RESEARCH ARTICLE

Anti-Vascular Endothelial Growth Factor Targeting by Curcumin and Thalidomide in Acute Myeloid Leukemia Cells

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Abstract

Acute myeloid leukemias (AMLs) are blood disorders that exhibit uncontrolled growth and reduction of apoptosis rates. As with other malignancies, progression may be result of induction and formation of new blood vessels influenced by disease conditions. Cancer cells produce a variety of factors which play important roles in angiogenesis. Vascular endothelial growth factor (VEGF) is critical for many malignancies, including AMLs. Curcumin, as a natural compound, is able to enhance apoptosis via a mechanism affecting regulatory genes. As a new strategy we here evaluated anti-VEGF properties of curcumin, alone and in combination with thalidomide, in leukemic cell lines. Growth inhibitory effects were assessed by MTT assay and apoptosis was detected by annexin/PI staining in U937 and KG-1 cell lines. mRNA expression levels of VEGF isoforms were evaluated by qRT-PCR. Curcumin inhibited proliferation and induced apoptosis in both KG-1 and U937 cells and this effect was stronger in combination with thalidomide. In KG-1 cells, the level of VEGF (A, B, C and D) mRNA was decreased in curcumin-treated as compared to untreated cells. Maximum effects were obtained at the concentration of 40 μM curcumin in U937 cells. Taken together, the results indicate that the VEGF autocrine loop may have an impact on AML development and progression and could be considered as a therapeutic target. Thalidomide as a VEGF inhibitor in combination with curcumin appears to have a synergistic impact on inhibition of cell proliferation and promotion of apoptosis.

Keywords: Curcumin- thalidomide- vascular endothelial growth factor- acute myeloid leukemia

Introduction

Leukemia is the most prevalent type of malignancy among children (Abrahamsson et al., 2011; Alizad Ghandforoush et al., 2016). Acute myeloid leukemia (AML) is the most common hematopoietic stem cell disorder which known through clonal proliferation of myeloid precursors (Dick, 1997; Lim and Jamieson, 2014). In spite of high dose chemotherapy however relapse is common after conventional therapy. Recent studies demonstrated that leukemic populations are extremely heterogeneous and leukemia caused by increasing a group of leukemic cells which called leukemic stem cells (LSC) (Lane and Gilliland, 2010; Ghasemi et al., 2015; Panah et al., 2017). LSC contact with hematopoietic niche, keep self-generality property and alleviate the effect of chemotherapy(Mohammadi et al., 2017). LSC population in Human AML can be detected by surface markers, including CD34+ CD38- and CD123+ (Mohammadi et al., 2016b; Panah et al., 2017; Mohammadi et al., 2017b). Molecular characteristics associated with LSC are including mutations in kinase domain, transcription factor, tumor suppressor or involving changes in cell growth and survival mechanisms. Although many pathways are still unknown, inhibition of well-known pathways may be considered as an effective therapeutic target for leukemia (Mirzaei et al., 2017). Curcumin (CUR) is a phytochemical which extracted from Curcuma longa (turmeric) (Cheng et al., 2001; Haghi et al., 2017a; Mirzaei et al., 2017a). This natural compound is known as an effective anticancer agent. CUR affect the biochemical and molecular cascades in malignant cells (Jha et al., 2010) and also is able to enhance apoptosis (Pesakhov et al., 2010; Mohammadi et al., 2016a) through affecting on regulatory genes involved in cell proliferation and apoptosis (Kuo et al., 1996). Likewise, CUR can suppress angiogenesis by suppressing of TNF-α and INF-γ (Wnendt et al., 1996; Corral et al., 1999; Majumdar et al., 2002) (Figure-1). Vascular Endothelial Growth Factor (VEGF) is one of the major mediators of angiogenesis which controls angiogenic budding by guiding filopodial extension from endothelial tip cells, as a first step of new vessels formation. (Barnhill et al., 1984; Li et al., 2002). This factor has also been introduced as vascular permeability factor (VPF) which released by tumor cells.(Gerhardt et al., 2003; Mimura et al., 2007; Smith et al., 2010). VEGF consider as a critical
factor for cancer cells, including AML (Spilsbury et al., 2000; Xu et al., 2003). Over expression of this factor has huge impact on the process of leukemic cell proliferation and subsequently disease progression. Based on anti-VEGF function of Thalidomide (THAL) (Spilsbury et al., 2000; Xu et al., 2003), we decided to evaluate the combination effect of CUR and THAL as a new strategy with unique anti-VEGF properties and induction of apoptosis in leukemic cell lines. Also, the effect of these compounds on mRNA expression level of different isoform of VEGF were evaluated in these cell lines.

