RESEARCH ARTICLE

Reciprocal Interactions of Leukemic Cells with Bone Marrow Stromal Cells Promote Enrichment of Leukemic Stem Cell Compartments in Response to Curcumin and Daunorubicin

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Abstract

A predominant challenge in developing curative leukemia therapy is interactions of leukemic cells with the bone marrow stromal microenvironment. We aimed to investigate the role of stromal cells, such as bone marrow mesenchymal stromal cells (BMSCs) and osteoblasts (OBs), in curcumin (CUR) and daunorubicin (DNR) induced apoptosis of acute myeloid leukemia (AML) cells. We used KG1 and U937 as leukemia cell line models and treated them with CUR and DNR. The cells were then co-cultured with BMSCs or a combination of BMSCs and OBs as feeders. After 24 hours of co-culture, BMSCs or OBs were sorted and separated from the leukemia cells and apoptosis levels were analyzed by annexin/propidium iodide (PI) staining on flow cytometry. Potentially involved molecular pathways were analyzed at gene and protein levels by Real time PCR and western blotting, respectively. The results showed AML cells co-cultured with BMSCs plus OBs to be more resistant to drug induced-apoptosis compared to co-culture with BMSCs alone or without co-culture. Expression levels of OPN, CXCL-12, IL-6, STAT-3 and VCAM-1 were also significantly up-regulated in OBs and AML cells, at both mRNA and protein levels after co-culture, with concurrent enrichment of CD34+ AML cells. Our data showed, in a stromal cell niche-based model, that OBs revoke the influence of BMSCs on leukemic cells and promote enrichment of both CD34+ and CD34- leukemic stem cell (LSC) compartments in response to CUR and DNR. Up-regulation of OPN, CXCL-12, IL-6, STAT-3 and VCAM-1 in OBs and AML cells in co-culture might be part of molecular mechanisms that block CUR or CUR+DNR-induced apoptosis and promote enrichment of CD34+ and CD34- LSCs.

Keywords: Curcumin- daunorubicin- enrichment- leukemic stem cells- stromal cells

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Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, which is heterogeneous in terms of morphological, cytogenetic and clinical features (Hasserjian, 2013; Shahjahani et al., 2015). Accumulating evidence indicates that relapse of AML is caused by a rare fraction of leukemic populations which are known as leukemic stem cells (LSCs) (Dick, 2008; Raaijmakers et al., 2010). LSCs exhibit the distinctive characteristics as stem cells, including quiescence and self-renewal within the bone marrow (BM) microenvironment(Warner et al., 2004). Recent studies demonstrated that BM niche components contribute to LSC engraftment, development, survival and drug resistance by providing the essential cytokines and variety of cell contact-mediated signals (Dick, 2008; Doan and Chute, 2012). Determining autocrine and paracrine signaling pathways in LSCs will help to identify a strategy to disrupt the protection of BM microenvironment for resistance of AML-LSCs and consequently effective treatment for LSCs eradication (van Rhenen et al., 2005). LSCs as well as hematopoietic stem cells (HSC) are present in both distinct BM niches: vascular and osteoblastic niches (Nwajei and Konopleva, 2013). Coordination between the vascular and osteoblastic niches regulates LSCs hemostasis in and out of the BM (Jin et al., 2006; Azizidoost et al., 2017). The critical cellular components of the osteoblastic niche include osteoblasts (OBs), osteoclasts and bone marrow mesenchymal stem cells (BMSCs) (Adams et al., 2006; Lo Celso et al., 2009). Studies about engraftment of leukemia in mice have revealed a preferential homing of CD34+CD38– LSCs populations into the osteoblastic niche (Ishikawa et al., 2007; Saki et al., 2011; Hanahan

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and Coussens, 2012). During the past few years, a number of studies have investigated the potential impact of curcumin (CUR) and another natural NF-kB inhibitor agents (alone or in combination with other anticancer agents) on cancer in vitro as well as in animal models (Liu et al., 2002; Bharti et al., 2003; Padhye et al., 2010; Shehzad et al., 2010; Lv et al., 2013; Zahedpanah et al., 2016). In previous study (Mohammadi et al., 2016b), we investigated the molecular effect of CUR treatment on AML cell lines. Our experiments showed that CUR treatment induced up-regulation of osteopontin (OPN) in a residual subpopulation of AML cells. The increase in OPN expression level at the end of therapy was strongly associated with the residual enriched chemo-resistance AML cells with LSCs phenotype. 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. Response to the chemotherapy in BM stromal niche is even more complicated, compared to in vitro, and is partly associated with the interactions of leukemic cells and the BM stromal microenvironment. The cross-talk between leukemia cells and BM stromal cells results in reciprocal modulation of each other's functions (Tabe et al., 2007; Konopleva et al., 2009; Ding et al., 2010; Nair et al., 2010; Jacamo et al., 2014). Understanding this reciprocal interaction in acquisition of drug resistance and LSCs enrichment might potentially provide a new strategy in the treatment of leukemia (Jacamo et al., 2014).

Even though the mechanism the protective effect of OBs on HSC and acute lymphoid leukemia (ALL) cells in vitro has been extensively studied (Iwamoto et al., 2007; Levesque et al., 2010; Ehninger and Trumpp, 2011), the molecular process by which leukemia-stromal interactions induce chemo-resistance to leukemia cells is not completely understood (Wu et al., 2005; Juarez et al., 2007); and the role of OBs in protecting AML cells has not been well- characterized. In present study, we focused on molecular interaction of BMSCs and OBs with leukemic cells; our study shows that these reciprocal interactions may promote a complex interplay of various signaling pathways in response to the drugs treatment (CUR and daunorubicin (DNR)) resulting in cytoprotection and enrichment of CD34+ LSC compartments.

