Original Article
The overexpression of uPA promotes the proliferation and fibrinolytic activity of human umbilical vein endothelial cells

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Abstract: The purpose of this article is to study whether the overexpression of urokinase-type plasminogen activator (uPA) can promote the proliferation and fibrinolytic activity of human umbilical vein endothelial cells (HUVECs). The recombinant adenovirus vectors containing the human uPA gene were constructed and transfected into HUVECs. In this study, the mRNA of uPA was detected by qPCR, and the uPA protein was measured by Western blot. The cell proliferation was measured using MTT. The fibrinolytic activity of uPA was quantified using a colorimetric assay. We also measured MMP2 (metalloproteinase-2), MMP9 (metalloproteinase-9), and VEGF (vascular endothelial growth factor) proteins using ELISA. The results showed that the levels of the uPA mRNA and the protein in the overexpression group were significantly higher compared to the other groups, (P < 0.05). The cell proliferation and uPA activity were increased significantly in the overexpression group, compared to the other groups, (P < 0.05). The secretions of MMP2, MMP9, and VEGF in the overexpression group were significantly higher than they were in the other two groups (P < 0.05). In conclusion, we successfully transfected a recombined adenovirus vector carrying uPA into a HUVEC. The exogenous uPA gene could transcribe and secrete the uPA protein in the HUVECs. The overexpression of uPA can increase cell proliferation and uPA activity. It can improve the invasion and angiogenesis ability in HUVECs by promoting their secretions of MMP2, MMP9, and VEGF.

Keywords: Overexpression, uPA, proliferation, HUVEC, MMP2, MMP9, VEGF

Introduction

Deep venous thrombosis (DVT) is a serious cardiovascular disease. Without proper treatment, DVT can easily develop into post-thrombotic syndrome, femoral bruising, femoral white swelling, and even death due to pulmonary embolism [1]. Every year, millions of people suffer from DVT, and the incidence of DVT is getting higher and higher [2]. At present, the treatment for DVT in the lower extremities is mainly anticoagulant therapy, thrombolytic therapy, interventional therapy, surgical thrombectomy and so on, but these treatments are risky, expensive, and have many disadvantages.

Therefore, it is of great significance to find a new treatment method, which enables the body itself to produce cytokines that respond to venous thrombosis, that is, to ablate the thrombus, to promote the remodeling of venous wall fibrosis, and not to cause significant negative effects on other tissues of the body.

UPA is a second generation thrombolytic agent and belongs to the serine proteases. It has two forms: one is an inactive single-chain structure, the other binds to urokinase-type plasminogen activator receptors (uPAR), enzymatic hydrolysis of membrane-bound plasminogen and other proteases and produces an active double-chain form, which does not require fibrin as a cofactor and can directly activate plasminogen to become plasmin [3-10]. Plasmin can degrade fibrin clot proteolytic enzymes, which is an important component of the fibrinolytic system. Once
the body produces a coagulation reaction, it activates the fibrinolytic system and removes excess thrombus [11]. UPA synthesis has been used in thrombolytic therapy in vitro, such as with acute myocardial infarction, cerebral embolism, peripheral arteriovenous thrombosis, etc.

However, there are few studies on promoting uPA secretion by human vein endothelial cells for thrombolytic therapy in vivo. Therefore, we transfected the adenovirus carrying the uPA gene into human umbilical vein endothelial cells and measure the secretion of uPA, so as to lay a foundation for the later thrombosis treatment of uPA in vivo.

**Materials and methods**

**Cell lines, cell cultures and reagents**

HUVECs were obtained from the American Type Culture Collection (Manassas, VA). Lipofectamine 2000, Rabbit monoclonal anti-human antibodies against uPA, and β-actin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). VEGF, MMP2, and MMP9 were purchased from Boster Biological Technology., LTD (Wuhan, China). LightCycler®96 Real-time Fluorescence Quantitative PCR (qPCR) is manufactured by the Roche Company in Switzerland.

**Construction of recombinant adenovirus vectors**

The recombinant adenovirus vectors containing exogenous uPA gene were designed and synthesized by the Wuhan Cell Marker Biotechnology Co., Ltd. in Wuhan, China. The genespecific uPA primer pair was as follows: Forward, 5'-CAAGCTTGGCCACCATGAGAGCCCTGGCG-3' and reverse, 5'-CAAGCTTCCCGAGGGCCAGC-3' (sense) and 5'-GTAGACGCGCTGGCTTGTCT-3' (antisense); In each reaction, 10 µl SYBR® Premix Ex Taq™, 0.4 µM reverse and forward, 1 µl cDNA in a total volume of 20 µl were used. The standard 2^ΔΔCT method was used to calculate the results.

**Western blot analysis**

A Western blot analysis was performed with reference to the reported literature [15]. In short, RIPA containing protease inhibitors and phosphatase inhibitors cleaved human umbilical vein endothelial cells, 10% SDS-PAGE electrophoresis, then the cells were transferred to a PVDF membrane, 3% BSA blocked 1 hour later, then the membrane was incubated with Rabbit monoclonal anti-human antibodies (uPA or β-actin 1:1000) overnight, After washing with TBST for 1 hour, we added goat anti-rabbit secondary antibody (1:8000) for 1 h, followed by a TBST wash, luminescent solution, exposure imaging, and β-actin was used as a protein load measurement.

