Altered Gene Expression of Angiogenic Factors Induced by Calcium-Mediated Dissociation of Retinal Pigment Epithelial Cells

Xue-Feng Wang, Jing Z. Cui, Shiv S. Prasad, and Joanne A. Matsubara

PURPOSE. To examine the effect of loss of cell–cell contacts on the gene expression of vascular endothelial growth factor (VEGF) and other factors in primary culture of human retinal pigment epithelial (RPE) cells with real-time reverse transcription-PCR.

METHODS. The dissociation of postconfluent RPE cells was induced by calcium chelation, low-calcium medium, anti-E-cadherin, and anti-N-cadherin antibodies. Total RNA was isolated from the cultured RPE cells and reverse transcribed to cDNA. VEGF was quantified by real-time PCR with a fluorescence detector. VEGF isoforms were differentially measured by specific exon-spanning primers. Besides VEGF, the gene expression levels of some other growth factors were also examined in calcium-mediated dissociation.

RESULTS. Disruption of cell–cell contacts of RPE cells was induced by calcium chelation and low-calcium medium, but not by anti-E-cadherin and anti-N-cadherin antibodies. Calcium-mediated dissociation of RPE cells significantly increased the gene expression levels of VEGF. The mRNA levels of VEGF increased by 6.3-fold on treatment with EGTA and by 4.7-fold in the low-calcium medium at 6 hours. Splice variants of VEGF showed the differential pattern of gene expression. Whereas the expression of VEGF121 and VEGF165 was upregulated on calcium-induced dissociation of RPE cells, that of VEGF145 and VEGF189 was unchanged. VEGF206 was not detected. On calcium-induced dissociation, bFGF, IL-6, matrix metalloproteinase (MMP)-1, and placental growth factor (PIGF) were upregulated, whereas acidic (α)FGF and pigment epithelium–derived factor (PEDF) were both downregulated.

CONCLUSIONS. The results show that loss of intercellular contacts promotes increased gene expression of VEGF and other angiogenic factors in human RPE cells. (Invest Ophthalmol Vis Sci. 2005;46:1508–1515) DOI:10.1167/iovs.04-0951

Choroidal neovascularization (CNV) in age-related macular degeneration (AMD) is the leading cause of severe visual impairment in the elderly population.1–3 The abnormally formed new vessels originate from the choroid, penetrate Bruch’s membrane, and grow into the subretinal space. Vascular endothelial growth factor (VEGF), also referred to as VEGF-A, is one of the most potent angiogenic factors identified to date and has been implicated in CNV.4–8 Other growth factors that promote angiogenesis include basic fibroblast growth factor (bFGF, also FGF-2),9–10 acidic fibroblast growth factor (aFGF, also FGF-1),11 angiogenin,1,2 angiopoietin-1,13 interleukin (IL)-6,14 leptin,15,16 matrix metalloproteinases (MMPs),17,18 placental growth factor (PIGF),19 and platelet-derived growth factor (PDGF).20 Angiogenesis is also regulated by inhibitors or angiostatic factors, such as pigment epithelium–derived factor (PEDF)21,22 and tissue inhibitor of metalloproteinase (TIMP)-1 and its family members.23–24 The exact role of these factors in CNV is still under debate.

RPE cells secrete angiogenic factors and modulators, such as VEGF and PEDF.25,26 This cell type has also been shown to have diverse functions in the maintenance of retinal integrity and probably plays a crucial role in the formation of CNV.25–53 However, the triggers that cause RPE cells to secrete angiogenic factors are still unknown. Oxidative stress is one factor that has been shown in experiments to cause the upregulation of angiogenic factors by RPE cells in vitro.34,35 The lipid peroxide 4-hydroxynonenal has been found to increase VEGF expression in RPE cells in vitro.56 Application of hydrogen peroxide to RPE cells in vitro does not change VEGF levels, but reduces the expression of PEDF in RPE cells, leading to an imbalance between angiogenic and angiostatic factors.22 Aging is also an important factor implicated in the pathogenesis of AMD. We have recently analyzed the differential gene expression levels of VEGF and other angiogenic factors and have found that there is no enhanced expression of known angiogenic factors in late passage (reproductively senescent) RPE cells, suggesting that aging, per se, is unlikely to be a causal factor in promoting CNV.

