Effects of Hypoxia on Biology of Human Leukemia T-cell Line (MOLT-4 cells) Co-cultured with Bone Marrow Mesenchymal Stem Cells

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Abstract

Background: One of the most significant problems in the treatment of leukemia is the expansion of resistance to chemotherapeutic agents. Therefore, assessing the drug resistance and especially the drug resistance genes of leukemic cells is important in any treatment. The impact of Mesenchymal Stem Cells (MSCs) and hypoxic condition have been observed in the biological performance of majority of leukemic cells.

Methods: MOLT-4 cells were co-cultured with MSCs in the hypoxic condition induced by Cobalt Chloride (CoCl2) for 6 and 24 hr. Then, apoptosis of cells was analyzed using annexin-V/PI staining and expression of the drug resistance genes including MDR1, MRP, and BCRP along with apoptotic and anti-apoptotic genes, including BAX and BCL2, was evaluated by real-time PCR.

Results: The hypoxic condition for MOLT-4 cells co-cultured with MSCs could significantly increase the expression of MDR1 and BCRP genes (p<0.05) which are involved in drug resistance. Also, the results indicated that this condition significantly increases the expression of BCL2 (p<0.05) and reduces the apoptosis in MOLT-4 cells co-cultured with MSCs in the hypoxic condition.

Conclusion: These effects can demonstrate the important role of hypoxia and MSCs on the biological behavior of Acute Lymphoblastic Leukemia (ALL) cells that may lead to particular treatment outcomes.

Keywords: Acute lymphoblastic leukemia, Drug resistance, Hypoxia, Mesenchymal stem cell

Introduction

Childhood malignancies are serious problems that can cause inexpiable damages to families and societies. About 35% of childhood malignancies are childhood leukemia and Acute Lymphoblastic Leukemia (ALL) involves 80-85% of them 1. Mesenchymal Stem Cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cells and have a critical role in hematopoiesis regulation and successful hematopoietic stem cell transplantation. There is some evidence that the MSCs have a supporting effect on tumor stroma through various activities such as producing growth factors, amplifying tumor vessel formation and forming niches for tumor stem cells especially in solid tumors like gastric and breast cancer. Thus, it shows that MSCs of tumor microenvironment can promote tumor growth in several ways 2. Besides, it has always been debatable that how the MSCs influence the leukemic cells. In some studies, apoptosis induction was supposed to be an answer to this question 3. Several studies have shown a proliferation-suppressing effect of MSCs on lymphoid lineage by secreting multiple cytokines that arrest these cells at G0 and G1 phases of cell cycle 4. Therefore, more investigations are needed to clarify the MSCs effects in lymphoid leukemia.

Hypoxia-Inducible Factor-1 (HIF-1), a heterodimeric transcription factor, is an essential part of the cellular response to the hypoxic condition 5. This element was swiftly destroyed under normoxic condition via proteasomal degradation, but in the hypoxic situation, its degradation is slow, which eventually leads to increased expression of its target genes. Cobalt chloride (CoCl2) has the potential of blocking the HIF-1 degradation and hence it can mimic the hypoxic situation 6. Based on many reliable reports in several different
kinds of cells, CoCl₂ can induce apoptosis 7-10.

It is a big challenge to prevent leukemic cells recurrence in the bone marrow to overcome treatment failure in ALL. Bone marrow environment is heterogeneous and has a hypoxic compartment 11-13. As a result, it can inhibit the hydroxylation of HIF-1 and give rise to activation of this transcription factor.

HIF-1 target genes are involved in erythropoiesis, angiogenesis, and energy metabolism. In a hypoxic environment, these genes have a lot of critical functions in controlling cellular homeostasis, which makes these genes involved in radio- and chemotherapy resistance of tumors, resulting in tumor survival and more aggressive phenotypes 14-16. Several other genes, as mitotic kinase family (Aurora A, B, and C), which are associated with tumor enhancement and cancer progression, are also influenced by hypoxia. Therefore, these genes are new targets for treatment strategies 7-14.

Although several different mechanisms have been found, one of the most dangerous problems in cancer patients is the drug resistance that frequently occurs and has a bad prognosis 20-22. Some of these mechanisms are the prevention of the drug from entering into the cell, pumping the drug out of the cell, enzymatic inactivation of drug, mutation or alteration of drug targets, apoptosis or senescence destructive, and deficient repair mechanisms. Drug resistance is the primary cause of unsuccessful treatment in patients with acute leukemia 23. Multi Drug Resistance (MDR) is a well-known protein that is one of the primary pumps which can actively excrete drugs from the cell 24. MDR-Associated Protein (MRP) acts like MDR toward excreting drugs out of the cell and when it is overexpressed in acute leukemia, tumoral cells show resistance against several classes of drugs such as anthracyclines, etoposide, and vinca alkaloids 25. In patients for whom MRP was low, overall survival was better than patients with moderate or higher MRP expression 26. Another protein that is reported to be involved in MDR phenotype is a newly characterized one and is called Breast Cancer Resistance Protein (BCRP, ABCG2) that is a member of ABC transporter family and seems to develop resistance against commonly used AML drugs 27.

