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CHAPTER 3

POLARIZED VASCULAR ENDOTHELIAL GROWTH FACTOR SECRETION BY HUMAN RETINAL PIGMENT EPITHELIUM AND LOCALISATION OF VEGF RECEPTORS ON THE INNER CHORIOCAPILLARIS: EVIDENCE FOR A TROPHIC PARACRINE RELATION

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ABSTRACT

The retinal pigment epithelium (RPE) maintains the choriocapillaris (CC) in the normal eye and is involved in the pathogenesis of choroidal neovascularisation in age-related macular degeneration. Vascular endothelial growth factor-A (VEGF) is produced by differentiated human RPE cells *in vitro* and *in vivo* and may be involved in paracrine signalling between the RPE and the CC. We investigated whether there is a polarized secretion of VEGF by RPE cells *in vitro*. Also, the localisation of VEGF receptors in the human retina was investigated. We observed that highly differentiated human RPE cells, cultured on Transwell filters in normoxic conditions, produced 2- to 7-fold more VEGF towards their basolateral side as compared to the apical side. In hypoxic conditions, VEGF-A secretion increased to the basal side only, resulting in a 3- to 10-fold higher basolateral secretion. By immunohistochemistry in 30 human eyes and in two cynomolgus monkey eyes, KDR (VEGFR-2) and flt-4 (VEGFR-3) were preferentially localized at the side of the CC endothelium facing the RPE cell layer, while flt-1 (VEGFR-1) was found on the inner CC and on other choroidal vessels. Our results indicate that RPE secretes VEGF towards its basal side where its receptor KDR is located on the adjacent CC endothelium, suggesting a role of VEGF in a paracrine relation, possibly in cooperation with flt-4 and its ligand. This can explain the known trophic function of the RPE in the maintenance of the CC and its fenestrated permeable phenotype, and points to a role of VEGF in normal eye functioning. Upregulated basolateral VEGF secretion by RPE in hypoxia or loss of polarity of VEGF production may play a role in the pathogenesis of choroidal neovascularisation.

INTRODUCTION

The retinal pigment epithelium (RPE) has important functions in both the normal eye as in pathological conditions. Experimental evidence indicates that the RPE is involved in the pathogenesis of choroidal neovascularisation (CNV) which occurs in the course of various eye conditions like age-related macular degeneration (AMD).¹⁻³ In normal healthy conditions, the RPE appears to have a positive survival effect in the maintenance of the highly vascularized, highly permeable fenestrated choriocapillaris (CC) on its outer basal aspect, while the photoreceptor layer internal to it is completely avascular. The anatomy of the choroidal vasculature and the preferential localisation of the CC fenestrations on the side of the RPE also suggest a modulating role of the RPE on the CC. In addition, clinical observations in AMD and experimental animal studies show that absence of the RPE can cause secondary atrophy of the choriocapillaris.^{1,2,3}

On the other hand, in the course of CNV, RPE cells are observed to encapsulate the new vessels, with their basal side towards the endothelial cells, which coincides with the arrest of further vascular growth.⁴ These morphological observations *in vivo* suggest that the RPE can have a function in controlling CNV.

Based on these observations it seems that the RPE has a dual role in its interactions with the CC, but how the integrity of the RPE/CC microunit is regulated in healthy conditions, how the photoreceptor layer remains avascular, and why pathological changes in the RPE layer either lead to choroidal atrophy or to an angiogenic response from the choroid is essentially unknown. It is likely, however, that mediator molecules play an essential role in these intercellular interactions.

Such interactions have previously been investigated in cell cultures of the constituent cells, but from these studies conflicting results have emerged. While some authors were able to demonstrate that RPE cells *in vitro* produce an inhibitor of endothelial proliferation,⁵ others found mitogenic activity for endothelium in RPE conditioned culture medium.⁶

We hypothesize that a polarized secretion of growth/inhibitory factors may explain these apparently conflicting *in vivo* and *in vitro* observations. Growth factors produced by RPE *in vitro* include platelet-derived growth factor-BB, transforming growth factor- β and vascular endothelial growth factor-A (VEGF).⁷ VEGF, a vascular permeability and angiogenesis factor, is a good candidate for a role in maintaining the CC. VEGF has been shown to increase vascular permeability in skin and muscle partly by inducing a fenestrated endothelial phenotype.⁸ Furthermore, it can act as a vascular survival factor *in vivo*.⁹ In a co-culture experiment of RPE and CC endothelium *in vitro*, RPE-derived VEGF was shown to stimulate tube formation of the underlying endothelial cells.¹⁰

The aim of the present study was to evaluate the polarity of VEGF secretion by cultured human RPE cells, and to investigate the localisation of VEGF receptors *in situ* in human and monkey eye tissue sections.