Materials and Methods

Reagents and antibodies

CUR and DNR were obtained from Sigma-Aldrich (St. Louis, MO). Annexin-V/ PI assay kit was purchased from Roche (Roche Applied Science, Penzberg, Germany), while other materials were received from BD Biosciences (San Jose, CA,USA), Abcam (Cambridge, UK) and R&D System (Minneapolis, MN, USA). The CD34-R-phycoerythrin (RPE) and Phycoerythrin (PE), CD38-fluorescein isothiocyanate (FITC), CD45 (FITC), CD44 (FITC) , CD90 (FITC) CD14 (PE), CD73 (PE), CD29 (PE), CD166 (PE) and CD123-peridinin Chlorophyll protein (per-CP) monoclonal antibodies were obtained from BD Biosciences, CD105-Allophycocyanin (APC), CD105 (FITC) from Abcam and antibodies against OPN, β-Actin, CXCL12, IL-6 and goat anti-mouse-horse radish peroxidase (HRP)-conjugated secondary antibody from R&D System . The CD105 Micro Bead Kit was purchased from Myletenyi Biotec Inc (Auburn, CA.USA).

Cell lines and cell culture

Human AML cell lines, KG-1 and U937, were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cell lines were cultured in RPMI medium with 10% FBS (Invitrogen, CA, USA), 2 mM L-glutamine, 100 units/mL penicillin and 100μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. CUR was dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 100 Mm. Culture media with 0.1% of DMSO was used as a control. DNR was dissolved in sterile double distilled water.

BMSCs culture

BM aspirate was collected from a patient at initial time of diagnosis and prior to any treatment during admission in Hematology, oncology ward. Informed consent was obtained and the study was approved in the Ethics Committee (No: ir.tums.horcsct.rec.1394.103.5) at the Hematology, Oncology and Stem Cell Research Center, Shariati Hospital. BMSCs were isolated from BM aspirate by density gradient centrifugation (Ficoll 1.077 g/mL; Lympho prep, STEM CELL after 1:1 dilution with Dulbecco's phosphate-buffered saline (D-PBS; STEM CELL, USA) and plated in non-coated 75 cm^2 polystyrene culture flasks (Greiner bio-one, Kremsmünster, Austria) at a density of 160,000/cm^2 in complete culture medium supplemented with 2 mM L-glutamine, and 10% FBS. Cultures were maintained at 37°C, in a 5% CO2 humidified atmosphere. After 48 h, non-adherent cells were discarded.

Osteoblast culture

For osteogenic differentiation, 60%–70% confluent BMSCs were incubated in DMEM supplemented with 10% FBS (Invitrogen, CA, USA), 2 mM L-glutamine, 0.1 mM dexamethasone, 10 mM β-glycerophosphate, 0.2 mM ascorbic acid (all reagents were from Sigma-Aldrich) for 8 days (Mohammadi et al., 2016c). Mineralized deposits were visualized by Alizarin Red staining.

Immunophenotypic characterization of BMSCs

FITC and PE labeled monoclonal antibodies specific for the following antigens: CD45, CD14, CD34, CD73, CD44, CD29, CD90, CD166 and CD105 were employed for the assessment of the surface phenotype on the BMSCs, using a Partec PAS III instrument (Partec, Munich, Germany) and analyzed by the Flow Max version 2.5 (Supplementary Figure 1).

Co-culture system

The indicated OBs and BMSCs lineage cells (5×105 of each cells) were washed with PBS prior to addition of either KG1 or U937 cells (5×10^5 cells/ml). Each treated cell lines were co-cultured with BMSCs alone and with BMSCs combined with OBs and maintained at 37°C in

RPMI supplemented with FBS for 24 h. Then, co-culture treated cells were separated and washed for further assay.

BMSCs separation after co-culture assay

The CD105 Micro Bead kit was used for isolation of OBs from BMSCs after co-culture with negative selection for molecular assessment. The purity of isolated cells was evaluated by anti-CD105 labeled APC (Abcam, Cambridge, UK) with flow cytometry (Supplementary Figure 2).

Measurement of apoptosis by Annexin V/PI analysis

Leukemic cells (5×10^5/ml) were co-cultured and treated in 6-well plates with indicated concentrations of CUR and DNR. Then, the cells were harvested and employed for Annexin V /PI staining as recommended by manufacturer's instruction.

Flow cytometry and characterization of LSCs sub groups

Cell lines were treated with CUR and DNR for 24 h in co-culture model. After separation of KG-1 cells form feeder layer by washing, cells were centrifuged at 200 g for 10 min and washed again in order to remove dead cells. The immune-phenotypes of the residual viable cell were determined by a Partec PAS III flow cytometer (Partec, Munich, Germany), and the data were interpreted by using the Flomax software. Leukemic population were defined by their phenotype such as: (CD34+/CD38-/CD123+, CD34+/CD38+/CD123+, CD34-/CD3-/CD123+, CD34-/CD38+/CD123+) (Mohammadi et al., 2016b).

RNA isolation and Real time PCR

Tripure isolation reagent (Roche Applied Science, Germany) was used for total RNA extraction from cell lines, BMSCs and OBs before and after co-cultures, according to the manufacturer's instruction. Complementary DNA (cDNA) synthesis was performed by using cDNA synthesis kit (Takara Bio Inc., Otsu, Japan). Real Time PCR was performed with Step One Plus™ (Applied Bio systems, Foster City, CA) using SYBR Premix Ex Taq technology (Takara Bio Inc., Otsu, Japan). HPRT mRNA expression levels were used to estimate the relative expression levels. The specificity of the PCR reactions was confirmed by melting curve analysis. The fold change for each mRNA in treated leukemic cell lines in comparison with untreated cells was computed by the $2^{-\Delta\Delta CT}$ method. The primers and their corresponding amplicon size are listed in Table 1.