In order to have a better understanding of how the hypoxia and MSCs affect leukemia cells, the effects of the hypoxia and MSCs on MOLT-4 cells (ALL cell line) were studied. The proliferation rate, apoptosis, cytotoxicity and expression profiles of the genes involved in the drug resistance process that have very important functions in leukemic cells' biological behavior were investigated.

Materials and Methods

Cell culture

The MOLT-4 cell line was purchased from Pasteur Institute Cell Bank (Tehran, Iran) and was cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY). Bone marrow MSCs (positive for CD44, CD90, STRO-1 and Negative for CD14, CD19, CD146) were purchased from Royesh Stem Cell Biotechnology Institute Cell Bank (Tehran, Iran) and were cultured in DMEM (Dulbecco's Modified Eagle Medium) (Gibco Laboratories). Both media contained 10% fetal bovine serum (FBS) (Gibco Laboratories) and were incubated at 37°C humidified atmosphere in 5% CO₂ incubator.

Determining the dose of CoCl₂ cytotoxicity

HIF1 induction via CoCl₂ is dose-dependent. To reach the maximum levels of HIF1 without significant cell death, the CoCl₂ induced cytotoxicity was assessed via cell counting by Trypan blue. To evaluate the CoCl₂ cytotoxicity on MOLT-4 cells, different concentrations of cobalt (0, 25, 50, 100, 150, and 200 µM CoCl₂) were employed.

Co-culture of MOLT-4 cells with MSCs

Second passage MSCs were seeded in plates containing DMEM at a density of 5×10⁴ cells/cm². The medium was changed every three days, till the MSCs feeder layer reached confluence. Subsequently, MOLT-4 cells in 2×10⁶ number were added into supernatant and incubated for 6 and 24 hr.

Hypoxic treatment

2×10⁶ MOLT-4 cells, with or without MSCs, were treated with 100 µM CoCl₂ in 5% CO₂ incubator at 37°C for 6 and 24 hr.

RNA extraction and cDNA synthesis

After removing the medium from plates, MSCs were adherent and MOLT-4 cells were suspended and RNA extraction was needed. High capacity kit (Bio-tek, Japan) was used to determine the mRNA levels of BAX, BCL-2, MDR-1, BCRP, and BCRP genes. The analysis of melting curves was performed using real-time PCR system (Rotor Gene 6000, Corbett Life Science). The supplemental table 1 shows the primers used for BAX, BCL-2, MDR-1, MRP, BCRP, β-actin and GAPDH genes. β-actin and GAPDH were used as an internal control, and duplicate analysis was performed for all samples. The list of the primers is given in table 1.

Apoptosis analysis using annexin V/ FIT/C/PI

Cells undergoing apoptosis were identified by annexin V and propidium iodide staining (BD Pharmingen, USA) according to the manufacturer’s instructions. Briefly, the cells were washed twice with cold PBS and then were resuspended in 1X binding buffer at a concentration of 1×10⁶ cells/ml. Then, 100 µl of
The solution (1×10^5 cells) was transferred to a 5 ml culture tube. 5 µl of annexin V-FITC and 5 µl of PI were also added. Then, the cells were vortexed gently and incubated for 15 min at RT (25°C) in the dark. Finally, 400 µl of 1× binding buffer was added to each tube and they were analyzed using FACSCalibur flow cytometer (Becton-Dickenson, Mountain View, CA, USA) and FlowJo software.

**Statistical analysis**

Our results were statistically analyzed by The SPSS v.19. Data statement was as means±SD. One-Way ANOVA was used to assess the observed statistical differences. The GraphPad Prism v.6 was employed for regression analysis of correlation and the response linearity (GraphPad Software Inc). Statistically significant data were considered for p<0.05.

**Results**

**Cell toxicity assay of CoCl2 treated cells**

According to our results, with less than 100 µM doses of CoCl2, cell growth was observed at 48 and 72 hr, and at the dose of more than 100 µM, CoCl2 was fatal for cells in each of the designated times. So, the optimum dosage was fixed at 100 µM concentration of CoCl2 within 24 hr (Figure 1).