MATERIALS AND METHODS

RPE Cell Culture

Four human donor eyes (age of the donors: 9, 15, 17 and 24 years), obtained from the corneabank, Amsterdam, The Netherlands, were used as a source of primary RPE cells. The RPE cells (further designated as 'RPE cell lines') were isolated within 24 hours post-mortem.¹¹ RPE cells were isolated as described by Holtkamp *et al.*¹¹ In short, the cornea, the anterior segment, the optic nerve, vitreous and neural retina were removed from the eye and the RPE cells were dissociated from the eye with trypsin by two subsequent incubations at 37°C. Cells obtained from the second incubation were plated in 24-well plates (Corning, Acton, MA, USA) at 10⁵ cells/well in Iscoves Modified Dulbecco's Medium (IMDM, Gibco BRL) supplemented with 20 % Fetal Calf Serum (FCS, Gibco BRL), penicillin (100 U/ml, Gibco BRL) and streptomycin (100 mg/ml, Gibco BRL). Non-adherent cells were removed after two days by washing and refreshing the culture medium. At confluence, cells were detached by trypsin treatment and passed to culture flasks at approximately 4·10⁴ cells/cm². For the present experiments, RPE cells were used between the sixth and the eleventh passage. To investigate whether the RPE cell lines

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were not contaminated during the isolation procedure the cells were analyzed morphologically and by immunohistochemistry.

For immunohistochemistry, cells were cultured on tissue chamber slides (Lab-tek, Nunc Inc. Naperville, IL, USA), and stained with antibodies specific to cytokeratin 8/18 (CAM 5:2, Beckton-Dickinson, San Jose, CA, USA) or specific to glucose transporter-1 protein (a kind gift from L. Andersson, University of Uppsala, Uppsala, Sweden). As a negative control an antibody against a non-human bacterial protein was used (Mouse negative control immunoglobulins, DAKO).

All cells were cultured at 37°C and at 5% CO₂. Medium was changed twice a week.

RPE Cell Monolayers on Transwell Filters

RPE cells were cultured on transwell filters (Corning, Acton, MA, USA, 12 mm diameter, 0.4 mm pore size) according to the method of Holtkamp *et al.*¹¹ Briefly, filters were coated with 160 ml of a 1:40 dilution of Matrigel (Collaborative Biomedical Products, Bedford, USA) in medium and air dried overnight. The RPE cell lines were seeded as 1.6×10^5 cells/cm² in a volume of 200 ml/filter, in IMDM supplemented with 1% NHS (CLB, Amsterdam, The Netherlands). In the lower compartment, 1000 ml medium was added thereby levelling the height of the liquid levels to prevent hydrostatic pressure. After 2 days IMDM/1%NHS was added to a final volume of 750 ml in the upper compartment and 1500 ml in the lower compartment.

Trans epithelial resistance (TER) was measured once a week using an Endohm chamber and an ohmmeter (World Precision Instruments, Sarasota, USA). TER measurements were corrected for background by subtracting the TER value of a matrigel coated filter without cells. Filters with RPE monolayers were used for experiments approximately 3 weeks after plating.

Microscopy of RPE Cell Monolayers on Transwell Filters

The possible formation of multi-layers of RPE cells was checked by light microscopy on sections of the filters. The filters were washed with PBS, frozen in liquid N₂, embedded in Tissue-Tek (Miles Inc., Elkhart, IN, USA), cut in 10 µm thick sections, fixed in acetone during 5 minutes and air dried. The sections were stained with haematoxylin for 10 minutes followed by staining with eosin for 1 minute.

The presence of tight junctions was determined by immunofluorescence microscopy. Filters were washed with phosphate buffered saline (PBS, pH 7.4) and fixed with 4 % paraformaldehyde, washed again with PBS, permeabilized by incubation with 0.2 % triton X-100 for 15 minutes, blocked with 5 % normal goat serum (Jackson Lab, USA) and incubated with polyclonal antibodies against the tight junction associated protein ZO-1 (Zymed Laboratories Inc, San Francisco, CA98540, USA, 1:200) for 1 hour. After washing with PBS, filters were incubated for 1 hour with FITC-conjugated swine anti-rabbit immunoglobulins (Nordic, The Netherlands,

1:100), washed again in PBS and sealed with vectashield before being observed in a fluorescence microscope (Leica).

As described previously,¹¹ the integrity of the RPE cell monolayers was analyzed by electron microscopy (EM). For scanning EM, filters were washed with Hanks buffered salt solution, after which the cells were fixed in an 80 mmol/L cacodylate-buffered solution containing 1% glutaraldehyde and 1.25% paraformaldehyde (pH 7.3). After fixation for several days, the filters were dehydrated with ethanol, critical-point dried with CO₂ as the intermediate, and coated with a few nanometres of platinum. The filters were inspected and micrographed with a Philips SEM 505 scanning electron microscope (Philips Industries, Eindhoven, The Netherlands) with a secondary emission detector.