Western Blot

In co-culture system (BMSCs plus OBs), KG-1 cell lines were treated with CUR and/or CUR+ DNR for 24 h without FBS. After that media were collect and secreted proteins were deposited in this media by Trichloroacetic acid (TCA) method (Santa et al., 2016). Protein solution were resolved on 10% SDS-PAGE and then transferred electrophoretically to the polyvinylidene difluoride membrane (PVDF, Perkin-Elmer, Life Science, MA, USA) as described before (Mohammadi et al., 2016b). Membranes were subjected to immunodetection procedure using specific antibodies against β-Actin, OPN, CXCL-12 and 1L-6. Proteins were detected using a BM chemiluminescence detection kit (Roche Applied Science, Peuzberg, Germany).

Statistical Analysis

All data were presented as means ± SE of triplicate determinants. Data were analyzed using an unpaired two-tailed t test or $\chi 2$ test. Statistical significance were defined at *P<0.05, **P<0.01, and ***P<0.001 compared to the corresponding controls.

Results

BMSCs enhance CUR- or CUR+DNR-induced apoptosis in AML cell lines

In previous study we demonstrated the poptotic effect of CUR(40 μM), DNR (0.5 μg/ml) and CUR+DNR (40 μM + 0.5 μg/ml) on two distinct cell types of AML, DNR-insensitive CD34+ AML cell lines (KG1) and DNR-sensitive AML cell lines (U937) (Mohammadi et al., 2016a; Mohammadi et al., 2016b). Since the effects of DNR alone is obvious based on recent studies (Come et al., 1999; Palucka et al., 1999) and also response of stromal to CUR alone and CUR+DNR was more considered in this study , so, in present study, the cytotoxic effect of CUR (40 μM) or CUR+DNR (40 μM + 0.5 μg/ml) was

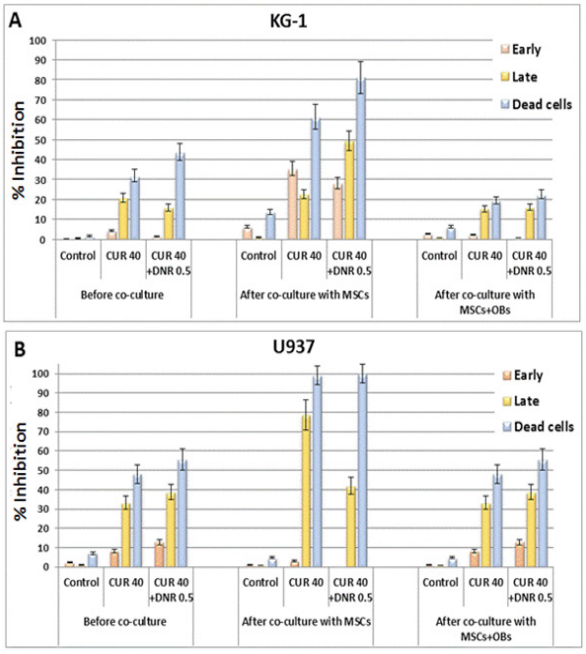

Figure 1. Result of Annexin/PI Staining in CUR- or CUR+DNR- Treated KG-1 and U937 Cells During co-Culture with BMSCs or OBs+BMSCs. (A) KG-1 or (B) U937 cells were cultured alone or co-cultured with BMSCs or OBs+BMSCs. Mono-cultured and co-cultured cells were treated for 24 h with either CUR (40 μM) or CUR+DNR (40 μM+0.5 μg/ml). The percentage of early apoptotic cells (Annexin+/PI-), apoptotic cells (Annexin+/PI+) and dead cells (early apoptotic+ apoptotic cells) were assessed by flow cytometry using Annexin V/PI staining. BMSCs enhance CUR or CUR+DNR-induced apoptosis in AML cell lines ($p<0.05$) (A-B). Furthermore, OBs plus BMSCs protect AML cells from CUR- or CUR+DNR-induced apoptosis ($p<0.05$) (A-B). Values are given as mean ± S.E. of three independent experiments

Figure 2. Alizarin Red Staining for Assessment of Osteoblast Cells Differentiation. for osteogenic differentiation, BMSCs were incubated in osteogenic media for 8 days. Mineralized deposits were visualized by Alizarin Red staining

evaluated in cited cell lines. The results show that CUR or CUR+DNR induced significant apoptosis on both cell lines; even though, the combination therapy did not show a significant enhanced effect on these leukemic cells (Figure 1: A, B).

We then examined whether co-culturing of AML cells with BMSCs, as a vascular niche model, could protect AML cells from CUR or CUR+DNR-induced apoptosis. The results showed that co-culturing of CUR-treated KG-1 or U937 cells with BMSCs not only did not protect AML cells from CUR or CUR+DNR-induced apoptosis, but also, it actually induced more apoptosis in AML cell lines compared to the cells treated in mono-culture model. The U937 cells were more susceptible to apoptosis by co-culturing with BMSCs than KG-1 cells (Figure 1B) and (Supplementary Figure 3B).

OBs plus BMSCs protect AML cells from CUR- or CUR+DNR-induced apoptosis

Since BMSCs and OBs coexist in the osteoblastic niche of BM (Kremer et al., 2014), we co-cultured BMSCs plus OBs with KG-1 or U937 cells to evaluated the role of combining these cells in mediating survival protection effects. Induction of differentiation to OBs was confirmed by alizarin red staining before co-culture (Figure 2). Our results showed that OBs plus BMSCs could protect AML cell from CUR and/or CUR+DNR-induced apoptosis even in the presence of BMSCs (Figure 1: A-B and Supplementary Figure 3C).

