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Human retinal epithelium produces and responds to placenta growth factor

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Abstract *Background:* Placenta growth factor (PlGF) is an important co-factor in retinal neovascularization. To examine whether retinal pigment epithelial (RPE) cells may represent a source for PlGF during retinopathy, we determined whether human RPE cells in vitro produce and respond to PlGF. In addition, we determined whether the cells express receptors for PlGF, i.e. flt-1 and neuropilins. *Methods:* Cultured human RPE cells of passages 3–5 were used. The regulation of the PlGF gene and protein expression by growth factors and cytokines was evaluated by quantitative PCR and ELISA. Proliferation rates and chemotaxis were determined by a bromodeoxyuridine and a Boyden chamber assay. *Results:* Human RPE cells express mRNAs for various members of the vascular endothelial growth factor family and for their receptors, including mRNAs for PlGF, flt-1, KDR, and neuropilins-1 and -2. The expression levels of the mRNAs for neuropilins-1 and -2 were significantly higher than those for flt-1 and KDR. Members of the transforming growth factor (TGF)-β superfamily of growth factors (BMP-4, TGF-β1, and β2) were strong inducers of PlGF gene expression, and evoked secretion of PlGF-2 protein by RPE cells. Exogenous PlGF-2 induced chemotaxis in RPE cells and reduced slightly the cell proliferation at high concentrations. *Conclusion:* The findings that RPE cells produce and respond to PlGF indicate that the factor exerts an autocrine/paracrine action on these cells. It is suggested that increased expression of TGF-β-related growth factors during diabetic retinopathy may cause PlGF secretion by RPE cells contributing to the stimulation of cell migration as a critical component of the progression of fibrovascular membranes.

Keywords Retinal pigment epithelial cells · Gene expression · Proliferation · Migration · VEGF and PlGF secretion

Abbreviations bFGF: basic fibroblast growth factor · BMP: bone morphogenetic protein · BrdU: bromodeoxyuridine · ERK: extracellular signal-regulated kinase · flt: FMS-related tyrosine kinase1 (VEGF-R1) · GAPDH: Glyceraldehyde-3-phosphate dehydrogenase · HB-EGF: heparin-binding epridermal growth factor-like growth factor · HGF: hepatocyte growth factor · IL: interleukin · KDR: kinase insert domain receptor (VEGF-R2) · MAPK: mitogen-activated protein kinase · PCR: polymerase chain reaction · PDGF: platelet-derived growth factor · PI3K: phosphatidylinositol-3 kinase · PlGF: placenta growth factor · RPE: retinal pigment epithelium · TGF: transforming growth factor · TNF: tumor necrosis factor · VEGF: vascular endothelial growth factor · VEGF-R: VEGF receptor

Introduction

Pathological angiogenesis is a serious consequence of important eye diseases such as diabetic retinopathy and age-related macular degeneration. Though the vascular endothelial growth factor (VEGF) and its receptors (VEGF-Rs) have been critically implicated in mediating pathological neovascularization [10, 20, 27], it became evident that the synergistic action of other proangiogenic factors and of their receptors is required for the angiogenic effect of VEGF [6]. One of the receptors expressed in fibrovascular tissues and in choroidal neovascular membranes is neuropilin-1 [7], and it has been found that the expression level of neuropilin-1 correlates with the degree of neovascularization [16]. Neuropilin-1 is a cell-surface receptor for different ligands: it is an isoform-specific receptor for VEGF-A_{165} [34], a receptor for the heparin-binding form of the placenta growth factor (PlGF), PlGF-2 [19, 23, 24], and a receptor for semaphorin-3A which is a member of the collapsin-semaphorin family of proteins functionally involved in axon guidance and cell migration [12, 38]. Neuropilin-1 acts as a co-receptor that enhances the binding affinity of VEGF to VEGF-R2 (KDR/flk-1) [11, 35], and therefore increases the bioactivity (proliferation and migration) of VEGF in endothelial cells [25, 34].

PlGF is a member of the VEGF family of growth factors and has at least three isoforms generated by alternative splicing of RNA, PlGF-1 ($PlGF_{149}$), PlGF-2 ($PlGF_{170}$), and PlGF-3 ($PlGF_{221}$) [4, 18]. There is evidence that PlGF is implicated in the formation of fibrovascular tissue during proliferative diabetic retinopathy: the vitreous level of PlGF is elevated during the development of proliferative diabetic retinopathy [17, 21]; PlGF immunoreactivity is expressed in fibrovascular membranes (but not in non-vascularized epiretinal membranes or in normal retinas) [17]; and the retinal expression of PlGF mRNA is increased during diabetic retinopathy [36]. Moreover, deficient expression of PlGF has been shown to prevent the development of experimental choroidal neovascularization [30]. PlGF facilitates endothelial cell proliferation and vascular permeability by potentiating the activity of VEGF [1, 8, 26], and the presence of PlGF is required for the angiogenic effect of VEGF in the ischemic retina [5]. One major source of VEGF in the retina is the retinal pigment epithelium (RPE) [3]. However, it is not known whether RPE cells produce (in addition to VEGF) also PlGF. Therefore, we investigated the production and secretion of PlGF by human RPE cells in vitro. We determined the expression of mRNA for different members of the VEGF protein family and for the receptors of PlGF and VEGF, flt-1, KDR, and neuropilins-1 and -2, and investigated the regulation of mRNA expression by various growth factors and cytokines. Additionally, we determined whether RPE cells secrete and respond to PlGF protein. The results suggest that RPE cells may represent one major source for PlGF during retinopathy.

