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The role of the tyrosine kinase Syk in retinoic acid-induced leukemic HL-60 cell differentiation

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SYK encodes the tyrosine kinase protein belonging to the signal-transducing molecules that interact with activated immunoreceptors. The current evidence indicates that deregulated Syk is involved in the pathogenesis of cancer. One of the most promising anticancer therapy strategies is the induction of cell terminal differentiation. In promyelocytic HL-60 cells, retinoic acid (RA) induces differentiation towards granulocytes. Increase in the activity of Syk during the process of differentiation suggests that Syk might play a role in directing HL-60 cells towards granulocytic differentiation. Therefore, we have studied the involvement of Syk tyrosine kinase in the process of retinoic acid-induced granulocytic differentiation of HL-60 cells. The 2.6-kb human SYK cDNA fragment harboring a complete coding sequence was inserted into the pLXSN retroviral vector in sense and antisense orientation and transfected into HL-60 cells. Following RA treatment, HL-60 cells transfected with SYK showed an increased differentiation to granulocytes when compared with the control cells transfected with an empty pLXSN vector. Reduction of the intracellular Syk level by antisense or short interfering RNA (siRNA) strategy yielded the inhibition of HL-60 cell differentiation. We propose that Syk is a positive regulator of retinoic acid-induced granulocytic differentiation of leukemic HL-60 cells and presents a potential target for the development of new anticancer therapies.

Key words: protein tyrosine kinase Syk, differentiation; HL-60; retinoic acid

INTRODUCTION

The mechanism-based target identification is a new strategy in the post-genomic cancer medicine, and induction of differentiation as well as apoptosis is a promising approach to cancer therapy [1–3].

Syk is a non-receptor protein-tyrosine kinase that is usually expressed in hematopoietic cells. It is involved in the transduction of signals of proliferation, differentiation, phagocytosis and apoptosis from activated immunoreceptors to downstream signaling events [4]. Recently, it has been found in cells and tissues outside the hematopoietic lineage, like fibroblasts, hepatocytes, epithelial cells, breast tissue, neuronal cells, and vascular endothelial cells [5]. Recent findings have revealed that deregulation of Syk expression or activation appears to be involved in the pathogenesis of various types of cancer: gastric carcinoma [6], breast cancer [7–10], T and B leukemias [11, 12]. SYK is considered to be a potential tumour suppressor and antimetastatic gene in various human cancers. Conversely, the growth-promoting, oncogenic role of Syk kinase has been demonstrated in lymphoma [13]. Anyhow, it may present a molecular target for the development of new anticancer therapy strategies.

Induction of cell differentiation is one of the strategies in cancer therapy [14]. Syk protein-tyrosine kinase was shown to be involved in neuron-like differentiation of embryonal carcinoma P19 cells [15]; also, it participates in the differentiation of 3T3-L1 mouse embryonic fibroblasts to adipocytes [16], deregulated Syk inhibits the differentiation and induces the growth factor-independent proliferation of pre-B cells [17]. Leukemic cell lines are useful tools to study the factors and processes associated with the differentiation of neoplastic cells, since leukemias are characterized as a deregulation of cell differentiation as well as proliferation and apoptosis

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[18]. In our experiments, we used the human promyelocytic leukemia HL-60 cell line. HL-60 cells are blocked at an early stage of the myelomonocytic maturation pathway. At the molecular level, HL-60 cell line is characterized by C-MYC amplification, TP53 deletion and N-RAS mutation [19]. In response to various differentiation agents, HL-60 cells undergo molecular changes that result in the cell phenotype with monocyte / macrophage or granulocyte characteristics. One of the agents used to induce granulocytic differentiation in HL-60 cells is retinoic acid (RA) – a compound proposed as a future radical therapeutics for the treatment of acute promyelocytic leukemia. RA acts through a subfamily of nuclear hormone receptors that regulate the expression of target genes [20, 21]. RA is known to induce differentiation as well as apoptosis in various cell types including tumour cells, although the mechanism underlying retinoic acid-induced differentiation remains mainly undefined.

There are data demonstrating a correlation between the increase in the activity of Syk and the level of differentiation of HL-60 cells towards granulocyte lineage [22]. Changes in the activity of Syk during the process of RA-induced differentiation suggest that Syk might play a role in directing HL-60 cells towards granulocytic differentiation.

