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SHORT COMMUNICATION

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VITAMIN E ACTIVATES EXPRESSION OF C/EBP ALPHA TRANSCRIPTION FACTOR AND G-CSF RECEPTOR IN LEUKEMIC K562 CELLS

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***Background*: Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder associated with the activity of *BCR-ABL* fusion oncogene. Tyrosine kinase inhibitors are the current treatment of CML, but secondary mutations finally contribute to therapy resistance and blast crisis of the disease. The search for the novel compounds for the effective control of CML is now in the spotlight. The progression of CML to blast crisis is correlated with down-modulation of C/EBP alpha. Therefore, C/EBP alpha may be considered as a putative target in differentiation therapies in myeloid leukemias. *The aim* of the study was to assess the potential of vitamin E as the possible inducer of C/EBP alpha expression in BCR-ABL-positive CML K562 cells. *Materials and Methods*: RNA extracted from K562 cells cultured with valproic acid or vitamin E was converted to cDNA, RT-PCR reactions were carried out using HotStarTaq DNA polymerase with primers for C/EBP alpha and granulocyte colony-stimulating factor receptor (G-CSFR). *Results*: We have not found detectable expression of C/EBP alpha in K562 cells. Upon 48-h culture with vitamin E at a dose of 100 μM, K562 cells expressed both C/EBP alpha and G-CSFR. *Conclusion*: Vitamin E restored the expression of C/EBP alpha mRNA in chronic myelogenous leukemia K562 cells. In this setting, G-CSFR expression in vitamin E treated K562 cells seems to suggest the activation to granulocytic differentiation. It should be further elucidated whether such effects of vitamin E on C/EBP alpha transcription factor are direct or mediated indirectly due to antioxidant properties of vitamin E.**

***Key Words:* chronic myeloid leukemia, granulocytic differentiation, C/EBP alpha transcription factor, G-CSFR, vitamin E.**

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder caused by a reciprocal translocation t(9:22) (q34;q11) fusing the *BCR* gene to *ABL* gene [1, 2]. The BCR/ABL fusion protein with elevated ABL tyrosine kinase activity is crucial for transformation of hematopoietic cell [3]. Tyrosine kinase inhibitors (imatinib, nilotinib and dasatinib) are the current treatment of CML. Nevertheless, BCR-ABL point mutations are the principal cause of resistance to the treatment resulting in CML relapse and progression [4, 5].

Thyrosine kinases as the products of the oncogenes and tumor suppressor phosphatases maintain the cell homeostasis by affecting cell growth, survival and differentiation. The imbalance between kinase and phosphatase activities is critical in many hematological malignancies [6].

Protein phosphatase 2A (PP2A) is one of the most abundant cellular proteins comprising up to 1% of the total cellular protein, a prominent tumor suppressor that regulates the activity of numerous kinases. It is commonly deregulated and deactivated in a variety of cancers [7].

Moreover, PP2A activity leads to growth suppression, enhances apoptosis, and restores differentiation of cancer cell. It was shown that in BCR/ABL transformed cell and CML blast crisis hematopoietic progenitors, the PP2A activity is strongly inhibited, while the pharmacological activation of PP2A suppresses BCL/ABL activity and induces BCR/ABL degradation [8]. The pharmacological modulation of PP2A activity is becoming an attractive strategy for cancer treatment. The substances of several different classes are known as PP2A activating compounds. Recently, vitamin E (α-tocopherol) and its analogues were reported to be among such compounds [7, 9].

Nevertheless, the effects of vitamin E on differentiation pathways in cells of CML, in particular those involving restoration of the expression of CCAAT-enhancer binding protein alpha (C/EBPα) and granulocyte colony-stimulating factor (G-CSF) receptor (G-CSFR) have not been yet studied. Expression of these proteins decreases drastically in CML (chronic phase) and during blast crisis. PP2A activation induces C/EBPα and G-CSFR expression which is consistent with differentiation of CML cells or BCR/ABL-transformed cell lines [10, 11].

In this regard, we evaluated the effect of vitamin E on RNA expression of crucial factors of myeloid differentiation, C/EBRα and G-CSFR, in BCR-ABL-positive CML K562 cells.

MATERIALS AND METHODS

K562 cell line originated from a CML patient in blast crisis was obtained from Depository of Cell Lines and

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Abbreviations used: AML – acute myeloid leukemia; CML – chronic myeloid leukemia; G-CSFR – granulocyte colony-stimulating factor receptor; PP2A – protein phosphatase 2A; VA – valproic acid.

Tumor Strains of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, the NAS of Ukraine. The cells were grown in suspension in RPMI-1640 medium supplemented with 10% of fetal calf serum.

Valproic acid (VA; Pharma GMBX, Austria) and vitamin E (Technolog, Ukraine) were used for the study. RNA was isolated from cultured cells by means of TRIzol reagent (Invitrogen, Gaithersburg, MD) according to manufacturer's recommendations. RNA concentration in samples was measured on Nanodrop 2000 spectrophotometer (Termo Scientific, USA). RNA was converted to cDNA using the Qiagen's QuantiTect Rev. Transcription Kit (Qiagen, Hilden, Germany).

