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# Culture of Endothelial Cells by Transfection with Plasmid Harboring Vascular Endothelial Growth Factor

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**Abstract** Vascular endothelial cells (ECs) are usually difficult to culture in a large scale because of their complicated requirements for cell growth. As the vascular endothelial growth factor (VEGF) is a key growth factor in the EC culture, we transfected human umbilical vein endothelial cells (HUVEC) using a plasmid containing VEGF gene and let them grow in a culture medium eliminated an important supplement, endothelail cell growth supplement (ECGS). The expression of VEGF by HUVEC tansfected with VEGF gene was not enough to stimulate the growth of HUVEC, only 40% of maximum cell density obtainable in the presence of ECGS However, when the culture medium was supplied with 2.5 ng/mL of basic fibroblast growth factor (bFGF), a synergistic effect of VEGF and bFGF was observed. In this case, the final cell density was recovered up to about 78% of maxium value.

*Keywords*: endothelial cell culture, vascular endothelial growth factor, transfection, autocrine

## INTRODUCTION

The endothelial cells (ECs) play an important role in physiologic hemostasis, blood vessel permeability and the response of the blood vessel to other physiologic and pathologic stimuli. ECs are also widely employed to seed on the surface of artificial organs which are usually made of synthetic polymers such as polyurethane, or polytetrafluoroethylene, which is so called endothelialization [1,2] or to fabricate a completely biological artificial blood vessel [3]. However, the culture of endothelial cells in a large scale is not easy because of the complicated requirements for the cell growth. The culture of ECs requires many kinds of growth factors or supplements such as gelatin coated surface, heparin or heparin binding proteins, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and/or mixed growth supplements. VEGF, a key angiogenic growth factor, is a naturally occuring protein that exists in four isoforms: 121, 165, 189 and 206 aminoacids. The physiological effects of VEGF include enhanced vascular permeability and nitric-oxide-induced vasodilation Several properties make VEGF a potential agent for the proliferation of vascular endothelium (angiogenesis) *in vitro* It is relatively specific for endothelial cells and the degree to which it binds to heparin may prolong exposure in the culture medium [4].

In this work, we tried to culture the human umbilical vein endothelial cell (HUVEC) by transfection and expression of VEGF using plasmid containing VEGF gene, so that the ECs can use the VEGF by themselves as an autocrine, replacing several complicated supplements. If the idea to transfect cells with the plasmids containing growth factor genes is feasible, instead of adding growth factors to the culture medium, we can save the cost for the purchase of growth factors, which are generally produced by genetic recombination technique and are usually very expensive.

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## MATERIALS AND METHODS

### Chemicals

Cationic lipids, DOTAP (dioleoyloxy-trimethylammonium propane) was obtained from Boehringer-Mannheim (Mannheim, Germany) The ELISA kit for VEGF immunoassay was purchased from R&D Systems (Minneapolis, USA).

### Isolation and Culture of HUVEC

HUVEC was obtained from umbilical veins by collagenase digestion according to the method of Jaffe *et al.* [5] Briefly, umbilical cords were sterilely collected from healthy newborns and kept in ice cold travel medium for 6 h or less. Veins from 15-30 cm long unclamped cords were cannulated at both ends, washed with cord buffer, and placed in a 37°C bath of sterile water. A warm collagenase solution was injected to rinse and fill the vein. After a 15 min incubation, the veins were gently massaged and vigorously perfused with two times 50 mL of M199 containing 10% FBS and antibiotics. The cell solution obtained was centrifuged and the cell pellet was resuspended in medium for endothelial cell. HUVEC were then plated in 25 $cm^2$ tissue culture plates

precoated with 0.2% gelatin. The growth medium consisted of Medium 199 supplemented with 10% FBS, 50 μg/mL endothelial cell growth supplement (ECGS), 100 U/mL heparin, 100 U/mL penicillin and 100 μg /mL streptomycin. Cultures were grown at 37°C in 5% $CO_2$. Confluent HUVEC were passaged with trypsin-EDTA and were routinely used between passage 2 and 6. Cultured cells were identified as endothelial by their cobblestone morphology and the presence of factorVIII related antigen