For transmission EM, filters were postfixated in OsO₄, dehydrated in a graded series of ethanol, and embedded in Epon. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and inspected and photographed in a Philips EM 400 electron microscope (Philips Industries) equipped with an EDAX PV9800 system and an ultrathin window detector (EDAX PV9760/53).

VEGF Secretion of RPE Cell Monolayers on Transwell Filters

Experiments were performed with 12 Transwell filters with confluent RPE cells from each donor. Medium was removed from the 48 filters and replaced with fresh medium containing 1% NHS just before the experiments. The filters were incubated in normal growth conditions (24 filters, 37°C, 20% O₂, 5% CO₂, moist environment) or in hypoxic conditions (24 filters, 37°C, 1% O₂, 5% CO₂, moist environment) for 24 hours (24 filters) or 48 hours (24 filters). Thus, each of the four conditions investigated was tested *in triplicate*. After 24 hours or 48 hours medium of both the upper and lower compartment was collected, snap frozen, and stored at -20°C until further analysis.

Levels of VEGF production of the RPE cells towards the basal or the apical side were assayed by ELISA (R&D systems, Abingdon, UK), according to the manufacturer's description. Each sample of medium was tested in triplicate.

Statistical Analysis

For statistical data analysis, the Independent Samples t-test was used after investigation of equality of variances using Levene's test for equality of variances (SPSS Software, SPSS Inc., USA).

Human and Primate Tissue Samples

Eyes from 30 persons with no known eye disease were obtained from the Corneabank Amsterdam, The Netherlands, within 5-15 hours after death. Furthermore, fresh normal eyes from two *Cynomolgus* monkeys (*Macaca fascicularis*) were kindly provided by the University of

Nijmegen, The Netherlands. All animal experiments in this study were performed in accordance with the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research. Samples of the posterior segment were dissected. All tissues were snap-frozen and stored at -70°C .

Immunohistochemistry

Air-dried cryo-sections ($10\ \mu\text{m}$) were fixed in cold acetone for 10 minutes and post-fixed for 2 minutes with Zamboni fixative (2% paraformaldehyde in a saturated picric acid solution) and stained by an indirect immunoperoxidase procedure. In order to reduce non-specific staining, sections were pre-incubated in 10% normal goat serum (Jacksons Immuno Research Laboratories, West Grove, USA) in PBS for 15 minutes and then incubated overnight at 4°C with the following antibodies: anti-endothelial MoAb PAL-E (1:1000), anti-VEGF receptor KDR, anti-VEGF receptor flt-1 (both a kind gift of Dr. H.A. Weich, Department of Gene Expression, Braunschweig, Germany, 1:200), and anti-VEGF receptor flt-4 (raised at the Haartman Institute, University of Helsinki, Helsinki, Finland; 1:400). Sections were washed and incubated with subsequent compounds of a sensitive avidin-biotin based Histostain plus kit (Zymed) according to the manufacturer's instructions. Sections were washed again and staining was performed using 3-amino-9-ethyl carbazole (AEC) containing 0.01% H_2O_2 .¹² The reaction was terminated by rinsing the sections with H_2O . Counter staining was performed with haematoxylin. Sections were washed and mounted in glycerol/glycerine (Sigma). In control sections the first antibodies were omitted.

All sections were examined by two independent observers in a masked fashion. Microvascular staining was graded as follows: no staining (-), weak staining (+/-), or evident staining (+).

RESULTS

Characterisation of RPE Cultures on Transwell Filters

After seeding on transwell filters (12 filters for each donor), the human retinal pigment epithelial (RPE) cell monolayers (passage between 6 and 11) showed a gradual increase in trans epithelial resistance (TER). The TER of the matrigel-coated filters without cells was $15\ \text{Ohm cm}^2$. Maximal net TER was reached after approximately 3 weeks. The TER of the 4 RPE cell lines ranged from $30\ +/-\ 1.2$ to $100\ +/-\ 4.3\ \text{Ohm cm}^2$, but showed little variation within each cell line (Figure 1).

The integrity of the RPE monolayers was verified by light microscopical examination of cross-sections of the filters (Figure 2A). Transmission electron microscopical studies revealed a monolayer of cells with microvilli and tight junctions at the side of the upper compartment of the Transwell system (Figure 2B), and deposition of basement membrane material between the lower side of the cells and the filter, indicating that the upper compartment represents the apical ('retinal') side of the RPE monolayer and the lower compartment the basal ('choroidal') side. The presence of tight junctional complexes was suggested by immunofluorescence staining of the cell