C/EBPα and G-CSFR mRNA expression was quantified by real-time RT-PCR using SYBR Green protocol. RT-PCR reactions were carried out using HotStarTaq DNA polymerase (Qiagen), 50 ng of cDNA and SYBR Green in a 1:60,000 dilution in triplicate. PCR conditions were: 95 °C initial activation for 15 min was followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s on an Bio-Rad Real-time PCR Detection System IQ5, CA. Real-time RT-PCR was performed and conducted by means of SYBR Green (BioLab) on termocycler CFX96 Real-Time System (Bio-Rad, USA) for C/EBPα and G-CSFR gene mRNA expression level detection.

The primers of corresponding genes was used as: C/EBPα forward: 5′-CAAGAACAGCAACGAGTACCG-3′; reverse: 5′-GTCACTGGTCAACTCAG-CAC-3′; G-CSFR forward — 5′-ACAAGC-CGCAGCGTGGAGAAG-3′; reverse — 5′-TTCT-GAAGGCAGGTGGAAGGTG-3′. GAPDH is a reviewer gene, forward — 5′-CGCTCTCTGCTCCTCCTGTT-3′; reverse — 5′-CCATGGTGTCTGAGCGATGT-3′. The gene expression was quantified using 2 Δ-Ct method with normalization to mRNA expression of GAPDH. Statistical significance of differences was evaluated by Student`s *t*-test.

RESULTS AND DISCUSSION

We studied the effects of VA with known differentiation properties towards myeloid cells and vitamin E in K562 cells. The toxicities and the possible growth inhibiting effects of the studied substances were first analyzed to select the appropriate concentrations. According to the literature, different effects of VA *in vitro* were analyzed in concentrations up to 2 mM [12, 13].

In our study, VA in a concentration of 4 mM for 48 h reduced the growth rate and cell viability and induced apoptosis in a fraction of K562 cells (up to 30%) (data not shown). As to vitamin E, in the series of our preliminary experiments, no evidence of toxicity has been demonstrated when K562 cells were cultured with vitamin E in a concentration of 100 μM for 48 h. These concentrations were further used in the experiments for assaying the expression of C/EBPα and G-CSFR mRNA.

Fig. 1 demonstrates that VA did not change significantly the level of mRNA C/EBP expression in K562 cells. On the contrary, vitamin E proved to be an effective inducer of mRNA C/EBP.

When mRNA G-CSFR expression in K562 cells was assessed, both VA and vitamin E induced mRNA of this receptor, with effect of vitamin E surpassing that of VA (Fig. 2).

The fold increases in C/EBPα and G-CSFR mRNA expression in K562 cells exposed to 100 μM vitamin E for 48 h calculated by $2^{-(\Delta\Delta Ct)}$ method are presented in Table.

Table. Fold increase (analyzed in triplicates) in gene expression in K562 cells line culture under 48-h vitamin E exposure (100 μM) calculated by $2^{-(\Delta\Delta Ct)}$ method

N (n=3)	C/EBPα		G-CSFR	
	Fold increase	Standard deviation, σ	Fold increase	Standard deviation, σ
1	8.395 ± 1.481	1.988	3.930 ± 1.843	1.988
2	9.854 ± 0.023	1.219	4.626 ± 0.853	1.988
3	11.381 ± 1.506	1.219	5.134 ± 1.357	1.988

Note: $\sigma = \sqrt{1/n\ \Sigma(x_i - x^-)^2}$.

Differentiation of hematopoietic cells is regulated by lineage-specific transcription factors. It has recently

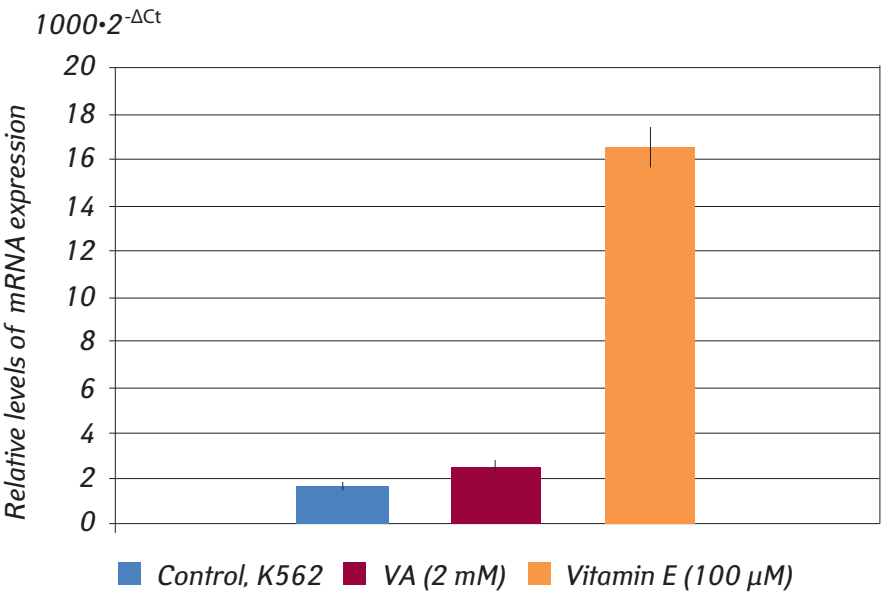

Fig. 1. The relative levels of C/EBPα mRNA expression in K562 cells exposed to VA (2 mM) or vitamin E (100 μM) for 48 h