Materials and Methods

Reagents
CUR, Annexin V-FITC apoptosis detection kit, dimethylsulfoxide (DMSO) and DEPC treated water were obtained from the Sigma-Aldrich,USA (Sigma-Aldrich, St. Louis, MO), and THAL was purchase from the Santa Cruz (Santa Cruz, Dallas, Texas). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Carlsbad, CA). The cDNA synthesis kit was purchased from Takara (Takara Bio Inc., Otsu, Japan). TRI pure was obtained from Roche (Roche Applied Science, Germany).

Cell lines and cell culture
The human leukemia cell lines U937 and KG-1 were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). These cell lines were cultured in complete RPMI-1640 medium with 10% and 20% heat inactivated FBS for U937 and KG-1 cell line respectively, and supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin. All cells were cultured in a humidified atmosphere at 37°C with 5% CO2 incubator. Cells were seeded at 1x10^5 cells/ml.

Cell proliferation assay
U937 and KG-1 cell line were seeded at an initial density 5 × 10^3 cells per well in 96-well plate and were treated with CUR, THAL and their combinations at 37°C and 5% CO2 atmosphere for 24, 48 and 72 hours. The proliferation rate of cells was analyzed by MTT assay. Results were expressed as a proliferation rate, with 100% representing control cells treated with 0.1% DMSO alone.

Annexin V/PI
U937 and KG-1 cell lines were seeded at density of 3 × 10^5 cell/well and incubated for 24 hours in absence and presence of CUR, THAL and their combinations. To assess the percentage of apoptosis induction by treated compounds, fluorescein-conjugated Annexin V (Annexin V-FITC) staining assay was accomplished based on manufacturer protocol. The percentage of apoptosis was determined as a percentage of the Annexin V+/PI- cells through flow cytometry by BD flow cytometer instrument and analyzed with flow jow program.(Vermes et al., 2000; Njoh et al., 2006; Edward, 2009; Rieger et al., 2010; Ganjalikhani-Hakemi et al., 2017).

DNA cell cycle analysis
Cells were treated with indicated concentrations of CUR and THAL, then fixed in 70% in ethanol and dye by PI. Cells were assessed by BD flow cytometer instrument analyzed with flow jow program. The apoptotic cell fraction could predict from hypodiploid sub-G0/G1 DNA fraction.

RNA isolation and Real time PCR
Total cellular RNA was isolated by phenol-chloroform method using Tri-Pure extraction solution (Roche Applied Science, Peuzberg, Germany) according to the manufacturer’s guidelines and quality control to determine concentration,a 260:280 assay was conducted. Individual qPCR reactions performed with the standard StepOnePlus (Applied Biosystems™) using SYBR green Premix Ex Taq technology (Takara Bio Inc.) according to manufactures instructions,for each qpr the result exported into excel file for statistical analysis based on2−ΔΔCT method to estimate copy number of selected genes, The cDNA concentration was then normalized in series of PCR by using HPRT primer (Nikbakht et al., 2017). The normalized cDNAs were subjected to amplification, using Step One Plus™ ABI instrument (Apply Bio systems, USA). The levels of HPRT mRNA expression were used to estimate the relative expression levels. The comparative Ct method was used to compute relative expression values. The primers and their corresponding amplicon lengths were provided in Table 1.

Statistical Analysis
All in vitro experiments were performed in triplicate, and results have been expressed as the mean ± standard deviation (SE). Student’s t test and one-way analysis of variance (one-way ANOVA) were used to determine statistical significances of difference. Statistical significance were defined at *P< 0.05, **P< 0.01, and ***P < 0.001 compared to corresponding control.

Results
Cell proliferation
The cytotoxic effect of CUR and THAL was evaluated through the ability to inhibit cell proliferation. The results showed that CUR and THAL had a significant inhibitory effect on cell proliferation compared to control group. The combination of CUR and THAL showed a synergistic effect on the inhibition of cell proliferation.

