Pituitary adenylate cyclase-activating polypeptide attenuates tumor necrosis factor-α-induced apoptosis in endothelial colony-forming cells

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Abstract. Endothelial colony-forming cells (ECFCs) are important in angiogenesis and vascular proliferation. Tumor necrosis factor (TNF)-α is a significant risk factor for the development of atherosclerosis and a key proinflammatory cytokine known to induce apoptosis in endothelial cells. Pituitary adenylate cyclase-activating polypeptide (PACAP) is one of the members of the vasoactive intestinal peptide/secretin/growth hormone-releasing hormone/glucagon superfamily and exists in two biological active forms, PACAP 38 and PACAP 27. PACAP has been reported to help prevent endothelial apoptosis via an anti-inflammatory mechanism. However, to the best of our knowledge, the anti-apoptotic potential of PACAP has not been investigated in ECFCs. The aim of the present study was to demonstrate the efficacy of PACAP for decreasing TNF-α-induced apoptosis in ECFCs. The results indicated that PACAP exerts a cytoprotective effect on ECFCs exposed to TNF-α. Furthermore, PACAP partially rescues the proliferation potential of ECFCs inhibited by prolonged TNF-α exposure. These findings support an anti-inflammatory role for PACAP in circulation diseases.

Introduction

Endothelial progenitor cells (EPCs) have been demonstrated as important in neovascularisation and contribute to vascular repair (1). Endothelial colony-forming cells (ECFCs) are a subset of EPCs, also referred to as late-outgrowth EPCs, which exhibit essential progenitor characteristics, including high proliferative potential and the capacity for self-renewal. ECFCs have received widespread attention as the primary progenitor population contributing to neovasculogenesis, in particular due to their reparative capacity to ameliorate vascular injury (2,3). Reduced numbers and altered function of ECFCs are associated with endothelial dysfunction under disease conditions, such as diabetic hyperglycemia and arterial hypertension (4-6). Furthermore, endothelial dysfunction is an early abnormality in the process that leads to atherosclerosis and its associated complications (7). Currently, the transfer of ECFCs is considered to be a therapeutic modality that is potentially effective in ischemia-associated conditions, such as myocardial infarction (8) and in non-ischemic inflammatory models, such as dilated cardiomyopathy (9). ECFC levels have been shown to be negatively correlated with risk factors for atherosclerosis (10), suggesting that decreased repair of vascular plaques may be the result of ECFC depletion or insufficient production (11). Repair of a damaged endothelium by ECFCs is likely critical in preventing or limiting blood vessel injury (12-14).

Evidence from *in vitro* and clinical studies indicates that inflammation and oxidative stress triggers ECFC apoptosis. Atherosclerosis develops through an inflammatory pathology, which results in plaque formation and acute coronary events. Tumor necrosis factor (TNF)-α is a proinflammatory cytokine that is central in the pathogenesis of atherosclerosis (15,16) and a contributing risk factor in metabolic disorders, such as insulin resistance and dyslipidemia. Primarily produced by activated macrophages (17), TNF-α regulates numerous immune cell functions, including rolling, adhesion, proliferation and apoptosis (18). Additionally, TNF-α is key as an inflammatory mediator and as an inducer of apoptosis in endothelial cells and ECFCs.

Pituitary adenylate cyclase-activating polypeptide (PACAP), encoded by the *ADCYAP1* gene, was originally isolated from an ovine hypothalamus extract (19), and has been identified in gastrointestinal, respiratory, cardiovascular

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and urogenital systems (20,21). PACAP is one of the members of the vasoactive intestinal peptide/secretin/growth hormone-releasing hormone/glucagon superfamily and exists in two biological active forms, PACAP 38 and PACAP 27, with three currently identified receptors: PAC1, VPAC1 and VPAC2. PACAP has been reported to promote survival in various types of cell, including lymphocytes, chondrocytes, endothelial cells and Schwann cells, as well as in liver, lung, and ovarian tissue samples (22,23). Adcyap1-deficient mice exhibited a markedly higher degree of, as well as more wide spread, inflammation (24), increased levels of proinflammatory cytokines, such as interleukin (IL)-6, TNF-α, interferon (IFN)-γ and decreased levels of IL-4 (25). Due to its broad distribution in tissues and its cytoprotective effects on many cell types, PACAP is considered to be an attractive therapeutic candidate possessing anti-inflammatory and anti-atherosclerotic potential.

