Original Article
Methylprednisolone suppresses the Wnt signaling pathway in chronic lymphocytic leukemia cell line MEC-1 regulated by LEF-1 expression

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Abstract: High dose methylprednisolone (HDMP) has been an effective salvage therapy for patients with relapsed chronic lymphocytic leukemia (CLL), while little is known about the exact mechanisms implicated in glucocorticoid-induced cell death. To explore the mechanism of glucocorticoid-induced cell death, we investigated the effect of HDMP on canonical Wnt signaling which emerged as a key pathway implicated in the pathogenesis of CLL. In this study, the human CLL cell line MEC-1 was incubated with various concentrations of methylprednisolone. Cell proliferation activity was detected by CCK8 assay, the apoptotic effect was evaluated by TUNEL assay. Western blot was used to detect active-caspase 3, and the key proteins in Wnt signaling pathway (LEF-1, β-catenin). RT-PCR was performed to assess the mRNA levels of β-catenin, LEF-1, c-myc and cyclin D1. We observed that high concentration of methylprednisolone could suppress the proliferation activity of MEC-1 cells, promote the relative expression of active-caspase 3, and induce apoptotic cell death. Furthermore, methylprednisolone could inhibit LEF-1 protein expression, consequently down-regulate mRNA levels of c-myc and cyclin D1, but could not affect the transcription level of β-catenin and LEF-1 mRNA. The results of this study indicate that methylprednisolone can suppress Wnt signaling pathway by down-regulating LEF-1 protein expression, indicating a novel mechanism for HDMP therapy in CLL.

Keywords: Methylprednisolone, chronic lymphocytic leukemia, wnt signaling pathway, β-catenin, LEF-1

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with significant variation in disease progression, response to therapy, and survival outcome [1, 2]. As the widely application of chemoimmunotherapy regimens, remarkable progress has been achieved in the treatment of CLL, it remains an incurable disease with significant relapse rates or resistance to conventional therapy. There is an urgent need to find a novel or effective regimen which should be possible to overcome the chemoresistance associated with p53 defects. Surprisingly, high dose methylprednisolone (HDMP) has been an effective salvage therapy for patients with relapsed CLL including those with unfavorable cytogenetic features [3].

The Wnt signaling pathway has been found to be active in CLL cells, especially in the aggressive CLL subgroup, playing a critical role in the pathogenesis of CLL [4, 5]. Binding of Wnt protein to its membrane-bound receptor complex leads to activation of the canonical Wnt signaling pathway. During the Wnt signaling activation, β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it cooperates with the transcription factors T-cell factor (TCF) and lymphoid enhancer factor-1 (LEF-1) to alter the production of proteins important for cell proliferation and survival, including c-myc, cyclin D1, and several antiapoptotic proteins [6, 7].

In this study, we investigated the influence of methylprednisolone on the proliferation, apop-
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Table 1. Primers used for the quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence (5' to 3')</th>
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<tbody>
<tr>
<td>C-myc</td>
<td>Forward TCAAGAGGCAGAACACACAC&lt;br&gt;Reverse GGCCTTTTCAATTTTTCCA</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>Forward ACCTGAGGAGCCCAACAA&lt;br&gt;Reverse TGTGGCTGTGAGGGG</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Forward GACGACAGCTTTACTT&lt;br&gt;Reverse ACAGAGCTTTGGAGGTAT</td>
</tr>
<tr>
<td>LEF-1</td>
<td>Forward CTTCGCGAGATCGATCA&lt;br&gt;Reverse CTGGCCTTTGCCTGGTAG</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward TCCATCATGAAAGGTGGACAT&lt;br&gt;Reverse GAGGAATGCTTGTTCAT</td>
</tr>
</tbody>
</table>

Figure 1. Effects of methylprednisolone on proliferation of MEC-1 cells. The proliferation activity was significantly reduced by 23.34%, 30.73%, 30.57% respectively with high concentrations of 50 µM, 100 µM, 500 µM after 24 h, and 28.48%, 42.35%, 44.56% after 48 h, compared with the untreated control. With the extension of culture time, the inhibitory effect on the proliferation activity increased gradually.

Materials and methods

Cell line and culture

The human CLL cell line MEC-1 (p53deleted/mutated) was cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) maintained in a humidified incubator containing 5% CO₂ at 37°C.

Proliferation assays

Logarithmically growing MEC-1 cells were plated in 96 well plate at a density of 3x10⁴ cells/100 µl/well, incubated with increasing concentrations of methylprednisolone (1 µM, 10 µM, 50 µM, 100 µM and 500 µM) for 12 h, 24 h, 48 h, 72 h and 96 h. Before proliferation detected, 10 µl of CCK8 (Beyotime Institute of Biotechnology) was added to the 100 µl cultured cell. After incubated for 2 h in a humidified incubator containing 5% CO₂ at 37°C, the absorbance was detected at a wavelength of 450 nm.

