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Acquired expression of osteopontin selectively promotes enrichment of leukemia stem cells through AKT/mTOR/PTEN/β-catenin pathways in AML cells

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ABSTRACT

Aims: Acute myeloid leukemia (AML) initiation and progression have been attributed to subpopulations of self-renewing leukemia stem cells (LSCs), which contribute to progression, recurrence and therapeutic resistance in leukemia. Osteopontin (OPN) plays an important role in promoting survival and drug resistance in LSCs. The aim of this study was to explore OPN roles in modulating curcumin-mediated LSC enrichment and survival in AML cell lines and primary CD34+/CD38− bone-marrow-derived AML cells.
Materials and methods: The growth inhibitory effects of curcumin (CUR) were evaluated by MTT assay in U937 and CD34+ KG-1 AML cell lines as well as primary CD34+/CD38− bone-marrow derived AML cells isolated by MACS technique. The proportion of LSC markers (CD34, CD38 and CD123) were evaluated by flow cytometry. The expression levels of OPN, AKT, mTOR, PTEN, β-catenin and NF-κB were investigated by qRT-PCR. Short interfering RNA (siRNA) against OPN was used in AML cells incubated with or without CUR.
Key findings: Proportions of CD34+/CD38−/CD123+ and CD34+/CD38+/CD123+ LSCs compartment co-expressing an increased level of OPN could be enriched in AML cell lines and in patient's primary cells by CUR treatment. The expression levels of AKT, mTOR, PTEN, and β-catenin and NF-κB1, were also significantly up-regulated concurrently with OPN in the enriched CD34+ AML cells.
Significance: The increased in CUR-mediated OPN level is involved in a complex interplay of various signaling pathways resulting in cytoprotection and enrichment of CD34+ LSC compartment in CUR-treated AML cells. AKT/mTOR/PTEN/β-catenin/NF-kB signaling pathways may play roles in modulating OPN-mediated LSC cell survival and enrichment.

1. Introduction

High-dose chemotherapy is only effective on survival of 30%–40% of AML patients, due to existence subset of malignant cells that are not effectively eliminated by current treatment regiments [1,2]. Leukemia stem cell (LSC) theory may explain this failure [3]. The exact phenotypes of LSCs remain controversial, however, it is suggested that they mainly reside within the CD34+/CD38− compartment of the leukemic clone [4] and CD34+/CD38+ and CD34− populations are thought as other fractions of LSCs [5]. Osteopontin (OPN), a metastasis-associated glycoprotein, is secreted by many cell types such as lymphocytes, osteoclasts, endothelial cells and tumoral cells [6,7]. It has several different functions, depending on the environment where it is expressed. OPN expression is observed in several solid tumors and correlated with development and progression of cancer [8]. In contrast to the well-defined functions for OPN in solid tumors, there is paucity of research on OPN in leukemia [8,9]. OPN is an important component of HSC niche [6] and deregulated expression of OPN may play an important pathogenic role in hematological malignancies [10]. The role of OPN in regulating cancer progression is the subject of intense investigations and targeting OPN might be an appropriate therapeutic strategy for the treatment of cancer. The PI3K/AKT/mTOR pathway is activated in majority of AML cases (via down-regulation of PTEN [11]); however, the effect of inhibition of this pathway on LSC has not been described in detail [12]. The Wnt/β-catenin pathway is implicated in LSC self-renewal through activation of NF-κB [13]. Curative treatment of leukemia probably will require to elimination of LSCs. It is shown that inhibitors of NF-κB are able to successfully eradicate LSCs [14,15]. Curcumin is (CUR) is one of different molecules in *Curcuma longa* plant. CUR inhibits cancer cell proliferation and induces apoptosis by blocking NF-κB [16]

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and AKT/mTOR [17–19]. Although, CUR induces apoptosis in AML cells, but the cytotoxic effect of CUR in immature CD34+ AML cells remain unclear [20,21].

OPN has been proposed to play a role in drug resistance; however, the contribution of OPN overexpression in the selection and enrichment of drug resistance LSCs during the chemotherapy, such as CUR, has not been defined. In this study, we examined the potential role of OPN in progression and survival of AML cells during CUR treatment using U937 and CD34+ KG-1 AML cell lines and primary CD34+/CD38− bone marrow derived AML cells. Our study demonstrates for the first time that acquired up-regulation of OPN might prevent CUR-induced apoptosis and promotes enrichment of CD34+ AML cells. Up-regulation of OPN in the enriched CD34+ AML cells was concurrently associated with the up-regulation of NF-κB1, AKT, mTOR, PTEN and β-catenin. These findings indicate that OPN is associated with the LSC enrichment, and may be considered as a useful molecular biomarker for detection of MRD and a suitable therapeutic target for the selective elimination of AML-LSCs.

