Effects of Xuezhikang on Proliferation and Adhesion Capacity of Cultured Endothelial Progenitor Cells: An In Vitro Study

Xiang-Quan Kong, MD¹; Meng-Zan Wang, MD¹; Le-Xin Wang, MD, PhD²; Jing-Bo Kong, MD¹; Xue-Wen Qi, MD¹; and Shuang-Feng Chen, MD¹

¹Department of Cardiology, Liaocheng Clinical School, Taishan Medical College, Shandong Province, China; and ²School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, New South Wales, Australia

ABSTRACT

BACKGROUND: Endothelial progenitor cells (EPCs) might be useful in the management of coronary artery disease (CAD).

OBJECTIVE: The aim of this study was to investigate the effects of xuezhikang, an extract of Chinese red yeast rice, on the proliferation and adhesion capacity of EPCs from the peripheral blood of patients with stable CAD.

METHODS: Mononuclear cells from 20 Chinese patients (14 men, 6 women; mean [SD] age, 64.5 [2.8] years [range, 60–69 years]) were isolated using density-gradient centrifugation. After 4 days in culture, the attached cells were treated with different concentrations of xuezhikang (50, 125, 250, and 500 ng/mL; 20 samples/group), atorvastatin (10 ng/mL; n = 20), or phosphate-buffered saline (control, n = 20) for 3 days. Cells that were positive for 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein and lectin were defined as EPCs. They were counted by 2 independent investigators in ≥4 randomly selected high-power fields per well. EPCs were then treated and adherent cells were counted by the independent investigators who were blinded to study drug administration.

RESULTS: The mean (SD) number of cultured EPCs in the xuezhikang 50-, 125-, 250-, and 500-ng/mL groups (205 [28], 244 [31], 283 [42], and 334 [43] cells, respectively; all, P < 0.001) was significantly increased in a dose-dependent manner compared with the control group (167 [36] cells). The adhesion capacity of the EPCs was significantly greater in the 4 xuezhikang groups (51 [9], 62 [10], 71 [11], and 83 [12] cells; all, P < 0.001) when compared with that of the control group (41 [7] cells). Both the number of EPCs (327 [49] cells) and the number of adhesive EPCs (84 [15] cells) in the atorvastatin group were also significantly increased compared with the control group (both, P < 0.001); however, these increases were not significantly different from those in the xuezhikang 500-ng/mL group.

CONCLUSIONS: Xuezhikang was associated with significantly enhanced proliferation and adhesion capacity of EPCs derived from the peripheral blood of these patients with stable CAD. These effects were not significantly different between xuezhikang...

**KEY WORDS:** xuezhi, endothelial progenitor cell, coronary artery disease, statins.

**INTRODUCTION**

Bone marrow–derived endothelial progenitor cells (EPCs) are capable of differentiating into endothelial cells in ischemic tissue, contributing to neovascularization and endothelial function.\(^1\)-\(^3\) There is growing evidence that EPCs isolated from peripheral blood migrate to the ischemic sites and disrupt endothelium.\(^4\) In experimental models of myocardial infarction, injection of ex vivo–expanded EPCs significantly improved blood flow and cardiac function and reduced left ventricular scarring.\(^5\),\(^6\) In addition, several small clinical trials have found that EPCs acquired from bone marrow or peripheral blood enhanced the blood supply of the ischemic tissues in the limbs and the heart.\(^7\),\(^8\)

Increasing the number or the adhesion capacity of circulating EPCs may be a useful strategy for managing coronary artery disease (CAD). Several studies\(^9\)-\(^11\) found that statins significantly increased the number of circulating EPCs and their adhesion capacity.

Xuezhi is an extract of Chinese red yeast rice. In a clinical trial\(^12\) of 83 patients with hyperlipidemia, oral xuezhi supplements (2.4 g/d) administered for 12 weeks reduced concentrations of serum triglycerides, total cholesterol, and low-density lipoprotein cholesterol by up to 30%. Xuezhi is also associated with improved preprandial and postprandial endothelial function through its potent anti-inflammatory and lipid-lowering effects.\(^13\)

In the present study, we investigated the effect of xuezhi (WBL Peking University Biotech Co., Ltd., Beijing, China) on the proliferation and adhesion capacity of EPCs isolated from the peripheral blood of patients with stable CAD.

**PATIENTS AND METHODS**

**Patient Characteristics**

Between January and May 2006, 32 Chinese patients were approached to participate in the study at the Department of Cardiology, LIAOCHENG People's Hospital (Shandong Province, China). After the initial screening, 20 patients (14 men, 6 women; mean [SD] age, 64.5 [2.8] years [range, 60–69 years]) with angiographically documented CAD and clinical evidence of stable angina pectoris were enrolled in the study. None of the patients had been treated with a statin in the 4 weeks immediately preceding the study.

Patients with unstable angina or myocardial infarction in the past 3 months were excluded from the study. Patients with concomitant inflammatory or malignant diseases were also excluded.