### Transfection

Plasmid containing CMV promoter and VEGF gene was obtained from Dr. Youngjin Nam (National Institute of Health). A given amount of DOTAP diluted in 100 μL of HBSS (Hanks' balanced salt solution) was mixed with the supplement protein, lactoferrin (LF), asialofetuin (AF), protamine (PT), and incubated at room temperature for 15 min, followed by incubation with 2 μg of plasmid diluted in 12 μL of HBSS for 15 min HUVEC grown in a 6-well plate to 40-50% confluence were washed once with serum-free medium and incubated in 1 mL of serum-free medium with lipoplex prepared. After 4 h cells were returned to culture with a medium containing 10% fetal bovine serum (FBS). Forty hrs after transfection, cells was washed with phosphate buffered saline (PBS) and lysed for 10 min with 200 μL of reporter lysis buffer. After brief centrifugation, fifty microliters of lysate was assayed for VEGF by enzyme linked immunosorbent assay (ELISA) method.

### Cell Proliferation and Cytotoxicity Assay

To assay the cell proliferation $3 \times 10^3$ cells/well were plated on 96 well plate coated with gelatin. And each growth factor (1-30 ng/mL) containing complete medium was added. In most experiments, the medium was renewed every 2 days. To estimate the number of viable cell, we used the colorimetric assay which measure the neutral red (NR) uptake reflecting the relative number of viable cells, as described [6].

## RESULTS AND DISCUSSION

### Effect of VEGF on the Growth of HUVEC

As VEGF is one of the major angiogenic factors, the effect of VEGF on the growth of HUVEC was studied and the results are shown in Fig. 1, which shows the effect of VEGF alone or VEGF and bFGF mixture on the growth of HUVEC. With VEGF or bFGF alone, at least 10 or 20 ng/mL of VEGF or bFGF is necessary to obtain the maximum growth of HUVEC (Fig. 1(a) and (b)). But in the presence of 2.5 ng/mL of VEGF, 2.5 ng/mL of bFGF is enough to obtain the same effect of growth stimulation (Fig 1(b)). This synergistic effect may be due to the fact that two different receptors stimulated by each growth factor amplify the signals for the proliferation of HUVEC through different pathway [9].

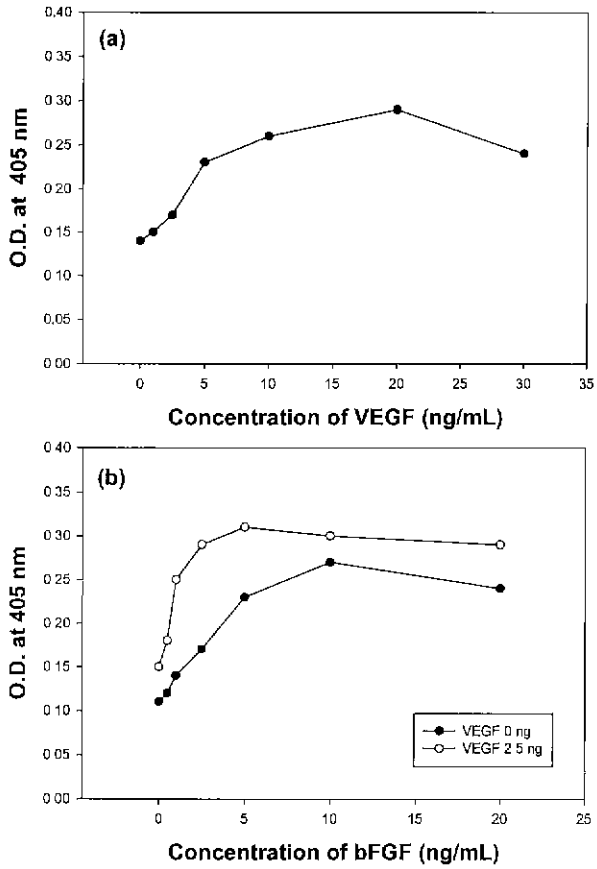


Fig. 1. (a) Effect of vascular endothelial growth factor (VEGF) on the growth of human umbilical vein endothelial cell (HUVEC). (b) Effect of basic fibroblast growth factor (bFGF) on the growth of HUVEC with or without VEGF. HUVEC was cultured in 96-well plate for 6 days and the final cell density was determined by NR assay method.