In this study, we analyzed the role of tyrosine kinase Syk in the retinoic acid-induced granulocytic differentiation of HL-60 cells. For this purpose, we have constructed retroviral vectors containing the cDNA of human tyrosine kinase Syk in sense and antisense orientation. HL-60 cells infected with the retroviral vector pLSYKSN harbouring the SYK gene in the sense orientation relative to the promoter showed an increased differentiation towards granulocytes after RA treatment. The downregulation of SYK expression by the antisense or siRNA interference strategy yielded opposite results. The obtained results propose Syk kinase as a molecular target for antileukemic therapies.

MATERIALS AND METHODS

Reagents

RPMI 1640, FBS (fetal bovine serum), penicillin, streptomycin, and geneticin (G418) were from Gibco BRL, and nitroblue tetrazolium (NBT), phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate), and retinoic acid (RA) were from Sigma, St. Louis, MO. The monoclonal anti-Syk antibody was kindly provided by Dr. P. Draber; monoclonal anti-β-actin, HRP-conjugated anti-mouse antibodies, and RNAi assay reagents were obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

Cell culture and differentiation analysis

HL-60 cells were maintained in an RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 U/ml streptomycin in 5% carbon dioxide air at 37 °C. In differentiation experiments, cells were treated with 500 nM all-trans retinoic acid prepared from a stock solution of 1 mM RA in 96% ethanol (stored at –20 °C). To monitor granulocytic differentiation, cells were washed with PBS and incubated with 0.1% NBT and 0.1 μg/ml phorbol ester TPA for 30 min at 37 °C. The differentiated cells produce superoxide to reduce nitroblue tetrazolium to a cytoplasmic precipitate. The percentage of cells containing reduced black formasan deposits was determined under the light microscope at the times indicated in the text. The number of differentiated cells was given by counting 500 cells per sample.

Retrovirus construction and transduction of target cells

The full-length human SYK cDNA insert cloned in the πH3M expression vector was released by Xho1 and recloned in sense and antisense orientations into the Xho1 site of retroviral vector pLXSN under the L-Moloney murine leukemia virus long terminal repeat promoter. To generate a recombinant retrovirus, the ecotropic packaging cell line ϕ2 was transfected with pLSYKSN, pLantiSYKSN and pLXSN by the calcium phosphate coprecipitation method. A stable vector-producing cell line was generated harvesting the ϕ2-produced virus and infecting the PA 317 virus packaging cell line. The virus produced by PA 317 cells was used to infect target HL-60 cells. After 24 hours, the infected cells were placed into a medium containing 1 mg/ml G418. About two weeks later, G418-resistant colonies were expanded into large cultures and maintained in the medium containing 1 mg/ml G418.

Western blot analysis of Syk

Whole cell lysates corresponding to an equivalent cell number were separated by SDS-PAGE (10%). The proteins were transferred electrophoretically onto polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp.) and blocked in a blocking reagent (5% nonfat dry milk powder in TBS). The membranes were probed with the anti-Syk antibody followed by the horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham). The membranes were stripped and reprobed for β-actin as a protein-loading control.

siRNA-mediated inhibition of Syk gene expression

The assay was performed according to the manufacturer's instructions. Briefly, 1×10^6 cells were transfected either with Syk siRNA or with a negative control siRNA. One microgram of siRNA in each case was diluted in 100 μl of siRNA transfection medium and then mixed with 100 μl of transfection medium containing 5 μl of the transfection reagent. The mixture was incubated for 30 min at room temperature. The cells were washed and resuspended in 0.8 ml of the transfection medium. The transfection mixture and

the cell suspension were mixed and incubated for 6 h. After incubation, the medium was replaced, and cells were maintained for additional 48 h before using for experiments. After transfection, cell viability was 85–90% as shown by the trypan blue dye exclusion test.

Statistical analysis

The values are expressed as the means of at least three experiments ±SD. The means were compared using the t-test. The p values <0.05 were regarded as statistically significant.