Figure 1. Molecular Pathway of Curcumin: Curcumin Suppresses the Activation of NF-κB via Inhibition of IκB Activity, Leading to Suppression of Many NF-κB-Regulated Genes Involved in Tumorigenesis.
in KG1 and U937. After treatment with different concentrations of CUR and THAL, growth suppressive effects were assessed by MTT assay for 24h, 48h and 72h. Our result indicated that CUR inhibited cell proliferation with IC50 value of 40 μM for both cell lines (Figure-3: B), (Figure-4: B). THAL inhibited cell proliferation with IC50 value of 60μM for U937 and 80μM for KG-1 cells (Figure-3: A), (Figure-4: A). Our results showed that CUR had a significant cytotoxic effect on both cell lines in dose dependent manners. To investigate the synergistic effect of CUR in combination with THAL, the proliferation rate of treated cells also were assessed at 24h and 48h post-treatments (72h had not significantly difference in compare with 48h). Moreover, the combination treatment was shown a significant effect on inhibiting cell proliferation in compare with single treatment in cited leukemic cells (Figure 3: C), Figure-4: C).

Apoptosis
To apoptotic induction effects of THAL and CUR in KG and U937 cells, were assessed by Annexin PI staining. For this reason the cells were treated and incubated with indicated concentration of THAL and CUR for 24 h. Therefore, the AML cell lines of this study have been treated with indicated concentration

| primer | sequence | Ref |
|--------|----------|-----|
| GAPDH  | TGAAGGGGAAGCTCAGTG | Kong et al., (2014a) |
| HPRT   | GCCTAAATCTTTTGACCTGCTG | Gusembauer et al., (2015) |
| VEGFA  | AGGCGAATCTACAGGAGT | Kong et al., (2014b) |
| VEGFB  | GAGATGTCCTGAGGAAACACAG | Yang et al., (2009) |
| VEGFC  | GAGGACGTACGGTCTGCTG | Awad et al., (2016) |
| VEGF-D | GATGGACTGCCTGTCAGCAT | Niki et al., (2000) |

Figure 3. Cell Proliferation in KG-1. Effects of Curcumin and Thalidomide on cell proliferation of KG-1 cell lines. The anti-proliferative effects of Curcumin (0-100 μM) (B), Thalidomide (0-100 μM) (A) and their combinations (C) on KG-1 were assessed by MTT assay after 24 h-48h and 72 h treatment. Significant difference between 48h and 72h was not observed. After detection of suitable doses for Curcumin (40μM) and Thalidomide (80μM), we evaluated combination effect of Curcumin and Thalidomide. Combination effect of Curcumin and Thalidomide compared to the control or even single compound could significantly decrease cell proliferation in KG-1 cell lines. Data are mean ± S.E of three independent experiments. Statistical significance were defined at *P<0.05, **P<0.01 and ***P<0.001 compared to corresponding control.

Figure 4. Cell Proliferation in U937. Effects of Curcumin and Thalidomide on cell proliferation of U937 cell lines. The anti-proliferative effects of Thalidomide (0-100 μM) (A), Curcumin (0-100 μM) (B), and their combinations (C) on U937 were assessed by MTT assay after 24 h-48h and 72 h treatment. Significant difference between 48h and 72h was not observed. After detection of suitable doses for Curcumin (40μM) and Thalidomide (60μM), we evaluated combination effect of Curcumin and Thalidomide. Combination effect of Curcumin and Thalidomide compared to the control or even single compound could significantly decrease cell proliferation in U937 cell lines. Data are mean ± S.E of three independent experiments. Statistical significance were defined at *P<0.05, **P<0.01 and ***P<0.001 compared to corresponding control.

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of cited compounds and their combination for different periods following analysis of percentage of apoptosis. We observed a significant increase in early apoptotic cells and minimum percentage of necrosis when cells were treated with concentration THAL-60 µM, CUR-40 µM and CUR-40 µM + THAL-60 µM for U937 and THAL-80 µM, CUR-40 µM and CUR-40 µM + THAL-80 µM for KG-1 cells as compared with control in both cell lines (Figure-5), (Figure- 6).

