THE EXPRESSION OF GROWTH FACTORS AND THEIR RECEPTORS IN RETINAL AND ENDOTHELIAL CELLS COCULTURED IN THE ROTATING BIOREACTOR

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Vascular endothelial growth factor (VEGF) has been strongly implicated in the development of choroidal neovascularization, which is seen in age-related macular degeneration. This study investigates whether retinal cells cultured individually or as cocultures with endothelial cells and maintained in the horizontally rotating bioreactor will express more VEGF and VEGF receptors. We measured the expression of VEGF isotypes and VEGF receptors for cells maintained in monolayer and horizontally rotating bioreactor culture at various times by using reverse transcription PCR and Western blot analysis. Retinal cells showed a twofold increase in VEGF-A mRNA expression after five days of culture in the bioreactor, compared with monolayer cultures (77±3 vs 42±2, $P<.006$). Further, we found that the expression of mRNA for VEGF-A growth factor was increased fivefold for retinal cells cocultured with endothelial cell (52±4 for one day vs 240±15, $P<.001$, cultured in the bioreactor for five days). Where the expression of VEGF receptors (FLK-1 and FLT-4) was low for monolayer retinal cultures, we found the expression of both VEGF receptors was higher after 5, 10, and 15 days of culture. Increased expression of these receptors was also found for cocultured retinal/endothelial cells. Further, we found that cultured retinal cells showed higher VEGF-C protein expression compared to monolayer cultures. Our protein analysis data showed that the expression of VEGF-A was increased by twofold (780±30 for one day vs 1520±36 for five days, $P<.001$) after five days in bioreactor cocultures. These findings suggest that retinal/endothelial cell coculture in the horizontally rotating bioreactor may be a very good model for investigating the role of growth factors in the neovascularization seen in human ocular disorders. (*Ethn Dis*. 2008;18[Suppl 2]:S2-44–S2-50)

Key Words: neovascularization, retinal cells, endothelial cells, co-culture and vascular endothelial growth factors

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INTRODUCTION

Neovascularization, or blood vessel formation, is a natural component of normal development and wound repair. However, it also occurs under a variety of pathologic states, such as cancer and retinal neovascularization.[1] Retinal neovascularization plays a critical role in several human ocular disorders, including diabetic retinopathy and choroidal neovascularization, seen in age-related macular degeneration. In the retina, neovascularization is regulated by two counterbalancing systems, stimulators of neovascularization (eg, vascular endothelial growth factor [VEGF]) and inhibitors (eg, angiostatin, pigment epithelium-derived factor).[2,3] Primary angiogenic factors expressed by various cell types are the VEGF isotypes (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor). VEGF exerts its cellular effects by interacting with its high-affinity tyrosine kinase receptors Flt-1/VEGFR-1, Flk-1/KDR/VEGF-2, and neuropolin-1. A third receptor, Flt-4/VEGFR-3, binds VEGF-C and VEGF-D but not VEGF-A. The expression of VEGF is regulated by several factors, including hypoxia, cytokines (eg, interleukin-1), and the activation of several oncogenes. Hence, VEGF and its receptors appear to be essential for blood vessel formation in several diseases.[4,5]

In the eye, the cell types implicated in neovascularization are retina, retinal pigment epithelium (RPE), endothelial cells, and pericytes. With most *in vitro* models of neovascularization, the formation of new vessel-like structures is slow, taking from two to six weeks, which makes analysis of early events in neovascularization difficult. In this study, we used a horizontally rotating bioreactor, which enabled us to maintain the two different types of cells at optimal concentrations of gases and nutrients with low shear stress. This coculture system promotes the co-spatial localization of different cell types to generate 3D tissue-like structures.[6,7] This culture system has not been used previously to examine the cell-cell interactions of retinal and endothelial cell cocultures, which may play a role in retinal neovascularization. The objective of the studies in this report was to ascertain whether the expression of the pro-angiogenic factor VEGF and other factors is enhanced in retinal cells cultured alone or in coculture with endothelial cells in the horizontally rotating bioreactor. Since the bioreactor promotes cell-cell interactions, growing retinal cells in the presence of endothelial cells in this culture system is thought to result in 3D tissue-like structures that may resemble some aspect of *in vivo* ocular tissue. Hence the expression of VEGF and other factors may be closer to what we expect *in vivo*. The retinal cells grown alone in the horizontally rotating bioreactor will give insight into the response of this cell line so that the response of cocultures with this cell line can be better interpreted.

