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Proliferation of endothelial cell on polytetrafluoroethylene vascular graft materials carried VEGF gene plasmid*

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Abstract: Objective: To investigate whether vascular endothelial growth factor (VEGF) gene plasmid carried by polytetrafluoroethylene (PTFE) vascular graft materials could transfect endothelial cells (ECs) and promote their growth. Methods: PTFE vascular graft materials carried with pCDI-hVEGF$_{121}$, pCDI or pEGFP were incubated in Tris-buffer solution and the values of optical density of 260 nm at different time were plotted, then the DNA controlled release curve was made. ECs derived from human umbilical vein were seeded on the pCDI-hVEGF$_{121}$/pCDI/pEGFP-PTFE materials or tissue culture plates, ECs numbers were counted and VEGF protein concentrations at different time were measured by enzyme-linked immunoadsorbent assay method. Green fluorescent protein (GFP) expression in ECs on pEGFP-PTFE materials was examined with fluorescence microscopy. Results: The controlled release curve showed that the gene released from PTFE materials was rapid within 8 h, then slowed down and that the gene released continuously even after 72 h. At 24, 72 and 120 h, ECs number and proliferation rate of pCDI-hVEGF$_{121}$-PTFE materials were higher than those of pCDI or pEGFP-PTFE materials (P<0.05). VEGF protein concentration of pCDI-hVEGF$_{121}$-PTFE materials was higher than that of pCDI or pEGFP-PTFE materials at 6, 24, 72 and 120 h (P<0.01). GFP expression in ECs on the pEGFP-PTFE materials could be detected by fluorescence microscopy. Conclusion: PTFE graft can be used as a carrier of VEGF gene plasmid, VEGF gene carried by PTFE can transfect ECs and promote ECs growth.

Key words: Polytetrafluoroethylene, Vascular endothelial growth factor, Vascular grafts, Gene, Endothelial cell
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INTRODUCTION

Synthetic biomaterials have been widely employed as vascular grafts in clinical practice over the past 30 years (Stansby *et al*., 1994), but none of the currently available small-caliber grafts (≤6 mm ID) have acceptable long-term patency (Anderson *et al*., 1987). The major cause of grafts failure is the inherent thrombogenicity of the synthetic graft surface and intima hyperplasia which leads to progressive occlusion and closure of the graft (Callow, 1982).

It is considered that the lack of endothelium is critical to both thrombosis and intima hyperplasia (Yu *et al*., 2003). The seeding of endothelial cells on synthetic grafts was studied to find ways to reduce surface thrombogenicity and increase the long-term patency of synthetic grafts (Herring *et al*., 1978; Eickhoff *et al*., 1987). Unfortunately, although improved patency had been reported in animal models (Pearce *et al*., 1987; Shindo *et al*., 1987), human clinical studies yielded mixed results (Jensen *et al*., 1994; Herring *et al*., 1994; Deutsch *et al*., 1999).

In the recent years, great progress has been made in gene therapy for vascular diseases. Some investigators began to use gene to modify the artificial blood

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vessel with the purpose of promoting endothelium growth. Several earlier studies demonstrated that the transfection of some growth stimulation and thrombolytic genes into endothelial cells could improve cellular adhesion, proliferation and enhance fibrinolytic capability in culture when cells were seeded onto prosthetic grafts (van Belle *et al*., 1997; Grandas *et al*., 2001; Pels *et al*., 2003; Yu *et al*., 2005). Although these methods were effective in inhibiting thrombosis, restenosis and increasing patency of synthetic grafts, the procedures including cell harvest and culture, gene transfer, cell seeding and graft implantation are quite complicated and expensive. Furthermore, several weeks are needed to implement the procedures and absolute caution is needed.

Polytetrafluoroethylene (PTFE) is a porous hydrophobic polymer with 10 μm to 90 μm inter pore mean diameter (Campbell *et al*., 1979; Anderson *et al*., 1987). These space of pores should be suitable for gene plasmid storage. However, there is no data available in the literature regarding PTFE as a gene carrier for improving endothelialization. The use of PTFE vascular grafts as a carrier for vascular endothelial growth factor (VEGF) gene may be effective. The localized delivery of VEGF gene into implanted vascular and muscular tissue could result in predominant superior expression of VEGF protein and improve the endothelialization of the graft.