### Expression of VEGF in HUVEC

HUVEC was transfected with 2 μg of VEGF plasmid complexed with the various proteins such as LF, AF or PT. After 40 h of transfection, cells were lysed and the concentration of VEGF was determined by ELISA method and the results are shown in Fig. 2. Without the supplemental proteins, the amount of VEGF expressed by HUVEC was less than 80 pg/mL. This concentration is too low to expect that VEGF stimulates the growth of HUVEC *in vitro* (see Fig. 1(a)). When the plamid was complexed with 3 μg of LF or AF, the concentration of VEGF in culture medium was increased up to 0.3 ng/mL and 0.6 ng/mL, respectively (Fig. 2(a) and (b)). The enhancement in transfection efficiency by LF is due to the net positive charge of LF in neutral pH [7]. However, the cause of enhancement by AF is not yet clear. Fetuin is an abundant protein in fetal bovine plasma and AF is a fetuin of which sialic acid is removed. AF receptors are found on the membrane surface of hepatic cells, but not for EC. However, since the AF-plasmid complex can enhance the transfection efficiency greatly, the complex seems to interact strongly with the membrane of EC, although how this occurs is not understood in detail.

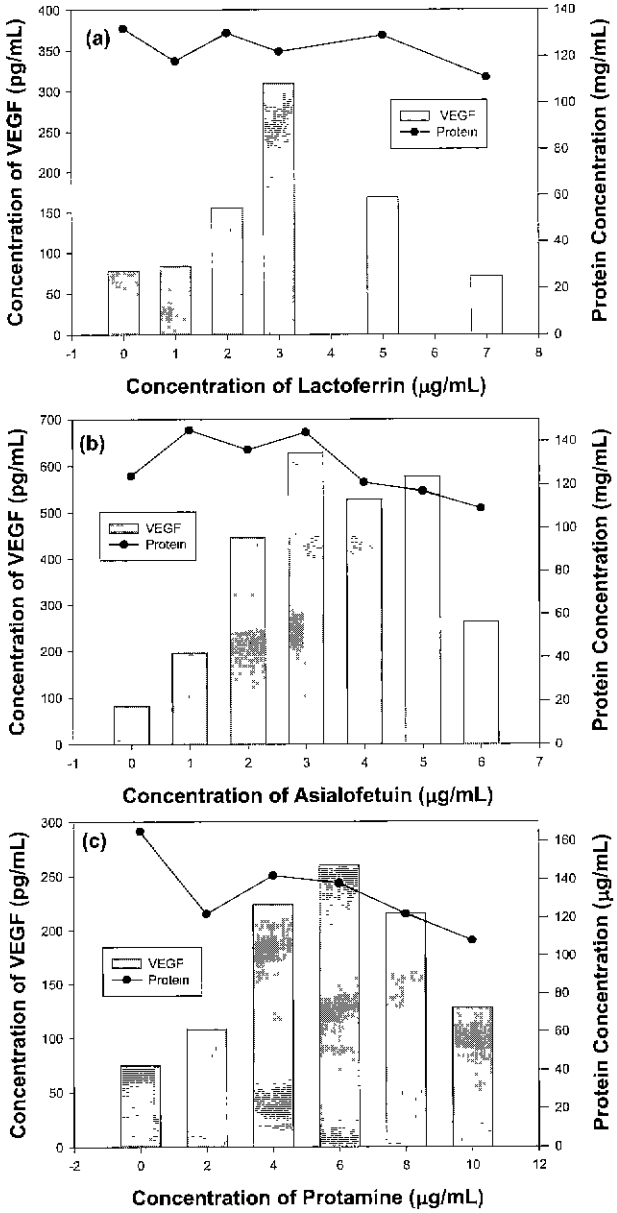


Fig 2 Expression of vascular endothelial growth factor (VEGF) by human umbilical vein endothelial cell (HUVEC) HUVEC was transfected with VEGF plasmid using poly cationic lipid, DOTAP in the presence of various supplemental proteins; (a) Lactoferrin, (b) Asialofetuin, (c) Protamine.

