Chapter

Vitamin E in Chronic Myeloid Leukemia (CML) Prevention

Lyudmyla Shvachko, Michael Zavelevich, Daniil Gluzman and Gennadii Telegeev

Abstract

The resistance to inhibitors of tyrosine kinase necessitates novel approaches to the therapy of chronic myeloid leukemia (CML). The progression of CML to blast crisis is associated with down-regulation of C/EBP-alpha being involved in the differentiation block in leukemic blast cells. Moreover, lowered C/EBP-alpha expression correlates with resistance to imatinib in CML. We have demonstrated that vitamin E up-regulates expression of C/EBP-alpha and down-regulates expression of Snail transcription factor in K562 cells in vitro contributing to the putative recovery of myeloid differentiation potential. In parallel with increased CEBP alpha expression, Vitamin E treatment results in the decreasing expression of placental-like alkaline phosphatase and increasing expression of tissue non-specific alkaline phosphatase. We suggest that vitamin E could be used as the plausible biological modulator to prevent the progression to blast crisis and to overcome drug resistance of leukemic cells in CML.

Keywords: chronic myeloid leukemia, vitamin E, C/EBP-alpha, Snail, K562 cells, drug resistance

1. Introduction

Chronic myelogenous leukemia (CML) is a clonal hematopoietic stem cell disorder associated with the activity of *Bcr-Abl* fusion oncogene that arises from the translocation of chromosomes 9 and 22 as t (9:22) (q34;q11) [1, 2]. The BCR/ABL fusion protein with elevated ABL tyrosine kinase activity is crucial for transformation of hematopoietic stem cells (HSCs) [3]. The constitutively active P210 BCR-ABL tyrosine kinase is considered as a key player in the molecular pathogenesis of CML [3, 4]. The disease begins with an indolent chronic phase that can last for several years. If untreated, it then progresses into accelerated phase and within a year into blast crisis phase. The survival of patients in blast crisis is less than one year. Because the preeminent rearrangement driving CML is *Bcr-Abl*, only BCR-ABL tyrosine kinase inhibitors such as imatinib (or nilotinib and dasatinib) are a known curative therapy of CML with extraordinarily successful 5-year survival rates greater than 90% [5–8]. Nevertheless, the secondary mutations finally contribute to the therapy resistance and blast crisis of the disease. The search for the novel compounds for the effective control of CML progression is now in the spotlight.

The free radical scavengers like alpha-tocopherol may be effective against cancer-associated oxidative stress. The mean serum vitamin E level significantly

decreased in CML patients that seems to be quite in agreement with free radical involvement in CML progression [9]. In contrast to antioxidant function of vitamin E in CML, we suggest new modulation mechanisms of vitamin E that could be operative in prevention of CML progression. In particular, we analyzed the modulation function of vitamin E for molecular unblocking of myeloid differentiation potential in CML cells via vitamin E-dependent induction of pivotal transcription factor CEBP alpha (CCAAT/enhancer-binding protein) as myeloid master regulator of myelopoiesis/granulopoiesis and consequently G-CSFR (granulocyte-colony stimulation factor receptor) [10]. Moreover, we have found that vitamin E could be involved in targeting epithelial-mesenchymal transition (EMT) mechanism in CML cells via SNAIL as EMT inducer [11, 12]. Therefore, we propose that vitamin E could be a therapeutic option when CML progresses in setting of imatinib therapy. Finally, since alkaline phosphatase is considered as a marker of stem cells [13], we studied the aberrant expression of placental-like alkaline phosphatase (PLAP) and discovered the potential of vitamin E in remodeling of CML-associated aberrant expression of this enzyme [14]. Vitamin E-dependent induction of tissue non-specific alkaline phosphatase (TNAP) is paralleled by restored CEBP alpha expression as myeloid master regulator in CML cells [14].

Taken together, these findings suggest that vitamin E shows ability of remodeling leukemic stem cell (LSC) phenotype in CML cells to hematopoietic stem cell (HSC) phenotype with myeloid differentiation potential development.

