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Culture of Human Umbilical Vein Endothelial Cells Using 96-well Microplates and Position Effects on Cell Growth

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Abstract When endothelial cells isolated from human umbilical veins were cultured for 6 days using 96-well microplates, the final cell density in each well was found not to be the same although the medium composition of each well was exactly the same Cell growth in the wells located at the periphery of a microplate was low, while that in the central area of the plate was high. A possible cause for different rate of growth was proposed as the uneven concentration of oxygen in the culture medium of each well.

Keywords. endothelial cell, culture, 96-well plate, oxygen concentration

INTRODUCTION

Endothelial cells (ECs) play an important role in physiological hemostasis, blood vessel permeability and the response of blood vessel to other physiological and pathological stimuli. ECs are also widely employed on the surface of artificial organs, which are usually made of synthetic polymers such as polyurethane and polytetrafluoroethylene, to reduce the risk of thrombogenesis [1,2] or to fabricate a completely biological artificial blood vessel [3]. However, culturing of ECs on a large scale is difficult because of the complicated requirements for cell growth. Many kinds of growth factors or supplements such as a gelatin coated surface, heparin or heparin binding proteins, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and/or mixed growth supplements are needed for culturing of ECS.

When we studied the effect of growth factors on human endothelial cell growth using 96-well microplates, we found that the final cell density in each well was not the same although the medium composition of each well was exactly the same. In this work, we tried to determine why the final cell density of each well in the microplate was different. The possible cause may have been the uneven concentration of oxygen in the culture medium of each well.

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MATERIALS AND METHODS

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical veins by collagenase digestion according to the method of Jaffe [4]. Briefly, the umbilical cords were sterilely collected from healthy newborns and kept in an ice cold travel medium for 12 h or less. The veins from the 15-30 cm long unclamped cords were cannulated at both ends, washed with cord buffer solution, and placed in a bath of sterile water at 37°C. A warm collagenase solution was injected into the veins to rinse and fill the veins. After 15 min of incubation, the veins were gently massaged and vigorously perfused two times with 50 mL of M199 containing 10% fetal bovine serum (FBS) and antibiotics. The cell solution obtained was centrifuged, and the cell pellet was resuspended in the medium for endothelial cell culture. HUVECs 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. Culturing was done at 37°C in 5% CO_2 incubator. Confluent ECs were passaged with trypsin-EDTA and were routinely used between passage 2 and 6. Cultured cells were identified as endothelial cells by their cobblestone morphology and the presence of factor VIII related antigen [4].

Culture in 96-well Microplates and Proliferation Assay

To assay cell proliferation, 3×10^3 cells/well was plated on 96-well microplates coated with gelatin. Two hun-

dred microliter of medium was added into each well. In most experiments, the medium was renewed every 2 days. After 6 days of culturing, to estimate the number of viable cells, we used the colorimetric assay which measured the neutral red uptake reflecting the relative number of viable cells, as described by Hockley and Baxter [5] ECs were also cultured using a 96-well microplate in which holes were made with a heated pin on the plastic cover just above each well. The microplate was then placed inn an anaerobic culture jar (GasPak, BD Science, NJ) to reduce the oxygen concentration of atmosphere. The initial oxygen concentration was adjusted to 5% using nitrogen-air gas cylinder, but the oxygen concentration fluctuated during the culturing period. Nitrogen gas was recharged every 2 days. Every experiment was repeated three times independently to confirm the results but the final data were taken for analysis.

