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Abstract: Objectives: To investigate the effects of Xuezhikang, an extract from Chinese red-yeast rice, on the proliferation and adhesive capacity of endothelial progenitor cells (EPCs) from the peripheral blood of patients with stable coronary artery disease. Methods: Mononuclear cells were isolated by density-gradient centrifugation from 20 patients. After four days in culture, the attached cells were treated with PBS (control, n=20) or different concentrations of Xuezhikang (50, 1125, 250 and 500 ng/ml, 20 samples per group) or atorvastatin (10 ng/ml, n=20) for additional three days. The numbers of the EPCs and their capacity of adhesion to extracellular matrix proteins were evaluated. Results: Compared to the control (167±36), the numbers of cultured EPCs in the Xuezhikang groups (205±28, 244±31, 283±42 and 334±43) were significantly increased (P< 0.001) in a dose-dependent manner. The adherence capacities of the EPCs in the four Xuezhikang groups (51±9, 62±10, 71±11 and 83±12) were greater than the control group (41±7, P< 0.001). Both the numbers (327±49) and adherence capacities (84±15) of the EPCs in the atorvastatin group were also increased in comparison with the control group (P< 0.001), but these increases were similar to that in the 500 ng/ml Xuezhikang group (P> 0.05). Conclusions: Xuezhikang enhances the proliferation and adhesive capacity of EPCs derived from the peripheral blood of the patients with stable coronary artery disease. These effects were similar between Xuezhikang and atorvastatin.

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**Effects of Xuezhikang on proliferation and adhesive capacity of cultured
endothelial progenitor cells**

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Abstract

Objectives: To investigate the effects of Xuezhikang, an extract from Chinese red-yeast rice, on the proliferation and adhesive capacity of endothelial progenitor cells (EPCs) from the peripheral blood of patients with stable coronary artery disease.

Methods: Mononuclear cells were isolated by density-gradient centrifugation from 20 patients. After four days in culture, the attached cells were treated with PBS (control, n=20) or different concentrations of Xuezhikang (50, 1125, 250 and 500 ng/ml, 20 samples per group) or atorvastatin (10 ng/ml, n=20) for additional three days. The numbers of the EPCs and their capacity of adhesion to extracellular matrix proteins were evaluated.

Results: Compared to the control (167±36), the numbers of cultured EPCs in the Xuezhikang groups (205±28, 244±31, 283±42 and 334±43) were significantly increased ($P<0.001$) in a dose-dependent manner. The adherence capacities of the EPCs in the four Xuezhikang groups (51±9, 62±10, 71±11 and 83±12) were greater than the control group (41±7, $P<0.001$). Both the numbers (327±49) and adherence capacities (84±15) of the EPCs in the atorvastatin group were also increased in comparison with the control group ($P<0.001$), but these increases were similar to that in the 500 ng/ml Xuezhikang group ($P>0.05$).

Conclusions: Xuezhikang enhances the proliferation and adhesive capacity of EPCs derived from the peripheral blood of the patients with stable coronary artery disease. These effects were similar between Xuezhikang and atorvastatin.

Key words: Xuezhikang; endothelial progenitor cell, coronary artery disease, statins.

Introduction

Bone marrow-derived endothelial progenitor cells (EPCs) are capable of differentiating endothelial cells in ischemic tissue, contributing to neovascularization and endothelial function (1-3). There is growing evidence that endothelial progenitor cells isolated from peripheral blood are homing to the ischemic sites and disrupted endothelium (4). In animal models of myocardial infarction, injection of ex vivo–expanded EPCs significantly improves blood flow and cardiac function and reduces left ventricular scarring (5, 6). In addition, small clinical trials have shown that EPCs acquired from bone marrow or peripheral blood can enhance blood supply of the ischemic tissues in the limbs or the heart (7, 8).

It seems that increasing the numbers or the functional activities of circulating EPCs may be a useful strategy for the management of coronary artery disease. Recently, several studies have demonstrated that statins increase the number of circulating EPCs and their functional activities (9-11). Xuezhikang, an extract from Chinese red-yeast rice, has pharmacological actions similar to HMG-CoA reductase inhibitors, such as atorvastatin (12). In a clinical trial on 83 patients with hyperlipidemia, oral Xuezhikang supplements for 12 weeks reduced blood triglycerides, total cholesterol and LDL-cholesterol by up to 30% (12). Xuezhikang also improves preprandial and postprandial endothelial function through its potent anti-inflammatory and lipid-lowering effects (13).

