p53 and nitric oxide are involved in cytokine-induced apoptosis in Kasumi-1 and Molt-4 Leukemics cells

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Summary

Background: **Immunotherapy has been developed to treat cancers. There are many signaling pathways involved in cytokine induced apoptosis of many cancers but their role remains unclear in some cancers such as leukemia.**

Objective: **To investigate the involvement of the nitric oxide (NO) and p53 tumor suppressor gene in apoptotic pathways induced by cytokines in leukemic cell lines.**

Methods: **Leukemic cell lines, Kasumi-1 (AML-M2) and Molt- 4 (ALL) were treated with cytokines, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ). The effect of cytokines on the induction cell apoptosis was analysed by flow cytometry. In addition, nitric oxide production and p53 protein levels were measured by using the Griess method and Western blot, respectively.**

Results: **Upon cytokine treatment, there was a significant increase in the percentage of cell apoptosis in both leukemic cell lines. The highest apoptosis was shown in 40 U/ml IFN-γ treated cells. In addition, nitric oxide and p53 protein increased in IFN-γ treated cells. There was a reduction of apoptosis and p53 level after adding the inducible nitric oxide synthase inhibitor, SMT.**

Conclusion: **p53 and nitric oxide are involved in the mediation of apoptosis induced by cytokines in Kasumi-1 and Molt-4 leukemic cell lines.** ***(Asian Pac J Allergy Immunol 2014;32:133-9)***

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Introduction

Cytokines are peptides that control the function, growth and differentiation of a variety of cells, including vascular endothelium, hematopoietic cells, macrophages, fibroblasts, and epithelial cells.[1] These proteins regulate the cell viability, multiplication and differentiation of hematopoietic cell lineages. Any abnormalities of this process could lead to hematological diseases, such as leukemia.

Much has been learned about the pathogenesis of hematological neoplasms but the understanding of the molecular mechanisms of these disorders is not complete. Also the functions of many established oncogenes remain unclear.[2] Altered forms of the c-myc, ras, RB1, and p53 tumor suppressor are examples of leukemogenic genes found in both hematological and non- hematological cancers. Altered forms of the anti-oncogenes RB1 and p53 are very widely distributed in acute hematological cancers, as well as in cancers of the colon, breast and lung. Mutations, rearrangements, or deletions lead to the inactivation of anti-oncogenes and the loss of tumor-suppressing function.[3] p53 is a tumor suppressor protein which functions as a transcription factor regulating downstream genes important in cell cycle arrest, DNA repair and apoptosis.[4] Under normal conditions p53 protein levels are kept low due to ubiquitin-dependent degradation, mediated by ubiquitin ligase murine double minute 2(MDM2).[5] It has been shown that the inactivation of p53 plays a critical role in cell transformation and tumor growth.[6] p53 could be related with other proteins or signaling pathways in anti-cancer functions. There is a report that RAW 264.7 macrophages and RINm5F cells demonstrate p53 protein accumulation in response to nitric oxide.[7]

One of the key advances in cancer research is the recognition that evasion of apoptosis is one of the hallmarks of cancers. Proper regulation of programmed cell death is important for

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hematological malignancies, because tissue homeostasis in cellular compartments with a high proliferative capacity, such as the hematopoietic system, depends on a tight balance between proliferation and cell death.[8] Also killing of tumor cells by cytotoxic therapies, such as chemotherapy, γ-irradiation, and immunotherapy, has been reported to depend on the induction of cell death in target cells. The same oncogenic alterations and defects in apoptosis programs that suppress cell death during tumor development can also confer resistance to cytotoxic therapies. In this regard apoptosis provides a conceptual framework to link cancer formation and cancer therapy.[9] Apoptosis targeted therapies may enhance the responsiveness of human cancers to conventional treatments that are currently used in the clinic, e.g., chemotherapy or radiotherapy, since these therapies primarily exert their anti-tumor activity by triggering apoptosis in cancer cells.[10] Studies of the role of apoptosis regulating factors in cytokine mediated immunotherapy can further improve the use of these products as anti cancer agents. Cytokines such as Interleukin-2 (IL-2), interferon-α (IFN-α), Tumor Necrosis Factor-α (TNF-α) and interleukin-12 (IL-12) have been successfully applied in the treatment of human cancer. There was evidences from previous work[11] that IL-1β, TNF-α and IFN-γ could induce apoptosis in leukemia. A recent report showed that cytokine mediated apoptosis could be involved in nitric oxide production. However, the mechanism of cytokines induced apoptosis remains unclear. The role of p53 in cytokine mediated nitric oxide induced apoptosis of leukemia cells was investigated in this study.