Materials and methods

Materials. TNF-α was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Synthetic PACAP38 was obtained from GL Biochem (Shanghai) Ltd. (Shanghai, China). Cluster of differentiation CD34 (dilution, 1:200; cat. no. 561440), CD31 (dilution, 1:200; cat. no. 562861) and KDR (dilution, 1:400; cat. no. 560494) antibodies were obtained from BD Biosciences (San Jose, CA, USA). The secondary antibody (goat anti-rabbit HRP-IgG; cat. no. sc-2048) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The FITC Annexin V Apoptosis Detection kit was purchased from BD Pharmingen (San Diego, CA, USA) and endothelial differentiation medium (EGM-2MV) was obtained from Clonetics Corp. (San Diego, CA, USA). Lymphoprep™ was obtained from Axis-shield (Oslo, Norway). The human umbilical cord blood (UCB) was obtained from six patients of the First Affiliated Hospital of Jinan University (Guangzhou, China) between May 2014 to January 2015. Patients with known genetic diseases, cancer, a history of anemia, or medical conditions that involved cardiopulmonary insufficiency, were excluded. All subjects provided written informed consent and the present study was approved by the Regional Ethics Committee of Jinan University.

Cell culture. ECFCs derived from human UCB were isolated by Lymphoprep™ density gradient centrifugation at 800 x g for 20 min at 4˚C. Following centrifugation, the mononuclear cell (MNC) layer was harvested and washed twice with 0.9% saline. The MNCs were cultured at 37˚C in 5% CO_2 in endothelial differentiation medium (EGM-2MV) containing 5% fetal bovine serum, vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor and insulin-like growth factor-1. Approximately two days after the initial plating, non-adherent cells were discarded and fresh medium was applied; thereafter, the medium was replaced on alternating days. Subsequent to 1-2 weeks of culture, the cells exhibited a paving stone-like morphology and were identified as ECFCs by immunohistochemical staining. In order to evaluate the effect of TNF-α on ECFCs, ECFCs were treated with TNF-α (10, 20 and 40 ng/ml) for 24 h and the cleaved caspase-3 expression level was determined by western blot analysis. Subsequently, flow cytometric apoptosis assays were performed on the control group (cells were cultured in EGM-2MV), the TNF-α group (cells were cultured in EGM-2MV, pulsed with 20 ng/ml TNF-α) and 3 PACAP groups [the ECFCs were treated with 20 ng/ml TNF-α for 5 min and the medium was replaced with fresh medium, which contained PACAP (1, 10 and 100 nM)]. The MTT assay and flow cytometry were performed to evaluate cell proliferation and cell cycle.

ECFC immunohistochemistry. ECFCs were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) buffer (0.1 mmol/l) for 20 min. After blocking with 10% goat serum for 20 min, cells were incubated with anti-CD31 (1:200), -CD34 (1:200), or -KDR (1:400) antibodies overnight at 4˚C. Cells were washed three times with PBS, which was followed by application of the goat anti-rabbit IgG secondary antibody (1:500) with an avidin-peroxidase conjugate. Visualization was performed using a DAB chromogen kit (cat. no. ST033; Beyotime Institute of Biotechnology, Shanghai, China) and images were obtained by bright-field microscopy (IX71; Olympus Corporation, Tokyo, Japan).