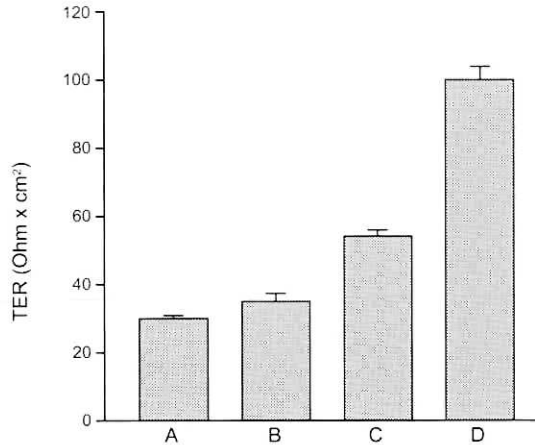


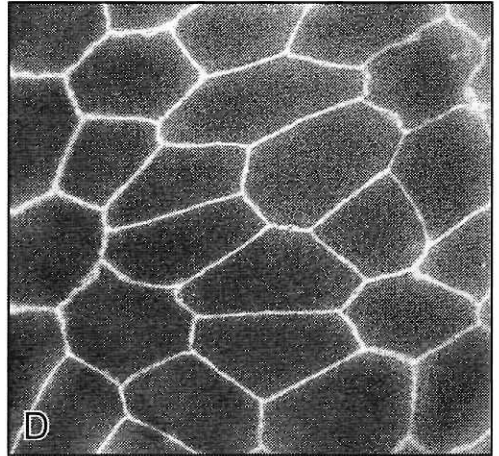
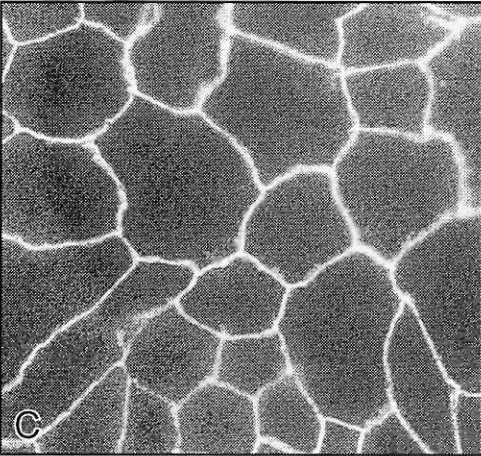
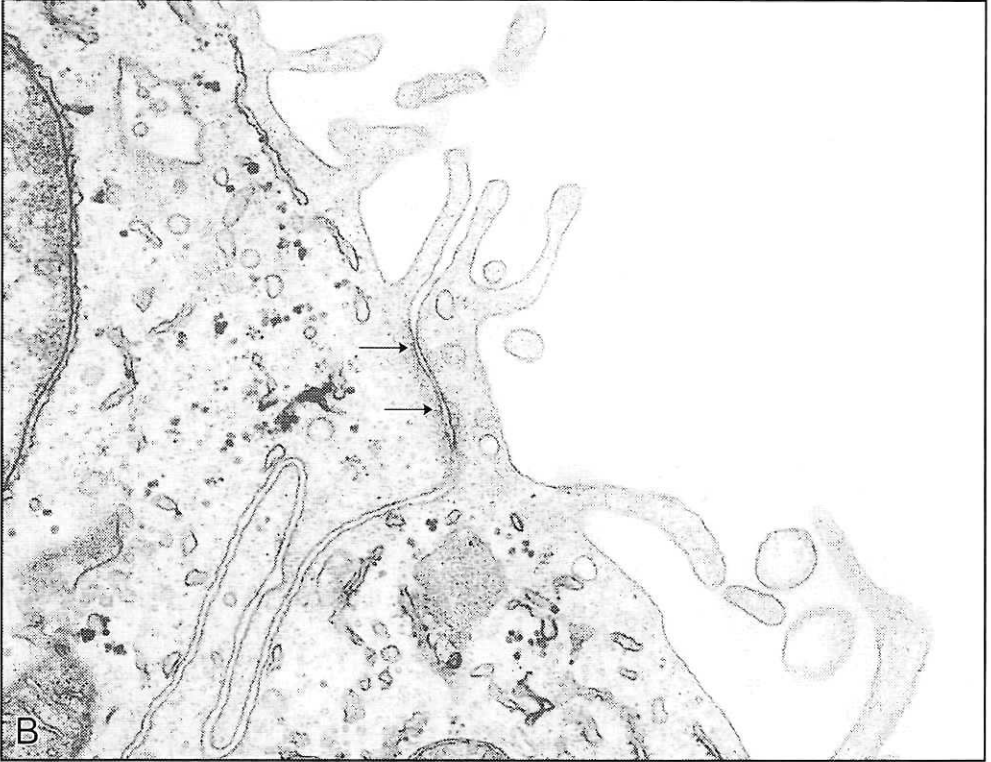
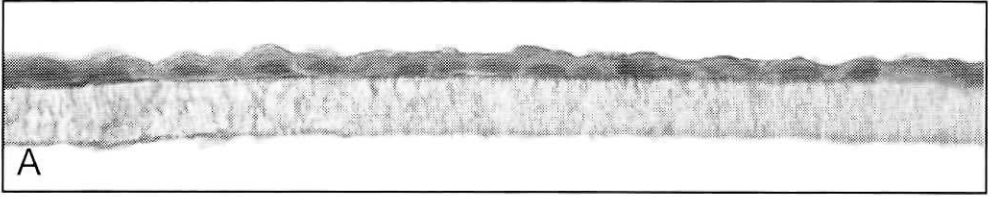
Fig 1. Trans epithelial resistance (TER, Ohm cm² +/- standard deviation) of RPE cells of the four donors A, B, C and D, cultured on transwell filters for three weeks (Each bar represents 12 filters).

