Oxymatrine suppresses MOVAS proliferation induced by platelet-derived growth factor via G0/G1 arrest.

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Abstract

Objective: The traditional Chinese medicine Oxymatrine (OMT) has numerous biological effects, such as anti-inflammatory, anti-fibrosis, anti-proliferation and anti-tumor. However, the underlying effect of OMT on vascular smooth muscle cells remains unclear. Herein, the aim of this study is to investigate the role and mechanism of OMT on MOVAS (a mouse vascular aortic smooth muscle cell line) proliferation induced by Platelet-Derived Growth Factor-BB (PDGF-BB).

Methods: Firstly, we assessed proliferation in MOVAS subjected to PDGF-BB induction or controlled intervention with/without OMT treatment. And then we detected cell cycle, cell apoptosis and the expression levels of cyclins, Cyclin-Dependent Kinases (CDKs) and p21 of each group.

Results: In the present study, we found that OMT remarkably restrained the proliferation induced by PDGF-BB stimulus. Additionally, it was demonstrated that cell population of MOVAS treated with OMT in the S phase was reduced, but that of MOVAS in the G0/G1 phase was increased when compared to the PDGF-BB group. However, OMT treatment showed no effect on MOVAS apoptosis. Mechanistically, the expression of cyclinD1-CDK4/6 and cyclinE2-CDK2 were inhibited, while the expression of p21 was increased in MOVAS treated with OMT.

Conclusion: These results demonstrated that the inhibitory effect of OMT on proliferation of MOVAS were in part due to G0/G1 arrest by inhibiting cyclinD1-CDK4/6 and cyclinE2-CDK2, and promoting p21 expression. Therefore, OMT might become a new strategy for treating excessive proliferation of vascular smooth muscle cells in the future.

Keywords: Oxymatrine, MOVAS, PDGF-BB, Proliferation, Cell cycle.

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Oxymatrine (OMT), one of the main bioactive ingredients of Saphorae flavescens radix, which is known as a traditional Chinese herbal medicine extracted from the dried root of Saphorae flavescens Aiton [14,15]. Previous studies have demonstrated that OMT shows a wide-range of pharmacological effects, including anti-inflammatory, anti-cancer, antiviral and immunomodulatory effects [14,16-19]. In recent years, the cardio-protective effects of OMT are becoming more and more arresting, and it has been used to treat cardiovascular diseases via anti-arrhythmic effect, vasodilative activity, hypolipidemic, positive inotropic effect and resistance of ventricular remodeling [20-25]. However, whether OMT exerts pharmacological effect on PDGF-BB-induced MOVAS (a mouse vascular aortic smooth muscle cell line) proliferation is not yet clear. Therefore, this study intends to explore the influence of OMT on MOVAS proliferation, and lay the theoretical basis for clinical intervention on atherosclerosis and post-angioplasty restenosis.
Materials and Methods

Materials

Cell Counting Kit-8 was bought from Dojindo Molecular Technologies, Inc. (CK04-11, Rockville, Maryland, USA). TRITon® Reagent was from Invitrogen Life Technologies (*15596018, Carlsbad, CA, USA). Transcriptor First Strand cDNA Synthesis Kit was purchased from Roche (*4896866001, Basel, Switzerland). Bicinchoninic acid (BCA) protein assay kit was from Thermo Scientific Pierce Biotechnology, Inc. (*23227, Rockford, IL, USA). Primary antibodies including anti-cyclinD1 antibody (#2978, 1:1000), anti-CDK4 antibody (#12790, 1:1000), anti-CDK6 (#3136, 1:1000) and anti-CDK2 antibody (#2546, 1:1000) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies including anti-p21 antibody (sc-397, 1:500) and anti-cyclinE2 antibody (sc-22777, 1:500) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (MB001, 1:10,000) was purchased from BioWorld Technology (St. Louis Park, MN, USA). HRP-conjugated goat anti-mouse (*074-1806, 1:10,000) or goat anti-rabbit (*074-1506, 1:10,000) secondary antibodies were purchased from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD, USA). PDGF-BB was from Prospec-Tany Technogene Ltd. (CYT-501, Ness-Ziona, Israel). OMT was purchased from Shanghai Jinsui Biotechnology Co. Ltd. (*16837-52-8, Shanghai, China).

