Osteopontin b and c isoforms: Molecular Candidates Associated with Leukemic Stem Cell Chemoresistance in Acute Myeloid Leukemia

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Abstract

Despite impressive advances in therapeutic approaches, long-term survival with acute myeloid leukemia (AML) is low as a result of treatment resistance and frequent relapse. Among multitude oncogenic proteins involved in the acquisition of a chemo-resistant phenotype, osteopontin (OPN) recently has attracted marked attention. In spite of the well-defined association between OPN expression and cure rate with solid tumors, there is a scarcity of information on any role of this protein in AML cases. Based on the critical role of OPN in cell survival, it seems reasonable to hypothesize that isoform expression levels may impact on the regulation of apoptosis in AML cells in response to conventional chemotherapeutic drugs and its relation to relapse. To investigate associations between induction of apoptosis and OPN isoform expression, two distinct AML cell lines (KG-1 as a leukemic stem cell model and U937) were treated with chemotherapy drugs, and cell viability and apoptosis were evaluated by MTT and Annexin/PI assay. After determination of appropriate drug doses, mRNA expression levels of OPN isoforms and OPN-related genes were investigated. Our results demonstrated for the first time that acquired up-regulation of OPN-b and c isoforms might prevent conventional chemotherapy regimen-induced apoptosis in AML cells. Moreover, upregulation of OPN-b and c in AML cells appears concurrent with upregulation of AKT/VEGF/CXCR4/STAT3/IL-6 gene expression. To sum up, this study suggests that OPN-b and c isoforms could be considered as unique beneficial molecular biomarkers associated with leukemic stem cell chemoresistance. Hence, they have potential as molecular candidates for detection of minimal residual disease (MRD) and determination of remission in AML patients. Further evaluation with quantitative real time PCR on patient samples for confirmation appears warranted.

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2004; Flamant et al., 2005; Nilsson et al., 2005; Mirza et al., 2008; Powell et al., 2009; Zduniak et al., 2015). More recently, it has been suggested that the serum expression level of OPN-b and OPN-c can be regarded as a biomarker for cancer diagnosis. In spite of the well-defined functions of OPN in solid tumors, there is a scarcity of analysis on the role of this protein in hematologic malignancies (Philip et al., 2001; Philip and Kundu, 2003; Rangel et al., 2008; Shevde and Samant, 2014). Our previous studies in monoculture and coculture model demonstrated that OPN appears to be a key gene not only for the detection of MRD but also for the selective elimination of AML-LSCs as a target candidate (Mohammadi et al., 2016b; Mohammadi et al., 2017a). Hence, in the present study, we analyzed the expression of OPN isoforms in both resistsants (KG-1) as an LSCs model (Zhang et al., 2010) and sensitive AML cell lines (U937) upon treatment with IDR or DNR in combination with Ara-C as a conventional regiment in AML chemotherapy in the clinic. Moreover, to confirm OPN gene expression data, we investigated the effects of simvastatin and OPN siRNA, as two OPN inhibitors, on the cell proliferation and induction of apoptosis in the indicated cell lines. As far we are aware, this study for the first time showed that OPN-b and c isoforms can be considered as unique beneficial molecular biomarkers which are associated with LSCs chemoresistance. In a nutshell, the findings of current probe suggest these isoforms as substantial molecular candidates for detection of minimal residual disease (MRD) and determination of remission in AML patients.

Materials and Methods

Cell Culture

KG-1 and U937 cell lines (Pasteur Institute, Tehran, Iran) were cultured in RPMI-1640 Medium (Invitrogen, CA, USA) that contained amino acid mixtures, including 4 male L-glutamine and 10% fetal bovine serum (Invitrogen, CA, USA), 1000 units/ml Penicillin, and 100 μg/ml streptomycin. Cells were cultured in humid atmosphere 5% CO2 in 37°C.

Drugs Preparation

Three-drug Daunorubicin (DNR), Cytarabine (Ara-C) and Idarubicin (IDR) (Sigma-Aldrich St. Louis, MO, USA) were dissolved in purified water and make a stoke for treat cell lines.

