped by the types of white blood cell are affected, myelocytic and lymphocytic leukemia. (1,2). Every year, more than 30,000 new cases of leukemia were diagnosed in the United State, and adult onset accounts for 90 percent of the new cases (3). In Thailand, it is ranked as the eighth most common cancer in males and tenth in females (4). The age-standardized incidence rates (ASR) of the leukemia is 3.9 per 100,000 in male and 3.2 per 100,000 in females. The highest incidence is found in Udon Thani (ASR 5.4) and lowest in Nakhon Phanom (ASR 0.3). Interestingly, in Rayong the incidence is much different in male and female (ASR 5 vs. 2.4) and in Songkhla, the incidence is higher in female (ASR 4.5 vs. 3.6). In adults, myeloid leukemia is more common than lymphoid leukemia (ratio 1.6:1 in male; 1.4:1 in female) (4). Lymphoid leukemia is significantly more common in males than in females (ratio 1.6:1). Chronic lymphoid leukemia is rare. Chronic myeloid leukemia is mainly a disease of middle age, with few cases before 20. CML accounts for 10-18% of all leukemias (4).

Leukemia can be treated by chemotherapy, radiation therapy, immunotherapy or stem cell therapy. Treatment for leukemia is limited in many conditions, which depends on type of cell affected. Some treatment such as chemotherapy may harm healthy blood cells, thus creating side effects (5) whereas radiotherapy does kill normal cells along with the cancer cells (6).

There is an evidence that the development of leukemic cell is due to the dysregulation of the balance between cell proliferation and apoptosis. In apoptosis, in contrast with necrosis, the stimulus of cell death is not directly but by activating a

cascade of events which results in the destruction of the cell and there is no inflammatory reaction (7). Apoptosis or programmed cell death is a process for killing cells through the activation of an intracellular pathway leading to cellular changes. It is essential in the homeostasis of normal tissues of the body. Abnormalities in cell death control can provide 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 normal apoptotic pathways (8). The majority of chemotherapeutic agents as well as radiation used to induce cancer cell death in the apoptotic pathways. Apoptotic signaling occurs through multiple independent pathways that are initiated either from activating events within or from outside the cell. Therefore, understanding the apoptotic signaling and the apoptotic mechanism may allow the development of better treatment of cancer (9).

Many types of anti-tumor therapy including radiotherapy act their effect by activating apoptosis (10). Apoptosis is executed by the activated caspases. However, there are some data studies in cell line system, that a caspase-independent pathway of apoptosis exists, which induced by a mitochondrial apoptosis-inducing factor (11).

Many cytokines have been isolated by their ability to induce growth such as transforming growth factor beta (TGF-β), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), Platelet-derived growth factor (PDGF), erythropoietin (EPO) and thrombopoietin (TPO) that called growth factors. In myeloid hematopoietic cells, lymphocytes and neuronal cells, *in vitro* and *in vivo* studies have shown the role of cytokines in inducing differentiation of different cell types to become mature cells (12). Some cytokines can act on more than one cell types. Cytokines can be used to decrease apoptosis in normal cells and inhibition of cytokine activity may improve cancer therapy by inducing apoptosis in cancer cells. The apoptosis suppressing function of cytokines is mediated by changing the balance in the activity of apoptosis inducing and suppressing genes (12). However, some cytokines have the antiproliferation effect and used as immunotherapy in cancer such as interferon gamma (IFN-γ), interferon alpha (IFN-α), tumor necrosis factor alpha (TNF-α) and some interleukins (13,16).

There is evident from a study that interleukin-1 beta (IL-1β) is a much more powerful and effective inducer of amniochorion apoptosis than IL-6 (13). IL-1β

induced initiator and effector caspase expression followed by increased caspase activity, and DNA fragmentation (13). It has been reported that both TNF-α and IL-1β induce apoptosis of the trophoblast cells through the Fas-FasL mediated pathway (14). IL-1β can activate TNF-α in immunocytes and human fetal membranes which raises the question of IL-1β independent role in fetal membrane apoptosis (15). These findings suggest that cytokines may use as immunotherapy in leukemic cells to induce cell apoptosis.

CHAPTER II
OBJECTIVES

The increase of abnormal cell proliferation and decrease in cell apoptosis results in cancers. Leukemia is the cancer of blood cells that develop from bone marrow and spread into the blood and other organs. Induction of apoptosis can reduce leukemic cell, by activating death signaling pathways such as caspase cascade. Cytokines have been used in some types of cancer including leukemia to induce apoptosis as an immunotherapy (16). There are many reports on cytokines treatment of leukemia (13). However, the effect of cytokines on apoptotic induction in myeloid leukemia remains unclear and needs for investigation.

The aims of this thesis are

1. To study the effect of cytokines, interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), on cell viability, growth inhibition, cell morphology and apoptosis of leukemic cell lines, HL60 (human acute promyelogenous leukemia) and K562 (human chronic myelogenous leukemia).
2. To study the possible signaling pathways of cytokine regulated apoptosis in leukemic cell lines.

CHAPTER III
LITERATURE REVIEW

1. Leukemia

Leukemia is the cancer that affects the blood-forming stem cells. The disease develops when the body produces large numbers of abnormal white blood cells. Leukemia starts in the bone marrow and then spread into the blood circulation, lymph node, spleen, liver, central nervous system and other organs. Leukemias that affect on white blood cells are myelocytic and lymphocytic leukemia. Myeloid leukemias are cancers that arise from myeloid stem cells, which normally mature into red blood cells, white blood cells, and platelet. Lymphoblastic leukemias are cancers that arise from lymphocyte stem cells, which normally mature into white blood cells, also known as leukocytes. The fast of leukemia progress can be classified into acute and chronic leukemia. Acute types refer to cancers increase rapidly in immature stem cells and are unable to function normally, while chronic types refer to cancers increases more slowly in mature stem cells and can carry out some of their normal functions (1,2).

The incidence of cancer in children between 0 and 14 years of age is 1 in 7000 in the countries of North America, Asia, Western Europe, and Australia. About 33% of cancers in children aged 0-14 years are leukemia, which the most common type is acute lymphocytic leukemia (ALL) that accounts for approximately 25%. Acute myelogenous leukemia (AML) accounts for approximately 5%, whereas chronic leukemia is extremely rare in children and only 2.4% are chronic myelogenous leukemia (CML) (17,18). Most cases of leukemia occur in older adults that more than half of all cases occur after age 67. The most common types of leukemia in adults are acute myelogenous leukemia (AML) accounts for 80% to 90% and chronic lymphocytic leukemia (CLL). Most cases of chronic myelogenous leukemia (CML) occur in adults than in children (18,19).

1.1 Type of Leukemia

Leukemias are categorized by how fast they progress and which types of white blood cells are effected. Acute leukemia spread quickly, while chronic leukemia spread more slowly. Lymphocytic leukemia affects lymphocytes, whereas myelogenous leukemia affect either granulocytes and monocytes (1,20). The four major forms of leukemia are:

1.1.1 Acute Myelogenous Leukemia (AML) that occurs more often in adults.

1.1.2 Acute Lymphocytic Leukemia (ALL) is most common during childhood and in early adulthood, although it is also diagnosed in adults older than 30 years old.

1.1.3 Chronic Myelogenous Leukemia (CML) is more common between ages 40 and 70 and is rare among young people.

1.1.4 Chronic Lymphocytic Leukemia (CLL) most often affects adults over the age of 55. It sometimes occur in younger adults, but it almost never affects children.

Acute leukemia can occur in children and adults. It involves an overgrowth of immature blood cells, that the bone marrow unable to produce healthy blood cells. The rapid progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body. This condition is life-threatening because there are not enough mature blood cells to prevent anemia, infection and bleeding. Immediate treatment is required in acute leukemia. If left untreated, the patient will die within months or even weeks (17,19).

Chronic leukemia mostly occurs in older people, but can theoretically occur in any age group. It involves an overgrowth of mature blood cells, but still abnormal. The cells are produced at a much higher rate than normal cells, resulting in many abnormal white blood cells in the blood. Commonly, people with chronic leukemia have enough mature blood cells to prevent serious bleeding and infection (18).

The most important types of leukemia that found in adult are acute myelogenous leukemia and chronic myelogenous leukemia.

1.2 Acute myelogenous leukemia (AML)

Acute myelogenous leukemia (AML) is a heterogeneous group of leukemias that arise in precursors of myeloid, erythroid, megakaryocytic, and monocytic cell lineages. It is a disorder of myeloid hematopoietic cells has been called a variety of names including acute myeloid leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute granulocytic leukemia and acute nonlymphocytic leukemia (21,22). These leukemias result from clonal transformation of hematopoietic precursors through the acquisition of chromosomal rearrangements and multiple gene mutations in hematopoietic stem cells, which display increased proliferation and resistance to apoptosis, as well as impaired differentiation (21,23). AML results from mutations in the DNA of a developing cell in the marrow and undergoes the leukemic change, it multiplies into many cells. These cells grow and survive better than normal cells and crowd out healthy cells (24). These alterations limit their ability to differentiate into erythrocytes, granulocytes, and platelets and lead to the proliferation of leukemic cells or blasts. Their are heterogeneous group of disorders (25,26). The uncontrolled growth leads to an accumulation of leukemic blast cells block production of normal marrow cells, leading to a deficiency of red cells (anemia), of platelets (thrombocytopenia), and of normal white cells, especially neutrophils (neutropenia), in the blood (24).

The subclassification of acute myelogenous leukemia is important, in different types of therapy may be used based on subtype and the course of the disease may be different. It is possible, although uncommon, for additional subtypes to occur (22). The identifying subtypes based on different types and patterns of cells in a patient's blood or marrow. Most people who are diagnosed with AML clearly have one of eight different patterns (26). These patterns are designated as the subtypes, shown in Table 1. The feature of AML blood smear is shown in Figure 1.

Table 1. Acute myelogenous leukemia cell subtype (27)

Classification	Cell subtype
M0	Myeloblastic, undifferential
M1	Myeloblastic, without maturation
M2	Myeloblastic, with maturation
M3	Promyelocytic
M4	Myelomonocytic
M5	Monocytic
M6	Erythroleukemia
M7	Megakaryocytic

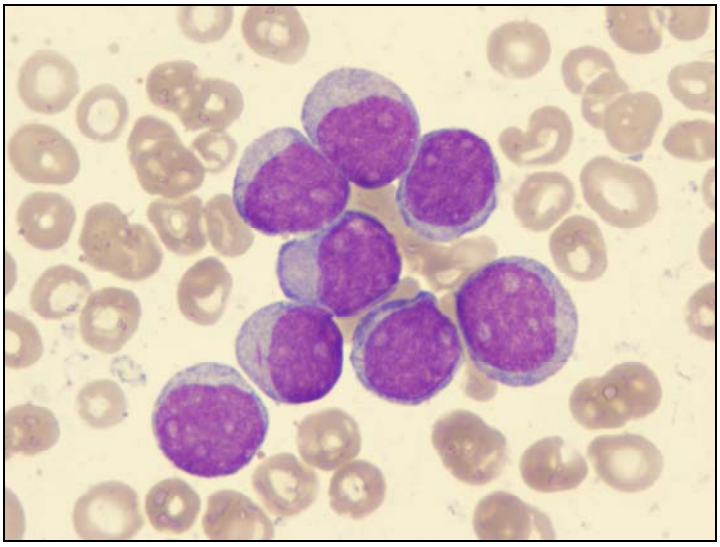

Figure 1. Acute myelogenous leukemia morphology

Myeloblast is a type of undeveloped white cell. If myeloblasts are the dominant leukemic cells in the marrow at the time of diagnosis, the leukemia is referred to as a myeloblastic type M0, M1. If there are many myeloblasts but some cells are developing toward fully formed blood cells, the added designation "with maturation" is used M2. M3 blasts are found in acute promyelocytic leukemia (APL) and have prominent granules. M4 blasts occur in acute myelomonocytic leukemia (AMMoL) and are a mixture of myeloblasts and monoblasts. If there are cells developing features of monocytes, red cells or platelets these designations are used M5, M6, M7 subtypes (17). Certain chromosomal changes can give important information for patient management. For example, three chromosomal changes indicate a relatively favorable prognosis, especially in younger patients. They are AML associated with a translocation between chromosomes 8 and 21 (t8;21), AML associated with an inversion or translocation of chromosome 16 (t16;16) and AML associated with a translocation between chromosomes 15 and 17 (t15;17). AML characterized by this translocation requires different treatment than other types of AML (19,27). The treatment of the acute promyelocytic leukemia (APL), M3 subtype of AML differs from the treatment for other AML subtypes. With APL, the cells that accumulate in the marrow can be identified as promyelocytes, the step in blood cell formation that follows the development of myeloblasts. These cells also have a balanced reciprocal translocation, t(15;17), the promyelocytic (PML) gene on chromosome 15 and the retinoic acid receptor-α (RARα) on chromosome 17 (28,29).

Recurring chromosomal translocations are a hallmark of human leukemias. There are more than 300 recurring chromosomal translocations, of which more than 100 have been cloned (30), providing important insights into the pathogenesis of disease. Chromosomal aberrations are found in about half of all AML cases and are grouped into two major subtypes, balanced and unbalanced aberrations (31). In this category of AML, molecular analyses have revealed mutations in the FMS-like tyrosine kinase 3 (*FLT3*), CCAAT/enhancer binding protein-α (*CEBPA*), CD117 (*KIT*) and nucleophosmin (*NPM*) genes, as well as mixed lineage leukemia (*MLL*) partial tandem duplication (31,32). The result of chromosomal translocations in AML is loss of function mutations in transcription factors that are required for normal hematopoietic development. Examples in myeloid leukemias include activating

mutations in RAS family members (N-RAS and K-RAS), in the hematopoietic tyrosine kinases *FLT3* and *c-KIT*, loss of neurofibromatosis type 1 (NF-1) function, and gain of function mutations in the hematopoietic Src homology 2-containing tyrosine phosphatase (SHP-2) (30). These mutations confer proliferative advantage to hematopoietic progenitors and cooperate with loss of function mutations in hematopoietic transcription factors to cause an acute leukemia phenotype characterized by proliferation and impaired differentiation (33,34). The correct balance of general and lineage-specific transcription factors is necessary to induce the gene expression profiles that are required to direct the development of hematopoietic progenitors into specific lineages. The inhibition of this process is a major hallmark of AML. Therefore, the realization that AML-specific chromosomal translocations and mutations often target these transcription factors strongly suggested their involvement in the differentiation block in AML (22). The best example of oncogene-targeted therapy in AML currently is all-*trans* retinoic acid (ATRA), which can specifically inhibit the transforming activities of the PML-RARα oncogene in acute promyelocytic leukemia (25). However, these mutations do not only influence the differentiation of myeloid cells, but also their proliferation and survival either by influencing the expression of important regulators of the cell cycle and apoptosis machinery, or by physical association with these factors and modification of their function (22).

1.3 Chronic myelogenous leukemia (CML)

Chronic myelogenous leukemia (CML) is called by several names, including chronic granulocytic, chronic myelocytic or chronic myeloid leukemia. CML does not completely interfere with the development of mature red cells, white cells and platelets; these cells can generally continue to function normally. This is an important distinction from acute leukemia and accounts for the less severe early course of chronic leukemias (18). Figure 2 show the feature of CML blood smear.

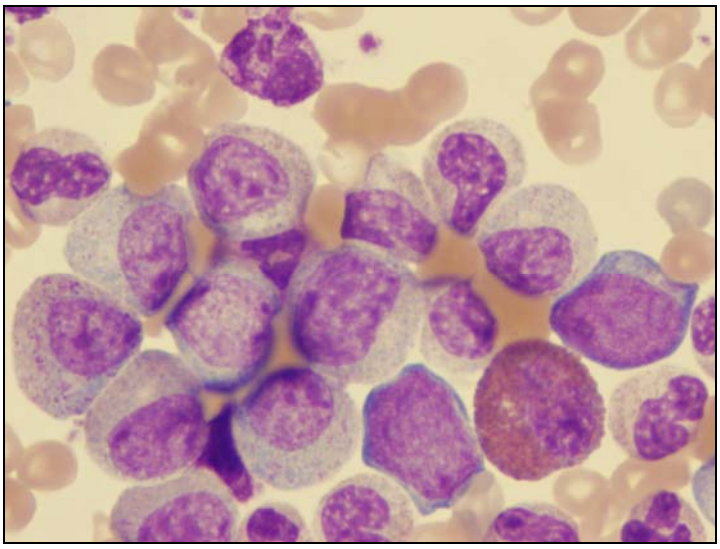

Figure 2. Chronic myelogenous leukemia morphology

CML is the member of myeloproliferative disorder (Polycythemia vera, Essential thrombocytosis, Primary or idiopathic myelofibrosis and Chronic myelogenous leukemia) resulting from the clonal expansion of a transformed hematopoetic stem cell. Following an initial chronic phase lasting a median of 4–5 years, CML progresses through a poorly defined accelerated phase to a terminal acute leukemia (35). There are three phases of CML progress; chronic phase, accelerated phase and blast crisis phase. Most patients are in the chronic phase of the disease when their CML is found. During this phase, CML symptoms are less intense. White cells can still fight infection. Once patients in the chronic phase are treated, red cells and platelets can do their functions. Most patients can go back to their usual activities. In the accelerated phase, the patient may develop anemia. The number of white cells may go up or down. The number of platelets may drop. The number of early bone marrow blast cells, increases. The spleen may swell and the patient may feel ill. During the blast crisis phase, the number of blast cells grows in marrow and blood (36). The blast phase is often preceded by a transition period which is marked by the acquisition of

new cytogenetic abnormalities in 50% to 80% of patients (37). The number of red cells and platelet cells drops. The patient may have bleeding and infections. The patient may be tired, have shortness of breath, stomach pain, bone pain or bleeding (38). The median age of disease onset is 67 years; however, CML occurs in all age groups. Untreated patients from chronic phase to a rapidly fatal blastic phase, generally over 3 to 5 years (39). CML was the first hematological malignancy to be associated with a specific chromosome abnormality, caused by constitutively activated tyrosine kinases. This abnormality known as the Philadelphia chromosome result from a reciprocal translocation between chromosome 9 and 22 or t(9;22)(q34;q11), which is confer a proliferative and survival advantage to hematopoietic progenitors but do not affect differentiation. These activated kinases are validated targets for therapy with selective tyrosine kinase inhibitors (30,33). The molecular consequence of this translocation event is the fusion of the abelson murine leukemia (*abl*) oncogene from chromosome 9 to sequences from chromosome 22, the breakpoint cluster region (*bcr*), giving rise to a chimeric *bcr-abl* gene (35,40).

1.4 Risk factors for leukemia

Several factors including improved diagnosis and longer life expectancy, resulting in increased environmental chemical exposures, are probably responsible for this development of leukemias (20). Certain genetic disorders predispose individuals to leukemia and viruses are closely allied to leukemia. Since, the damage on DNA within the cell could causes cancer. DNA damages in the white blood cells which protect the body from infections could cause leukemia. DNA damage can occur through chromosome translocations or mutations. Any one type of leukemia can have several genetic abnormalities at its core (30). However, in most cases, the DNA damage that finally results in the onset of leukemia is brought about by interactions between genes, age, and a variety of environmental or lifestyle factors such as nutrition and exposure to chemicals (41). Risk factors for leukemia include;

1.4.1 Age can be considered the biggest risk factor for developing leukemia, up to 70 percent of leukemia cases are over 50 years old (42). The chromosomes of white blood cells in older people are more fragile than those in young adults and are more vulnerable to the types of DNA damage (e.g., by free radical) that cause leukemia (43).

1.4.2 Nutrition, diets lacking in essential micronutrients (mineral or vitamins) are as harmful as cigarette smoking in the cause of cancer and can cause the same kind of DNA damage as exposure to radiation. Micronutrients provide to leukemia include folic acid and vitamins B12 and B6 (44, 45).

1.4.3 Chemotherapy used for the treatment of other cancers can cause DNA damage and make increase the risk of developing some forms of leukemia. For example, chemotherapy for treatment of other cancers is the major recognized cause of AML in the young, referred to by clinicians as secondary or treatment-related AML (46).

1.4.4 Radiation is the best known and most studied risk for leukemia. Studies have been done of people exposed to radiation in war, on the job, or as part of medical treatment. CLL is one of the little leukemia that has not been linked with radiation exposure. The other three major leukemias were found during studies of ionizing radiation in survivors of the atomic bombing in Japan. The atomic bomb in Japan and near by area of the nuclear reactors in the Chernobyl disaster of 1986, people who survivors and the radiation exposure were got leukemia (47). After exposure, survivors developed leukemia at a minimum of two years and at an average period of ten years. People receiving high doses of radiotherapy for solid tumors have relative risks for leukemias as well. In the population studies show incidence of leukemia was high in the years during radiation exposure and immediately after the nuclear testing between 1951 and 1962 in the United States and the onset of leukemia. Exposure to radiation is linked to acute myeloid leukemia in children. The exposure to high doses of radiation causes leukemia by inducing DNA damage through translocations (48).

1.4.5 Chemicals exposure, such as benzene, in long-term or in occupational is a cause of acute leukemia as same as exposure to herbicides, pesticides, and other agricultural chemicals is linked to an increased risk of developing leukemia (49). Hair

dyes contain chemicals that cause cancer and are associated with leukemia as in the long-term use of permanent dyes (50). AML is most strongly linked with these exposures, but there may also be a link to CML with benzene exposure. There also is a link to CLL. It must be noted that the industrial use of benzene has been outlawed in the US since the end of World War II; however, it is still used in some third world countries (51).

1.4.6 Cigarette contains leukemia-causing chemicals like benzene. Although smoking in the young-adult is associated with increases in the risk of developing leukemia, in those over 60 years old smoking is associated with a twofold increase in risk for AML and a threefold increase in the risk for ALL. Nevertheless, total leukemia incidence has not risen relative to the time during which the use of cigarettes and exposure to cigarette smoke climbed steadily. Lung and other smoking related cancers have rising incidence rates that can be attributed to smoking. Therefore, it would appear that the risk might have been overestimated for the leukemias. A few studies have shown increased risk for lymphoid leukemia among cigarette smokers, but most show a relationship to the myeloid types (51,52).

1.4.7 Genetics disease, such as Down's syndrome has a 10 - 20 times higher risk of developing leukemia than the general population. There are also inherited disorders, such as Fanconi's anemia and Bloom's syndrome, that are characterized by genetic instability and inability to repair DNA damage and are associated with an increased risk of leukemia (53,54).

1.4.8 Viral diseases have been implicated in the development of leukemia. The Epstein-Barr virus (EBV) is a herpes virus that can inhabit B-lymphocytes and nasopharyngeal cells. The human T-cell lymphoma/leukemia virus (HTLV-1) is closely associated with T-cell lymphocytic leukemia responsible for regulating cell growth and corrupts their functions resulting in the uncontrolled cell growth of leukemia, found in Japan, Africa, and the Caribbean. Only a small fraction of those infected with this virus develop leukemia (55).

1.5 Medical therapy of leukemia

Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient. Similarly in leukemia treatment, the effectiveness of treatment depends on the type of leukemia.

1.5.1 Chemotherapy. Leukemia in general responds to the use of combination of chemotherapy and radiotherapy. Chemotherapy agents attack rapidly dividing cells, but they can not differentiate leukemia cells from other rapidly non-cancerous dividing cells. Chemotherapy harms healthy red blood cells, white blood cells, blood-clotting platelets, hair follicles, and cells lining the gastrointestinal tract, thus creating terrible side effects. The damage to white blood cells increases the risk of infection (5). Medications known as colony-stimulating factors (CSFs) increase white blood cell counts and are often given in combination with chemotherapy (56). Some healthy cells are destroyed as well, which is what causes the side effects, but normal cells are often able to repair themselves after treatment. Different types of drugs are used for the different types of leukemia (5).

1.5.2 Radiation therapy is helpful in treating cancer because cancer cells reproduce faster than most normal cells (6). Radiotherapy damages DNA of leukemic cells by exposing them to ionizing radiation. Radiation is treatment with high-energy rays that destroy cancer cells. In high doses radiation therapy kills cells or keeps them from growing and dividing. However, radiation is not the primary treatment for leukemia that is used in 4 percent of leukemia cases in clinical (57). Although radiation does kill normal cells along with the cancer cells. This is due to chemotherapy alternatives. Sometime use irradiation in the treatment of the enlarged spleen of leukemia patients (6).