Western blot analysis. ECFCs were homogenized in PBS containing a protease inhibitor cocktail (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 10% glycerol). Samples were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 30 V for 20 min. The membranes were blocked with 5% bovine serum albumin (w/v) at room temperature for 1 h, and incubated with primary anti-cleaved caspase 3 antibody (1:1,000; Abcam, Cambridge, MA, USA; cat. no. ab-2302) at 4˚C overnight, and then incubated with secondary antibody (1:3,000; Santa Cruz Biotechnology, Inc; cat. no. sc-2048) at room temperature for 1 h, developed with chemiluminescence ECL reagent (LumiGold; SignaGen Laboratories, Rockville, MD, USA) and exposed to Hyperfilm MP (Beyotime Institute of Biotechnology). The results of each time point in each group were normalized to GAPDH. The relative band intensities of the blots were determined using Adobe Photoshop software (Adobe Photoshop 6.0; Adobe, San Jose, CA, USA).

Apoptosis analysis. ECFCs were harvested, washed with ice-cold PBS, resuspended in binding buffer (500 μl), and incubated with propidium iodide (PI; 5 μl) and Annexin V-fluorescein isothiocyanate (5 μl) at 4˚C in the dark for 15 min. The cells were then washed and resuspended in PBS (500 μl) and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

MTT assay. ECFCs were seeded in 96-well plates (4,000 cells per well). At a series of time points (24, 48, 72 and 96 h) following treatment with TNF-α and/or PACAP, depending on

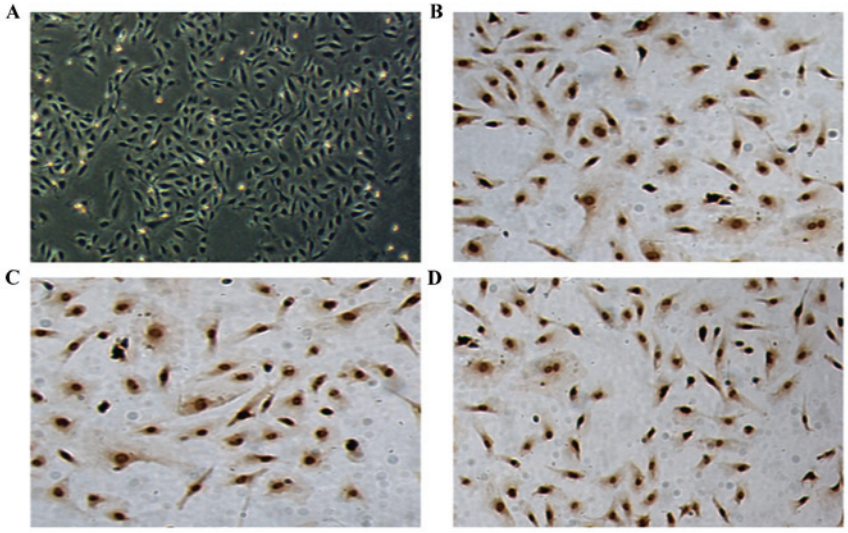

Figure 1. Cell morphology and identification of ECFCs. (A) Bright field microscopic images of cell morphology (magnification, x100). (B) CD31, (C) CD34 and (D) KDR staining as markers of ECFCs (magnification, x100). ECFC, endothelial colony-forming cells; CD, cluster of differentiation.

the group, the cells were incubated with MTT (5 mg/ml) in PBS for 3 h, and dissolved with 50% N,N dimethylformamide (Beyotime Institute of Biotechnology) and 10% SDS for 3 h at 37˚C. The optical density was measured at 570 nm. Data are representative of three biological replicates.

Cell cycle analysis. The cell cycle was determined using a Cell Cycle Assay kit from GenMed Scientifics Inc. (Shanghai, China). Briefly, cells were washed twice with PBS and fixed in 80% ethanol. The fixed cells were washed with PBS, incubated in PI (50 μg/ml) at room temperature for 20 min and immediately analyzed by flow cytometry according to the manufacturer's instructions.

Statistical analysis. An unpaired Student's t-test was used to evaluate statistical differences between the groups. Data are expressed as the mean ± standard deviation and $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 19 software (IBM SPSS, Armonk, NY, USA).

Results

Culture and identification of ECFCs. MNCs were isolated from human UCB and seeded in collagen-coated tissue culture plates in EGM-2MV media. Following ~1 week of culture, the cells exhibited a paving stone-like morphology (Fig. 1A). Immunocytochemical staining was positive for CD34 (dilution, 1:200), CD31 (dilution, 1:200) and KDR (dilution, 1:400; Fig. 1B-D), identifying the cells as ECFCs.