Microculture Tetrazolium Test (MTT)
The inhibitory effect of three drugs was measured using MTT assay. Each two cell lines were cultured in 96-well plates (SPL Life sciences, Pocheon, Korea) with 5000/100 μL cells per well concentration and then treated with different doses of DNR (0-2 μM), Ara-C (0-20 μM) and IDR (0-4 μM) for 24-72 hours. MTT (Sigma-Aldrich, St. Louis, MO, USA) solution (0.5 mg/ml) was added to each well and the plate was incubated at 37°C in 5% CO2 atmosphere. DMSO was added to each well and incubated for 4 hours to liquify the dye crystals. The optical density was read at a wavelength of 570 nm in an ELISA reader.

Annexin / PI Assay

Cells were cultured at 1× 10^5/ml per well in 6-well plates and treated with the cited drug. After 48 hours, each two cell lines were harvested and combined with Annexin/PI according to the manufacturer’s instructions (Roche Applied Science, Penzberg, Germany). The stained cells were examined by flow cytometry (Partec, Munich, Germany).

Inhibition of OPN by Simvastatin and Short interfering RNA (siRNA) Transfection

The effect of OPN mRNA expression and also the efficacy of cited drugs in the induction of apoptosis were tested in both cell lines using siRNA and simvastatin (Sigma-Aldrich, St. Louis, MO, USA). siRNA transfection was performed based on the described method (Mohammadi et al., 2016b). Along with siRNA, Simvastatin was used as a natural element for inhibition of OPN too.

RNA Extraction, cDNA Synthesis and Gene Expression Analysis by Real Time PCR

Total RNA was isolated from the cells using Tripure Isolation Reagent (Roche Applied Science, Peuzberg, Germany) according to manufacturer’s instructions. The quantity of RNA samples was assessed spectrophotometrically using Nano drop ND-1000 (Nanodrop Technologies, Wilmington, DE). The cDNA synthesis kit (Takara Bio Inc., Otsu, Japan) was used for generating cDNA from RNA. With using Step One Plus™ (Applied Biosystems, CA, USA) Real Time PCR was completed and we used SYBR Premix Ex Taq technology (Takara Bio Inc., Otsu, Japan). For estimate the relative expression levels we investigated HPRT1 mRNA expression levels and the relative expression was calculated based on the 2−ΔΔCT method (Nikbakht et al., 2017). Nucleotide sequences of the primers were used for Real Time PCR and siRNA sequence (Table.1).

Statistical analysis

All data were presented as means ± SE of triplicate determinants. Data were analyzed using an unpaired two-tailed t-test or χ² test. Statistical significance was defined at *P<0.05, **P<0.01, and ***P<0.001 compared to the corresponding control.

Results

DNR, Ara-C and IDR dose Determination for Molecular Assesment

The cytotoxic effect of three drugs, IDA, DNR, and Ara-C were evaluated in two cell lines KG-1 and U937 to determine the suitable dose for further molecular assessment. In this regard, growth suppressive effects and apoptosis were evaluated by MTT and Annexin/PI assay after treatment with different concentrations of drugs for 24–48 h. Our result showed that IDA, DNR, and Ara-C inhibited cell proliferation with IC50 values of 0.4, 0.5 and 2 μM for KG-1737 cells and 0.8, 0.5 and 4 μM for KG-1 cells, respectively. The results revealed that these chemotherapeutic drugs had a significant cytotoxic effect on both cell lines Considering dose and time-dependent
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The OPN isoforms Expression Level was Increased in Treated AML cells

KG-1 and U937 cells were treated with IDA, DNR, and Ara-C for 48h and then examined for expression of OPN isoforms using Real Time PCR. As shown in Figure 3, the expression level of OPN isoforms was markedly increased in Ara-C-plus DNR and IDA plus DNR-treated KG-1 cells as compared to the untreated cells as well as cells treated with IDR and Ara-C were much more effective than DNR.

Since these drugs are used in combination in clinics, the synergistic activities of drugs in combination with each other as well as the viability of treated cells were also assessed 24 and 48 h following the treatments. Taken together, the combination therapy demonstrated significant enhanced effect on these leukemic cells...
with IDA, DNR or Ara-C alone; however, the difference was significant. Separately, Ara-C decreased each three OPN isoforms; however, it increased each three isoforms in KG-1 cells in combination with two other drugs, while only OPN-c increased in U937 cell line (Figure 3).