In the present study, VEGF plasmid DNA was impregnated into a porous PTFE material. The kinetics of DNA release from the impregnated PTFE material was evaluated. The transfection efficiency of plasmid DNA carried by PTFE in cultured human umbilical vein endothelial cells (HUVECs) and the growth and proliferation of HUVECs on VEGF gene impregnated PTFE material were further investigated.

MATERIALS AND METHODS

Plasmid preparation

The vector pCDI-hVEGF$_{121}$ was kindly provided by Peking University Center for Human Disease Genomics (China), and consisted of human VEGF$_{121}$ complementary deoxyribonucleic acid (cDNA) full-length sequence and highly efficient eukaryotic expression plasmid pCDI. The vector pEGFP, coding for enhanced green fluorescence protein, was purchased from Clontech Corporation (Clontech, USA). These plasmids were amplified in *E. coli* DH5a, and retrieved and purified using QIAGEN Plasmid Maxi Kit (QIAGEN, Germany) according to the manufacturers' instructions.

Preparation of PTFE vascular grafts carried with plasmid DNA

pCDI-hVEGF$_{121}$, pCDI and pEGFP were mixed with fibronectin (Sigma, USA) in TBS (10 mmol/L Tris/HCl pH 7.4, 150 mmol/L NaCl) respectively. The final concentration of each plasmid and fibronectin was 1 mg/ml. These three solutions were injected into 4 mm-diameter ePTFE grafts respectively. Two ends of each graft were clipped, then the graft was pressed until the external walls appeared as liquid drops in that plasmids solution impregnated through the wall and entered into the fine pores of the grafts. These grafts were dried and cut into small disks (6.5 mm in diameter). As control, one graft was provided only with fibronectin TBS without any plasmid. The PTFE disks were named pCDI-hVEGF$_{121}$-PTFE disks, pCDI-PTFE disks, pEGFP-PTFE disks, and fibronectin-PTFE disks according to the different substances they carried.

Plasmid DNA release in vitro

PTFE disks carrying pCDI-hVEGF$_{121}$, pCDI or pEGFP (5 samples per plasmid) were incubated in 1 ml TBS separately on rocking bed at 37 °C. Two microlitres solution was taken out from each sample at 0.5, 1, 2, 4, 8, 24, 48, 72, 120, 168 h. The concentrations of DNA released in the solution at different time were measured spectrophotometrically at 260 nm. The value of optical density was converted into DNA concentration. The controlled release curve was made using those results.

HUVEC culture

HUVECs were harvested from freshly obtained human umbilical cord using the collagenase isolation technique described by Jaffe *et al*.(1973) and Marin *et al*.(2001) with slight modification. Briefly, the umbilical vein was rinsed with phosphate buffer solution (PBS, pH 7.4), filled with 0.1% collagenase I (Sigma, USA) and incubated for 15 min at 37 °C. After harvesting, cells were placed in 25 cm^2 tissue culture flasks (Falcon, USA). The complete growth medium

consisted of 80% (v/v) MCDB131 medium and 20% (v/v) fetal bovine serum, supplemented with 100 U/ml penicillin, 100 mg/L streptomycin, 4 mmol/L L-glutamine (above substances were all purchased form GibcoBRL, USA), 50 mg/L endothelial cell growth supplement (ECGS, Sigma, USA) and 100 mg/L heparin (Sigma, USA). The cells were cultured in the medium at 37 °C in an atmosphere with 5% CO_2. The medium was firstly changed after 8 h and then every 2 d until the cells were confluent. At confluence, cells' passage referred to traditional trypsin-EDTA method. In this study, passage 1-3 HUVECs were used for experiments.

Cell adhesion and proliferation

PTFE disks carrying pCDI-hVEGF$_{121}$, pCDI, pEGFP or fibronectin were fixed to the bottom of 96-well tissue culture plates (TCP, Falcon, USA). HUVECs were seeded onto the various PTFE materials and empty 96-well TCP at a density of 25000 cells per well in 200 μl culture medium (without ECGS). The medium was first changed after 6 h resulting the removal of unattached cells, then every 2 d. At 6, 24, 72 and 120 h after seeding, the cells were detached by using 0.25% trypsin-0.02% EDTA in PBS. The number of cells was counted under hemocytometer. The cell adhesion ratio is defined as the ratio of cell number at 6 h and cell number at seeding. Five replicate samples of various PTFE materials or TCP were evaluated at each time.

