·Basic Research·

Effect of NF-κB p65 antisense oligodeoxynucleotide on transdifferentiation of normal human lens epithelial cells induced by transforming growth factor-β2

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Abstract

• AIM: To study the inhibition of nuclear factor kappa-B p65 (NF-κB p65) antisense oligodeoxynucleotide (ASODN) on transdifferentiation of normal human lens epithelial cells induced by transforming growth factor-β2 (TGF-β2) *in vitro*.

• METHODS: NF-κ B p65 ASODN and NF-κ B p65 missense oligodeoxynucleotide (MSODN) were designed and synthesized. Human lens epithelial cell line (HLE B-3) cells were prepared for study and divided into 7 groups. Control group was HLE B-3 cells cultured *in vitro* in dulbecco´s modified eagle medium (DMEM). T1, T2, and T3 group were HLE B-3 cells cultured *in vitro* in DMEM with 10 ng/mL TGF-β2 for 6h, 12h, 24h respectively. A+T group was HLE B-3 cells cultured with 10 ng/mL TGF-β2 for 24h after transfected by NF-κB p65 ASODN for 24h. M+T group was HLE B-3 cells cultured with 10 ng/mL TGF-β2 for 24h after transfected by NF-κB p65 MSODN for 24h. The negative control group was HLE B-3 cells cultured with 10 ng/mL TGF-β2 for 24h after cultured with transfer agent (HiPerFect) for 24h. Cell morphology was observed at different time points using an inverted microscope. The expression of NF-κB p65 mRNA was detected with reverse transcription-polymerase chain reaction (RT-PCR), and the expression of α-smooth muscle actin (α-SMA) protein was assayed with ELISA.

• RESULTS: With the TGF-β2 stimulation prolongation, the expression of NF-κB p65 mRNA and a-SMA protein increased in T1, T2, T3 groups compared with the control group, and the difference was statistically significant (P<0.05). NF-κB p65 ASODN lowered the expression of NF-κB p65 mRNA and α-SMA protein induced by TGF-β2. NF-κB p65 MSODN and HiPerFect did not lower the expression of NF-κB p65 mRNA and α-SMA protein induced by TGF-β2. The difference between control group and A+T group was not statistically significant (P>0.05), but the difference among A+T group and other groups was statistically significant (P<0.05).

• CONCLUSION: NF-κB p65 ASODN could lower the expression of NF-κB p65 mRNA and α-SMA protein induced by TGF-β2, and antagonized TGF-β2-induced transdifferentiation of HLE B-3 *in vitro*. NF-κB p65 ASODN could be used as a new biological therapeutic target of posterior capsular opacification.

• KEYWORDS: nuclear factor kappa-B p65; antisense oligodeoxynucleotide; transforming growth factor-β2; α-smooth muscle actin; lens epithelial cells
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INTRODUCTION

Posterior capsular opacification (PCO) refers to the lens capsule opacification after the extracapsular cataract extraction or ultrasonic phacoemulsification, and is the most common complications of modern cataract surgery which may lead to vision loss. The proliferation, migration and differentiation of postoperative residual lens epithelial cells in the posterior capsular membrane are the main reasons for the formation of PCO [1]. Park *et al* [2] found that nuclear factor kappa-B (NF-κB) seems to be related to the proliferation, migration of lens epithelial cells.

Antisense oligodeoxynucleotide (ASODN) was a synthetic nucleotide, and can effectively regulate gene expression by inhibiting translation, maturation, or blocking transcription,

which had been widely used in many fields. ASODN of STAT6 could inhibit the allergic airway inflammation by transfecting of lung epithelial cells[3]. ASODN of STAT5 had been testified effectively in treating certain leukemias/ lymphomas [4]. Both phase I study of the c-raf-1 antisense oligonucleotide and phase II study of the GTI-2040 antisense oligonucleotide had proven the efficacy of ASODN as targeted therapy in preclinical studies and been able to modulate target protein's expression [5-6]. Li *et al* [7] found that NF-κB p65 ASODN can inhibit gastric tumors proliferation and induce apoptosis of tumor cells *in vivo* and *in vitro*. Zhou *et al* [8] found that NF-κB p65 ASODN can inhibit fibrosis of liver cells *in vivo*.

ASODN had also been studied extensively in the field of ophthalmology. ASODN can affect the lens cells and corneal cells [9-10]. NF-κB ASODN can effectively inhibit graft rejection and prolong the survival time of corneal allografts[10]. The inhibitors of NF-κB could block lens epithelial cells migration from the equatorial to the posterior capsule [11]. Here, we examined the impact of NF-κB p65 ASODN on the transdifferentiation induced by transforming growth factor-β2 (TGF-β2) in Human lens epithelial cell line (HLE B-3) cells *in vitro*, and studied the gene therapy of PCO experimentally.

MATERIALS AND METHODS

Culture of Human Lens Epithelial Cell Line The HLE B-3 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells (2×10^5 cells/well) were cultured in 24-well plates in dulbecco's modified eagle medium (DMEM) including 10% inactivated fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37℃. When the cells reached 80% confluence, the culture medium was replaced with serum-free medium (SFM). Cultivated for another 24h in FSM, the cells achieved synchronization for experiment. Coverslips of 0.8×0.8-cm^2 size were placed in 6 well plates, the HLE B-3 cells (2×10^5 cells/well) were cultured in it to produce the cell climbing pieces.

