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Epidermal growth factor: the driving force in initiation of RPE cell proliferation

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Abstract

Background—To analyze whether epidermal growth factor (EGF) exerts regulatory effects on proliferation and differentiation in ARPE19 cells after different incubation periods (24 vs. 48 h) for obtaining ideal conditions for feasible rejuvenation and autologous transplantation of retinal pigment epithelial cells (RPE cells).

Methods—To evaluate gene expression patterns of RPE-specific differentiation and proliferation markers as well as transcriptional and translational changes of beta-catenin (β -catenin)-signaling markers by fluorescence activated cell sorting (FACS) and reverse transcription – polymerase chain reaction (RT-PCR) after 24 h of EGF treatment.

Results—After 24 h of EGF treatment, a significant decrease of retinal pigment epithelium-specific protein 65 (RPE 65), cellular retinaldehyde-binding protein (CRALBP) and cytokeratin 18 in ARPE-19 cells was scaled. In addition, an increase of cyclin D1 expression and a significant decrease of glycogen synthase kinase-3 β (GSK-3 β) and beta-catenin (β -catenin) were equally observed after 24 and 48 h of EGF treatment. Cell-cycle studies revealed an increase of ARPE cells in S-G2/M phase after 24 h of EGF treatment.

Conclusions—Our data demonstrate the induction of proliferation and upregulation of the β -catenin signaling pathway by EGF even after 24 h of incubation. As ideal cell culture conditions

are essential for maintaining RPE-specific phenotypes, short incubation times enhance RPE cell quality for feasible rejuvenation and subsequent autologous transplantation of RPE cells.

Keywords

Retinal pigment epithelium; Age-related macular degeneration; Ageing; Differentiation; Proliferation; Epidermal growth factor; β -catenin signaling pathway

Introduction

The transplantation of retinal pigment epithelial (RPE) cells is one main approach to the treatment of retinal degenerations as age-related macula degeneration (AMD). Transplantation of homologous RPE cells has always been rejected by the patients' immune system so far whereas autologous RPE cells have already been successfully transplanted in human eyes [1–8]. In the animal model, even recovery of vision has been observed, but in human with AMD, only limited results have been obtained. The literature describes many possible factors for this limited outcome such as source of RPE (cultured versus fresh), high age of donor, type of delivery of transplanted cells (cell sheets better than suspension), status of host (typically late-stage disease), influence of melanosomes on phototoxicity (young melanosomes protect RPEs but aged ones are detrimental) and the influence of lipofuscin on phototoxicity [9, 10].

All of these factors demonstrate that autologous RPE cells from aged donors result in a senescent, dysfunctional RPE phenotype, which might not have a major impact on recovering vision in AMD patients [6, 7] as they are genetically programmed and environmentally induced seniors. Therefore young or rejuvenated cells would be the best candidates for successful RPE transplantation so that AMD patients might recover vision again [11, 12]. In our first study, we examined that the first step of such a rejuvenation process is the “push back” of cells into G1 phase of the cell cycle to provide an insight into an active cell cycle for those cells indicating induced proliferation of cells and reduced differentiation [13]. Therefore, we chose different RPE-specific differentiation markers (retinal pigment epithelium-specific Protein 65 (RPE 65), cellular retinaldehyde-binding protein (CRALBP), cytokeratin 18), proliferation markers (cyclin D1) and Wnt markers (beta-catenin (β -catenin), glycogen synthase kinase-3beta (GSK-3 β)) to investigate proliferation vs. differentiation in ARPE-19 cells on the translational level via fluorescence activated cell sorting (FACS) and on the transcriptional level via reverse transcription – polymerase chain reaction (RT-PCR). In addition, we evaluated cell-cycle studies to prove whether epidermal growth factor (EGF) is a possible candidate for promoting cell proliferation and restriction of differentiation via activation of the beta-catenin signaling pathway, part of the canonic Wnt pathway, as EGF is known to induce proliferation in other epithelial cell types [14, 15]. Our data identified EGF as a potent initiator of RPE proliferation via activation of the β -catenin signaling pathway after 48 h of treatment, showing a significant decrease of RPE 65, CRALBP and cytokeratin 18 in ARPE-19 cells [13]. In addition an increase of cyclin D1 expression and a significant decrease of GSK-3 β and β -catenin were observed. Cell-cycle studies revealed an increase of ARPE cells in S-G2/M phase after 48 h of EGF treatment [13].