2. Material and method

2.1. Reagents and antibodies

Curcumin (CUR) and Daunorubicin (DNR) were obtained from the from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO); CD34 Multi Sort Micro Bead kit were purchased from Miltenyi biotec Inc (Miltenyi biotec Inc, Auburn, CA); while Anti-β-actin, anti-OPN antibodies and goat anti-mouse horse radish peroxidase (HRP)-conjugated secondary antibody were obtained from R&D System. (R&D System, Minneapolis, MN). CD34, CD38 and CD123 monoclonal antibodies were purchased from BD Biosciences (BD Biosciences, San Jose, CA).

2.2. Cell lines and cell culture

KG-1 and U937 were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). KG-1 and U937 cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA) at 37 °C in 5% CO_2 incubator. CUR was dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 50 mM. Culture media with 0.1% of DMSO was used as a control. DNR was dissolved in sterile double distilled water.

2.3. Cell separation, cell sorting and primary culture

Bone marrow aspirate were collected from 10 patients at initial time of diagnosis during admission in the Hematology, oncology ward. Informed consent was obtained from all participants and the study was approved in the Ethics Committee at the Hematology, Oncology and Stem Cell Research Center. The clinical characteristics of the leukemic patients are shown in Table 1. BMNCs were obtained by Ficoll-Hypaque density gradient centrifugation. CD34+/CD38− cells were purified from these mononuclear cells by Multi Sort CD34 MACS Column Technology. The isolated cells were treated with CUR and DNR and cultured in RPMI 1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA) for indicated time.

2.4. MTT assay

MTT assay was carried out to estimate cell viability after treatment with CUR and DNR. Briefly, the cells were cultured in 96-well plates at a density of 5000/100 μL cells per well in the presence of the above-mentioned compounds. After 24–48 h, 50 μL MTT solutions (0.5 mg/mL) (Sigma-Aldrich, St. Louis, MO) was added to each well and then incubated at 37 °C and 5% CO_2 for 2 h. The precipitated formazan was dissolved with 100 μL of DMSO was added and the optical density was read at a wavelength of 570 nm in an ELISA reader.

2.5. Flow cytometry analysis and characterization of leukemic stem cells subgroups

KG-1 cells were treated with CUR (40 μM) for 24 h and dead cells were removed by centrifugation at 200 g/10 min. Then, plated cells were incubated with CD34, CD38, and CD123 antibodies and analyzed in Partec PAS III flow cytometer (Partec, Munich, Germany), Leukemic populations were defined by their immunophenotypes: CD34+/CD38+/CD123+, CD34+/CD38−/CD123+,CD34−/CD38+/CD123+ and CD34−/CD38−/CD123+.

2.6. RNA isolation and real time PCR

Total RNA was isolated with Tripure Isolation Reagent (Roche Applied Science, Peuzberg, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse transcribed by using cDNA synthesis kit (Takara Bio Inc., Otsu, Japan) as described before [22]. Real Time PCR was performed with Step One Plus™ (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq technology (Takara Bio Inc.). HPRT1 mRNA expression levels were used to estimate the relative expression levels, and the relative expression was calculated based on the $2^{-\Delta\Delta CT}$ method. The primers and their corresponding amplicon size and also siRNA against all variants of OPN are listed in Table 2.

2.7. Short interfering RNA (siRNA) transfection

siRNA were used to evaluate its effect on mRNA expression and also efficacy of CUR in induction of apoptosis. Maximal gene knockdown was attained 24 h post-transfection by using siRNAs at a final concentration of 40 (pM). The impact of OPN gene knockdown on cells number and

Table 1
Patient's characteristic.

Patient	Age/sex	FAB	%CD34 in BMC	Source	Cytogenetic	FLT3	WT-1 copy number
P_1	35Y/M	M1	32%	BM	46,XY[8]	Neg	905
P_2	40Y/M	M0	38%	BM	46,XY[20]	Neg	1810
P_3	30Y/M	M1	43%	BM	50,XY add(3)(p12), − 5,+8,+21,+21, +marI[4]/51,XY,idem,+18, +marI[3]/50,XY,idem,+22[2]/51,XY,idem,22,marI[2]/52,xy,IDEM, +18,marI,+marII[4]/46,xy[5],45,xx,-7[49]/46,XX[1]	Neg	701
P_4	50Y/F	M0	61%	BM	45,XX,-7[49]/46,XX[1]	Neg	4302
P_5	40Y/M	M2	77%	BM	45,XY,del(5) (q14;q34),der(15),t(15;17)(p11.1;q11.1),-17[12]/46,XY[38]	Neg	3066
P_6	28Y/M	M4	15%	BM	45,XY,[20]	Neg	780
P_7	38Y/M	M2	40%	BM	45,XY,[20]	Neg	7331
P_8	36Y/M	M2	45%	BM	46,XY,t(6;9)(p23;q3),t(9;18)(q34;q21)[20]	Neg	5080
P_9	49Y/M	M4	30%	BM	46,XY,del(11q23)[10]	Neg	28,911
P_{10}	30Y/F	M2	43%	BM	46,XX,[20]	Neg	3532

P: patient, Y: year, M: male, FAB: French-American-British, BMC: bone marrow cells, Percentage of CD34+ cells in bone marrow cells of AML patients before sorting, FLT3: fms like tyrosine kinase, WT-1: Wilms Tumor-1.