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Research Article

Protein Expression of Mesenchymal Stem Cells after Transfection of pcDNA3.1^{-}-hVEGF$_{165}$ by Ultrasound-Targeted Microbubble Destruction

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Ultrasound-targeted microbubble destruction (UTMD) has been proposed as a new technique for organ-specific gene transfer and drug delivery. This study was performed to investigate the effect of UTMD on marrow mesenchymal stem cells (MSCs) transfected with pcDNA3.1^{-}-hVEGF$_{165}$.pcDNA3.1^{-}-hVEGF$_{165}$ were transfected into the third passage of MSCs, with or without UTMD under different ultrasound conditions. Protein expression was quantified by hVEGF$_{165}$-ELISA kit after transfection for 24, 48, and 72 hours. UTMD-mediated transfection of MSCs yielded a significant protein expression. UTMD of mechanic index (MI) 0.6 for 90 seconds led to the highest level of protein expression.

1. Introduction

Heart disease currently remains the leading cause of death worldwide. With the development of tissue engineering, stem cell technology has been widely used and highlights the latest advances in these exciting fields [1]. Mesenchymal stem cells (MSCs) have demonstrated the ability to differentiate into cardiomyocytes, but are still limited to construct the vessels [2, 3]. Vascular endothelial growth factor (VEGF) could induce vascular endothelial cell proliferation and angiogenesis [4]. Because of its short half-life, VEGF could not maintain effective concentration in blood after injection [5]. In recent years, Ultrasound-targeted microbubble destruction (UTMD) has been proved to be a promising technique for organ-specific gene and drug delivery [6]. In this experiment, we transferred pcDNA3.1^{-}-hVEGF$_{165}$ into MSCs by UTMD and observed the effect of the protein expression.

2. Materials and Methods

2.1. Separation and Cultivation of MSCs. Our experiment was performed in the Clinical Research Center, the Second Affiliated Hospital, School of Medicine, Zhejiang University, China. Five male Sprague-Dawley rats, weighing 80–100 g, were provided by the animal center of Zhejiang University. All experiments have adhered to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications no. 8023, revised 1978). Approval from the Institutional Animal Care and Use Committee at Zhejiang University Health Science Centre was also obtained to perform the described experiments. MSCs were harvested from the bone marrow of femurs of these rats. Briefly, bone marrow cells were flushed out with 30 mL complete Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 5 mg/mL glutamine (Gibco, USA), 100 U/mL penicillin (Gibco, USA), and 100 U/mL streptomycin (Gibco, USA). The cells were grown in a humidified atmosphere containing 5% CO_2 and 95% O_2. The medium was replaced 24 hours later and refreshed every 2 days. Cells were subcultured according to 1 : 2 ratio when they reached approximately 80% confluence by trypsinization (0.25% trypsin, Gibco, USA). The third passage of MSCs was adopted for transfection.

2.2. Recombinant pcDNA3.1$^-$-hVEGF$_{165}$ Gene Transfer into MSCs by UTMD. The third passage of MSCs were planted into three 6-well plate (Becton Dickinson, USA) at 1.0×10^5 cells/per well and cultured for 24 hours in 37°C, 5% CO_2 conditions. Before transfection, 5 mL normal saline were added to the microbubble contrast agent SonoVue (Bracco, Italy) powder (25 mg) and thoroughly mixed for 20 seconds. 4 μg/per well pcDNA3.1$^-$-hVEGF$_{165}$ recombinant (Future Biotech, China) were mixed with 10 mL lipofectamin 2000 transfection reagent (Invitrogen, USA) for 20 s [7].

In this study, all the cells were divided intothe following five groups:

(1) the blank control group: MSCs with culture fluid,

(2) the control group A: 4 μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant were transfected into MSCs,

(3) the control group B: 4 μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant mixed with 300 μl SonoVue microbubble were transfected into MSCs,

(4) the control group C: 4 μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant were transfected into MSCs by ultrasonic exposure (illustrated by the example of mechanic index (MI) 1.0 and exposure time (ET) 60 s),

(5) the UTMD group: 4 μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant were transfected into MSCs by UTMD (MI 1.0, ET 60 s).

The UTMD group was also divided into three groups according to different MI and ET.