The study was approved by the institutional review board of LIAOCHENG Clinical School of Taishan Medical College, Shandong Province, China. Written informed consent was obtained from all participants.
Mononuclear cells were isolated from the peripheral blood of patients by density-gradient centrifugation using previously described methods. Immediately after isolation, mononuclear cells were plated on 24-well culture dishes coated with human fibronectin (Gibico, Grand Island, New York) and maintained in endothelial basal medium (EBM) (Clonetics, Lonza, Inc., Allendale, New Jersey). This medium was supplemented with 20% fetal calf serum, 50 ng/mL vascular endothelial growth factor (Sigma-Aldrich Corp., St. Louis, Missouri), 50 ng/mL stem cell factor (Sigma-Aldrich Corp.), 100 U/mL benzylpenicillin, and 100 U/mL streptomycin.

After 4 days in culture, nonadherent cells were removed by a thorough washing with phosphate-buffered saline (PBS). Adherent cells were maintained and treated with different concentrations of xuezhikang (50, 125, 250, or 500 ng/mL), atorvastatin* (10 ng/mL), or PBS (control group) for 3 days.

The investigators reviewing the characterization of EPCs were blinded to the group allocation. To detect the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiLDL) (Molecular Probes, Invitrogen Corporation, Carlsbad, California), adherent cells were incubated with DiLDL (2.4 μg/mL) at 37°C for 1 hour. Cells were then fixed with 2% paraformaldehyde for 10 minutes and incubated with *Ulex europaeus* agglutinin I conjugated with fluorescein isothiocyanate (FITC) (FITC-lectin, 10 μg/mL) (Sigma-Aldrich Corp.) at 37°C for another hour. Cells were then observed with an inverted fluorescent microscope (Olympus Corp., Tokyo, Japan). Cells that were positive for both DiLDL and lectin were defined as EPCs. They were counted by 2 independent investigators in ≥4 randomly selected high-power fields per well.

After 7 days, EPCs were washed and gently detached with 0.1% trypsin in PBS. EPCs were then spun, resuspended in EBM-2 with 5% fetal calf serum, and counted. The same numbers of EPCs were replated onto fibronectin-coated culture dishes and incubated for 30 minutes at 37°C. Adherent cells were counted by independent investigators who were blinded to the treatment. In each blood sample, ≥4 randomly selected high-power fields were counted per well.

Data were expressed as mean (SD). Comparisons of numbers of cells between groups were performed using the Student t test. $P < 0.05$ was considered statistically significant.

*Trademark: Lipitor® (Pfizer Inc., Dalian, China).
RESULTS
MORPHOLOGY AND IDENTIFICATION OF EPITHELIAL PROGENITOR CELLS
The initially seeded cells were round. The EPC colonies with spindle-shaped cells radiating around the round cells in the core were observed after 4 to 5 days of culture. EPCs were identified as positive for uptake of both DiLDL and lectin in all samples.

EFFECT OF XUEZHIKANG ON EPITHELIAL PROGENITOR CELL PROLIFERATION
After 7 days, the mean number of EPCs was significantly increased in the xuezhikang-treated groups (50, 125, 250, 500 ng/mL) (205 [28], 244 [31], 283 [42], 334 [43] cells, respectively) compared with the control group (167 [36] cells; all, P < 0.001). The number of EPCs was also significantly increased in the atorvastatin-treated group (327 [49] cells) compared with the control group (P < 0.001). The mean number of EPCs in the 500-ng/mL xuezhikang group was not significantly different from the atorvastatin group (Table). However, the mean number of EPCs in other xuezhikang groups was significantly less than that observed in the atorvastatin group (all, P < 0.01).

EFFECTS OF XUEZHIKANG ON EPITHELIAL PROGENITOR CELL ADHESION CAPACITY
Compared with the control group (41 [7] cells), the mean number of adhesive EPCs was significantly higher in the xuezhikang-treated groups (50, 125, 250, 500 ng/mL) (51 [9], 62 [10], 71 [11], 83 [12] cells, respectively; all, P < 0.001), with a peak in the 500-ng/mL group (2-fold increase). A significantly higher cell count was also observed in the atorvastatin group (84 [15] cells) compared with the control group (P < 0.001). No significant difference in the number of adhesive EPCs was found between the 500-ng/mL xuezhikang group and the atorvastatin group, but the number of adhesive cells in other xuezhikang groups was significantly less than in the atorvastatin group (P < 0.01) (Table).

DISCUSSION
A search of English-language articles in MEDLINE and PubMed (1980–2007) using the combined search terms xuezhikang and EPCs found that this was the first published study to suggest that xuezhikang exerts a dose-dependent effect on the proliferation and adhesion capacity of EPCs in patients with stable CAD. This study also found that atorvastatin was associated with increased EPCs and improvement of adhesive capacity in a manner similar to the highest dose of xuezhikang (500 ng/mL).