layers for the tight junction associated protein ZO-1. ZO-1 was present at one level around every cell, both in the cell lines with low (35 Ohm cm², Figure 2C) and high TER (100 Ohm cm², Figure 2D). Staining for ZO-1 also showed that the density of cells on the filters was similar for all four RPE cell lines (approximately 10⁶ cells/well). Taken together, these results indicated that the cultured RPE cells are very similar to differentiated resting RPE *in vivo*.

VEGF Production by RPE Cell Lines in Normoxic and Hypoxic Culture Conditions

The RPE cell lines produced vascular endothelial growth factor-A (VEGF) under normal culture circumstances (20% O₂, 5% CO₂). The total production of VEGF varied between the different RPE cell lines, ranging from 1056 +/- 64 to 7410 +/- 456 pg/ filter (10⁶ cells) (24 h incubation) (Figure 3). Interestingly, the TER values of the four RPE cell lines correlated with the total VEGF production (compare Figures 1 and 3).

VEGF secretion in all filters examined was highly polarized. This was statistically significant for each donor, both in hypoxic and in normoxic conditions and at both time points ($p < 0.05$). In normoxic conditions, a 2- to 7-fold higher accumulation of VEGF was found at the basal side of the cells compared to the apical side at both time points (Figure 4). When RPE monolayers were kept in hypoxic conditions (1% O₂, 5% CO₂), VEGF production increased between 1.3 and 1.5-fold to the basal side but much less to the apical side. At 24 hours, this basal increase (and consequently higher polarization) was statistically significant for donors B, C and D ($p < 0.05$, Figure 4). As a consequence, in hypoxic conditions the amount of VEGF secreted towards the basal side was 3 to 10 times higher than the amount secreted to the apical side.



Localisation of VEGF Receptors KDR, flt-1, and flt-4 in the Human and Monkey Eye

Retina and choroid of 30 human donor eyes and two monkey eyes were stained with antibodies against VEGF receptors flt-1 (VEGFR-1), KDR (VEGFR-2) and flt-4 (VEGFR-3) and with a marker for fenestrated endothelium, PAL-E. Staining with the antibody against PAL-E strongly outlined the endothelial cells of the choriocapillaris as well as the endothelial cells of the other choroidal vessels in both the human donor eyes and the monkey eyes (Figure 5A). Staining of the sections with antibodies against the VEGF receptor KDR and flt-4 showed a particular staining pattern of the choriocapillaris in both human and monkey eyes (Figures 5C and 5D). Only on the side of the choriocapillaris endothelium which faces the RPE, a granular staining for KDR and flt-4 was observed. On the scleral side of the choriocapillaris and in the larger choroidal vessels only sporadic staining for KDR and flt-4 was seen (Figures 5C, 5D). The microvasculature of adjacent retina was negative. The staining for flt-4 in the choriocapillaris was even more pronounced than that for KDR. In contrast, staining with anti-flt-1 antibodies showed, in addition to marked staining of the inner CC, weak staining in the other choroidal vessels (Figure 5B), and also staining of the entire retinal microvasculature in a pattern suggestive of expression of this receptor by both endothelial cells and pericytes (results not shown).

DISCUSSION

This study indicates that tight monolayers of cultured human retinal pigment epithelial cells (RPE) preferentially secrete vascular endothelial growth factor-A (VEGF) to their basal (choroidal) side. Furthermore, in human and monkey tissue sections of the eye the VEGF receptors KDR and flt-4 were found to be specifically located at the retinal side of the choriocapillaris endothelium facing the RPE, while flt-1 had a more widespread distribution. These findings suggest that VEGF is involved in a paracrine relation between the choriocapillaris and RPE and this may help to explain the well known role of the RPE in maintaining the survival and fenestrated morphology of the choriocapillaris. Furthermore, these findings may have important implications for understanding the pathogenesis of choroidal neovascularisation (CNV).

