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# Choroidal endothelial cells transmigrate across the retinal pigment epithelium but do not proliferate in response to soluble vascular endothelial growth factor

Pete Geisen, Janet R. McColm, M. Elizabeth Hartnett *

*Department of Ophthalmology, University of North Carolina Chapel Hill, 103 Mason Farm Road, CB No. 7041, 6135 NSRB, Chapel Hill, NC 27599, USA*

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**Abstract**

The purpose of this study was to investigate the effects of soluble VEGF on human choroidal endothelial cell (CEC) transmigration across an RPE monolayer as it relates to choroidal neovascularization in AMD.

In coculture assays, ARPE-19 (ARPE) was plated on the undersides of Transwell inserts having 0.4 μm pores. Primary human CECs were then plated into the insert. CECs in the Transwell inserts were counted after 72 hr of growth. CEC proliferation was also measured after culturing CECs in ARPE–CEC coculture-conditioned media or in media with exogenous $VEGF_{121}$ and/or $VEGF_{165}$ added. Transmigration assays were performed on Transwells with 8.0 μm pores: green-labelled CECs were plated in Transwell inserts with or without red-labelled ARPE plated on the undersides of the insert. In some transmigration assays, ARPE was plated into the wells to provide a chemotactic gradient for CEC transmigration. After 72 hr CECs were plated, green cells were counted either within the well media as CECs that transmigrated the epithelial monolayer, or on the underside of the insert as CECs that transmigrated the Transwell insert to but not beyond the ARPE monolayer. A neutralizing antibody to VEGF was added to the wells of Transwells at the time the CECs were plated in the insert and transmigrated CECs were counted. VEGF protein was measured in the conditioned media of ARPE and CEC coculture and in transmigration assays.

Compared to control, CEC proliferation significantly increased when CECs were cultured in coculture conditioned media ($p=0.001$) or in coculture assays ($p<0.001$). However, there was no effect on CEC proliferation when $VEGF_{121}$, $VEGF_{165}$, or both were added to solo CECs. Antibody to VEGF did not reduce the proliferative effects of coculture conditioned media on CEC. ARPE plated in the well significantly increased CEC transmigration ($p<0.001$) compared to transmigration assays without ARPE in the well. VEGF protein measured in the well media of transmigration assays having ARPE within the well was significantly greater than in the assays without ARPE within the well ($p<0.004$). Exogenous neutralizing antibody to VEGF significantly reduced transmigration, and this effect was dose-dependent.

VEGF provides a chemotactic gradient for human CECs to transmigrate across a monolayer of ARPE. Neutralization of VEGF in the media partially reduces transmigration. Whereas soluble VEGF does not increase proliferation of solo CECs, coculture conditioned media enhances proliferation, suggesting that growth factors other than VEGF cause CEC proliferation. These findings may have relevance to the transformation of occult CNV into CNV within the neurosensory retina in AMD.


*Keywords:* choroidal neovascularization; age-related macular degeneration; vascular endothelial growth factor

## 1. Introduction

Choroidal neovascularization (CNV) in the neurosensory retina causes severe central vision loss in age-related macular degeneration (AMD), and it continues to be a leading cause of blindness despite multiple treatment trials over the years, including radiation (Marcus and Peskin, 2004), photodynamic therapy (Blinder et al., 2003), and treatments to block the actions of angiogenic factors (D'Amico et al., 2003; Gragoudas et al., 2004). In contrast, CNV that remains beneath the RPE, or is 'occult' (Hartnett et al., 1992; Lafaut et al., 2000), can remain stable with preservation of visual acuity (Bressler et al., 1988; Chang et al., 1994; Macular Photocoagulation Study Group, 1996) or even improvement in vision (Jalkh et al., 1990; Chang et al., 1994). When loss of vision occurs in occult CNV, it is

* Corresponding author.
*E-mail addresses:* pgeisen@email.unc.edu (P. Geisen), jmccolm@email.unc.edu (J.R. McColm), hartnet@med.unc.edu (M. Elizabeth Hartnett).

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often associated with the transition from occult CNV (under the RPE) to classic CNV (in the neurosensory retina), which is believed to occur from transmigration of endothelial cells (EC) across the retinal pigment epithelium (RPE) into the neurosensory retina (Stevens et al., 1997). In addition, vision loss in occult CNV can occur from the development of other retinal vascular anomalous complexes/retinal angiomatous proliferations within the inner retina (Kuhn et al., 1995; Hartnett et al., 1996; Yannuzzi et al., 2001; Gass et al., 2003) or from increasing outer retinal and RPE degeneration (Stevens et al., 1997).