In the present study we investigated the effect of Xuezhikang on the proliferation and functional activity of EPCs isolated from the peripheral blood in patients with stable coronary artery disease.

Materials and methods

General characteristics of patients

Between January and May 2006, 32 patients were approached for the study at the Department of Cardiology, Liaocheng People's Hospital. After the initial screening, 20

patients (mean age 64.5 ± 2.8 years, ranged 60-69 years) with angiographically documented coronary artery disease and clinical evidences of stable angina pectoris were enrolled in the study. None of the patients had been treated with a statin in the past four weeks of the study.

Patients with unstable angina or myocardial infarction in the past three 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. Written informed consent was obtained from all participants.

EPCs culture

Mononuclear cells were isolated by density-gradient centrifugation from peripheral blood of patients using methods as previously described (6, 9). Immediately after isolation, mononuclear cells were plated on 24-well culture dishes coated with human fibronectin (Gibico) and maintained in endothelial basal medium (EBM, Clonetics). This medium was supplemented with 20% fetal calf serum, 50 ng/ml vascular endothelial growth factor (VEGF, Sigma), 50 ng/ml stem cell factor (SCF, Sigma), 100 U/ml benzylpenicillin, and 100 U/ml streptomycin.

After four days in culture, nonadherent cells were removed by a thorough washing with PBS. Adherent cells were maintained and treated with PBS (control) or different concentrations of Xuezhikang (50, 125, 250 or 500 ng/ml) (WBL Peking University Biotech Co, Ltd), or atorvastatin (10 ng/ml) for additional three days.

Characterization of EPCs

The investigators reviewing the characterization of EPC's 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 Probe), adherent cells were incubated with DiLDL (2.4 µg/mL) at 37°C for one 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) at 37°C for another hour. Cells were then observed with an inverted fluorescent microscope (Olympus). Cells that were double positive for DiLDL and lectin were defined as EPCs. They were counted by two independent investigators in at least four randomly selected high-power fields per well. The average cell counts from the blood samples of the 20 patients were listed in Table 1.

Cell adhesion assay

After treatment with Xuezhikang, atorvastatin or PBS, EPCs (on day 7) were washed and gently detached with 0.1 % trypsin in PBS. EPCs were spun down, re-suspended 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 min at 37 °C. Adherent cells were counted by independent investigators who were blinded to the treatment. In each blood sample, at least four randomly selected high-power fields were counted per well. The average cell counts from the blood samples of the 20 patients were listed in Table 1.

Statistical analysis

Data were expressed as mean ± SD. Comparison in cell numbers between groups was performed by a student *t* test. $P < 0.05$ was considered to be statistically significant.

Results

Morphology and identification of EPCs

The initially seeded cells were round cells. The EPCs colonies with spindle-shaped cells radiated around the round cells in core were observed after 4-5 days of culture. EPCs are identified as double positive for uptake of DiLDL and lectin reactivity.

Effect of Xuezhikang on EPCs proliferation

As shown in Table 1, the number of EPCs was significantly increased with in Xuezhikang treated groups ($P<0.001$). Compared with the control group, the number of EPCs were also increased in the atorvastatin-treated group (Table 1, $P<0.001$). The average number of EPCs in the 500 ng/ml Xuezhikang group was similar to the atorvastatin group ($P>0.05$).

Effects of Xuezhikang on EPCs adhesiveness

As illustrated in Table 1, the average number of adhesive EPCs was higher than the control group ($P<0.01$), with a peak at 500 ng/ml (2-fold increase). A greater cell count was also found in the atorvastatin group (Table 1, $P<0.01$). No significant difference of adhesive EPCs count was observed between the 500 ng/ml Xuezhikang and atorvastatin group ($P>0.05$).

Discussion

To the best of our knowledge, this the first study to demonstrate that Xuezhikang exerts a dose-dependent effect on the proliferation and adhesive capacity of EPCs in patients with stable coronary artery disease. This study also showed that atorvastatin increases the number of EPCs and improved their adhesive capacities similar to Xuezhikang.