The reciprocal molecular effects of leukemic cells with BMSCs on the transcription of genes associated with niche signaling during CUR or CUR+DNR treatments.

Molecular changes in BMSCs: To identify possible molecular pathways involved in BMSCs and leukemic cells interactions during the CUR and/or CUR+DNR treatment, we performed expression profile of several main genes involved in the niche signaling in both BMSCs (before and after co-cultured with AML cells; KG-1 or U987) and in AML cells (before and after co-cultured with BMSCs). As shown in Fig-3B, co-culture of BMSCs and KG-1 cells induces CXCL12 gene over expression in BMSCs (CUR: P<0.05; CUR+DNR: P< 0.001). However, a significant decrease in the expression levels of IL-6, STAT3 and VCAM-1 genes was observed in

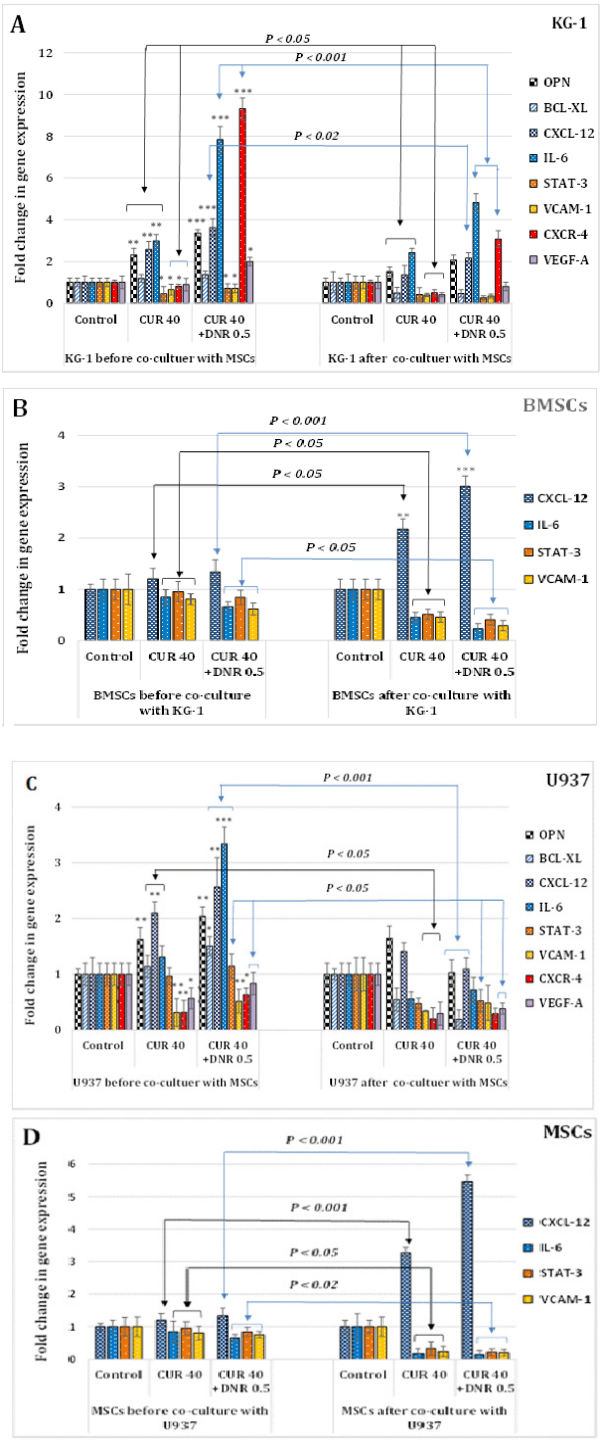

Figure 3. The Reciprocal Molecular Effects of Leukemic Cells with Bmscs on Transcriptional Changes of Genes Involved in the Niche Signaling During CUR or CUR+DNR Treatments. KG-1 or U937 cells were cultured (mono-cultured and co-cultured) and treated (CUR-40 µM and CUR-40 µM +DNR 0.5 µg/ml) for 24 hours with BMSCs (A-D). After separating the AML cells from BMSCs (as indicated in Methods), total RNA from BMSCs and AML cells were extracted. Expression levels of CXCL-12, IL-6, STAT3, and VCAM-1 in BMSCs (B,D) and also OPN, BCL-XL, CXCL-12, IL-6, STAT3, VCAM-1, CXCR-4 and VEGF-A in KG-1(A) or U937 cells(C) were determined by qRT-PCR. 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 controls. Arrows show significant difference between CUR or CUR+DNR-treated cells before and after co-culture.