Materials and methods

Materials

The recombinant growth factors and cytokines transforming growth factor-β1 (TGF-β1), TGF-β2, bone morphogenetic protein-4 (BMP-4), VEGF-A_{165}, platelet-derived growth factor-BB (PDGF-BB), heparin-binding epidermal growth factor-like growth factor (HB-EGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and tumor necrosis factor-α (TNFα) were purchased from R&D Systems (Wiesbaden, Germany). PlGF-2 and interleukin-1β (IL-1β), were obtained from RELIATech (Braunschweig, Germany). The following antibodies were used: a rabbit anti-human p44/p42 mitogen-activated protein kinase (MAPK) (New England Biolabs, Frankfurt/M., Germany; 1:1000), a rabbit anti-phosphorylated p44/p42 MAPK (New England Biolabs; 1:1000), a rabbit anti-human Akt (New England Biolabs; 1:1000), a rabbit anti-human phosphorylated Akt (New England Biolabs; 1:1000), a rabbit anti-human p38 MAPK (New England Biolabs; 1:1000), a rabbit anti-human phosphorylated p38 MAPK (New England Biolabs; 1:750) and an anti-rabbit IgG conjugated with alkaline phosphatase (Chemicon, Hofheim, Germany; 1:2000).

Cell culture

Human RPE cells were obtained from several donors within 48 h of death, and were prepared as described previously [9]. The use of human material was approved by the ethics committee of the University of Leipzig Medical Faculty, and was performed according to the Declaration of Helsinki. The cells were suspended in Ham F-10 medium containing 10% fetal bovine serum, glutamax II, and gentamycin, and were cultured in tissue culture flasks (Greiner, Nürtingen, Germany) in 95% air/5 % CO_2 at 37°C. Cells of passages 3–5 were used. The epithelial nature of the RPE cells was identified routinely by immunocytochemistry using the monoclonal antibodies AE1 (which recognizes most of the acidic type I keratins) and AE3 (which recognizes most of the basic type II keratins) (Chemicon, Hampshire, U.K.). All tissue culture components and solutions were purchased from Gibco BRL (Paisley, UK).

DNA synthesis rate

The cells were seeded at 3×10^3 cells per well in 96-well microtiter plates (Greiner), and were allowed to attach for 48 h. Thereafter, the cells were growth arrested in medium without serum for 5 h, and subsequently, medium containing 0.5% serum with and without test substances was added for another 24 h. The incorporation of

Table 1 Primer pairs used for PCR to detect mRNA for members of VEGF protein family and for their receptors

Gene and accession no.	Primer sequence (5'?3')	Amplicon length (bp)
hGAPDH	GCAGGGGGGAGCCAAAAGGGT	219
XM_006959	TGGGTGGCAGTGATGGCATGG	
hVEGF-A	CCTGGTGGACATCTTCCAGGAGTA	407; 347; 275
AH001553	CTCACCGCCTCGGCTTGTCACA	
hVEGF-B	ACCGGATCATGAGGATCTGCA	223
U52819	CTCTCAAGGCCCCAAACCA	
hVEGF-C	CTCTCAAGGCCCCAAACCA	152
NM_005429	AGGTCTTGTTCGCTGCCTGA	
hVEGF-D	GATCGCTGTTCCCATTCCA	152
NM_004469	ATCATGTGTGGCCCACAGAGA	
hPlGF	GGCCATGAGAATCTGCACTGT	164
X54936	CACCTTTCCGGCTTCATCTTC	
hflt	TCCCTTATGATGCCAGCAAGT	161
AF063657	CCCCTCTTTCAGCATTTTCAC	
hKDR	CTTCGAAGCATCAGCATAAGAAACT	156
AF063658	TGGTCATCAGCCCACTGGAT	
h neuropilin-1	CGCTCCCGCCTGAACTACCCT	521
NM_003873	TGAGGTGCGGGTGGAAGTGCC	
h neuropilin-2	GGTGCGGGAAGCCAGCCAGGA	447
NM_018092	GCCCCCAGGAGGACGCCCAGT	

bromodeoxyuridine (BrdU) into the genomic DNA was measured by using the Cell Proliferation ELISA BrdU Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. BrdU (10 μM) was added to the culture medium 5 h before fixation. To compare possible effects of the factors tested with the action of a known mitogen for RPE cells, and to reveal possible modulating effects on growth factor-evoked proliferation, PDGF was used as positive control.

Chemotaxis

Measurement of chemotaxis was performed by using a modified Boyden chamber assay. Suspensions of RPE cells (100 μl; 5×10^5 cells/ml serum-free medium) were seeded onto polyethylene terephthalate filters (pore size 8 μm; Becton Dickinson, Heidelberg, Germany) coated with fibronectin (50 μg/ml) and gelatin (0.5 mg/ml). Within 4 h after seeding, the cells attached to the filter and formed a semiconfluent monolayer. Thereafter, the medium was changed into medium without addivitves in the upper well and medium containing PlGF-2 or VEGF-A_{165} in the lower well. After incubation for 16 h, the inserts were washed with buffered saline, fixed with Karnofsky`s reagent, and stained with hematoxylin. Non-migrated cells were removed from the filters by gentle scrubbing with a cotton swab. The migrated cells were counted, and the results were expressed relative to the cell migration rate in the absence of test substances.