HMG-CoA reductase inhibitors (statins) have been developed as lipid-lowering drugs and are known to reduce morbidity and mortality in patients with coronary artery disease.

There is growing evidence to support that the beneficial effects of statins on the coronary artery disease outcomes may go beyond the lipid-lowering actions. Dimmeler et al. (9) demonstrated that statins potently augment endothelial progenitor cell differentiation in mononuclear cells and CD34-positive hematopoietic stem cells isolated from peripheral blood. Assmus et al. (14) reported that atorvastatin dose-dependently enhances the inhibition of EPCs senescence. Atorvastatin also induces EPCs proliferation *in vitro*, which may lead to improvement in the functional activity of EPCs (14). Furthermore, statins facilitate the migration and potency of EPCs to form vessel structures and to contribute to vasculogenesis (15).

The mechanisms mediating the effects of statins on EPCs remain to be determined. Recently, phosphatidylinositol 3-kinase (PI3K)/Akt has been shown to mediate the effect of statins on EPCs. Activation of the PI3K/Akt pathway by statins may have multiple protective effects on EPCs, including increase in numbers, inhibition of apoptosis, improvement of functional activity, and the prevention of senescence (14). Also, EPC's migration and tube formation can be blocked by phosphatidylinositol 3-kinase inhibitors, indicating that the effects of statins on EPCs involves PI3K signaling pathway (9, 15). Apart from the PI3K/Akt pathway, an increased availability of endothelial nitric oxide is pivotal for statin-induced improvement in EPC mobilization, and myocardial neovascularization after myocardial infarction (16).

The effect of Xuezhikang on the EPCs was assessed from two perspectives in the present study. First, Xuezhikang was associated with a dose-dependent increase in the number of cultured EPCs. At a higher dose of Xuezhikang, the increase in the average EPCs was similar to the increase following atorvastatin treatment. Second, the function of the EPCs was assessed by measuring the average number of adhesive cells following treatment. Several studies have confirmed that the homing of EPCs to the sites of ischemia and the

resultant neovascularization involves several key processes, such as adhesion to endothelial cells, incorporation to capillary, and transendothelial migration into extravascular space (17, 18). Adhesion to fibronectin, cultured endothelial cells, and cardiomyocytes is a critical function for EPCs to participate in trafficking in ischemic muscle (17, 19). In the present study, Xuezhikang has led to a 2-fold increase in the average number of adhesive EPCs, indicating an enhancement of the adhesive capacity. The study also showed that at 10ng/ml, atorvastatin also led to a 2-fold increase in the adhesive capacity of EPCs.

A potential limitation of this study is that Xuezhikang is not a single chemical entity; it contains HMG-CoA reductase inhibitors, fatty acids and isoflavones (12). It has lipid-lowering as well as anti-inflammatory actions (13). Therefore, although Xuezhikang and atorvastatin showed similar results in EPC numbers and adhesive capabilities when compared to the control group, the mechanisms resulting in these outcomes may be different and require further investigation.

In conclusion, the present study demonstrated that at a high dose, Xuezhikang has a similar effect to atorvastatin in increasing the proliferation and adhesive capacity of EPCs from peripheral blood of patients with stable coronary artery disease. These findings thus provide further explanation to the previously reported beneficial effects of Xuezhikang on the endothelial function in patients with coronary artery disease. Further studies are required to investigate whether the effects of Xuezhikang on the cultured EPCs are also present *in vivo*. More importantly, whether the beneficial effects of Xuezhikang on the proliferation and functional activities of EPCs can be translated into improvement in the clinical outcomes of coronary artery disease needs to be further investigated.

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Table1. Effect of Xuezhikang (XZK) on the proliferation and adherence capacities of endothelial progenitor cells (EPCs)

	Control (n=20)	XZK 50ng/ml (n=20)	XZK 125ng/ml (n=20)	XZK 250ng/ml (n=20)	XZK 500 ng/ml (n=20)	AS 10ng/ml (n=20)
Number of EPCs	167±36	205±28*	244±31*	283±42*	334±43*	327±49*
EPC Adherence	41±7	51±9*	62±10*	71±11*	83±12*	84±15*

XZK: Xuezhikang; AS: Atorvastatin. * $P < 0.001$ compared to the control group.