RESULTS AND DISCUSSION

Growth Pattern of HUVEC in 96-well Microplates

Ninety six-well microplates were used mostly to test the effect of growth factors on the cultured cells *in vitro* out of convenience. When we cultured HUVEC for 6 days using 96-well microplates with the same medium composition in each well, the final cell density according to the position of the well was found as shown in Fig. 1. A ninety six-well microplate made up with 8 rows (A-H) and 12 columns (1-12), and Fig. 1 shows the final cell density measured from one quarter of a microplate (row, A-D, column; 1-6). We did not expect that the growth of HUVEC would be severely dependent on the position of the well in the microplate Cell growth in the wells located at the periphery of the plate was low, while that in the central area of the plate was high, although the initial composition of medium in each well was exactly identical. The disparity of cell growth among the wells was apparent even after two days of culturing and was enhanced with the culture time (data not shown). However, when human fibroblasts isolated from foreskin were cultured in a 96-well microplate, this pattern of disparity was not observed (Fig. 2). These results meant that the growth of HUVEC in microplates is influenced by a factor other than growth factors in the culture medium.

Possible Cause of Uneven Cell Growth of HUVEC Cultured in 96-well Microplates

The influence of oxygen concentration on the growth of HUVEC is well known. When HUVEC were cultured under conditions of hypoxia, various growth factors including VEGF were induced and the growth of ECs was stimulated [6-9]. According to Yamagishi *et al.* [10], when microvascular Ecs were cultured under below 10% O_2, the growth of ECs was enhanced by more than 50%. This increase was due to the VEGF induced by the

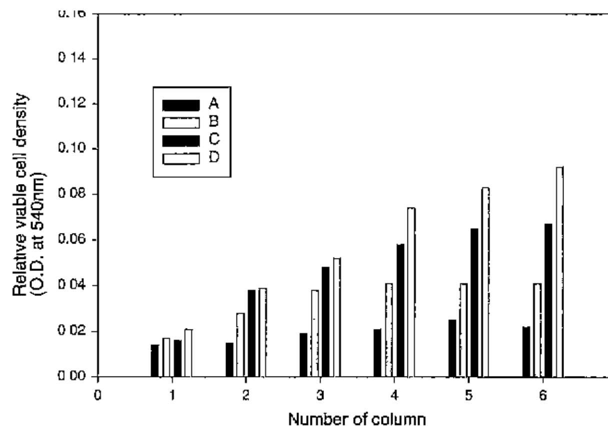

Fig. 1. Relative viable cell density of human umbilical vein endothelial cells cultured for 6 days using a 96-well microplate. Cell density shown here is measured from one quarter of the microplate (row; A-D, column; 1-6)

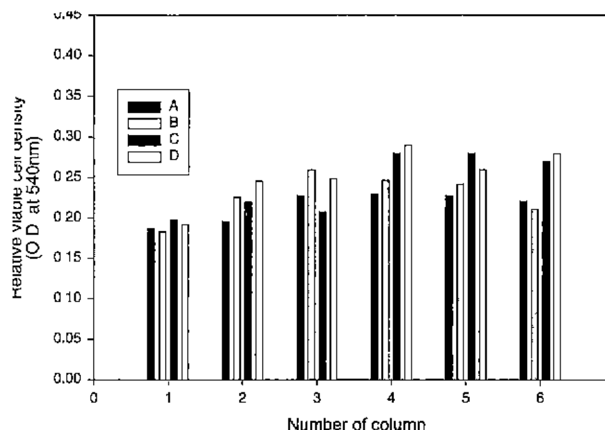

Fig. 2. Relative viable cell density of human foreskin fibroblasts cultured for 6 days using a 96-well microplate. Cell density shown here is measured from one quarter of the microplate (row; A-D, column; 1-6)

ECs, and ECs utilize VEGF as an autocrine [10]. The physiological effects of VEGF include enhanced vascular permeability and nitric-oxide-induced vasodilatation. 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 [11]. For this reason, we supposed that the difference in cell density of HUVEC according to the well position was due to the different oxygen concentration in each well, which meant a different concentration of VEGF in each well. The following experimental results proved this assumption.