1.5.3 Antioxidant agents. They are reduced in leukemia patients undergoing chemotherapy, with the low levels of antioxidant intake are associated with increases in adverse effects of chemotherapy in children with ALL (58). Vitamins C, E, and beta-carotene are associated with reduced toxicity from chemotherapy and lower frequencies of infections (58,59).

1.5.4 Stem cell therapy. Stem cell therapy fills up bone marrow that is the transplantation of stem cells into the patient's bone marrow following chemotherapy or radiation therapy to kill the leukemia cells also damages the rapidly dividing blood-forming cells. Stem cells may be obtained from the patient (autologous) or from a donor (allogeneic) who is a close tissue match to the patient (60). Autologous stem-cell therapy is ensuring that the removed stem cells are not contaminated with leukemia cells. Stem cells can be obtained either by bone marrow aspiration or by apheresis (peripheral blood stem-cell transplant), through which the cells are removed from the peripheral blood system (61).

1.5.5 Cell signaling pathway inhibitor. In early disease progression, many types of leukemia produce certain inflammatory and immunosuppressive cytokines (chemical messengers) and use cell-signaling pathways. Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine significantly elevated in all leukemias except for AML and myelodysplastic syndromes (62). The method to regulate normal cell growth with the drug that effect slows proliferation and causes apoptosis in *bcr/abl* cell lines and fresh leukemic cells from "Philadelphia chromosome positive" (Ph+) CML. Gleevec® (imatinib mesylate) is drug of choice for treat the patients with Ph+ CML in blast crisis, accelerated phase, or chronic phase after failure of interferon-α therapy (20).

1.5.6 Immunomodulators and immune enhancers. They are biological therapy treatment with substances that affect the immune system's response to cancer. Immunotherapy uses the body's immune system to fight cancer, using antibodies to target and destroy leukemia cell. These are three main substances :

1.5.6.1 Hematopoietic growth factors

Growth factors such as granulocyte-colony stimulating factor (G-CSF) use in chemotherapy to elevate the number of normal white blood cells, thus allow patients to tolerate high chemotherapeutic doses and reducing infections (5, 56). G-CSF treats neutropenia during CML therapy. Granulocyte-macrophage-colony stimulating factor (GM-CSF) blocks the migration of myeloid cells and leukemia spread (63).

1.5.6.2 Cytokines

Interferons (IFNs) are a group of naturally occurring substances that sometimes used in the treatment of chronic leukemia (13,64). They enhance the immune response and reduce the growth and reproduction of leukemia cells. It is useful when used as a maintenance therapy in patients. They were the study of Interleukin-1 beta (IL-1β) induces tumor necrosis factor alpha (TNF-α) expression on mouse myeloid mulipotent cell line that can inhibited their proliferation (65).

1.5.6.3 Immunotoxins

Antibodies to the molecules present on the surface of AML cells show anti-leukemic responses in clinical studies (66). Antibodies are binding to leukemic cell marks the cell as a target for destruction. Antibodies can be attached to cytotoxic agents and can be transferred to leukemia cells (67). Antibody therapy is beneficial in treating CLL (68).

2. Cytokines

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They generally act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell, like hormones and neurotransmitters, which are used for inter-cell communication. Cytokines are produced by a many types of cell in both hemopoietic and non-hemopoeitic and can effect on nearby cells or throughout the organisms. They have various names as lymphokines, interleukins or chemokines, based on their supposed function, cell of secretion or target of action. Responses to cytokines include increasing or decreasing expression of membrane protein (including cytokine receptors), proliferation, and secretion of effector molecules (69).

Interleukins (ILs) are not only secreted by leukocytes but also able to affect the cellular responses of leukocytes. Specifically, interleukins are growth factors targeted to cells of hematopoietic origin (69). The interleukins and interferons are listed in Table 2-3.

Table 2. List of Interleukins (69)

Interleukins	Principal source	Primary activity
IL-1α IL-1β	macrophages antigen presenting cells (APCs)	- hematopoiesis - co-stimulation of APCs and T cells - inflammation and fever - acute phase response
IL-2	activated TH_1 cells NK cells	- proliferation of B cells and activated T cells - NK functions
IL-3	activated T cells	- growth of hematopoietic progenitor cells
IL-4	TH_2 cells mast cells	- B cell proliferation - eosinophil and mast cell growth and function - IgE and class II MHC expression on B cells - inhibition of monokine production
IL-5	TH_2 cells mast cells	- eosinophil growth and function
IL-6	activated TH_2 cells APCs somatic cells	- acute phase response - B cell proliferation - thrombopoiesis - synergistic with IL-1 and TNF on T cells
IL-7	thymic cells marrow stromal cells	- T and B lymphopoiesis
IL-8	macrophages somatic cells	- chemoattractant for neutrophils and T cells
IL-9	T cells	- hematopoietic and thymopoietic effects
IL-10	activated TH2 cells CD8+ T and B cells macrophages	- inhibits cytokine production - promotes B cell proliferation and antibody production - suppresses cellular immunity - mast cell growth

Table 2. List of Interleukins (continued)

Interleukins	Principal Source	Primary Activity
IL-11	stromal cells	- synergisitc hematopoietic - thrombopoietic effects
IL-12	B cells macrophages	- proliferation of NK cells - IFN-γ production - promotes cell-mediated immune functions
IL-13	TH_2 cells	IL-4-like activities
IL-15	epithelial cells monocytes	- proliferation of B cells and activated T cells - NK functions
IL-16	CD8 T cells	- chemoattracts CD4 T cells
IL-17	activated memory T cells	- promotes T cell proliferation
IL-18	macrophages	- induces IFN-γ production

Table 3. List of Interferons (69)

Interferons	Principal source	Primary activity
IFN-α and -β	macrophages neutrophils some somatic cells	- antiviral effects - induction of class I MHC on all somatic cells - activation of NK cells and macrophages
IFN-γ	activated TH_1 NK cells	- induces of class I MHC on all somatic cells - induces class II MHC on APCs and somatic cells - activates macrophages, neutrophils, NK cells, - promotes cell-mediated immunity, antiviral effects

Cytokines may have stimulatory, inhibitory, or several different effects on hematopoietic progenitor and stem cells. Interleukin-4 represents a classic cytokine with a dual effect on hematopoiesis (70). The hematopoietic effect can be direct, as seen with most of these cytokines, or indirect and mediated by the induction of other cytokines. IL-17 represents a classic cytokine whose hematopoietic activity is largely indirect (71). It has been difficult to settle on a system classifying cytokines into distinct groups because of the overlapping activity and redundancy seen in the cytokine world. The hematopoietic cytokines can grouped into a late-acting, lineage-specific group that includes granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), IL-5, and erythropoietin, and an intermediate-acting, lineage-nonspecific cytokine group that includes IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4, as well as a group promoting the cycling of dormant progenitors, which includes IL-6, G-CSF, IL-11, stem cell factor (SCF), leukemia inhibitory factor (LIF), and IL-12 (72). The hematopoietic cytokines showed in Table 4.

Table 4. Hematopoietic cytokines (73)

Colony-stimulating factors Granulocyte colony-stimulating factor (G –CSF) Granulocyte-macrophage colony-stimulating factor (GM-CSF) Macrophage colony-stimulating factor (M-CSF)
Interleukin-3 (IL-3)
Erythropoietin (Epo)
Thrombopoietin (Tpo)
Interleukin-5 (IL-5)
Co-stimulatory cytokines Stem cell factor (SCF) Flt-3 Ligand (FL)

Table 4. Hematopoietic cytokines (continued)

Interleukin-6 (IL-6) family
IL-6
IL-11
Leukemia inhibitory factor (LIF)
Oncostatin M (OSM)
Other interleukins with hematopoietic activities
IL-1
IL-2
IL-4
IL-7
IL-9
IL-10
IL-12
IL-17
IL-20
Novel factors with hematopoietic activities
Notch ligands
Wnt family
Vascular endothelial growth factor (VEGF) family
Classic growth factors with hematopoietic activities
Insulin-like growth factor-1 (IGF-I), IGF-II
Basic fibroblast growth factor (b-FGF)
Hepatocyte growth factor (HGF)
Platelet-derived growth factor (PDGF)
Suppressive cytokines
Chemokines
Interferons (IFNs)
Tumor necrosis factor-α (TNF-α)
Transforming growth factor-β (TGF-β)

2.1 Cytokine receptors

Cytokine receptors are glycoproteins that bind their ligands in a specific manner and at low cytokine concentration to transduce signals leading to biologic responses (74). These receptors display significant conservation in their structure and activity, which places them within the cytokine receptor superfamily (75). There are examples of newly cloned proteins that were deduced to be cytokine receptors based on the presence of conservative domains in their structures. Cytokine receptors function as dimeric or oligomeric complexes consisting of two to four chains. Ligand binding results in dimerization or oligomerization of the receptor chains, which causes receptor activation and signal transduction. This mechanism of receptor chain clustering appears to be universal among hematopoietic cytokine receptor and receptor tyrosine kinase families (76).

2.2 Interleukin-1β

Interleukin-1 (IL-1) is one of the most important cytokine in immune response. The predominant function of IL-1 is to enhance the activation of T-cells in response to antigen. The IL-1s are secreted primarily by macrophages but also from neutrophils, endothelial cells, smooth muscle cells, B- and T-cells, fibroblasts and keratinocytes. Production of IL-1 by these different cell types occurs only in response to cellular stimulation (77). IL-1α and IL-1β are the most highly investigated cytokines of the IL-1 family. IL-1β is a cytokine that is secreted by macrophages, monocytes and dendritic cells that important for the inflammatory response of the body against infection (78). This cytokine is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (79). IL-1β along with TNF-α are key inflammatory cytokines in many disease such as rheumatoid arthritis, dermatomyositis and skin disease (69).

IL-1β is synthesized as precursors (31 kD). Processing of pro–IL-1β to mature forms of 17 kD requires specific cellular proteases. IL-1β share 22% amino acid homology to IL-1α and bind to the same receptor. Pro–IL-1α is fully active as a precursor and remains intracellular. The opposite with the pro–IL-1β (IL-1β

precursor), which is not fully active. Pro-IL-1β remains primarily cytosolic until it is proteolytically processed to its active form by caspase 1 or IL-1-converting enzyme (ICE) that cleaved behind aspartate at position 116 and transported out of the cell (69). Other IL-1 family member is IL-1 receptor antagonist (IL-1ra) that acts as a competitive inhibitor of IL-1α and β actions by binding to the IL-1 receptor without inducing an intracellular signal (80).

There are two of IL-1 receptors exist, type I receptors, which transmit the intracellular signal, (80) and type II receptors, which do not transmit signal and may work as a decoy receptor (81). IL-1 does not induce hematopoietic colony formation when used alone. *In vivo*, IL-1 induces neutrophilia, and a single low-dose injection of IL-1 accelerates multilineage recovery after bone marrow ablation (79). At higher doses, IL-1 suppresses myelopoiesis *in vivo* (82), probably because the induction of interferons, TNF-α and prostaglandin E2 that inhibit progenitor cell proliferation. Mature IL-1β exerts its effects by binding to IL-1 receptor. While releasing from cells, the mature IL-1β encounters two antagonistic molecules that are the soluble form of the type II receptor (tightly binds IL-1β) and IL-1 receptor antagonist (competes with IL-1β for cell surface receptor occupancy) (83). Figure 3 show the IL-1 signaling pathway (80).

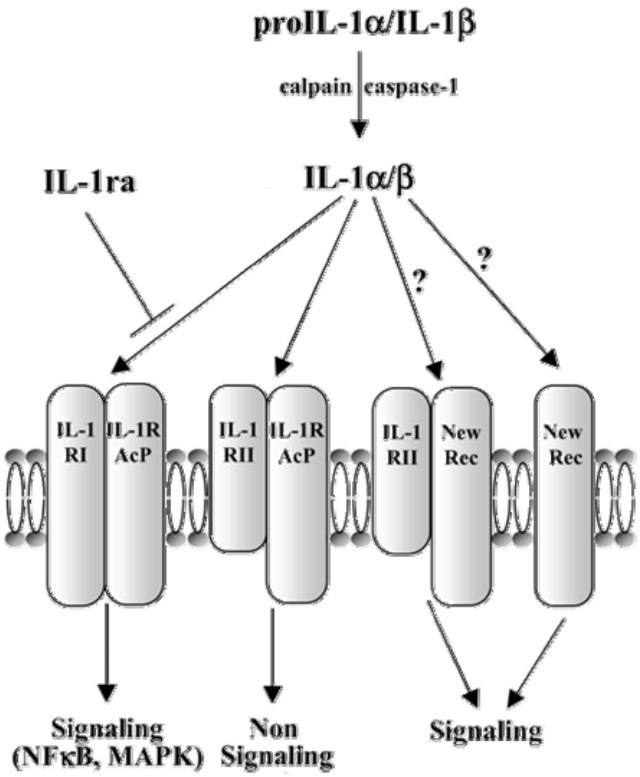

Figure 3. Interleukin-1 signaling pathway (80). The representation of classical and possible existence novel IL-1β receptor and IL-1 signaling pathway. IL-1ra (interleukin-1 receptor antagonist), IL-1RAcP (interleukin-1-receptor accessory protein), MAPK (mitogen-activated protein kinase) NFκB (nuclear factor-kappa B).

IL-1 can increase the production of various CSFs and stem cell factors for expansion of bone marrow, either by increasing their transcription or by stabilizing their mRNA. In addition, IL-1 may have protective effects after irradiation or cytotoxic drugs. However, normal hematopoiesis can take place in the absence ICE, IL-1β or IL-1 receptor type I in mice with specifically targeted gene deletions (77). IL-1 is not necessary for normal hematopoiesis but plays a role in hematopoietic responses to disease states. The spontaneous expression of IL-1 in blood cells circulation from normal volunteers could not be detected even using sensitive polymerase chain reaction methods in AML. IL-1β is spontaneously expressed, but IL-1 receptor antagonist gene expression is suppressed even when stimulated with GM-CSF. In CML patients with advanced disease and poor survival have suppressed IL-1 receptor antagonist with high IL-1β (69). The production of IL-1α, IL-1β, and IL-1 receptor antagonist in solid tumors (melanomas, hepatoblastoma, sarcomas, squamous cell carcinomas transitional cell cancers, and ovarian carcinomas) has been described as well, and in some cases, may contribute to metastatic potential (79). However, the relationship between IL-1 and tumor growth is complex. For some cell types, IL-1 can have growth inhibiting properties (69).

2.3 Tumor Necrosis Factor-α

Tumor necrosis factor-α (TNF-α), like IL-1 is a major immune response, is a potent cytokine produced by many cell types, including macrophages, monocytes, lymphocytes, keratinocytes, and fibroblasts in response to inflammation, infection, injury, and other environmental challenges. TNF-α is named for its ability to regress tumor masses when they get bacterial infections. TNF-α was isolated in 1984 and was found to be a pleiotropic cytokine that is expressed as a transmembrane protein and can be cleaved into a soluble form. It belongs to a family of about 20 factors that are grouped together in the TNF super-family (85). TNF-α transmits its signal through two receptors: TNFR1 or p55TNFR (TNF-receptor type 1 or p55 TNF-receptor) and TNFR2 or p75TNFR (TNF-receptor type 2 or p75 TNF-receptor) (86). TNF-α plays a bifunctional role in that it directly inhibits hematopoietic progenitors (87), at least partially by downregulating CSF receptors (88).

In its soluble form, TNF acts as a homotrimer with molecular mass of 17 kDa (157 amino acids) in each subunit. It is first synthesized as a 26 kDa (233 amino acids) membrane bound pro-peptide (pro-TNF) and is released after cleavage by the TNF-converting enzyme (TACE). TACE is a member of the a-disintegrin-and-metalloproteinase (ADAM) family. It can also release TNF receptors from the cell surface: these circulating cytokine-binding proteins represent an important mechanism of negative regulation for the biological activity of soluble TNF (89). The TNF and TNF-receptor were shown in Figure 4 (76). TNF family receptors can be classified into three major groups. The first group contains a death domain (DD) in the cytoplasmic tail. Activation of these DD containing receptors by their corresponding ligands leads to the recruitment of intracellular DD-containing adaptors such as Fas-associated DD (FADD/MORT1) and TNF-R associated DD (TRADD), which together form death inducing signaling complex (DISC). These molecules cause activation of the caspase cascade and induction of apoptosis, but can also recruit TNF-R associated factor (TRAF) family members. The second group of receptors contains one or more TRAF-interacting motifs (TIM) in the cytoplasmic tail. Activation of TIM containing TNF-R leads to the direct recruitment of TRAF family members, which ultimately activate multiple signal transduction key mediators, such as mitogen-activated protein kinases (MAPK) (e.g. c-Jun N-terminal kinase (JNK), p38 (p38MAPK), extracellular signal-related kinase (ERK)), inhibitor of nuclear factor kappa-B (NFκB) kinase (IKK) and phosphatidyl-inositol 3-kinase (PI3K). The third group of TNF receptor family members does not contain functional intracellular signaling domains or motifs. Although these decoy receptors cannot provide intracellular signaling, they can effectively compete with the other two receptor groups for their corresponding ligands (89).

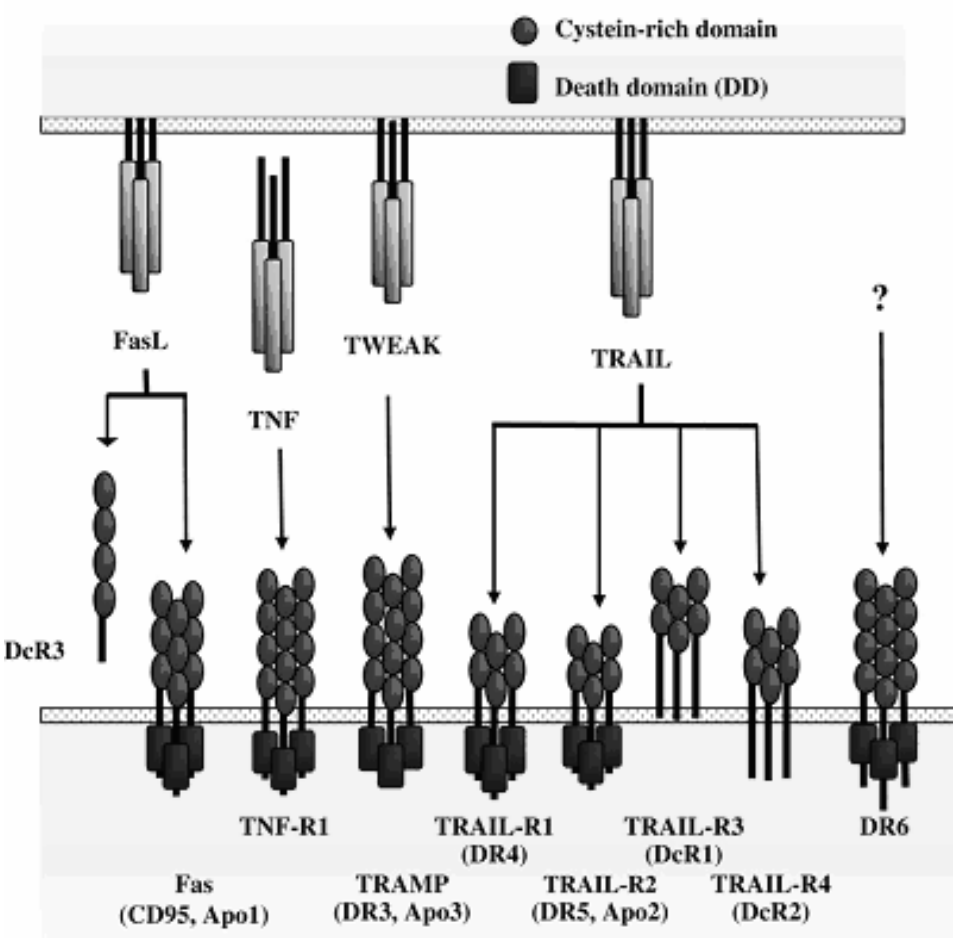

Figure 4. Tumor necrosis factor family and their receptors (76). All death receptors are type I proteins containing cysteine-rich domains. Death receptors contain a cytoplasmic sequence named death domain (DD). Decoy receptors (DcR) lack the DD. TNFSF (Tumor necrosis factor ligand superfamily member), FASL (Fas ligand), APO-1 (Apoptosis antigen ligand 1), TRAIL (TNF-related apoptosis-inducing ligand; Apo2 ligand), TWEAK (TNF-like weak inducer of apoptosis; Apo3 ligand)

3. Apoptosis and apoptotic signaling pathways

Apoptosis is a physiological process for killing cells which critical for the normal development and function of multicellular organisms. Apoptosis is an important process in different biological systems, including embryonic development, cell turnover, and immune response against tumorigenic or virus-infected cells (9). The apoptotic features include the loss of cell volume or cell shrinkage, chromatin condensation, plasma membrane blebbing, internucleosomal DNA fragmentation, and the formation of apoptotic bodies that are phagocytized by macrophages or neighboring cells to get rid of dying cells (90). Abnormal survival of cells that should be killed can cause autoimmune disease or tumorigenesis. Conversely, abnormally increased apoptosis has been implicated in certain degenerative disorders (91).

Under either physiological or pathological conditions, apoptotic process is regulated by interactions among several families of protein, i.e. caspases, Bcl-2 family proteins, and inhibitor of apoptosis proteins (IAP) that show in Figure 5 (92). Apoptosis signaling occurs through multiple independent pathways that are initiated either from triggering events within the cell or outside the cell, by ligation of death receptors. All apoptotic signaling pathways converge on a common machinery of cell destruction that is activated by a family of cysteine proteases or caspases that cleave proteins at aspartate residues (9).

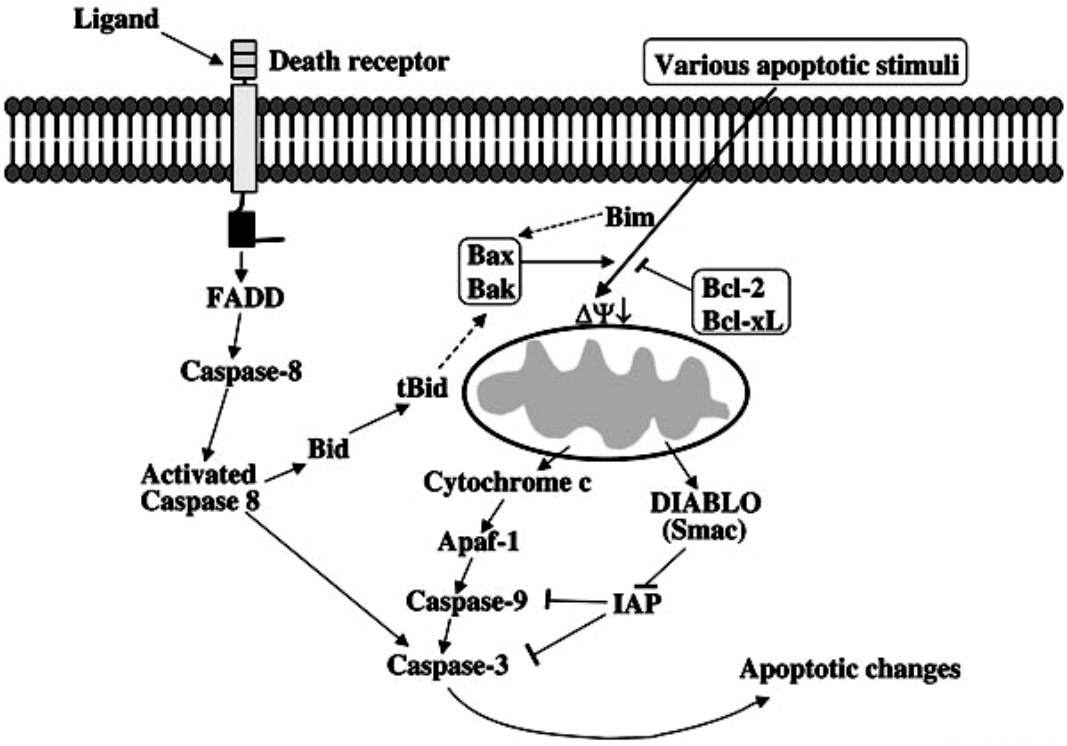

Figure 5. Apoptosis pathways (92). FADD (Fas-Associated protein with Death Domain), Bcl-2–associated X protein (Bax), Bcl-2 homologous antagonist/killer (BAK), Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), inhibitor of apoptosis protein (IAP), apoptotic protease activating factor 1 (Apaf-1)

3.1 Caspase Family

Caspase stands for cysteine-dependent aspartate-specific protease. There are at least 14 members of this family have been identified in mammals. Members of the caspase family can be divided into three subgroups depending on inherent substrate specificity, domain composition or the presumed role in apoptosis (92) that show in Table 5 and Figure 6.