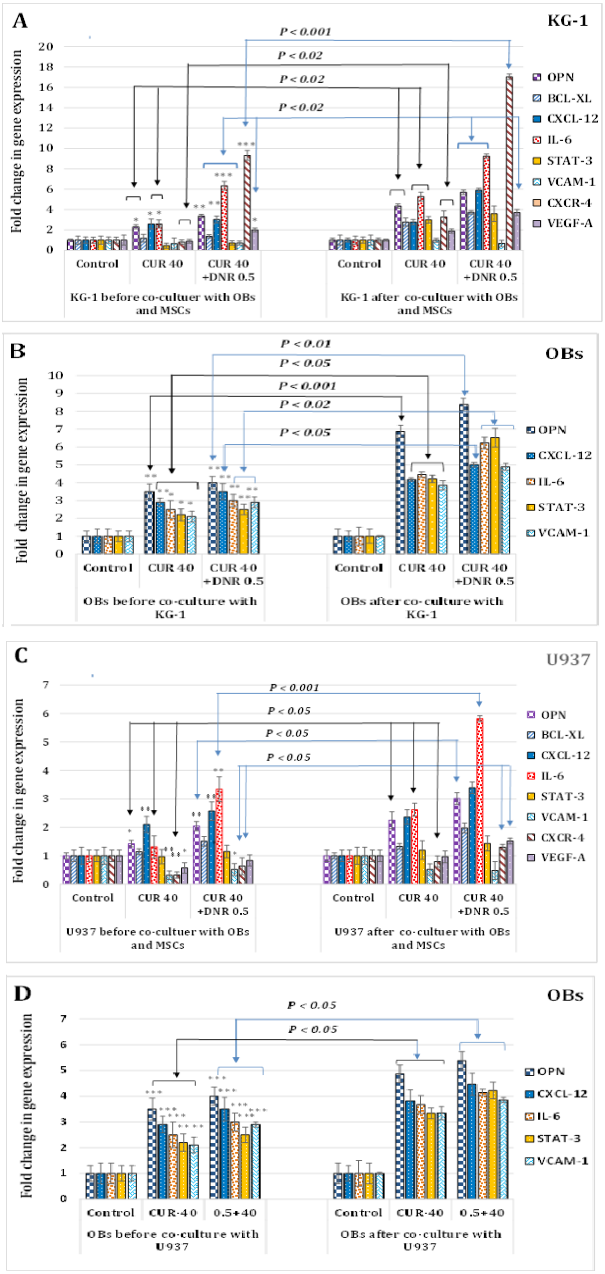

Figure 4: The Reciprocal Molecular Effects Of Leukemic Cells With Obs And Obs+Bmscs On Transcriptional Changes Of Genes Involved In The Niche Signaling During CUR Or CUR+DNR Treatments. OBs were cultured alone (mono-cultured) or OBs+BMSCs co-cultured for 24 hours with (B) KG-1 or (D) U937 cells that were either untreated or treated with CUR (40 μM) and CUR+DNR (40 μM+0.5 μg/ml). After separating the cells (as indicated in Methods), total RNA from OBs was extracted. Expression levels of OPN, CXCL-12, IL-6, STAT3, and VCAM-1 in OBs were determined by qRT-PCR. KG-1 or U937 cells were cultured alone or co-cultured for 24 hours with OBs+BMSCs that were either untreated or treated with CUR and CUR+DNR. After separating the cells (as indicated in Methods), total RNA from AML cells was extracted. Expression levels of OPN, BCL-XL, CXCL-12, IL-6, STAT3, VCAM-1, CXCR-4 and VEGF-A in KG-1(A) or U937(C) cells were determined by qRT-PCR. 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 controls. Arrows show significant difference between CUR- or CUR+DNR-treated cells before and after co-culture.

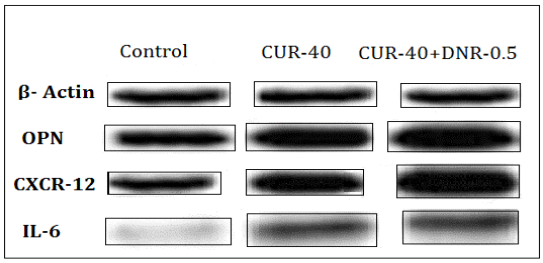

Figure 5. Expression of OPN (48KDa), CXCL-12 (11KDa) and IL-6 (28KDa) Secreted Proteins were assessed in Media of Treated KG-1 with BMSCs Plus OBs in Co-Culture Model by Western Blot Analysis. β-Actin was used as internal loading control. The levels of proteins were significantly increased in these conditions compared to corresponding controls. β-Actin was used as internal loading control.

BMSCs (P<0.05). The increase in the expression level of CXCL-12 in BMSCs co-cultured with treated-U937 was significantly higher compared to the BMSCs co-cultured with treated-KG-1 (Figure 3D vs 3B).

Molecular changes in AML cells: As shown in Figure 3A and 3C, first in a mono-culture, KG-1 or U937 cells were treated with CUR or CUR+DNR for 24h and then examined for the expression of levels of OPN, BCL-XL, CXCL-12, CXCR-4, STAT3, IL-6, VEGF-A, VCAM-1 by real time RT-PCR. The expression levels of OPN, CXCL-12 and IL-6 mRNA were markedly increased in CUR or CUR+DNR-treated KG-1 and U937 cells compared to the untreated cells; while, STAT3, VCAM-1, CXCR-4 and VEGF-A were decreased. In co-culture condition (Figure 3A) CUR or CUR+DNR-treated KG-1 cells co-cultured with BMSCs presented a considerable decrease in the expression levels of all genes, especially in the IL-6 (CUR: P<0.05 and CUR+DNR: P< 0.001) and CXCR-4 expressions level (CUR-40: P<0.05 and CUR +DNR: P<0.00.1). As shown in Fig-3C, results for genes expression in co-culture model of U937 with BMSCs were in line with KG-1 co-culture model to large extent. Although, the expression level of CXCR-4 in CUR+DNR-treated U937 in both mono- and co-culture was significantly lower compared to KG-1 (Figure 3C vs 3A).

The reciprocal molecular effects of leukemic cells with OBs+BMSCs on the transcription of genes associated with niche signaling during CUR or CUR+DNR treatments.

To elucidate the role of OBs in our osteoblastic niche model (OBs+BMSCs) (Lo Celso et al., 2009; Toscani et al., 2015), possible molecular pathways were evaluated in AML cells and OBs. In this regard, we performed expression profile of mentioned genes in OBs before co-culture with AML cells and also isolated OBs from BMSCs (in OBs+BMSCs model) after co-culture with AML cells, via negative selection by MACS technique. Likewise molecular assessment were performed for AML cells before and after co-cultured with OBs+BMSCs.