The change of total protein concentration in Fig. 2 indicates the cytotoxicity of added proteins to EC. The toxicity of lactoferrin or asialofetuin was negligible within the range of experiments whereas that of protamine was not. Fig. 3 shows the change of VEGF concentration with the function of time after transfection. The expression of VEGF by HUVEC was rapid that the concentration of VEGF was 0 37 ng/mL at day 1 and was maximum at day 2 with 0.48 ng/mL but began to decrease thereafter. We tried to increase or to prolong the transfection efficiency of VEGF further using various transfection enhancers such as poly-L-lysine, inactivated influenza virus or viral peptide, hemagglutinin, phospholipase *etc.* [8], but never better than AF. On the other hands, when we cultured HUVEC in the presence of bFGF in addition to VEGF, a synergistic effect on the growth of HUVEC was observed.

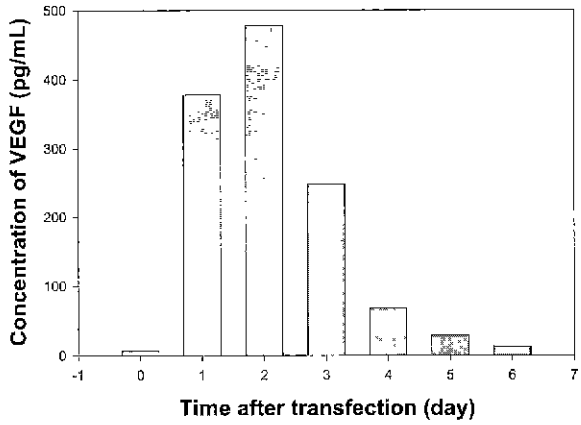


Fig. 3 Change of vascular endothelial growth factor (VEGF) expression by human umbilical vein endothelial cell (HUVEC) with the function of culture time HUVEC was transfected with VEGF plasmid using poly cationic lipid, DOTAP in the presence of Asialofetuin, 3 μg/mL

## Proliferation of HUVEC by VEGF

When HUVEC was cultured without plasmid transfection, the influence of bFGF addition (2.5 ng/mL) on cell growth was negligible (Fig. 4(a)), which is already shown in Fig 1(b). On the other hand, when HUVEC was cultured after the transfection using VEGF plasmid, the effect of bFGF on the cell growth was prominent, especially at the beginning of cell growth (Fig. 4(b)). This enhancement is due to the synergistic effect of bFGF supplemented externally and VEGF expressed internally by HUVEC ECGS is the most influential and expensive supplement in the HUVEC culture medium When ECGS concentration was decreased from 50 to 0 μg/mL, the final cell density of HUVEC cultured for 6 days also diminished progressively (Fig. 5), which indicates that ECGS is indispensible for the growth of HUVEC If ECGS was completely removed from the culture medium (0 μg/mL of ECGS), the final cell density of HUVEC was only 23% of maximum cell density obtainable with 50 μg/mL of ECGS. When 2.5 ng/mL of VEGF was supplemented externally or VEGF was expressed internally by transfection, the final cell density was recovered up to 40% of maximum value obtainable with 50 μg/mL of ECGS. If HUVEC was transfected with VEGF plasmid and at the same time bFGF was supplemented to the culture medium, the final cell density was about 78% of maximum value. From these results, we can conclude that VEGF transfection to HUVEC may replace partially the role of ECGS in cell growth but further study is necessary to enable the culture of animal cells by transfecting them using plasmid containing growth factor gene.