Western blotting

The cells were seeded at 5×10^5 cells per well in six-well plates in 1.5 ml serum-containing medium, and were allowed to grow up to a confluency of 80%. After growth arrest for 16 hours, the cells were pre-treated with blocking substances for 30 min and thereafter with test substances for 10 min. Then, the medium was removed, the cells were washed twice with prechilled phosphate-buffered saline (pH 7.4; Invitrogen, Paisley, UK), and the monolayer was scraped into 150 μl lysis buffer (Mammalian Cell Lysis-1 Kit; Sigma-Aldrich, Taufkirchen, Germany). The total cell lysates were centrifuged at 10,000 *g* for 10 min, and the supernatants were analyzed by immunoblots. Equal amounts of protein (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with primary and secondary antibodies, and immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Cytokine ELISA

The cells were cultured at 3×10^3 cells per well in 96-well plates (100 μl culture medium per well). At a confluency of ~80%, the cells were cultured in serum-free medium for 16 h. Subsequently, the culture medium was changed, and the cells were stimulated by growth factors and cytokines at doses indicated, in the presence of 0.5% serum. The supernatants were collected after 6 or 24 h, and the levels of

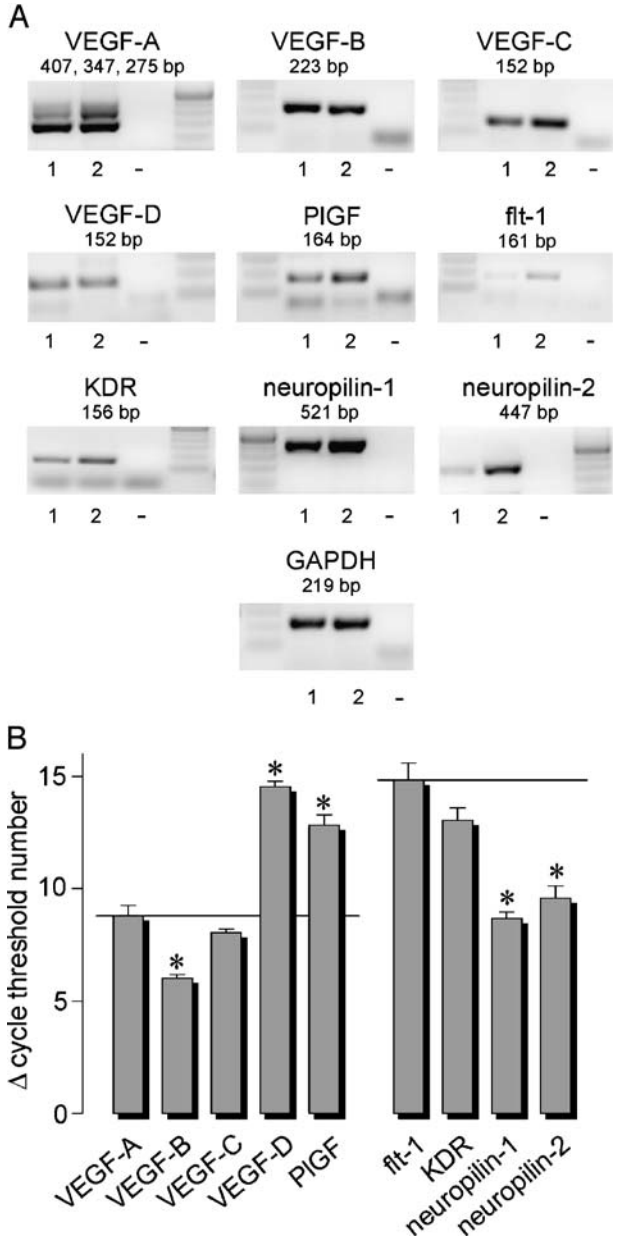

Fig. 1 Cultured human RPE cells express mRNA for different members of the VEGF family of growth factors, as well as for their receptors. **a** RT-PCR analysis revealed the presence of mRNA for VEGF-A, -B, -C, -D, and PlGF, as well as for the receptors VEGF-R1 (flt-1), VEGF-R2 (KDR), neuropilin-1, and neuropilin-2. The cells were investigated after 16 (1) or 40 h (2) of serum-free culturing. The negative control (-) was done by adding water instead of cDNA. **b** Mean±SD number of cycles necessary to detect the mRNAs for the members of the VEGF family of growth factors (*left*) and for their receptors (*right*) by qPCR in cells which were cultured for 16 h. The detection thresholds were normalized to the relative cycle number necessary for the detection of GAPDH mRNA. A lower relative cycle number means a higher expression level of the mRNA. Significant differences versus. the cycle number for VEGF-A (*left*) and flt-1 mRNAs (*right*), respectively: *P<0.001

PlGF-2 or VEGF-A_{165} in the cultured media (50 µl or 200 µl) were determined by ELISA (R&D Systems).