Several authors have previously demonstrated that cultured human RPE cells produce VEGF, among other growth factors.^{7,13-18} In fact, VEGF was found to be responsible for most, if not all, mitogenic activity for endothelial cells in RPE conditioned medium.⁶ *In vivo*, RPE

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Fig 2. Characterisation of RPE monolayers cultured on Transwell filter for three weeks. Light microscopy of RPE cell monolayer (donor B), stained with haematoxylin-eosin (Fig. 2A); electron microscopy of the apical side of the RPE cell monolayer (Fig. 2B). Note the presence of apical microvilli and between the cells a structure (indicated by arrows) suggesting the presence of a tight junction; immunofluorescence microscopy of RPE cell monolayers of donor B (low TER: 35 Ohm cm²) (Fig. 2C) and donor D (high TER: 100 Ohm cm²) (Fig. 2D) stained for tight junction associated protein ZO-1. Note staining for ZO-1 at the intercellular junctions.

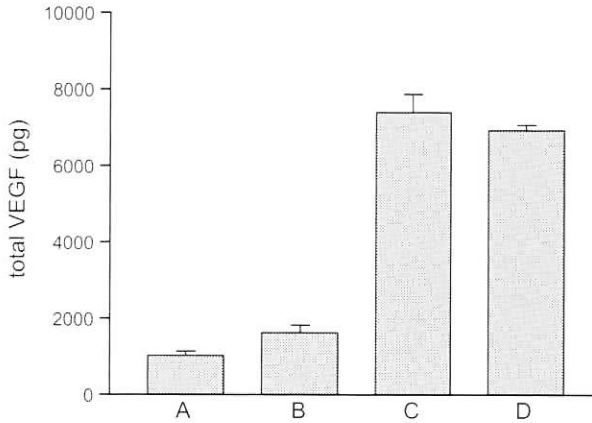


Fig 3. Total VEGF secreted (basal and apical side together, +/- standard error of mean) in 24 hours by RPE cells of the four donors A, B, C and D cultured on Transwell filters for three weeks (each bar represents three filters).

can dedifferentiate and act as multipotent cells in ocular wound healing and proliferative vitreoretinopathy. Therefore, one may argue that human RPE *in vitro* produce VEGF as a result of the specific culture conditions, which may induce an 'injury-type' phenotype of cells, comparable to those observed *in vivo* in wound healing and other pathological conditions. However, several *in vivo* studies indicate that also in the resting normal eye,^{1,6,17,18} VEGF is produced constitutively by RPE, suggesting a physiological role of the growth factor in the eye. Furthermore, of our four different RPE cell lines those with a higher TER produced higher

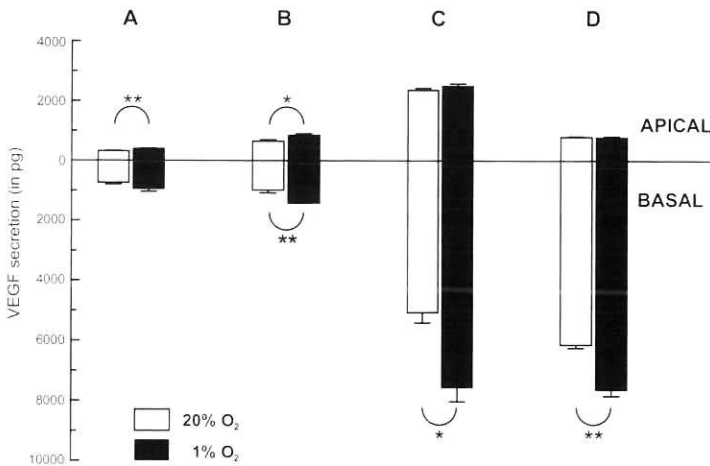


Fig 4. Differential VEGF secretion (+/- standard error of mean) towards the apical and basal side of Transwell filters by RPE cell monolayers of the four donors A, B, C, and D in normal (37°C, 20% O₂, 5% CO₂, moist environment) and in hypoxic conditions (37°C, 1% O₂, 5% CO₂, moist environment) in 24 hours (each bar represents three filters). Note that VEGF secretion is higher to the basal side than to the apical side (statistically significant for all conditions, p<0.05). In hypoxic conditions, VEGF secretion is higher, mainly to the basal side (statistical significance indicated by: * = p<0.05 and **=p<0.01).

amounts of VEGF. This may indicate that a higher degree of differentiation of RPE cells *in vitro*, as reflected by a higher TER,¹⁹ leads to a higher production of VEGF. In these tight RPE monolayers with the highest TER, VEGF secretion was also most polarized, with up to 10 times more VEGF produced to the basal side as compared to the apical side. Previous *in vitro* studies have found that RPE cells secrete between 0.6 and 70 ng VEGF per 10⁶ cells.^{6,20} This correlates well with our results, as the concentrations of VEGF found in the RPE conditioned medium ranged from 0.2 ng/ml on the apical side to 11 ng/ml on the basal side in hypoxic conditions. These concentrations of VEGF are in the biologically active range as observed in experiments with cultured endothelium.²¹⁻²³ From such experiments, VEGF has emerged as an important permeability and angiogenesis factor.²¹⁻²³ *In vitro*, VEGF is mitogenic for endothelial cells and stimulates their migration. When injected *in vivo* in muscle or skin, it rapidly induces endothelial fenestrations in the continuous endothelium of these tissues.⁸ In other conditions, like in the developing retina, VEGF has been shown to act as a survival factor for endothelium.⁹