Methods

Cell culture and cytokine treatment

Kasumi-1 (Acute Myeloid Leukemia-AML-M2) was obtained from Dr. Setzuko Miyanishi and Mrs. Yaowalak U-pratya (Siriraj Hospital, Bangkok, Thailand), Molt-4 (Acute lymphoblastic leukemia) cell lines were purchased from Cell Line Services (Eppelheim, Germany). Cell lines were cultured in RPMI 1640 (Invitrogen Corporation, NY, USA), supplemented with 15 % fetal bovine serum (Invitrogen Corporation, NY, USA) and 2% penicillin-streptomycin in a humidified atmosphere of 5% CO_2. Cells were treated with 40 and 400 U/ml of IL-1β, TNF-α and IFN-γ (Chemicon International, CA, USA). The cells were then cultured for 24 and 48 hr at 37 °C in a humidified atmosphere of 5% CO_2. Untreated cells were used as controls. The morphology of the cells was evaluated using cytospin slide preparations.

Apoptosis Assay

Cytokine-treated and untreated cell lines were suspended in Annexin V binding buffer and incubated with Annexin V-FITC and Propidium Iodide (BD Bioscience, San Diego, CA, USA) for apoptosis determination using a flow cytometer (Beckton-Dickinson, Franklin Lake, NJ, USA).

Nitric oxide production determination

Nitric oxide production was based on measurement of nitrite concentration in cell cultures as determined using the Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% H_3PO_4; Sigma].[12] A 50-µl aliquot culture supernatant was mixed with 100 µl of Griess reagent and the mixture was incubated for 5 min at 25° C. Absorbance at 570 nm was measured in an automated plate reader (Beckman Coulter, Fullerton, CA, USA). A standard solution of sodium nitrite was used to generate a calibration curve.

P53 protein level by western blot analysis

For whole cell lysates, cells were harvested and placed in radioimmunoprecipitation assay buffer (Millipore Corporation, Temecula, CA, USA). Aliquots were removed for determination of protein concentration by utilizing the Bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA), prior to the addition of Laemmli buffer (Bio-Rad Laboratories Inc, Hercules, CA, USA). After the samples were prepared, proteins were separated by size using SDS-PAGE. Following gel electrophoresis, the separated protein mixtures were transferred to a nitrocellulose membrane (Amersham Biosciences, UK) for further analysis. Ponceau S stain was applied to the membrane to ensure that efficient transfer of proteins had taken place. For the blocking step the nitrocellulose membrane was agitated in 3% Bovine serum albumin (BSA) at 4°C. Proteins were visualized following incubation with primary and horse-radish peroxidase (HRP)-conjugated secondary antibodies using a chemiluminescent detection kit (Thermo Scientific, Rockford, IL, USA) on X-ray film. Analysis was performed using antibodies to p53 (DO-1) [Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA]. GAPDH (6C5) [Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA] which were obtained from Dr. Pimpicha Patmasiriwat (Mahidol University, Bangkok, Thailand).

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Statistical analysis

Results are expressed as the mean ± SD. Statistical analysis was performed using Student's t test, and significant difference was set at a *p*-value <0.05.

