ORIGINAL RESEARCH

Effects of adrenomedullin on the cell numbers and apoptosis of endothelial progenitor cells

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Abstract

Purpose: To investigate the effect of adrenomedullin on the cell numbers and apoptosis of endothelial progenitor cells (EPCs).

Methods: Mononuclear cells were isolated from peripheral blood by Ficoll density gradient centrifugation. The cells were stimulated with adrenomedullin, before and after the treatment of adrenomedullin-receptor antagonist, adrenomedullin 22-52, or a PI3K inhibitor LY294002.

Results: Adrenomedullin dose-dependently increased the number of EPCs ($P<0.05$). Adrenomedullin also significantly decreased apoptosis rate of EPCs in a concentration-dependent manner ($P<0.05$). In the isolated human mononuclear cells pretreated with adrenomedullin 22-52 or LY294002, adrenomedullin failed to increase the number of EPCs or to reduce the level of apoptosis.

Conclusions: Adrenomedullin increases the number of EPCs and decreases their apoptosis. These actions are likely mediated by PI3K signaling pathways. The clinical importance of these favourable effects on EPCs remains to be determined.

There is growing evidence that endothelial progenitor cells (EPCs) isolated from peripheral blood are able to travel to the sites of ischemia and endothelial disruption, enhancing endothelial function and

neovascularization.¹ A recent study suggests that the level of circulating EPCs predicts the occurrence of cardiovascular events and death from cardiovascular causes and may help to identify patients with increased cardiovascular risk.² Augmentation of EPCs with improved functional activity might represent a useful strategy for clinical therapy of coronary artery disease.³

Adrenomedullin is a potent and long lasting vasodilating peptide, comprising 52 amino acids. It is secreted from endothelial cells, smooth muscle cells and adventitial fibroblasts in various organs, such as the heart, lung, kidney, adipose tissues, and the central nervous system.^{4,5} Adrenomedullin signaling is of particular importance in endothelial cell biology, since this peptide protects cells from apoptosis, promotes angiogenesis, and affects vascular tone and permeability.⁵ Adrenomedullin has also been shown to enhance the angiogenic potency of bone marrow-derived mononuclear cell transplantation, and to improve the cardiac function in rats with myocardial infarction.⁶ These beneficial effects are believed to be mediated partly by the angiogenic property of adrenomedullin itself and by its antiapoptotic effect on

mononuclear cells.⁶ However, whether adrenomedullin has any direct effect on EPCs is unclear.

The primary aim of the present study is to investigate the effect of adrenomedullin on the cell numbers and apoptosis rate of EPCs in patients with existing coronary artery disease.

Methods

Patient selection

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.

Between January and May 2006, about 30 patients with angina pectoris were approached for the study at the Cardiology Department of Liaocheng Clinical School. After excluding those with unstable angina or myocardial infarction in the past three months, 20 patients (14 male, average age 64.5 ± 2.6 , range, 60-69 yr) with angiographically documented coronary artery disease were recruited. These patients had a normal renal and liver function, and had no concomitant inflammatory or malignant diseases.

Isolation of EPCs

The total mononuclear cell fraction was isolated by density-gradient centrifugation from peripheral blood as previously described.⁷ Immediately after isolation, mononuclear cells were plated on 24-well culture dishes coated with human fibronectin (Gibico) and maintained in endothelial basal medium (Clonetics). The endothelial basal medium was supplemented with 20% fetal bovine 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 four different concentrations of adrenomedullin (10^{-6} mol/L, 10^{-7}

mol/L, 10^{-8} mol/L, 10^{-9} mol/L) for seven days. In a separate group, mononuclear cells were first treated with adrenomedullin 22-52, a selective adrenomedullin-receptor antagonist at 10^{-7} mol/L for three days. Adrenomedullin (10^{-7} mol/L) was added to the adrenomedullin 22-52 treated dishes for seven days. In another group, mononuclear cells were treated with LY294002, a PI3K inhibitor at 10^{-5} mol/L, for three days then followed by adrenomedullin treatment at 10^{-7} mol/L for seven days.