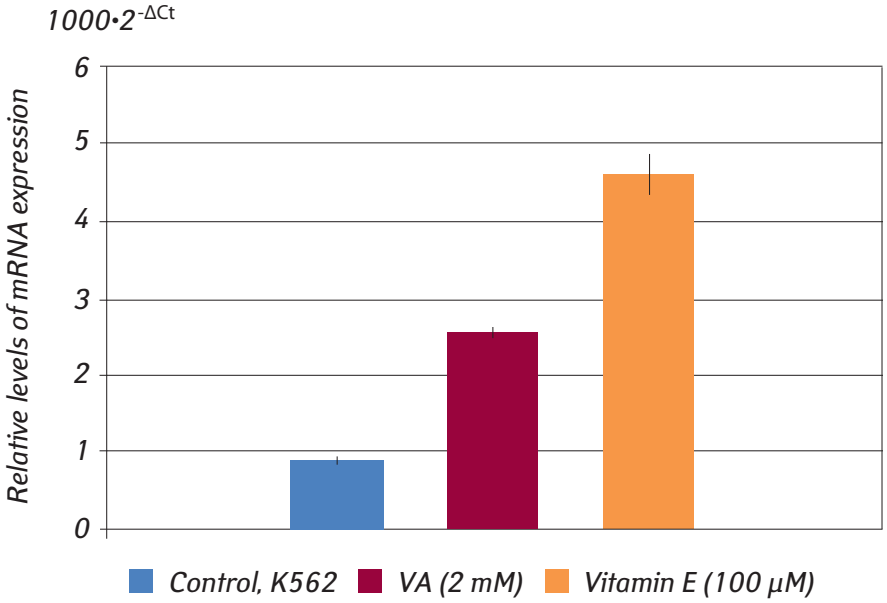

Fig. 2. The relative levels of G-CSFR mRNA expression in K562 cells exposed to VA (2 mM) or vitamin E (100 μM) for 48 h

been proposed that loss of C/EBPα transcription factor expression or function may contribute to the differentiation block, enhanced proliferation, and development of acute myeloid leukemia (AML) [14]. C/EBPα is mainly involved in cell fate decisions for myeloid differentiation [15]. The level of C/EBPα expression was significantly declined in CML patients [16]. Therefore, C/EBPα is a critical regulator of myeloid cell development directing granulocyte and monocyte differentiation.

Tavor *et al.* [17] first demonstrated that the restoration of C/EBPα expression in BCR-ABL-positive KCL22 blast cell line transfected with *C/EBPα plasmide vector (pMTα)* triggered a proliferative arrest, a block in the G2/M phase of the cell cycle and a gradual increase in apoptosis. Therefore, C/EBPα may be considered as a putative target in differentiation therapies in myeloid leukemias.

While VA is known as the inducer of apoptosis and differentiation in K562 cells [18], the mechanisms have not been yet elucidated in details. The effect of VA on the induction of C/EBPα in AML cells has been demonstrated unquestionably but the data relating the effects of VA and C/EBPα in CML setting remain controversial [19]. In fact, our data do not demonstrate the statistically significant increase in C/EBPα expression in K562 cells exposed to VA.

C/EBPα directly activates G-CSFR transcription during common myeloid progenitor lineage committing activation [20–22]. Therefore, C/EBPα loss is causally connected with early block in myeloid maturation, suggesting that C/EBPα is a master regulator of hematopoietic differentiation. The malignant transformation in CML and AML is associated with the loss of G-CSFR as direct neutrophyl granulocytic differentiation marker and C/EBPα as major myeloid master regulator [23, 24].

Our data suggest that vitamin E restores the expression of C/EBPα mRNA in K562 cells. It should be further elucidated whether such effects of vitamin E on myeloid transcription factor C/EBPα are direct or mediated indirectly due to the antioxidant properties of vitamin E.

Nevertheless, our data suggest vitamin E-associated hematopoietic differentiation-like potential. Further studies are needed to prove whether C/EBPα-dependent activation as well as an increased G-CSFR expression in vitamin E treated K562 cells could be associated with gain of any particular features of granulocytic differentiation.

Our findings may be very important for future studies of imatinib resistance in clinical setting taking into account the recent report by Kagita *et al*. [25] demonstrating correlation of C/EBPα expression with response and resistance to imatinib in CML.

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