Results

Effects of cytokines on cell apoptosis of leukemic cell lines

The apoptotic response of cell lines to cytokines, IL-1β, TNF-α and IFN-γ was determined using Annexin V-FITC and analysed by flow cytometry. In addition to the changes in cell morphology that occur during apoptosis, a major event is the loss of phosphatidylserine (PS) from the inner leaflet of the phospholipid bilayer to the cell surface. Annexin V has a high affinity for PS containing phospholipid bilayers.[13] Fluorochrome conjugates of Annexin- V can be used to monitor changes in the cell membrane phospholipid asymmetry, thereby providing a convenient tool for detection of apoptotic cells.[14] Leukemic cells, Kasumi-1 and Molt-4, treated with cytokines for 48 hrs had greater effect on cell apoptosis than treatment for 24 hrs. The highest percentage of apoptosis was seen in cells treated with IFN-γ at 40 U/ml for 48 hr (Figure 1).

Effects of cytokines on nitric oxide production

To determine whether the cell lines generated nitric oxide in response to stimulation with the cytokines, IL-1β, TNF-α and IFN-γ, supernatants from leukemic cell lines were treated with cytokine for 24 and 48 hrs and nitric oxide levels were quantified. Nitrite is a stable nonvolatile breakdown product of nitric oxide which is measured using the Griess reagent system. In Kasumi-1 cell line, the highest level of nitrite was generated when it was treated with IFN-γ at 40 U/ml for 48 hrs (Figure 2a). In Molt-4 cell line, the highest level of nitrite was generated when they were treated with IFN-γ at 400 U/ml for 48 hr (Figure 2b). However, there was no statistically significant difference from 40 U/ml IFN-γ treatment for 48 hrs.

Effect of cytokine on p53 protein level

The p53 protein expression level was determined by Western blotting using the p53 mouse monoclonal antibody DO-1, which recognizes an epitope that resides between amino acid 11-25 of the p53 protein of both wild-type and mutant p53.[15] Western blot results showed that Molt-4 and Kasumi-1 cell lines expressed p53 protein.

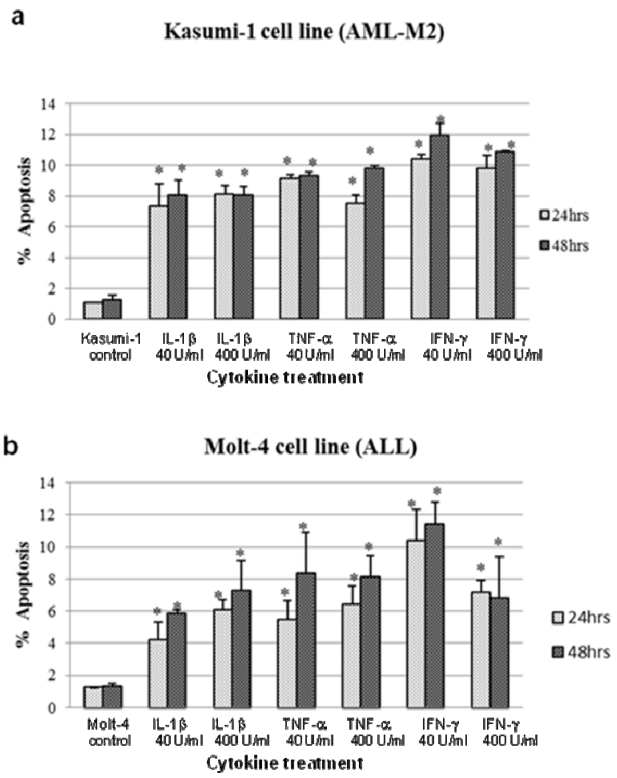

Figure 1. Percent apoptosis of Kasumi-1(a) and Molt-4 (b) cell lines. Cells were treated with various concentrations of IL-1β,TNF-α and IFN-γ for 24 and 48 hrs. Annexin V-FITC intensity was analysed by flow cytometry and the percentage of cell apoptosis was calculated.*, $p < 0.05$ and compared with the control group.