Molecular changes in OBs: As shown in Fig-4B, the expression levels of OPN, CXCL-12, IL-6, STAT3, VCAM-1 genes were up-regulated in drug-treated OBs before co-culture compared to the untreated-controls. Also after co-culture with KG-1 the expression levels these

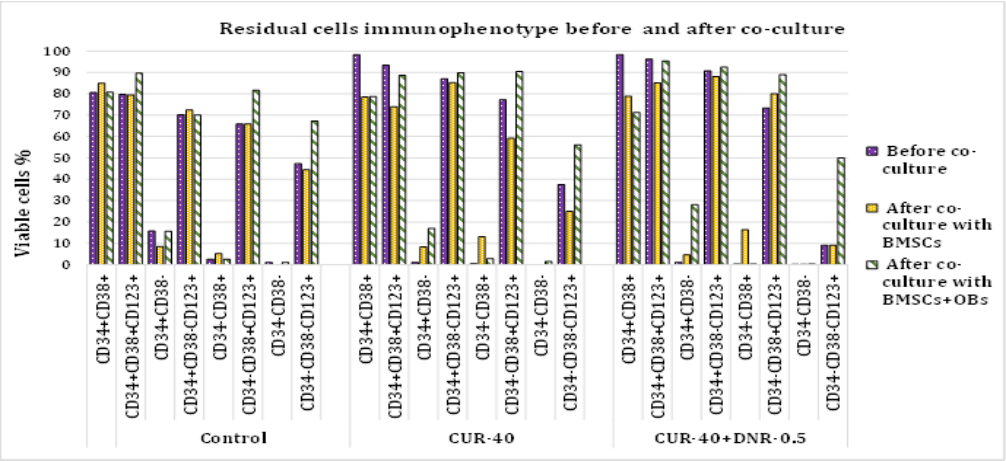

Figure 6. Immunophenotypes of Leukemic Subpopulation in CUR- Or CUR+DNR-Treated KG-1 Cells before and after Co-Culture with Bmscs or Obs+Bmscs. KG-1 cells were treated with CUR (40 µM) or CUR+DNR (40 µM+0.5 µg/ml) for 24 h, before and after co-culture with BMSCs or OBs+BMSCs, incubated with CD34, CD38 and CD123 antibodies and then analyzed by a flow cytometer. Bar chart shows that BMSCs induce enrichment of CD34+/CD38+/CD123+ and CD34+/CD38-/CD123+ LSCs compartments. Unlike BMSCs, osteoblasts induce enrichment of CD34+/CD38+/CD123+, CD34+/CD38-/CD123+ accompanied by CD34-/CD38+/CD123+ LSCs compartments in co-culture model.

Table 1. Nucleotide Sequences of the Primers Used for Real-Time PCR Sequence

Gene	Accession Number	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (bp)
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	111
OPN	NM_001251830	ACCCTTCCAAGTAAGTCCAACG	GGTGAGAATCATCAGTGTCATCTAC	139
VEGFA	NM_001025366	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA	75
BCL-XL	NM_138578	GAGCTGGTGGTTGACTTTCTC	TCCATCTCCGATTCAGTCCCT	119
CXCR-4	NM_003467	CTCCTCTTTGTCATCACGCTTCC	GGATGAGGACACTGCTGTAGAG	127
CXCR-12	NM_000609	GGCAGAAAGCTTGTCTCAACCC	CTCCTTCAGGAACAGCCACCAA	135
VCAM-1	NM_001078	CTTAAAATGCCTGGGAAGATGGT	GTCAATGAGACGGAGTCACCAAT	143
IL-6	NM_000600	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG	132
STAT-3	NM_139276	ATCACGCCTTCTACAGACTGC	CATCCTGGAGATTCTCTACCACT	176

genes were even significantly increased as compared to the untreated-controls as well as to the mono-culture OBs. Similarly, in OBs before co-cultured with U937 cell lines (Figure 4D), the expression levels of OPN, CXCL-12, IL-6, STAT3, VCAM-1 genes were up-regulated in drug-treated OBs, as compared to the untreated-controls. Likewise, up-regulation of these genes was even significantly increased after co-culture with U937, as compared to the OBs before co-culture (Figure 4D).

Molecular changes in AML cells: In U937 cells co-cultured with OBs+BMSCs (Figure 4C), except CXCL-12 (in CUR) and VCAM-1 (in both CUR+DNR), all evaluated genes were relatively overexpressed in drugs treated-U937 cells ($P < 0.05$). The overall comparison between mono and co-culture of KG-1 cells with OBs+BMSCs revealed that, except CXCL-12 (in CUR) and VCAM-1 (in both CUR+DNR), all evaluated genes were relatively overexpressed in drugs treated-KG-1 cells ($P < 0.05$) (Figure 4A). Interestingly, the rate of increase in expression level of all mentioned genes in drugs treated-KG-1 cells after co-culture with OBs+BMSCs were significantly higher compared to the U937 (Figure 4A vs 4C).

The reciprocal effects of leukemic cells with OBs+BMSCs on the translation of genes associated with niche signaling during CUR or CUR+DNR treatments.

To clarify whether transcriptional up-regulation of OPN, BCL-XL, CXCL-12, CXCR-4, STAT3, IL-6, and VEGF-A molecular loop genes also concurs to its translational expression, secreted proteins expression were also detected by western blotting in co-culture media. As shown in Figure 5, the levels of OPN, CXCL-12 and IL-6 proteins were significantly increased under these conditions as compared to the untreated control group.