Since oxygen transfer occurred through the edge of the microplate's plastic cover, the microplate was wrapped with parafilm to block oxygen transfer to the periphery of the microplate. When this was done, the difference in cell density among the wells did not decrease, while the cell density in each well almost dou-

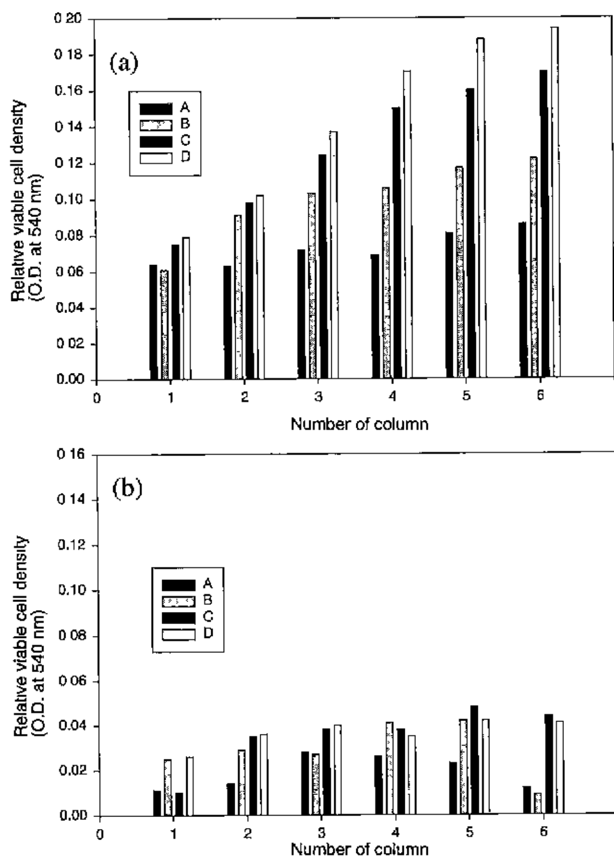

Fig. 3. Relative viable cell density of human umbilical vein endothelial cells cultured for 6 days (a) using a 96-well microplate which was wrapped with parafilm or (b) using a 96-well microplate in which its plastic cover was pinholed just above each well. Cell density shown here is measured from one quarter of the microplate (row; A-D, column; 1-6).

bled (Fig. 3(a)). However, when we cultured HUVEC using a microplate in which its plastic cover had one pinhole above each well, so as to eliminate the oxygen Gradient developed across the microplate, the difference in cell density of each well was reduced, and at the same time, the cell growth was also diminished by half comparing to that from the microplate without parafilm wrapping (Fig. 3(b)). These results confirmed that the difference in cell density according to the well position in the microplate was mainly due to the different oxygen concentration in each well. The first result shows that the hindered oxygen transfer provoked a low oxygen concentration in each well, which induced growth factors including VEGF and then enhanced growth of ECs. The second result shows that high oxygen concentration acquired with the aid of the pinholed cover can inhibit the growth of ECs regardless of the well position. The oxygen gradient across the microplate is usually negligible and almost all of the cell culture using microplate is not problematic as shown in the culture of human fibroblasts (Fig. 2). However, ECs are so sensitive to oxygen concentration that even a slight difference in oxygen concentration among the wells resulted

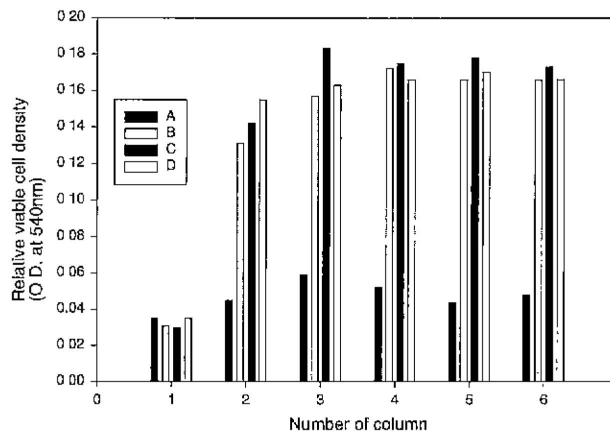

Fig. 4. Relative viable cell density of human umbilical vein endothelial cells cultured for 6 days using a 96-well microplate in which its plastic cover was pinholed and it was put in an anaerobic culture jar. Cell density shown here is measured from one quarter of the microplate (row, A-D, column; 1-6).

a different cell density after 6 days of culturing in the microplate.