It has been reported that the Kasumi-1 cell line and Molt-4 cell lines express wild-type p53,[16] while, HL-60 and K-562 cell line are negative for p53 protein.

In Molt-4 cell lines, the level of p53 protein was increased when treated with IFN-γ at 24 hrs and further increased at 48hrs. A statistically significant difference was found in cells treated with IFN-γ for 48hrs as compared to controls. In the Kasumi-1 cell line, p53 protein expression was increased after treatment with IFN-γ at 40 U/ml for 24 hrs and 48hrs. The highest intensity was shown in cells treated with IFN-γ for 48 hrs (Figure 3).

Effect of iNOS inhibitor (SMT) on apoptosis

Leukemic cell lines, Molt-4 and Kasumi-1, were treated with IFN-γ 40U/ml alone and combined with 1 ng/ml S-methylisothiourea (SMT) which is a selective iNOS inhibitor. The percentage of apoptosis was measured by flow cytometry. The result showed the percentage of apoptosis was reduced in SMT treated cells of both cell lines (Figure 4).

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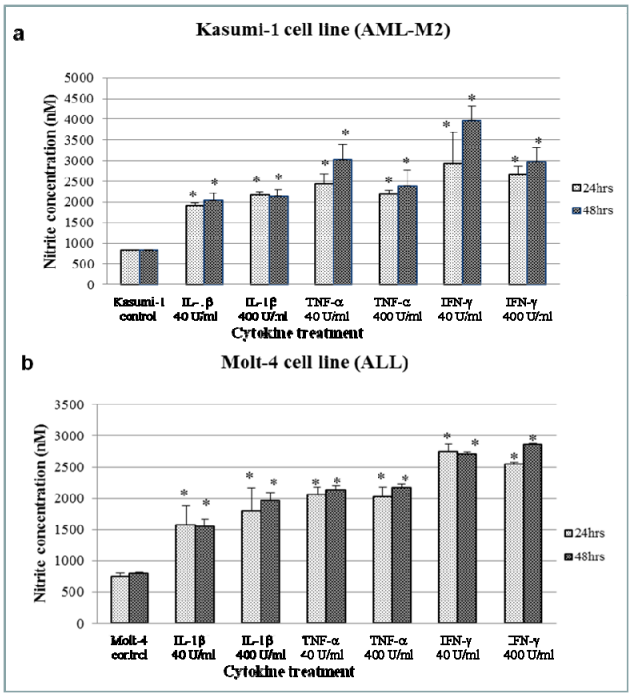

Figure 2. Nitrite concentrations in Kasumi-1(a) and Molt-4 (b) cell lines. Cells were treated with various concentration of IL-1β, TNF-α and IFN-γ for 24 and 48 hrs. Cell culture supernatants were assayed for relative levels of nitrite (NO_2^-) by the Griess assay.*, $p < 0.05$ compared with the control group.

Effect of iNOS inhibitor (SMT) on p53 protein level

In Kasumi and Molt-4 cell lines, p53 protein was reduced after treatment with the SMTand iNOS inhibitor. A statistically significant difference was found in Molt-4 cell line treated with IFN-γ for 48 hr compared with controls (Figure 5).

Discussion

Apoptosis plays an important role in cell differentiation, elimination of cells that have genetic damage or display uncontrolled cellular proliferation. Proper regulation of programmed cell death is important in hematological malignancies because tissue homeostasis in the hematopoietic system critically depends on a tight balance between proliferation and cell death.[17] With recent advances in molecular genetics, it has become evident that malignant cells commonly have defects in cell death control and apoptosis. Induction of programmed cell death in cancer cells can be achieved by a variety of agents, including cytotoxic cancer chemotherapy compounds,[18] heat shock,[19] antibodies to certain cell-surface antigens[20] and transfection with the tumor-suppressor gene wild-type p53.[21] Recently, cytokines have been used as an apoptosis induction based therapies.