PCR

The total RNA of cultured cells was extracted by using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and treated with DNase I (Roche). cDNA was synthesized from 1 µg total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St Leon-Roth, Germany). PCR was carried out using the Taq PCR Master Mix Kit (Qiagen) and the primer pairs described in Table 1. A 1-µl aliquot of the first-strand mixture and 1 µM of each gene-specific sense- and anti-sense primer were used for amplification in a final volume of 20 µl. Amplification was performed for 40 cycles with the PTC-200 Thermal Cycler (MJ Research, Watertown, Mass., USA). Each cycle consisted of 30 s at 94°C, 60 s at 60°C, and 2 min at 72°C.

Quantitative PCR (qPCR) was performed with the Single-Color Real-Time PCR Detection System (BioRad, Munich, Germany). The PCR solution contained 1 µl cDNA, specific primer set (1 µM each) and 10 µl of QuantiTect SYBR Green PCR Kit (Qiagen) in a final volume of 20 µl. The PCR parameters were initial denaturation and enzyme activation (one cycle at 95°C for 15 min); denaturation, amplification and quantification, 45 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for one minute; melting curve, 55°C with the temperature gradually increased (0.5°C) up to 95°C. The amplified samples were analyzed by standard agarose gel electrophoresis. The mRNA expression was normalized to the levels of GAPDH mRNA, by using the mathematical model of Pfaffl [29].

Statistics

The rates of BrdU incorporation, migration, and VEGF secretion are expressed as percent of untreated control (100%). For each test, at least three independent experiments were carried out in triplicate. Data are expressed as means±SD (mRNA analysis) or SEM; statistical significance (Student's *t*-test, non-parametric Mann-Whitney *U*-test) was accepted at P<0.05.

Results

mRNA expression

To investigate whether RPE cells may produce and respond to PlGF, we performed firstly a RT-PCR analysis to determine whether cultured human RPE cells express mRNA for different members of the VEGF family of growth factors and their receptors. As shown in Fig. 1a,

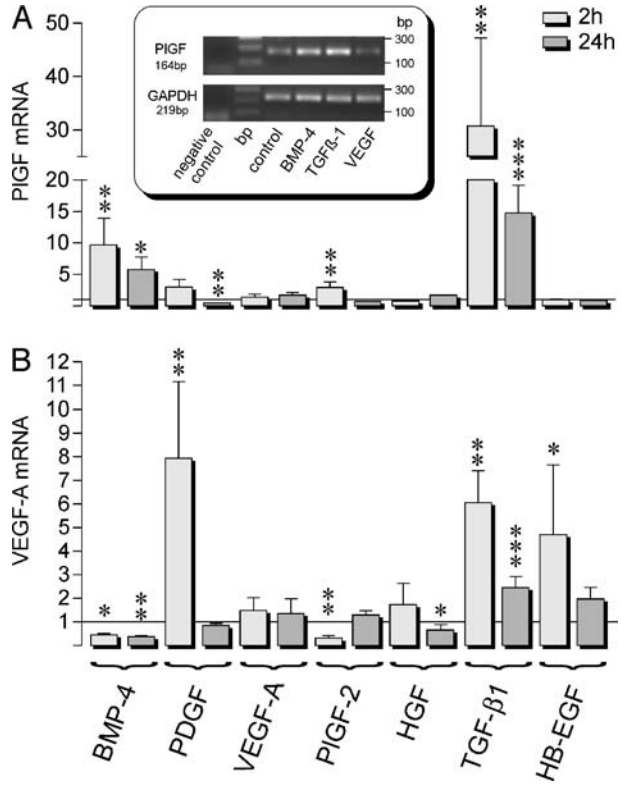

Fig. 2 Effect of different growth factors and cytokines on the gene expression of PlGF (**a**) and VEGF-A (**b**) in human RPE cells. The factors were applied at 10 ng/ml for 2 or 24 h. The mRNA levels were determined by qPCR, and are expressed as folds of control. Means±SEM of three to six independent experiments using cells from different donors. Significant differences versus. untreated control: **P*<0.05; ***P*<0.01; ****P*<0.001

gene expression for VEGF-A, -B, -C, -D, and PlGF was detected. The expression level of the mRNA for VEGF-B was significantly higher compared with the level of VEGF-A mRNA, whereas no difference was observed in respect to the gene expressions of VEGF-A and -C (Fig. 1b). On the other hand, significant lower mRNA levels were observed for VEGF-D and PlGF compared to VEGF-A mRNA (Fig. 1b). The cells expressed the mRNAs for the receptors of these factors, VEGF-R1 (flt-1), VEGF-R2 (KDR/flk-1), neuropilin-1, and neuropilin-2 (Fig. 1a). The expression levels of the mRNAs for neuropilins were higher when compared with the mRNAs for flt-1 and KDR, as indicated by the significantly (*P*<0.001) lower cycle threshold numbers necessary for the detection of the mRNAs by qPCR (Fig. 1b). In addition to the cultured RPE cells, we investigated the mRNA expression in acutely isolated RPE cells. We found that, similarly to cultured cells, acutely isolated cells expressed the various mRNAs for the members of the VEGF family and their receptors (data not shown). The expression of the mRNAs for PlGF and its receptors suggest that RPE cells may produce and respond to this factor.