In the present study, we investigated the effects of cell–cell interaction on the expression of angiogenesis-associated factors in RPE cells. Cell death of RPE occurs in aging eyes and pathologic conditions, such as oxidative stress, resulting in a partial loss of contacts with neighboring cells.58–60 Although it has been reported that disruption of cadherin-related adherens junctions increases VEGF expression in aortic endothelial cells,61 the effects of cell–cell adhesion on the expression of angiogenesis-associated factors in RPE cells have not been investigated. Cadherins are a superfamily of calcium-dependent cell adhesion molecules.62 The dynamic expression and distribution of cadherins, particularly E- and N-cadherin, in RPE cells have been extensively studied by Burke et al.43,44 In the present study, we used real-time, quantitative reverse transcription-PCR to quantify the gene expression levels of VEGF and other factors on calcium-mediated cell dissociation in primary RPE cells. The effects of anti-E- and N-cadherin antibodies on cell dissociation were also examined.

MATERIALS AND METHODS

Tissue Culture of Primary RPE Cells

The primary human RPE cells were obtained from donor tissues as described previously.27 The cells of passages 3 through 5 were used. They were cultured in laminin-coated flasks and maintained in a hu-
midified atmosphere with 5% CO₂. The culture medium is Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). RPE cells were plated at 1 × 10⁵ cells per well in six-well plates for both control and experimental groups. The cells reached confluence after approximately 1 week and were used after further culturing of 5 to 10 days.

**Calcium-Dependent Dissociation of Confluent RPE Cells**

Three methods were used to disrupt calcium-dependent intercellular adhesion of confluent RPE cells: calcium chelation by EGTA, low-calcium medium, and calcium-switch with anti-E- and N-cadherin antibodies. One day before these treatments, the medium of all cultures, including the control cultures, was replaced by 1% FBS supplemented DMEM. For EGTA treatment, DMEM was added to the medium at a final concentration of 1.8 mM, equivalent to the calcium level of normal DMEM. Samples were collected at different time intervals.

For low-calcium (10 μM) treatment, the medium was changed to calcium-free DMEM with 1% FBS. For the calcium-switch experiment, antibodies to anti-E-cadherin (Clone SHE78-7; Calbiochem, La Jolla, CA) and anti-N-cadherin (Clone GC-4; Sigma-Aldrich, St. Louis, MO) were used. In this experiment, the culture medium was first changed to low-calcium medium at a final concentration of 4 mM on the day of the experiment. For high-calcium (10 μM) treatment, the medium was changed to calcium-free DMEM with 1% FBS. For the calcium-switch experiment, antibodies to anti-E-cadherin (Clone SHE78-7; Calbiochem, La Jolla, CA) and anti-N-cadherin (Clone GC-4, Sigma-Aldrich, St. Louis, MO) were used. In this experiment, the culture medium was first changed to low-calcium medium at a final concentration of 4 mM on the day of the experiment. For high-calcium (10 μM) treatment, the medium was changed to calcium-free DMEM with 1% FBS. For the calcium-switch experiment, antibodies to anti-E-cadherin (Clone SHE78-7; Calbiochem, La Jolla, CA) and anti-N-cadherin (Clone GC-4, Sigma-Aldrich, St. Louis, MO) were used. In this experiment, the culture medium was first changed to low-calcium medium at a final concentration of 4 mM on the day of the experiment.

**Immunohistochemical Staining of Actin and Cadherins**

Cultured RPE cells were fixed in 4% formaldehyde solution and permeated by 0.1% Triton X-100 in PBS. Cells were preincubated in PBS containing 1% FBS for 30 minutes to reduce nonspecific binding. Fluorescent Texas red-X phalloidin (Molecular Probes, Eugene, OR) was then used to stain F-actin. For immunostaining of the cadherins, fixed monolayers were incubated with primary antibodies of anti-E-cadherin (1 μg/mL) or anti-N-cadherin (1:100) overnight at 4°C. FITC-conjugated anti-mouse IgG was used as secondary antibody to visualize the stained cells.