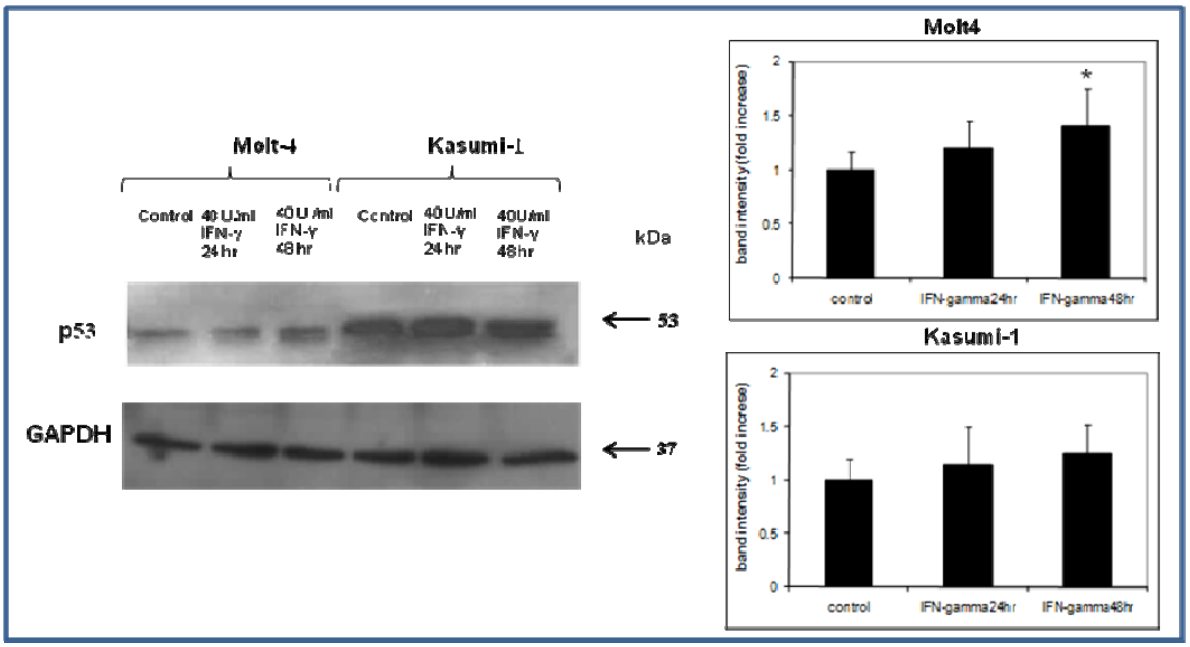

Figure 3. Levels of p53 protein after treatment with 2 ng/ml IFN-γ. Molt-4 and Kasumi-1 cells were treated with 40U/ml of IFN-γ for 24 hrs and 48 hrs. Cell lysates (40μg/each lane) were fractionated on 12% SDS-Polyacrylamide gel and analysed by Western blotting with p53 (DO-1) antibody. Untreated cells were used as controls.

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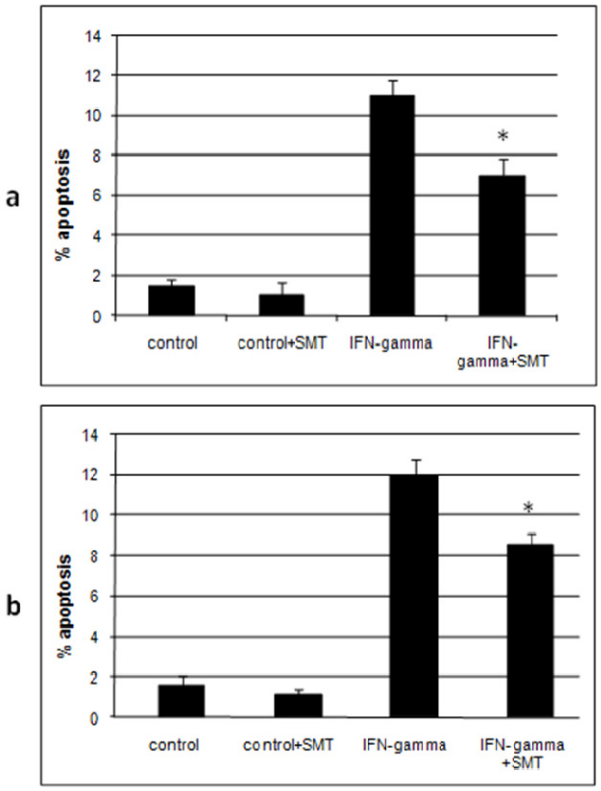