Regulation of mRNA expression

Since various growth factors and cytokines have been implicated in the progression of fibrovascular disease, we investigated using qPCR analysis whether the expression of mRNA for PlGF is regulated by different factors in RPE cells. The cells were treated with the factors for 2 or 24 h, and the relative mRNA levels in comparison to unstimulated control was determined. As shown in Fig. 2a,

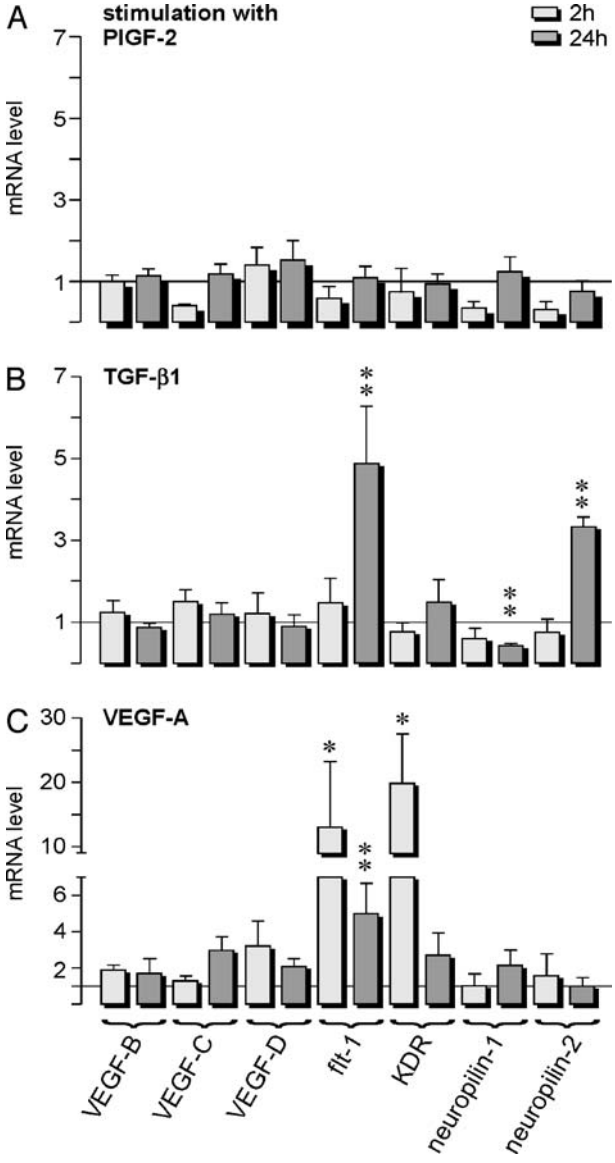

Fig. 3 Effects of exogenous PlGF-2 (**a**), TGF-β1 (**b**), and VEGF-A_{165} (**c**), respectively, on the gene expression of members of the VEGF protein family and of their receptors. The factors were applied at 10 ng/ml for 2 or 24 h. The mRNA levels were determined by qPCR, and are expressed as folds of control. Means ±SEM of three to six independent experiments using cells from different donors. Significant differences versus untreated control: **P*<0.05; ***P*<0.01

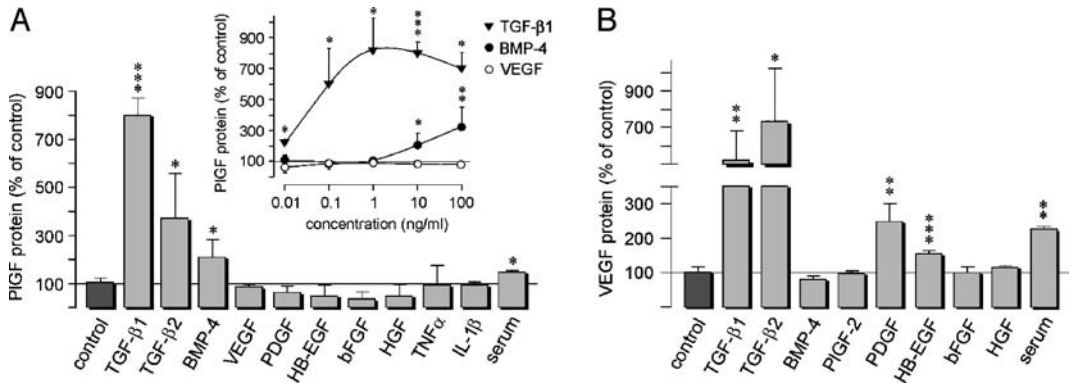

Fig. 4 Human RPE cells secrete PlGF and VEGF proteins upon stimulation with members of the TGF-β superfamily of growth factors. **a** Secretion of PlGF-2. *Inset:* Concentration dependence of the effects of TGF-β1, BMP-4, and VEGF on the secretion of PlGF-2. **b** Secretion of VEGF-A_{165}. The cells were stimulated with different factors (each at 10 ng/ml) and serum (5%), respectively, for 24 h; subsequently, the protein concentration in the cultured media was measured by ELISA. Data are expressed as percentage of untreated control (100%). Means±SEM of three to six independent experiments carried out in duplicate. Significant differences versus. untreated control: **P*<0.05; ***P*<0.01; ****P*<0.001

members of the TGF-β superfamily of growth factors (BMP-4 and TGF-β1) significantly increased the expression of mRNA for PlGF both at 2 and 24 h of culturing. Various other growth factors examined (VEGF, HGF, HB-EGF, PDGF) showed no influence on the expression of PlGF mRNA. This stimulation pattern was different from the pattern of the cytokine-dependent expression of mRNA for VEGF-A (Fig. 2b). PDGF, TGF-β1, and HB-EGF increased significantly the gene expression of VEGF-A after 2 h of incubation, while gene expression was decreased in the presence of BMP-4 and PlGF-2. The data suggest that the genes for PlGF and VEGF-A are differentially regulated in RPE cells by cytokines.