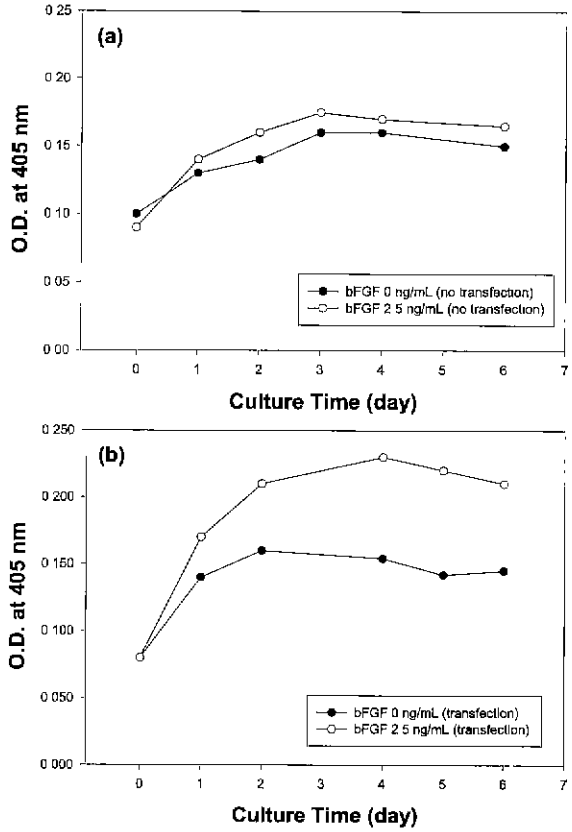


Fig. 4 (a) Growth curve of human umbilical vein endothelial cell (HUVEC) without transfection, (b) Growth curve of HUVEC transfected with vascular endothelial growth factor (VEGF) plasmid HUVEC was cultured in 96-well plate for 6 days using culture medium with or without basic fibroblast growth factor (bFGF) and the cell density with the function of culture time was determined by NR assay method.

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## REFERENCES

[1] Stemerman, M. B. and T. H. Spaet (1972) The subendothelium and thrombogenesis *Bull. NY Acad. Med.* 48: 289-293

[2] Meinhart, J., M. Deutsch, and P. Zilla (1997) Eight years of clinical endothelial cell transplantation *ASAIO J.* 43: M515-M521.

[3] L'Heureux, N., S. Paquet, R. Labbe, L. Germain, and F. Auger (1998) A completely biological tissue-engineered human blood vessel *FASEB J.* 12: 47-56.

[4] Ferrara, N., H. Heinsohn, C. E. Walder, S. bunting, and G. R. Thomas (1995) The regulation of blood vessel growth by VEGF. *Ann. NY Acad. Sci.* 27: 246-256.

[5] Jaffe, E., R. Nachman, C. Becker, and C. Minick (1973) Culture of humna endothelial cells derived from umbilical veins; identification by morphologic and immunologic criteria *J. Clin. Invest.* 52: 2745-2756

[6] Hockley, K. and D. Baxter (1986) Use of the 3T3 cell neutral red uptake assay for irritants as an alternative to the rabbit test *Fd. Chem. Toxic.* 24. 473-475

[7] Choe, T. B., I. C. Park, and S. I. Hong (1998) Enhancement of cationic liposome-mediated transfection by lactoferri, *Biotechnol. Tech.* 12. 577-581.

[8] Jang, S. J. (1999) MS thesis, Development of transfection methods using cationic liposomes Konkuk University.

[9] Goto, F., K. Goto, K. Weindel, and J. Folkman (1993) Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels *Lab. Invest.* 69. 508-517.

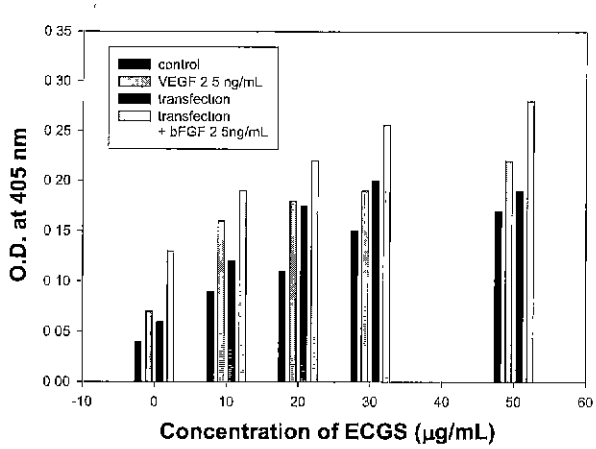


Fig. 5 Effect of transfection or growth factors (VEGF or bFGF) on the growth of human umbilical vein endothelial cell (HUVEC) HUVEC was cultured in 96-well plate for 6 days using culture medium containing endothelial cell growth supplements (ECGS, 0-50 μg) and the final cell density was determined by NR assay method Control, no transfection or no addition of growth factor, transfection; HUVECs were transfected with VEGF plasmid

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