OBs+BMSCs induced enrichment of both CD34+ and CD34- LSCs compartments

Based on clinical study, the percentage of CD34+/CD38-/CD123+ leukemic cells, as a surrogate of LSCs, at diagnosis was significantly correlated with response to treatment and survival (Vergez et al., 2011). Hence, to investigate, the immunophenotypes of residual viable cells after treatment with CUR or CUR+DNR before and after co-culture with BMSCs or OBs+BMSCs, the treated and untreated KG-1 cells as s LSCs model (Hauswirth et al., 2007; Zhang et al., 2010) were incubated with mouse anti-human CD34, CD38, and CD123 antibody, and then the percentage of each LSCs subpopulations were analyzed by flow cytometry. (Figure 6 and Supplementary Figure 4: A-B). The untreated KG-1 cells in mono-

culture were dominated by 79.80 % CD34+/CD38+/CD123+ subpopulation; and the percentage of CD34+/CD38-/CD123+ subset was 70.27%, accompanied by 65.89% CD34-/CD38+/CD123+ cells. However, after treatment with CUR, KG-1 cells were enriched by 93.41 % in CD34+/CD38+/CD123+, and 87.04% in CD34+/CD38-/CD123+ subpopulations, accompanied by 65.89% CD34-/CD38+/CD123+ cells. Moreover, after treatment with CUR+DNR, KG-1 cells were enriched by 96.28 % in CD34+/CD38+/CD123+, and 90.74% in CD34+/CD38-/CD123+ subpopulations, accompanied by 73.33% CD34-/CD38+/CD123+ cells. Likewise, immunophenotypes of leukemic subpopulation in CUR or CUR+DNR-treated KG-1 cells before and after co-culture with BMSCs were determined. CD34+/CD38+/CD123+, CD34+/CD38-/CD123+ and CD34-/CD38-/CD123+ LSCs subpopulations were enriched by 73.95%, 85.3% and 59.15% for CUR-40 μM and 85.11%, 88.11% and 80% for CUR+DNR, respectively. Next, we studied the effects of OBs+BMSCs on KG-1. CD34+/CD38+/CD123+, CD34+/CD38-/CD123+ and CD34-/CD38-/CD123+ LSCs populations were enriched by 89.63%, 89.86% and 90.41% for CUR and 95.44%, 92.48 and 89% for CUR+DNR, respectively. Collectively, the results (as summarized in Figure 6 and Supplementary Figure 4:A-C) demonstrate that BMSCs induced the enrichment of CD34+/CD38+/CD123+ and CD34+/CD38-/CD123+ LSCs compartments. Unlike BMSCs, OBs induce the enrichment of CD34+/CD38+/CD123+, CD34+/CD38-/CD123+ and also CD34-/CD38+/CD123+ LSCs compartments in co-culture model.

Discussion

The unequal responses to chemotherapy in vivo, compared to in vitro, are partly associated with the interactions of leukemic cells and the BM stromal microenvironment. Understanding this reciprocal interaction of stromal cells, BMSCs and OBs, in acquisition of drug resistance and LSCs enrichment might potentially provide a new strategy to eradicate leukemia. In this study we established 2D niche-based culture model to elucidate molecular mechanisms by which leukemia-stromal cells interaction within the BM microenvironment could confer cytoprotection and chemoresistance to leukemia cells. Our results revealed that co-cultured with BMSCs alone not only did not protect AML cells from CUR and/or CUR+DNR-induced apoptosis, but also stimulated apoptosis of AML cells. On the other hand, OBs which coexist with BMSCs in the osteoblastic niche of BM (Kremer et al., 2014), protected AML cells from CUR or CUR+DNR-induced apoptosis even in the presence of BMSCs. It seems that OBs selectively revoked the influence of BMSCs on leukemic cells. Although CUR and/or CUR+DNR were effective inhibitor of cell growth and induction of apoptosis in both cell lines after co-culture with BMSCs; however, our flow cytometry analyses clarified that BMSCs and OBs are able to induce enrichment of residual LSCs compartments in response to treatment. Our results for the first time show that CUR and/or CUR+DNR selectively enrich LSCs in AML in co-culture mode. Furthermore, we established that BMSCs and OBs during CUR and/or CUR+DNR treatment affect leukemia cells and also leukemia cells change the gene expression profile of cytokines and proteins produced by BMSCs and OBs.

In line with our results, several studies have also described protective features of OB-lineage cells that enhance the ability of primary normal and leukemic hematopoietic cells to survive and grow in vitro in either the presence or absence of chemotherapeutic drugs (Iwamoto et al., 2007; Cao et al., 2013; Yang et al., 2013; Kremer et al., 2014). Our data showed the impacts of these stromal cells on leukemia cell lines were somewhat different. OBs had more protective effect on KG1 cells against CUR or CUR+DNR-induced apoptosis as compare to U937. Similar observation has been reported in De Toni et al study. They demonstrated that OBs show a limited protection of U937 cells in the presence of DNR. The difference in the impacts could be because of the differences in leukemic cell types. KG1 is known as DNR-insensitive CD34+ AML cell lines and U937 as DNR-sensitive AML cell lines (De Toni et al., 2006).

To identify possible molecular pathways involved in leukemia-stromal interactions during the co-culturing and drugs treatment, we performed expression profile of several key genes involved in the niche signaling using qPCR analyses. Our expression analysis suggested that CXCL-12, STAT-3, IL-6, VCAM-1 genes in BMSCs (Figures 3B,3D) and OPN, CXCL-12, STAT-3, IL-6, VCAM-1 in OBs (Figs 4B, 4D) were differentially expressed after co-culture in AML-treated cells.