Ultrasound-targeted microbubble was ruptured as following: Acuson Sequoia 512 ultrasound's 3V2C transducer (Siemens, German) was placed on the bottom of each well plate according to the preset ultrasonic exposure condition. The ultrasound parameters were set as follows: the frequency was 4 MHz, the depth was 4 cm, MI was 0.6, 1.0, and 1.4 respectively, and ET was 30 s, 60 s, and 90 s, respectively.

MSCs cultural supernatant was collected after transfection for 24, 48, and 72 hours, respectively. Five samples were applied in each group.

2.3. Detection of VEGF$_{165}$ Protein Expression after Transfection by ELISA Quantitative Assay. hVEGF$_{165}$-ELISA kit (Jingmei, China) was used to determine VEGF$_{165}$ protein expression after transfection for 24, 48, and 72 hours according to the instructions.This was repeated five times in this experiment.

2.4. Statistical Analysis. All the parameters were expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA), followed by a LSD (least significant difference) test was used to compare VEGF$_{165}$ protein expression among different groups. All analyses were performed using SPSS statistical software, version 13.0 (SPSS, Inc., USA). A two-sided $P < .05$ was considered statistically significant.

Table 1: Protein expression of VEGF$_{165}$ in mesenchymal stem cells supernatant after transfection ($n = 25$, ng/mL).

Groups	Protein expression of VEGF$_{165}$		
	24 h	48 h	72 h
(1) The blank control group	12.5 ± 1.8	12.1 ± 0.6	11.8 ± 0.1
(2) The control group A	73.1 ± 0.4	74.0 ± 1.2	70.4 ± 1.0
(3) The control group B	67.3 ± 2.1	79.4 ± 0.8	74.1 ± 1.5
(4) The control group C	63.7 ± 2.6	82.1 ± 1.7	76.3 ± 1.3
(5) The UTMD group	218.6 ± 0.9*	269.2 ± 2.2*	199.4 ± 2.1*

* $P < .05$, versus each other non-UTMD groups.
(1) The blank control group: MSCs with culture fluid.
(2) The control group A: 4 μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant were transfected into MSCs.
(3) The control group B: 4 μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant mixed with 300 μl SonoVue microbubble were transfected into MSCs.
(4) The control group C: 4 μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant were transfected into MSCs by ultrasonic exposure (illustrated by the example of Mechanic index (MI) 1.0 and exposure time (ET) 60 s).
(5) The UTMD group: 4μg pcDNA3.1$^-$-hVEGF$_{165}$ recombinant were transfected into MSCs by UTMD (MI 1.0, ET 60 s).

3. Results

The results showed that the VEGF$_{165}$ protein expression increased at 24 hours and reached the maximum level at 48 hours, then decreased at 72 hours (Table 1). Compared with the control group, protein expression of the UTMD group was significantly increased ($P < .05$).

Table 2 also demonstrated that VEGF$_{165}$ protein level varied according to different ultrasound conditions. The group with ET 90 s and MI 0.6 showed the highest protein level at 48 hours, which has statistical significance compared with every group with ET 30 s and MI 0.6, 1.0, and 1.4, respectively ($P < .05$).

4. Discussion

The lack of suitable autologous grafts has produced a need for artificial grafts, but the patency of such grafts is limited compared to natural materials. Tissue engineering, whereby living tissue replacements can be constructed, has emerged as a solution to some of these difficulties [8]. MSCs have demonstrated the ability to differentiate into cardiomyocytes, This, in turn, is limited by the availability of MSCs to construct the vessels [9].

VEGF, a class of molecular weight of 34∼45 KD glycoprotein, could induce vascular endothelial cell proliferation and angiogenesis. VEGF$_{165}$ protein-induced differentiation of MSCs directional vascular endothelial cells plays a vital role in neovascularization of ischemic tissues [10, 11]. However, because of its short half-life, VEGF could not maintain

Table 2: Protein expression of $VEGF_{165}$ in mesenchymal stem cells supernatant after transfection under different ultrasound conditions ($n = 25$, ng/mL).