Table 5. Subfamily members of caspase family (92).

Role	Members
Apoptosis activator (initiators caspases)	Caspase 2 Caspase 8 Caspase 9 Caspase 10
Apoptosis executioner (executioners caspases)	Caspase 3 Caspase 6 Caspase 7
Inflammatory mediator (cytokine activation caspases)	Caspase 1 Caspase 4 Caspase 5 Caspase 11 Caspase 12 Caspase 13 Caspase 14

A relationship is exist between the initiators and executioners in that the initiators act upstream of the executioners. The activated executioners cleave key proteins required for the maintenance of homeostasis, leading to the collapse and decease of the cell. All the initiator and executioner caspases have either a direct or indirect role in the processing, propagation and amplification of apoptotic signals that results in the destruction of cellular structures (92).

Initiator caspases play a role in initiating the apoptotic pathway. A different combination of initiator caspases, adaptors and regulatory proteins are required for the control and execution of different death stimuli. Caspase 8, Caspase 10 and its adaptor, FADD (Fas-associated death domain), are needed for Fas and TNF-R1-transduced apoptosis, although they are dispensable for other cell death pathways (94). In thymocytes and embryonic fibroblasts, caspase-9 and its adaptor apoptotic protease activating factor-1 (Apaf-1) are required for DNA damage, corticosteroid and staurosporine-induced cell death but not Fas and TNF-R1-transduced apoptosis (93). Subsequent to the recruitment and autocatalytic cleavage of caspase 8 and caspase 9, a second subpopulation of caspase 3, 6 and 7 are activated. These are executioner caspases that play role in the enzymatic cleavage of a variety of cellular proteins. Caspase-3 is essential for embryonic development. Moreover, caspase-3 and caspase-7 have been demonstrated to be almost synonymous in their substrates and inhibitor specificity. Their activation usually ensures the completion of the apoptotic process (92). Caspase 6 and caspase 7 are highly homologous to caspase 3. Procaspase 6 can be activated by caspase 3. Caspase 6 can also activate procaspase 3 by a positive feedback pathway. The substrates of caspase-6 include Poly (ADP-ribose) polymerase (PARP), lamin and procaspase-3 (93).

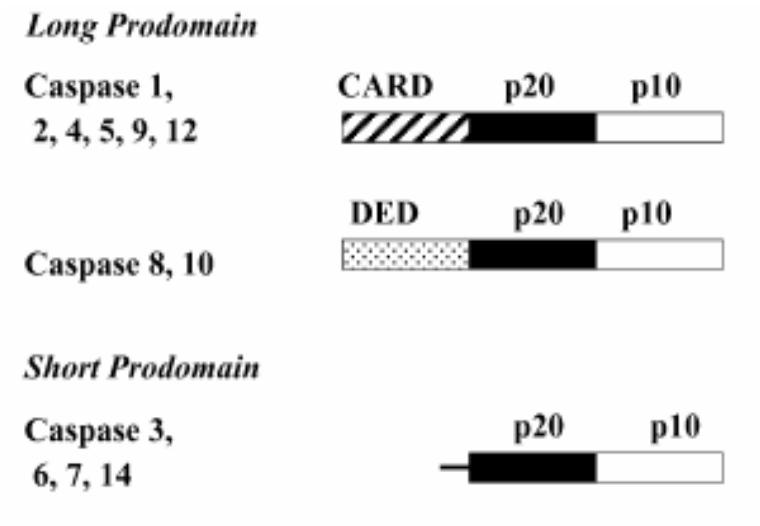

Figure 6. A schematic diagram of mammalian caspases (94). Caspases can be identified by a long prodomain and either a death effector domain (DED) as observed in the initiator caspases 8 and 10 or a caspase recruitment domain (CARD) as present in the initiator caspase 9 or inflammatory caspases 1, 2, 4, 5 and 12. Alternatively, the executioner caspases 3, 6, 7 and 14 are represented by a short prodomain

3.2 Caspase activation

Caspases exist within cells as immature pro-enzymes that must undergo proteolytic cleavage at a minimum of two sites containing aspartate residues to become activated (Figure 7). Processing appears to follow a specific pattern where the initial cleavage event follows an aspartate residue separating the large and small subunits followed by a second cleavage event to remove the pro-domain (95,96). Pro-caspases have a low level of intrinsic activity and it is now recognized that when brought into close proximity and in the absence of other processing, pro-caspases can transactivate themselves (95).

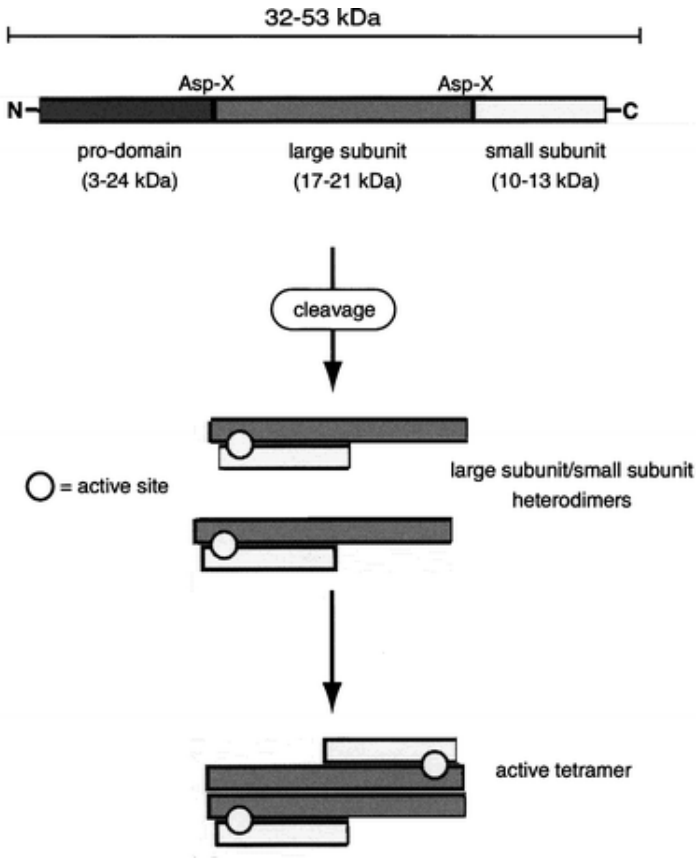

Figure 7. Caspase pro-enzyme organization and activation (84). Caspases require proteolytic cleavage following aspartate residues to liberate the small and large subunits and to release the pro-domain. Following cleavage, the large and small subunits join to form a heterodimer containing the active site (hollow circle). Two heterodimers then join to form the active tetrameric enzyme with the active sites facing away from each other.

Two major pathways of caspase activation have been revealed are the receptor-mediated apoptosis pathway where the TNF family of death receptors activate upstream caspase 8 and the mitochondrial-mediated apoptosis pathway where cytochrome c is released from the mitochondria and activates upstream caspase 9 (10).

Caspase activation plays a central role in the execution of apoptosis. They are two pathways of caspase activation. The first pathway is the cell surface death receptor pathway or extrinsic pathway (Figure 8). The receptor-mediated pathway of apoptosis begins with the TNF family of cytokine receptors that includes Fas (CD95), DR4 (TRAIL-R1) and TNFRI (CD120a), and TNFRII. These receptors differ in their ligand specificity, activating binding partners and downstream effectors. Upon ligand binding, activated TNFRI and Fas receptors recruit and bind the death effector protein FADD. Bound FADD recruits procaspase 8. Procaspase 8 is converted to its active form and is released back into the cytosol, activation of caspase-8 following its recruitment to the death-inducing signaling complex (DISC) is the critical event that transmits the death signal. Activated caspase 8 can activate downstream caspases by direct cleavage the effecter caspases such as caspase 3, 6 and 7 or indirectly by cleaving Bid and inducing cytochrome c release from the mitochrondria (93,96).

Caspase activation in the second pathway is the mitochrondrial-mediated pathway or intrinsic pathway (Figure 9), is initiated by mitochondrial damage that leads to cytochrome c–release. Cytochrome c is normally sequestered between the inner and outer membranes of the mitochondria. In response to a variety of proapoptotic stimuli, cytochrome c is released into the cytosol. Cytochrome c then binds and activates Apaf-1. Apaf-1 activates procaspase 9, which in turn cleaves procaspase-3. This pathway is dependent upon activated Bak/Bax and inhibited by Bcl-2 and its antiapoptotic family members, and inhibition of caspase activation by the proteins that belong to the inhibitors of apoptosis protein (IAP) (10,97).

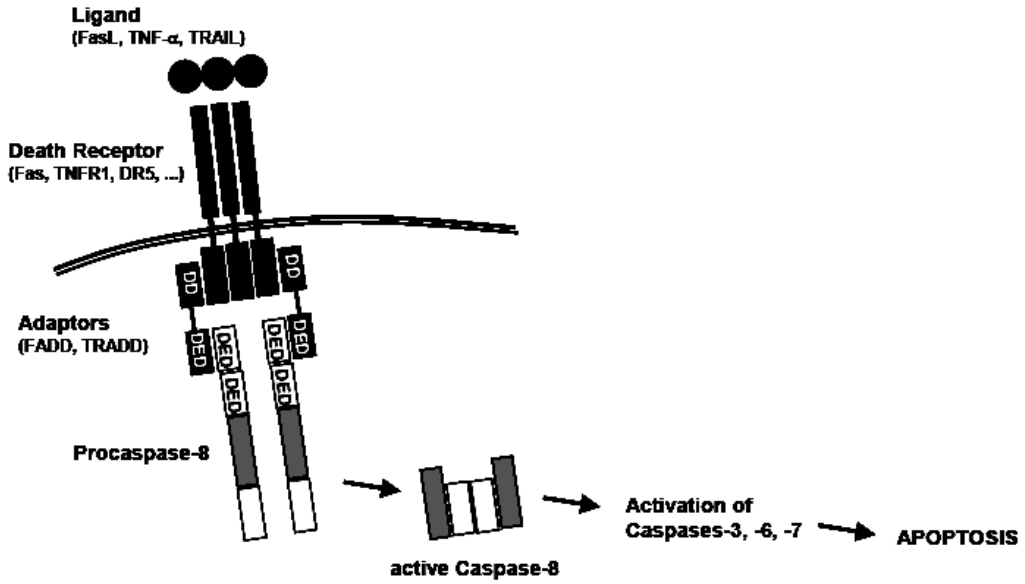

Figure 8. Receptor-mediated caspase activation at the DISC (extrinsic pathway).

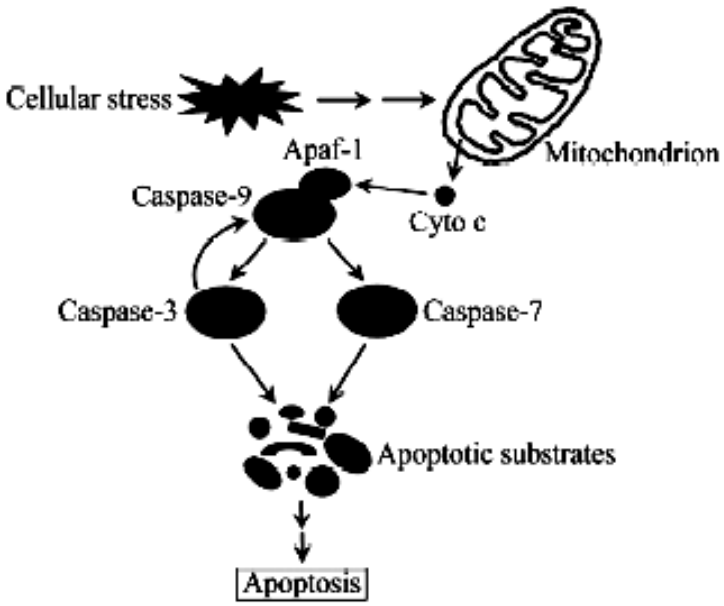

Figure 9. Mitochrondria-mediated and caspase-dependent pathways (intrinsic pathway) (93).

4. Apoptosis and leukemia

The mechanism of apoptosis can be studied either *in vitro* in a cell line model or *in vivo* in animal models or in humans. The *in vivo* methods have the advantage that they reflect the effect of the environmental factors, e.g. the cytokines, too. The apoptotic pathways may be induced by several exogenous injuries such as heat shock, cytokine defects, ionizing radiation, immunotherapy, and drugs as glucocorticoid, DNA topoisomerase I and II inhibitors, antimetabolites, tubulin inhibitors, akylating factors and resveratrol (*trans*-3,4,5-trihydroxystilbene), which has an apoptotic effect both in normal and leukemic cycling hemopoietic cells. Cytokines level decreased also has a proapoptotic effect (7).

Spontaneous apoptosis examined by annexin V was higher in normal CD34+ cells compared to that lower in AML CD34+ cells, if examined all bone marrow mononuclear cells. The apoptosis in acute lymphoid leukemia (ALL) is significantly lower than in AML and shows no significant difference from normal peripheral blood mononuclear cells (98,99). The effect of anticancer drugs on tumor cells was attributed to their crippling action on rapidly proliferating cancer cells (100). The drug-target interaction would lead to irreparable damage, and tumor cell death would be a consequence of the disruption of vital metabolic functions (100,101). However, over the past few years it has become clear that anticancer drugs are able to induce apoptosis and that this process is involved in the mediation of their cytotoxic effects (102). Furthermore, the induction of apoptosis was found to be a common event for different classes of anticancer agents, and because apoptosis induced by distinct classes of anticancer agents converges into similar downstream mechanisms, disruption of such mechanisms can lead to broad drug resistance (103). Nevertheless, the lack of specificity of cytotoxic drugs for tumors cells and the resulting toxicity to normal tissue hampers an additional exploitation of their apoptotic effects (102).

The induction of apoptosis in leukemia is increased by cytostatic drugs, corticosteroids, and radiation. The inducer damage cellular DNA initiating a cascade of events leading to cell death. The effect of the drugs on the apoptosis shows a significant even in identically classified patients (104). The apoptosis inducer may induce apoptosis through the death receptors (TNFR, Fas, TRAIL) and by activating

proteases or directly damaging DNA or the microtubules (105). In apoptosis the interaction with intracellular modulators is important. Induction of apoptosis is at the transcriptional level counterbalancing the cell proliferation effect of the cytokines. The ability of antineoplastic agents to induce apoptosis of neoplastic transformed cells represent a positive prognostic parameter in the treatment of malignity (106,107). Also, cytotoxic therapy and immunotherapy of leukemia and lymphoma predominantly mediates cell death through induction of apoptosis (108).

CHAPTER IV
MATERIALS AND METHODS

1. Reagents and instruments

1.1. Reagents for leukemic cells culture

1.1.1 RPMI 1640 (GIBCO^{TM}, Invitrogen Corporation)

1.1.2 Fetal Bovine Serum; FBS (GIBCO^{TM}, Invitrogen Corporation)

1.1.3 Penicillin-Streptomycin (GIBCO^{TM}, Invitrogen Corporation)

1.1.4 Sodiumbicarbonate (Merck, Germany)

1.2. Reagents for analysis

1.2.1 Trypan blue (Sigma-Aldrich, USA)

1.2.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphemyltetrazoliumbromide (MTT assay) (Chemical international)

1.2.3 Anexin V-FITC Apoptosis detection kit (BD Biosciences, USA)

1.2.4 Cycle TEST^{TM} plus DNA Reagent kit (Beckton Dickinson, USA)

1.2.5 Caspase-3 Detection Kit (FITC-DEVD-FMK) (Calbiochem, Germany)

1.2.6 Caspase-8 Detection Kit (FITC-IETD-FMK) (Calbiochem, Germany)

1.2.7 Caspase-9 Detection Kit (FITC-LEHD-FMK) (Calbiochem, Germany)

1.2.8 Wright – Giemsa (Merck, Germany)

1.3. Instruments

1.3.1 Hemocytometer

1.3.2 Hand counter

1.3.3 Fluorescence Microscope BX51 (Olympus, Japan)

1.3.4 Centrifuge (Hettich Zentrifugen universal 320R, Germany)

1.3.5 Light microscope (Olympus, Japan)

1.3.6 FACSCalibur E6361 Flow cytometer (Beckton-Dickinson, NJ)

1.3.7 CO_2 incubator (SL shel lab, USA)

1.3.8 Auto pipette

1.3.9 Autoclave

1.3.10 Microflow advance Biosafety Cabinet-class II (Science Tech)

1.3.11 Water bath (JulaboTW8, Germany)

1.3.12 Microplate Reader Multimode detector DTX800 (Beckman Coulter, USA)

1.4. Special equipments

1.4.1 6 wells cell culture plates (Corning Incorporation, USA)

1.4.2 24 wells cell culture plates (Corning Incorporation, USA)

1.4.3 96 wells cell culture plates (Corning Incorporation, USA)

1.4.4 25 cm^2 cell culture flasks (Corning Incorporation, USA)

1.4.5 75 cm^2 cell culture flasks (Corning Incorporation, USA)

1.4.6 15 ml centrifuge tube (Corning Incorporation, USA)

1.4.7 50 ml centrifuge tube (Corning Incorporation, USA)

1.4.8 5 ml polystyrene round button tube (Flacon, France)

1.5. Glassware, Plastic ware and supplies

1.5.1 Beaker, glass, 50, 100, 250, 500 and 1000 ml

1.5.2 Bottle, glass, 250, 500 and 1000 ml

1.5.3 Cylinder 250, 500 and 1000 ml

1.5.4 Glass slide and cover glass

1.5.5 Microcentrifuge tube (Appendrof) 1.5 ml

1.5.6 Millipore filter 0.22 μm

1.5.7 Pipette tip 20, 200, 1000 μl

1.5.8 Seropipette 5, 10 ml

1.5.9 Rack

1.5.10 70% alcohol

2. Human leukemic cell lines and culture conditions

2.1. Cell lines

2.1.1 K562 (Human chronic myelogenous leukemia)

K562 cell line was obtained from Prof.Dr.Watchara Kasinrerk, Faculty of Associated Medical Sciences, Chiangmai University.

2.1.2 HL60 (Human acute promyelogenous leukemia)

HL60 cell line was purchased from Cell Lines Services, Germany.

2.2. Culture medium (Appendix 1)

2.2.1 Cells were cultured in RPMI 1640 medium supplement with 10% fetal bovine serum and 2% penicillin-streptomycin.

2.2.2 Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

3. Cytokines and cytokines treatment

3.1. Cytokines

3.1.1 Interleukin 1 beta ; IL-1β (Chemical international)

IL-1β is a potent immuno-modulator that mediates a wide range of immune and inflammatory responses including the activation of B and T-cells. Human IL-1β is a 17.3 kDa protein containing 153 amino acid residues.

3.1.2 Tumor necrosis factor alpha ; TNF-α (Chemical international)

TNF-α is a potent lymphoid factor with exerts cytotoxic effects on a wide range of tumor cells and certain other target cells. The mature form of human TNF-α has 157 amino acid residues.

3.2. Cytokines treatment

K562 and HL60 cell lines were treated with 0.2, 2 and 20 ng/ml of IL-1β and TNF-α, then cultured for 12, 24 and 48hr at 37 °C in a humidified atmosphere of 5% CO_2. Untreated cells were used as control. After incubation time, the cells were harvested and prepared for analysis.

4. Growth curve

Generation of a growth curve can be useful in evaluating the growth characteristics of a cell line. From a growth curve, the lag time, population doubling time and saturation density can be determined.

4.1 Cultured leukemic cells were collected by centrifugation at 400 x g for 7 min at RT

4.2 Discard supernatant, resuspend the pellet in 5 ml of RPMI 1640 medium and count total cells under light microscope.

4.3 Dilute the cell suspension in order to have an appropriate amount of medium and cells to 10^4 and 10^5 cell/ml.

4.4 Mix well and seed the well-plate with the 1 ml of diluted cell suspension.

4.5 Count some of the leftover cell suspension in order to determine the actual total cell count. Record the number of cells in Day 0.

4.6 Put the plates in 37°C incubator with 5% CO_2.

4.7 Count the duplicate plates every 24 hours for 7 days.

4.8 Plot the results on a linear scale. The population-doubling time can be determined by identifying a cell number along the exponential phase of the curve, tracing the curve until that number has doubled.

5. Cell morphology

Wright's stain is a Romanowsky type metachromatic stain made by mixing methylene blue dye with eosin in a methanol diluent. Basic components of the cell, such as hemoglobin or certain inclusions or granules, will combine with the acidic portion of the stain, eosin, and are said to be eosinophilic. These components are stained shades of pink or red. Acidic cell components, such as nucleic acids and reactive cytoplasm, take up the basic dye components, methylene azure, and stain blue or purple. pH must be carefully controlled through the use of a buffer of 6.4-6.7. If the pH is too acidic the stain will take on a pinkish tint, and nuclear structures will be

poorly stained. A basic pH will cause all intracellular structures to be blue-black in color, with poorly defined structure. (Appendix 1)

5.1 After cytokines treatment, cells were harvest and prepared for cytospin.
5.2 Overlay a prepared cytospin slide with Wright's-Giemsa stain.
5.3 After 5 minutes, layer the slide with an equal amount of buffer solution. Blow gently to mix and leave for 5 minutes.
5.4 After 5 minutes, wash the slide for 30 seconds with distilled water.
5.5 Allow slide to dry at room temperature.
5.6 Observed the cell morphology under light microscope.

6. Cell viability assay

Trypan Blue is a vital stain recommended for use in estimating the proportion of viable cells in a population. The reactivity of this dye is based on the fact that the chromophore is negatively charged and does not react with the cell unless the membrane is damaged. Staining facilitates the visualization of cell morphology. Live (viable) cells do not take up the dye and dead (non-viable) cells do. Trypan blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted and resuspended in protein-free medium or salt solution prior to counting. (Appendix 1)

6.1 After cytokines treatment, 10^5 cells/ml were harvest and prepared for trypan blue staining.
6.2 Transfer 20 µl of cell suspension into appendrof.
6.3 Mix cells in ratio 1:1 with 20 µl of trypan blue solution. Allow the cell suspension mixture stand at least 5 minutes.
6.4 With the cover chamber in place, transfer the mixture to chamber of the hemacytometer. Carefully touch the edge of the cover chamber with the pipette tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.

6.5 Count cells on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at the bottom and right sides. Viable cells exclude trypan blue, while non-viable cells will stain blue due to trypan blue uptake.

6.6 Count all cells in each squares of the hemacytometer (Figure 8), represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. This is the conversion factor for the hemocytometer.

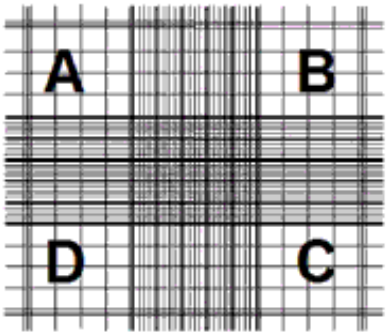

Figure 10. Corner squares on hemocytometer

6.7 Since 1 cm^3 is approximately 1 ml, the subsequent cell concentration/ml and total cell number can be determined using the following calculations;

Number of cells/ml = (average cells count/square) x (DF) x 10^4

DF = dilution factor

10^4 = chamber convertion factor

Total cells = (cells/ml) x (the original or predilution volume)

Total viable = (viable cells/ml) x (original volume)

$$\text{Cell viability (\%)} = \frac{\text{total viable}}{\text{total cells}} \times 100$$

7. Cell growth inhibiton

3-(4,5-dimethylthiazol-2-yl)-2,5-diphemyltetrazoliumbromide or MTT colorimetric assay assess the cell cytotoxicity. MTT is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria to cleave significant amounts of MTT.

