Reduction of Atherosclerosis in Cholesterol-Fed Rabbits and Decrease of Expressions of Intracellular Adhesion Molecule-1 and Vascular Endothelial Growth Factor in Foam Cells by a Water-Soluble Fraction of Polygonum multiflorum

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Abstracts. Polygonum multiflorum stilbeneglycoside (PMS) is a water-soluble fraction of Polygonum multiflorum Thunb., one of the most famous tonic traditional Chinese medicines, that has protective effects on the cardiovascular system. The purpose of the present study is to elucidate the effects of PMS on macrophage-derived foam cell functions and the reduction of severity of atherosclerosis in hypercholesterolemic New Zealand White (NZW) rabbits. NZW rabbits were fed for 12 weeks with a normal diet, a high cholesterol diet, or a high cholesterol diet associated with irrigation with different doses of PMS (25, 50, or 100 mg/kg). Treatment of NZW rabbits fed with high cholesterol diet with 100 mg/kg PMS attenuated the increase in plasma cholesterol, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol, and plasma triglyceride. Treatment with 50 and 100 mg/kg PMS caused 43% and 60% decrease in atherosclerotic lesioned area ratio to total surface area, respectively. In U937 foam cells, PMS could decrease the high expression of intercellular adhesion molecule (ICAM)-1 protein and the vascular endothelial growth factor (VEGF) protein levels in the medium induced by oxidized lipoprotein when analyzed by flow cytometry. The results proved that PMS is a powerful agent against atherosclerosis and that PMS action could possibly be through the inhibition of the expression of ICAM-1 and VEGF in foam cells.

Keywords: atherosclerosis, Polygonum multiflorum, growth factor, cell adhesion molecule, macrophage

Introduction

Atherosclerosis can be considered to be a modified form of chronic inflammation induced by lipids (1), and much evidence is consistent with this, including findings indicating the presence of numerous cell adhesion molecules and growth factors in the atherosclerotic plaques. Recent studies have stressed the close interactions among the adhesion molecules and growth factors in the inflammatory responses, in which intercellular adhesion molecule-1 (ICAM-1) and vascular endothelial growth factor (VEGF) have been considered to be the marker molecules in the inflammatory response to atherosclerosis (2–4). More and more drugs were applied in the intervention studies of atherosclerosis, which were aimed directly at these marker molecules (5, 6). Our laboratory has focused on the expressions of ICAM-1 and VEGF to support ongoing functional studies on the pathogenesis of atherosclerosis. Up-regulated ICAM-1 (7) and VEGF (8, 9) expression kinetics have been demonstrated in the formation of macrophage-derived foam cells, which indicated the difference of expression curves of two genes in response to oxidized lipoprotein (10).

Polygonum multiflorum Thunb., the root tuber of polygonum plant Polygonum multiflorum, is one of the most famous tonic traditional medicines in China and

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Japan (11, 12), and it has been confirmed to have a protective effect on the cardiovascular system. The compounds of Polygonum multiflorum Thunb. have anti-oxidant and anti free radical properties, which may explain its protective role against atherosclerosis. Recently our laboratory purified a water-soluble fraction of Polygonum multiflorum Thunb., Polygonum multiflorum stilbeneglycoside (PMS). The purpose of the present study is to elucidate the effects of PMS on macrophage-derived foam cell functions and the reduction of severity of atherosclerosis in hypercholesterolemic rabbits.

Materials and Methods

Animals and reagents

Animals were maintained in accordance with guidelines of the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW publication No. [NIH] FS-23) on Animal Care. Male New Zealand White (NZW) rabbits were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade II, Certified SCXK Hu2002-0010).

PMS was prepared by the following processes: grinding the Polygonum multiflorum Thunb. into coarse powder; percolating the powder with 80% ethanol at room temperature; concentrating the percolate to aqueous extract; chromatographic separation of the resulting aqueous extract with macroporous resin D101 using water, 30% ethanol, and 95% ethanol as eluant in turn; collecting the 30% ethanol fraction and concentrating it to dryness (11). The obtained product, PMS, is a brownish-yellow powder containing more than 60% stilbene.