**Simvastatin and OPN Specific siRNA Reduces OPN Gene Expression in AML Cell Lines**

In order to prove the effectiveness of drugs on gene expression of OPN isoforms and also to determine whether suppression of these isoforms has an effect on the response to the above-mentioned drugs in our cell lines or not, we used two OPN inhibitors in the present study. We used simvastatin (3-hydroxy- 3- methylglutaryl coenzyme A reductase inhibitor) as a natural OPN inhibitor (Matsuura et al., 2010) and OPN specific siRNA for OPN gene expression inhibition. To determine the effect of simvastatin on OPN expression in vitro, two cell lines were treated with simvastatin (Figure 4A -B) and simvastatin plus three chemotropic drugs for 24-48h (Figure 5). Our result revealed that simvastatin inhibited cell proliferation with IC50 values of 4μM and 8μM for U937 and KG-1 cells, respectively (Figure 4 A-B). Total RNA was then isolated from the cells, and OPN isoforms mRNA expression of AML cell lines was determined by Real Time PCR. According to our results, declared OPN gene isoforms expression was significantly decreased in simvastatin treated groups in KG-1 and U937 cell lines. Likewise, the levels of OPN isoforms mRNA expression were significantly increased in the simvastatin plus drug treated groups in KG-1 cells (*P<0.05) (Figure 6).

Furthermore, we used the specific siRNA against OPN. The obtained data showed that combination of drug with siRNA can result in a decrease in cell count and viability of cells, more than either drug treatment alone (Figure 7). On the other hand, our results demonstrated that IDR, DNR, and Ara-c can be nullified siRNA and drug -induced apoptosis in AML cell lines by enhancement of OPN.
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isofoms gene at mRNA level (Figure 8).

**OPN isofoms Likely Prevent Drug-induced Apoptosis Through AKT/VEGF/STAT3/CXCR4/IL-6 Molecular Loop**

OPN is a secreted protein and may act as both an autocrine/paracrine manner in AML. It may affect several survival signaling pathways and angiogenesis within the cell stimulating cell proliferation and inhibiting cell apoptosis. Therefore, we examined drugs treatment effect on the expression levels of OPN isofoms/AKT/VEGF/STAT3/CXCR4/IL-6 (Figure 7).

As shown in Figure 9, the mRNA levels of OPN isofoms concurrent with AKT/VEGF/STAT3/CXCR4/IL-6 were increased significantly in the treated-KG-1 cells (P<0.05) after 48 h, as well as U937, treated cells. Furthermore, our result illustrated significant increases in the mRNA levels of AKT/VEGF-A/VEGF-C/STAT3/CXCR4/IL-6 in the 3 drug regimen plus simvastatin-treated KG-1 cells (P<0.05) in parallel with OPN isofoms enhancement (Figure 9 A-B). 

**Figure 6.** The Effect of Simvastatin on Expression Level of Opn Isoforms was Determined by Qrt-Pcr Analysis. Simvastatin can reduce the OPN isofoms in two cell lines treated with drugs and drugs by enhancement of OPN isofoms gene at mRNA level could be nullified drug -induced apoptosis in AML cell lines. Simvastatin with reduction of the expression of OPN might has a significant contribution to the effectiveness of the drugs. Values were normalized using the expression of the housekeeping HPRT. 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 control.

**Figure 7.** The Effect of Sirna OPN on Cell Viability in KG-1 and U937 Cells. The suppressive effect of siRNA OPN (40pM) for KG-1 and U937 on viability was assessed by MTT assay after 48 h in both cell lines. Combined treatment has result a decrease in cell count and viability of cells, more than either drug treatment alone. The suppression of OPN gene by siRNA increased the susceptibility of two cell lines to apoptosis. Data are mean ± SE of three independent experiments. Statistical significance was defined at *p<0.05 compared to corresponding control.

**Figure 8.** The Effect of Sirna OPN on Expression Level of OPN Isoforms was Determined by Qrt-PCR Analysis. OPN specific siRNA used to knockdown the OPN function and investigated the effect on the OPN isofoms-mediated enhancement of AML cells survival and sensitivity to drugs. The result shows that the suppression of OPN gene by siRNA increased the susceptibility of KG-1 cell line to apoptosis. Moreover, the combination of drugs with siRNA increased the percentage of apoptosis in KG-1 cells. These results suggest that conventional regimen by enhancement of OPN isofoms gene at mRNA level could be nullified siRNA and drug -induced apoptosis in AML cell lines. Values were normalized using the expression of the housekeeping HPRT. 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 control.