7.1 The 10^5 cells/ml were placed within 96-well culture plates for 100μl in each wells. The cells were treated with 0.2, 2 and 20 ng/ml of IL-1β and TNF-α, incubated for 12, 24 and 48hr at 37 °C in a humidified atmosphere of 5% CO_2. Untreated cells were used as control. After incubation time, the cells were harvested and prepared for MTT assay.

7.2 The MTT assay was measured after various incubation time. Add 10 μl of 50mg/ml MTT solution into each well and incubated for 4 hr at 37 °C.

7.3 The reaction results in the reduction of MTT by the mitochondrial dehydrogenases of viable cells to a purple formazan product.

7.4 The MTT-formazan product dissolves in HCl/isoproplanol was estimated by measuring absorbance at 570 nm in an microplate reader.

7.5 The OD of formazam products were calculated for percentage of growth inhibition, using the following calculation;

$$\% \text{ cell inhibition} = \frac{(\text{OD of control} - \text{OD of sample})}{\text{OD of control}} \times 100$$

8. Cell apoptosis by Annexin-V-FITC and Propidium iodide staining

The cellular apoptosis was measured by membrane redistribution of phosphatidyl serine (PS), using an anexin V-FITC apoptosis detection kit, bases on the binding properties of Annexin-V to PS and on the DNA-interacting capabilities of propidium iodide (PI).

8.1 The 10^5 cells/ml were treated with 0.2, 2 and 20 ng/ml of IL-1β and TNF-α, incubated for 12, 24 and 48hr at 37 °C in a humidified atmosphere of 5% CO_2.

8.2 After incubation time, the cells were harvested by centrifugation at 12,000 rpm for 5 min and then wash the cell pellets with PBS and centrifugation at 12,000 rpm for 5 min.

8.3 Cell pellets were added with 1X binding buffer and stained with Anexin-V-FITC and Propidium iodine (PI), then leave at RT for 15 min in dark.

8.4 Analyze the stained cells by Flow cytometry.

8.5 The number of cell stained with Annexin-V-FITC was measured for percentage of cell apoptosis.

9. Cell cycle analysis

Cell cycle of the treated cells was analyzed using a flow cytometer. The method involves dissolving the cell membrane lipids with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA with an enzyme, and stabilizing the nuclear chromatin with spermine. Propidium iodide (PI) is stoichiometrically bound to the clean, isolated nuclei which are then run on a flow cytometer with electronic doublet-discrimination capability. Propidium iodide-stained nuclei emit fluorescent light primarily at wavelengths between 580 and 650 nm. The resulting fluorescence histograms may be analyzed to detect the presence of an abnormal DNA aneuploidy.

9.1 After treatment cells with various concentration of cytokines, cells were harvested by centrifuge at 3000 rpm for 5 min.

9.2 Wash pellet with PBS and centrifuge at 300 x *g* for 5 minutes, RT

9.3 Discard the supernatant, leaving approximately 50 µl of residual fluid in the tube to avoid disturbing the pellet.

9.4 Resuspend the pellet in 1 ml of buffer solution by gently vortexing at low speed. Count the cells by using a hemacytometer. Adjust the concentration to 1.0 x 10^6 cells/ml with buffer solution.

9.5 The cell suspensions were centrifuged at 400 x *g* for 5 minutes, RT. Carefully decant all the supernatant, and tap off the last drop onto a tissue paper.

9.6 Add 250 µl of trypsin buffer to each tube and gently mix by tapping the tube by hand. Do not vortex. Allow to react for 10 minutes RT.

9.7 Add 200 µl of trypsin inhibitor and RNase buffer to each tube and gently mix by tapping the tube by hand. Do not vortex. Incubate for 10 minutes at RT.

9.8 Add 200 µl of cold (2° to 8°C) propidium iodide stain solution to each tube. Gently mix as above and incubate for 10 minutes in the dark on ice or in the refrigerator (2° to 8°C).

9.9 Run samples on the flow cytometer within 3 hours after addition of propidium iodide stain solution.

9.10 The histogram plot of cell cycle analysis was measured for sub G1 population.

10. Cell apoptotic signaling pathway

In the present of active caspases, the substrate was cleaved and its bioluminescence was increased.

10.1 Caspase 3 FITC

FITC-DEVD-FMK (FITC-Asp-Glu-Val-Asp-fluoromethyl ketones), is a cell-permeable, non-toxic and irreversibly binds to activated caspase 3 in apoptotic cells. The FITC label allows for direct detection of activated caspase 3 in apoptotic cells by flow cytometry.

10.1.1 After treated cells 10^6 cells/ml, concurrently incubate a control culture without induction. Aliquot 300 µl each of the induced and control cultures into appendrof.

10.1.2 Add 1 µl of FITC labeled DEVD-FMK to each tube and incubate for 1 hr in a 37°C incubator with 5% CO_2.

10.1.3 Centrifuge cells at 3000 rpm for 5 min and remove supernatant.

10.1.4 Resuspend cells in 0.5 ml of wash buffer and centrifuge again.

10.1.5 Repeat wash step

10.1.6 Proceed to quantification by flow cytometry, resuspend cells in 300 µl of wash buffer. Put samples on ice and analyze samples by flow cytometry.

10.1.7 The percentage of FITC intensity was calculated for caspase 3 activation.

10.2 Caspase 8 FITC (extrinsic pathway)

FITC-IETD-FMK (FITC-Ile-Glu-Thr-Asp-fluoromethyl ketones), is a cell-permeable, non-toxic and irreversibly binds to activated caspase 8. The FITC label allows for detection of activated caspase 8 by measurement of fluorescence intensity by flow cytometry.

10.2.1 After treated cells 10^6 cells/ml, concurrently incubate a control culture without induction. Aliquot 300 µl each of the induced and control cultures into appendrof.

10.2.2 Add 1 µl of FITC label IETD-FMK into each tube and incubate for 1 hr in a 37°C incubator with 5% CO_2.

10.2.3 Centrifuge cells at 3000 rpm for 5 min and remove supernatant.

10.2.4 Resuspend cells in 0.5 ml of wash buffer and centrifuge again.

10.2.5 Repeat wash step

10.2.6 Proceed to quantification by flow cytometry, resuspend cells in 300 µl of wash buffer. Put samples on ice and analyze samples by flow cytometry.

10.2.7 The percentage of FITC intensity was calculated for caspase 8 activation.

10.3 Caspase 9 FITC (intrinsic pathway)

FITC-LEHD-FMK (FITC-Leu-Glu-His-Asp-fluoromethyl ketones), is cell permeable, nontoxic, and irreversibly binds to activated caspase 9. The FITC label allows for detection of activated caspase 9 by measurement of fluorescence intensity by flow cytometry.

10.3.1 After treated cells 10^6 cells/ml, concurrently incubate a control culture without induction. Aliquot 300 µl each of the induced and control cultures into appendrof.

10.3.2 Add 1 µl of FITC-LEHD-FMK to each tube and incubate for 1 hr in a 37°C incubator with 5% CO_2.

10.3.3 Centrifuge cells at 3000 rpm for 5 min and remove supernatant.

10.3.4 Resuspend cells in 0.5 ml of wash wuffer and centrifuge again.

10.3.5 Repeat wash step

10.3.6 Proceed to quantification by flow cytometry, resuspend cells in 300 µl of wash buffer. Put samples on ice and analyze samples by flow cytometry.

10.3.7 The percentage of FITC intensity was calculated for caspase 9 activation.

11. Statistical Analysis

The experiments of cell viability, growth inhibition, cell apoptosis, cell apoptotic signaling pathway and cell cycle analysis were performed in triplicate compare the effect among control (untreated cells) and treated cells and the results were expressed as mean ± S.E.M. The statistical analysis was performed by paired-t test significant statistic difference at $p\ value < 0.05$.

CHAPTER V
RESULTS

1. Growth curve of leukemic cell lines

K562 (chronic myelogenous leukemia) and HL60 (acute promyelogenous leukemia) cell lines (10^4 and 10^5 cell/ml) were cultured in RPMI 1640 medium supplement with 10% fetal bovine serum along with 2% penicillin-streptomycin and grown at 37 °C in a humidified atmosphere of 5% CO_2. The total cells were counted duplicate everyday for 7 days on hemocytometer under light microscope and the numbers of cell were plotted on linear scale. From the growth curve, the lag time, population doubling time, and saturation density were determined. In K562 cell, the doubling time of 10^4 and 10^5 cells/ml cultured were 20.6 hr and 31.7 hr, respectively (Figure 11, Appendix 2). In HL60 cell, the doubling time of 10^4 and 10^5 cells/ml cultured were 29.0 hr and 26.0 hr, respectively (Figure 12, Appendix 2). After that, the optimal concentrations of K562 and HL60 cell lines were determined by MTT assay. The various cell concentrations (10^4, $5x10^4$, 10^5, $5x10^5$ and 10^6 cells/ml) were cultured in 96 wells plate for 24 hr and measured the OD values. The suitable density of both cell lines was 10^5 cells/ml that the OD value nearly 1.0 (Figure. 13, Appendix 3). This suitable cell concentration was then used for cell cultured and treatment of cytokines.

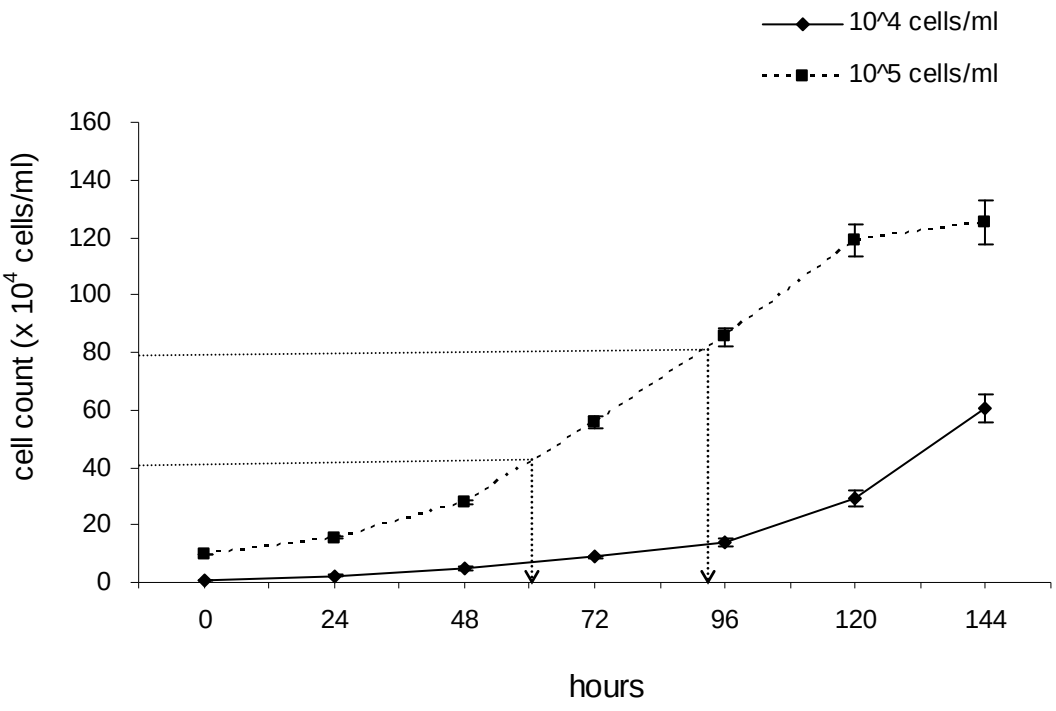

Figure 11. Doubling time of K562 cells.

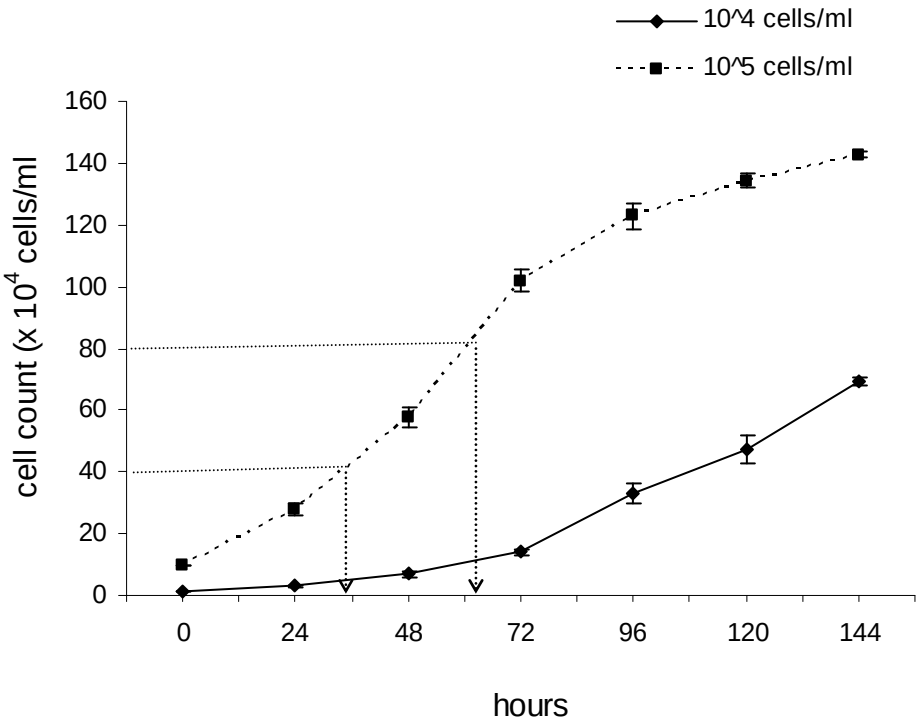

Figure 12. Doubling time of HL60 cells

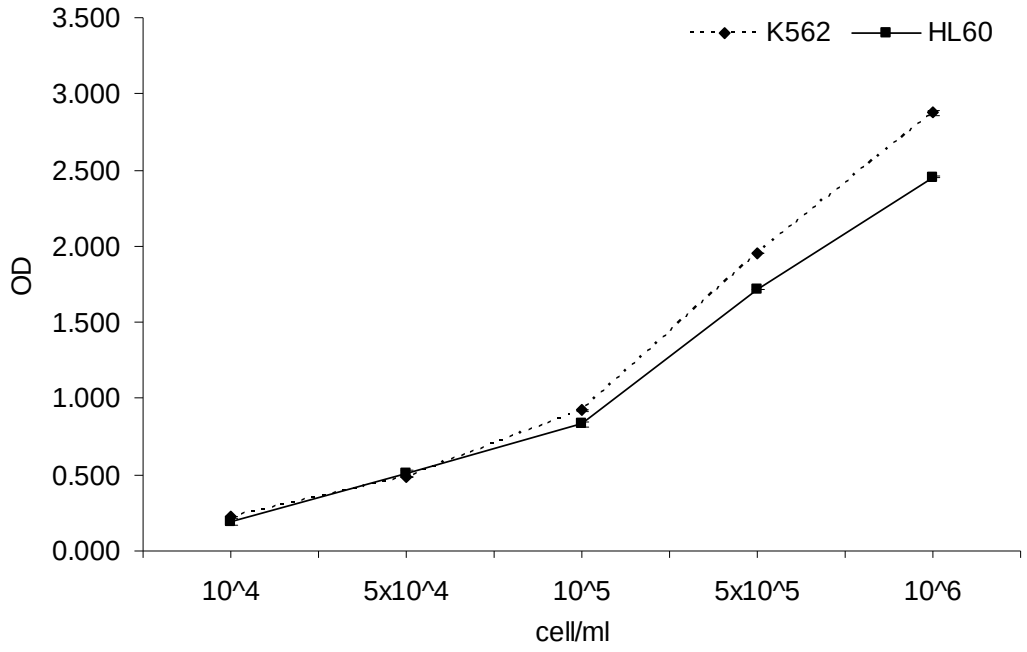

Figure 13. The optimal concentration for K562 and HL60 cell lines.

2. Effect of cytokines on cell viability of leukemic cell lines

Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor alpha (TNF-α) are pro-inflammatory cytokines that can promote apoptosis in many cells, which might be used as immunotherapy in cancers (13-16). In order to investigate the effect of cytokine on reduction of cell viability in leukemic cell lines, various concentrations of IL-1β and TNF-α at 0.2, 2 and 20 ng/ml were chosen. K562 and HL60 cell lines were cultured with these cytokines for 12, 24 and 48 hr. The percentage of cell viability was determined by trypan blue staining and compared with untreated cell as control. The results indicated that the percentage of cell viability of K562 and HL-60 were decreased after treatment with IL-1β and TNF-α. The lowest percentage of cell viability of K562 was 58.2±2.5% after treatment with 2 ng/ml of IL-1β at 24hr (Figure 14A) and 54.6±3.6% after treatment with 20 ng/ml of TNF-α at 48hr (Figure 14B, Appendix 4). Whereas, the lowest percentage of cell viability of HL60 was 58.4±4.0% after treatment with 2 ng/ml of IL-1β at 24hr (Figure 15A) and 55.2±2.8% after treatment with 20 ng/ml of TNF-α at 48hr (Figure 15B, Appendix 4).

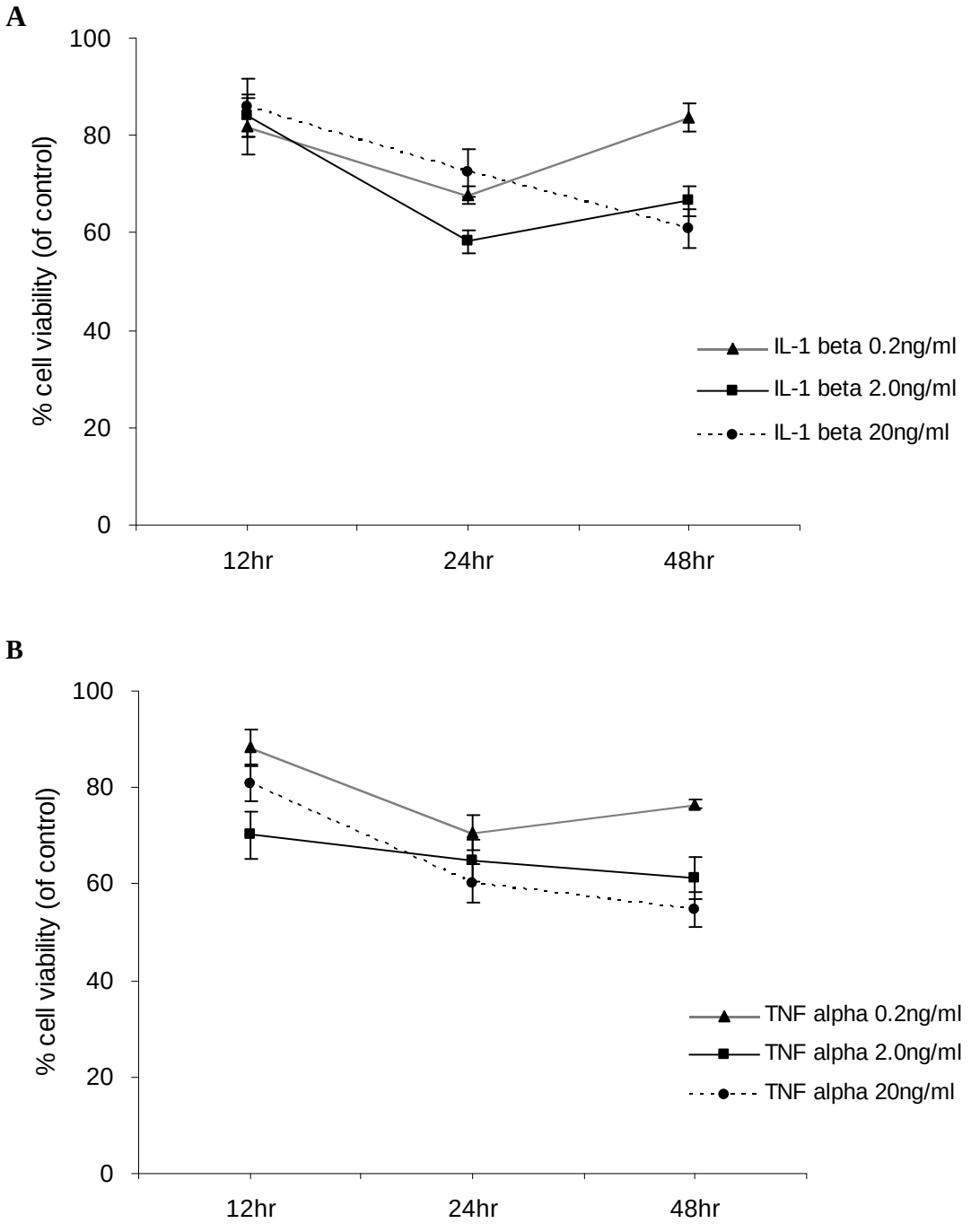

Figure 14. Percentage of cell viability of K562 cell line. Cells were treated with various concentrations of IL-1β (A) and TNF-α (B) in difference incubation time. Cells were stained with trypan blue and the number of viable cells were counted and calculated in percentage of cell viability.

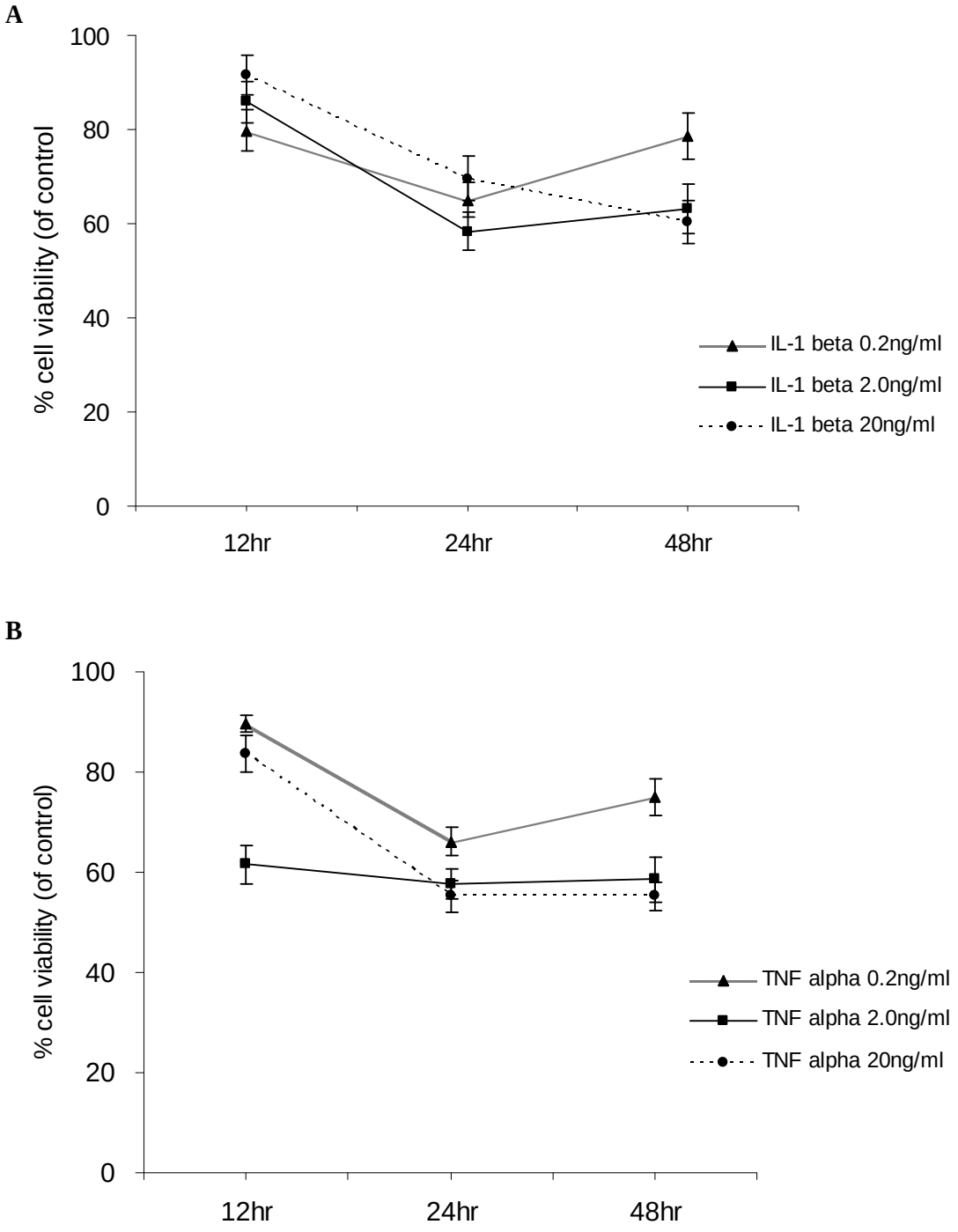

Figure 15. Percentage of cell viability of HL60 cell line. Cells were treated with various concentrations of IL-1β (A) and TNF-α (B) in difference incubation time. Cells were stained with trypan blue and the number of viable cells were counted and calculated in percentage of cell viability.