**RT-PCR and Quantitative Real-Time PCR**

Total RNA was isolated from the cultured RPE cells using TRIzol (Invitrogen, Carlsbad, CA). The RNA sample was then treated with DNase I to remove genomic DNA contaminants. The RNA yields for both control and experimental groups did not display any significant difference. The cDNA was synthesized by reverse transcription (SuperScript II reverse transcriptase; Invitrogen) and random primers.

The splice variants of VEGF were amplified by PCR and visualized in agarose gel. The VEGF gene is composed of eight exons. The reported splice variants of VEGF contain VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206, according to the lengths of amino acids in the mature proteins. The common sequence of all variants is exons 1 to 5 and exon 8 (Fig. 1). Therefore, the following primers, positioned on exons 1 and exon 8, respectively, were used to amplify all isoforms: 5’-TGCTGTCTTGGGTGCATTGGA 3’ (forward) and 5’-GAGGATCCTTCCCGAAA 3’ (reverse). The cDNA samples were amplified by TaqDNA polymerase (Invitrogen) in 50 mM KCl, 20 mM Tris-HCl, 1.5 mM MgCl₂, 0.2 mM dNTP and 0.4 μM of each primer. PCR was performed at 94°C for 2 minutes, followed by 35 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 1 minute.

The primers for quantifying VEGF isoforms in real-time PCR are shown in Figure 1 and Table 1. The mRNA sequences of VEGF121, VEGF145, VEGF165, and VEGF189 are from Keck et al. (GenBank accession no. M27281; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), whereas that of VEGF206 is from Houck et al. (GenBank accession no. S85192). The only difference of these two reported sequences is the length of exon 6, with the latter designated as exon 6. Primer synthesis was performed with the manufacturer’s protocol. The reactions were performed at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The correct amplification of a specific product was confirmed by the dissociation temperature of the product and agarose gel electrophoresis. Relative quantitation of gene expression was performed using a standard curve method. Briefly, the analysis was based on the threshold cycle (Ct) values of the target genes with glycolaldehyde-3-phosphate dehydrogenase (GAPDH), as the endogenous control. The standard curve for each amplified product was constructed from the four serially diluted samples in duplicate, starting with a 1:20 dilution of cDNA and 1:2 diluted to 1:40, 1:80, and 1:160. Each standard curve was used to calculate the relative amount of unknown samples for that product.
values of the experimental samples to that of the control. The effects of calcium-mediated cell dissociation on the gene expression of some other angiogenic or angiostatic factors were also examined. The primers for these factors are listed in Table 2.

**RESULTS**

We first examined the effects of three experimental methods on cell–cell adhesion of RPE cells. Shortly after administration of 4 mM EGTA to the RPE cell cultures, disruption of cell–cell contacts was evident, as observed under phase-contrast microscopy and immunostaining with F-actin antibody (Fig. 2). Dissociation of intercellular junctions also occurred when free Ca\(^{2+}\) concentration was lowered to approximately 10 \(\mu M\) in low-calcium medium. The cadherins, such as E- and N-cadherin, are major homophilic cell–cell adhesion molecules. Immunocytochemical staining of the RPE cultures with antibodies to E- and N-cadherin showed their existence at intercellular sites (data not shown). These antibodies have been used to induce the disruption of intercellular adherens junctions.\(^{43,50}\)