Protein collection and Western blot

MEC-1 cells were incubated with methylprednisolone for different time periods, then active-caspase 3, LEF-1 and β-catenin protein expressions were detected by Western blot. β-actin was detected as a loading control for Western blot analysis.

Cells were washed three times and were lysed in RIPA Lysis Buffer. The concentration of extracted protein was measured by the BCA protein assay (CWbio.Co.Ltd, Beijing, China) following the instructions. For Western blot, equal amounts of total protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking by 3% skim milk for 1 hour at room temperature, the PVDF membranes were incubated with the primary antibodies as anti-LEF1 antibody 1:500 (ABGENT), anti-β-catenin antibody 1:500 (BD) and anti-cleaved caspase 3 antibody 1:500 (Abcam) at 4°C overnight. The membranes were then rinsed and incubated with HRP-conjugated secondary antibody (anti-rabbit IgG, 1:5000, CWbio.Co.Ltd, Beijing, China) for 1 hour at room temperature. After staining, the blots were developed using Enhanced Chemiluminescence Detection Kit (CWbio.Co.Ltd, Beijing, China). Quantification of the autoradiograms was done using the Quantity One software (Bio-Rad).

TUNEL assay

MEC-1 cells were incubated with 100 µM methylprednisolone on a slide paved with poly-L-lysine (PDL) for 12 h, 24 h and 48 h. The MEC-1...
cells on slides were fixed with 4% paraformaldehyde (PFA). TUNEL labeling of apoptotic cells was performed using an In Situ Cell Death Detection Kit (Roche), according to the instructions of the manufacturer.

**Quantitative RT-PCR**

MEC-1 cells were incubated with 100 µM methylprednisolone for 24 h and 48 h. Cells from one culture flask were collected or quickly stored at -80°C until use. Total RNAs was extracted using Ultrapure RNA Kit (CWbio.Co.Ltd, Beijing, China) according to the manufacturer's recommendations. RNA quality was assessed by electrophoresis confirmed that no degradation in RNA. Reverse transcription to complementary DNA (cDNA) was performed using HiFi-MMLV cDNA First Strand Synthesis Kit according to the manufacturer's instructions (CWbio.Co.Ltd, Beijing, China). Amplification was performed with UltraSYBR Mixture (CWbio.Co.Ltd, Beijing, China) in a total volume of 20 µl which contained 10 µl of UltraSYBR Mixture, 0.4 µl of each primer (10 µM), 2 µl of cDNA sample and 7.2 µl of dH2O. The mRNA levels of objective gene and housekeeping gene (β-actin) were quantified by RT-PCR on the ABI Prism 7900 sequence detection system (Applied Biosystems) by using the primer sets as follows in list (Table 1). The PCR reactions were cycled 40 times after initial denaturation (95°C, 10 min) with the following parameters: 95°C for 15 seconds and 60°C for 60 seconds.
The data were analyzed by using the comparative Ct ($2^{-\Delta\Delta Ct}$) method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The normalized expression level of objective genes in methylprednisolone treated cells was compared with that in the corresponding untreated cells at a particular time point. RT-PCR for each gene sample was performed in triplicate.

Statistical analysis

All statistical analyses were performed by using the SPSS18.0 software. The data were expressed as mean ± standard deviation (SD). Of MEC-1 cells

To better evaluate the apoptosis inhibition of methylprednisolone, the MEC-1 cells were incubated with 10 µM, 50 µM and 100 µM methylprednisolone respectively for 12 h, 24 h and 48 h. Western blot analysis revealed that treatment with concentrations above 50 µM methylprednisolone could not suppress the proliferation activity of MEC-1 cells. While treatment with concentrations above 50 µM could significantly reduce the proliferation activity by 23.34%, 30.73%, 30.57% after 24 h, and 28.48%, 42.35%, 44.56% after 48 h respectively (Figure 1). In addition, the inhibitory effect on proliferation activity of MEC-1 cells increased gradually with time.

Methylprednisolone induces apoptotic cell death

The significance of differences between groups was determined by using the Student’s t test or ANOVA. A P value less than 0.05 ($P<0.05$) was considered statistical significance.

Results

Methylprednisolone suppresses the proliferation activity of MEC-1 cells

The MEC-1 cells were incubated with different concentrations of methylprednisolone for 12 h, 24 h, 48 h, 72 h and 96 h. CCK8 assay revealed that treatment with 1 µM and 10 µM methylprednisolone could not suppress the proliferation activity of MEC-1 cells. While treatment with concentrations above 50 µM could significantly reduce the proliferation activity by 23.34%, 30.73%, 30.57% after 24 h, and 28.48%, 42.35%, 44.56% after 48 h respectively (Figure 1). In addition, the inhibitory effect on proliferation activity of MEC-1 cells increased gradually with time.