METHODS

Cell Cultures

Human retinal cells (passages 40–42) were provided by Dutt (Morehouse School of Medicine, Atlanta, Ga),[8] and bovine aortic endothelial cells (BAEC, passages 4–6) were obtained from Cambrex (East Rutherford, NJ). Both cells were maintained in Dulbecco modified Eagle medium F-12 (Invitro-

gen, Carlsbad, Calif) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 2 mM L-glutamine, .075% sodium bicarbonate, penicillin 100 units/mL, and streptomycin 100 μg/mL.

Horizontally Rotating Bioreactor Cultures

Retinal and endothelial cells were cultured individually or as cocultures on laminin (100 μg/mL) coated Cytodex-3 microcarrier beads as previously reported.[7,9,10] A retinal cell density of 18×10^6 and BAEC densities of 2×10^6 (1:10 ratio) were used to establish cocultures of these cell lines. Cultures were grown for 1–15 days in the bioreactor with a 55-mL vessel (Synthecon, Houston, Texas) at 37°C and 5% (v/v) CO_2 as previously reported. The bioreactor rotation was started at 3 rpm and gradually increased each day, reaching 10 rpm on day 3, and maintained at this speed for the duration of the experiment.

Western Blot Analysis

Cells/beads were harvested from bioreactor cultures at specified times as previously reported.[7] Monolayer cultures were used as controls. Harvested cells/beads were washed several times with phosphate-buffered saline and solubilized in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-100, .2 mM AEBSF, .5 mM Benzamidine, 2 μg/mL aprotinin, .5 mM leupeptin. Total protein in the whole cell lysates was determined by using the Bio-Rad BCA method. Equal amounts of protein sampled from whole cell lysates were subjected to electrophoresis on 4%–20% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline, the membranes were incubated with primary antibodies (VEGF-A, VEGF-B, VEGF-C, FLK-1, and nuclear factor-κB [NFκB]) overnight at 4°C. All primary antibodies were diluted 1:100. Membranes were washed and incubated for one hour with a horseradish peroxidase-conjugated secondary antibodies at room temperature. All primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). The protein bands were developed with an enhanced chemiluminescent assay (Amersham Biosciences, Piscataway, NJ) and visualized by autoradiography. Western blots were quantitated by densitometry scanning and analyzed by using gel-pro software (Media Cybernetics, Silver Spring, Md).

Table 1. Gene-specific primers for reverse transcription PCR

Genes	Forward Sequence	Reverse Sequence
VEGF-A	GCACCCATGGCAGAAGGAGGAGG	TTCCCGAAACCCTGAGGGAGGCT
VEGF-C	AAGGAGGCTGGCAACATAAC	CCACATCTGTAGACGGACAC
FLK-1	TTGTGACCCAAGAATGTGTCTGTG	CGAACTCTACTTTAGCCCAACTCG
FLT-4	AGCCATTCATCAACAAGCCT	GGCAACAGCTGGATGTCATA

RNA Analysis

Total RNA was isolated from harvested cells/beads by using the Triazol reagent (Invitrogen) according to the manufacturer's protocol. The amount of total RNA was determined from the A260/280 nm ratio. The expression of angiogenic factors was confirmed by reverse transcription (RT)-PCR as previously detailed.[7] The oligonucleotide primer sequences obtained from GenBank for the forward and reverse primers of these factors are shown in Table 1. All reagents were obtained from Invitrogen. The PCR reaction was carried out in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Calif) for 35 cycles at the appropriate annealing temperature; 18S rRNA was used to verify RNA quantity. The PCR efficiency was evaluated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. The RT-PCR bands were quantitated by densitometry scanning and analyzed by using gel-pro software.

Statistical Analysis

All experiments were performed in three different independent experiments, data were pooled, and results are expressed as means plus or minus standard deviation. Student *t* test was used to analyze data, and a *P* value $<.05$ was considered significant.