**DNA cell cycle analysis**

DNA content of KG-1 and U937 cells was evaluated treated with concentration THAL-60 µM, CUR-40 µM and CUR-40 µM + THAL-60 µM for U937 and THAL-80 µM, CUR-40 µM and CUR-40 µM + THAL-80 µM for KG-1 cells as compared with control in both cell lines (Figure-5), (Figure- 6).

**Figure 5.** Apoptosis Assay in KG-1 Cell Line after 24h. Cells in the Lower Right Quadrant Represented Apoptosis while in the Upper Right Quadrant Indicated Post-Apoptotic Necrosis. Data are mean ± S.E of three independent experiments. Statistical significance were defined at *P<0.05, **P<0.01 and ***P<0.001 compared to corresponding control.

**Figure 6.** Apoptosis Assay in U937 Cell Line after 24h. Cells in the lower right quadrant represented apoptosis while in the upper right quadrant indicated post-apoptotic necrosis. Data are mean ± S.E of three independent experiments. Statistical significance were defined at *P<0.05, **P<0.01 and ***P<0.001 compared to corresponding control.

**Figure 7.** The concentration-dependent effects of Thalidomide and Curcumin and their combination on cell cycle. A) Following 24h of Thalidomide and Curcumin and their combination treatment flow cytometric analysis was used to measure of cellular DNA content. B) Percentages of cell populations in different phases of the cell cycle for U937 cells are plotted at different concentrations. Escalated doses of Thalidomide and Curcumin and their combination caused a significant accumulation of cells in the G0/G1 phase.

**Figure 8.** The Effects of Curcumin and Thalidomide and Their Combination on the mRNA Level of VEGF-A, VEGF-B, VEGF-C and VEGF-D in U937 Cells(A). The effect of curcumin (40 µM) and thalidomide (80 µM) and combination (curcumin 40 µM thalidomide 80 µM) on the expression levels of VEGF-A, VEGF-B, VEGF-C and VEGF-D was determined by qRT-PCR analysis in KG-1 cells(B). Values are given as mean ± S.E. of three independent experiments. Statistical significance was defined at *P < 0.05, **P < 0.01 and ***P < 0.001 compared to corresponding untreated controls.
during cell cycle to get information about cell cycle progression. In present study after treatment of U937 the cell population in G0/G1 phase increased in all doses especially when cells were treated with CUR-40 µM +THAL-60 µM for 24h, the cell population in G0 phase increased from 1.44% to 8.2%. Moreover the cell population in G0/G1 phase increased in treated cited cell lines for all doses especially when cells were treated with CUR-40 µM +THAL-80 µM for 24h, the cell population in G0 phase increased up to 48.7% (Figure-8).

**VEGF mRNA expression level was increased in Curcumin and Thalidomide treated AML cells**

The expression of VEGF isoforms in treated cells were evaluated by Real-time PCR. KG-1 cells were treated with selective concentrations of CUR (20µM - 100 µM), THAL-80µM and also their combination for 24h. We observed that the mRNA expression of VEGF (B, C and D) significantly downregulated when KG-1 cells were treated with CUR-40µM while significant change in mRNA expression of VEGFA was not observed. The significant downregulation VEGF isoforms observed when cells treated with 40 µM CUR in U937 cells. (Figure-8: A). The mRNA level of VEGF (B, C and D) was decreased in CUR-treated KG-1 cells as compared to the untreated cells (Figure-8: B).

The mRNA expression of VEGF-A decreased when cells treated with these concentrations and also their combination. In addition, the expression of VEGF-B mRNA declined when cells treated with CUR-40 µM, THAL-80 µM and their combination. In addition we observed that VEGF-C expression was down-regulated in response to CUR-40 µM treatment respectively but the THAL and its combination with CUR increased the expression level of VEGF-C mRNA. Furthermore the expression level of VEGF-D mRNA inhibited via CUR-40µM and decreased by THAL and its combination with CUR. In KG-1 cells the mRNA expression of VEGF-A decreased in THAL and combination of CUR-40 µM +THAL-80 µM. We also indicate that the level of VEGF-B, VEGF-C and VEGF-D mRNA expression decrease in response to all compounds and their combination (Figure-8: A-B).