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before 12 hours, but increased to 62±4% at 24 h and 90±6% at 48 h respectively (Figure 3).

Methylprednisolone inhibits LEF-1 protein expression and down-regulates mRNA levels of c-myc and cyclin D1 in MEC-1 cells

To further assess whether methylprednisolone-induced apoptosis was associated with the suppression of Wnt signaling pathway in MEC-1 cells, LEF-1 and β-catenin protein expressions were detected by Western blot (Figure 4), the mRNA levels of β-catenin, LEF-1, c-myc and cyclin D1 were measured by quantitative RT-PCR (Figure 5). Following treatment with 100 uM methylprednisolone for 24 h and 48 h, we observed β-catenin had no obvious change in the methylprednisolone treatment process, either protein expression or the mRNA level (P>0.05). LEF-1 protein expression was reduced by 31.94% after 24 h and 38.89% after 48 h in MEC-1 cells (P<0.05), which consequently down-regulated mRNA levels of downstream target genes as c-myc and cyclin D1, while there was no significant effect on LEF-1 mRNA level.

Discussion

CLL is predominantly a heterogeneous disease of the elderly, and often associated with a high comorbidity rate. Management of relapsed/refractory CLL represents a major challenge because of the poor prognosis. High-dose glucocorticoids appear to play an important role in the management of highly pretreated relapsed/refractory CLL [8, 9]. In addition, HDMP combined with monoclonal antibodies seems to improve further therapeutic efficacy in the management of high-risk CLL [10-13]. Experiments in vitro also confirmed that glucocorticoids combined with rituximab could induce synergistic direct anti-proliferative and pro-apoptotic effects [14].

MEC-1 cells were obtained from a CLL patient who was already in prolymphoblastic transformation [15], carrying both 17p deletion and P53 mutation. Our present experiments in vitro revealed that high concentration of methylprednisolone can suppress the proliferation activity of MEC-1 cells, and the inhibitory effect increase gradually with time. Additionally, high concentration of methylprednisolone can also promote the expression of active-caspase 3, indicating the occurrence of glucocorticoid-induced apoptosis as TUNEL assay confirmed.

As demonstrated, glucocorticoids (GCs) exert their effects through both genomic and non-genomic signaling [16]. According to the classic
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It was reported that glucocorticoids may suppress Wnt signaling pathway by affecting multiple aspects as enhancing the expression of sFRP1 [23], Dkk-1 [24] and Dkk-2 [25]. Moreover, glucocorticoids may also promote the glycosyn synethase kinase-3β (GSK-3β) activity [26], leading to β-catenin phosphorylated and degraded subsequently, which indirectly inhibit Wnt signaling mediated by β-catenin. Wallace K [27] demonstrated treatment with glucocorticoid resulted in a transient loss of constitutive Wnt-3a expression, phosphorylation and depletion of β-catenin, loss of β-catenin nuclear localization, and significant reductions in TCF/LEF transcriptional activity, associated with a reduction in Wnt signaling activity. But surprisingly, extending glucocorticoid treatment resulted in the re-expression of Wnt-3a from around 21 days. And a return to higher TCF/LEF transcriptional activity was correlated with the re-expression of Wnt-3a. However, direct TCF/LEF inhibition did not substitute for glucocorticoid, suggesting a TCF/LEF-independent mechanism was also involved. Furthermore, Ohnaka K [28] found that glucocorticoid could suppress the Wnt3a-induced TCF/LEF-dependent transcriptional activity in a dose-dependent manner. While the addition of anti-Dkk-1 specific antibody partially restored the transcriptional activity suppressed by glucocorticoid. They thought the inhibitory action was in part attributed to the increase of Dkk-1 expression by glucocorticoid.

LEF-1 is a nuclear protein overexpressed in primary CLL cells. It has been identified as an important regulator of pathophysiologically relevant genes in CLL, which can serve as an excellent target for therapeutic intervention in CLL [29, 30]. Our further experiments in vitro focus on the effects of methylprednisolone on the key proteins (β-catenin, LEF-1) and target genes involved in Wnt signaling pathway. In the present study, we demonstrated that methylprednisolone can inhibit LEF-1 protein expression in MEC-1 cells, consequently down-regulate mRNA levels of downstream target gene as c-myc and cyclin D1, but cannot affect the transcription level of β-catenin and LEF-1 mRNA. These data suggested that methylprednisolone can suppress Wnt signaling pathway by down-regulating LEF-1 protein expression.

Nevertheless, there are other aberrant signaling pathways involved in CLL [31]. Further experiments are needed to explore the mechanisms.
implicated in glucocorticoid-induced cell death of CLL cells.

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Disclosure of conflict of interest

None.

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