After the various treatments, the attached cells were cultured by serum-free media for 24 hr. EPCs were characterized as adherent cells double positive for DiLDL (1, 1-dioctadecyl-3, 3, 3, 3-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein, Molecular Probe) up- take and lectin binding by direct fluorescent staining.

Cellular staining

To detect the uptake of DiLDL, adherent cells were incubated with DiLDL (2.4 μ g/mL) at 37°C for 1 hour.^{8,9} Cells were then fixed with 2% paraformaldehyde for 10 min, and incubated with Ulex europaeus agglutinin I conjugated with fluorescein isothiocyanate (FITC) (FITC-lectin, 10 μ g/ml; Sigma) at 37°C for another one hour. Cells that were double positive for DiLDL and lectin were judged to be EPCs. They were counted by two independent investigators in at least four randomly selected high-power fields per well.

Quantitative determination of apoptosis

Apoptosis rates of EPCs were measured by DNA flow cytometry and DNA electrophoresis.^{10,11} Annexin V binding was assessed using bivariate flow cytometry, and cell staining was evaluated with fluorescein isothiocyanate (FITC)-labelled Annexin V (green fluorescence), simultaneously with dye exclusion of propidium iodide (PI) (negative for red fluorescence).^{10,11}

FIGURE 1. : Endothelial progenitor cells were incubated with DiLDL (red, A) and lectin (green, B). Double positive cells were identified as endothelial progenitor cells (yellow, C).

Statistical analysis

Data were expressed as mean $\pm$ SD, and assessed by one-way ANOVA for single comparisons. Categorical data were analyzed with *Chi-square* test. $P < 0.05$ was considered to be statistically significant.

Results

Characterization of EPCs

The initially seeded cells were round cells. The EPC 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 (Fig 1). Their numbers were manually counted by two independent investigators who were blinded to the treatment group allocations.

Effect of adrenomedullin on the numbers of EPCs

The average number of EPCs in adrenomedullin treated cells was higher than in the control group (Table 1, $P < 0.05$). The average EPC numbers in the 10^{-7} mol/L group was greater than in the 10^{-8} mol/L, 10^{-9}

TABLE 1. Effect of adrenomedullin on the numbers and apoptosis of endothelial progenitor cells.

	Number of EPCs	Apoptosis rate
Control (n=20)	168 $\pm$ 36	44.6 $\pm$ 6.1%
AM 10^{-9} mol/L (n=20)	264 $\pm$ 31*	34.4 $\pm$ 6.8%*
AM 10^{-8} mol/L (n=20)	284 $\pm$ 42*	29.5 $\pm$ 5.2%*
AM 10^{-7} mol/L (n=20)	328 $\pm$ 49*	21.8 $\pm$ 4.5%*
AM 10^{-6} mol/L (n=20)	206 $\pm$ 28*	21.8 $\pm$ 4.5%*
AM 22-52 (n=20)	178 $\pm$ 33	43.2 $\pm$ 5.9%
LY294002 (n=20)	178 $\pm$ 37	44.6 $\pm$ 6.1%

AM: adrenomedullin. EPCs: endothelial progenitor cells.

* $P < 0.05$ compared with the control group.

mol/L or 10^{-6} mol/L group (Table 1, $P < 0.05$).

The average EPC count in the adrenomedullin 22-52 and LY294002 pretreated group was similar to the control group (Table 1, $P > 0.05$).

Effect of adrenomedullin on the apoptosis rate of EPCs

There was a reduction in the rate of apoptosis in the adrenomedullin treated cells (Fig 2a and 2b). As shown in Table 1, the average apoptosis rate in the adrenomedullin treated group was lower than in the control group ($P < 0.05$). The apoptosis rate in the 10^{-6} mol/L and 10^{-7} mol/L group was lower than in the 10^{-8} mol/L and 10^{-9} mol/L group ($P < 0.05$).