2. Vitamin E activates expression of C/EBP alpha transcription factor and G-CSF receptor in CML blast crisis leukemic K562 cells

C/EBPα is mainly involved in cell fate decisions for myeloid differentiation [15]. The progression of CML to blast crisis is correlated with down-modulation of C/EBP-alpha contributing to the differentiation block, enhanced proliferation, and development of acute myelogenous leukemia [16, 17]. The level of C/EBPα expression is significantly declined in CML patients [18]. Currently, the deregulation of C/EBP alpha is considered as a paradigm of leukemogenesis [19]. Therefore, C/EBPα is a critical regulator of myeloid development guiding granulocyte and monocyte differentiation.

We have studied the modulating potential of vitamin E as the possible inducer of C/EBP-alpha expression in BCR-ABL-positive CML K562 cells. K562 cell line originated from a CML patient in blast crisis progression is recognized as a model for leukemia research. We studied the effects of vitamin E in K562 cells in comparison with valproic acid with known differentiation properties towards myeloid cells [20–22].

Valproic acid 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%). 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/EBP-alpha and G-CSFR mRNA. **Figure 1A** demonstrates that valproic acid 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 with about 10-fold increase in expression as compared with non-treated K562 cells. When mRNA G-CSFR expression in K562 cells was assessed, both valproic acid and vitamin E induced mRNA of this receptor, with effect of vitamin E surpassed that of VA (**Figure 1A** and **Table 1**).