RESULTS

Morphological Assessment

The retinal cells grown alone or cocultured with BAEC in the horizontally rotating bioreactor formed large (20–30 beads) 3D structures; the viability of cells in these cultures was >80%. More aggregation was observed for cocultured cells than for cells grown alone. Figure 1 shows phase contrast photomicrographs of retinal cells, grown alone or in coculture with BAEC, in a horizontally rotating bioreactor for one to five days. The cells started forming aggregates with a tissue-like assembly between the beads by day 5. Hoffman modulation photomicrographs clearly show the 3D assembly of the retinal/endothelial cell coculture maintained in the bioreactor for one and five days (Figure 2). Cord or capillary-like structures were also seen; the well-defined capillary-like structures, with some branching, were observed in the coculture system. Double immunostaining of retinal cells and BAEC for neuronal-specific enolase and von Willebrand factor indicated both cell types in coculture (data not shown).

Western Blot Analysis

We found that VEGF-A was expressed more in monolayer cultured

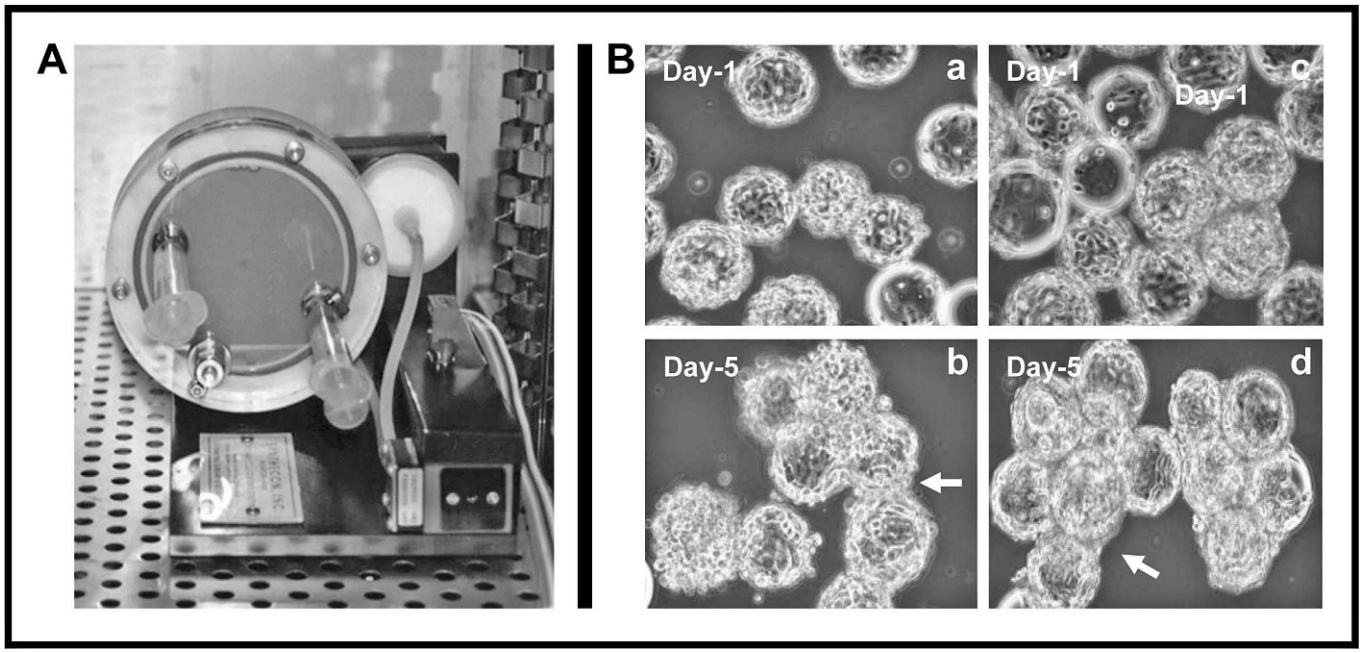

Figure 1. A) NASA-developed horizontally rotating bioreactor is designed to distribute cells uniformly and to provide the optimal concentrations of gases and nutrients. B) Phase micrographs of Retinal cells were cultured in horizontally rotating bioreactor (a-b) and Retinal cells were cocultured with endothelial cell (c-d) in horizontally rotating bioreactor for day 1 and 5, respectively.