However, treatment with blocking antibodies against E-cadherin (3 \(\mu g/mL\)) or N-cadherin (1:30) in calcium-switch experiments did not induce dissociation of RPE cells (Fig. 2). Other concentrations of the anti-E-cadherin (0.1–3 \(\mu g/mL\)) and anti-N-cadherin (1:1000–1:30) antibodies, as well as the combinations of the anti-E- and anti-N-cadherin antibodies in calcium-switch experiments, did not change with treatments with anti-E- and anti-N-cadherin antibodies in calcium-switch experiments. These results suggest that gene expression of VEGF is highly regulated by intercellular adhesion of RPE cells whose dissociation is induced by calcium chelation and low calcium concentration, but not by cadherin blocking. The time course of VEGF expression was examined in RPE cultures treated with EGTA and low-calcium medium (Fig. 4). Total VEGF gene expression increased at 3 hours after addition of 4 mM EGTA and low-calcium medium significantly increased, whereas VEGF levels did not change with treatments with anti-E- and anti-N-cadherin antibodies in calcium-switch experiments. The increased expression reached peak levels at approximately 6 hours and gradually decreased within 24 hours.

There are several VEGF isoforms generated by alternative splicing from a single gene. The splice variants, including V\(_{121}\), V\(_{145}\), V\(_{165}\), V\(_{189}\), and V\(_{206}\), have been identified in human tissue. These VEGF species have been implicated in different functions in a variety of tissues. In the present experiment, we used specific boundary-spanning primers to quantify the VEGF isoforms in calcium-dependent cell dissociation. First, the existence of VEGF isoforms in RPE cell cultures was examined. Total RNA from cultured RPE cells was reverse transcribed to cDNA. Standard RT-PCR was used to amplify all VEGF splice variants with primers on exons 1 and 8. Four amplified species, cell–cell adhesion and VEGF gene expression, real-time PCR was used to measure VEGF gene expression under different conditions. Total VEGF mRNA was quantified with the primers on exon 3 to measure all splice variants of VEGF. GAPDH was used as the internal control. As shown in Figure 3, the total VEGF mRNA levels at 6 hours after treatment of EGTA and low-calcium medium significantly increased, whereas VEGF levels did not change with treatments with anti-E- and anti-N-cadherin antibodies in calcium-switch experiments. These results suggest that gene expression of VEGF is highly regulated by intercellular adhesion of RPE cells whose dissociation is induced by calcium chelation and low calcium concentration, but not by cadherin blocking. The time course of VEGF expression was examined in RPE cultures treated with EGTA and low-calcium medium. The increased expression reached peak levels at approximately 6 hours and gradually decreased within 24 hours.

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TABLE 2. Primer List Used for Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward Primer (5’–3’)</th>
<th>Reverse Primer (5’–3’)</th>
<th>Amplicon Length (bp)</th>
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<tr>
<td>aFGF</td>
<td>X65779</td>
<td>CCTTGGGGATGCGACAGT</td>
<td>ACATTCCCTCATGTTGTTGGTGT</td>
<td>71</td>
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<tr>
<td>Angiogenin</td>
<td>M11567</td>
<td>TTCTGAGCAGCGAGCATATGATG</td>
<td>GCTGCTGGCATGAGTGGTCCAC</td>
<td>74</td>
</tr>
<tr>
<td>Angiopoietin</td>
<td>U83508</td>
<td>GGAAGAATGTCATGAGAAATATACACTCA</td>
<td>GGCGCATTCCAGCAGTTGAT</td>
<td>83</td>
</tr>
<tr>
<td>bFGF</td>
<td>J04513</td>
<td>CCGAGGCGAGTTGACGAGAGACACAGAT</td>
<td>TGACGGTACGACTGACACACTTTCAC</td>
<td>112</td>
</tr>
<tr>
<td>GAPDH</td>
<td>X01677</td>
<td>CATCCATGAGCAAACTTGGTATGCTG</td>
<td>GAATCTTGGGTTGGGATGGA</td>
<td>74</td>
</tr>
<tr>
<td>IL-6</td>
<td>X01960</td>
<td>GGATGACCTCCAGCAGCGATC</td>
<td>GTGGCTCTGTGGCTGGCTGCCCAC</td>
<td>81</td>
</tr>
<tr>
<td>Leptin</td>
<td>U18915</td>
<td>TCAAGGGCATTGAATGAGCTTTCAC</td>
<td>CCCAGGAGAATGAGCTCCAACC</td>
<td>80</td>
</tr>
<tr>
<td>MMP-1</td>
<td>X54925</td>
<td>TGGAGAATGTTTCTGATCAGATGCTG</td>
<td>CTGTCCTAGGAGCAGGGGATCAT</td>
<td>78</td>
</tr>
<tr>
<td>MMP-2</td>
<td>J03210</td>
<td>TGGTCTCCTCCTCCCTTGGTCTG</td>
<td>GAGTCGGTGGTGCTGCACTGTA</td>
<td>112</td>
</tr>
<tr>
<td>PEDF</td>
<td>M76097</td>
<td>TGGGACAGGCGGAAATGCTG</td>
<td>GACGCTGAGTCGACTGAAATTA</td>
<td>131</td>
</tr>
<tr>
<td>PIGF</td>
<td>X54936</td>
<td>TGGGAGAATGCGCCAGTCTG</td>
<td>GAATCTTGGGTTGGGATGGA</td>
<td>74</td>
</tr>
<tr>
<td>PDGF</td>
<td>X02811</td>
<td>CGATCGCCCTCCTTTGATGAT</td>
<td>TCCAACTGGCCGCATCT</td>
<td>73</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>X03124</td>
<td>CGCTGACATCCGCTTCCTG</td>
<td>GTTGTGGGAGCTGTTGGGATATC</td>
<td>70</td>
</tr>
</tbody>
</table>