Nevertheless, several studies in the field of RPE cell biology verify the fact that ideal cell culture conditions are essential to maintain RPE-specific phenotype [16, 17]. Short incubation times enhance RPE cell quality for feasible rejuvenation and subsequent autologous transplantation of RPE cells [18]. As EGF turned out to be that powerful after 48 h incubation [13], we investigated its mitogenic effect on the induction of RPE cell proliferation after shorter incubation times (24 h).

Materials and methods

Cell culture

ARPE-19 cells were obtained from ATCC [13, 19], and cultured in Ham's F10, 10% FCS. ARPE-19 cells of passages 3–8 were used for growth factor stimulation.

Stimulation of ARPE-19 cells with EGF

Cells were plated out at a density of 1×10^6 /T₂₅ flasks. Subconfluent cells were starved in 0% FCS medium for 30 min followed by incubation with the growth factor (Sigma Aldrich) in 2% FCS medium for 24 h and harvested before cells reached confluency. Each experiment was performed five times ($n=5$), and EGF was respectively used in one control and two different dilutions (no EGF, 50 ng/ml, 100 ng/ml).

Flow cytometry (FACS)

After growth factor treatment cells were harvested and fixed and permeabilized using Fix and Perm intracellular staining kit (An Der Grub, Germany). Cells were incubated with primary antibodies against cytokeratin 8/18 (Dako), RPE65 (abcam), CRALBP (abcam), cyclin D1 (Cell Signalling Technology), β -catenin (Cell Signalling Technology) and GSK-3 β (Cell Signalling Technology). Thereafter, cells were washed and incubated for 30 min with an FITC-labeled anti-mouse antibody (Caltag). FACS analysis was performed on FacScan (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson).

Real-time reverse transcription (RT)-polymerase chain reaction (PCR)

Preparation of mRNA from ARPE-19 cells was performed using the QIAGEN RNeasy kit (QIAGEN, Hilden, Germany), followed by reverse transcription into cDNA with oligo-dT primers (Clontech, Palo Alto, CA); 5 μ l of cDNA was amplified for 40 cycles with specific primers for G3PDH, cytokeratin 18, RPE65, CRALBP, β -catenin, cyclin D1, and GSK-3 β (Table 1) [15, 20, 21]. PCR reactions containing SYBR-green were amplified on a Corbett real-time PCR machine (Rotor-Gene 2000, Corbett Research).

Data were acquired as cycle threshold (Ct) value, which denotes the starting cycle for the amplification. As internal standard to normalize mRNA levels for differences in sample concentration and loading, amplification of G3PDH was used. Standard curves were constructed from standard reactions for each target gene and internal control by plotting Ct values vs. log cDNA dilution. Because the amplification efficiencies of target genes and internal control were equal, the relative change of target gene expression in stimulated ARPE-19 cells compared unstimulated ARPE-19 cells (Δ Ct-calibrator values) was calculated using the equation $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t(\text{calibrator value}) - \Delta C_t(\text{target value})$ [22]. The Δ Ct values were determined by subtracting the average G3PDH Ct value from the average target gene Ct value. The standard deviation (SD) of the difference was calculated from the standard deviations of target gene and β -actin values.

After each real-time RT-PCR, a melting profile and agarose gel electrophoresis of each sample were performed to rule out non-specific PCR products and primer dimers.

Cell cycle analysis

Cell cycle analysis was performed by standard protocols. Briefly, cells were fixed in ice-cold 70% ethanol (4°C, 30 min) followed by RNaseA digestion (37°C, 30 min). Cells were stained with propidium iodide for 5 min at RT. DNA content of the cells was measured by FACS analysis and cell-cycle distribution was calculated using the CellQuest software.

Statistics

Means and standard deviations (standard error) were calculated. For statistics, paired Student's *t* test was performed.