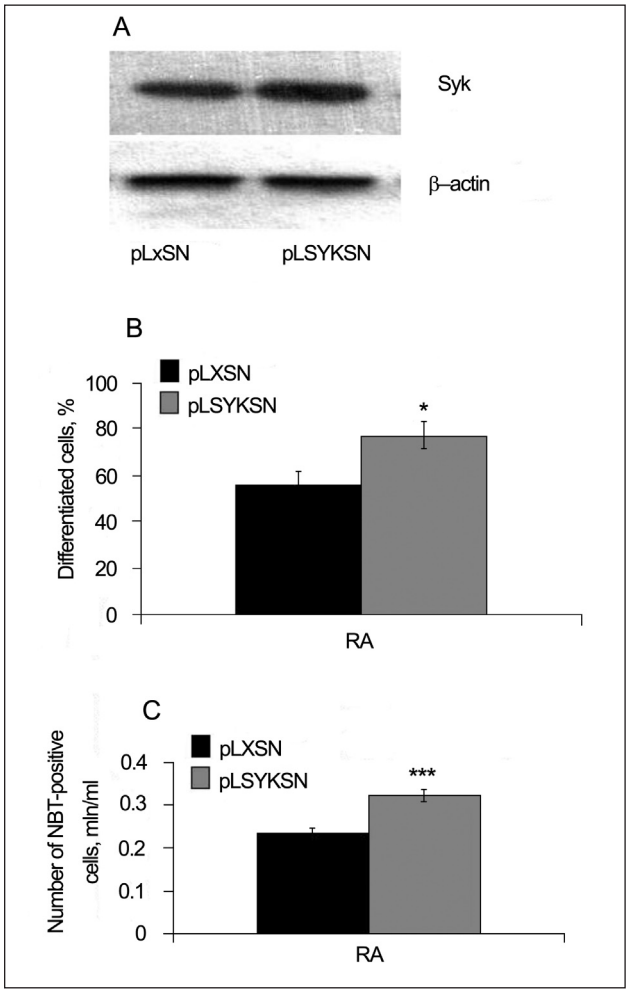

Fig. 1. Overexpression of protein tyrosine kinase Syk potentiates retinoic acid-induced differentiation of HL-60 cells. (A) Increased expression of Syk in pLSYKSN-transfected HL-60 cells. Whole cell extracts from an equal number of control pLXSN and pLSYKSN-transfected HL-60 cells were analyzed by SDS-PAGE and immunoblotted with anti-Syk antibody. The membrane was reprobed with anti-β-actin antibody to confirm equal loading. (B) Percentages of NBT-positive, differentiated, cells in control (pLXSN) and SYK (pLSYKSN)-transfected cell populations at 72 hour after retinoic acid treatment. Cells were exposed to retinoic acid (500 nM), and the differentiation was measured by NBT test. Data are presented as means ±SD, * $p < 0.05$. (C) Absolute number of differentiated cells in control (pLXSN) and SYK (pLSYKSN)-transfected HL-60 cell populations at 72 hour after retinoic acid treatment. Data are presented as means ±SD, *** $p < 0.001$

RESULTS

In this study, we were interested in the role of Syk kinase in the process of the granulocytic differentiation of HL-60 cells. To investigate the role of Syk tyrosine kinase in HL-60 cell differentiation, the retroviral vector pLSYKSN containing a full-length human SYK cDNA fragment was constructed. After selection with G418, HL-60 cells stably expressing pLSYKSN were generated. An increased expression of tyrosine kinase Syk was confirmed by Western blotting assay (Fig. 1A). Next, we examined the intensity of granulocytic differentiation in HL-60 pLXSN and HL-60 pLSYKSN cells after RA (500 nM) treatment. To evaluate cell differentiation, we used the nitroblue tetrazolium dye reduction test. Our results indicate the functional differences between the cells harboring different levels of Syk protein: HL-60 cells transfected with pLSYKSN showed an increased percent-