In total, 12 angiogenesis-related genes were selected, and GAPDH was used as the internal control.
corresponding to V121, V145, V165, and V189, were shown in the gel (Fig. 5A). No amplified product corresponding to V206 was found. VEGF isoforms were also examined by real-time quantitative RT-PCR with their corresponding primers, as shown in Table 1. Consistently, the amplified products of V121, V145, V165, and V189, but not V206, were identified in agarose gel (Fig. 5B). The same results were obtained from the RPE cell samples treated with EGTA and low-calcium medium (data not shown).

The changes of VEGF isoforms under the condition of calcium-dependent cell–cell dissociation were quantified by real-time quantitative RT-PCR. The primers for amplifying total VEGF and its splice variants were shown in Figure 1 and Table 1. The cultured RPE cells were treated with 4 mM EGTA and low-calcium medium for 6 hours. GAPDH was used as the internal control. Leptin cDNA was not detected. The results showed that the gene expression levels of bFGF increased and that of aFGF decreased significantly in RPE cells treated by both EGTA and low-calcium medium (Table 3). RPE cells treated with EGTA, but not low-calcium medium, showed the upregulated expression of IL-6, MMP-1, and PlGF and downregulated expression of PEDF. It is unknown why the changes of these factors were not observed in the treatment of low-calcium medium. The gene expression levels of other factors, including angiogenin, angiopoietin, MMP-2, PDGF, and TIMP-1 were unchanged in calcium-mediated dissociation.

**DISCUSSION**

Angiogenesis is a highly regulated process that is under the control of angiogenic stimulators and inhibitors. Unregulated

![Figure 3](image3.png)

**Figure 3.** Changes of VEGF expression induced by calcium chelation, low calcium and anti-cadherin antibodies. Postconfluent RPE cells were treated for 6 hours under the following conditions: EGTA (4 mM), low-calcium medium, anti-E-cadherin (3 μg/mL), and anti-N-cadherin (1:30) antibodies in calcium-switch experiments. The cDNA was synthesized by reverse transcription. The expression levels of total VEGF (including all splice variants) were quantified by real-time PCR with the primers on exon 3 as shown in Figure 1. Values are expressed as x-fold changes compared with the control. Data represent the mean ± SEM of duplicate samples in four independent experiments. *Significantly different from the control (P < 0.01).