Cell culture and treatment

Briefly, the MOVAS cells (ATCC® CRL-2797™), a mouse vascular aortic smooth muscle cell line, were cultured with DMEM/high glucose (SH30022.01, Hyclone) supplemented with 10% fetal bovine serum (SH30084.03, Hyclone), and 1% penicillin-streptomycin (15140-122, ThermoFisher Scientific) at 37°C. The cells were stimulated with 20 ng/ml PDGF-BB to induce MOVAS proliferation [26,27]. In the present study, to detect the appropriate concentration of OMT, the effect of several concentrations (0, 5, 10, 20, 40 μmol/L) of OMT on MOVAS proliferation were evaluated following exposure for 48 h. Next, with the appropriate concentration, the cells were treated with different times (0, 12, 24, 48 and 72 h) to choose the appropriate treatment time. Upon the appropriate concentration and time of OMT, the cells were divided into the following groups: Phosphate Buffered Saline (PBS)+Dimethyl Sulfoxide (DMSO), PBS+OMT, PDGF-BB+DMSO and PDGF-BB+OMT.

Cell proliferation assay

Cell proliferation assay was performed according to the manufacturer’s instructions of Cell Counting Kit-8 (CCK8). Firstly, cell suspension was inoculated in a 96-well plate and incubated. The MOVAS cells were then incubated with the CCK8 solution at 37°C for two hours. And then the absorbance of each well was measured at 450 nm (A450) by using a microplate reader.

Cell flow cytometry analysis

The MOVAS cells (about $2.5 \times 10^6$ cells) were collected by trypsin digestion, and these cells were washed with PBS for twice, then centrifugation to harvest cells. After re-suspending the cells with PBS, 5 ml of pre-cold 70% ethanol was added to fix cells overnight at 4°C. Next day, the cells were washed with PBS after discarding ethanol. Then, 0.3 mg of Ribonucleic Acid (R5125, Sigma-Aldrich) and 0.015 mg of PI (P4864, Sigma-Aldrich) were applied to stain cells in darkness. To avoid clumps, the stained cells were sorted by using BD FACS Aria™ III sorter for cell cycle analysis (n=3).

Real-time polymerase chain reaction (PCR) assay

Briefly, total mRNA was extracted by using TRIZol reagent according to the manufacturer’s instructions. Then, the cDNA synthesis was performed by using the Transcriptor First Strand cDNA Synthesis Kit. The relative mRNA levels of each gene were detected by CFX connect™ real-time PCR detection system (Bio-Rad) using iQ™SYBR® green supermix (n=4). GAPDH gene expression was used as an internal control. The sequences of the primers pairs used in this study were designed by Primer 3.0 and displayed as follows: GAPDH forward primer 5'-GGTGAAGGTCGGTGTGAACGGATT-3' and GAPDH reverse primer 5'-GCAGAAGGGGCGAGATGATGA-3'; Ki67 forward primer 5'-CAGTACTCGGAATGACGAGA-3' and Ki67 reverse primer 5'-CAGTCTTCAGGGGCTCTGTC-3'; PCNA forward primer 5'-CTGTTGCTGTGCTTTGGTAA -3' and PCNA reverse primer 5'-CCCAATACACACTCCACT-3'.

Western blot analysis

The total protein of the MOVAS cells was extracted by RIPA. After the protein denaturation, 20 μg of total protein was loaded and separated by using SDS-PAGE at 120 V for 120 min. Subsequently, the protein was transferred to a Polyvinyl Difluoride (PVDF) transfer membrane (IPVH00010, Millipore) at 4°C, which then blocked with 5% non-fat milk at room temperature. Following this, the membranes were incubated with indicated primary antibodies including cyclinD1, CDK4, CDK6, cyclinE2, CDK2, p21 and GAPDH overnight at 4°C with gentle shaking. The membranes were washed with TBST, and incubated with secondary antibodies respectively at room temperature with shaking. The protein signals were detected by using ChemiDoc™ XRS® system (Bio-Rad) (n=3). And GAPDH was used as a loading control.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Statistical analyses were performed using SPSS version 19.0 software. Comparisons between each group were performed using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.
Results