At 6 and 120 h after seeding, cell growth status on the different PTFE materials was observed under scanning electronic microscopy (SEM). Various PTFE disks fixed in 2.5% glutaral solution were postfixed for 1 h in 10 ml/L OsO_4, dehydrated in a graded series of alcohol, CO_2 critical-point dried, mounted on aluminum tubs and sputter-coated with gold. Samples were examined with an SEM (Stereoscan 260, Cambridge, UK) operated at 25 kV.

Determination of pEGFP gene transfection and the transfection efficiency

At 6, 24, 72 and 120 h after seeding, cells grown on the pEGFP-PTFE disks were detached by 0.25% trypsin-0.02% EDTA in PBS. Cell GFP expression was detected by fluorescence microscopy. The transfection efficiency was determined by counting cells expressing GFP in three non-overlapping vision fields in relation to the total cell number determined by phase contrast microscopy. Five replicate samples were evaluated at each time.

Enzyme-linked immunoadsorbent assay (ELISA) of VEGF expression

At 6, 24, 72 and 120 h after cell seeding, secretion of VEGF in the supernatant of the cultured HUVECs on different materials was measured using ELISA kits for human VEGF (R & D, USA) and following the manufacturer's instructions. Optical density was determined at wavelength of 450 nm, VEGF concentration was determined by comparison with standard curves. Each material had five replicated samples each time.

Statistical analyses

Data were expressed as means±standard deviation (*SD*) and at the same time the differences between different materials were assessed by two factors analysis of variance or Kruskal-Walllis *H* test (SPSS V.10.0). *P* values less than 0.05 were considered to be statistically significant.

RESULTS

The release rates of various plasmid DNA from PTFE materials were almost identical, the difference of various plasmid DNA concentration at the same time was not statistically significant (Fig.1a, $P>0.05$). Take the cumulative release curve of pCDI-hVEGF$_{121}$ for instance (Fig.1b), the release of pCDI-hVEGF$_{121}$ from PTFE disk in vitro was characterized by an early burst, with more than 50% total pCDI-hVEGF$_{121}$ release within the first 8 h of incubation, then the speed of release decreased obviously. Thought DNA released continuously even after 5 d, the increase of DNA release between 3~7 d had no statistical significance ($P>0.05$). Therefore, the total dose of plasmid that could be carried by one 6.5 mm diameter PTFE disk by our method was about 6 μg.

The differences of HUVECs adhesion ratios at 6 h on PTFE disks carrying pCDI-hVEGF$_{121}$, pCDI, pEGFP or fibronectin were not statistically significant (0.41±0.1, 0.43±0.1, 0.44±0.09, 0.44±0.09, respectively, $P>0.05$, Fig.2), these adhesion ratios were far lower than that on TCP (0.91±0.08, $P<0.01$). At 24, 72 and 120 h, the number of HUVECs on pCDI-hVEGF$_{121}$-PTFE disks was far more than that on

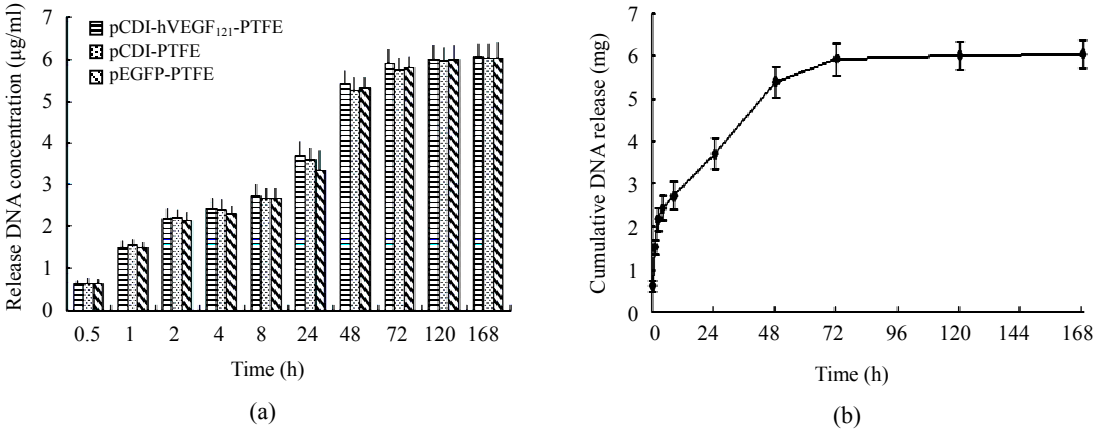

Fig.1 Plasmid DNA controlled release. (a) The cumulative release of various plasmid DNA from PTFE materials at different time, the difference of various plasmid DNA concentration at the same time was not statistically significant (P>0.05); (b) The cumulative release curve of pCDI-hVEGF$_{121}$