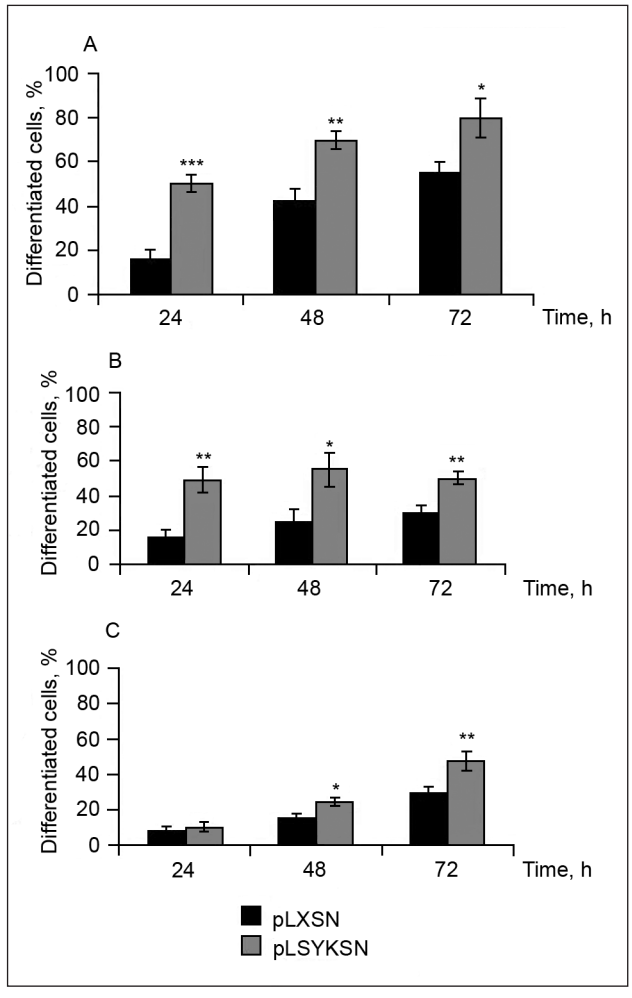

Fig. 2. SYK-transfected HL-60 cells undergo accelerated retinoic acid-induced differentiation. (A), (B), (C) Percentages of differentiated control (pLXSN) or SYK (pLSYKSN)-transfected cells from three different cell infection experiments are shown. Cells were exposed to retinoic acid (500 nM), and the differentiation was measured by NBT test at indicated times. Data are presented as means ±SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

age of NBT-positive cells ($p < 0.05$) differentiated towards granulocytes, as compared with the control (pLXSN-transfected) cells (Fig. 1B). Correspondingly, on day 3 after RA exposure, the absolute number of differentiated cells was significantly higher ($p < 0.001$) in the SYK-transfected cell population compared to the control (pLXSN-transfected) cell population (Fig. 1C).

Similar results were obtained by using cells from other infection experiments (Fig. 2A, B, and C). The experiments demonstrate the possible involvement of tyrosine kinase Syk in the retinoic acid-induced granulocytic differentiation of HL-60 cells.

Another approach used in this study was the reduction of the intracellular level of Syk by antisense and RNA interference strategies. As shown in Fig. 3B, introduction of SYK in antisense orientation (pLantiSYKSN) as well as siRNA transfection (Fig. 4B) resulted in a partial decrease of Syk tyrosine kinase expression. Despite the fact that Syk was still detectable by Western blotting, its downregulation affected cell differentiation. We found that after RA treatment, there were less NBT-positive cells in pLantiSYKSN- (Fig. 3A) and Syk siRNA-transfected (Fig. 4A) HL-60 cell populations compared to control cells. These observations suggest that Syk participates in the granulocytic differentiation of HL-60 cells.