**Discussion**

AML is a progressive disease which characterized (Rubnitz et al., 2010; Mohammadi et al., 2017c) by high proliferation of leukemic cells with repression of normal hematopoiesis in the bone marrow. Leukemic cells produce various factors in hypoxic condition to promote vascular formation. Among those factors, VEGF has critical role in enhancing proliferation, migration and differentiation of endothelial cells (Santos and Dias, 2004; Haghi et al., 2017). The level of VEGF mRNA expression, its receptors and also microvessel density as index of angiogenesis are admitted element in solid tumor (Ferrara et al., 2003). Recent studies indicated that growth of leukemias like solid tumor can be depend on angiogenesis (Schuch et al., 2002). Endothelia cell of bone marrow release leukemic growth factor including macrophage colony-stimulating factor, granulocyte, interleukin (IL-6) and IL-10. Perez-Atayde and his colleagues for the first time explained significant increase in microvessel density in the bone marrow in childhood ALL. They indicated that leukemic cells secrete VEGF and also other cytokines (Perez-Atayde et al., 1997). Fiedler and his colleagues indicated that AML blasts express VEGF as well as its receptors (Fiedler et al., 1997). Anti-angiogenic therapy known as one of the most promising strategy for human neoplasia (Li et al., 2002). Most of anti-angiogenesis agents are able to inhibit VEGF, TNF-α and bFGF production and consequently decrease the formation of new capillaries (KENYON et al., 1997). In present study for the first time we reported that combination of CUR and THAL can effectively decrease VEGF expression and consequently inhibit AML progress.

THAL is known to be a potent inhibitor of angiogenesis which used for the treatment of cancer, leprosy and chronic graft versus host disease (Komorowski et al., 2006). THAL has been proven to block VEGF-induced angiogenesis (Kenyon et al., 1997). Various studies demonstrated that CUR (the active principle of turmeric) as a phytochemical inhibits proliferation in different type of cancer cells via targeting multiple cellular signaling pathways such as NF-kB, the mitogen-activated protein kinase, Wnt, phosphoinositide-3 kinase/Akt/mammalian target of rapamycin and Notch-mediated signaling pathways (Wang et al., 2006; Yu et al., 2008). Furthermore, CUR could enhance apoptosis in various cancers cell line including human leukemia cell lines through downregulated NF-kB activity (Anto et al., 2002). Moreover CUR is able to inhibit VEGF induced angiogenesis through MAPKs to prevent cancer pathogenesis (Binion et al., 2008). We observed that THAL and CUR promote apoptosis, repress cell proliferation and also decrease VEGF mRNA expression in KG-1 and U937 cell lines. In addition we observed significant difference in combination of compounds as compare with single agent. We also demonstrated that CUR in combination with anti-VEGF (THAL) enhanced anti-angiogenic activity as compared with CUR alone.

Komorowski et al., (2006) indicated that anti-angiogenic feature of THAL is related to direct inhibitory function of VEGF secretion and capillary microvessel formation in human endothelial cell line EA.hy 926. Li et al., (2002) in a research which focus on lung cancer cell line shown that THAL highly declined the protein levels of VEGF and bFGF in a dose- and time-dependent manner. Moreover, Steins et al., (2003) reported that THAL has significant anti-leukemic function with some evidence for anti-angiogenic activity in bone marrow.

Chakraborty et al., (2008) showed that CUR represses OPN-induced VEGF expression and tumor angiogenesis. In another study Yousungnoen and his colleague explained that treatment with this phytochemical significantly reduce the tumor-induced over-expression of COX-2 and serum VEGF in hepatocellular carcinoma cell which exhibit that CUR is able to inhibit tumor angiogenesis (Yousungnoen et al., 2006). In addition Gururaj et al., (2002) confirmed angio-inhibitory effect of this natural compound at the level of gene expression. furthermore Chen et al., (2005)

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indicated anti-angiogenesis effect of CUR through suppressing VEGF in U937 leukemia cell line.

In conclusion, to sum up, the VEGF autocrine loop may have potential impact on AML development and progression and could be considered as a therapeutic target. Thalidomide as a VEGF inhibitor in combination with Curcumin has a synergistic impact on inhibition of cell proliferation and promotion of apoptosis.

Conflicts of interest
The authors declare no conflicts of interest.

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