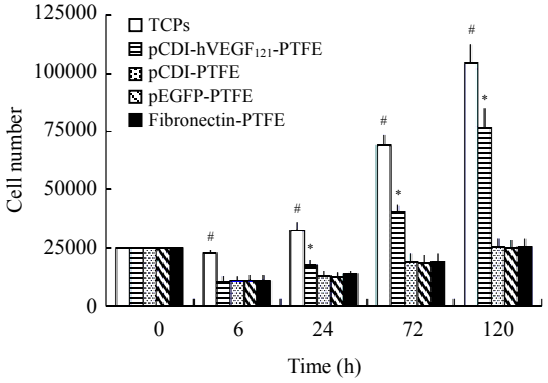

Fig.2 HUVEC number on different materials at different time

# P<0.01 compared with pCDI-hVEGF$_{121}$-PTFE/pCDI/pEGFP/fibronectin-PTFE disks; * P<0.05 compared with pCDI/pEGFP/fibronectin-PTFE disks

pCDI/pEGFP/fibronectin-PTFE disks (P<0.05), but less than that on TCP (P<0.01). However, the proliferation rate of HUVECs in pCDI-hVEGF$_{121}$-PTFE disks in 6~120 h was (7.56±0.82), which was not only higher than that of pCDI-PTFE disks (2.39±0.16), pEGFP-PTFE disks (2.31±0.15) and fibronectin-PTFE disks (2.36±0.14) but also higher than that of TCP (4.63±0.19) obviously (P<0.01, Fig.2). The differences of cell numbers between pCDI/pEGFP/fibronectin-PTFE disks at 6, 24, 72 and 120 h were not statistically significant.

Representative SEM pictures of HUVECs cultured on the pCDI-hVEGF$_{121}$-PTFE disks and fibronectin-PTFE disks are shown in Fig.3. At 6 h, cell numbers on pCDI-hVEGF$_{121}$-PTFE disk and fibronectin-PTFE disk were almost identical. However, at 120 h, cell number on pCDI-hVEGF$_{121}$-PTFE disk was more than that on fibronectin-PTFE disk. Though cell morphology was not very well, with some cell clusters, and many cells did not have HUVEC's typical spindle-shape because of absence of flow environment, these pictures generally demonstrated that HUVECs could quickly proliferate to a certain extent on the pCDI-hVEGF$_{121}$-PTFE disk.

The proportion of GFP-positive cells on pEGFP-PTFE disks is positively related to the culture time. At 6, 24, 72 and 120 h, the proportions were (1.19%±0.31%), (2.89%±0.42%), (4.78%±0.73%) and (7.14%±0.94%) respectively. Fig.4 shows representative pictures of fluorescence microscopy and phase contrast microscopy at 120 h.

VEGF concentrations in supernatant of the cultured HUVECs on each material at different time are shown in Table 1. VEGF concentration of pCDI-hVEGF$_{121}$ group was higher than that of TCP, pCDI, pEGFP and fibronectin group (P<0.01). The difference of VEGF concentration between TCP, pCDI-PTFE disks, pEGFP-PTFE disks and fibronectin-PTFE disks was not statistically significant (P>0.05). For pCDI-hVEGF$_{121}$-PTFE disk group, expression of VEGF by HUVECs increased continuously. The VEGF level reached 539×10^{-12} g/ml at 120 h after cell culture, (23.16±1.64) times higher than that at 6 h.