Besides VEGF, many other growth factors have been implicated in angiogenesis. The effects of calcium-mediated intercellular dissociation on the gene expression of these factors were investigated. Twelve angiogenesis-related factors were selected for real-time PCR examination (Table 2). GAPDH was used as the internal control. Leptin cDNA was not detected. The results showed that the gene expression levels of bFGF increased and that of aFGF decreased significantly in RPE cells treated by both EGTA and low-calcium medium (Table 3). RPE cells treated with EGTA, but not low-calcium medium, showed the upregulated expression of IL-6, MMP-1, and PlGF and downregulated expression of PEDF. It is unknown why the changes of these factors were not observed in the treatment of low-calcium medium. The gene expression levels of other factors, including angiogenin, angiopoietin, MMP-2, PDGF, and TIMP-1 were unchanged in calcium-mediated dissociation.

![Figure 4](image4.png)

**Figure 4.** Time course of VEGF expression on calcium-dependent cell dissociation. The postconfluent cultures were treated with EGTA and low-calcium medium. The samples were collected at different time intervals. Total VEGF levels were measured by real-time PCR with the primers on exon 3. Data are expressed as changes (x-fold) with respect to the control. Data represent the mean ± SEM of duplicates in four independent experiments. *Significantly different from the control (P < 0.01).
angiogenesis is seen under pathologic conditions, including CNV in the wet form of AMD. As discussed earlier, RPE cells have been found to secrete angiogenesis-associated factors and modulate the formation of choroidal capillaries. It is important, therefore, to know the potential external or internal stimuli that may alter the homeostasis of these growth factors. In the present study, we investigated the effects of disruption of cell–cell contacts on the gene expression of some selected angiogenesis-associated factors in cultured RPE cells. Our results show that gene expression levels of a number of factors are significantly affected by calcium-dependent intercellular dissociation. VEGF, the major angiogenic factor, has the highest increase of gene expression. Other factors, such as bFGF, 

**FIGURE 5.** Identification of amplified products of VEGF isoforms. The cDNA sample of confluent RPE cells was amplified by PCR and real-time PCR. (A) PCR products amplified with primers on exons 1 and 8. The expected lengths (base pairs) of the VEGF isoforms are 485 (V121), 557 (V145), 617 (V165), 689 (V189), and 740 (V206). (B) Real-time PCR products amplified with each pair of primers shown in Table 1. V260 products were not detected in both cases. Similar results were also observed from RPE cells treated with EGTA and low-calcium medium (not shown).

**TABLE 3.** Relative Gene Expression of Angiogenesis-Related Factors in Calcium-Mediated RPE Cell Dissociation

<table>
<thead>
<tr>
<th>Gene</th>
<th>EGTA/Control</th>
<th>Change</th>
<th>Low Ca/Control</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>aFGF</td>
<td>0.24 ± 0.02</td>
<td>▼</td>
<td>0.54 ± 0.09</td>
<td>▼</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>1.52 ± 0.23</td>
<td>↑</td>
<td>1.17 ± 0.14</td>
<td>↑</td>
</tr>
<tr>
<td>Angiopoietin</td>
<td>0.78 ± 0.24</td>
<td>↑</td>
<td>1.04 ± 0.11</td>
<td>↑</td>
</tr>
<tr>
<td>bFGF</td>
<td>1.60 ± 0.07</td>
<td>↑</td>
<td>1.63 ± 0.14</td>
<td>↑</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.86 ± 0.19</td>
<td>↑</td>
<td>0.88 ± 0.34</td>
<td>↓</td>
</tr>
<tr>
<td>MMP-1</td>
<td>2.16 ± 0.28</td>
<td>↑</td>
<td>1.56 ± 0.24</td>
<td>↑</td>
</tr>
<tr>
<td>MMP-2</td>
<td>0.85 ± 0.08</td>
<td>↑</td>
<td>1.08 ± 0.11</td>
<td>↑</td>
</tr>
<tr>
<td>PEDF</td>
<td>0.69 ± 0.04</td>
<td>↓</td>
<td>1.21 ± 0.05</td>
<td>↓</td>
</tr>
<tr>
<td>PIGF</td>
<td>2.32 ± 0.12</td>
<td>↑</td>
<td>1.12 ± 0.16</td>
<td>↑</td>
</tr>
<tr>
<td>PDGF</td>
<td>1.18 ± 0.05</td>
<td>↑</td>
<td>1.67 ± 0.19</td>
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<tr>
<td>TIMP-1</td>
<td>1.06 ± 0.07</td>
<td>↑</td>
<td>1.11 ± 0.05</td>
<td>↑</td>
</tr>
</tbody>
</table>