Results

Protein expression profile after 24 h of EGF treatment

After 24 h of EGF stimulation, ARPE19 cells showed a decrease of translation of cytokeratin 18 (82.35 ± 17.67 MCF (mean cell fluorescence) vs. 61.71 ± 20.14 MCF; $p=0.15$), RPE 65 (82.51 ± 22.80 MCF vs. 67.22 ± 21.17 MCF; $p=0.29$) and CRALBP (87.12 ± 18.67 MCF vs. 52.77 ± 15.45 MCF; $p=0.11$) (Fig. 1). In addition, a significant increase of cyclin D1 protein amount was found (55.51 ± 5.62 MCF vs. 95.33 ± 23.61 MCF; $p=0.03$), whereas a significant decrease of β -catenin (88.53 ± 29.58 MCF vs. 37.30 ± 7.38 MCF; $p=0.05$) and GSK-3 β (78.27 ± 21.06 MCF vs. 50.41 ± 3.83 MCF; $p=0.03$) protein translation could be demonstrated (Fig. 1).

Target gene expression profile after 24 h EGF treatment

24 h EGF treatment clearly inhibited the transcription of Cytokeratin (22.85 ± 3.50 and 21.15 ± 4.43 ; $p=0.77$) and RPE65 (27.42 ± 1.31 and 26.44 ± 1.24 ; $p=0.14$) mRNAs. In addition a significant decrease of CRALBP mRNA amount (30.89 ± 1.68 and 30.63 ± 1.58 ; $p=0.03$) could be scaled, resulting in remaining 72% Cytokeratin-, 76% RPE65- and 58% CRALBP mRNA in relation to controls (Fig. 2). Furthermore a significant decrease in the transcription of Cyclin D1 (28.24 ± 0.73 and 27.45 ± 2.20 ; $p=0.04$), β -Catenin (26.07 ± 2.94 and 25.91 ± 2.28 ; $p=0.05$) and GSK-3 β (27.16 ± 4.70 and 24.67 ± 1.95 ; $p=0.05$) mRNAs was measured, resulting in residual 45% of cyclin D1-, 73% of β -catenin- and 35% GSK-3 β mRNA in the cells nuclei.

Cell cycle analysis

Cell cycle analysis shed light upon the cells' current cell-cycle stage due to growth factor treatment. After 24 h, EGF incubation resulted in a decrease of G1/G0 stage cells (32.59 ± 5.78 ; $p=0.25$) whereas percentage of cells cycling in G2/M phase increased significantly (27.53 ± 3.50 ; $p=0.03$; Fig. 3). No changes were observed in the relative amount of S-phase cells (39.88 ± 8.21 ; $p=0.58$).

Conclusions

Our recent data demonstrate the induction of proliferation via activation of the β -catenin signaling pathway by EGF even after 24 h of incubation.

Flow-cytometry data together with RT-PCR data confirmed that incubation of cells in EGF resulted in a decrease of β -catenin and GSK-3 β transcription and translation after 48 h [13] even after 24 h of incubation. EGF is known to inhibit GSK-3 β , leading to accumulation and translocation of β -catenin into the nucleus via activation of β -catenin signaling pathway [23, 24]. As demonstrated by our cyclin D1 data, target gene expression is boosted at the end of the β -catenin signaling cascade according to 24-h EGF treatment, indicating induction of cell proliferation and progression through the cell cycle. Additionally, the down-regulation of RPE65-, CRALBP-, and cytokeratin gene expression is also a hint for cell proliferation rather than differentiation. Cell-cycle analyses confirm that EGF is a powerful tool for boosting RPE cell proliferation after 24-h incubation time, making cells progressing further through the active cell cycle.

ARPE-19 is a well-characterized RPE cell line that expresses differentiation, cytoskeleton, and proliferation proteins involved in proliferation of RPE cells [19, 25, 26] and therefore served as a model for investigating RPE cell proliferation and differentiation in different studies. Our data derived from ARPE-19 cells indicate how proliferation of RPE cells can be boosted and differentiation is inhibited even though these are already proliferating cells with minor differentiation abilities.