**MTT assay**

MTT was performed with reference to the reported literature [16]. In short, HUVEC was put into 96-well plates the night before transfection. The density of the plates was 2000 cells/well, and we added a 100 μL medium containing 10% FBS into each well. The next day, four 96-well plates were transfected with the cells.
uPA in HUVEC

Then one plate was taken out after being transfected for 24 h, 48 h, 72 h, 96 h, and then 20 μL MTT (5 mg/ml) was added into each well. After 4 h, 150 μL/well of dimethyl sulfoxide (DMSO) was added to the culture solution, the wells were shaken for 10 min, and the optical density (OD) of the sample was measured on a microplate spectrophotometer (Thermo Multiscan MK3, USA) at 490 nm.

Statistical analysis

The experimental results were expressed as the mean ± the standard error of the mean (S.E.M). SPSS 18.0 was used for the statistical analysis. The statistical analyses were performed with one-way ANOVA and Student's t-test. Differences of data were considered statistically significant when a P-value < 0.05.

Results

The recombined adenovirus vector carrying uPA was successfully transfected into HUVECs

The recombined adenovirus vector carried the enhanced green fluorescent protein (EGFP) gene, which could be seen under a fluorescence microscope after it was transfected. Most of the cells in the overexpression group and the empty vector group showed green fluorescence, but no green fluorescence was observed in the blank control group after 48 h of transfection (MOI 50). It is suggested that the transfection was successful, and the transfection efficiency was about 85±2.6% (Figure 1).

The overexpression of uPA promoted uPA mRNA expression in the HUVECs

The expressions of the uPA mRNA were measured using a qPCR analysis after the cells were transfected for 48 hours. The expression of uPA mRNA in the overexpression group was significantly higher than the expression of the blank control and empty vector groups (P < 0.05), but there was no significant difference between the blank control group and the empty vector group (P > 0.05, Figure 2A).

The overexpression of uPA promoted uPA protein expression in the HUVECs

The expressions of the uPA protein were measured using Western blot analysis after the cells were transfected for 48 hours. The expression of uPA protein in the HUVECs in the overexpression group was significantly higher than in the blank control group and the empty vector group (P < 0.05). However, there was no significant difference
between the blank control group and empty vector group (P > 0.05, Figure 2B and 2C).

The overexpression of uPA promoted proliferation in the HUVECs

The MTT results showed that the proliferation of HUVECs in the overexpression group was significantly higher than it was in the empty vector and blank control groups after the cells were transfected 48 hours. (P < 0.05), but there was no significant difference between the blank control group and the empty vector group (P > 0.05, Figure 3).

The overexpression of uPA promoted the secretions of MMP2, MMP9 and VEGF in the HUVECs

The secretions of MMP2, MMP9, and VEGF were detected by ELISA after the cells were transfected for 48 hours. The secretions of MMP2, MMP9, and VEGF in the overexpression group were significantly higher than they were...
The mechanism of DVT is complex, and the important reason behind DVT’s complexity is the injury of the vascular wall. Vein endothelial cells, as the main component of the vascular wall, can regulate the relationship between blood and blood vessels by providing physical barriers on the one hand, and by playing an anti-thrombotic role by secreting fibrinolytic cytokines (such as uPA) [17]. Moreover, uPA, when combined with uPAR in vivo, can activate the signal transduction pathways that mediate various cellular physiological processes and participate in cell migration, adhesion, proliferation, and differentiation [18]. UPA is one of the most suitable thrombolytic agents in vivo. Gene-level therapy for venous thrombosis has advantages that traditional therapy does not have. It can promote the secretion of endogenous uPA by its own tissue, leading to thrombolytic ablative. In the treatment of venous thrombosis, it is necessary not only to restore the patency of blood vessels, but also to restore the endothelial cell intima of the venous wall [13]. Vascular endothelial growth factor (VEGF) is a growth factor that can specifically act on vascular endothelial cells and is a functional protein that promotes vascular endothelial cell proliferation [19, 20]. It is involved in thromboembolization and in the intimal repair of venous thrombosis [21].

Stepanova V studies have shown that uPA promotes the secretion of VEGF [22]. Waltham et al. transfected the expression plasmid containing the vascular endothelial growth factor gene into the animal model of inferior vena cava thrombosis and established a model of vascular recanalization after embolization [23]. Matrix metalloproteinases (MMPs) promote cell invasion by degrading the extracellular matrix. MMP 2 and MMP9 are important members of its family and promote cell invasion and metastasis [24, 25]. Studies have shown that uPA can promote cell invasion and angiogenesis by degrading the extracellular matrix and basement membrane components, such as fibronectin, laminin and collagen, when tissue secretes a
large amount of uPA [26-29]. Our study showed that the secretions of MMP2, MMP9 and VEGF in the overexpression group were significantly higher than those in the empty vector group (P < 0.05). These results suggest that the overexpression of uPA can improve the invasion and angiogenesis abilities in HUVECs by promoting their secretions of MMP2, MMP9, and VEGF.

Conclusion

We recombined an adenovirus vector carrying uPA and successfully transfected it into HUVECs. The exogenous uPA gene can transcribe and secrete the uPA protein in HUVECs. The overexpression of uPA can increase cell proliferation and uPA activity. It can improve the invasion and angiogenesis abilities in HUVECs by promoting their secretions of MMP2, MMP9, and VEGF. However, the thrombolytic process is very complex in vivo. We have not carried out animal experiments, and the specific effect is not clear. We will further study the thrombolytic effect on animal models.

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Disclosure of conflict of interest

None.

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