3. Effect of cytokines on growth inhibition of leukemic cell lines

Next, the effect of cytokines on cell growth inhibition was determined by MTT assay that used to investigate active mitochondria in living cells. The cells were treated with 0.2, 2 and 20 ng/ml of IL-1β and TNF-α , then incubated for 12, 24 and 48hr. After incubation time, the inhibition of cell growth was calculated. The results indicated the percent cell growth inhibition of K562 and HL-60 were increased after treatment with IL-1β and TNF-α compared with untreated cell as a control. The treatment of leukemic cell lines, K562 and HL60 with various cytokines concentrations showed a dose dependent inhibition of growth together with evident from viable cell count by trypan blue staining method. The highest percentage of cell growth inhibition of K562 cell was 11.6±0.7% after treatment with 2 ng/ml of IL-1β at 24hr and 13.2±2.1% after treatment with 20 ng/ml of TNF-α at 48hr (Figure 16, Appendix 5). Whereas, the highest percentage of cell growth inhibition of HL60 cell was 9.8±1.0% after treatment with 2 ng/ml of IL-1β at 24hr and 11.9±1.9% after treatment with 20 ng/ml of TNF-α at 48hr (Figure 17, Appendix 5). From the results of cell viability and cell growth inhibition, the suitable concentrations were used to examine the next study.

The effect of combination of cytokines, 2 ng/ml of IL-1β and 20 ng/ml of TNF-α on cell growth inhibition was also investigated. The result of the use of cytokines combination in K562 were 6.5±2.6%, 7.1±0.6 and 9.5±1.7% at 12hr, 24hr and 48hr,respectively (Figure 16, Appendix 5) and in HL60 were 7.8±1.6%, 7.6±1.0 and 8.1±1.8 at 12hr, 24hr and 48hr,respectively (Figure 17, Appendix 5).

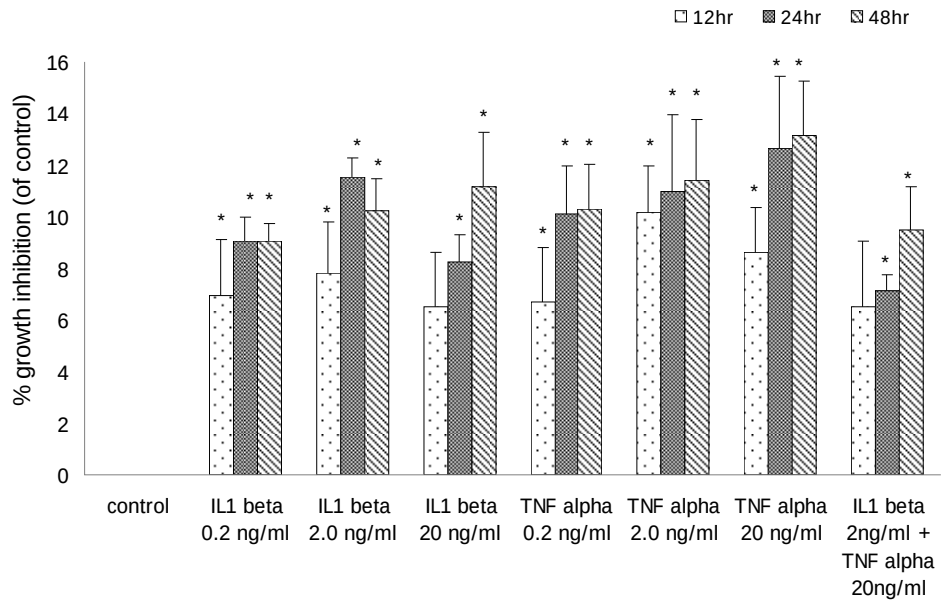

Figure 16. Percentage of cell growth inhibition of K562 cell line. Cells were treated with IL-1β or TNF-α in different concentration and incubation time. MTT assay was analyzed and calculated for the percentage of cell growth inhibition. (*, p <0.05 compare with control group)

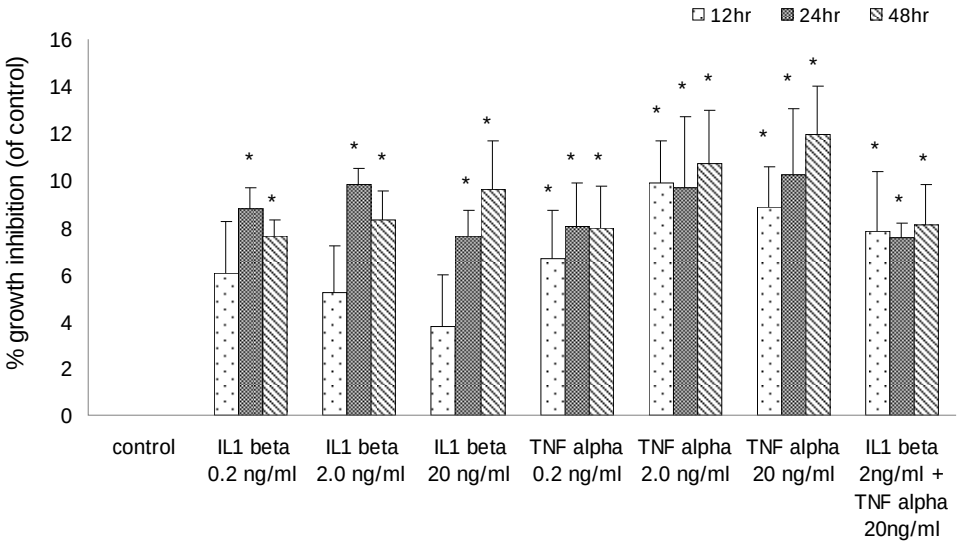

Figure 17. Percentage of cell growth inhibition of HL60 cell line. Cells were treated with IL-1β or TNF-α in different concentration and incubation time. MTT assay was analyzed and calculated for the percentage of cell growth inhibition. (*, p <0.05 compare with control group)

4. Effect of cytokines on apoptosis in leukemic cell lines

Apoptosis has been implicated in a wide range of pathological conditions. Increased apoptosis has been associated with acute ischemic diseases, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, AIDS, diabetes and hepatitis. Decreased apoptosis is involved in cancer and autoimmune disorders. Mutations and deletions of apoptotic genes play important roles in carcinogenesis, tumor growth, and tumor regression (109). Apoptotic cells have been observed in cancers. Increased apoptosis was observed in irradiated tumors and those treated with cytotoxins, implying that, increased the rate of apoptosis could be used for cancers treatment (8). To reduce leukemic cell by induce cells undergo apoptosis is widely use to treatment in leukemic patients. To measure apoptotic cells, 0.2, 2 and 20 ng/ml of IL-1β and TNF-α were treated with K562 and HL60 cell lines and incubated for 12, 24 and 48hr. After incubation time, cells were stained with Annexin-V labeled FITC and PI. The Annexin-V-FITC intensity was measured by using the flow cytometry. Since the redistribution of phosphatidylserine (PS) from the internal to the external membrane surface represents an early indicator of apoptosis, Annexin-V-FITC and its conjugates could be used for the detection of apoptosis because they interact strongly and specifically with exposed PS (7). The percentage of cell stain with Annexin-V-FITC was collected form upper and lower right quadrant and calculated for percentage of cell apoptosis (Figure 18 and 20, Appendix 6). In K562, the highest percentage of FITC stained was 13.8% after treatment with 2 ng/ml of IL-1β at 24hr and 17.6% after treatment with 20 ng/ml of TNF-α at 48hr, compare with control 6.8% and 6.5% at 24hr and 48hr, respectively (Figure 19). In HL60, the highest percentage of FITC stained was 13.0% after treatment with 2 ng/ml of IL-1β at 24hr and 11.0% after treatment with 20 ng/ml of TNF-α at 48hr, compare with control 7.5% and 6.4% at 24hr and 48hr, respectively (Figure 21). The results indicated that IL-1β and TNF-α treatment increased percent cell apoptosis of K562 and HL60.

The effect of the cytokines combination on cell apoptosis was also investigated. The FITC intensity in K562 were 5.1±0.4% and 6.0±2.0% at 24hr and 48hr, respectively and in HL60 were 4.0±0.7% and 2.4±0.1% at 24hr and 48hr, respectively. Thus, the combination of IL-1β and TNF-α are less effect on cell

apoptosis than each cytokines alone. In addition, mononuclear cells were used as normal cell to study the effect of cytokine on cell apoptosis. Mononuclear cells were isolated from peripheral blood of healthy sample by using histopaque. CD34 positive cells were isolated by MACCS® column and cultured in RPMI 1640 with IL-3, SCF and GM-CSF for myeloid cells (Appendix 1). After culture for 3 day, the normal cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α for 24hr and 48hr. The treated cells were stained by Annexin-V-FITC and the percentage of FITC intensity was analyzed by flow cytometry. The percentage of Annexin-V-FITC in cytokines treatment of normal cell was lower than control untreated cell in 24hr and 48hr. In 24hr, the FITC intensity in control was 11.21%, whereas in IL-1β and TNF-α treatment of normal cells were 10.85% and 9.46%, respectively. In 48hr, the FITC intensity in control was 9.08%, whereas in IL-1β and TNF-α treatment of normal cell were 9.04% and 8.12%, respectively (Figure 22, Appendix 7).

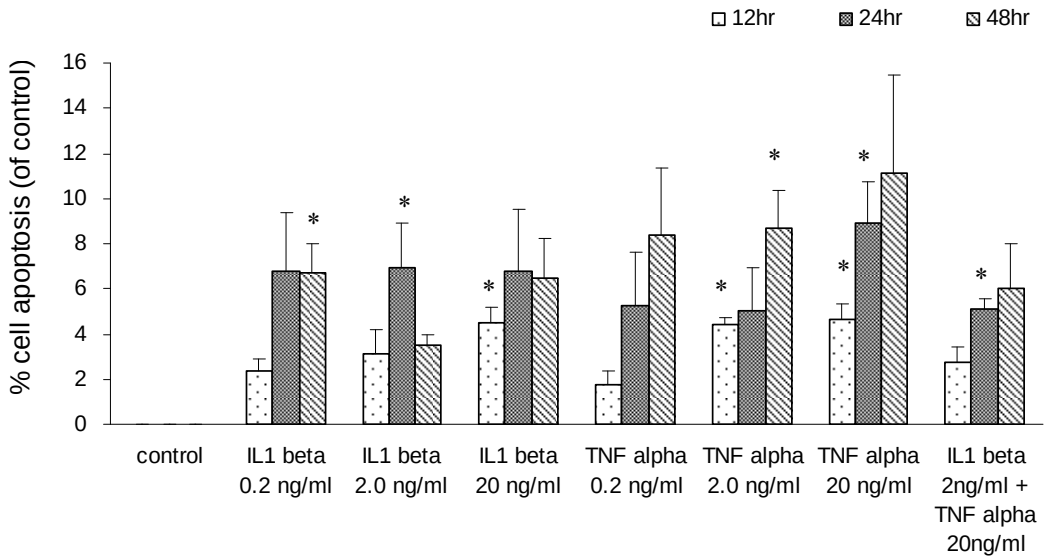

Figure 18. Percentage of cell apoptosis of K562 cell lines. Cells were treated with IL-1β or TNF-α in different concentration and incubation time. The percentage of cell apoptosis was calculated from Annexin-V-FITC intensity. The highest percentage of apoptosis (of control) was shown in cell treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24 and 48hr, respectively. (*, $p < 0.05$ compare with control group)

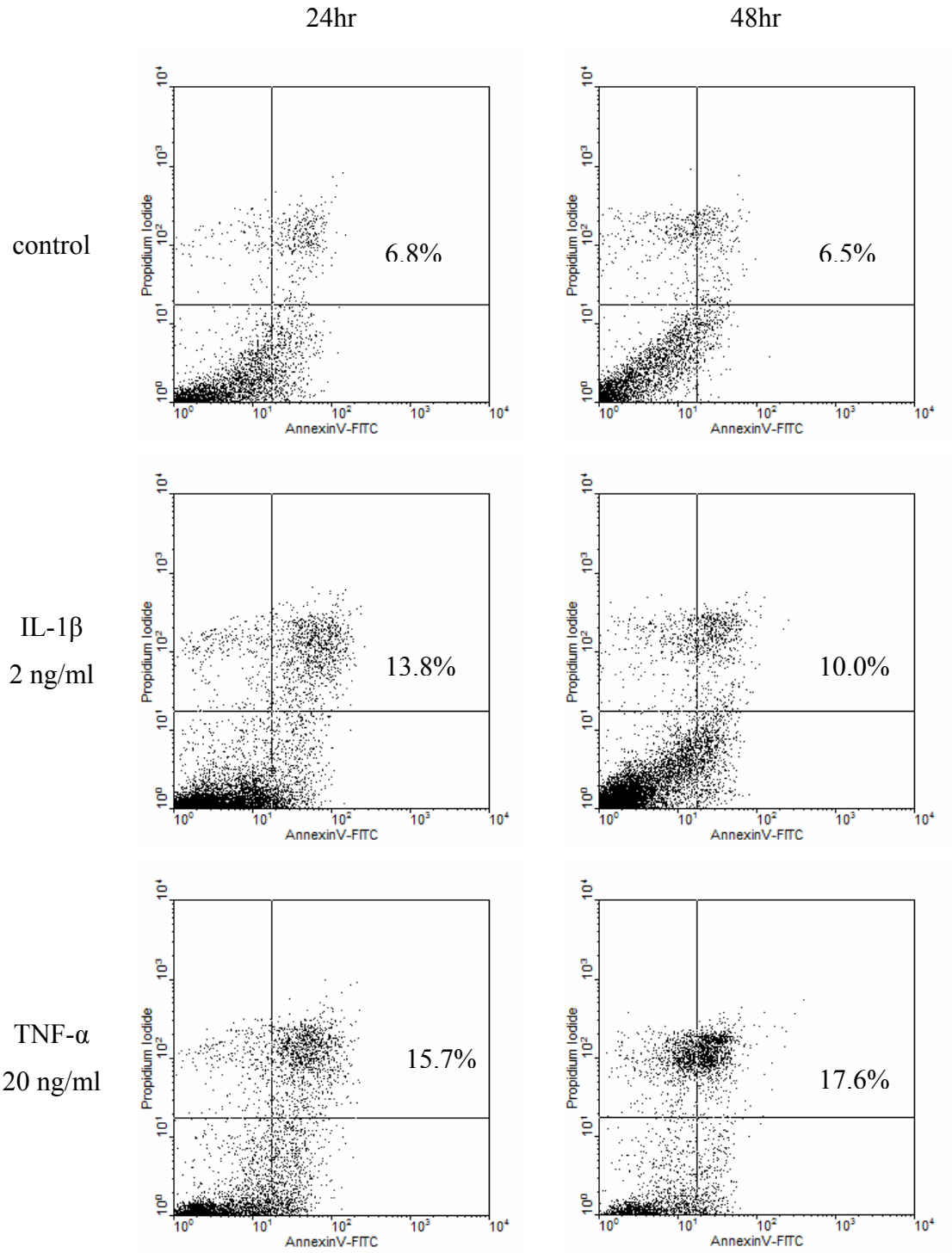

Figure 19. Flow cytometry analysis of Annexin-V-FITC and PI staining of K562 cell line. Cells were treated with IL-1β or TNF-α in different concentration and incubation time. Annexin-V-FITC intensity was analyzed by flow cytometry and calculated for the percentage of cell apoptosis (of control).

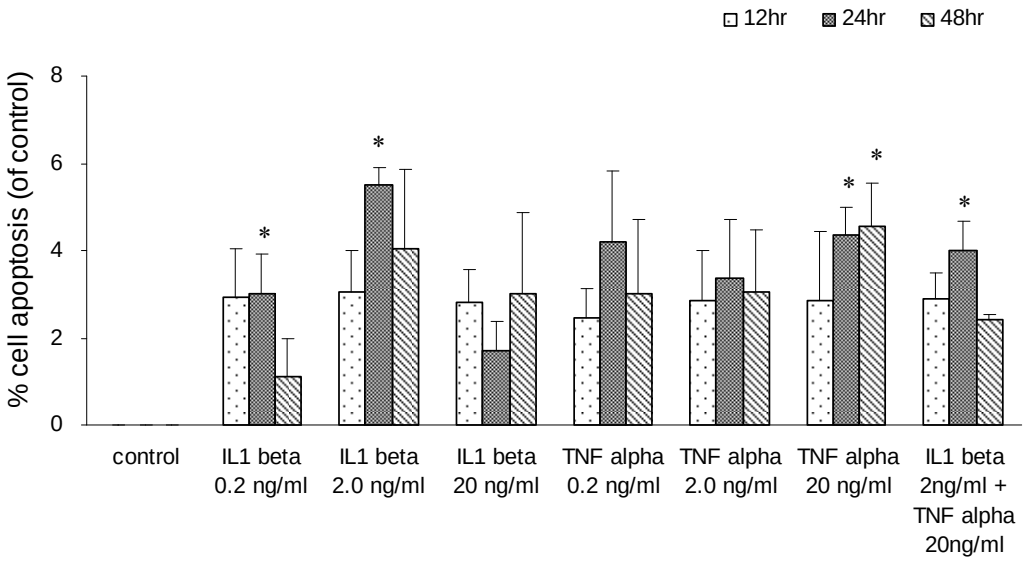

Figure 20. Percentage of cell apoptosis of HL60 cell lines. Cells were treated with IL-1β or TNF-α in different concentration and incubation time. The percentage of cell apoptosis was calculated from Annexin-V-FITC intensity. The highest percentage of apoptosis (of control) was shown in cell treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24 and 48hr, respectively. (*, $p < 0.05$ compare with control group)

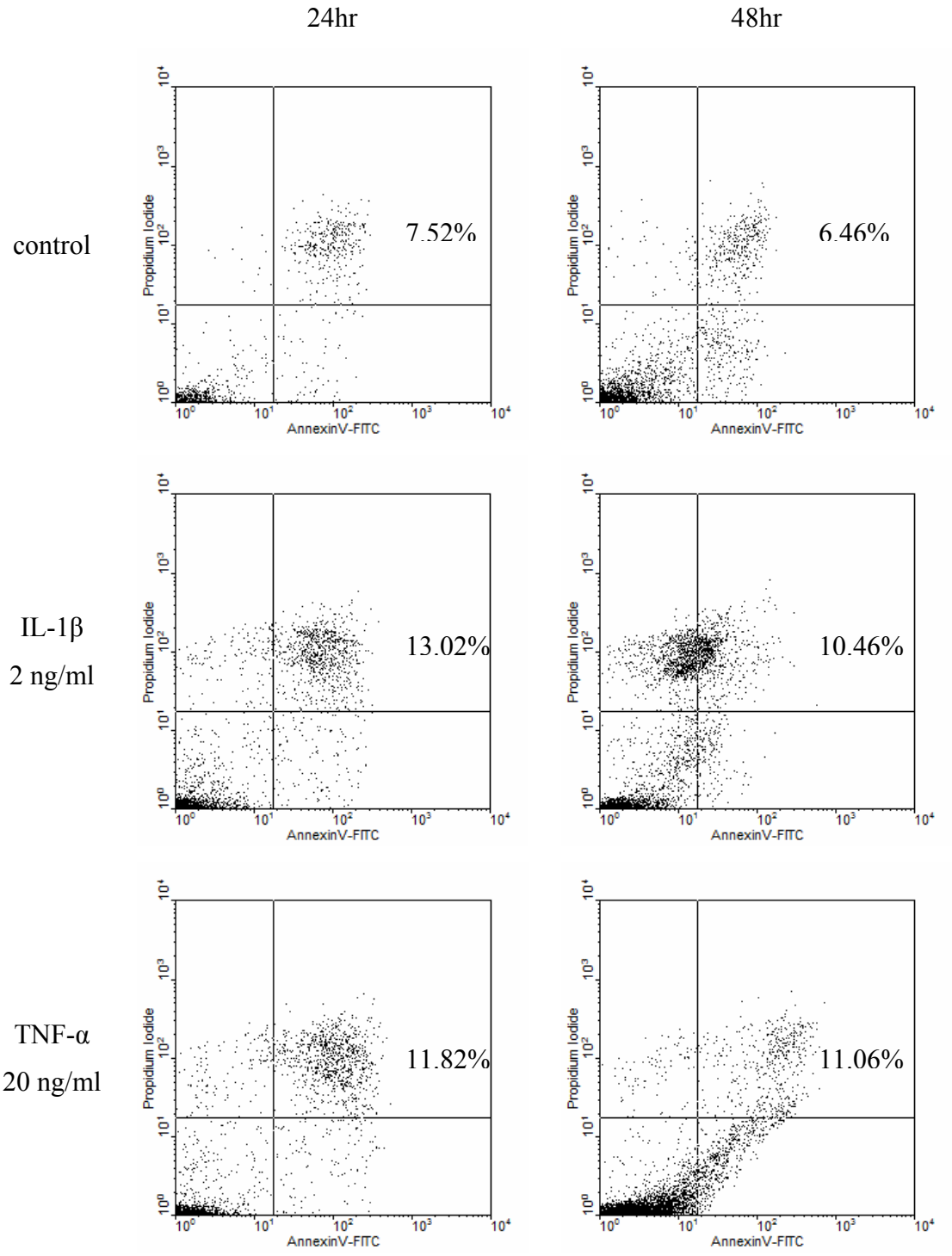

Figure 21. Flow cytometry analysis of Annexin-V-FITC and PI staining of HL60 cell line. Cells were treated with IL-1β or TNF-α in different concentration and incubation time. Annexin-V-FITC intensity was analyzed by flow cytometry and calculated for the percentage of cell apoptosis (of control).

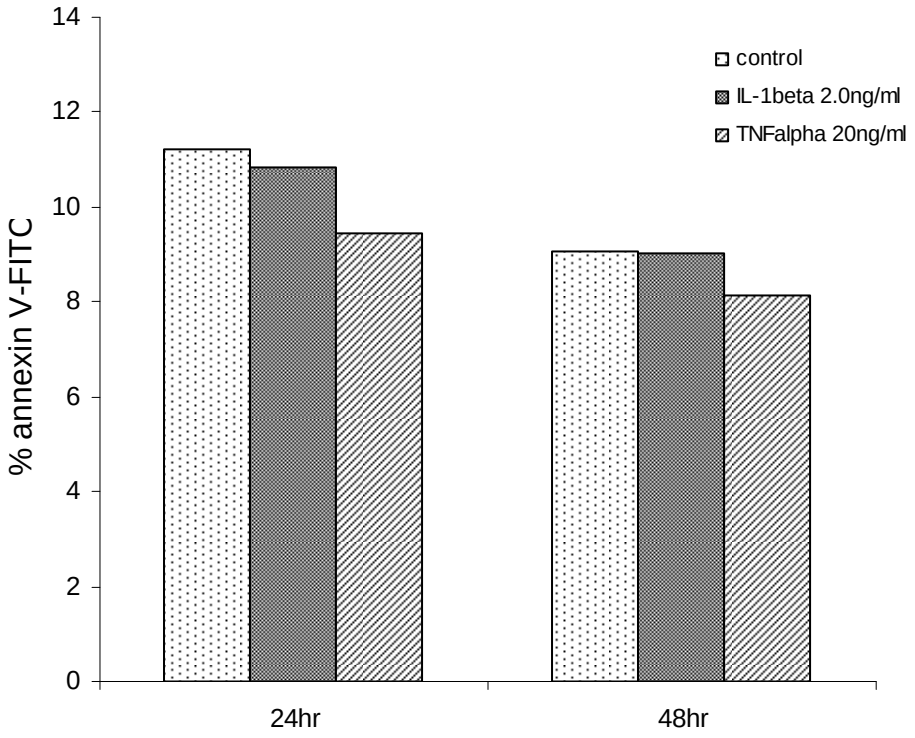

Figure 22. Percentage of Annexin-V-FITC intensity in normal cells. Normal cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α in 24hr and 48hr. Cells were analyzed by flow cytometer for percentage of Annexin-V-FITC intensity.