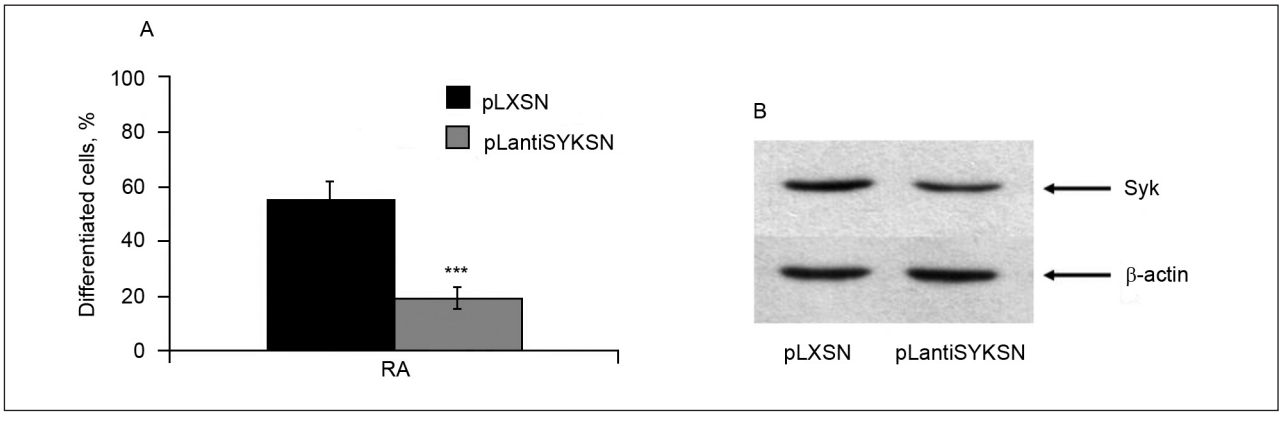

Fig. 3. Antisense-mediated inhibition of Syk expression suppresses retinoic acid-induced granulocytic differentiation. (A) HL-60 pLXSN and pLantiSYKSN cells were treated with 500 nM retinoic acid (RA), and the differentiation was examined by NBT test at 72 h. The data are means ±SD of three independent experiments, *** $p < 0.001$. (B) Reduction of Syk protein level. HL-60 pLXSN and pLantiSYKSN whole cell extracts were subjected to immunoblotting analysis with anti-Syk antibody. The membrane was stripped and reprobed with anti-β-actin antibody as a protein-loading control

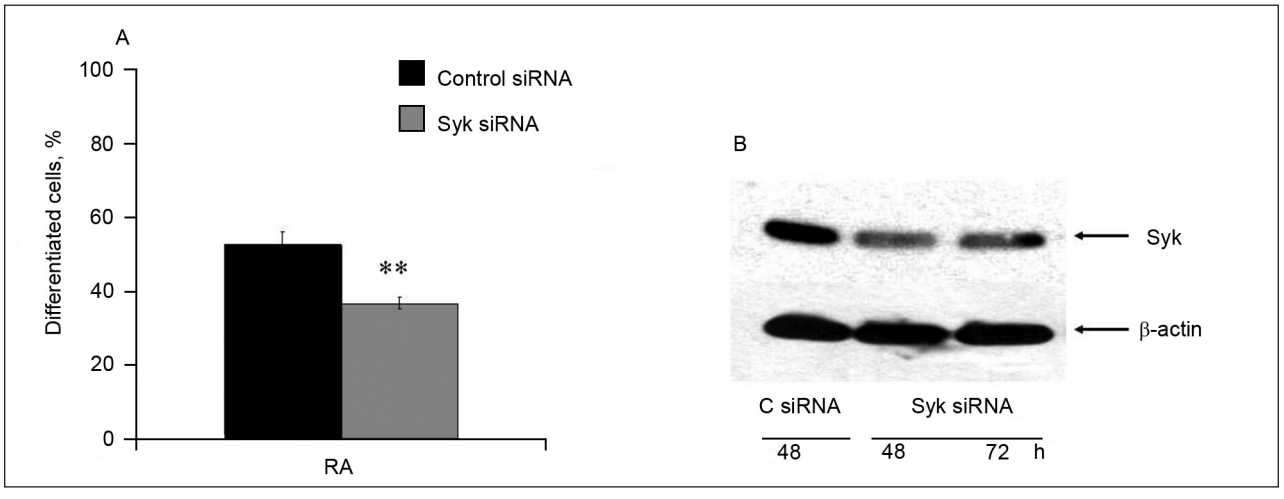

Fig. 4. Effect of siRNA-mediated inhibition of Syk expression on the retinoic acid-induced granulocytic differentiation of HL-60 cells. (A) Control siRNA or Syk siRNA-transfected cells were treated with 500 nM retinoic acid (RA), and the differentiation was examined by NBT test at 72 h. The data are means ± SD of three independent experiments, ** $p < 0.01$. (B) Reduction of Syk protein level by Syk siRNA. HL-60 cells were transfected with Syk siRNA or control siRNA (C siRNA), whole cell extracts were prepared at indicated times after transfection and then subjected to SDS-PAGE for protein expression using anti-Syk antibody. Equal loading was confirmed with a monoclonal anti-β-actin antibody