When HUVEC were cultured again using a microplate with a pinholed cover but now in a low oxygen atmosphere using an anaerobic culture system, the final cell density in each well was recovered to be normal and the difference in cell density among the wells was remarkably reduced (Fig. 4). The most exterior wells still exhibited poor cell growth, but the wells in the central area produced a normal cell concentration. Nonetheless, we could not confirm that this stimulating effect on cell growth with the low oxygen condition was mainly due to the growth factor, VEGF, because when we addedanti-VEGF-antibody to each well (2 μg/well) of the microplate, the rate of inhibition of HUVEC growth by the antibody was less than 15% (data not shown).

We studied the effect of endothelial cell growth supplement (ECGS), an important supplement of culture medium, on the growth of HUVEC using 96-well microplates, and the results are summarized in Fig. 5. Case 1 is the culture of HUVEC using microplates with the pinholed covers in a low oxygen atmosphere, where the concentration of ECGS was changed from zero to 50 μg/mL. Cases 2 and 3 are the cultures of HUVEC using microplates with the covers without holes in a normal oxygen atmosphere, *i.e.* the concentration of oxygen at 20%. For Case 2, the concentration of ECGS was increased from the outside wells to the inside wells (from C2 to C6) while for Case 3, the concentration of ECGS was increased from the inside wells to the outside wells (from C7 to C11). In Case 1, the growth of ECs increased linearly with the increase of ECGS concentration, showing a direct influence of ECGS on the growth of HUVEC. In Case 2, the effect of ECGS at high concentration, above 30 μg/mL, seemed to be exaggerated by the position effect, *i.e.* the effect of relatively low oxygen concentration in the central area of the microplate (C5 and C6). However, in Case 3, the effect of

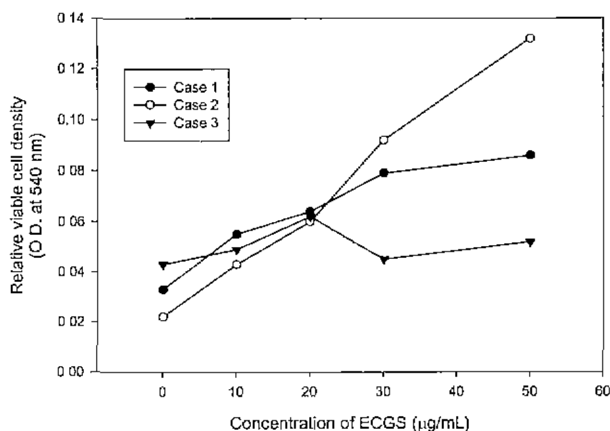

Fig. 5. Effect of endothelial cell growth supplement (ECGS) on the growth of human umbilical vein endothelial cells. ECGS was changed from zero to 50 μg/mL. Case 1; Cells were cultured for 6 days using a 96-well microplate in which its plastic cover was pinholed, and then microplate was placed in an anaerobic culture jar. Case 2, Cells were cultured for 6 days using a 96-well microplate in normal conditions. ECGS was increased from C2 to C6. Case 3; Cells were cultured for 6 days using a 96-well microplate in normal conditions. ECGS was increased from C7 to C11

effect of ECGS in the two wells in the central area of the microplate (C7 and C8) seemed to be exaggerated by the low concentration of oxygen, while the effect of ECGS above 30 μg/mL seemed to be eliminated by the relatively high oxygen concentration in the periphery area of the microplate (C10 and C11).

From these results, we concluded that the culturing of HUVEC using 96-well microplate should be done under a condition of an even oxygen concentration in each well in order to obtain correct data on the growth of HUVEC.

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