To investigate whether PlGF-2, TGF-β1, or VEGF-A_{165} also regulate the expression of the genes for other members of the VEGF protein family and for their receptors, a comparative gene expression analysis was carried out using qPCR. Exogenous PlGF-2 did not significantly alter the gene expression of VEGF-B, C, and D, and of the receptors (Fig. 3a). Similarly, exogenous BMP-4 had no effects on the gene expression of the other members of the VEGF protein family and of the receptors investigated (not shown). Exogenous TGF-β1 stimulated the gene expression of flt-1 and neuropilin-2 after 24 h of incubation, and decreased the mRNA level for neuropilin-1 (Fig. 3b). VEGF-A strongly enhanced the gene expression of the VEGF receptors, flt-1 and KDR, while it had no effects on the gene expression of neuropilins and the other VEGF subtypes investigated (Fig. 3c). The data suggest that members of the TGF-β superfamily of growth factors, but not VEGF-A, stimulate gene expression of PlGF in RPE cells.

Secretion of PlGF-2

Since RPE cells express mRNA for PlGF (Fig. 1a) we investigated whether the cells secrete PlGF protein. The cells were stimulated with different growth factors and cytokines, as well as with serum, for 24 h, and subsequently, the concentration of PlGF-2 in the cultured media was measured by ELISA. The cells constitutively secreted PlGF-2 protein; the concentration of PlGF-2 in the supernatants at unstimulated conditions was ~5 pg/ml. Addition of various members of the TGF-β superfamily of growth factors (BMP-4, TGF-β1, TGF-β2) to the culture medium significantly enhanced the content of PlGF-2 in the supernatants (Fig. 4a). Different growth factors (VEGF, PDGF, HB-EGF, bFGF, HGF) and pro-inflammatory cytokines (TNFα, IL-1β) did not enhance the release of PlGF-2 by the cells, while serum displayed a slight stimulating effect (Fig. 4a). The TGF-β1- and BMP-4-evoked stimulation of PlGF secretion was dose-dependent (Fig. 4a, inset). TGF-β1 evoked secretion of PlGF-2 at concentrations above 0.01 ng/ml, while the effect of BMP-4 was observed only at higher concentrations above 1 ng/ml. The data suggest that the secretion of PlGF is evoked mainly by TGF-β-related cytokines.

Secretion of VEGF-A

RPE cells are a major source of VEGF in the retina [3]. To investigate whether PlGF may alter the secretion of VEGF by RPE cells, we measured the content of VEGF-A protein in the supernatants that were derived from RPE cells cultured in the absence and presence of various growth factors and cytokines. The cells constitutively secreted VEGF-A, with concentrations between 100 and 250 pg/ml measured in the supernatants at untreated conditions. As shown in Fig. 4b, TGF-β1 and TGF-β2 strongly increased the VEGF-A content of the cultured media while BMP-4 and PlGF-2 were without effects. PDGF and HB-EGF evoked secretion of VEGF while bFGF and HGF were ineffective (Fig. 4b). Similar results were obtained in