In our previous study, we suggested how human senile RPE cells could be preceded by re-entry of the cell cycle according to activation of the β -catenin signaling pathway via EGF treatment after 48 h, leading to “rejuvenation” of these senile RPE cells [13]. Our chosen differentiation-, proliferation-, and Wnt/ β -catenin signaling markers offered a good overview of how proliferation of aged cells could be promoted and differentiation could be suppressed. Nevertheless, the ideal human RPE phenotype for autologous transplantation is strongly connected to ideal cell-culture conditions. Therefore, short incubation times have a beneficial impact on the enhancement of RPE cell quality for feasible rejuvenation and subsequent autologous transplantation of RPE cells. EGF turned out to be a powerful actor in boosting cell proliferation after 48 h of incubation [13]. In this study, we verified the activation of β -catenin signaling pathway and its associated biochemical cascade even after 24 h of incubation, pointing out the powerful impact of EGF on RPE cells. Our findings confirm the potential for “rejuvenation” of RPE cells within short incubation times without penalty of RPE-specific phenotype/morphology. The other growth factors used in our first study turned out to be less powerful for this purpose, indicating no statistically significant effect on RPE cell-cycle progression and associated progress of RPE cell proliferation. Considering the mathematical/statistical point of view, the 24-h data's significance values in FACS and RT-PCR are lower compared to those in our previous study, but beyond doubt they are unalterably still statistically significant. Additionally, preservation of ideal conditions for feasible rejuvenation and autologous transplantation of RPE cells can be guaranteed by observing the same mitogenic effect on the induction of RPE cell proliferation after shorter incubation times (24 h). Therefore, 24-h data serve the same outcome as the 48-h data plus additionally offer better conditions for obtaining RPE-specific phenotype, suggesting these young or rejuvenated cells to be the best candidates for successful RPE transplantation so that AMD patients might recover vision again.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Impact of EGF on Translation of RPE specific Differentiation and Proliferation Markers

Fig. 1.

The impact of EGF on the translation of RPE-specific differentiation and proliferation markers after 24 h. Compared to our previous 48-h data (Steindl-Kuscher et al., 2009) EGF activated the β -catenin signaling pathway even after 24 h of incubation, resulting in a significant decrease of the negatively regulated Wnt proteins (β -catenin and GSK-3 β) and a significant increase of cyclin D1 protein in the cell's cytoplasm. Additionally, the amount of RPE-specific differentiation proteins (cytokeratin, RPE65, CRALBP) was reduced. * $p < 0.05$, $n = 5$

Fig. 2.

The impact of EGF on the transcription of RPE-specific differentiation and proliferation markers after 24 h. RT-PCR data confirmed activation of the β -catenin signaling pathway by indicating a significant decrease of transcription of the Wnt markers (β -catenin and GSK-3 β). CRALBP transcription was also significantly inhibited. Additionally, the amounts of cytokeratin-, RPE65-, and cyclin D1 mRNAs were reduced as well in the cells nuclei. * $p < 0.05$, $n = 5$

Fig. 3.

Cell cycle analysis. The impact of EGF on the progression of RPE cell cycle after 24 h of EGF incubation. Percentage of counted cells cycling at G1/G0 stage of the cell cycle decreased whereas percentage of G2 stage cells increased significantly after 24 h of EGF treatment, indicating the proliferation of cells. * $p < 0.05$, $n = 5$

Table 1
Primers of RPE-specific differentiation and proliferation markers used for RT-PCR

Marker	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product length (bp)
G3DPH	CCCATCAGGATCTTCCAG (Krugluger et al., 2007)	CCTGCTTCACCACCTTCT	590
Cytokeratin 18	CACACAGTCTGCTGAGGTTG (Krugluger et al., 2007)	TAAAGTCCTCGCCATCTTCC	332
RPE65	GTGTAGTTCTGAGTGTGGTG (Krugluger et al., 2007)	CACAGAGGAAGTATGATTAT	369
GSK-3	AACTGCCCCGACTAACAACAC (Krugluger et al., 2007)	ATTGGTCTGTCCACGGTCTC	253
β -catenin	TGCGGACTCAGAAGGAACATGAC (Krugluger et al., 2007)	ACTAGTCGTGGAATGGCACC	162
CRALBP	TGG-CAA-AGT-CAA-GAA-ATC-ACC (Schlunck et al., 2002)	CGT-GGA-CAA-AGA-CCC-TCT-CA	313
Cyclin D1	CCA-TGG-AAC-ACC-AGC-TCC (Hecquet et al., 2002)	GGA-GCT-GGT-GTT-CCA-TGG	270