retinal cells than in BAEC monolayer (Figure 3). Further, a threefold higher expression of VEGF-A was seen when retinal cells were cultured for one day in the horizontally rotating bioreactor, compared with control monolayer cultures (132±8 for monolayer vs 387±13 for horizontally rotating bioreactor, $P<.002$). No significant differences were observed in the expression of VEGF-C when retinal cells were cocultured with BAEC in five days. On the other hand, BAEC expression of VEGF-A was very low for monolayer cultures and did not show any change in expression when BAEC were cultured for one day in the horizontally rotating bioreactor. However, five days of culture in the bioreactor resulted in 11-fold higher expression of VEGF-A in BAEC (590±22) when compared with one day of bioreactor culture (55±4). We also noticed a twofold higher level (780±30 for one day vs 1520±36 for five days) of VEGF-A expression for retinal/BAEC cocultures maintained for five days in the horizontally rotating bioreactor.

In the horizontally rotating bioreactor, the expression of VEGF-B was elevated for retinal cells, whether cultured alone or in coculture with BAEC (Figure 3). The upregulation of VEGF-C was also seen for BAEC cultured alone or in coculture with retinal cells for five days in the horizontally rotating bioreactor. However, the VEGF receptor, FLK-1, was downregulated in bioreactor cells, compared with monolayer cultured cells. We did not find a difference in the level of NFκB in any of the bioreactor cultures (Figure 3).

RT-PCR Analysis

The expression of VEGF-A mRNA was low in retinal cells, whether cultured in monolayer or in the horizontally rotating bioreactor, after one and five days; however, a threefold increase was seen by 10 days in bioreactor-cultured cells (48±6 for 1 day vs 128±9 for 10 days, $P<.001$, Figure 4). Fivefold increased expression of VEGF-A was seen when both retinal cells and BAEC were cocultured for five days (52±4 for one day vs 240±15 for five days, $P<.001$). In addition to the above changes in VEGF-A mRNA, the expression of its receptor was also altered. Low FLK-1 expression was found for retinal cells grown as a monolayer or bioreactor cultures for 1 and 10 days. However, more than threefold higher FLK-1 mRNA expression was seen with retinal cells cocultured with BAEC in the bioreactor for five days (805±23) as compared with one day of coculture (232±11). We found low VEGF-C expression for retinal cells whether grown in monolayer or in the bioreactor. VEGF-C expression was upregulated by twofold when retinal cells were cocultured with BAEC in the bioreactor for five days (35±2 for retinal cells grown alone vs 75±3 for retinal cells coculture with BAEC, $P<.005$). We also found that FLT-4 (a VEGF receptor) mRNA was low for retinal cells grown as

