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The fibroblast growth factor receptors, FGFR-1 and FGFR-2, mediate two independent signalling pathways in human retinal pigment epithelial cells

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Abstract

To examine the effects and potential implications for the expression of the two basic fibroblast growth factor (bFGF) receptors, FGFR-1 and FGFR-2, in retinal pigment epithelial (RPE) cells, bFGF-dependent changes in gene expression and RPE cell function were studied. bFGF increased L-type Ca^{2+} channel activity of RPE cells, which in turn resulted in an increase of vascular endothelial growth factor A (VEGF-A) secretion from RPE cells. Also, both bFGF and direct stimulation of L-type Ca^{2+} channels by BayK8644 increased the expression of c-fos in RPE cells, to the same extent. bFGF-induced-c-fos expression was reduced by inhibition of FGFR-1, but not by L-type Ca^{2+} channel inhibition, demonstrating that stimulation of FGFR-1 results in a Ca^{2+} channel-independent change of gene expression. In contrast, stimulation of FGFR-2 results in a Ca^{2+} channel-dependent stimulation of VEGF secretion. Furthermore, immunohistological investigation of neovascular tissues obtained from patients with age-related macular degeneration (AMD) revealed FGFR-1 and FGFR-2 expression in the RPE of the diseased tissue. Our findings support the hypothesis that there are two different FGFR-1- and FGFR-2-dependent pathways that modulate the role of bFGF in induction of neovascularisation in AMD.

Keywords: Fibroblast growth factor receptors; L-type calcium channels; bFGF-induced-c-fos expression; Neovascularisation; Age-related macular degeneration; Retinal pigment epithelial cells

Basic fibroblast growth factor (bFGF or FGF2) is expressed in the retina where it can function as a rescue factor and is up-regulated in degenerative diseases of the retina [1–9]. It is found in high concentration in neovascular tissue in age-related macular degeneration (AMD) and is up-regulated in laser-induced choroidal neovascularisation (CNV) pointing to its potential role in the development of CNV [1,10–18]. Studies on the role of bFGF in the development of CNV have shown that bFGF can indirectly induce neovascularisation (NV), by up-regulating vascular endothelial growth factor (VEGF) and increasing the proliferation of endothelial cells [10,16,19–24]. This finding suggests that the FGF and VEGF signalling systems interact. This is further supported by a study showing that VEGF induces expression of fibroblast growth factor receptor-1 (FGFR-1) in retinal pigment epithelial (RPE) cells [20]. An indirect rather than causative role for bFGF in initiation of NV is further supported by the fact that bFGF deficient mice still develop experimentally induced

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NV [25]. The lack of bFGF in these mice may be compensated by the activity of other growth factors such as insulin-like growth factor-1 (IGF-1) [26–28], which can also induce VEGF expression. Nonetheless, bFGF may play an important role in CNV since it can induce NV in the eye when cell damage, such as photoreceptor or RPE injury, is present [1–3,8,16,23,29]. This is significant and suggests bFGF's role in the induction of NV may be similar to that of other growth factors such as VEGF. A recent study demonstrated that VEGF overexpression by the RPE alone is not sufficient to induce CNV, while VEGF overexpression in combination with a loss of the integrity in the RPE cell layer leads to CNV [30]. It follows, therefore, that an up-regulation of bFGF in response to cell injury is responsible for this effect.

Since the RPE is a major source for VEGF in CNV [10,13,27,31,32] and expresses both FGFR-1 and FGFR-2 [9,22,33–36], it is plausible that the RPE functions as a target for the bFGF-dependent enhancement of NV. The functional significance for the expression of the two FGF receptors in the RPE is not known. One possibility is that the two receptors activate different pathways as suggested by an investigation of tyrosine kinase-dependent regulation of L-type Ca^{2+} channels in the RPE, which demonstrated that FGFR-2 selectively activates L-type Ca^{2+} channels by direct interaction, whereas FGFR-1 has no effect on channel activity [35]. The aim of the present study was to describe changes in RPE cell function caused by different FGF receptor signalling pathways that lead to CNV development. We found that both receptors are expressed in RPE cells in CNV membranes and that stimulation of FGFR-1 leads primarily to changes in gene expression whereas stimulation of FGFR-2 increases the rate of VEGF secretion from RPE cells.

Materials and methods

Immunohistology. Indirect immunohistochemistry was used to demonstrate the localisation of bFGF and FGF receptors in CNV membranes surgically excised from patients with exudative age-related macular degeneration (AMD; $n = 3$), during macular translocation surgery with 360° peripheral retinectomy. Cryosections (10 μm) were treated with acetone (5 min), dried at ~45 °C (10 min), rehydrated (5 min) with phosphate-buffered saline (PBS), blocked with 20% rabbit serum (Jackson Immunoresearch, West Grove, PA) in PBS/0.5% Triton for 2 h at room temperature, and then incubated overnight with a primary antibody (polyclonal goat raised against human FGFR-1, FGFR-2 or bFGF; R&D systems, Minneapolis, MN) at 4 °C. The following day, sections were incubated with secondary antibody (rabbit anti-goat-biotinylated IgG; Jackson Immunoresearch) for 1 h. Between incubations, sections were washed three times with 2% rabbit serum in PBS for 5 min each. Signal amplification was obtained with a Vectastain ABC kit, followed by colour development with a peroxidase substrate-diaminobenzadine kit (DAB; Vector laboratories, Burlingame, CA). Slides probed with non-immune serum or irrelevant primary antibodies served as negative controls.