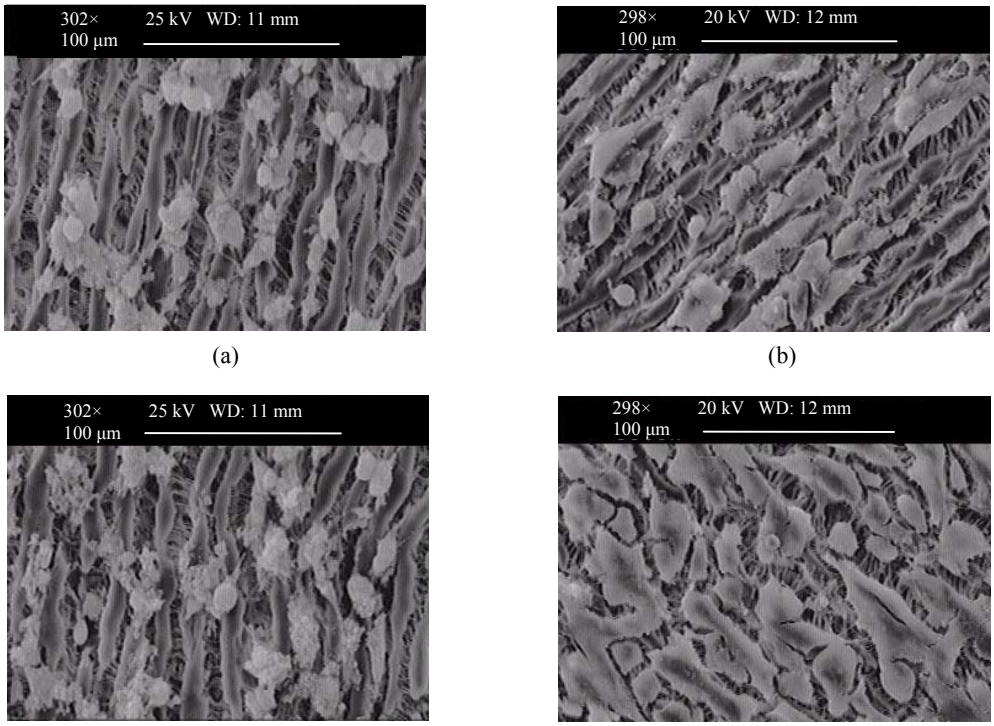

Fig.3 Representative SEM pictures of HUVECs cultured on fibronectin-PTFE disks at (a) 6 h and (b) 120 h and HUVECs on pCDI-hVEGF$_{121}$-PTFE disks at (c) 6 h and (d) 120 h

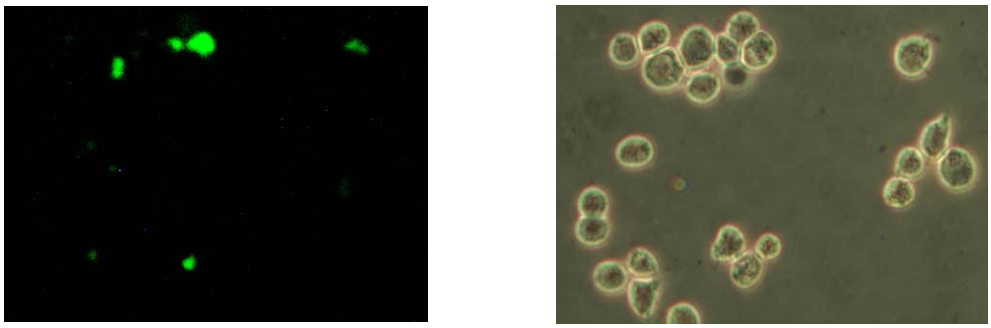

Fig.4 HUVECs on the pEGFP-PTFE disks at 120 h. (a) Fluorescence microscopy; (b) Phase contrast microscopy

Table 1 VEGF protein concentration at different time (ELISA, $\times10^{-12}$ g/ml, n=5, $\bar{x}\pm s$)

Group	6 h	24 h	72 h	120 h
TCP	10.72±0.74	11.06±0.90	11.44±1.13	10.8±0.70
pCDI-hVEGF$_{121}$	23.36±1.51$^{\#}$	191.84±17.49$^{\#}$	303.24±13.06$^{\#}$	539.22±16.78$^{\#}$
pCDI	11.74±1.34*	11.5±1.22*	12.52±1.16*	11.3±1.21*
pEGFP	11.28±1.45	11.4±1.34	12.94±1.45	11.7±1.54
Fibronectin	11.56±1.45	11.26±1.55	12.72±1.57	11.9±1.79

$^{\#}$ P<0.01 compared with pCDI/pEGFP/fibronectin-PTFE disks and TCP at the same time; * P>0.05 compared with pEGFP/fibronectin-PTFE disks and TCP at the same time

DISCUSSION

The development of a small diameter artificial vascular graft is limited by rapid thrombus formation upon blood contact and subsequent intima hyperplasia after implantation (Esquivel and Blaisdell, 1986). Rapid and controlled endothelialization and neoarterial regeneration via perianastomotic as well as transmural tissue ingrowth play a critical role in the patency of implanted vascular grafts (Clowes *et al*., 1986). Herring *et al*.(1978) studied endothelial cell seeding in synthetic vascular grafts, but the results of this method used in clinical trials are still controversial.