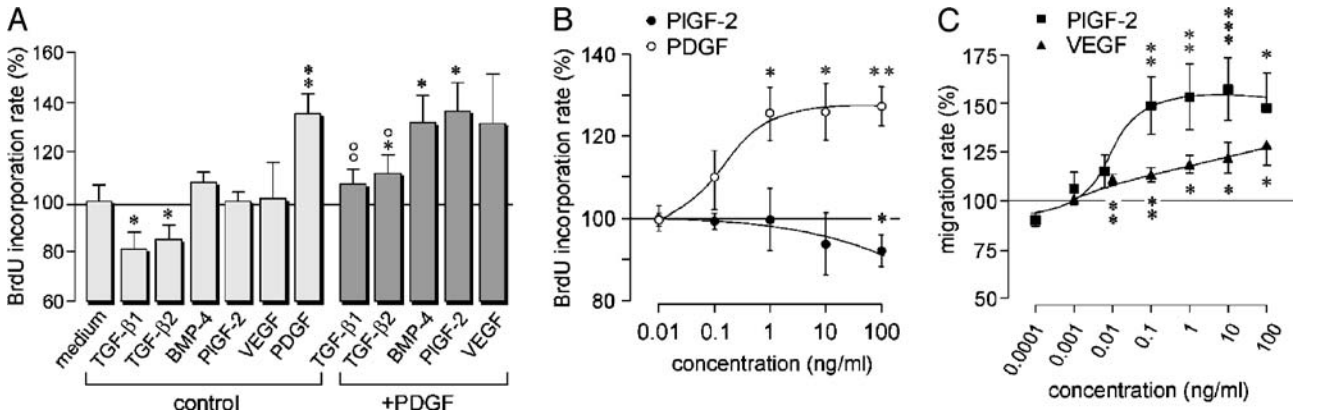

Fig. 5 PlGF is a motogen but not a mitogen for human RPE cells. **a** PDGF but not PlGF-2 nor BMP-4 stimulated the proliferation of RPE cells. TGF-β1 and -β2 decreased control proliferation, and inhibited the proliferation evoked by PDGF. All factors were tested at 10 ng/ml. **b** Dose-dependence of the effects of PlGF-2 and PDGF on the cell proliferation rate. **c** Dose-dependence of the chemotactic effects of PlGF-2 and VEGF-A_{165}. Means±SEM of three to six independent experiments using cells from different donors. Significant differences versus. untreated control: *P<0.05; **P<0.01; ***P<0.001. Significant differences versus stimulation with PDGF: °P<0.05; °°P<0.01

cultures that were stimulated for 6 or 24 h (not shown). It is concluded that PlGF does not alter the secretion of VEGF by RPE cells.

Cell proliferation

Since RPE cells express receptors for PlGF (Fig. 1) we determined whether exogenously applied PlGF may modulate cell responses which are important in the growth of fibrovascular tissue, i.e. cell proliferation and migration. Cell proliferation was determined by measurement of the DNA synthesis rate. Addition of PlGF-2 (at 10 ng/ml) to the culture medium did not alter the proliferation of RPE cells (Fig. 5a). In contrast, PDGF, a known mitogen for RPE cells [14], enhanced cell proliferation, while TGF-β1 and β2 decreased the proliferation at control conditions and in the presence of exogenous PDGF (Fig. 5a). BMP-4 and VEGF-A were without effects on cell proliferation. PDGF evoked a dose-dependent increase of RPE cell proliferation

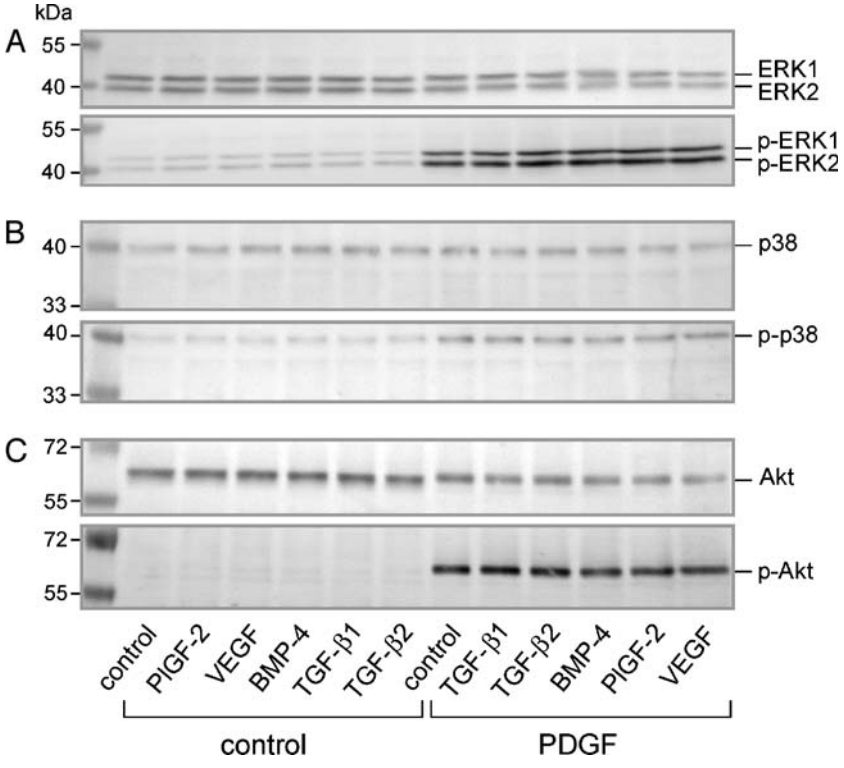

Fig. 6 PDGF but not PlGF-2, VEGF-A_{165}, BMP-4, TGF-β1, or TGF-β2 evoke phosphorylation of ERK1/2 (**a**), p38 (**b**), and Akt proteins (**c**) in human RPE cells. Amounts of total proteins are shown above; amounts of phosphorylated proteins are shown below. The cultures were stimulated with the growth factors (10 ng/ml) for 10 min. Similar results were obtained in three independent experiments using cells from different donors

(Fig. 5b). PlGF-2, at a high concentration of 100 ng/ml, slightly decreased the cell proliferation (Fig. 5b).

Chemotaxis

The chemotaxis of RPE cells was investigated by using a modified Boyden chamber assay. As shown in Fig. 5c, exogenous PlGF-2 strongly enhanced the chemotaxis of RPE cells, with significant effects at concentrations above 0.01 ng/ml. In contrast, VEGF-A evoked a significantly smaller chemotactic response. The data indicate that PlGF-2 is not a mitogen but a relatively strong motogen for RPE cells.