5. Effect of cytokines on the apoptotic feature of leukemic cell lines

In the induction of morphological features of apoptotic cell were also evident upon microscopic examination. The morphological features of cell apoptosis such as cell shrinkage, chromatin condenses and presence of apoptotic bodies (109). From the results of cell viability and cell growth inhibition, 2 ng/ml of IL-1β and 20 ng/ml of TNF-α were used to treatment K562 and HL60 cells for 24hr for 48hr, respectively, compare with untreated cell at 24hr. Then the cells were observed fresh preparation under inverted microscope, Wright's-Giemsa stain under light microscope and Annexin-V-FITC stain under fluorescence microscope. From the fresh preparation, in both cells treated with suitable concentration of IL-1β and TNF-α to induce cell apoptosis shown apoptotic feature including membrane blebbing and apoptotic body occurred whereas control cell shown normal feature and healthy cell (Figure 23). From Wright's-Giemsa stain, the morphological of treated cell shown membrane blebbing, apoptotic body, the reduction in the volume and nuclear chromatin condensation where as the control cell shown normal feature of leukemic cell (Figure 24). The differentiation between apoptotic and necrotic cells can be performed by simultaneous staining with Annexin-V labeled FITC and propidium iodide (PI). The treated cells shown green color stained on the cells with Annexin-V-FITC indicated those cells underwent apoptosis, while the cells stained red color of PI indicated those cells were necrosis cells. Where as, the control cell was less stained with Annexin-V-FITC or PI (Figure 25). The arrows indicate the death cells or apoptotic cells.

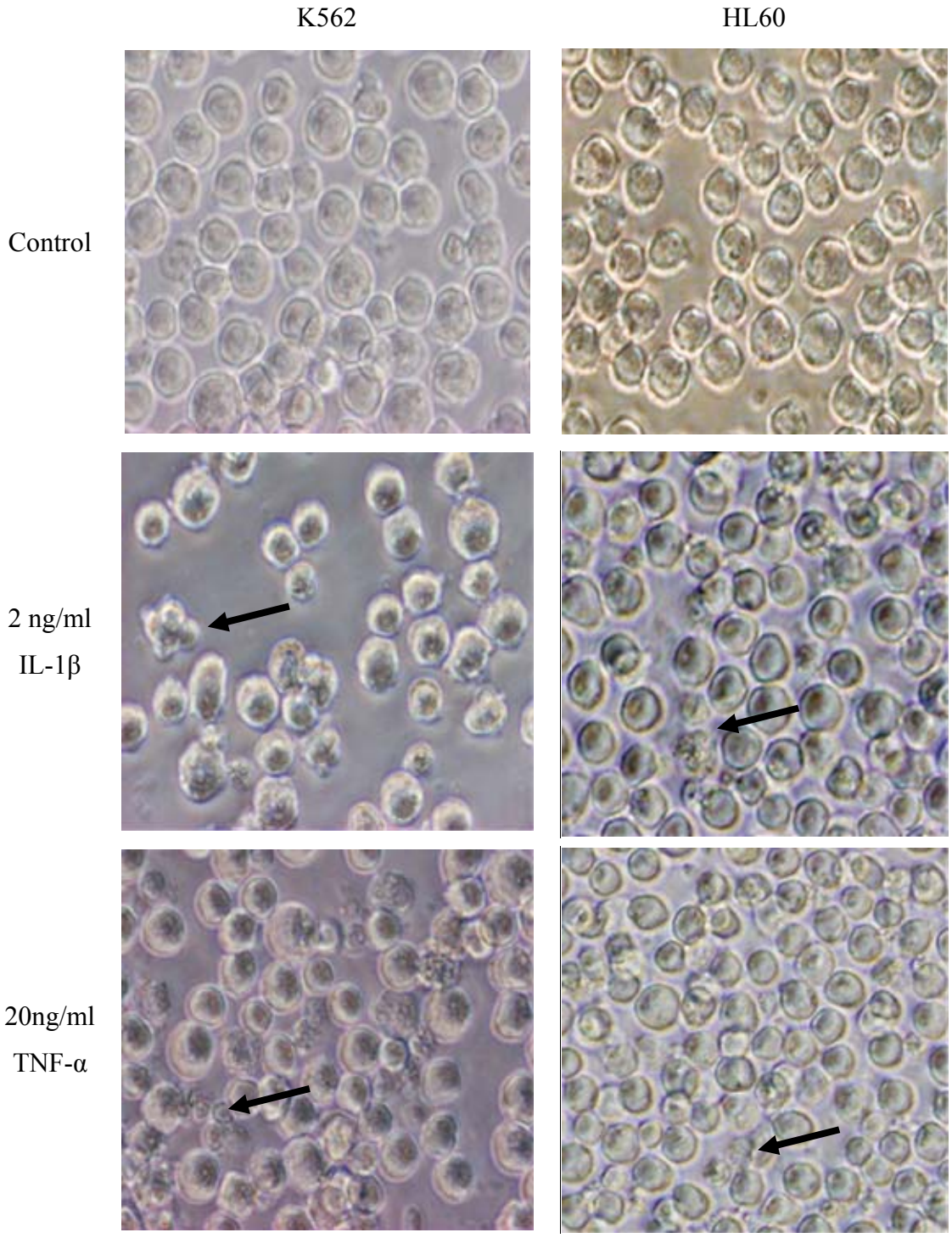

Figure 23. Fresh preparation of K562 and HL60 cells. Cells were treated with 2 ng/ml of IL-1β for 24hr and 20 ng/ml of TNF-α for 48hr compared with control at 24hr. After that, cells were observed apoptotic feature under inverted microscope (40X).
← indicate apoptotic cells.

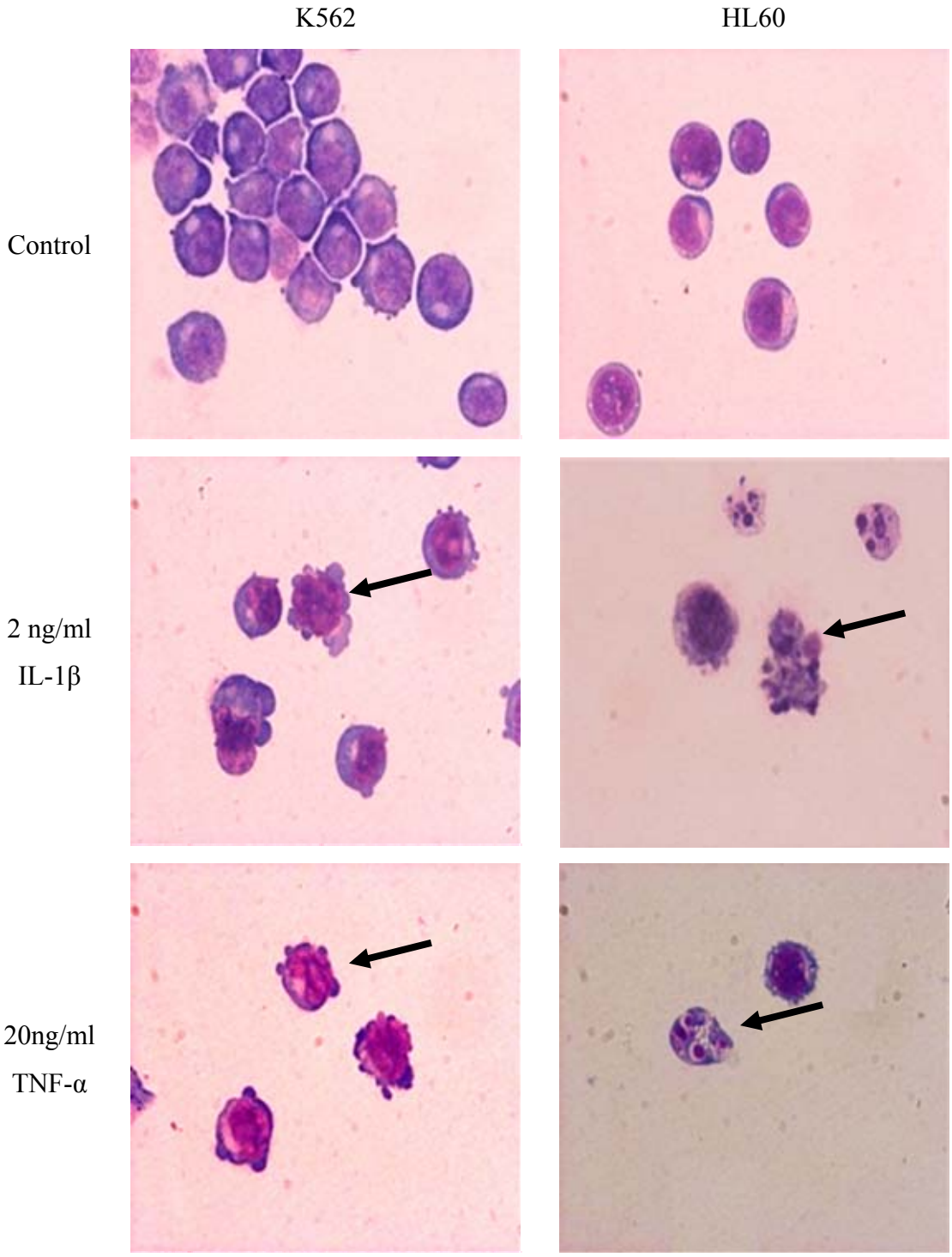

Figure 24. Wright-Giemsa staining of K562 and HL60 cells. Cells were treated with 2 ng/ml of IL-1β for 24hr and 20 ng/ml of TNF-α for 48hr compared with control cells at 24hr. After that, cells were stained and observed apoptotic feature under light microscope (40X). ← indicate apoptotic cells.

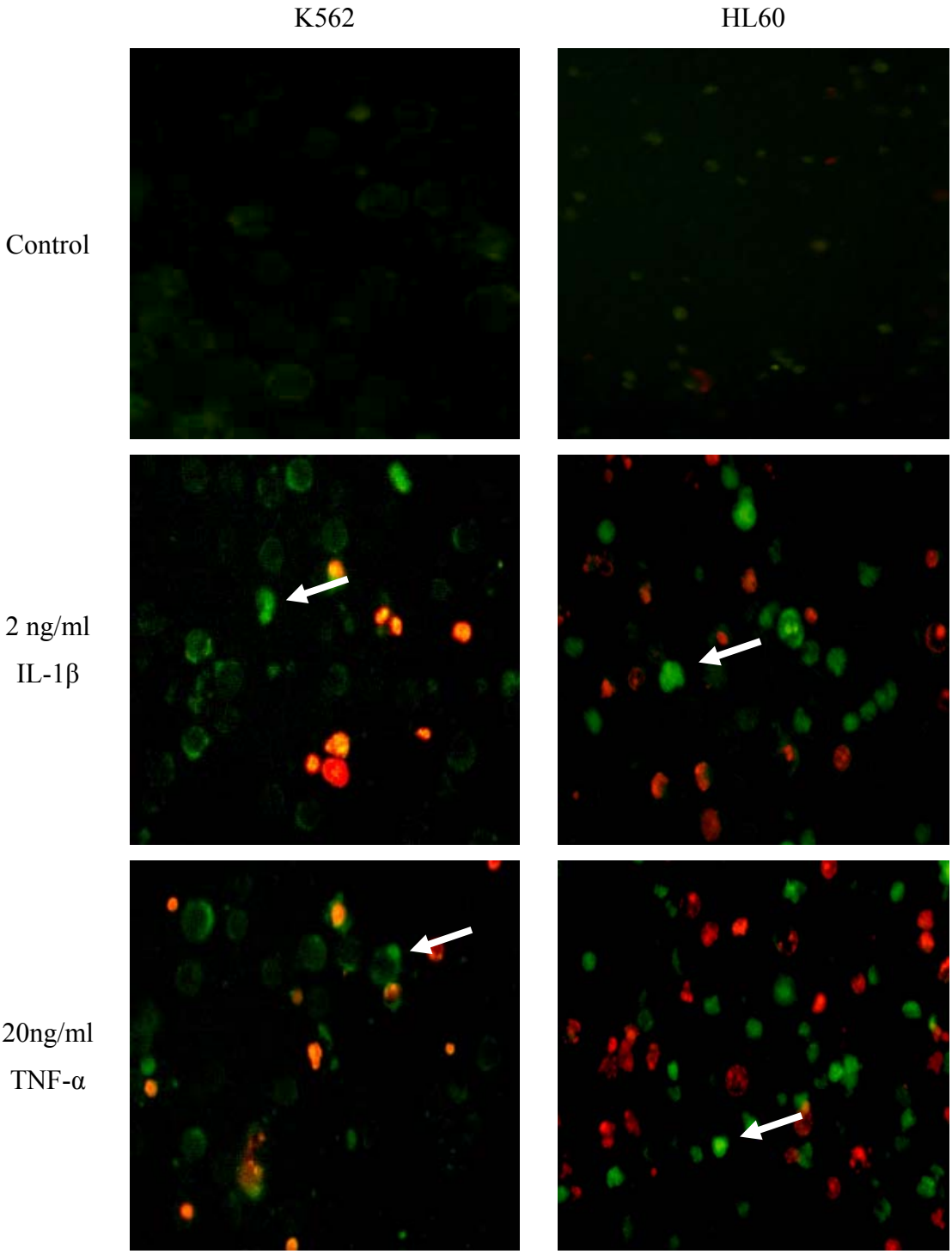

Figure 25. Annexin-V-FITC and PI staining of K562 and HL60 cells. Cells were treated with 2 ng/ml of IL-1β for 24hr and 20 ng/ml of TNF-α for 48hr compared with control cells at 24hr. After that, cells were stained and observed apoptotic feature under fluorescence microscope (40X). ← indicate apoptotic cells.

6. Effect of cytokines on DNA content of cell cycle in leukemic cell lines

Cell cycle is the series of events that take place in a eukaryotic cell leading to its replication. These events can be divided in two phase, interphase and mitotic. Interphase periods during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA that consists of four distinct phases: G1 phase, S phase and, G2 phase. The mitotic or M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm divides forming distinct cells. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing entered a state of quiescence called G0 phase (109,110). Apoptotic cells appear in a hypodiploid sub G0/1 peak as a consequence of partial DNA loss. Because the nucleus becomes fragmented during apoptosis and numerous individual chromatin fragments may be present in a single cell, the percentage of objects with a fractional DNA content is represented by the subG1 peak (111). Cells with DNA content less than G1 in the cell cycle distribution were counted as hypodiploid cells. The hypodiploid cells in the subG1 population after cytokines treatment were determined by flow cytometry analysis. All cycle phase populations were calculated (Appendix 8). The results indicated that increased subG1 poppulation of K562 and HL60 after treatment with IL-1β and TNF-α. The percentages of subG1 population in K562 incubation with concentration of 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24hr were 11.4±0.4% and 17.5±0.5%, respectively and at 48hr were 8.5±0.4% and 11.5±0.5%, respectively (Figure 26,27). While, the percentages of subG1 population in HL60 at 24hr were 4.3±0.1% and 4.0±0.1%, respectively and at 48hr were 3.2±0.4% and 3.3±0.2%, respectively (Figure 28,29). The subG1 population of control of K562 at 24hr and 48hr were 3.1±0.1% and 3.7±0.6%, respectively and the subG1 population of control of HL60 at 24hr and 48hr were 1.7±0.1% and 1.4±0.3%, respectively. The resulted in the appearance of a hypodiploid peak, probably due to the presence of apoptosing cells and/or apoptotic bodies with DNA content less than 2*n*.

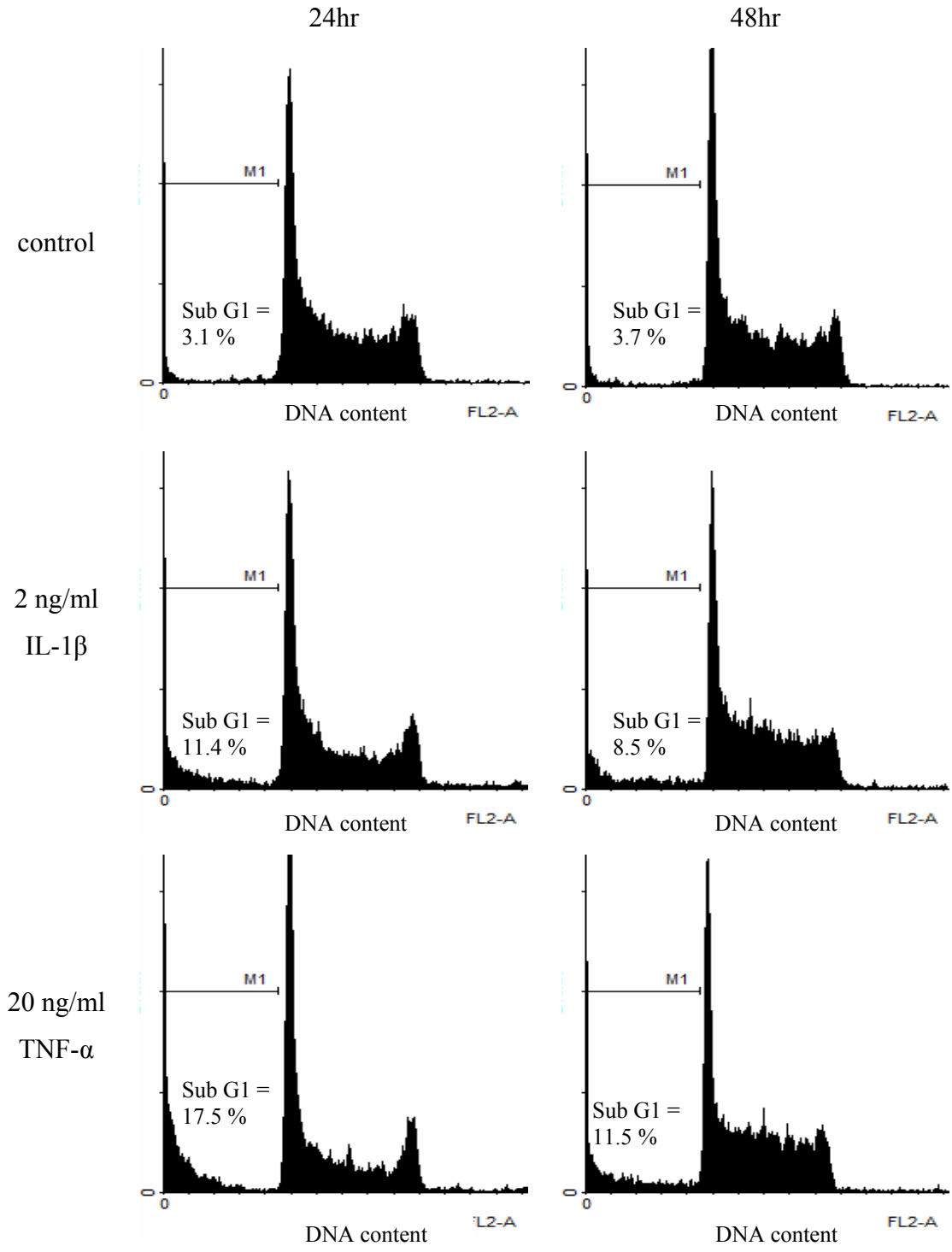

Figure 26. Flow cytometry analysis of cell cycle of K562 cell line. Cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α in 24hr and 48hr. Cell cycle phase was analyzed by flow cytometry and Sub-G1 populations were calculated.

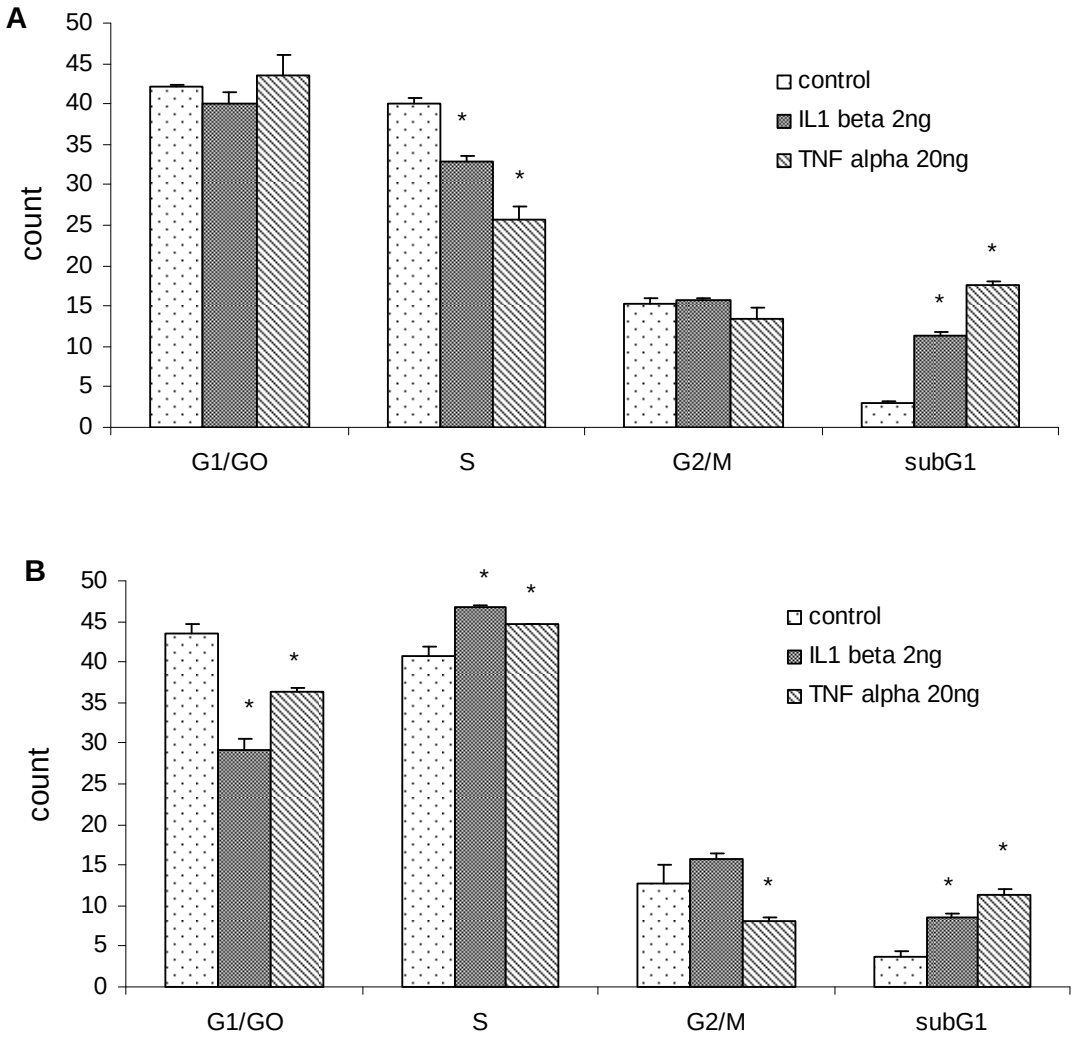

Figure 27. Percentage of cell in cycle phase fractions of K562 cell line. Cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24 (A) and 48hr (B). Cell cycle phase was analyzed by flow cytometry and all cycle phase populations were calculated.