TNF-α induces ECFC apoptosis. TNF-α significantly increased the cleaved caspase-3 expression level in ECFCs in a concentration-dependent manner (Fig. 2A). In addition, a time course test of caspase-3 expression in ECFCs was performed by treating ECFCs with TNF-α (20 ng/ml) for 12, 24 and 36 h. The results indicated that TNF-α treatment significantly increased the cleaved caspase-3 expression level in a time-dependent manner (Fig. 2B).

PACAP inhibits TNF-α-induced apoptosis of ECFCs. Flow cytometric apoptosis assays indicated that PACAP significantly decreased the number of apoptotic cells in a concentration-dependent manner compared with the TNF-α (20 ng/ml) group. The higher concentrations of PACAP (10 and 100 nM) induced a statistically significant reduction in apoptotic cell number compared with the TNF-α (20 ng/ml) group ($P<0.05$ and $P<0.01$). The lower concentration of PACAP (1 nM) reduced the apoptotic cell number, but this was not statistically significant when compared with the TNF-α (20 ng/ml) group ($P=0.078$; Fig. 3A).

PACAP restored cell proliferation, which was reduced by TNF-α. The viability of ECFCs was evaluated by MTT assay. The proliferation of TNF-α (20 ng/ml) group was markedly inhibited compared with the control group ($P<0.01$). High concentrations of PACAP (10 and 100 nM) rescued cell proliferation when compared with the TNF-α (20 ng/ml) group ($P<0.01$). No significant differences were observed in cell proliferation between the TNF-α (20 ng/ml) group and the low concentration PACAP (1 nM) group (Fig. 3B).

Effect of PACAP on cell cycle progression. A dose-dependent effect of PACAP on the cell cycle was observed in the present study. The effect of PACAP on cells appears to be dose-dependent, as a higher dosage of PACAP resulted in a greater number of cells in the G2/M phase. After 48 h of 100 nM PACAP treatment, cells in the G2/M population increased from 8.27 to 10.75% compared with the TNF-α group, whereas the group of cells treated with 10 nM PACAP, the G2/M population increased to 10.17%. The increase of cell population at the G2/M phase was accompanied by a decrease of cell population in the G1 phase of the cell cycle (Fig. 3C).

Discussion

Increased expression levels of inflammation mediators have been implicated in numerous types of vascular disease and these mediators are known to negatively impact endothelial