The relation between the choriocapillaris and the RPE has long been a matter of attention of both clinicians and basal scientists. Anatomically, the choroidal vascular plexus is modelled towards the RPE, as if drawn to it by some mysterious force. Furthermore, the choriocapillaris endothelial cells have fenestrations only on their side facing the RPE. It is also clear from clinical observations that the choriocapillaris will disappear after loss of the RPE in geographic age related macular degeneration (AMD) and in other conditions.^{24,25} Animal models also demonstrate that RPE loss leads to choriocapillaris atrophy, preceded by loss of the endothelial fenestrations.^{2,26} All these data suggest a strong modulating influence of the RPE on the choriocapillaris.

It has previously been suggested that VEGF may be involved in this respect, based on the observed production of VEGF by cultured RPE. Furthermore, in a co-culture experiment, VEGF derived from RPE monolayers induced capillary tube formation of underlying endothelial cells in a type I collagen gel.³ These studies did not specify the degree of differentiation of the RPE cells or the direction of secretion of VEGF, however. In fact, these results were confusing, because, when VEGF would be secreted by RPE towards the retina *in vivo*, this would be expected to induce retinal neovascularisation. Such neovascularisation was demonstrated recently in a transgenic mouse model with constitutive VEGF production by photoreceptor cells.²⁷ However, in the normal eye, the outer retina is of course completely avascular. The data presented in this study suggest that the constitutive secretion of VEGF by RPE *in vivo* may well be highly polarized and be restricted towards the basal choroidal side.

Our observations suggest that VEGF is involved in a physiological paracrine relation between the RPE and the choriocapillaris. The observed specific localisation of the VEGFR-2, KDR, at the inner choriocapillaris, which is facing the basal side of the RPE cells, further supports this notion as expression of VEGFR-2 by these cells would allow paracrine signalling by VEGF-A. In contrast, immunohistochemical staining for the VEGFR-1, flt-1, was also found in other vessels of the choroid and retina, in a pattern suggestive of both endothelial and pericyte