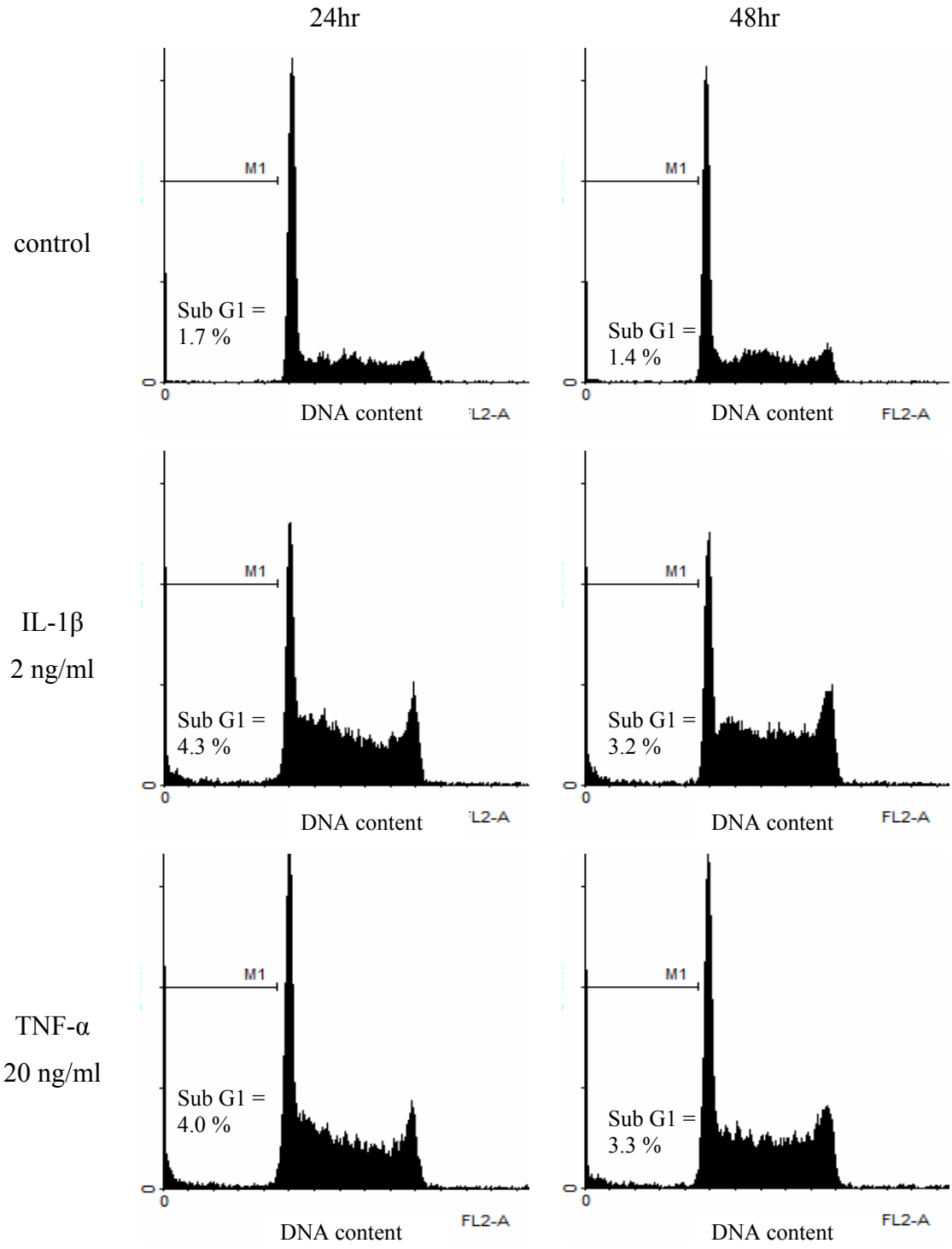

Figure 28. Flow cytometry analysis of cell cycle of HL60 cell line. Cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α in 24hr and 48hr. Cell cycle phase was analyzed by flow cytometry and Sub-G1 populations were calculated.

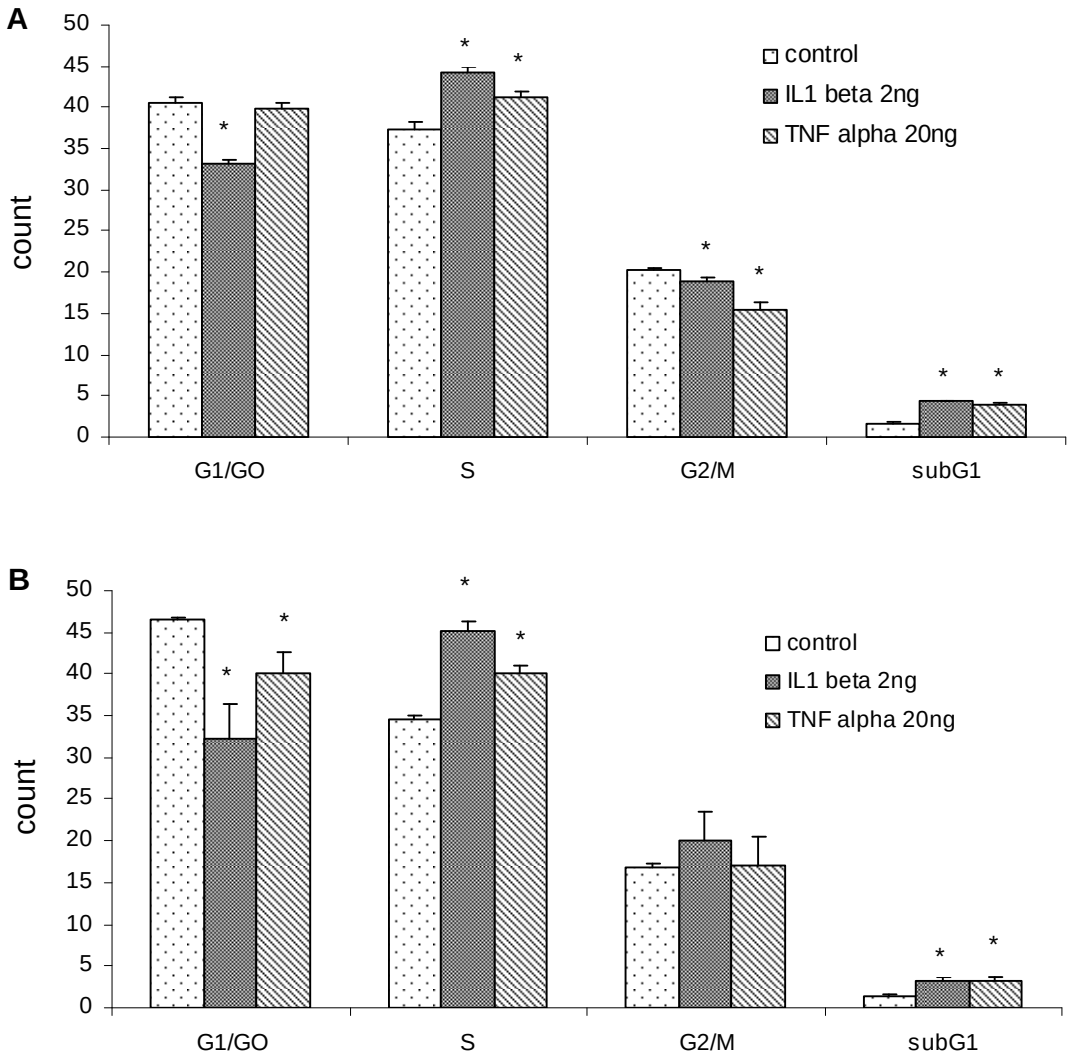

Figure 29. Percentage of cell in cycle phase fractions of HL60 cell line. Cells were treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24 (A) and 48hr (B). Cell cycle phase was analyzed by flow cytometry and all cycle phase populations were calculated.

7. Effect of cytokines on caspases activation in apoptotic signaling pathway

Next, the apoptotic signaling pathways induced by cytokines were investigated, there are two major apoptotic pathways involved in caspase activation. The death receptor-mediated pathway (extrinsic pathway) through the deathinducing signaling complex as an activating complex for pro-caspases 8 and the mitochondria-mediated pathway (intrinsic pathway) through the apoptosome as activating complex for procaspase-9. (95). Both pathways activate the enzymatic caspase cascade, which carries out numerous proteolytic events that mediate caspase 3 to the apoptotic cell death program (112). To study the involvement of caspases in apoptotic pathways of K562 and HL60 cells, assessment of caspase 3, 8, and 9 fluorimetric assay was performed by flow cytometry. Caspase detection kit measured the amount of activated caspase in living cells by using FITC as a fluorescent marker and irreversibly binds to activated caspase in apoptotic cells. K562 and HL60 cells treated with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α in 24hr and 48hr. FITC, as the fluorescent marker, was stained and measured the percentage of FITC intensity as the activity of active caspase. In K562, the activation of caspase 3 was increased to 4.8±0.8% after treatment with IL-1β in 24hr and decreased to 3.1±0.7% in 48hr, whereas the activation of caspase 3 was increased from 3.1±0.4% in 24hr to 7.3±0.1% in 48hr after treatment with TNF-α (Figure 30A, Appendix 9). In HL60, the activation of caspase 3 was increased to 4.5±1.2% after treatment with IL-1β in 24hr and decreased to 3.9±0.4% in 48hr, whereas the activation of caspase 3 was increased from 3.5±0.3% in 24hr to 6.8±0.8% in 48hr after treatment with TNF-α (Figure 30B, Appendix 9). These results indicated that IL-1β and TNF-α induce caspase 3 activation mainly in 24 and 48hr, respectively. To investigate whether caspase 8 (extrinsic) or caspase 9 (intrinsic) play role in upstream apoptotic signaling pathways. The activated caspase 8 and 9 was also determined by flow cytometer. In K562, the activated caspase 8 in IL-1β treatment was 1.4±0.1% and 1.1±0.3% at 24hr and 48hr, respectively (Figure 31A, Appendix 10). While, the activated caspase 9 of IL-1β treatment was 1.6±0.2% and 0.9±0.1% at 24hr and 48hr, respectively (Figure 31B, Appendix 11). In the activated caspase 8 of TNF-α treatment was 3.8±0.1% at 24hr and decrease to 2.5±0.4% at 48hr (Figure 31A, Appendix 10). While, the activated caspase 9 in TNF-α treatment was

1.1±0.2% and 1.3±0.1% at 24hr and 48hr, respectively (Figure 31B, Appendix 11). From these result 2 ng/ml of IL-1β could induced caspase 8 and caspase 9 activation in K562 in low level. However, 20 ng/ml of TNF-α could induce the activation of caspase 8 but not caspase 9 activation (Figure 31, Appendix 10-11). In HL60, the caspase 8 activation in IL-1β treatment was increased to 3.2±0.4% at 24hr and decreased to 2.0±0.4% ar 48hr (Figure 32A, Appendix 10). As same as, the caspase 9 activation of IL-1β treatment was increased to 3.6±0.6% at 24hr and decreased to 1.5±0.4% at 48hr (Figure 32B, Appendix 11). In the caspase 8 activation of TNF-α treatment was 3.6±0.2% at 24hr and decrease to 2.6±0.2% at 48hr (Figure 32A, Appendix 10). While, the caspase 9 activation in TNF-α treatment was 1.5±0.2% and 1.5±0.2% at 24hr and 48hr, respectively (Figure 32B, Appendix 11). From the result, it suggests that 2 ng/ml of IL-1β induced caspase 8 and caspase 9 activation and 20 ng/ml of TNF-α treatment induced the activation of caspase 8 but not caspase 9 activation (Figure 32, Appendix 10-11).

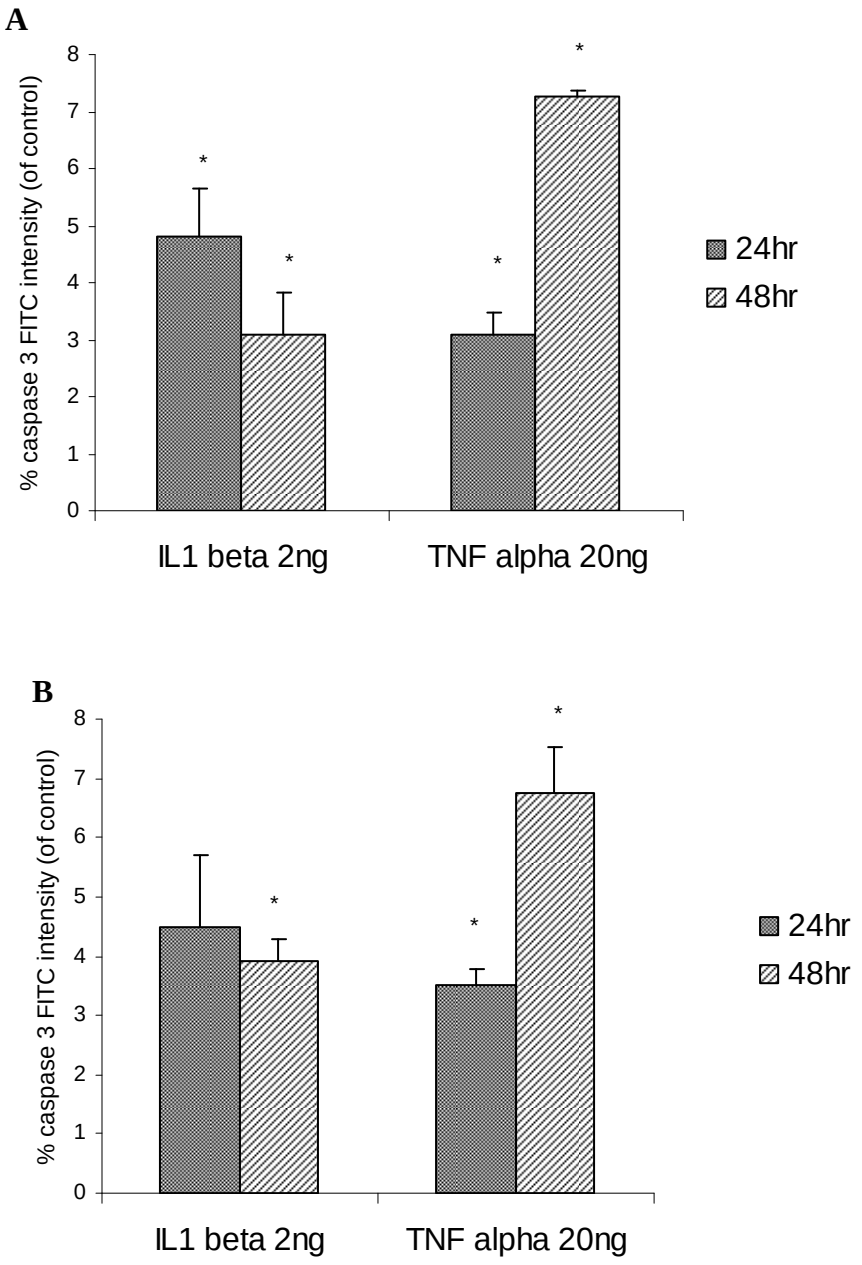

Figure 30. Percentage of FITC intensity of caspase 3 in K562 (A) and HL60 (B) cell line. Cells were treated with IL-1β and TNF-α in 24hr and 48hr. FITC as the fluorescent marker was stained and measured the intensity by flow cytometry.

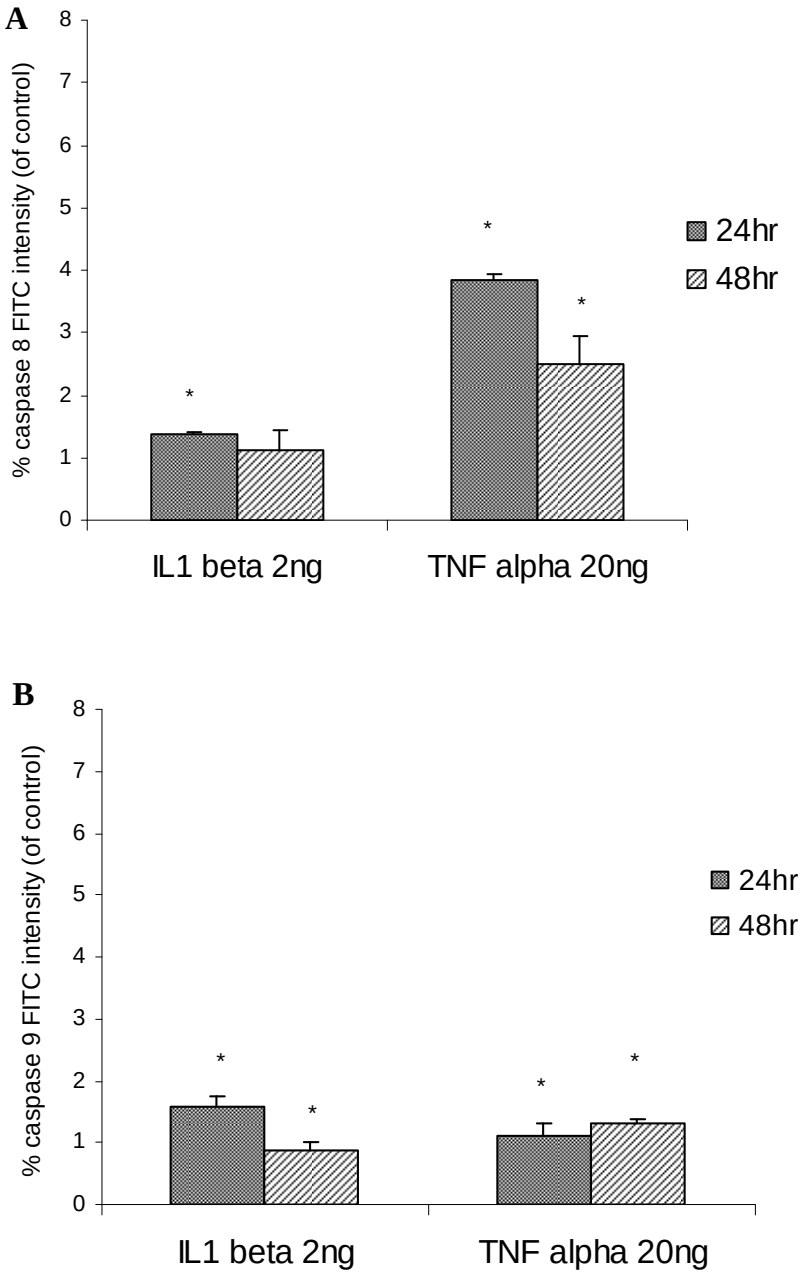

Figure 31. Percentage of FITC intensity of caspase 8 (A) and caspase 9 (B) in K562 cell line. Cells were treated with IL-1β and TNF-α in 24hr and 48hr. FITC as the fluorescent marker was stained and measured the intensity by flow cytometry.

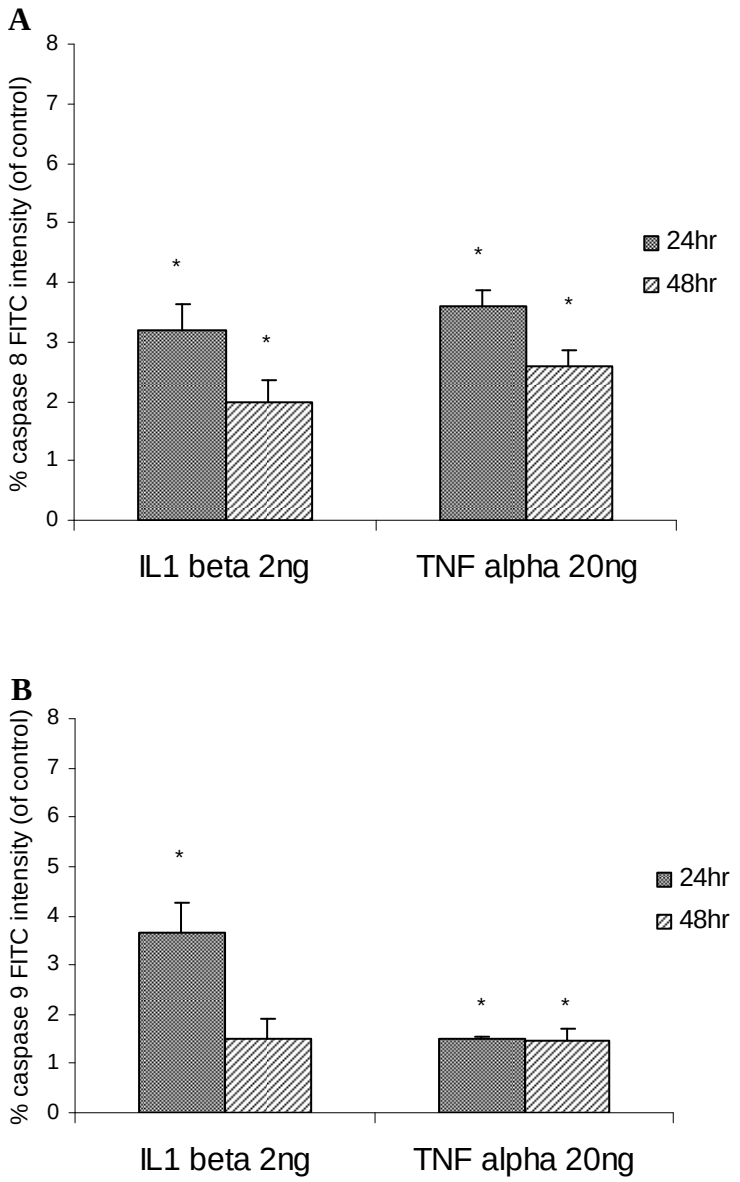

Figure 32. Percentage of FITC intensity of caspase 8 (A) and caspase 9 (B) in HL60 cell line. Cells were treated with IL-1β and TNF-α in 24hr and 48hr. FITC as the fluorescent marker was stained and measured the intensity by flow cytometry.

CHAPTER VI
DISCUSSION

Leukemia develops via the dysregulation of the balance between cell proliferation and apoptosis (7). Antiproliferative effect of cytokine in human leukemic cell has been reported to be involved in some signaling pathway (16). Interferon-α (IFN-α) was the first-line therapy for patients with chronic-phase CML. There are both clinical and laboratory evidence suggesting imatinib therapy alone is not curative in CML, whereas IFN has induced a low but reproducible curative effect in some patients (13). IL-1β and TNF-α are the cytokines that have been report to be related with apoptosis in many cells (69). IL-1β regulates human trophoblastic cell growth by induction of cell cycle arrest by delaying the G0/G1 phase and triggered apoptotic cell death (113). However, there are controversial report in concerning the involvement IL-1β and TNF-α in cancer promotion and progression (114,116).

In this study, we report that IL-1β and TNF-α could induce apoptosis in leukemic cell lines, K562 and HL60 cell, and the apoptosis was induced through caspase cascade. The percentage of cell viability of treated cells was reduced in dose and time dependent. The suitable concentration and incubation time was 2 ng/ml of IL-1β at 24hr and 20 ng/ml of TNF-α at 48hr in both cell lines. Apoptotic feature was observed by Wright's staining and percentage of apoptosis was measured by Annexin-V labeled FITC staining and analyzed by flow cytometry. The morphological changes of treated cells showed apoptotic bodies including condensation of chromatin and nuclear fragmentation (90), implying that the mode of cell death is mediated through apoptosis. In early apoptosis, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner face of the plasma membrane to the cell surface, and exposed to the external environment. Annexin-V is a phospholipid-binding protein that has a high affinity to PS, and easily binds to cells which exposed PS. Annexin-V may be conjugated with FITC, to serve as a sensitive probe for flow cytometric

analysis of cells that are undergoing apoptosis (7). Percentage of apoptotic cell was increased after treatment K562 and HL60 cell with IL-1β and TNF-α.

It is known that cell proliferation is mediated by cell cycle progression. Thus, the inhibition of cell cycle progression might be an appropriate target for the regulation of cancer. The cell cycle consists of several phases. In the G1 phase, the cell grows and becomes larger. When it has reached a certain size it enters the S phase, in which DNA-synthesis takes place. The cell duplicates its hereditary material (DNA-replication) and a copy of each chromosome is formed. During the next phase (G2 phase) the cell checks that DNA-replication is completed and prepares for cell division. The chromosomes are separated (mitosis, M) and the cell divides into two daughter cells. Through this mechanism the daughter cells receive identical chromosome set ups. After division, the cells are back in G1 and the cell cycle is completed (109). DNA content of cells with fragmented nuclei is lower than at G1 phase and is present in the sub-G1 compartment. IL-1β and TNF-α in suitable concentration could induce cells undergoing apoptosis. The sub-diploidy cells with fragmented nuclei thus were increased compare with the untreated control cells.