**STAT3/CXCR4/IL-6**

**Figure 9.** The Effect of Simvastatin on OPN Isoforms Expression Level was Determined by Qrt-Pcr Analysis. Values were normalized using the expression of the housekeeping HPRT. 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 control.

**Figure 10.** The Effect of Sirna OPN on Expression Level of OPN Isoforms was Determined by Qrt-PCR Analysis. Values were normalized using the expression of the housekeeping HPRT. 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 control.
Discussion

In the recent years, the effective impact of OPN on cancer cell growth and survival is well recognized in various genetic and cancer biology studies (Robertson and Chellaiah, 2010; Mohammadi et al., 2017b). OPN has been found to be expressed in different types of human tumors, including breast, ovarian, lung, and gastric cancers. Moreover, the raised up OPN serum levels have been described in CML and AML (Powell et al., 2009); however, the involvement of this multifunctional protein in the pathogenesis of AML is not fully understood. Because, there is an association between OPN expression and tumor cells sensitivity to chemotherapeutic drugs, the researchers’ desire to study the role of this protein in various cancers has increased. Based on the critical role of OPN in regulating cell proliferation in HSC niche (Azizidoost et al., 2017), it seems reasonable to hypothesis that the high expression of OPN may participate in disrupting the regulation of cell survival in AML cells (Kwak et al., 2000; Takemoto et al., 2001; Wong et al., 2002; Minoretti et al., 2006). Our previous studies showed that the acquired up-regulation of OPN might prevent curcumin-induced apoptosis and promote enrichment of CD34+/CD38- /CD23+ AML cells as an LSCs surrogate. While activation of the pathway AKT/mTOR/PTEN/β-catenin/NF-xB1 is related to the OPN activity, it is likely to be part of the anti-apoptotic autocrine signaling pathways. (Mohammadi et al., 2016b). Likewise in another study on 2D model, Mohammadi et al demonstrated that upregulation of OPN/CXCL-12/IL-6/STAT-3 and VCAM-1 in AML cell lines could be a part of the molecular mechanism that induces chemoresistance and enrichment of both LSCs (Mohammadi et al., 2017a).

Herein, based on these data, we aimed to investigate the association between chemotherapeutic drugs-induced apoptosis and OPN isoforms expression in two distinct acute myeloid leukemia cell lines with conventional AML chemotherapy drugs.

The findings showed that following the combined treatment of KG-1 cells with the most well-known chemotherapeutic drugs used in AML treatment (DNR, IDA, and Ara-C), the expression level of all three isoforms of OPN was increased significantly, while maximum elevation observed in OPN isoform c. On the other hand, in U937 cells, only the transcription of OPN isoform c was increased upon treatment with the chemotherapeutic drugs. It is worth to mention that treating both cell lines with the single agent of Ara-c reduced the mRNA expression level of all OPN isoforms; however, the combination of this agent with the other chemotherapeutic drugs had an inductive effect on the gene expression level of OPN isoforms. Our results demonstrated that the mRNA levels of OPN isoforms concurrent with AKT/VEGF/STAT3/CXCR4/IL-6 were increased significantly in both cell lines. Furthermore, our result illustrated significant increases in the mRNA levels of AKT/VEGF-A/VEGF-C/STAT3/CXCR4/IL-6 in the 3 drug regimens plus simvastatin-treated KG-1 in parallel with OPN isoforms enhancement. Inline with our finding, Tilli et al., showed that isoform OPN-c stimulates the proliferation of epithelial ovarian IOSE cells through PI3K/AKT signaling pathways indicating the tumorigenic role for this isoform (Tilli et al., 2011). Moreover, other investigations have demonstrated that OPN-c can be considered as a prognosis marker for breast cancer since this isoform takes place in disability of breast cells in the extracellular matrix (He et al., 2006; Mirza et al., 2008). It should be noted that the role of oncogenic OPN-b in gastric cancer cells has been well documented in some research, including Tang et al. (Tang et al., 2013). According to the study conducted by Tang et al., OPN-b strongly induces gastritis cell survival by regulating Bcl-2 apoptotic genes, whilst OPN-c effectively activates metastatic GC activity by increasing the secretion of MMP-2, uPA, and IL-8 (Tang et al., 2013). Based on these notions,
our results suggest that increased expression level of OPN isoforms b and c upon treatment with conventional chemotherapeutic drugs in AML probably contributes to the reduced sensitivity of AML cells to the toxic agents. We also found that treatment of KG-1 cells with either simvastatin or OPN siRNA resulted in the decreased expression level of OPN isoforms, which in turn led to stimulation of apoptotic cell death.

