Methylprednisolone Induce Terminal Differentiation in the U-937 Human Myelomonocytic Leukemia Cells

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ABSTRACT
Differentiation studies have shown that methylprednisolone have some roles onto the leukemia cell line such as HL-60 and K-562. Methylprednisolone (MP), a steroid compound, binds the nuclear glucocorticoid receptor and regulates the transcription. In this study, differentiation effect of methylprednisolone on human monocytic leukemia (U-937) cell line was investigated for two types of cluster differentiation (CD11b, CD68) markers by using flow cytometry. It was observed that the significant terminal differentiation of U-937 cells occurred after treatment with high dose (10^-3 M) MP for 24 and 48 hrs. Our data points out that MP is a differentiation inducing agent for myelomonocytic leukemic cells and could be potential treating compound in other type of leukemic cell differentiation.

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INTRODUCTION
Methylprednisolone (MP), a member of the family of steroid hormones is known to play a critical role in the cellular processes such as proliferation, differentiation and apoptosis mechanisms by binding to members of the zinc-finger containing superfamily of nuclear hormone receptors. When coupled with MP, these nuclear hormone receptors play a role as a transcription factor by binding directly to specific DNA recognition sequences in the promoter region of target genes, resulting in the alteration of the transcription initiation of differentiation genes. Hence, steroid compounds suggest that they might play a role of differentiation on leukemic cells since they have been shown to induce the differentiation of granulocytic and monocytic stage of mouse blast cells. Besides valuable data from Lotem’s group, it has been demonstrated that other steroid compound such as dexametasone and prednisone also induced differentiation on leukemic cells.

Myelomonocytic leukemia (MMoL) is a part of the acute myeloid leukemia characterised by the accumulation of malignant myelomonocytic cells. Experimental differentiation studies of myelomonocytic leukemia dates back to the 1974 when Sundström and Nilsson established the U-937 cell line from a diffuse histiocytic lymphoma of a 37 year old male patient in Rudbeck Laboratory, Uppsala-Sweden. In this study, the U937 cell line was chosen since it serves as an in-vitro model for monocyte/macrophage differentiation.

In-vitro effect of steroid molecule in myelomonocytic and in other types of leukemia cells dates back to the late of 1970s and beginning of the 1980s. The cells which contain glucocorticoid receptors are related to response of steroid molecules in-vitro.
In addition, the increased effect of dexamethasone was shown in RA (Retinoic Acide) induced differentiation of HL-60 cells to neutrophils. In the end of 1990s, He and Jiang showed that another part of steroid compounds induced differentiation of HL-60 cells in a dose dependent manner. Last decade it has been shown that many differentiation agents had been induced the differentiation of HL-60 cells. Recently, a study from Turkey demonstrated that different signal transduction pathways were used during differentiation of HL-60 and K-562 cells by MP or arsenic tri-oxide (As2O3). Unlike As2O3, MP-induced granulocytic differentiation was related with serine/threonine protein phosphatases type 2A subunit upregulation. In addition, the combination of As2O3 and MP has also a synergistic effect on differentiation of HL-60 cells.

Short course (3-7 days) of high dose methyleprednisolone (HDMP) (20-30 mg/kg/day) treatment has been shown to induce in vivo differentiation of myeloid leukemia cells to mature granulocytes and apoptosis of myeloid leukemic cells in children with different subtypes (AML-M1, -M2, -M3, -M4, M7) of AML. Moreover HDMP has been shown to effect the differentiation of primary blastic cells.

The objective of the present study was to evaluate the in-vitro differentiation effect of 6α-methylprednisolone in U-937 (human myelomonocytic leukemia) cells which is one of the rare cell lines displaying many monocytic characteristics and has thus served as a best model for monocyte/macrophage differentiation experiment. The U-937 cells are committed to the macrophage branch of the myeloid lineage and can be induced by a variety of agents to mature from a promonocytic into a monocytic stage of development. In this study, cell surface expression of myelomonocytic and macrophage like markers, CD11b and CD68 were analysed to demonstrate the effect of MP on the differentiation of U937 cells in to a further stage of development.