Figure 4. Effect of iNOS inhibitor or SMT on percentage apoptosis of leukemic cell lines. Molt-4 (a) and Kasumi-1 (b) cell lines were treated with IFN-γ 2 ng/ml alone or combined with 1 ng/ml S-methylisothiourea (SMT). Thye percentage of cell apoptosis was measured by flow cytometry. *, $p < 0.05$ compared with IFN–γ treated cells.

The role of cytokines in immunotherapy is the subject of an enormous research effort. TNF, IFN, and IL-2 have been used singly or in combination to treat a variety of solid tumors, including melanoma, renal cell carcinoma, multiple myeloma, and ovarian cancer.[22] Cytokines have also been used as adjuncts to improve the effectiveness of chemotherapy or radiotherapy. The inflammatory cytokines, IL-1β, TNF-α and IFN-γ, have been implicated in inducing apoptosis in various cell types. IFN-γ up-regulates the expression of apoptosis-related proteins in different cell types.[23] Reports indicate that TNF-α is a mediator involved in inflammatory reactions other than those induced by endotoxin. TNF-α is chemotactic for monocytes and stimulates phagocytosis, and adherence to endothelium.[24] TNF-α appears to be a mediator of the cytocidal activity of natural cytotoxic cells and of activated macrophages.[25] The cytokines TNF-α, IL-1α and IL-1β are defined as 'alarm cytokines' that are secreted by macrophages and initiate inflammation.[26] IL-1β has been reported to cause inflammation and induce the expression of pro-inflammatory genes, such as cyclooxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOS), IL-6 and other cytokines and chemokines.[27]

It is important to elucidate the mechanism by which cytokines induce apoptosis in the leukemic cell lines used in this study. This study has shown that cytokines induced apoptosis in leukemic cell lines, as defined by an increase in cell apoptosis. The percentage of cell apoptosis of cytokine treated cells increased in a time dependent manner. Furthermore the study showed that treatment with cytokines leads to an increase in the nitric oxide generated by the cells. The mechanisms by which nitric oxide induces cell death have been a matter of ongoing debate. The range and strength of the effects of NO are by-products of its extensive chemistry, which can be coupled to the local cellular environment.[28] An increase in oxidants in the presence of NO causes the formation of the highly reactive molecule peroxynitrite which can activate p38 and c-Jun N-terminal kinase (JNK) to initiate the intrinsic apoptotic pathway. In addition, BAX and BAK activation is regulated by BH3-only proteins which could be involved in NO-induced cell death.[29] In our previous study[11] we showed that nitric oxide is the effector of cytokine-mediated apoptosis and that the selective iNOS inhibitor, SMT, could prevent both nitric oxide production and apoptosis in leukemic cells.