VEGF is a strong mitogenic and migration-promoting factor for vascular endothelial cells and therefore a major initiator of angiogenesis (Ferrara and Henyel, 1989; Leung *et al*., 1989). Increased NO and prostacyclin secretion induced by VEGF is known to inhibit smooth muscle cell proliferation and platelet aggregation in animal models as well (Tsurumi *et al*., 1997; Laitinen *et al*., 1997; Dorafshar *et al*., 2003). Some experimental and clinical studies proved that naked VEGF gene plasmids directly injected into myocardium or skeletal muscle could express VEGF protein and promote angiogenesis (Losordo *et al*., 1998; Isner *et al*., 1996; Shyu *et al*., 2003). Recent trials showed that the transfection of VEGF gene could promote vascular endothelial cell regeneration, decrease thrombosis, reduce proliferation of muscle cells and vascular stenosis rate after arterioplasty and stent implantation (Riessen *et al*., 1993; Walter *et al*., 2004). In the present study, VEGF gene was employed to improve the endothelialization and patency of artificial vascular grafts. As for a carrier for VEGF gene plasmid, vascular grafts was investigated in terms of a simple and practical approach for transferring VEGF gene into the cells of the implant bed tissue, in other words, pCDI-hVEGF$_{121}$ was impregnated into the wall of vascular grafts that were used not only as reconstruction material but also as gene carrier.

PTFE is a fine porous hydrophobic polymer with a highly electronegative surface for vascular graft (Esquivel and Blaisdell, 1986). We hypothesized that VEGF gene can permeate into and be stored in the pores of PTFE with controlled release. Fibronectin was used besides a substrate matrix for cell attachment as cross-linking agent of PTFE and gene plasmid to increase the retention of the gene on the grafts. The release curve showed gene released rapidly at the beginning because of rapid dissolution of gene attached on the surface and then released slowly when gene hidden in the pores of PTFE began to release after 8 h. These results suggested PTFE vascular graft carrying plasmid DNA has controlled release effect to some extent.

The growth of endothelial cells seeded on PTFE is usually very slow. However, in our experiment, HUVECs cultured on pCDI-hVEGF$_{121}$-PTFE disks grew very fast, with a (7.56±0.82)-fold increase in cell number after 120 h, this speed of cell proliferation was not only significantly higher than that on the pCDI-PTFE disks, pEGFP-PTFE disks and fibronectin-PTFE disks but also higher than that on the TCP. ELISA verified that HUVECs cultured on pCDI-hVEGF$_{121}$-PTFE disks produced a great quantity of VEGF protein. These results proved VEGF gene plasmid carried by PTFE materials could transfect HUVECs successfully and result in VEGF protein expression which consequently improve the proliferation of HUVECs. The GFP expression in the HUVECs cultured on pEGFP-PTFE disks also certified that plasmid DNA carried by PTFE material could transfect cells cultured on them. It had been reported that direct contact of cells with pure plasmid DNA added in the culture does not result in gene transfection in the cell (Klugherz *et al*., 2000). However, in the present study, DNA transfection occurred without the use of any enhancing agent such as lipofectamine or calcium phosphate precipitation. The possible mechanisms are supposed to be: (1) PTFE materials impregnated with DNA, when in intimate contact with cell membranes, will lead directly to transfection (Klugherz *et al*., 2000); (2) When DNA is mixed with fibronectin which may interact with some adhesive molecule receptors (such as integrin $\alpha_v\beta_3$) on cell membranes, internalization of fibronectin-DNA complex can be realized via receptor-mediated endocytosis (Hood *et al*., 2002), although the transfection efficiency of this method was much lower than that when enhancing agent was used. However, if a desired gene product is a secreted protein, the transfection of only a small percentage of target cells may result in production of a biologically relevant concentration of protein since the transfected

cells can act as secretory "factories" for the protein of interest (Losordo *et al*., 1994; Nabel *et al*., 1993; Takeshita *et al*., 1996; Pels *et al*., 2003). This hypothesis is well supported by our study result (fast proliferation of HUVECs cultured on pCDI-hVEGF$_{121}$-PTFE disks).

In conclusion, we found that the PTFE graft can be used as a carrier of VEGF gene plasmid. VEGF gene releasing from the graft can transfect HUVECs and promote HUVECs proliferation in vitro successfully. In further research, we will investigate whether the VEGF gene carried by PTFE graft could transfect vascular beds tissue and promote endothelialization after graft transplantation.

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