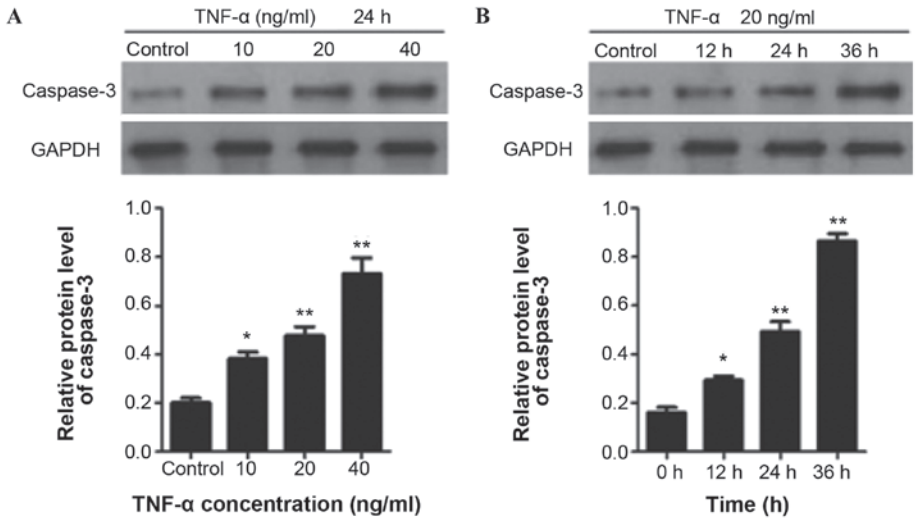

Figure 2. TNF-α increases cleaved caspase-3 expression levels in ECFCs in a concentration- and time-dependent manner. (A) Western blot analysis of cleaved caspase-3 expression levels in ECFCs treated with TNF-α at concentrations of 10, 20 and 40 ng/ml for 24 h. (B) Western blot analysis of cleaved caspase-3 levels in ECFCs treated with TNF-α (20 ng/ml) for 12, 24 and 36 h. Values in the bar charts are presented as means ± standard deviation. *P<0.05 and **P<0.01 vs. Control. TNF-α, tumor necrosis factor-α; ECFC, endothelial colony-forming cell.