Data are expressed at the mean ± SD. RPE cultures were treated with 4 mM EGTA and low-calcium medium for 6 hours. In total, 12 angiogenesis-related genes were examined, and GAPDH was used as the internal control. Leptin was not detected. Values represent mean ± SEM of four independent experiments in duplicate. †, increased significantly (P < 0.01); ‡, decreased significantly (P < 0.01). Others show no significant change.

**FIGURE 6.** Expression of individual splice variants of VEGF on calcium-dependent cell dissociation. Postconfluent RPE cells were treated with EGTA and low-calcium medium for 6 hours. The cDNA was synthesized by reverse transcription. Total VEGF and each splice variant were quantified by real-time PCR with the specific primers shown in Table 1. V260 was not amplified in all the samples. Data are expressed as relative changes (x-fold) in comparison with the corresponding control cultures. Data represent the mean ± SEM of duplicates in four independent experiments. *Significantly different from the control (P < 0.01).
of certain areas of confluent cells, needs to be developed to clarify the role of Ca\textsuperscript{2+} ions.

RPE cells on Ca\textsuperscript{2+}-mediated dissociation show the differential gene expression of VEGF isoforms in our study. Of the five reported splice variants of VEGF, V\textsubscript{206} was not detected in RPE cells. V\textsubscript{121} and V\textsubscript{165} were significantly upregulated on Ca\textsuperscript{2+}-mediated disruption of cell–cell contacts, whereas V\textsubscript{145} and V\textsubscript{189} were unchanged. This result reflects the different biological activities of each VEGF isoform. Consistent with this observation, other reports have shown the distinguished function of V\textsubscript{121} and V\textsubscript{165} in promoting angiogenesis.\textsuperscript{47,54–56} For example, using transiently expressed VEGF clones, Houck et al.\textsuperscript{47} found that V\textsubscript{121} and V\textsubscript{165}, but not V\textsubscript{189} and V\textsubscript{206}, possess endothelial cell mitogenic activity. A clinical study showed that VEGF\textsubscript{121} and VEGF\textsubscript{165} are more significantly related to synovial endothelial cell mitogenic activity. A study showed that angiogenic stimulators and inhibitors displayed a synergistic effect of multiple angiogenic factors in different cell types. For example, combined administration of VEGF and bFGF to endothelial cells in three-dimensional collagen gels results in much greater and more rapid capillary tubule formation than the additive effects of either mitogen alone.\textsuperscript{63,64} This synergism of VEGF and bFGF was also found in the capillary formation in an animal model of hind limb ischemia.\textsuperscript{65} The VEGF isoforms have different heparin-binding properties. The VEGF isoforms have different heparin-binding properties.

Calcium-Mediated Dissociation of RPE Cells

Besides VEGF, many other factors are involved in the angiogenesis process. Of the limited number of growth factors we examined, angiogenic stimulators and inhibitors displayed a differential expression pattern. bFGF is another major angiogenic factor that has been described.\textsuperscript{59–61} In our study, bFGF was upregulated in Ca\textsuperscript{2+}-mediated dissociation of RPE cells. Other genes that showed increased gene expression included PlGF, MMP-1, and IL-6. PlGF shares 53% amino acid sequence identity with VEGF and is capable of stimulating endothelial cell growth in vitro.\textsuperscript{13} MMPs are a family of degradative enzymes involved in the breakdown of ECM in normal physiological functions and disease processes, including neovascularization.\textsuperscript{13,62} IL-6 also plays a role in angiogenesis.\textsuperscript{14} Our study shows the decreased gene expression of aFGF and PEDF on calcium chelation. The reason for the downregulation of aFGF is unknown, because aFGF is considered an angiogenic factor.\textsuperscript{11} PDEF is a potent inhibitor of angiogenesis.\textsuperscript{21} Ohno-Matsui et al.\textsuperscript{22} reported that H\textsubscript{2}O\textsubscript{2} induces a dose-dependent decrease of PEDF expression in differentiated RPE cells, whereas VEGF expression is unchanged. They suggested that the oxidative-stress-induced vessel formation results from the imbalance of VEGF and PEDF, rather than a single-factor action.