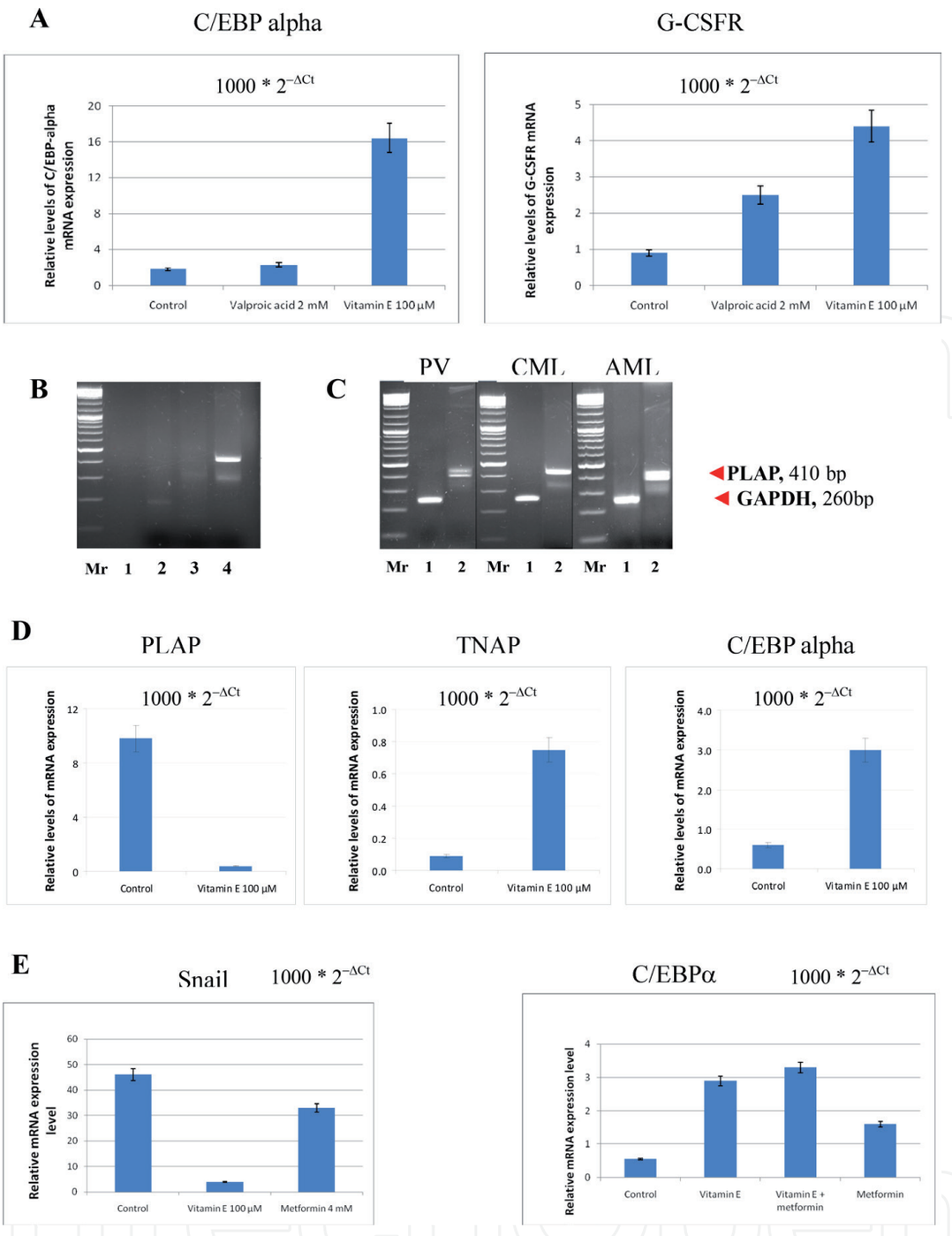

Figure 1.
mRNA expression in CML cells modified by vitamin E. (A) The relative levels of mRNA C/EBP-alpha and G-CSFR expression in K562 cells exposed to valproic acid (2 mM) or vitamin E (100 μM) for 48 h. (B) The aberrant AP mRNA detected by qRT-PCR in leukemic cells of the patient with CML blast crisis: 1 – control without primers; 2 – primers to PAP; 3 – primers to TNAP; 4 – primers to IAP. (C) Ectopic gene expression of embryonic PLAP mRNA in peripheral blood cells of the patient with CML, acute myeloid leukemia (AML), and polycytemia vera (PV): 1 – GAPDH, reference gene; 2 – aberrant PLAP. (D) The relative levels of mRNA expression of PLAP, TNAP, and CCAAT-enhancer binding protein alpha (C/EBPα) in K562 cells exposed to vitamin E (100 μM) for 48 h by real time RT-PCR $2^{-(\Delta Ct)}$ method. (E) Relative mRNA expression level of transcription factor Snail and transcription factor CEBPα in CML blast crisis K562 cells exposed to vitamin E (100 μM) and metformin (4 mM) for 48 h. The relative levels of mRNA expression were analyzed by qRT-PCR and calculated by $2^{-(\Delta Ct)}$ method.

Thease findings are quite confirmed by Tavor et al. [23] have first shown that the restoration of C/EBP-alpha expression in BCR-ABL-positive KCL22 blast cell line provided by transfection with C/EBPα plasmid vector caused a block in the G_2/M phase of the cell cycle with gradual increase in apoptosis suggesting that C/EBP-alpha may be considered as a putative target in differentiation therapies in acute myeloid

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

Table 1.
Fold increase C/EBP alpha and G-CSFR mRNA expression (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. Note: $\sigma = \sqrt{1/n \sum(x_i - x^-)^2}$.

leukemia. C/EBPα directly activates G-CSFR transcription in lineage committing activation of common myeloid progenitor [24–26]. 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 transcription factor C/EBPα is known as a critical regulator of myeloid development, directing granulocyte, and monocyte differentiation [27].

Our findings gave evidence of C/EBP alpha-dependent activation to granulocytic differentiation via targeted increase in G-CSFR expression in vitamin E treated K562 cells. It should be further elucidated whether such effects of vitamin E on myeloid transcription factor C/EBP-alpha are direct or mediated indirectly due to the antioxidant properties of vitamin E. Nevertheless, our data suggest vitamin E-associated hematopoietic differentiation-like potential associated with C/EBPα and G-CSFR up-regulation. Our findings might be very important for future studies of imatinib resistance in CML clinical setting taking into account the recent report by S. Kagita et al. demonstrating correlation of C/EBPα expression with response and resistance to imatinib in CML [28].