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) have been developed as lipid-lowering drugs and have been reported to reduce morbidity and mortality in patients with CAD.14 There is growing evidence that the beneficial effects of statins on CAD outcomes may go beyond the lipid-lowering actions. Dimmeler et al9 found that statins potently augmented EPC differentiation in mononuclear cells and CD34+ hematopoietic stem cells isolated from peripheral
Table. Effect of xuezhikang* and atorvastatin† on endothelial progenitor cell (EPC) proliferation (number of EPCs) and adherence capacity (number of adherent EPCs). Data are mean (SD).

<table>
<thead>
<tr>
<th>Variable</th>
<th>50 ng/mL (n = 20)</th>
<th>125 ng/mL (n = 20)</th>
<th>250 ng/mL (n = 20)</th>
<th>500 ng/mL (n = 20)</th>
<th>Atorvastatin 10 ng/mL (n = 20)</th>
<th>Control† (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPCs, no.</td>
<td>205 (28)!</td>
<td>244 (31)!</td>
<td>283 (42)!</td>
<td>334 (43)!</td>
<td>327 (49)!</td>
<td>167 (36)</td>
</tr>
<tr>
<td>Adherent EPCs, no.</td>
<td>51 (9)!</td>
<td>62 (10)!</td>
<td>71 (11)!</td>
<td>83 (12)!</td>
<td>84 (15)!</td>
<td>41 (7)</td>
</tr>
</tbody>
</table>

*WBL Peking University Biotech Co., Ltd., Beijing, China.
†Trademark: Lipitor® (Pfizer Inc., Dalian, China).
††Phosphate-buffered saline.
§§P < 0.001 versus the control group.
blood. Assmus et al\textsuperscript{15} reported that atorvastatin dose-dependently enhanced the inhibition of EPC senescence. Atorvastatin also induced EPC proliferation in vitro, which might lead to improvement in the functional activity of EPCs.\textsuperscript{15} Furthermore, statins were found to facilitate the migration and potency of EPCs to form vessel structures and to contribute to vasculogenesis.\textsuperscript{16}

The mechanisms mediating the effects of statins on EPCs remain to be determined. Phosphatidylinositol 3-kinase (PI3K)/Akt was found to mediate the effect of statins on EPCs.\textsuperscript{9} Activation of the PI3K/Akt pathway by statins may have multiple protective effects on EPCs, including an increase in numbers, inhibition of apoptosis, improvement of functional activity, and the prevention of senescence.\textsuperscript{15} Also, migration and tube formation of EPCs may be blocked by PI3K/Akt inhibitors, indicating that the effects of statins on EPCs involve the PI3K/Akt signaling pathway.\textsuperscript{9,16} Apart from the PI3K/Akt pathway, increased availability of endothelial nitric oxide is pivotal for statin-induced improvement in EPC mobilization and myocardial neovascularization after myocardial infarction.\textsuperscript{17}

The effect of xuezhikang on EPCs was assessed from 2 perspectives in the present study. First, xuezhikang was associated with a dose-dependent increase in the number of cultured EPCs. At the highest dose (500 ng/mL) of xuezhikang, the increase in the mean number of EPCs was not significantly different from the increase after atorvastatin treatment. Second, the function of the EPCs was assessed by measuring the mean number of adhesive cells after treatment. Several studies found that migration of EPCs to the sites of ischemia and the resultant neovascularization involved several key processes, including adhesion to endothelial cells, incorporation into capillaries, and transendothelial migration into extravascular space.\textsuperscript{18,19} Adhesion to fibronectin, cultured endothelial cells, and cardiomyocytes is critical for EPCs to participate in trafficking in ischemic muscle.\textsuperscript{18,20} In the present study, xuezhikang was associated with a 2-fold increase in the mean number of adhesive EPCs, indicating enhancement of the adhesion capacity. The study also found that atorvastatin 10 ng/mL was associated with a 2-fold increase in the adhesion capacity of EPCs.

A potential limitation of this study was that xuezhikang is not a single chemical entity: it contains statin-like compounds that inhibit the actions of HMG-CoA reductase, fatty acids, and isoflavones.\textsuperscript{12} It has lipid-lowering as well as anti-inflammatory actions.\textsuperscript{13} Therefore, although xuezhikang and atorvastatin were associated with similar results in the increase in the number and adhesion capacity of EPCs compared with the control group, the mechanisms resulting in these outcomes may be different and require further investigation. Another limitation of the study was the small sample size.

Further studies are required to investigate whether the effects of xuezhikang on cultured EPCs are also present in vivo. Whether the beneficial effects of xuezhikang on the proliferation and functional activities of EPCs can be translated into improvement in the clinical outcomes of CAD also needs to be further investigated.

**CONCLUSIONS**

The present study found that at a high dose, xuezhikang was not statistically different from atorvastatin in increasing the proliferation and adhesive capacity of EPCs from
peripheral blood in these patients with stable CAD. These findings provide further explanation of the previously reported beneficial effects of xuezhikang on the endothelial function in patients with CAD.

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**ADDRESS CORRESPONDENCE TO:** Le-Xin Wang, MD, PhD, School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia. E-mail: lwang@csu.edu.au