Phosphorylation of ERK1/2, p38, and Akt

To determine whether PlGF activates intracellular signaling pathways in RPE cells that may be associated with the stimulation of cell migration, the phosphorylation levels of extracellular signal-regulated kinases 1/2 (ERK1/2), p38 MAPK, and Akt (protein kinase B) proteins were examined by using Western blotting. Exogenous PlGF-2 did not enhance the amount of the phosphorylated proteins (Fig. 6), suggesting that the chemotactic effect of this factor is not mediated by activation of the ERK1/2, p38 MAPK, or phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways. Similarly, VEGF, BMP-4, TGF-β1, or TGF-β2 failed to evoke phosphorylation, while exogenous PDGF evoked phosphorylation of all three signaling proteins (Fig. 6). Similar results were obtained after extension of the stimulation time up to 3 h or using a higher concentration (50 ng/ml) of PlGF-2 or VEGF (data not shown).

Discussion

It has been shown that the co-expression of receptors for VEGF and PlGF, KDR and neuropilin-1, in fibrovascular tissues correlates with the degree of neovascularization in young patients with proliferative diabetic retinopathy [16]. PlGF facilitates endothelial cell proliferation and vascular permeability by potentiating the activity of VEGF [1, 8, 26], and the presence of PlGF is required for the angiogenic effect of VEGF in the ischemic retina [5]. Here, we show that human RPE cells produce, in addition to VEGF, also PlGF, and that PlGF stimulates the chemotaxis of RPE cells. The PlGF secreted by RPE cells may facilitate the formation of fibrovascular membranes in the course of the proliferative diabetic retinopathy, via stimulation of endothelial cell proliferation and RPE cell migration.

The present results confirm previous findings that human RPE cells in vitro express both VEGF receptors, flt-1 and KDR (Fig. 1a) [15]. In addition to the VEGF receptors, we found that RPE cells express receptors for PlGF, the neuropilins, and that the gene expression of neuropilins is significantly greater compared to the gene expression of VEGF receptors, flt-1 and KDR (Fig. 1a,b).

Despite the fact that TGF-β inhibits the proliferation of cultured endothelial cells [2, 13], it has been found to be a strong inducer of angiogenesis in vivo [31], probably via induction of angiogenic factors in cell types surrounding proliferating vessels [28]. Here, we describe that members of the TGF-β superfamily of growth factors strongly stimulate the gene expression of PlGF (Fig. 2a) and the secretion of PlGF protein in human RPE cells (Fig. 4a). In contrast, various other growth factors and pro-inflammatory cytokines displayed no effects on the expression of PlGF by RPE cells. In addition to PlGF, the RPE cells also produce and secrete VEGF. However, the cytokine-dependent regulation of PlGF and VEGF expression differed markedly. TGF-β induced elevation of gene and protein expression of both PlGF and VEGF (Figs 2 and 4), while BMP-4 increased the gene expression of PlGF and decreased the gene expression of VEGF. Distinct other growth factors (PDGF and HB-EGF) stimulated the expression of VEGF but not of PlGF (Figs 2 and 4). The present results support previous findings of a major role of TGF-β in the induction of VEGF secretion by RPE cells [22], and indicate that TGF-β plays also a crucial role in the expression of PlGF by RPE cells. It has been shown that the retinal expression of the mRNA for TGF-β1 is increased during the development of diabetic retinopathy, along with an increased gene expression of VEGF and PlGF [36]. The present results suggest that RPE cells stimulated by TGF-β-related growth factors may represent one source of the increased expression of PlGF in the diabetic retina. We found that PlGF did not induce secretion of VEGF (Fig. 4b), and vice versa, VEGF did not increase the secretion of PlGF (Fig. 4a), disclosing a direct relationship between PlGF and VEGF at the level of RPE cells. Apparently, TGF-β induces relatively independently the production and secretion of VEGF and PlGF in RPE cells. However, further studies are necessary to evaluate the relationships of the action of TGF-β on different cell types involved in the process of fibrovascular disease.

It has been shown previously that the receptor for PlGF, neuropilin-1, stimulates the migration of vascular endothelial cells, via activation of PI3K, p85, and RhoA [39]. We found that PlGF stimulates the chemotaxis of RPE cells (Fig. 5c), while it had only a slight inhibiting effect on cell proliferation at high concentration (Fig. 5b). The intracellular signaling pathways that are stimulated in RPE cells after activation of neuropilins by PlGF, and that stimulate cell migration, remains to be determined. As expected, the failure of an effect of PlGF on cell proliferation (Fig. 5a,b) is associated with an absence of enhanced phosphorylation of ERK1/2 by PlGF (Fig. 6). In various cell systems, cell migration is supported by activation of the p38 MAPK [32,

33, 37]. However, we did not found enhanced phosphorylation of p38 after stimulation with PlGF (Fig. 6). Likewise, Akt protein did not show phosphorylation in the presence of extracellular PlGF (Fig. 6), suggesting that PlGF did not stimulate the PI3K-Akt pathway in RPE cells.

In summary, we show that TGF-β-related growth factors induces the production and secretion of both VEGF and PlGF by RPE cells. Moreover, the migration of RPE cells stimulated by PlGF may contribute to the growth of fibrovascular tissue. It remains to be proven whether RPE cell-derived PlGF contributes to the progression of pathological neovascularization in situ.

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