3. Aberrant expression of placental-like alkaline phosphatase in chronic myeloid leukemia cells in vitro and its modulation by vitamin E

LSCs in CML do not depend on BCR-ABL signaling for their survival [29, 30], and their persistence remains a major obstacle to curing CML [31, 32]. The search for new biological markers of LSC phenotype is still relevant today. Placental-like alkaline phosphatase (PLAP) is expressed by many tumors. Its aberrant expression has been considered to be potentially useful as tumor marker [33]. However, the biological background of the role of this aberrant alkaline phosphatase (AP) in cancer is still unclear. The AP activity in blood serum known as nonspecific marker of bone metastasis [33] is also of potential significance for the identification of stem cell phenotype [13, 34]. Moreover, AP activity is a widely accepted marker of stem cells associated with embryonic stem cell pluripotency [35]. The expression of various forms of AP in CML cells has not yet been studied. Therefore, we aimed to analyze the expression patterns of various AP forms in cells originated from CML patients in blast crisis and to modify their expression by vitamin E (100 μM) in K562 cells. We used the primers to three known tissue AP, namely placental AP (PAP), non-specific AP (TNAP) (expressed in bone, kidney, liver) and intestinal AP (IAP) [36] to analyze the mRNA expression of these APs in CML cells by qRT-PCR. We have observed the aberrant expression of mRNA IAP in cells of CML patient in blast crisis (**Figure 1B**) that upon sequencing (data not shown) demonstrated the significant alignment with known cancer-associated PLAP sequence, while no gene homology with tissue PAP was detected. This fact gave reason to

consider revealed PLAP as embryonic-like placental AP (ELAP), to be more precise, the aberrant PLAP in blast cells of CML patients (**Figure 1C**). Indeed, such PLAP is expressed in early embryo pre-implantation period as was detected in studying mouse embryonic cell development, while tissue TNAP begins to express in post-implantation period [35]. We have not detected TNAP in cells of CML patients. Only the embryonic-like PLAP was detected, which expression also increased in acute myeloid leukemia (**Figure 1C**). Recently, TNAP recognized ultimately as mesenchymal stromal cell antigen-1 (MSCA-1) [13] was described as a biomarker associated with normal hematopoiesis as well as with terminal myeloid differentiation [37]. The decreased TNAP synthesis is a classical feature of CML used as one of diagnostic cytochemical markers in differential diagnosis [2]. We have observed vitamin E targeted decrease in aberrant embryonic-like PLAP expression at mRNA level with increased TNAP mRNA expression. Moreover, along with down-regulation of aberrant PLAP the up-regulation of C/EBP alpha mRNA expression was restored by vitamin E in exposed K562 cells as we founded (**Figure 1D** and **Table 2**).

Taken together, we have concluded that the loss of TNAP and CEBP alpha in CML may contribute to pathogenesis of this disease whereas aberrant embryonic-like PLAP may be considered as a new CML biomarker of LSC pluripotent phenotype in CML progression. Therefore, aberrant embryonic-like PLAP may be considered as a putative target in differentiation therapies in myeloid neoplasms. Our findings suggest the biomodulation role of vitamin E as the available inducer of differentiation potential of CML leukemic cells. The ectopic PLAP expression in leukemic cells of different myeloid neoplasms suggests its importance in biology of these malignancies.

Conclusivelly, to analyze whether ectopic PLAP expression in CMLcells *in vitro* may be modulated, we studied PLAP and TNAP expression in CML blast crisis K562 leukemic cells incubated with vitamin E for 48 h. In fact, vitamin E treatment affects expression of PLAP and TNAP in opposite ways. Namely, PLAP expression decreased significantly while TNAP expression increased. The increase in TNAP expression was paralleled with increased CEBP alpha expression (**Figure 1D** and **Table 2**). **Figure 1**, D demonstrates that vitamin E targeted aberrant PLAP expression is closely related to the restoration of CEBP alpha and TNAP expression. These key regulators tightly contribute to potential reactivation of myeloid differential as we studied in K562 leukemic cells. Therefore, we point out that vitamin E could be able to affect leukemic blast stem cell phenotype remodeling.