**Growth curve analysis of MOLT-4 cells co-cultured with MSC under the hypoxic condition**

Cobalt exposed (100 µM, 24, 48, and 72 hr) and untreated MOLT-4 cells were cultured in mutual numbers on 6 cm cell culture plates. After 24, 48, and 72 hr, the cells of each plate were counted using trypan blue. The MOLT-4 cells were cultured under different conditions (with MSCs, with CoCl2, with MSCs and CoCl2) and were counted by trypan blue in 1:1 ratio at 24, 48, and 72 hr. Our findings revealed that MOLT-4 cells with MSC and CoCl2 alone, and MSC along with CoCl2 had inhibitory effects on growth of them in 24 hr (Figure 2).

**Comparison of BAX and BCL2 gene expression levels in MOLT-4 cells co-cultured with MSC under hypoxic condition**

BAX and BCL2 were evaluated to determine apoptotic and anti-apoptotic changes in different conditions (MOLT-4+MSC, MOLT-4+CoCl2 and MOLT-4+MSC+CoCl2). BCL2 expression was increased in the presence of MOLT-4+MSC and MOLT-4+CoCl2 and was the highest in MOLT+MSCs+CoCl2 (p<0.05). However, BAX mRNA expression level did not differ significantly between different groups (Figures 3A and 3B).

**Comparing the multiple drug resistance genes expression levels in MOLT-4 cells co-cultured with MSC under the hypoxic condition**

Table 1. Summary of primer sequences. All primer sequences are presented in 5’ to 3’ orientation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5'-3')</th>
<th>Amplicon length (bp)</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>F: ATTGCCTACCCCTGTCCACC</td>
<td>90</td>
<td>57</td>
</tr>
<tr>
<td>MRP</td>
<td>F: CGTGTGGTCTCTGTGTTCTG</td>
<td>181</td>
<td>57</td>
</tr>
<tr>
<td>BCRP</td>
<td>F: CGAATCTGGTCTGGTCTGC</td>
<td>169</td>
<td>59</td>
</tr>
<tr>
<td>Bax</td>
<td>F: TGGCCAGAAACTGTGTCCTCA</td>
<td>194</td>
<td>59</td>
</tr>
<tr>
<td>Bcl2</td>
<td>F: CCGTCCGATTGCTGACTGATTCC</td>
<td>128</td>
<td>55</td>
</tr>
<tr>
<td>Jactin</td>
<td>F: CAGTACTACGACTCTCGTGCTGAC</td>
<td>184</td>
<td>57</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: GGCGTGAACAGCAGAGAAGTATAA</td>
<td>119</td>
<td>59</td>
</tr>
</tbody>
</table>

The MOLT-4 cells exposed to various doses of CoCl2 (0, 25, 50, 100, 150, 200 µM) during 0, 24, 48 and 72 hr time courses. In these periods, to detect the toxic dose of CoCl2, cells were counted using trypan blue in 1:1 ratio.
MDR1, MRP, and BCRP were evaluated to determine the expression level changes of drug resistance genes in different conditions (MOLT-4+MSC, MOLT-4+CoCl₂, and MOLT-4+MSC+CoCl₂). MDR1 expression was increased in the presence of MOLT-4+MSC and MOLT-4+CoCl₂ and was the highest in MOLT-4+MSC+CoCl₂ (p<0.05).

BCRP expression was increased in the presence of MOLT-4+MSC and MOLT-4+CoCl₂ and was the highest in MOLT-4+MSC+CoCl₂ (p<0.05). However, MRP mRNA expression level did not differ significantly between different groups (Figures 4A and 4B).

Hypoxia-induced apoptosis of MOLT-4 cells co-cultured with MSC

Hypoxia-induced apoptosis of MOLT-4 cells co-cultured with MSC was assessed using annexin V/PI...
Effect of Hypoxia and MSCs on Biology of MOLT-4

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staining. Treatment of MOLT-4 cells co-cultured with MSC with 100 µM CoCl₂ resulted in a statistically signif-
ificant increase in the number of apoptotic cells after 24 hr. After 24 hr of CoCl₂ treatment, apoptotic cells con-
tained about 20.80±0.66, 11.91±0.95, 8.10±0.44, and 2.21±0.42% of the totally measured cell population, 
that is MOLT-4, MOLT-4+MSC, MOLT-4+CoCl₂ and MOLT-4+MSC+CoCl₂, respectively (Figures 5A and 5B).