Analytical pure cholesterol and sodium cholate were purchased from Serva Co. (Heidelberg, Germany). CuSO\textsubscript{4} and ethylenediamine tetracetic acid disodium salt were purchased from Sigma Chemical Co. (St. Louis, MO, USA); VEGF ELISA kit was purchased from Cytimmune Co. (Rockville, MD, USA); ICAM-1 R-phycocerythrin (R-PE)-conjugated mouse anti-human monoclonal antibody (Cat No. 555511) was obtained from BD Biosciences Co. (San Jose, CA, USA); VEGF\textsubscript{165} and ICAM-1 cDNA probe was provided by Beijing University.

Animal treatment

Fifty male NZW rabbits were randomly divided into five groups (n = 10). The 10 rabbits in the normal group were fed a normal diet. The 10 rabbits in the high cholesterol group were fed a high cholesterol diet (normal diet supplemented with 0.5% cholesterol, 2.5% yolk powder, and 4% corn oil). Rabbits in the PMS-treated groups were fed a high cholesterol diet and simultaneously were irrigated with different doses of PMS (25, 50, or 100 mg/kg). Diets and drinking water were provided ad libitum. Blood was sampled periodically for measurement of plasma cholesterol and triglycerides. During the 12-week feeding period, we adhered to the guidelines for care and use of laboratory animals.

Plasma cholesterol and lipoprotein analysis

Rabbits were fastened overnight and then the plasma levels were obtained from animals after 0, 4, 8, and 12 weeks of feeding. Concentrations of plasma and lipoprotein cholesterol, as well as plasma triglyceride, were determined enzymatically (13) using COBAS FARA II (Roche, Indianapolis, IN, USA) and commercially available kits (Randox, Antrim, UK).

Extent of atherosclerosis

After 12 weeks of feeding, six animals in each group were killed, and the aortas from the aortic arch to iliac bifurcation were excised and cleaned of adhering tissue. Then the aortas were stained with a solution of oil red O to visualize the lesion area. The positive areas stained by oil red O were photographed and measured by computer-assistant planimetry (CA6300 planimetry, Chinese Academy of Automatization), in which the ratio of area of atherosclerosis to the whole aortic area was obtained.

Cell culture

The human monocyte line U937 was obtained from the cell bank of the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. U937 cells were cultured in RPMI1640 containing 10% fetal bovine serum at 37°C in a 5% CO\textsubscript{2} humidified incubator, counted, and diluted to 5 x 10\textsuperscript{6} cells per ml, after which 1 ml was transferred to each well of a 24-well tissue-culture plate. For experiments, the U937 cells were incubated with ox-LDL at concentrations of 100 mg/L for 24 h. Then, the U937 cells were incubated with 100 mg/L oxidized lipoprotein (ox-LDL) for 24 h in addition to PMS (25, 50, or 100 μg/L).

Flow cytometry analyzed the ICAM-1 protein levels

U937 cells (1 x 10\textsuperscript{6}) were washed twice with PBS solution, fixed in 0.1% paraformaldehyde for 15 min, and incubated in 200 μL PBS containing 20 μL R-PE-conjugated ICAM-1 antibody in the dark at 4°C for 30 min. The background fluorescence was adjusted with U937 blank cells. Analyses were done on a Coulter flow-cytometer (Miami, FL, USA).
**ELISA determined the VEGF protein levels**

The supernatants were collected and used for the measurement of VEGF protein quantification after the cells were centrifuged at 1000 × g for 10 min. ELISA procedures and the standard curve were the same as those described previously (8).

**Statistics**

All the experiments were performed with triplicate measurements and the results are shown as the mean ± S.D. Statistical analysis of data was performed firstly with repeated measures analysis of variance (ANOVA) and then compared between two groups using Tukey’s method multi-comparison test. *P<0.05 and **P<0.01 were considered to be significant.

**Results**

**Animal plasma cholesterol and triglyceride levels**

No differences in body weights were seen at the end of the 12-week feeding periods. Hypertriglyceridemia and hypercholesteremia occurred in all groups of animals fed a high cholesterol diet. Concentration of cholesterol levels in plasma and lipoproteins were increased during the feeding period. Treatment with PMS at 100 mg/kg significantly attenuated the increase in plasma cholesterol, low-density lipoprotein cholesterol, and very low-density lipoprotein cholesterol (see Table 1). In particular, significant reduction of plasma triglyceride was observed in the PMS treated group even at the low dose of 25 mg/kg, while higher doses (50 or 100 mg/kg) were required for the reduction of plasma cholesterol.