To sum up, we have demonstrated increased aberrant PLAP expression in leukemic cells of myeloid origin (CML) in the setting of the decreased TNAP expression. The aberrant expression of embryonic PLAP may be considered as

N(n=3)	Fold decreasing	Standard deviation, σ	Fold increasing	Standard deviation, σ	Fold increasing	Standard deviation, σ
	PLAP (M ± m)		CEBPα (M ± m)		TNAP (M ± m)	
1	4.088 ± 0.322	1.42	2.972 ± 1.594	1.35	6.023 ± 2.809	1.98
2	6.292 ± 1.883		4.377 ± 0.117		1.690 ± 1.524	
3	2.848 ± 1.561		6.207 ± 1.713		1.931 ± 1.283	

Table 2.
The relative fold decreasing PLAP corresponding to fold increasing CEBP α and TNAP mRNA expression (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. Note: $\sigma = \sqrt{1/n \sum (x_i - x^-)^2}$.

one of the putative markers of myeloid cell undifferentiated state. On the other hand, potential of PLAP as one of the possible target for controlling LSC phenotype should be further explored. More attention is needed to explore the potential of the bioactive molecules such as vitamin E that may induce granulopoiesis reprogramming.

4. Vitamin E suppresses EMT-SNAIL transcription factor and restores CEBP alpha transcription factor as master regulator of myelopoiesis in K562 cells

The persistence of LSC remains a major obstacle to cure CML [38, 39]. Epithelial mesenchymal transition (EMT) mechanism is known to contribute to tumor stem cell progression [40, 41]. Although EMT has been studied in relation to epithelium-derived tumors, there is increasing evidence implicating the involvement of EMT activators in hematopoietic malignancies [42, 43]. The expression of some EMT modulators has been demonstrated in Ph + leukemia cells [44]. EMT inducer Snail if of most important role in maintaining stemness properties in tumor progression [45, 46]. It was shown that Snail also drives LSC phenotype in leukemia progression [44, 47]. Earlier, we revealed that alpha-tocopherol might be an effective inducer of mRNA CEBP alpha in K562 cells *in vitro* [10]. The loss of C/EBPα contributes to leukemogenesis [16, 19] and CEBP alpha expression prevents from appearance of EMT phenotype [48].

We have determined the relationship between EMT-Snail suppression and restored CEBP alpha myeloid differentiation potential in CML blast crisis K562 cells exposed to vitamin E. Metformin as known substance mediating EMT reversal [49] was used to compare EMT suppression effect of vitamin E in K562 cells.

We have found highly detectable Snail1 mRNA expression and down-regulated CEBP alpha in K562 cells (**Figure 1E**). Vitamin E suppressed EMT-Snail mRNA expression and up-regulated myeloid master regulator CEBP alpha mRNA expression (**Figure 1E** and **Tables 3**, **4**). Such reactivation of CEBP alpha is enhanced by metformin pointing to the possible synergistic effect with alpha-tocopherol. We observed that vitamin E is a modulator of gene expression that affects Snail1 and CEBP alpha mRNA expression in K562 cells in opposite directions. One could suggest the causal relationship between EMT-Snail1 suppression and restoration of CEBP alpha expression that seems to contribute to recover myeloid differentiation potential of CML blast cells. As seen in **Figure 1E**, myelopoietic master regulator C/EBPα is also restored upon metformin treatment, although the effect of vitamin E is more pronounced (**Table 4**).

Taken together, schematic model of the Vitamin E modulation effects in CML blast crisis progression with Snail-EMT phenotype is presented (**Figure 2**).

N(n=3)	Fold decreasing	Standard deviation, σ	Fold decreasing	Standard deviation, σ
	Vitamin E/Snail M ± m		Metformin/Snail M ± m	
1	14.160 ± 0.437	0.408	1.579 ± 0.110	0.086
2	13.176 ± 0.547		1.366 ± 0.103	
3	13.833 ± 0.110		1.464 ± 0.005	

Table 3.
Fold increase EMT-inducer transcription factor SNAIL mRNA expression (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. Note: $\sigma = \sqrt{1/n \sum (x_i - x^-)^2}$.