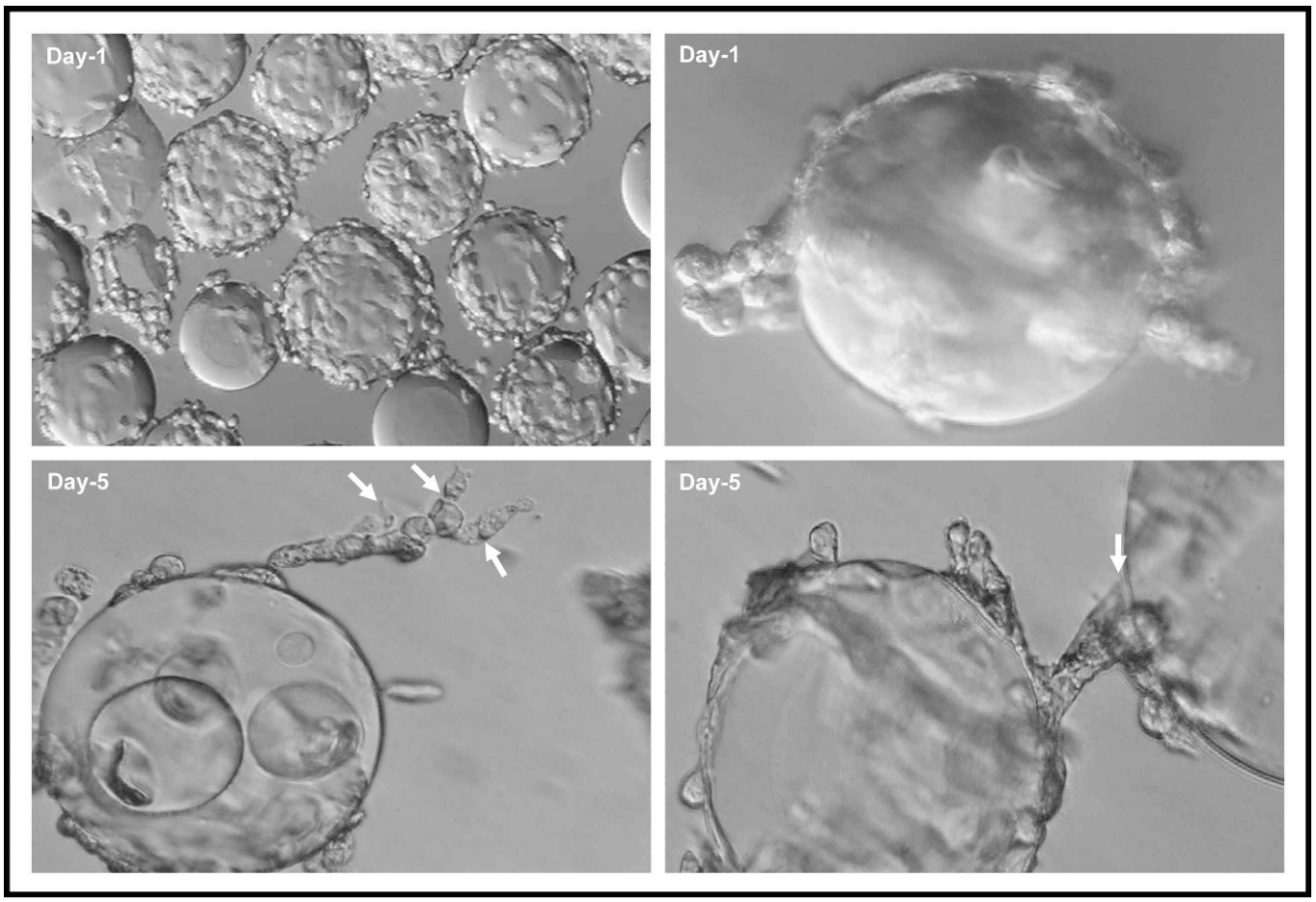

Figure 2. Photomicrographs of retinal and endothelial cell cocultures (days1 & 5) viewed by Hoffman modulation, showing the 3-D structure of these horizontally rotating bioreactor cocultures. The arrowheads point to areas where sprouting of newly formed capillaries can be seen.

monolayer (Figure 4), but fourfold higher (26±2 for monolayer vs 116±5 for five days in the bioreactor) in retinal cells cultured alone.

DISCUSSION

Several different cell types, including retinal, RPE, and endothelial cells participate in the pathogenesis of retinopathies and associated neovascularization seen in various disease states of the eye.[11–13] However, the effects of only one cell type or their secreted factors have been examined *in vitro* at a time. In this study, we used a 3D model that allowed us to investigate the interactions of multiple cell types.

In most *in vitro* models of neovascularization, formation of new capillary or vessel-like structures is slow, on the order of one to six weeks. We have previously shown that conditioned medium from RPE and retinal cells is capable of inducing the formation of capillary-like structures.[9] Additionally, we have shown that when retinal cells are cocultured with BAEC in a 3D model, capillary formation may begin as early as 24–36 hours. In this study, we found that retinal cells and BAEC, seeded in coculture in a 1:10 ratio on laminin-coated microcarrier beads, formed large 3D aggregates by five days of culture in the horizontally rotating bioreactor. These 3D assemblies showed evidence of capillary-like structures with some branching. This finding suggests that the microenvironment of the horizontally rotating bioreactor promoted the co-spatial localization of these two cell types that resemble the early stages of neovascularization seen in retinal diseases.