Our results declared a connection between angiogenesis and OPN b and c isoforms. It would seem, OPN b and c are two main OPN isoforms that they have involved in angiogenesis by STAT3, VEGF-C, KDR and β-catenin genes, and leading to drug resistance. Evidence and results from this research are based on the fact that the sensitivity of AML cells to the apoptosis impelled by chemotherapeutic treatments is strongly related to the disorder of STAT-3, β-catenin, VEGF-C and KDR genes. It is important to know that, OPN regulates VEGF expression, and VEGF regulates OPN expression that they control tumor angiogenesis via autocrine, paracrine pathways (Haghi et al., 2017). Regarding the points mentioned above, there is a positive relationship between OPN and VEGF that they can play an important role in growth, development, tumor progression and angiogenesis. (Chakraborty et al., 2008). According to the results of Zhao et al., (2016), IL-6 is also able to induce the VEGF-C production by the JAK-STAT3 signal pathway. Overexpression of CXCR4 is relevant to colorectal cancer (Schimanski et al., 2015), AML (Mohammadi et al., 2017a) and renal cell carcinoma (Wang et al., 2009) and is associated with chemotaxis, invasion, angiogenesis, and cell proliferation.

STAT3 is a key pathway that able to regulate metastasis in cancerous cells (Devarajan and Huang, 2009; Abroun et al., 2015). Also, activation of STAT3 leads to angiogenesis, cell proliferation, and resistance to apoptosis (Yu and Jove, 2004). Over expression of CXCR4/STAT3/STAT3/VEGF-A, and finally, the correlation between CXCR4 and VEGF-A was reported by Wang et al in non -small cell lung cancer. (Wang et al., 2011). Padro et al. presented that VEGF2R (KDR) commonly express on myeloblasts and VEGF inhibits chemotherapy-induced apoptosis in hematopoietic cells by inducing the anti-apoptotic factor in patients suffering AML (Padro et al., 2002). Our result showed that simvastatin is able to down-regulate STAT3 and β–catenin in KG-1 and VEGF-C, β-catenin, and KDR in U937 cells. In good agreement with our result, Robertson and colleagues reported that OPN promotes cancer cell progression and resistance to apoptosis by activating the AKT/β-Catenin pathway (Robertson and Chellaiah, 2010). KG-1 cells have highly express LSC-associated surface marker CD34 with high clonogenicity (Koeffer et al., 1980; Mohammadi et al., 2016a; Panah et al., 2017 ). U937 cells have mostly CD34+ populations with little clonogenic activity compared with CD34+/CD38- leukemic compartment in KG-1 (Tausig et al., 2010). Hence we hypothesized that U937 cells are depleted of CD34+ LSCs populations with high clonogenicity and it may justify far more β-catenin down-regulation in KG-1 cells as a CD34+ LSCs model (Figure-9A-B). Thus, the most straightforward interpretation of our results is that probably the relationship between acquired up-regulation of OPN isoforms b and c in CD34+ KG-1 cells as well as OPN isoform c in CD34- U937 cells with drug resistance is mediated through regulation of the indicated genes.

In conclusion, taken all together, acquired up-regulation of OPN b and c isoforms in KG-1 probably leads to impedes conventional chemotherapy drugs-induced apoptosis, enrichment of CD34+ AML cells and also its indicative of the significant role of OPN and OPN-related signaling networks for selection and retention of AML-LSCs. Moreover, since LSCs is the main cause of increasing trend of OPN-b, OPN-c, STAT3, VEGF-C, and KDR expression, it can be inferred that OPN isoforms b and c in CD34+ AML and OPN- c in CD34- AML can be regarded as appropriate markers for MRD detection and as a target candidate for the selective elimination of AML-LSCs.

Conflicts of inters
The authors declare no conflicts of interest.

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