These data demonstrate that synergistic efforts from angiogenic factors and inhibitors are necessary for the multistep process of angiogenesis that consists of degradation of basement membrane, migration, and proliferation of endothelial cells, canalization, and branching, for example.

There are several lines of evidence that show the possible interaction among multiple angiogenic factors in different cell types. For example, combined administration of VEGF and bFGF to endothelial cells in three-dimensional collagen gels results in much greater and more rapid capillary tubule formation than the additive effects of either mitogen alone.\textsuperscript{53,64} This synergism of VEGF and bFGF was also found in the capillary formation in an animal model of hind limb ischemia.\textsuperscript{65} The combinations of other growth factors, such as bFGF and PlGF or VEGF and insulin-like growth factor (IGF)-1, have shown similar synergistic effects on cell migration and proliferation of retinal endothelial cells.\textsuperscript{66} These results demonstrate the convergent action of multiple growth factors in processes leading to angiogenesis. Though the mechanism is largely unknown, some growth factors stimulate the production of receptors for other growth factors and act synergistically at this level.\textsuperscript{59,60}

Growth factors may also regulate the gene expression levels of other growth factors in an autocrine or paracrine manner. It has recently been reported that VEGF induces MMP-1 and IL-6 expression, and these findings suggest that VEGF is an autocrine stimulator in chondrocytes.\textsuperscript{67} It was also found that bFGF could upregulate VEGF expression in different cell types, such as endothelial cells,\textsuperscript{50} mesothelial cells,\textsuperscript{71} and smooth muscle cells.\textsuperscript{72} It is still unknown whether RPE cells share similar regulatory mechanisms, but it is highly possible. These studies point to the complex interactions among angiogenic factors, and highlight the need for further understanding of the synergistic effects, as well as the individual effects, of these growth factors.

Upregulation of a variety of growth factors, or a balance shift toward angiogenesis, was also reported in the wound-healing response. Mainly studied in skin wound-healing models, the family members of growth factors, including VEGF, FGF, PDGF, IGF, and EGF, were shown to have increased expression at the wound site.\textsuperscript{73} Similarly, induction of experimental CNV by laser burns increases the expression levels of VEGF and its receptor, as well as bFGF, in RPE cells.\textsuperscript{74,75} There is evidence that wound healing may be a factor associated with the pathogenesis of AMD.\textsuperscript{76} In the wound-healing models, cells at the edges of wounded monolayers not only lose cell–cell contacts, but also initiate an inflammatory response. Thus, in the wound-healing models, multiple factors may be associated with the balance shift toward angiogenesis. However, in the present study, we focused on the role of cell–cell contacts and its relationship to the balance shift toward angiogenesis.

Evidence has been obtained from human donor eyes that RPE cells may undergo apoptosis due to aging and/or oxidative stress caused by such injuries as UV light damage or trauma.\textsuperscript{77} The loss of cell–cell contacts within the RPE monolayer may be one of the triggers leading to a balance shift toward angiogenesis. If surrounding cells do not migrate into and replace the dead or dying cells, a chronic upregulation of angiogenic factors from the surviving cells may ensue. There is indirect evidence of this process, in that it has been found that focal CNV is associated with surviving RPE cells.\textsuperscript{78} More efforts are needed to clarify the role of cell–cell contacts on the RPE cell in CNV and other angiogenic processes in the eye.

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References