In BMSCs after co-culture with either KG-1 or U937, CXCL-12 was the most up-regulated genes; and STAT-3, IL-6 and VCAM-1 were significantly down-regulated in both CUR and CUR+DNR treated AML cells. Our data showed co-culturing BMSC with CUR- or CUR+DNR-treated KG1 cells resulted in a significant increase in CXCR12 expression level in BMSC ($P< 0.01$, Figures 3B, 3D). So, it appears that BMSCs through induction of considerable increase in CXCL12 and decrease in STAT3, VCAM-1 and IL-6 genes expression induced CUR- or CUR+DNR-driven apoptosis in both KG-1 and U937 co-culture model. In line with our finding, Kremer et al; shown that CXCL12 (also known as SDF-1), which is the sole endogenous ligand for the CXCR4 chemokine receptor, strongly induces apoptotic cell death through the intrinsic pathway in AML cell lines and in patient samples expressing CXCR4 on the cell surface (Kremer et al., 2013; Kremer et al., 2014).

On the contrary, OPN, CXCL-12, IL-6, STAT-3 and VCAM-1 were all up-regulated in OBs; and the degrees of these changes were greater in OBs co-cultured with KG-1. So, we hypothesized OBs may through induction of OPN, CXCL-12, STAT-3, IL-6, VCAM-1 expression level decrease CUR- or CUR+DNR-driven apoptosis rate in our co-culture model. It has been reported that OBs during differentiation potently inhibit the CXCL12-driven apoptotic pathway of CXCR4-expressing AML cells (Kremer et al., 2014). Thus, BMSCs seems incapable of inducing apoptosis in either KG-1 or U987 cells in presence of OBs, even with increased expression levels

of CXCL12-CXCR4. Likewise, BMSCs may be capable of inducing apoptosis in CXCR4-expressing AML cells if the survival signal of the osteoblastic niche could be inhibited (Kremer et al., 2014).

The analysis co-culture model of BMSCs plus OBs supernatant after treatment by western blot confirmed our gene expression data and showed that the levels of the OPN, CXCL-12 and IL-6 proteins were increased significantly. Our results suggest that leukemia cells after co-culture and treatment might respond to BMSCs and OBs through various ways such as change in either genes expression profile or proteins level. The variance in the impacts of these stromal cells on leukemia cell lines might contribute to differences among leukemia types. Our study demonstrated that both BMSCs and OBs affect AML cells; likewise, AML cells effect BM stromal cells and cause changes in the gene expression profile of cytokines and proteins produced by BMSCs and OBs. Also it has reported that much of the change in BMSCs and OBs induced by leukemia cells is likely due to soluble factors secreted by leukemia cells (Kremer et al., 2014). Based on our results, it seems environment-mediated drug resistance and LSCs enrichment comprises a combination of soluble factors and adhesion, and can be divided into soluble factor mediated drug resistance and cell adhesion mediated drug resistance. These findings are confirmed by Hui et al., study (2015) .

Our previous experiments showed that CUR treatment increased OPN expression in AML cells. The acquired up-regulation of OPN might prevent CUR-induced apoptosis and promote 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 (Mohammadi et al., 2016b). In the present study, we demonstrated that in a co-cultured-niche based model the CUR- or CUR+DNR treatment increased OPN, CXCL-12, STAT-3, IL-6, VCAM-1 levels in OBs cells; up-regulation of these genes by OBs was concurrently associated with the significant up-regulation of not only OPN but also BCL-XL, CXCL-12, IL-6, STAT3, CXCR-4, VEGF-A in drugs-treated AML cells. All these, as we show in KG-1 cells, were associated with the enrichment of CD34+ KG-1 cells. Collectively, reciprocal interactions of leukemic cells with BM stromal cells may promote a complex interplay of various signaling pathways during the chemotherapy resulting in cytoprotection and enrichment of CD34 LSC compartments.

Our flow cytometry data clarified that despite of BMSCs; OBs induce enrichment of both CD34+ and CD34- LSCs compartments in response to treatment and these residual cells might contribute to the chemo-resistance. Oh et al., demonstrated that CD34+/CD38- leukemic compartment are capable of initiating human AML in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Oh et al., 2000). It is now well established that N-Cadherin and Tie2 positive CD34+/CD38-/CD123+ LSCs compartments were highly enriched by chemotherapy. Therefore, these cited markers are probably the potential markers for identification of LSCs.(Zhi et al., 2010). Our results show that even though co-culture with BMSCs might increase CUR or CUR+DNR-induced apoptosis of KG-1 cells; however, certain fraction of KG-1 cells, mostly CD34+ fraction, were survived and enriched in these conditions. In line with our result, Chandran showed that BMSCs derived from patients with AML could limited the differentiation potential of CD34+ cells and also maintaining early progenitors (CD34+/CD38− cells) (Chandran et al., 2015) . OBs induced enrichment of both CD34+ and CD34- LSCs compartments in response to the treatments. Our study is the first to address that CUR or CUR+DNR selectively enrich LSCs of AML in both BMSCs and OBs co-culture models. Most of remaining treated-KG-1 cells was LSCs with co-expression significant levels of OPN, IL-6 and CXCR-4 after co-culture with BMSCs+ OBs cells.

Taken together, our study shows that the interaction between leukemia cells and BM stromal cells results in reciprocal modulation of each other's functions in response to the CUR and DNR treatments. In 2D stromal cells niche-based model, OBs revoke the influence of BMSCs on leukemic cells and promote enrichment of both CD34+ and CD34- LSCs compartments in response to CUR and DNR. Our study may point to the potential involvement of several factors in enhancing of the OB-mediated protection of AML cells. Furthermore, OPN, CXCL-12, IL-6, STAT-3 and VCAM-1 up-regulation in OBs and AML cells in co-culture might be a part of molecular mechanism that preclude CUR or CUR+DNR-induced apoptosis and promote enrichment of CD34+ and CD34- LSCs. These findings may provide some understanding of the protective mechanism of OBs and BMSCs during the acquisition of stroma-mediated drug resistance and LSCs enrichment.

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Conflict of interests

The authors declare no competing interests.

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