N(n=3)	Fold increasing	Standard deviation, σ	Fold increasing	Standard deviation, σ	Fold increasing	Standard deviation, σ
	Vitamin E M ± m		Vitamin E + Metformin M ± m		Metformin M ± m	
1	5.156 ± 0.328	0.232	5.564 ± 0.075	0.371	2.841 ± 0.007	0.272
2	4.634 ± 0.194		5.00 ± 0.489		3.164 ± 0.330	
3	4.696 ± 0.132		5.902 ± 0.413		2.498 ± 0.336	

Table 4.
The fold increase of relative levels of the transcription factor CEBP alpha mRNA expression compared with metformin (4 mM) under decreasing of relative levels of the transcription factor Snail mRNA expression by vitamin E (analyzed in triplicates) in K562 cells line culture under 48-h vitamin E exposure (100 μM) calculated by $2^{-(\Delta\Delta Ct)}$ method. Note: $\sigma = \sqrt{1/n \sum (x_i - x^-)^2}$.

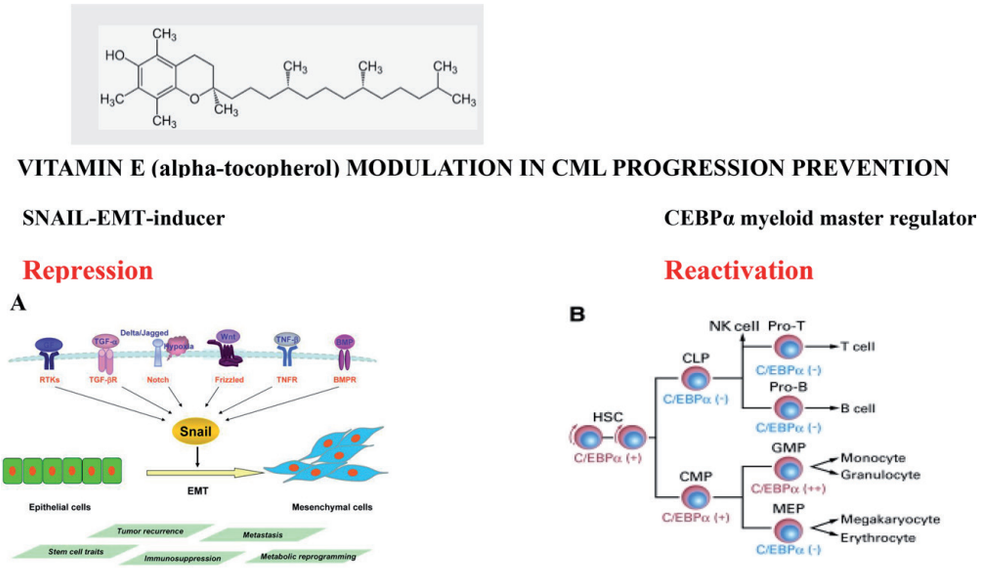

Figure 2.
Schematic model of the vitamin E modulation in CML progression with EMT phenotype.

5. Discussion

CML is characterized by an accelerated and unregulated proliferation of predominantly myeloid cells in the bone marrow with their accumulation in the blood. CML develops as a result of malignant transformation and clonal proliferation of pluripotent hematopoietic stem cells (HSCs), leading to overproduction of immature myeloid progenitor cells that results in blast cell crisis. The CML blast crisis resembles acute leukemia. Because the preeminent mutation driving CML is Bcr-ABL thyrosine kinase oncogene, the use of Bcr-Abl kinase inhibitors (TKIs), such as imatinib, dasatinib, and nilotinib, significantly improves treatment outcomes and extends the life expectancy of CML patients. However, imatinib resistance drives blast crisis progression. The persistence of LSCs remains a major obstacle to cure CML. The clinical CML blast crisis progression with LSC phenotype is practically incurable. Therefore, the blocking of terminal myeloid differentiation and LSC phenotype development defines a putatively new strategy for CML prevention.