Vascular endothelial growth factor (VEGF), a known chemotactic (Sakamoto et al., 1995; Ohno-Matsui et al., 2001) and angiogenic factor (Klagsbrun and D'Amore, 1991), is produced by the RPE (Adamis et al., 1993), and has been localized to human CNV (Frank, 1997). VEGF expression in the RPE is increased by hypoxia (Blaauwgeers et al., 1999; Ghiso et al., 1999; Mousa et al., 1999) and cytokines (Slomiany and Rosenzweig, 2004). In AMD, VEGF is postulated to cause CNV and reduce vision through exudation. CNV requires choroidal EC migration and proliferation and has been experimentally linked to overexpression of VEGF (Ishibashi et al., 1997). Animal models, in which VEGF is overexpressed in the RPE (Spilsbury et al., 2000; Schwesinger et al., 2001) or outer retina (Ohno-Matsui et al., 2002), develop retinal neovascularization. In one model, a recombinant adenoviral vector expressing $VEGF_{164}$ was injected through the sclera, puncturing Bruch's membrane, and into the subretinal space. RPE overexpressed VEGF and caused EC from the choroid to transmigrate the RPE (Spilsbury et al., 2000).

There are several steps necessary for choroidal EC transmigration into the neurosensory retina, such as compromise in the blood retinal barrier of the RPE, activation and proliferation of choroidal EC, enzymatic degradation of extracellular matrix, and production of a chemotactic gradient into the neurosensory retina, promoting EC migration. Currently, several clinical trials are testing compounds with anti-VEGF action on human CNV (Krzystolik et al., 2002; Tolentino et al., 2000). A recent study using an aptamer to $VEGF_{165}$ had a modest effect on CNV, as have other clinical studies (Blinder et al., 2003; Gragoudas et al., 2004). These studies suggest that additional treatments for CNV, targeting at other steps in the development of it, will be important to study. In addition, VEGF is an important survival factor for choriocapillaris EC (Li et al., 2000; Hoffmann et al., 2000), and when $VEGF_{165}$ was inhibited using a chimeric toxin, not only choroidal EC, but also RPE proliferation was reduced (Hoffmann et al., 2000). These data raise the concern that widespread inhibition of VEGF's actions may lead to visual compromise through adverse effects on the choriocapillaris and RPE. Therefore, it is important to understand the events that occur in specific steps in the development of CNV, such as when choroidal EC transmigrate across the RPE. We have used an in vitro coculture method to control a specific step involved in the development of neovascular AMD in the neurosensory retina and to study the effect of VEGF on some of the steps involved in the EC transmigration across the RPE.

## 2. Materials and methods

### 2.1. Cell culture

#### 2.1.1. Retinal pigment epithelial cells

ARPE-19 (ARPE, an immortal, non-transformed cell line from a human donor) was obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F12, 1:1 mixture (Invitrogen, IL), with HEPES buffer (DMEM) containing 10% fetal bovine serum (FBS) (Dunn et al., 1996). Cultures of ARPE were routinely subcultured by trypsinization (0.25%, Invitrogen, IL), and plated at $1.5\times10^4/cm^2$. Passages 10–22 were used in the experiments. ARPE was maintained in DMEM with 10% FBS at 37°C in 5% $CO_2$ and the media was replaced three times a week.

#### 2.1.2. Human choroidal endothelial cells

Donor eyes were obtained from the North Carolina Eye Bank, Inc. (Winston-Salem, NC). Primary human choroidal endothelial cells (CECs) were isolated using a modified method of Penfold (Penfold et al., 2002). The cornea, lens, vitreous, and retina were excised and most of the RPE removed by repeated washings within the eyecup with Hank's balanced salt solution (HBSS) (Invitrogen, IL). The choroidal tissue was separated from the sclera, minced into small pieces with a scalpel, and washed three times with cold HBSS containing 0.5 mg $ml^{-1}$ penicillin–streptomycin. The choroidal pieces were digested in a mixture containing 500 μg $ml^{-1}$ collagenase 1A (Sigma, MO) and 1.2 U $ml^{-1}$ Dispase II (Sigma, MO) for 45 min at 37°C in a rotating shaker.

To isolate human CECs and minimize RPE and fibroblast contamination, CD31-coated DynaBeads (Dynal Biotech, Inc., NY) were prepared for use following the manufacturer's protocol. Briefly, the beads were washed in 1 ml PBS/0.1% BSA and resuspended in 10 μl HBSS. After incubation, the choroidal digests were double filtered through 70 and 44 μm meshes. Enzymes were neutralized by adding two times the volume of DMEM with 10% FBS. Cells were centrifuged at 400 *g* at 4°C and resuspended in 100 μl HBSS with 5% FBS. The cell suspension was incubated with the washed beads for 15–30 min at room temperature (RT) with gentle rotation. The bead–endothelial cell complexes were then washed five times in HBSS with 5% FBS, mixed by gentle agitation for 1 min, and separated from the mixture with the Dynal Magnetic Particle Concentrator (MPC). The bead–CEC complexes were resuspended in 150 μl EGM-2 (endothelial growth media with growth factors: human epidermal growth factor, hydrocortisone, VEGF, basic fibroblast growth factor, insulin-like growth factor-1, and heparin, Cambrex, MD) with 10% FBS and 0.5 mg $ml^{-1}$ penicillin–streptomycin. The isolated bead–CEC complexes from each eye were plated onto a 1 $cm^2$ area and incubated overnight at 37°C and 5% $CO_2$. The HBSS and floating dead cells were removed after 24 hr and fresh EGM-2 with 10% FBS and 0.5 mg $ml^{-1}$ penicillin–streptomycin were added. CECs were confirmed as EC and not fibroblasts by 100% immunostaining with von Willebrand Factor