In some experimental models, conditioned media from retina and RPE cells was shown to induce capillary formation in bovine aortic or human umbilical vein endothelial cells, suggesting that factors secreted by retina or RPE could act as a trigger.[11–13] In choroidal neovascularization, subretinal neovascularization precedes ischemia, and substantial data show that RPE may be important in choroidal angiogenesis.[14] We are also aware of the fact

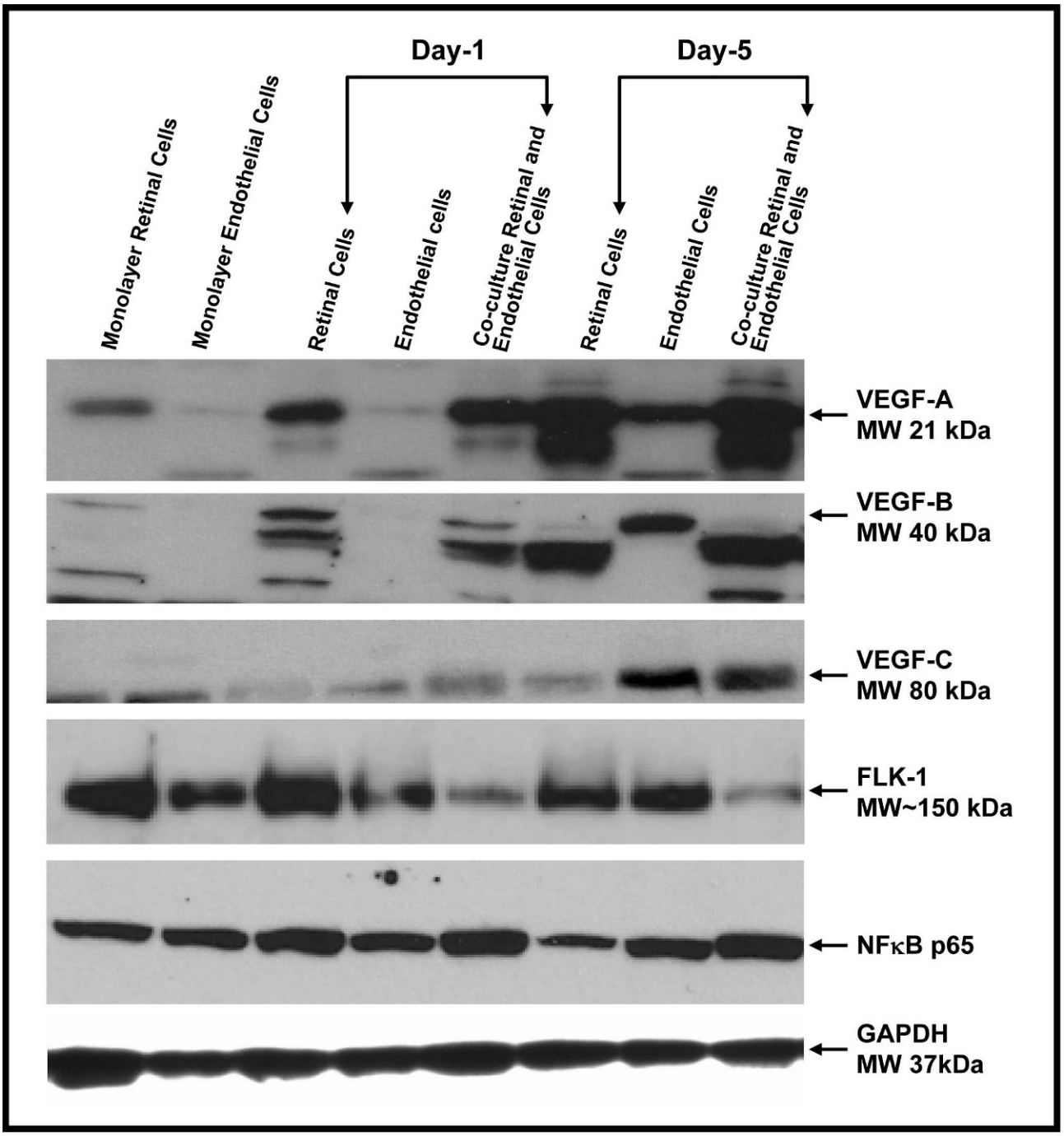

Figure 3. Western Blot Analysis of protein extracted from individual or in cocultures retinal and endothelial cell cultures monolayer and horizontally rotating bioreactor. These data are representative of three independent experiments.