The apoptosis rate in the adrenomedullin 22-52 and LY294002 treated cells was similar to the control group (Table 1, $P > 0.05$).

Discussion

The main findings of the present study are: 1) Adrenomedullin, a vasodilating peptide, increases the number of EPCs isolated from the peripheral blood of patients with coronary artery disease; 2) Adrenomedullin also reduces the rate of apoptosis of EPCs in a dose-dependent manner; 3) Adrenomedullin 22-52, a selective adrenomedullin-receptor antagonist, diminishes the anti-apoptotic actions of adrenomedullin. The present study is the first to demonstrate that adrenomedullin is associated with a dose-dependent in-

FIGURE 2a. Apoptosis rate of endothelial progenitor cells in the control group.

FIGURE 2b. Apoptosis rate of endothelial progenitor cells in 10^{-7} mol/L adrenomedullin group.

crease in the numbers of EPCs and a decrease in their apoptosis.

These findings may have important clinical implications. Intravenously injected EPCs may home to the sites of nascent neovascularization and differentiate into mature endothelial cells.¹ Exogenously administered EPCs have been shown to accelerate revascularization and promote limb salvage in mice with hindlimb ischemia.¹² Exogenously administered EPCs are also found to home to foci of myocardial neovascularization, augmenting vascularity and improving left ventricular function.^{13,14} Therefore, by pharmacologically enhancing EPCs numbers and reducing their apoptosis, one would hope that more EPCs will reach the ischemic sites in the heart and other vital organs and therefore, improves angiogenesis and preserves the function of these organs.

Adrenomedullin has been shown to exert an endothelium-dependent vasodilation in cardiovascular

tissues from several animal species.⁵ The vasodilation effect is due to activation of phosphatidylinositol 3-kinase (PI3K) and Akt via the Ca^{2+} /calmodulin-dependent pathway, which leads to increased production of nitric oxide through phosphorylation of endothelial nitric oxide synthase.¹⁵ Adrenomedullin also promotes proliferation and migration of cultured vascular endothelial cells, resulting in vascular regeneration.¹⁶ Furthermore, adrenomedullin inhibits serum deprivation-induced apoptosis of cultured rat vascular endothelial cells,¹⁷ human umbilical vein endothelial cells¹⁸ and rat bone marrow-derived mononuclear cells.¹¹

The cell number increase and antiapoptotic effects of adrenomedullin are associated with several signaling pathways. Adrenomedullin activates the PI3K/Akt-dependent pathway in vascular endothelial cells.¹⁹ This pathway seems to regulate multiple critical steps in angiogenesis, including endothelial cell survival,

proliferation, migration, and capillary-like structure formation.²⁰ In cardiomyocyte, adrenomedullin inhibits apoptosis through a cAMP-dependent mechanism.²⁰ However, in vascular endothelial cells, cAMP-independent mechanisms, such as nitric oxide-dependent pathways, may be involved in the antiapoptotic effect of adrenomedullin.^{17,18}

LY294002, a PI3K inhibitor, inhibited the cell increase and the antiapoptotic actions of adrenomedullin. These results indicate that PI3K signaling pathway plays an important role in the apoptosis of EPCs derived from peripheral mononuclear cells.

An interesting observation from this study was that the cell number increase was the highest and the apoptosis rate was the lowest when adrenomedullin was at a concentration of 10^{-7} mol/L. At 10^{-6} mol/L, adrenomedullin did not result in further increases in the cell numbers or further reduction in the apoptosis rate. These results may indicate that concentrations higher than 10^{-7} mol/L may be toxic and offset the beneficial effects of adrenomedullin on the EPC numbers or apoptosis.

Conclusion

This study has demonstrated that adrenomedullin increases the cell numbers and suppresses the apoptosis of EPCs derived from peripheral mononuclear cells in patients with coronary artery disease. These actions on EPCs seem to be mediated by the PI3K signaling pathway. Further studies are required to investigate whether the adrenomedullin-induced cell increase and reduction in apoptosis can be translated into therapeutic effects.

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