**MATERIALS AND METHODS**

**Reagents**

DMEM medium, 6α-Methylprednisolone, dimethylsulfoxide (DMSO), trypan blue, were purchased from Sigma Chemical Co. Fetal bovine serum (FBS), L-Glutamin and Penicilin/Streptomycin were purchased from Biochrome (Berlin, Germany). Stock solutions of the methylprednisolone were prepared in ethanol and diluted in fresh medium. PE-conjugated mouse anti-human CD11b and FITC-conjugated mouse anti-human CD68 monoclonal antibodies were purchased from Becton Dickonson (Mount View, CA). The U937 cell line was kindly gifted from Dr. Hande Canpinar from Hacettepe University Institute of Oncology, (Ankara, Turkey).

**Cell Culture**

The cell line U-937 was cultured in DMEM medium containing 15% heat inactivated fetal bovine serum, 2 mM L-glutamine, 10,000 units of penicilin per ml, 10 mg/ml of streptomycin at 37°C and in 5% CO2. Cells in logarithmic growth phase were used for the study. U-937 cells (1 x 10^6 cell/ml) were treated with 10^-6 M and 10^-3 M concentration of MP for 24 and 48 hours, in six well culture plates.

**Cytotoxicity Assay**

In order to determine viability and toxicity of MP, the cells were seeded 1x10^6 cell/ml in 6 well plates. After adding high (10^-3 M) and low (10^-6 M) dose of MP, U-937 cells were incubated under the humidified atmosphere and 5% CO2 conditions at desired periods of time. Cell viability was measured by either trypan blue dye exclusion assay and CBC. The concentration of drug which affected less than 50% of cell population were considered as non-toxic dose and used for the study.

**Determination of Differentiation**

Before and after treatment with MP, the determination of differentiated cells was assessed by the measurement of cell surface antigens CD11b and CD68 by flow cytometry. For testing CD11b and CD68 cell surface antigens, the U-937 cells were stained with PE-conjugated mouse anti-human CD11b and with FITC-conjugated mouse anti-human CD68 antibodies respectively. After incubation at +4°C in dark atmosphere for 30 minutes, the stained cells were diluted with 1 ml PBS and analysed by flow cytometry.
Statistical Analysis
The cell surface markers experiments were performed in triplicates and the data were evaluated as the mean plus or minus standart deviation (mean±S.D.). Two ratio comparing Z- test in flow cytometric assays were used for the statistical analysis. A “p value” less than 0.05 was considered as statistically significant.

RESULTS
The cytotoxic effect of MP on U-937 cells
We observed that high dose MP reduced the viability of U937 cells to almost 50% in 48 hours whereas MP led to more than 75% viability in other conditions (Table 1). The cultures with greater than 50% viability were considered in the study.

Induction of terminal differentiation of U-937 cells by MP
MP significantly decreased CD11b expression in time and dose dependent manner (Figure 1).

CD11b expression was dramatically reduced with 10^{-3} M MP in both 24 and 48 hours (p<0.001) while slight decrease was detected with 10^{-6} M MP.

On the other hand, it was observed that CD68 expression levels on U-937 cells in response to MP were elevated time and dose dependently. CD68 expression was increased with 10^{-3} M MP in both 24 and 48 hours (p<0.001) while statistically insignificant increase was detected with 10^{-6} M MP.

DISCUSSION AND CONCLUSION
The role of corticosteroids on differentiation mechanisms has first been reported by Lotem and Sachs4. They demonstrated that myeloid leukemic cells in-vivo can differentiate into macrophages and granulocytes when treated with dexamethasone. Since then, in-vitro and in-vivo differentiation effect of some steroid compounds such as dexamethasone and prednisolone have been reported by different groups5,21. In Turkey, high dose methylprednisolone (20-30 mg/kg/day) has been used in

Table 1: Effect of MP on U937 cell viability.

<table>
<thead>
<tr>
<th></th>
<th>CBC (x 10^6 /ml)</th>
<th>TBE (x10^5 /ml)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hour 48 hour</td>
<td>24 hour 48 hour</td>
<td>24 hour 48 hour</td>
</tr>
<tr>
<td>Control</td>
<td>1.4 1.8</td>
<td>1.3 1.7</td>
<td>92 94</td>
</tr>
<tr>
<td>10^{-6} M MP</td>
<td>1.2 1.5</td>
<td>1.3 1.5</td>
<td>88 77</td>
</tr>
<tr>
<td>10^{-3} M MP</td>
<td>0.7 1.1</td>
<td>1.0 1.3</td>
<td>80 52</td>
</tr>
</tbody>
</table>

Figure 1. Dose and time dependency of MP-induced changes of CD11-b expression on U-937 myelomonocytic cells. Two concentrations of MP (10^{-6} M and 10^{-3} M) were applied into the U937 cells for 24 and 48 hours. CD11b cell surface marker was tested on U937 cells by flow cytometry. The concentration of 10^{-3} M MP was observed decreasing of the level of CD11b.