Discussion

Today, the hypoxia and drug resistance are consid-
ered to play roles in the progression and growth of tu-
mor. There are clear shreds of evidence about the posi-
tive effect of HIF1 on the proliferation of leukemic 
cells, and it is one of the key regulators of the cell re-
response to hypoxia ²⁸,²⁹. It seems that hypoxia is a posi-
tive regulator of gene expression than a negative one 
and HIF1 might play a fundamental role in the regula-
tion of the genes related to the hypoxia ³⁰. In the field 
of studies on ALL cells, it has been suggested that 
mesenchymal cells in co-culture with these cells led to 
some changes in their signaling process. Some studies 
have demonstrated that MSCs have inhibitory effects 
on proliferation of ALL cells in the co-culture experi-
ments ¹,³¹. Others have reported that BCL2 gene which 
was significantly upregulated may play a role in the 
mechanism of the drug resistance induced by MSCs in 
the leukemic cells. Our results also showed that in co-
culture of MOLT-4 cells, the BCL2 gene expression 
was increased, whereas BAX gene expression did not 
show significant changes. Our observations also con-
firmed previous studies and revealed the inhibitory 
effect of MSCs on MOLT-4 cell growth ¹,³². Besides, 
the impact of hypoxia was examined on MOLT-4 cells 
co-cultured with MSCs. Our results showed that the 
hypoxia induced by 100 µM CoCl₂ significantly in-
creased the expression of BCL2 gene, whereas the 
BAX gene expression displayed no significant altera-
tion. Our outcomes presented that hypoxia, as well as 
co-culture with MSCs, leads to an increased expression 
of BCL2 gene in MOLT-4 cells. Unsurprisingly, the 
co-culture of this cancer cell line accompanied by hy-
poxia had the greatest increase in the BCL2 gene ex-
pression.

Several studies have been done on ALL lineage 
cells co-cultured with MSCs to explore the changes in 
the expression of drug resistance genes, which repre-

Figure 5A. Recognition of apoptosis in MOLT-4 cells co-cultured 
with MSC treated with CoCl₂. Figure 5A depicts a representable 
sample of assessment of apoptosis using Annexing-V staining and PI. 
(A) Change of phosphatidylserine externalization induced by CoCl₂ 
in MOLT-4 cells cocultured with MSC (flow cytometry analysis, 
Annexin V-FITC/PI staining). MOLT-4 cells were incubated with 
100 µM CoCl₂ for 24 hr. The data presents the three independent 
experiments. Annexin V positive/PI negative cells (Annexin V+/PI-) 
showed in the bottom right quadrant of each dot plot signifies cells 
corresponding to early apoptosis, whereas the Annexin V positive/PI 
positive (Annexin V+/PI+) cells showed in the upper right quadrant 
signifies cells corresponding to late apoptotic/necrotic cells.

Figure 5B. Recognition of apoptosis in MOLT-4 cells co-cultured 
with MSC treated with CoCl₂. (B) Apoptotic cells Percentage (de-

mONSTRATING phosphatidylserine externalization) induced by CoCl₂ 
(flow cytometry analysis, Annexin V-FITC/PI staining). MOLT-4 
cells co-cultured with MSC were incubated with 100 µM CoCl₂ for 
24 hr. Data is presented as means±SD of three independent experi-
ments. * Statistically significant difference compared to the respec-
tive data of control (untreated cells), p<0.05.

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as the MRP gene expression did not change significantly in none of the studied situations. It has been demonstrated that the hypoxia increases the expression of anti-apoptotic protein BCL-2, which can result in the drug resistance of leukemic cells and treatment failure. MSCs have a potential to retard the cell cycle, prevent the proliferation and reduce the apoptosis in leukemic cells. So, MSCs shield these cells against unfavorable conditions and eventually maintain their viability. The data obtained from flow cytometry studies revealed a decline in MOLT-4 cells apoptosis affected by MSCs and hypoxia. Considering the increase of the BCL2 gene expression during the MOLT-4 cells co-cultured with MSCs under the hypoxic condition, the apoptosis lowering was expectable.

A limitation of the present study was using CoCl2 as a hypoxia mimetic agent, that increases HIF1 expression and its DNA binding activity, instead of a low oxygen environment. Obviously, it doesn’t induce true hypoxia due to the toxic influence of CoCl2 on the cells and it is expectable to get the conflicting results compared with the real hypoxic condition that can cause numerous various changes in the cell biology.

Of course, because of the different study designs, cell line class, oxygen tension, and hypoxia duration, there are disagreements in determining whether CoCl2 acts as a supportive or suppressive agent for leukemic cells development or not.

**Conclusion**

Our results showed that the hypoxia induced by CoCl2 has an inhibitory effect on proliferation of ALL lineage MOLT-4 cells co-cultured with MSCs that can be a result of CoCl2 toxicity rather than the effect of hypoxia. Also, hypoxia and MSCs can contribute to increased expression of drug resistance genes and less apoptosis in ALL cells. Hence, the induction of anti-apoptotic proteins expression impressed by hypoxia and MSCs can be involved in the leukemic cells’ drug resistance phenomenon and subsequently in the treatment failure. Further studies are required to demonstrate the role of hypoxia and MSCs on the biological behavior of ALL cells, which may lead to particular treatment outcomes.

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**Conflict of Interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

**References**