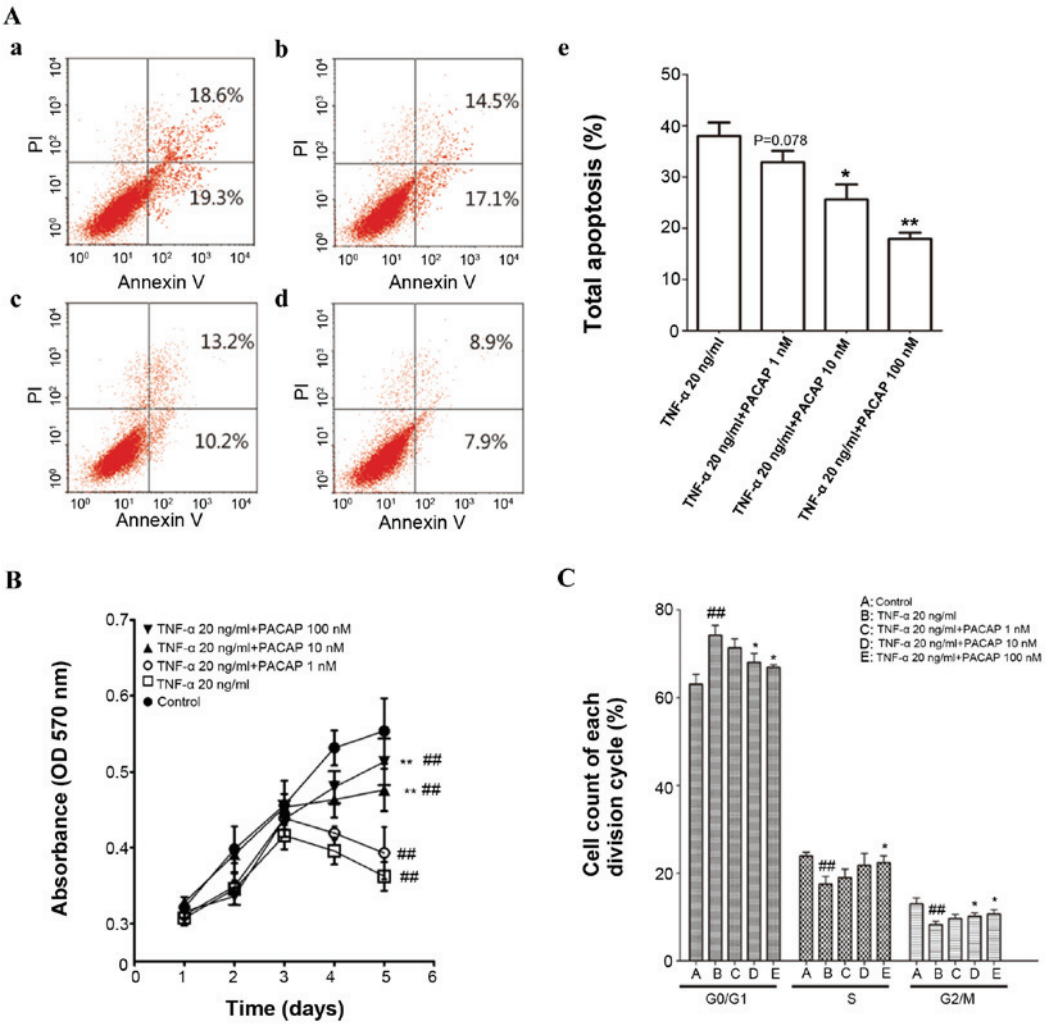

Figure 3. PACAP restores TNF-α-induced apoptosis in ECFCs. (A) Effect of TNF-α and PACAP on apoptosis of ECFCs. The cell populations in early and late apoptosis were determined using flow cytometry: (Aa) 20 ng/ml TNF-α; (Ab) 20 ng/ml TNF-α + 1 nM PACAP; (Ac) 20 ng/ml TNF-α + 10 nM PACAP; (Ad) 20 ng/ml TNF-α + 100 nM PACAP; (Ae) Bar chart of ECFC apoptosis. (B) Cell proliferation was evaluated by MTT assay. PACAP partially restored proliferation of TNF-α-treated ECFCs. (C) Proportion of cells in each cell cycle phase determined by flow cytometry. $^{\#\#}$P<0.01 vs. Control group; *P<0.05 and **P<0.01 vs. 20 ng/ml TNF-α group. PACAP, pituitary adenylate cyclase-activating polypeptide; TNF-α, tumor necrosis factor-α; ECFC, endothelial colony-forming cell; PI, propidium iodide.

cell function in atherosclerosis. Cytoprotective agents present as a promising preventative therapeutic strategy for these conditions, as they negate the apoptotic effects of inflammation. During the pathological progression of atherosclerosis, monocytes and T-lymphocytes are recruited to blood vessel walls and release chemokines, such as monocyte chemoattractant protein-1, TNF-α and IL-6, which are involved in maintaining the inflammatory process. ECFCs are recruited from the bone marrow to these sites of inflammatory signals. Previous studies indicated that acute exposure to low concentrations of TNF-α increases the adhesive properties of ECFCs to vascular endothelial cells (19), although chronic inflammatory stimulation induces ECFC apoptosis (26,27).

PACAP has been shown to limit the effects of chronic inflammation in rheumatoid arthritis and osteoarthritis (28,29). Furthermore, there is strong evidence that PACAP is involved in repair of nerve injury and damage induced by inflammation and oxidative stress (30-32). In addition, our previous studies demonstrated a role for PACAP in atherosclerosis in rabbits and identified receptors for PACAP in cardiovascular tissue (33-35). As a powerful stimulator of inflammation, TNF-α has been reported to induce apoptosis and senescence of ECFCs or other stem cells *in vitro* and *in vivo* (36-40); however, to the best of our knowledge, no previous studies have linked PACAP to attenuation of TNF-α-induced apoptosis in ECFCs. Caspase-3 is activated in the apoptotic cell by extrinsic and intrinsic signaling pathways (41). A positive correlation was identified between TNF-α and cleaved caspase-3 expression levels in ECFCs in the present study, indicating that TNF-α-mediated inflammation induced ECFC apoptosis. Furthermore, PACAP was shown to exert a direct anti-apoptotic effect on ECFCs treated with TNF-α and enhance proliferation of ECFCs that are inhibited by TNF-α. It has been reported that low concentrations of PACAP cross the blood-brain barrier and may stimulate neurons directly, as well as stimulate astrocytes and microglia to secrete neuroprotective factors (42). During the process of cytoprotection, PACAP may regulate the dynamic balance between the growth factor-activated extracellular signal-regulated kinases and stress-activated c-Jun N-terminal kinases-p38 signaling pathways in neuronal systems (43). However, the anti-apoptosis mechanism of PACAP has not yet been elucidated in ECFCs. Further studies are required to identify the molecular pathways mediating the effects of PACAP and identify the PACAP receptors in ECFCs.

In conclusion, the present results indicate that TNF-α induced apoptosis in ECFCs in a concentration- and time-dependent manner; however, PACAP partially blocks the negative effects of prolonged TNF-α exposure, including ECFC apoptosis, growth inhibition and cell cycle distribution, indicating its therapeutic potential for the treatment of circulatory diseases.

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