Figure 2. Dose and time dependency of MP-mediated CD68 expression on U-937 myelomonocytic cells. Two concentrations of MP (10^{-6} M and 10^{-3} M) were applied into the U937 cells for 24 and 48 hours. CD68 cell surface marker was tested on U937 cells by flow cytometry. The concentration of 10^{-3} M MP was observed increasing of the level of CD68.
children with AML since 1987 and clinically successful results were observed in the past two decades. It was shown for the first time, that HDMP treatment induces differentiation and apoptosis of leukemic cells in children with APL and in other with different morphological subtypes of acute myeloblastic leukemia (AML) in-vivo by Hicsönmez et al.\textsuperscript{9, 16-17}. Especially, the effect of pure form of MP (6α- methylprednisolone 21-hemisuccinate) studies in primer culture from AML blasts has first been shown by Özbek et al.\textsuperscript{20}. They successfully demonstrated that low (10\textsuperscript{-6} M) and high dose (10\textsuperscript{-3} M) 6α-MP 21-hemisuccinate induced mature granulocytic form and apoptosis in the blast cells from AML patients at different sub-types (M1, M2, M3, and M7) following 24 hours of incubation\textsuperscript{20}. However, showing more differentiated and apoptotic cells after treatment with HDMP (10\textsuperscript{-3} M) points out the clinical effects of high dose steroids\textsuperscript{20}.

In the present study, we evaluated the in-vitro effects of MP onto the differentiation of promonocytic leukemia cells. As a result of CD11b analyses, low dose (10\textsuperscript{-6} M) concentration of MP did not show any significant effect on U-937 cells but high dose (10\textsuperscript{-3} M) concentration of MP sharply decreased this myelomonocytic cell surface marker on U-937 cells. In other words the myelomonocytic cells were reduced by the effect of high dose MP. On the contrary, increased expression of CD68 supported the effect of MP onto the terminal differentiation of U-937 myelo-monocytic cells. A possible explanation for the suppressive effect of MP on CD11b expression could be decrease in mononcotic cells since terminal differentiated cells accumulated in the whole cell population as it is seen CD68 terminal differentiation marker accumulation (Figure 2).

The another differentiation study group from Turkey investigated the differentiation process on different types of myeloid leukemic cells (HL-60 and K-562) by showing signal regulation of methylprednisolone and arsenic trioxide compounds. They have tested the effect of arsenic trioxide and methylprednisolone alone and together. The valuable synergistic effect has been demonstrated in terminal differentiation of HL-60 and K562 cells with the significant increase of CD11b and CD11c\textsuperscript{15, 22}. In contrast, we did not show the CD11b cell surface antigen expression. We only showed the CD68 marker expression. This situation could be explained by the accumulation of macrophage-like cells in the whole population.

In a valuable study, it was shown that the steroid compounds such as guggulsterone isomers and 16-dehydroxyprogesterone have been shown to induce differentiation of myeloid leukemic cells. It was demonstrated that trans-guggulsterone highly effected HL-60 cells by increasing both CD11b and CD14 cell surface antigens while 16-dehydroxyprogesterone and cis-guggulsterone promoted only increase in the expression of CD14 antigen\textsuperscript{23}. These results suggested that different compounds of steroids might be acting in different ways.

The effect of high dose MP has been initially shown to induce in-vivo differentiation of myeloid leukemic cells to mature granulocytes in patients with AML. We also showed the increased CD68 expression at 24 and 48 hours in U-937 cells treated with high-dose concentrations of MP which is considered as macrophage-like differentiation. The results of our study suggest the importance of clinical use of HDMP therapy in patients with all types of AML. Moreover, this study would be enlarged to work with other leukemia and lymphoma counterpart cell lines except that U-937, HL-60 and K-562 by using other differentiation inducers with steroid for the best curing of lymphoma and leukemia. It would also be interesting to search if the changes in the signaling pathway of differentiation are related to the particular regulation of gene and protein expression processes.

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