that neovascularization occurs in diabetic retinopathy, whereas choroidal neovascularization occurs in age-related macular degeneration. Considerable data suggest that factors secreted from retina, RPE, pericytes, and endothelial cells themselves may all play a role in new blood vessel formation. A variety of growth factors have been identified within the eye that may play a role in neovascularization; three of these factors (bFGF, TGFβ, VEGF) have also been shown to induce neovascularization in animal models.[15–17] However, their precise roles in the pathophysiology of vasoproliferative retinopathies remain largely undefined. In the present study, we focused on VEGF and its receptors.

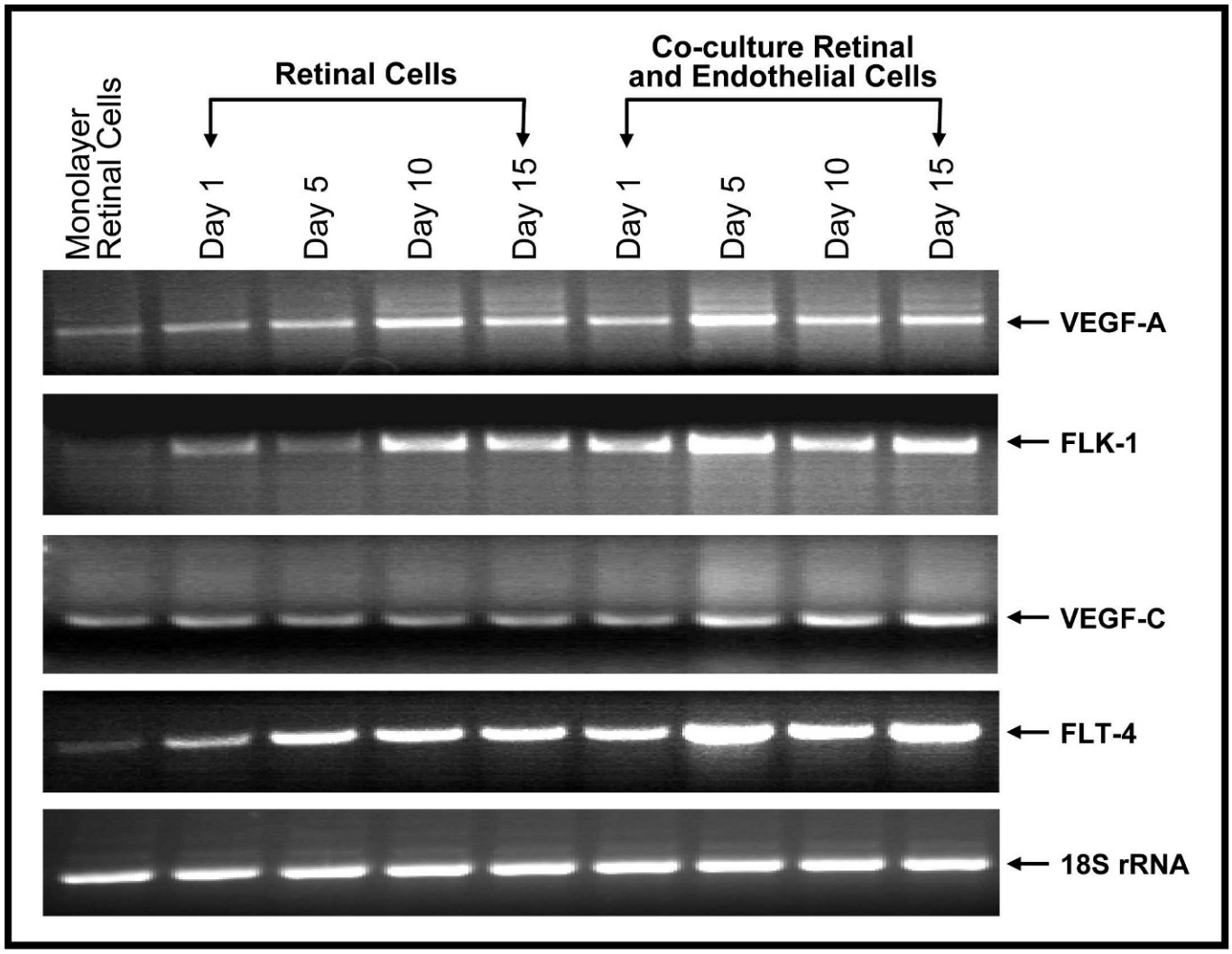

Figure 4. The expression of mRNA for different isotypes of VEGF, and the VEGF receptors, by retinal cell, individually and in co-culture with endothelial cell in the horizontally rotating bioreactor. Experimental detail has been given in the Methods section. Data are from one representative experiment of three. 18S RNA was used to normalize the amount of total RNA actually applied to the RT-PCR.

Our data suggest that interactions between retinal cells and endothelial cells, as well as the culture environment of the horizontally rotating bioreactor, modulated the expression of different VEGF isotypes both at the transcriptional and translation level. This complex pattern of expression provides additional evidence that retinal cells produce and secrete angiogenic growth factors, which is enhanced by coculture with BAEC or by culture in the horizontally rotating bioreactor. We found that retinal cells alone showed increased expression of VEGF-A mRNA with increasing culture time in the bioreactor, peaking at 10 days. These cells cocultured with endothelial cells produced maximal expression of VEGF-A mRNA at five days of bioreactor culture. This findings suggests 3D cell-cell interactions upregulate the transcriptional expression of this VEGF isotype. However, the addition of a second cell type to retinal cell cultures apparently enhanced the temporal expression of VEGF-A. Similarly VEGF-C mRNA expression is not affected by bioreactor culture for retinal cells alone but is upregulated when endothelial cells are cocultured with retinal cells. This relationship is not completely reflected in the expression of VEGF-A and VEGF-C protein. The expression of VEGF-A protein by cocultures was similar to what we observe for retinal cells alone, whereas VEGF-C expression patterns closely resembled what we observed for endothelial cells alone. Taken together, our data suggest that the interactions between endothelial and retinal cells may regulate the transcrip-