The potential of vitamin E in regulation of these interdependent mechanisms in CML progression was hinted by several observations that were reported earlier. Sangodkar et al. [50] showed that vitamin E activates PP2A phosphatase resulting in Bcr-Abl thyrosine kinase inhibition and re-activation of myeloid differentiation

pathway. In BCR/ABL transformed cells 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 [51]. 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, vitamin E (α-tocopherol) and its analogues having been reported among such compounds [50, 52].

Nevertheless, the effects of vitamin E on differentiation pathways in cells of blast crisis CML, in particular those involving restoration of the expression of CCAAT-enhancer binding protein alpha (C/EBPα) and granulocyte colony-stimulating factor receptor (G-CSFR) have not been yet studied. The expression of these proteins decreases drastically in chronic phase and blast crisis of CML [53, 54]. 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 blast crisis K562 cells. Our data demonstrate that vitamin E restores the expression of C/EBPα and concequently G-CSFR. Our results are consistent with Tavor et al. [23] who demonstrated that the restoration of C/EBPα expression in BCR-ABL-positive KCL22 blast cell line triggered a proliferative arrest, a block in the G2/M phase of the cell cycle and a gradual increase in apoptosis suggesting the activation of differentiation. Therefore, C/EBPα stimulated by vitamin E may be considered as a putative target in differentiation therapies in myeloid leukemias.

The second effect of vitamin E potentially useful for CML treatment was reported by Nieborowska-Skorska et al. [55] who demonstrated that vitamin E prevents accumulation of imatinib-resistant BCR-ABL1 kinase mutations in mice CML xenografts. The authors stressed anti-oxidant function of vitamin E in this processes. We use vitamin E as modulating factor in CML that involves vitamin E-dependent EMT mechanism of repression taking into account the pivotal role of EMT in the development of LSC phenotype. In this connection, we observed Snail1 overexpression suggesting some features of EMT phenotype in K562 cells seemingly contributing to CML pathogenesis. Furthermore, we have determined down-regulation of CEBP alpha transcription factor representing the master regulator of myelopoiesis in CML cells coinciding with Snail1 overexpression. Our findings are quite consistent with the recent report by Lourenço et al. [43] who suggest that C/EBP α is crucial determinant of epithelial maintenance by preventing EMT. Indeed, we have found that CEBP alpha is repressed by overexpression of EMT-inducer Snail in CML blast crisis K562 cells. Consequently, the reactivation of CEBP alpha by vitamin E is paralleled by suppression of Snail.

Therefore, our findings make deeper understanding of the role of vitamin E in suppression of CML LSC phenotype. In addition, we have revealed a new marker – aberrant placental-like alkaline phosphatase (PLAP) that expressed ectopically in CML progression. Moreover, its suppression by vitamin E consequently re-activates CEBP alpha and TNAP as myeloid differentiation factors. Taken together, our findings presented in this Chapter stress the role of vitamin E in modifying expression profile of CML cells towards restoration of myeloid differentiation potential.

6. Conclusion

Vitamin E is a complex group of lipid-soluble antioxidants comprising four tocopherols and four tocotrienols. It prevents production of reactive oxygen species (ROS) that are elevated in majority of tumor cells leading to lipid peroxydation, changing signaling pathways that control cell proliferation and apoptosis, expression of several transcription factors, epigenetic modulators, resistance to treatment,

etc. We have suggested the causal relationship between EMT-Snail1 suppression and restoration of CEBP-alpha myeloid master regulator expression that seems to contribute to recover myeloid differentiation potential of CML blast cells by vitamin E. We first observed that vitamin E is an effective modulator of down-regulation of transcription factor Snail EMT-inducer and up-regulation of pivotal myelopoietic transcription factor CEBP alpha resulting in restoration of TNAP expression. Taken into account the data of literature and our findings, we can postulate that vitamin E might be used as a potential pharmacopoeian biological modulator capable of preventing the onset of blast crisis development, ameliorating disease progression and possibly overcoming drug resistance of leukemic cells in CML patients.

Additional information

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