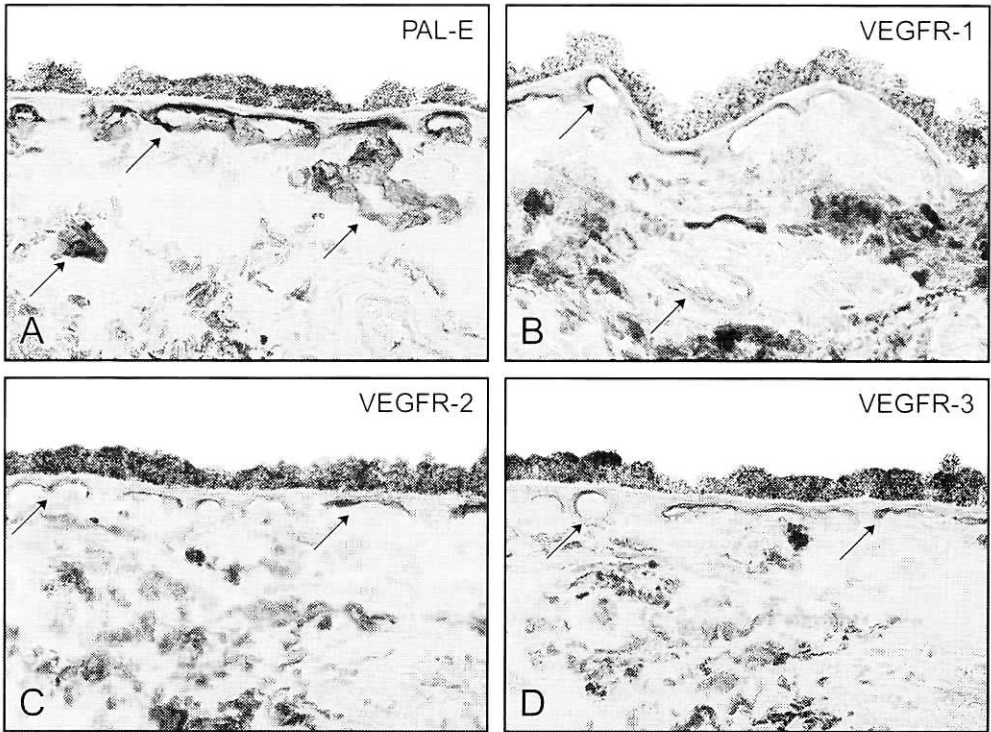


Fig 5. Immunoperoxidase staining of frozen tissue sections of human choroid with monoclonal antibody PAL-E, recognizing fenestrated endothelium (Fig. 5A), and antibodies recognizing VEGFR-1 (flt-1) (Fig. 5B), VEGFR-2 (KDR) (Fig. 5C), and VEGFR-3 (flt-4) (Fig. 5D). Note the intense staining of the entire choriocapillaris and other choroidal vessels for PAL-E (Fig. 5A), in contrast to the localized staining of the choriocapillaris at the side of the RPE cell layer for VEGFR-2 (KDR) (Fig. 5C) and VEGFR-3 (flt-4) (Fig. 5D). VEGFR-1 (flt-1) staining can be observed in the inner CC and in a large vessel in the choroid (Fig. 5B). Staining of vascular structures indicated by arrows.

staining. It has been proposed that these two VEGF receptors (KDR and flt-1) mediate different functions of VEGF-A.²⁸ Therefore, differential expression of the receptors in tissues may allow to regulate VEGF-A activity. A third member of the VEGF receptor family, flt-4, uses other gene products, VEGF-C and VEGF-D, as ligands, while its affinity for VEGF-A is negligible.^{29,30} Our finding that flt-4 is present in the same vessel segments of the choriocapillaris that contain KDR suggests that cooperativity may occur between different types of VEGF molecules. It is unknown however, whether RPE cells indeed produce VEGF-C or VEGF-D.

In the light of the known effects of VEGF on endothelial cells *in vivo* and *in vitro*, VEGF may well be very important in both the trophic influence of RPE on the choriocapillaris, as well as in inducing its highly permeable phenotype, reflected by the abundant fenestrations located on the RPE side of the choriocapillaris.³¹ Recently, Roberts and Palade⁸ and Esser *et al.*³² found that VEGF is able to induce endothelial fenestrations, both *in vivo* and *in vitro*. Likewise, secretion of VEGF by RPE cells to the basal side could induce the fenestrations of

the choriocapillaris. It has to be resolved whether the secretion of VEGF-A is sufficient or that VEGF-A and VEGF-C or other factors cooperate in this effect. A recent study indicates that such a cooperation exists in endothelial proliferation and tube formation *in vitro*.³³ Moreover, the VEGF receptors KDR and flt-4 are mainly localized on the RPE side of the choriocapillaris providing a selectively responsive area of the existing blood vessels. By such a paracrine mechanism VEGF secreted by the RPE cells may also act as a trophic factor on the choriocapillaris.

These findings may also have important implications for the understanding of the pathogenesis of choroidal neovascularisation as occurs in age-related macular degeneration (AMD). Choriocapillaris atrophy and an increased thickness of Bruch's membrane and relative impermeability to water-soluble compounds, like VEGF, have been reported in aging and AMD.^{34,35} One may speculate that the latter changes in Bruch's membrane may not only lead to CC atrophy resulting from insufficient amounts of VEGF reaching the choroid, but may lead on the other hand to accumulation of VEGF on the basal side of the RPE, as has indeed been observed in AMD in immunohistochemical studies.³⁶ Here, VEGF may reach a critical concentration and induce CNV under the RPE. This mechanism may be further enhanced by local hypoxia, as we observed upregulated basolateral production of VEGF by RPE cells in hypoxic conditions. In fact, a relative outer retinal hypoxia has been suggested to occur in AMD as a result of CC atrophy and thickening of Bruch's membrane.^{34,36} In other situations with CNV, defects in the RPE monolayer could lead to misdirection of secreted VEGF and subsequent classical subretinal neovascularisation.

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The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every entry, no matter how small, should be recorded to ensure the integrity of the financial data. This includes not only sales and purchases but also expenses and income. The document provides a detailed explanation of how to categorize these transactions and how to use a double-entry system to ensure that the books balance.

Next, the document covers the process of reconciling bank statements with the company's records. It explains that this is a crucial step in identifying any discrepancies or errors that may have occurred. The document provides a step-by-step guide on how to perform a bank reconciliation, including how to compare the bank's records with the company's ledger and how to investigate any differences.

The document also discusses the importance of regular audits and reviews. It explains that these are essential for ensuring the accuracy and reliability of the financial statements. The document provides a list of key areas to focus on during an audit, such as the accuracy of the accounting records, the completeness of the data, and the proper application of accounting principles.

Finally, the document provides a summary of the key points discussed and offers some final thoughts on the importance of maintaining accurate financial records. It concludes by stating that a well-maintained set of books is essential for the success of any business and that it is the responsibility of the management to ensure that these records are kept up-to-date and accurate.