**Reduction of atherosclerotic area ratio in aortas by PMS**

Severe atherosclerotic lesions were observed after feeding of a high cholesterol diet for 12 weeks. The atherosclerotic area ratio in the aorta was 78.9 ± 5.5% in high-cholesterol diet group. Treatment with PMS at 50 mg/kg caused a 43% decrease in atherosclerotic area.

### Table 1. The levels of serum TC, TG, HDLC, LDLC, and VLDLC in high-fat diet rabbits and the effects of PMS (mmol/L)

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
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<tbody>
<tr>
<td><strong>TC</strong></td>
<td></td>
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<tr>
<td>HCD</td>
<td>1.77 ± 0.87</td>
<td>30.93 ± 1.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.66 ± 0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.79 ± 0.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 25 mg/kg</td>
<td>1.47 ± 0.31</td>
<td>29.20 ± 3.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.98 ± 1.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.74 ± 5.54&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>HCD + PMS, 50 mg/kg</td>
<td>1.40 ± 0.29</td>
<td>27.47 ± 6.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.57 ± 3.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.16 ± 6.19&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>HCD + PMS, 100 mg/kg</td>
<td>1.47 ± 0.30</td>
<td>20.35 ± 4.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.97 ± 1.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.70 ± 2.49&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><strong>TG</strong></td>
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<tr>
<td>HCD</td>
<td>1.17 ± 0.85</td>
<td>2.27 ± 0.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.07 ± 0.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.71 ± 0.95&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 25 mg/kg</td>
<td>1.18 ± 0.61</td>
<td>0.95 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.76 ± 1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.73 ± 1.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 50 mg/kg</td>
<td>1.01 ± 0.34</td>
<td>0.98 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.66 ± 0.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.34 ± 1.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 100 mg/kg</td>
<td>0.93 ± 0.36</td>
<td>1.00 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.39 ± 0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.27 ± 0.77&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><strong>HDLC</strong></td>
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<tr>
<td>HCD</td>
<td>0.81 ± 0.34</td>
<td>2.12 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.71 ± 0.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00 ± 0.21</td>
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<tr>
<td>HCD + PMS, 25 mg/kg</td>
<td>0.69 ± 0.18</td>
<td>2.22 ± 0.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.15 ± 0.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.03 ± 0.21</td>
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<tr>
<td>HCD + PMS, 50 mg/kg</td>
<td>0.69 ± 0.20</td>
<td>2.06 ± 0.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.85 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.97 ± 0.18</td>
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<tr>
<td>HCD + PMS, 100 mg/kg</td>
<td>0.62 ± 0.15</td>
<td>2.17 ± 0.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.82 ± 0.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.97 ± 0.23</td>
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<tr>
<td><strong>LDLC</strong></td>
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<tr>
<td>HCD</td>
<td>0.57 ± 0.24</td>
<td>19.86 ± 2.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.32 ± 2.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.77 ± 3.97&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 25 mg/kg</td>
<td>0.47 ± 0.11</td>
<td>17.27 ± 3.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.36 ± 3.97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.34 ± 3.56&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 50 mg/kg</td>
<td>0.53 ± 0.13</td>
<td>18.56 ± 4.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.15 ± 2.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.81 ± 4.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 100 mg/kg</td>
<td>0.55 ± 0.09</td>
<td>14.32 ± 4.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.51 ± 2.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.31 ± 3.26&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><strong>VLDLC</strong></td>
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<tr>
<td>HCD</td>
<td>1.20 ± 0.65</td>
<td>11.07 ± 3.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.33 ± 2.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.02 ± 3.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 25 mg/kg</td>
<td>1.00 ± 0.25</td>
<td>11.93 ± 5.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.62 ± 3.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.40 ± 4.69&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 50 mg/kg</td>
<td>0.87 ± 0.21</td>
<td>8.91 ± 5.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.42 ± 3.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.35 ± 5.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCD + PMS, 100 mg/kg</td>
<td>0.91 ± 0.25</td>
<td>6.03 ± 2.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.47 ± 2.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.39 ± 4.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

n = 10. Mean ± S.D. *P<0.05, vs the levels at 0 weeks; **P<0.05, vs HCD group. TC: plasma cholesterol; TG: triglyceride; HDLC: high-density lipoprotein cholesterol; LDLC: low-density lipoprotein cholesterol; VLDLC: very low-density lipoprotein cholesterol.
Inhibitory Effect of PMS on AS in Rabbit

Ratio, and PMS at 100 mg/kg caused a 60% decrease (Fig. 1). Both decreases were statistically significant ($P<0.05$, $n=6$) compared with the high-cholesterol group values.
Inhibitory effects of PMS on ICAM-1 levels in foam cells

The U937 foam cells expressed high protein levels of ICAM-1 induced by ox-LDL when analyzed by flow cytometry. PMS decreased the ICAM-1 level in a dose-dependent manner (Fig. 2). Especially, after treatment with PMS at 100 µg/L, a 18.8% decrease in ICAM-1 was observed.