In this study, the involvement of p53 tumor suppressor gene and nitric oxide mediated cytokine induced apoptosis were investigated. The results show that after Molt-4 and Kasumi-1 cell lines are treated with the cytokines especially IFN-γ, there is a subsequent increased generation of nitric oxide and p53 protein. The highest p53 protein levels were shown when cells were treated with 40 U/ml IFN-γ for 48 hrs. The increased p53 level was seen along with a decrease in MDM2, which is a key negative regulator of p53 (data not shown). When cells were treated with IFN-γ 40 U/ml and subsequently treated with the nitric oxide inhibitor, S-methylisothiourea, there was a significant reduction in apoptosis and p53 expression. This suggests the involvement of p53 and the nitric oxide pathway in cytokine induced apoptosis. However, the level of p53 expression was significantly up-regulated in Molt-4

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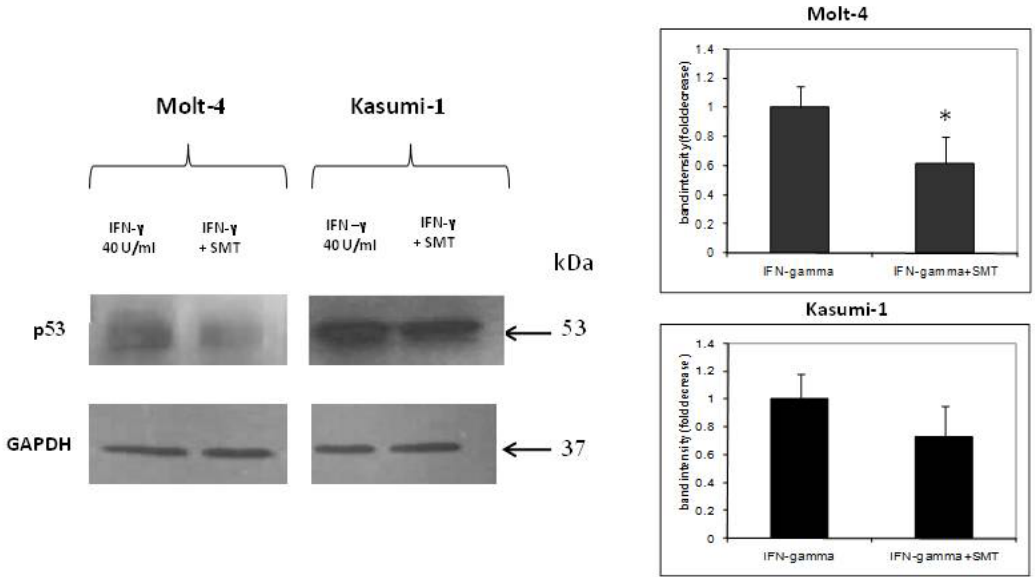

Figure 5. Levels of p53 protein after nitric oxide inhibitor (S-methylisothiourea) treatment. Molt-4 (a) and Kasumi-1 cells (b) were treated with 40U/ml of IFN-γ and 2ng/ml IFN-γ with a nitric oxide inhibitor (SMT) for 48 hr. Cell lysates (40μg/each lane) were fractionated on 12% SDS-Polyacrylamide gel and analyzed by Western blotting with p53 (DO-1) antibody

cell lines (ALL) but not in the than Kasumi-1 cell line (AML). It is possible that the p53 is response to cytokine induced apoptosis is cell type and lineage specific. Kastan MB and colleagues found that leukemic cell lines express p53 abnormally in a lineage-specific manner: lymphoid leukemia cell lines express p53 in higher levels than myeloid leukemia cell lines. As p53 is involved in two mechanisms, tumorigenesis as oncogene or tumor suppressor, then lineage-specific di fferences of p53 expression could affect these mechanisms.[30] In addition, the level of NO production and its signaling pathways were found to be involved in p53 expression. There was report suggesting that the tumor suppressor gene, p53, is associated with apoptotic responses to numerous stimuli.[31] Apoptotic responses are regulated at several different levels, for example at the activated receptor, along the mitochondrial pathway, caspase activation[32] and regulation by anti-apoptotic and pro-apoptotic proteins.[33]

From the results of this study we conclude that the cytokines IL-1β, TNF-α and IFN-γ induce apoptosis which is mediated by p53 and nitric oxide in the leukemic cell lines, Molt-4 (ALL) and Kasumi-1 (AML-M2).

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