The proliferation of MOVAS cells was inhibited by OMT to identify the role of OMT on VSMCs, we firstly treated the MOVAS cells with different concentrations of OMT to assess the best dosage. Our results of CCK8 showed that the cell proliferation was significantly inhibited by OMT at concentrations of 5 μM, 10 μM, 20 μM, 40 μM, and no obvious difference was observed between 20 μM and 40 μM (Figure 1A). Therefore, to lower the potential toxicity of OMT on MOVAS cells, 20 μM of OMT was selected for further experiments in this study. Next, MOVAS cells were treated with 20 μM of OMT for different times to investigate whether the proliferation inhibition effects of OMT on MOVAS cells is time-dependent manner. Our result demonstrated that the proliferation of MOVAS cells was inhibited by OMT as early as 12 h, and the best inhibition effect was observed at 48 h (Figure 1B). Thus, in subsequent experiments, we treated the MOVAS cells with 20 μM of OMT for 48 hours to study its role and mechanism on VSMCs. As shown in Figure 2A, 20 ng/ml of PDGF-BB dramatically promoted the proliferation of MOVAS cells, but the cell number of MOVAS was significantly reduced by 20 μM of OMT treatment for 48 h. The CCK8 assay also demonstrated that OMT suppressed PDGF-BB induced MOVAS cells proliferation (Figure 2B). Furthermore, the mRNA levels of proliferation markers PCNA and Ki67 were evaluated by RT-PCR. Compared with DMSO treated group, the expression levels of PCNA and Ki67 were significantly increased in PDGF-BB induced MOVAS cells, but this increment was dampened by OMT treatment (Figure 2C).

OMT treatment induces MOVAS cell cycle arrest in G0/G1 phase

Cell proliferation is tightly related to cell cycle progression, to elucidate the mechanism of MOVAS proliferation regulated by OMT, we evaluated cell cycle progression of MOVAS. Synchronized with serum starvation, the MOVAS cells were caused cell cycle arrest at G0/G1, and subsequently labeled with PI and analysed by flow cytometry. It was found that S-phase population was significantly increased and G0/G1 phase was significantly decreased in PDGF-BB group, while G0/G1 phase of MOVAS in PDGF-BB treated with OMT group was remarkably increased and S-phase population was significantly decreased. These results indicated that OMT treatment induces MOVAS cell cycle arrest in G0/G1 phase (Table 1).

OMT treatment shows no effect on MOVAS apoptosis

Reduction of cell number may result from inhibition of cell growth or induction of cell apoptosis [26]. To investigate whether OMT induced apoptosis in MOVAS stimulated with PDGF-BB, flow cytometry analysis was applied here. It was showed that there was no significant difference among groups, which indicated that the inhibitory effect of OMT on PDGF-BB-stimulated MOVAS might not be associated with cell apoptosis (Figure 3).

OMT inhibits MOVAS enter to S phase via downregulating cyclinD1-CDK4/6, cyclinE2-CDK2 and upregulating p21

G0/G1-phase to S-phase transition is primarily regulated by cyclinD1-CDK4/6, cyclinE2-CDK2 [28,29]. Additionally, p21 could downregulate cyclinE2 and CDK2, which contributed into cell cycle arrest [30]. To investigate the levels of these proteins in OMT-treated MOVAS induced by PDGF-BB, the protein levels of them were checked by Western blot. It was shown that the expression levels of cyclinD1, CDK4, CDK6, cyclinE2, and CDK2 were decreased in MOVAS treated with OMT and PDGF-BB, while the level of p21 was increased when comparing to PDGF-BB group, which suggested that
OMT inhibited the G1/S-phase transition probably via downregulating cyclinD1-CDK4/6 and cyclinE2-CDK2, and upregulating p21 (Figure 4).

**Table 1.** OMT treatment induces MOVAS cell cycle arrest in the G0/G1-phase (%).

<table>
<thead>
<tr>
<th>Phase</th>
<th>PBS+DMSO</th>
<th>PBS+OMT</th>
<th>PDGF-BB+DMSO</th>
<th>PDGF-BB+OMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>80.08 ± 0.45</td>
<td>80.70 ± 0.52</td>
<td>67.03 ± 0.27</td>
<td>78.19 ± 0.71</td>
</tr>
<tr>
<td>S</td>
<td>19.06 ± 0.37</td>
<td>18.50 ± 0.48</td>
<td>32.16 ± 0.24</td>
<td>21.05 ± 0.75</td>
</tr>
<tr>
<td>G0/M</td>
<td>0.86 ± 0.09</td>
<td>0.80 ± 0.04</td>
<td>0.81 ± 0.03</td>
<td>0.76 ± 0.04</td>
</tr>
</tbody>
</table>

The population of cell cycle phases in each group (n=3 samples per group).

**References**
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