Inhibitory effects of PMS on VEGF levels in foam cells

The U937 foam cells secreted increasing levels of VEGF in the medium after being incubated with ox-LDL in a dose-dependent manner. PMS significantly decreased the secretion of VEGF protein from foam cells induced by ox-LDL (Fig. 3). Treating foam cells, incubated with ox-LDL, with 100 µg/L PMS resulted in a decrease of as much as 56.9% of VEGF concentration in the medium compared to untreated cells.

Discussion

Here we investigated the reduction of atherosclerosis in cholesterol-fed rabbits and decreases in the expressions of intercellular adhesion molecule-1 and vascular endothelial growth factor in macrophage-derived foam cells by PMS. In particular, the atherosclerotic lesioned area was suppressed by 60% due to PMS at a dose of 100 mg/kg.

This anti-atherosclerosis effect of PMS firstly depends on its ability to decrease lipids, by which PMS could attenuate the increase in plasma cholesterol, plasma triglyceride, low-density lipoprotein cholesterol, and very low-density lipoprotein cholesterol. The effect of PMS on serum triglyceride level was most significant, which might be applicable for the treatment of hyperlipidemic disease in the future.
In cholesterol-fed rabbits, increased serum total cholesterol level has been reported to be due, in most part, to increased chylomicron remnant and/or \( \beta \)-VLDL cholesterol, which might be involved in the promotion of atherosclerosis (13). However in this study, the hyperlipidemic pattern induced by high fat diet showed both increased cholesterol and triglyceride levels. Especially, the high cholesterol diet was supplemented with 2.5% yolk powder and 4% corn oil, in addition to 0.5% cholesterol, in which the yolk powder and corn oil can increase the triglyceride level.

The mechanism by which PMS suppressed the increase in serum cholesterol and triglyceride levels is complicated. PMS may affect cholesterol and triglyceride excretion in the large intestine (14), because in the clinical experience of Chinese Traditional Medicine, Polygonum multiflorum Thunb. can promote detoxification of an organism (15).

Recently, numerous studies focused on the anti-inflammatory effects (16) of anti-atherosclerotic drugs such as statins (17). Therefore we also focus on the macrophage-derived foam cell functions in atherogenesis. Accumulation of ox-LDL in foam cells is a characteristic feature of atherosclerotic lesions and plays a fundamental role in the pathogenesis and progression of disease. Considering atherosclerosis as a problem of chronic inflammation, the involvement of macrophages is not surprising, and macrophage transformation to lipid-loaded foam cells is a primary event in the disease program (1).

The role of the foam cell is to act not only as a scavenger cell (18), but also as the principal, inflammatory mediator of cells in the atheromatous plaque microenvironment (19). The up-regulating expressions of ICAM-1 and VEGF were determined in foam cells, which confirmed that both ICAM-1 and VEGF participated in the inflammatory reaction during the formation of foam cells in lesions of atherosclerosis. During atherogenesis, increased ICAM-1 expression was associated with a marked monocyte and T lymphocyte intimal recruitment (20 – 22). The basic function of VEGF can induce endothelial cell migration and growth, differentiation/regeneration, and angiogenesis (23), which however can induce migration and activation of monocytes through its receptor FLT-1 (24), adhesion molecule (25), or monocyte chemoattractant protein-1 (26), and administration of VEGF to hypercholesterolemic animals enhances atherosclerotic lesions. As well-known, Ginkgo biloba extract (GbE) can inhibit ICAM-1 (27) and VEGF (28) expression, which play an important role in its anti-atherosclerosis effects. Our results suggested that both ICAM-1 and VEGF expressions were inhibited by PMS, which confirmed that PMS may have potential use in atherosclerosis treatment.

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References

13 Kurosawa T, Itoh F, Nozaki A, Nakano Y, Katsuda S, Osakabe