Various ultrasound conditions		Protein expression of $VEGF_{165}$		
ET	MI	24 h	48 h	72 h
30 s	0.6	118.2 ± 0.7	133.1 ± 0.3	112.7 ± 0.8
	1.0	140.5 ± 1.1	142.0 ± 0.5	131.5 ± 0.1
	1.4	136.6 ± 0.7	154.1 ± 1.1	121.8 ± 0.9
60 s	0.6	177.6 ± 1.2	168.8 ± 2.3	159.1 ± 0.8
	1.0	218.6 ± 0.9	269.2 ± 1.2	199.4 ± 2.1
	1.4	254.6 ± 0.7	289.6 ± 3.6	249.1 ± 0.8
90 s	0.6	289.9 ± 1.5*	319.1 ± 2.1*	268.7 ± 1.4*
	1.0	161.2 ± 1.8	186.5 ± 0.8	151.6 ± 1.3
	1.4	160 .0 ± 3.5	175.2 ± 1.6	148.2 ± 2.5

* $P < .05$, versus groups with ET 30 s and MI 0.6, 1.0, and 1.4, respectively.

effective concentration in blood after injection because of rapid degradation of deoxyribonucleic acid (DNA) [12–14]. Thus, intravenous injection of plasmid DNA does not lead to detectable transfection [15]. In the present study, UTMD, a promising technique for organ-specific gene and drug delivery, was tried aiming to transfer VEGF into MSCs efficiently.

UTMD has evolved as a promising tool for organ-specific gene and drug delivery [16]. This technique has initially been developed as a method in myocardial contrast echocardiography, destroying intramyocardial microbubbles to characterize refill kinetics. When loading similar microbubbles with a bioactive substance, ultrasonic destruction of microbubbles may release the transported substance in the targeted organ [17]. Furthermore, high-amplitude oscillations of microbubbles increased capillary and cell membrane permeability and facilitated tissue and cell penetration of the released substance [18–20].

As the target cell of gene transfer, MSCs could promote expression of VEGF protein and vascularization of tissue engineering bone by transfected $VEGF_{165}$. $VEGF_{165}$ was a kind of secretary protein, whether the transfected gene could express effectively was the critical point of the present experiment.

Table 1 showed that $VEGF_{165}$ protein production increased after MSCs was transfected with $VEGF_{165}$ by UTMD. The $VEGF_{165}$ protein expression reached maximum at 48 hours and decreased later, which had statistical significance compared with all other non-UTMD group at all set moments ($P < .05$). It could be explained by three mechanisms: firstly, electron microscopy has demonstrated pore formation on cell membranes immediately after destruction of microbubbles, the pores are transient and disappeared after 24 hours [21]. Such "sonoporation" effects may help facilitating gene or drug entry into the cell. Studies on single bubbles in vitro have shown that even linear bubble oscillations are sufficient to achieve rupture of lipid membranes [22]. Secondly, sudden violent collapse of microbubbles (inertial cavitation) can produce high-velocity fluid microjets that may penetrate adjacent membranes [23]. Thirdly, inertial cavitation, which is dependent on microbubble shell composition, ultrasound frequency, pulse duration, and acoustic power, can lead to secondary shock waves, transient local high temperatures, and shear stress, all of which could potentially contribute to gene or drug delivery by UTMD [24, 25].

Table 2 showed that $VEGF_{165}$ protein level changed under different ultrasound conditions. The group with UTMD of MI 0.6 for 90 s showed the highest peak protein level at 48 hours, which has statistical significance compared with other groups with ET 30 s. Studies have confirmed that the disruption force of microbubbles is greater when the ultrasound frequency used matches the resonant frequency of microbubbles. Even low acoustic pressures can result in microbubble destruction, but higher pressures will lead to more forceful reactions [26]. However, too higher acoustic pressure will hurt the cells, this is why the $VEGF_{165}$ protein level of groups with MI 1.4, ET 90 s was lower in this study.

5. Limitations

The first limitation of this present study is that the number of samples is small. However, even with this small number of samples, we were able to reach our primary goal of investigating the protein expression of UTMD on MSCs transfected with pcDNA3.1^{-}-h$VEGF_{165}$. Secondly, the cell proliferation and angiogenesis of transfected MSCs by UTMD will not be traced, which is very important for tissue engineering. Thirdly, this study is limited in vitro. So further investigation, especially in larger animal models, is needed.

6. Conclusion

UTMD-mediated transfection of MSCs yielded a significant protein expression. UTMD of mechanic index (MI) 0.6 for 90 seconds led to the highest level of protein expression.

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