DISCUSSION

Differentiation induction as a treatment for hematological malignancies is widely used in modern medicine. Retinoic acid is known as a differentiation-inducing agent in many different cell types. The anti-cancer activity of retinoids is exerted through the activation of yet fully unidentified program that modulates cell differentiation as well as cell proliferation and / or apoptosis [23–25]. Since the exact sequence of events induced by RA is not fully understood, the identification and characterization of the signaling mechanisms leading to RA-induced differentiation would suggest the means to manage this pathway. The HL-60 myeloid leukemia cell line serves as a prototype model for studying the antileukemic effect of retinoids. Retinoids are known to exert their action by signaling through nuclear receptor heterodimers that activate or silence specific gene networks [26]. Retinoid receptors require functional interactions with transcriptional repressors and co-activators as well as with other transcription factors for their effects in cells. Moreover, interactions between retinoid receptors and the components of classical intracellular signaling pathways initiated by cell surface receptors are shown [27]. These interactions can occur through genomic and nongenomic mechanisms [28–30]. In the present study, we have focused on the role of cytoplasmic protein tyrosine kinase Syk in the process of RA-induced leukemic cell differentiation. The upregulation of Syk kinase tyrosine phosphorylation and activity were detected during RA-induced differentiation into granulocytes, although the Syk amount was not altered [22]. The data showing an increase in Syk phosphorylation during granulocytic but not monocytic differentiation point to its role in this process. We have found that the forced expression of Syk accelerates RA-induced HL-60 cell differentiation. Although the mechanism of the role of Syk overexpression in potentiating cell differentiation is unknown, it is likely that it acts through Vav protein, guanine nucleotide exchange factor for Rho family GTPases. Vav proteins link cell surface receptors to downstream signaling proteins, leading to actin cytoskeletal rearrangements and transcriptional alterations [31]. A crucial role of Vav in modulating the process of morphological maturation after RA treatment was shown in myeloid cells [32–34]. Authors have shown that in tumoral promyelocytes induced to granulocytic maturation, the upregulation of tyrosine phosphorylation of Vav is dependent on the tyrosine kinase Syk and is essential for the induction of a number of all-trans retinoic acid-induced responsive genes, as well as for the maturation-related changes of cell morphology. Authors show the role of Syk-dependent tyrosine phosphorylation of Vav1 in regulating the ability of this protein to form molecular complexes recruited by the CD11b promoter [35]. Consequently, the amount of Syk protein could influence the formation of intracellular complexes with Vav and thereby determine the signaling events leading to granulocytic maturation.

Conversely, Syk was found to play a role in counteracting differentiation in primary acute myeloid leukemia (AML) cells and AML cell lines. The pharmacological or genetic inhibition of Syk induced spontaneous differentiation in AML [36].

The results of our study show that during retinoic acid-induced differentiation, Syk tyrosine kinase may be an important component directing HL-60 cells towards granulocytes. Thus, in our case, Syk acts as a tumour suppressor. On the other hand, there are findings to show another, pro-survival, role of Syk kinase in leukemic cells [37, 38]. Syk participates in the activation of the Akt survival pathway [39, 40] thereby favouring tumour cell growth.

In conclusion, our data show Syk as a potential regulator of leukemic cell differentiation. The increase in the amount of Syk kinase accounts for the enhanced RA-induced granulocytic differentiation of HL-60 cells. The obtained results suggest that manipulation of the Syk protein level or its activity could be a potential approach in developing new anticancer therapies involving a combination of conventional and targeted therapies.

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TIROZINO KINAZĖS SYK VAIDMUO RETINOINĖS RŪGŠTIES INDUKUOTOJE LEUKEMINIŲ HL-60 LĄSTELIŲ DIFERENCIACIJOJE

Santrauka

Sutrikusi tirozino kinazės Syk reguliacija lemia vėžio vystymąsi. Kadangi viena iš priešvėžinės terapijos strategijų yra ląstelės galutinės differenciacijos indukcija, mes tyrėme Syk vaidmenį retinoinės rūgšties indukuotai promielocitinių HL-60 ląstelių diferenciacijai į granuliocitus. Šiame darbe buvo sukonstruoti retrovirusiniai vektoriai su prasmine ir priešprasme promotoriaus atžvilgiu SYK geno seka ir transfekuoti į HL-60 ląsteles. Po retinoinės rūgšties poveikio nustatyta, kad ląstelės su didesniu Syk kiekiu efektyviau diferencijavosi į granuliocitus lyginant su ląstelėmis, transfekuotomis tuščiu vektoriu. Ir atvirkščiai, viduląstelinio Syk kiekio sumažinimas panaudojus mažų interferuojančių RNR ar priešprasmių sekų strategijas slopino retinoinės rūgšties indukuotą HL-60 ląstelių diferenciaciją į granuliocitus. Syk yra potencialus retinoinės rūgšties indukuotos promielocitinių HL-60 ląstelių diferenciacijos reguliatorius ir gali būti tyrimų plėtojant naujas priešvėžines terapijas objektas.

Raktažodžiai: tirozino kinazė Syk, diferenciacija, HL-60, retinoidinė rūgštis