Cell culture. Cell cultures from human eyes were established using the method of Aronson [37]. These cells and the human RPE cell line, ARPE19 (ATCC), were cultured in DMEM supplemented with 20% foetal calf serum (FCS), 100 μg/ml kanamycin, and 50 μg/ml gentamycin. All cell cultures were maintained at 37 °C with 5% CO_2 and the medium was changed twice a week. Confluent cultures were passaged using the trypsin/EGTA method and split in a ratio 1:2. Only RPE cells of early passages (2–4) were used for the experiments. For the use of human material, tenets of the Declaration of Helsinki were followed, informed consent was obtained, and Institutional Human Experimentation Committee approval was granted for the studies.

RNA isolation and Quantitative RT-PCR (qRT-PCR). Total RNA was isolated from confluent ARPE cell cultures using RNAzol B (Wak-Chemie Medical GmbH, Steinbach, Germany) according to the manufacturer's protocol. One microgram of total RNA per reaction was reverse- transcribed using an Omniscript RT Kit (Qiagen, Hilden, Germany) as described by the manufacturer. c-fos cDNA was amplified for 30 cycles using the following primer pair:

c-fos (human)	+	5′-CGAGATTGCCAACCTGCTGAA-3′
	−	5′-CACTGGGCCTGGATGATGC-3′

The amplified products were verified by agarose gel electrophoresis and showed single bands of predicted sizes for each sample and no products in negative controls. PCR products were gel purified and ligated into pCR2.1-TOPO (Invitrogen, Groningen, Netherlands) for further amplification and DNA sequence analysis. Quantitative PCR was performed using the FastStart DNA Master SYBR Green I Kit according to the manufacturer's instruction (Roche, Mannheim, Germany) on a Roche LightCycler. Briefly, 6 μl cDNA was used per reaction with a final $MgCl_2$ concentration of 4 mM. Reactions were denatured for 10 min at 95 °C and subjected to 40 cycles in a three-step PCR (95 °C/15 s, 60 °C/5 s, and 72 °C/10 s). Detection of fluorescence occurred at the end of the 72 °C elongation step. Specificity of PCR products was verified by melting curve analysis subsequent to the amplification. Amplification, data acquisition, and analysis were carried out by LightCycler. c-fos mRNA copies were expressed relative to the control (100%) value.

Measurement of $[Ca^{2+}]_i$ with fura-2. Intracellular-free calcium ($[Ca^{2+}]_i$) was measured using the Ca^{2+}-sensitive dye, fura-2AM (Sigma, Deisenhofen, Germany), based on methods described by Grynkiewicz et al. [38]. Before each experiment, semi-confluent cells were incubated in a control solution consisting of 130 mM NaCl, 3 mM KCl, 0.3 mM $CaCl_2$, 0.6 mM $MgCl_2$, 14 mM $NaHCO_3$, 1 mM Na_2HPO_4, 33 mM Hepes, and 6 mM glucose (pH 7.2 with Tris) with 10 mM fura-2AM for 30 min at room temperature. Following incubation, cells were perfused with the control solution for at least 30 min to remove any extracellular dye. Fluorescence of fura-2 was excited at two excitation wavelengths of 340 and 380 nm and recorded at 510 nm using a photomultiplier (Hamamatsu 928 SF, Hamamatsu Photonics, Herrsching, Germany). Data storage and processing was performed using TIDA for Windows software (HEKA, Lamprecht, Germany). Changes in the 340 nm/380 nm fluorescence ratio represent relative changes in $[Ca^{2+}]_i$. Absolute $[Ca^{2+}]_i$ was calculated using cellular calibration, and the equation and dissociation constant from Grynkiewicz et al. [38].

Patch-clamp recordings. Patch-clamp recordings were made in the perforated patch configuration with K^+-free solutions. The bath solution consisted of control solution w/o KCl plus 3 mM TEACl, 10 mM $BaCl_2$, and the pipette solution contained 100 mM CsCl, 10 mM NaCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM EGTA, 10 mM Hepes (pH 7.2 with Tris), and 150 μg/ml nystatin. To activate currents through L-type channels, the cells were depolarised from a holding potential of −70 mV. Depolarisation consisted of 9 voltage-steps of 50 ms duration and 10 mV increasing amplitude. Currents were measured using an EPC-9 patch-clamp amplifier (HEKA, Lamprecht, Germany) in conjunction with TIDA software (HEKA) for electrical stimulation, data storage, and analysis.

VEGF secretion by RPE cells. To measure VEGF secretion, approximately 10^5 RPE cells were plated in each chamber of a 12-well plate containing 500 μl DMEM without FCS. The concentration of VEGF-A (VEGF-165) secreted into the media was measured every 4 h by ELISA (Biosource International, Solingen, Germany) according to the manufacturer's protocol.

Reagents. All other chemicals or culture media were of analytical grade and were purchased from Sigma (Deisenhofen, Germany) or Biochrom (Berlin, Germany).