tion but not translational expression of proangiogenic growth factors.

In contrast, the expression of VEGF receptor protein (Flk-1) by endothelial cells was unchanged by bioreactor culture but was downregulated by coculture with retinal cells. The Flk-1 expression by cocultures may reflect more the response of retinal cells than endothelial cells, since the cocultures contained 10-fold more retinal cells than endothelial cells. However, retinal cells showed higher mRNA expression of Flk-1 and Flt-4 for bioreactor cultures than for monolayer cultures. Clearly, these studies suggest a complex regulation of angiogenic factors and their receptors in the bioreactor model. Further, these studies indicate that the transcriptional control is not reflected in protein expression.

In summary, our studies found that retinal and endothelial cells grown alone or in coculture in the horizontally rotating bioreactor formed 3D assemblies; cocultures developed capillary-like structures with evidence of branching. Second, culture in the bioreactor modulated the expression of VEGF isotypes and VEGF receptor, both mRNA and protein; coculture of the two cell lines in the bioreactor showed that the resultant cell-cell interactions modulated the expression of VEGF isotypes. Finally, the resultant VEGF expression was complex and suggested that regulation at the transcription level may be different from translational regulation by cells in bioreactor cocultures. Retinal and endothelial cells cocultured in the bioreactor may have an ideal combination of cell to cell interactions in three dimensions between similar and different cell types that leads to enhanced expression of proangiogenic growth factors and growth factor receptors. These cocultures showed evidence of angiogenic outgrowth much earlier than what has been found for similar monolayer coculture models. Hence, bioreactor cocultures may provide an excellent *in vitro* model for studying the mechanisms for ocular neovascularization.

Implications for Improving Health Disparities

The complications of hypertension and diabetes are prevalent in the minority community, which will ultimately lead to serious clinical disorders (stroke, cardiovascular disease, and eye diseases, eg, diabetic retinopathy). The main goal of our studies is to understand the factors involved in retinal neovascularization that could lead to therapies for retinal damage in humans, which ultimately will help the health of minorities and under-served populations.

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