Apoptotic signaling pathway was determined through caspase cascade; caspase 3, 8 and 9. It is known that caspase 3, the main executioners or effecter caspase (92), can be activated by initiator caspase 8 and/or caspase 9. The extrinsic pathway involves the activation of caspase 8 by receptor-mediated signaling, whereas intrinsic pathway involves the activation of caspase 9 by cytochrome c release from mitochondria (10). In this study, we found that cytokines participate in caspases induced apoptosis in K562 and HL60 cells. IL-1β and TNF-α induce activation of caspase 3, leading the cells to undergo apoptosis. The upstream caspase 8 and 9 might be involved in the pathway. In K562, IL-1β induced caspase 8 and caspase 9 activation in low level, and TNF-α induces activation of caspase 8 but not caspase 9. However, in HL60 cell, IL-1β induced caspase 8 and caspase 9 activation, and TNF-α induces activation of caspase 8 but not caspase 9. The reason why IL-1β induced caspase 8 and 9 activation in K562 in low level remains unclear. It could be resulted from different amount and types of IL-1β receptor on different type of cell lines, leading to the induction of different downstream caspase signaling. Results from the caspases pathway analysis suggest that TNF-α induced apoptosis might be involved in

extrinsic pathway of apoptosis in both cell lines. However, IL-1β might be involved in both extrinsic and intrinsic pathways in HL60 cell line. Many anti-cancer drugs trigger the extrinsic pathway in which Fas receptor-mediated signals induce caspase 8 activation (112). The degree of induction of apotosis via extrinsic pathway depends on the receptor on the cell surface. TNF-α and IL-1β have their receptors that can transmitted the signal into the cell and activate of caspase-8 by FADD to start the caspase cascade. Another apoptosis execution pathway activated by TNF is the evolutionarily conserved lysosomal death pathway, which mainly mediated by the cathepsin protease family (89). The initial step in signaling for the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily is the association of ligand, receptor and co-receptor. This enables recruitment of the adapter molecule, MyD88 which then recruits the serine/threonine kinases, namely IL-1R-associated kinase-1 (IRAK-1), IRAK-2, IRAK-M and IRAK-4 to the activated complex through interactions of death domains in the kinases and in the N-terminal portion of the adapter protein (83). However, there is evidence that mature form of IL-1β may directly pass through the plasma membrane using molecularly undefined transporters (115), then IL-1β could activated caspase pathway and lead to apoptosis *via* intrinsic pathway.

CHAPTER VIII
CONCLUSION

In summary, the present study described the cytokines IL-β and TNF-α induced apoptosis in K562 and HL60 cell lines *via* caspase signaling pathway. These cytokines decrease cell viability and increase cell growth inhibition of leukemic cell lines. Moreover, the combination of IL-1β and TNF-α at the suitable concentration could not induce more effect on cell growth inhibition than use IL-β or TNF-α alone. Similar to cell apoptosis induction, IL-1β or TNF-α alone have higher effect on induction apoptosis than the cytokines combination. The subG1 peak in cell cycle analysis also confirmed that the apoptotic cells were increased in cytokines-treated leukemic cells. To investigate the involvement of caspase in apoptotic signaling pathways, the activated caspase 3 and upstream signaling caspase 8 and 9 were measured. IL-1β induction mediated intrinsic pathway and extrinsic pathway, which are mitochrodria release of cytochrom *c* and receptor mediated pathways in HL60 cell, but TNF-α mediated in extrinsic pathway that is receptor mediated pathway in both leukemic cell lines. This finding suggested that IL-1β and TNF-α in induction apoptosis of K562 and HL60 cells involved in caspase signaling. These evidences may contribute to anti-leukemic immunotherapy or could be used as a good candidate for therapeutic agents of leukemia. The possible signaling pathway involved in cytokine induces apoptosis in K562 and HL60 cell lines show in Figure 33.

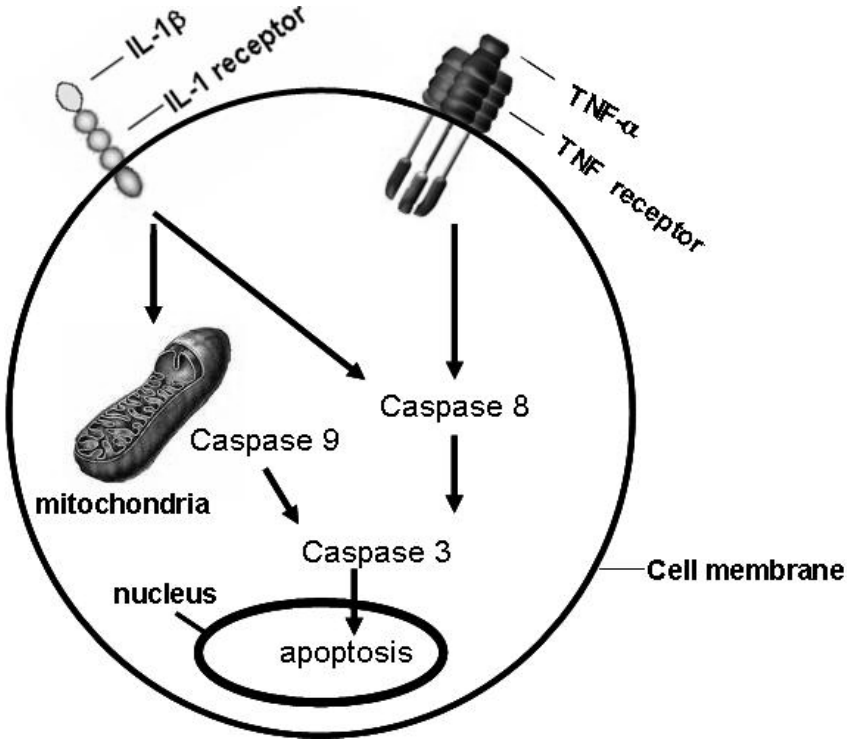

Figure 33. Possible apoptosis signaling pathway induction by IL-1β and TNF-α in K562 and HL60 cell lines.

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APPENDIX

Appendix 1

1. Reagent for cell cultivation

1.1 Preparation of 1 L of RPMI 1640 medium (incomplete medium)

Measure out 200 ml of sterile distilled water into 1000 ml beaker. Add powdered medium and gentle stirring. Rinse out inside of package to remove all traces of powder. Add 2.0 g of $NaHCO_3$ per liter of medium. Add 2% of penicillin-streptomycin antibiotic solution. Add distilled water to 1 L. Stir until dissolved. Adjust pH of medium to 7.1. After pH has been adjusted, keep container closed until medium is filtered. Sterilize immediately by membrane filtration with positive pressure. Keep cold in 4°C refrigerator.

1.2 Preparation of 1 L of RPMI 1640 medium (complete medium) + 10% Fetal Bovine Serum

Add 100 ml of sterile fetal bovine serum into 900 ml of incomplete RPMI 1640 medium, keep in sterile bottle at 4°C.

1.3 Preparation of 1 L of Phosphate Buffer Saline pH 7.4

Mix the reagent of 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, 8.0 g of NaCl and 0.2 g of KCl into 1 L of distilled water. Adjust pH to 7.4, then sterile by autoclave.

1.4 Preparation of peripheral blood mononuclear cells (PBMC)

Twenty ml of peripheral blood was collected in the sodium heparin vacutainer tube. The blood cell were centrifuged at 600 x *g* for 7 min at 20°C to remove the plasma, and then the packed red cells were diluted with 4 volumes of PBS/2mM EDTA. Diluted blood was then layered on Ficoll-Hypaque density 1.077 (Histopaque®-1.077; Sigma,USA) in ratio of 10:14. Then the layer was centrifuged at

400 x *g* for 30 minute at 20°C. After centrifuged, PBMC were carefully isolated from interface layer by Pasteur pipette in new 15 ml conical tubes. PBMC were then diluted in 5 ml of PBS/2mM EDTA and centrifuged at 400x *g* for 10 min at 20°C to get the pellet. The pellet was then mixed with 1 ml of red cell lysis buffer and incubated at 37°C for 5 min and then 10 ml PBS/2mM EDTA was mixed. Finally it was centrifuged at 400x *g* for 7 min at 20°C to get rid of the contaminated red cells. Clear pellet was mixed with 10 ml of buffer (0.5% HAS in DPBS). Total PBMC were counted by using hemocytometer. After cell counted, final washing step was done by centrifuged at 400x *g* for 10 min at 4°C. Supernatant was discard and 100µl of buffer (0.5% HAS in DPBS) was mixed in the pellet.

1.5 Isolation of CD34 positive progenitor cells

After PBMC preparation, progenitor cell were collected by CD34 positive selection technique (MACCS® column technology; Milternyi Biotec, USA) . FcR blocking reagent and anti-CD34 antibody labeled immunomagnetic micro bead (Milternyi Biotec, USA) were added to the cells in the ratio 10µl of each reagent per 10^8 cells, then mixed and incubated for 30 minute at 4°C. The LS column (Milternyi Biotec, USA) was placed in the magnetic socket. Pass twice 3 ml of cold buffer (0.5% HAS in DPBS) through the column to wash it. The cell suspension was passed through the column and washed 3 timed with 2 ml of buffer to get rid of unbounded non CD34 positive cells. The retained CD34 positive cells in the column were collected by talking column out of the magnetic socket and pressing the cell suspension with plunger of column. CD34 positive cells were kelp in the 15 ml conical tubes at 4°C.

1.6 CD34 positive myeloid progenitor cells culture

CD34 positive cells ($2x10^5$ cells/ml) in RPMI 1640 medium (incomplete medium) were supplemented with 15% fetal bovine serum. The cytokines were added at 5 ng/ml of interleukin-3, 20 ng/ml of stem cell factor and 10 ng/ml of granulocyte macrophage - colony stimulating factor (GM-CSF) (Chemical,USA). Cells were then incubated at 37°C in 5% CO_2. After 3 days of culture, cell suspension was divided into different

wells of culture dishes for the experiment with and without treatment of cytokines (2 ng/ml of IL-β and 20 ng of TNF-α).

1.7 Preparation of 500 ml of PBS/2mM EDTA

Add 2 ml of 0.5 M EDTA into 498 ml of 1X Dulbecco's Phosphate Buffered Saline (DPBS), mix and sterile by autoclave

1.8 Preparation of 500 ml of buffer (0.5% Human albumin serum in DPBS)

Add 10 ml of 2.5% human albumin serum and 2 ml of 0.5 M EDTA into 488 ml of 1X DPBS. Then mix the reagent.

1.9 Preparation of 500 ml of red cell lysis buffer

Add the reagent of 3.024 g of $CHKO_3$, 4.28 ml of NH_4Cl and 5 ml of 0.5 mM EDTA into 495 ml distilled water. Mix well, then sterile by autoclave.

2. Reagent for cell morphology

2.1 Wright's-Giemsa solution

Three grams of Wright's powder and 0.33 g of Giemsa powder are added into the bowl. Gentle pour 40 ml of glycerol and mix with the powders. Add 960 ml of methanol into the mixture and warm the solution at 37°C to complete dissolve the mixture. Keep the Wright's-Giemsa solution in the brown bottle and filter before use.

2.2 Sorensen's buffer

Solution A is 9.1 g/l of KH_2PO_4 in distill water and solution B is 9.5 g/l of Na_2HPO_4 in distilled water. The mixture of two solutions depending on pH required.

pH	Solution A (ml)	Solution B (ml)
6.4	73.0	27.0
6.6	63.0	37.0
6.8	50.8	49.2
7.0	38.9	61.1
7.2	28.0	72.0
8.0	5.5	94.5

3. Reagent for cell count

3.1 Trypan blue, 0.4%

Adding 0.4g of trypan blue into 100 ml of distill water. The solution was mixed completely and filtered before keep in the bottle at room temperature.

Appendix 2

Data of leukemic cell lines count in 7-day. The data use to calculate the percentage of growth curve and doubling time.

Day	Number of K562 cell count			
	$x10^4$ cells/ml		$x10^5$ cells/ml	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
0	1.0±0.0		1.0±0.0	
1	2.3±0.2	0.01	1.6±0.1	0.00
2	4.8±0.2	0.00	2.8±0.1	0.00
3	8.8±0.1	0.00	5.6±0.1	0.00
4	14.1±0.7	0.00	8.6±0.2	0.00
5	29.2±1.3	0.00	11.9±0.3	0.00
6	60.8±2.4	0.00	12.5±0.4	0.00

Day	Number of HL60 cell count			
	$x10^4$ cells/ml		$x10^5$ cells/ml	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
0	1.0±0.0		1.0±0.0	
1	3.0±0.3	0.02	2.8±0.1	0.00
2	6.9±0.6	0.01	5.8±0.2	0.00
3	14.2±0.6	0.00	10.2±0.2	0.00
4	32.9±1.9	0.00	12.3±0.3	0.00
5	47.3±2.5	0.00	13.4±0.1	0.00
6	69.2±0.7	0.00	14.3±0.1	0.00

Appendix 3

Data of optical density (OD) of leukemic cell lines in various concentrations. The data use to calculate for optimal concentration.

Concentration (cells/ml)	OD of K562 cell			Mean±SD
	1	2	3	
$1x10^4$	0.200	0.221	0.241	0.221±0.020
$5x10^4$	0.467	0.485	0.507	0.486±0.020
$1x10^5$	0.902	0.939	0.925	0.922±0.019
$5x10^5$	1.948	1.946	1.977	1.957±0.017
$1x10^6$	2.851	2.889	2.884	2.875±0.021

Concentration (cells/ml)	OD of HL60 cell			Mean±SD
	1	2	3	
$1x10^4$	0.178	0.213	0.187	0.193±0.019
$5x10^4$	0.489	0.497	0.539	0.508±0.027
$1x10^5$	0.841	0.839	0.816	0.832±0.014
$5x10^5$	1.701	1.722	1.711	1.711±0.011
$1x10^6$	2.436	2.474	2.450	2.453±0.019

Appendix 4

Data of percentage of cell viability.

The K562 and HL60 cells was cultured and treated with IL-1β and TNF-α in various concentration and incubation time. After harvested time, the cell was counted by typan blue staining and calculated for percentage of cell viability, untreated cell use as control.

cytokine	% cell viability of K562 cell					
	12hr		24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	100±0.0		100±0.0		100±0.0	
IL-1β 0.2ng	82.0±5.9	0.03	67.6±1.8	0.00	83.6±2.9	0.00
IL-1β 2.0ng	84.0±4.4	0.02	58.2±2.5	0.00	66.5±3.1	0.00
IL-1β 20ng	85.7±6.1	0.07	72.3±4.9	0.00	60.8±4.0	0.00
TNF-α 0.2ng	88.5±3.5	0.02	70.7±3.6	0.00	76.5±0.9	0.00
TNF-α 2.0ng	70.3±4.9	0.00	64.9±4.3	0.00	61.3±4.3	0.00
TNF-α 20ng	80.9±3.7	0.00	60.3±4.0	0.00	54.6±3.6	0.00

cytokine	% cell viability of HL60 cell					
	12hr		24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	100±0.0		100±0.0		100±0.0	
IL-1β 0.2ng	79.8±4.3	0.01	65.0±3.7	0.00	78.6±5.0	0.01
IL-1β 2.0ng	85.8±4.4	0.02	58.4±4.0	0.00	63.1±5.3	0.00
IL-1β 20ng	91.7±4.2	0.11	69.6±4.8	0.00	60.4±4.5	0.00
TNF-α 0.2ng	89.6±1.6	0.00	66.1±2.8	0.00	75.1±3.7	0.00
TNF-α 2.0ng	61.5±3.8	0.00	57.8±3.0	0.00	58.6±4.4	0.00
TNF-α 20ng	83.5±3.6	0.01	55.2±3.1	0.00	55.2±2.8	0.00

Appendix 5

Data of percentage of cell growth inhibition.

The K562 and HL60 cells were cultured and treated with IL-1β and TNF-α in various concentration and incubation time. After harvested time, O.D. of treated leukemic cell lines were analysed by MTT assay and calculated for percentage of cell growth inhibition, untreated cell use as control.

cytokine	% cell growth inhibition of K562 cell					
	12hr		24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0		0±0.0	
IL-1β 0.2ng	6.9±2.2	0.05	9.1±0.9	0.00	9.0±0.7	0.00
IL-1β 2.0ng	7.8±1.9	0.02	11.6±0.7	0.00	10.2±1.2	0.00
IL-1β 20ng	6.5±2.2	0.06	8.3±1.1	0.00	11.2±2.1	0.01
TNF-α 0.2ng	6.7±2.1	0.04	10.1±1.8	0.01	10.3±1.7	0.00
TNF-α 2.0ng	10.2±1.8	0.00	11.0±3.0	0.03	11.4±2.3	0.01
TNF-α 20ng	8.6±1.7	0.01	12.7±2.8	0.01	13.2±2.1	0.00
IL-1β 2.0ng + TNF-α 20ng	6.5±2.6	0.10	7.1±0.6	0.00	9.5±1.7	0.01

cytokine	% cell growth inhibition of HL60 cell					
	12hr		24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0		0±0.0	
IL-1β 0.2ng	6.1±2.2	0.07	8.8±1.0	0.00	7.6±1.4	0.01
IL-1β 2.0ng	5.2±1.9	0.07	9.8±1.0	0.00	8.3±1.0	0.00
IL-1β 20ng	3.8±1.3	0.07	7.6±1.7	0.01	9.6±1.6	0.00
TNF-α 0.2ng	6.6±1.0	0.00	8.1±1.5	0.01	8.0±0.7	0.00
TNF-α 2.0ng	9.9±1.2	0.00	9.7±1.1	0.00	10.7±1.4	0.00
TNF-α 20ng	8.8±0.7	0.00	10.3±1.8	0.00	11.9±1.9	0.00
IL-1β 2.0ng + TNF-α 20ng	7.8±1.6	0.01	7.6±1.0	0.00	8.1±1.8	0.01

Appendix 6

Data of percentage of cells apoptosis.

After treatment and harvested time, cells were stain with Annexin-V-FITC and Propidium iodide (PI) and analysed by flow cytometry. The percentage of Annexin-V-FITC intensity was measured and compared with control.

cytokine	% cell apoptosis of K562 cell					
	12hr		24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0		0±0.0	
IL-1β 0.2ng	2.3±0.5	0.06	6.8±2.6	0.08	6.7±1.3	0.03
IL-1β 2.0ng	3.1±1.1	0.06	7.0±2.0	0.04	3.5±0.5	0.06
IL-1β 20ng	4.5±0.7	0.01	6.8±2.7	0.09	6.5±1.8	0.07
TNF-α 0.2ng	1.7±0.6	0.07	5.3±2.3	0.11	8.3±3.0	0.11
TNF-α 2.0ng	4.5±0.3	0.01	5.0±1.9	0.08	8.7±1.7	0.04
TNF-α 20ng	4.6±0.7	0.01	8.9±1.8	0.02	11.1±4.3	0.12
IL-1β 2.0ng+ TNF-α 20ng	2.7±0.7	0.11	5.1±0.4	0.02	6.0±2.0	0.15

cytokine	% cell apoptosis of HL60 cell					
	12hr		24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0		0±0.0	
IL-1β 0.2ng	3.0±1.1	0.07	3.0±0.9	0.04	1.1±0.9	0.14
IL-1β 2.0ng	3.1±0.9	0.05	5.5±0.4	0.01	4.0±1.8	0.06
IL-1β 20ng	2.8±0.7	0.06	1.7±0.7	0.08	3.0±1.9	0.09
TNF-α 0.2ng	2.4±0.7	0.07	4.2±1.6	0.08	3.0±1.7	0.08
TNF-α 2.0ng	2.9±1.2	0.09	3.4±1.3	0.09	3.1±1.4	0.06
TNF-α 20ng	2.8±1.6	0.17	4.3±0.6	0.01	4.6±1.1	0.01
IL-1β 2.0ng+ TNF-α 20ng	2.9±0.6	0.10	4.0±0.7	0.02	2.4±0.1	0.07

Appendix 7

The percentage of cell apoptosis of cytokines treatment in normal cell.

After treatment with 2 ng/ml of IL-1β and 20 ng/ml of TNF-α at 24hr and 48hr incubation, cells were stain with Annexin-V-FITC and Propidium iodide (PI) and analysed by flow cytometry. The percentage of Annexin-V-FITC intensity was measured.

cytokine	% Annexin-V-FITC intensity	
	24hr	48hr
control	11.21	9.08
IL-1β 2.0ng	10.85	9.04
TNF-α 20ng	9.46	8.12

Appendix 8

Data of percentage of cell cycle phases.

The suitable concentrations and times were selected. Cells were treated and investigated the cell cycle and analysed by flow cytometry. The percentage of cell cycle phases were measured and compared with control.

cytokines	% cell cycle phase of K562 at 24hr			
	G0/G1	S	G2/M	subG1
control	42.2±0.3	40.2±0.7	15.2±0.8	3.1±0.1
IL-1β 2ng	40.1±1.3	32.9±0.7	15.7±0.4	11.4±0.4
TNF-α 20ng	43.5±2.6	25.8±1.5	13.4±1.4	17.5±0.5

cytokines	% cell cycle phase of K562 at 48hr			
	G0/G1	S	G2/M	subG1
control	43.4±1.2	40.8±1.1	12.8±2.3	3.7±0.6
IL-1β 2ng	29.1±1.5	46.8±0.3	15.6±0.8	8.5±0.4
TNF-α 20ng	36.2±0.5	44.6±0.2	8.2±0.3	11.5±0.5

cytokines	% cell cycle phase of HL60 at 24hr			
	G0/G1	S	G2/M	subG1
control	40.7±0.7	37.4±0.8	20.2±0.3	1.7±0.1
IL-1β 2ng	33.2±0.5	44.2±0.7	19.0±0.5	4.3±0.1
TNF-α 20ng	39.9±0.7	41.2±0.6	15.5±0.9	4.0±0.1

cytokines	% cell cycle phase of HL60 at 48hr			
	G0/G1	S	G2/M	subG1
control	46.6±0.2	34.7±0.2	16.9±0.4	1.4±0.3
IL-1β 2ng	32.3±4.0	45.1±1.1	20.0±3.6	3.2±0.4
TNF-α 20ng	40.2±2.5	40.0±1.0	17.0±3.4	3.3±0.2

Appendix 9

Data of percentage of FITC intensity of caspase 3.

After treatment and harvested time, cells were stain with Annexin-V-FITC specific for caspase 3 and analysed by flow cytometry. The percentage of FITC intensity was measured and compared with control.

cytokine	% FITC intensity of caspase 3 on K562 cell			
	24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0	
IL-1β 2.0ng	4.8±0.8	0.03	3.1±0.7	0.05
TNF-α 20ng	3.1±0.4	0.01	7.3±0.1	0.01

cytokine	% FITC intensity of caspase 3 on HL60 cell			
	24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0	
IL-1β 2.0ng	4.5±1.2	0.07	3.9±0.4	0.01
TNF-α 20ng	3.5±0.3	0.01	6.8±0.8	0.01

Appendix 10

Data of percentage of FITC intensity of caspase 8

After treatment and harvested time, cells were stain with Annexin-V-FITC specific for caspase 8 and analysed by flow cytometry. The percentage of FITC intensity was measured and compared with control.

cytokine	% FITC intensity of caspase 8 on K562 cell			
	24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0	
IL-1β 2.0ng	1.4±0.1	0.05	1.1±0.3	0.07
TNF-α 20ng	3.8±0.2	0.01	2.5±0.4	0.03

cytokine	% FITC intensity of caspase 8 on HL60 cell			
	24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0	
IL-1β 2.0ng	3.2±0.4	0.02	2.0±0.4	0.03
TNF-α 20ng	3.6±0.2	0.01	2.6±0.2	0.01

Appendix 11

Data of percentage of FITC intensity of caspase 9 .

After treatment and harvested time, cells were stain with Annexin-V-FITC specific for caspase 9 and analysed by flow cytometry. The percentage of FITC intensity was measured and compared with control.

cytokine	% FITC intensity of caspase 9 on K562 cell			
	24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0	
IL-1β 2.0ng	1.6±0.2	0.01	0.9±0.1	0.02
TNF-α 20ng	1.1±0.2	0.03	1.3±0.1	0.01

cytokine	% FITC intensity of caspase 9 on HL60 cell			
	24hr		48hr	
	Mean±SEM	*p-value*	Mean±SEM	*p-value*
control	0±0.0		0±0.0	
IL-1β 2.0ng	3.6±0.6	0.03	1.5±0.4	0.07
TNF-α 20ng	1.5±0.2	0.01	1.5±0.2	0.02

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