Human Lens Epithelial Cell Line Cultured with Transforming Growth Factor-β2 Cultivated in FSM for 24h, 10 ng/mL TGF-β2 was added to the culture medium. The cell morphology was observed and the cells were collected after cultured 6, 12, and 24h. The HLE B-3 cells cultured with 10 ng/mL TGF-β2 for 6h was the T1 group, for 12h was the T2 group, and for 24h was the T3 group. The HLE B-3 cells cultured in FSM were the control group.

Synthesis of Antisense Oligodeoxynucleotide and Missense Oligodeoxynucleotide In search of the full-length sequence of human NF-κB p65 mRNA in National Center for Biotechnology Information (NCBI), its ASODN and MSODN sequence were synthesized according to the literature [12]. ASODN's sequence is 5'GGG GAA CAG TTC GTC CAT GGC 3', MSODN's sequence is 5'GTA CGC GGT GAA GCTGCG ATC 3'. According to the Gene Bank, ASODN and MSODN had no homology with other genes besides NF-κB p65 gene. They were synthesized by Shanghai Biological Engineering Company (China) with phosphorothioate modification technology followed by PAGA purification.

Transfection of the Human Lens Epithelial Cell Line Cells would be 80% confluence at the time of transfection. Transfer agent (HiPerFect) was mixed with ASODN and MSODN in FSM, respectively. The final concentration of ASODN and MSODN was 5 nmol/L, and the volume of HiPerFect was 3.0 μL. The mixer added to each well and the cells were co-cultured for 24h. Then 10 ng/mL TGF-β2 was added and cultured for another 24h. The cells co-transfected by HiPerFect and NF-κB p65 ASODN were the A+T group, the cells co-transfected by HiPerFect and NF-κB p65 MSODN were the M+T group, and the cells transfected by HiPerFect only were the negative control group.

Reverse Transcription-Polymerase Chain Reaction Analysis of Nuclear Factor Kappa-B p65 mRNA Total RNA were extracted from the cells using the kit step (MBI Ferment), and were followed by PCR amplification with primers. The amplified PCR products were detected by standard agarose gel electrophoresis. The relative expression of NF-κB p65 mRNA was the quantitative absorbance values compared with the absorbance values of β-actin which was the internal reference (NF-κB p65 primers: upstream: 5'-CG CGGATCCTGGCTCGTCTGTAGTGCAC G-3', downstream: 5'-CCCAAGCTTTAGAAGCCATCCCG- GCAGTC-3', the product had 364 bp, the internal reference β-actin primers: upstream: 5'-GTGGACATCCGCAAAGA C-3', downstream: 5'-TCATAGTCCGCCTAGAAGC-3', the product had 281 bp). The two primers were synthesized by Shanghai Biological Engineering Company, China. PCR profile consisted of 2min of initial activation at 94℃, followed by 35 cycles of 40s at 94℃, 30s at 55℃, and 50s at 72℃ and final 10min annealing and extension at 72℃.

Detection of α-smooth Muscle Actin Protein The cells climbing pieces on the coverslips were detected by ELISA Kit (Zhuokang Biotechnology Co. Ltd., Shanghai, China). The absorbance value at 450 nm wavelength measured by enzyme-linked instrument was the optical density of each well, which indicated the content of α-SMA protein. The study adhered to the Declaration of Helsinki.

Statistical Analysis Data were presented as mean±standard deviation (SD), and *P* values were determined by unpaired Student's *t*-test using commercial software (Excel, Microsoft 2007). $P<0.05$ was considered statistically significant.

RESULTS

Morphological Changes of Cells The HLE B-3 cells shaped from oval or polygon to long spindle with the TGF-β2 stimulating, stretched out bulbe, and finally to

became similar fibroblasts. The distance between cells increased, and a large number of floating cells appeared. HLE B-3 cells were cultivated with the 10 ng/mL TGF-β2 for 12h (Figure 1).

Content of Nuclear Factor Kappa -B mRNA The expression of internal reference β-actin was stable in the supernatant of each group. The standard agarose gel electrophoresis results showed that NF-κB p65 mRNA appeared at 364 bp, internal reference β-actin appeared at 28l bp (Figure 2). The relative quantitative absorbance values of NF-κB p65 mRNA in each group were shown in Table 1. Compared with the control group, the values of NF-κB p65 mRNA in the T1, T2, T3, M+T and negative control groups increased, and the difference was statistically significant. Compared with the T1, T2, T3, M+T and negative control groups, values of NF-κB p65 mRNA in the A+T group was significantly lower, and the difference was statistically significant. The difference between the control group and the A+T group was not statistically significant. And the differences among the T3 group, the M+T group and the negative control group were also not statistically significant either.

Content of α -smooth Muscle Actin Protein The absorbance values of α-SMA protein in each group were shown in Table 1 too. Compared with the control group, the content of α-SMA protein in T1, T2, T3, M+T and negative control groups increased, and the difference was statistically significant. Compared with the T1, T2, T3, M+T and negative control groups, the content of α-SMA protein in A+T group was significantly lower, and the difference was statistically significant. The difference between the control group and the A+T group was not statistically significant. And the difference among the T3 group, the M+T group and the negative control group was not statistically significant either.

DISCUSSION

PCO is usually caused by the proliferation, migration, differentiation, collagen deposition, and lens fiber regeneration of residual lens epithelial cells [13]. Therefore, postsurgical medical inhibition of proliferation, migration and differentiation of the residual lens epithelial cells would be an option for preventing PCO. Usually, proliferation of the remaining lens epithelial cells starts within a few hours after cataract surgery [14]. Treatment with aldose reductase (AR) inhibitors significantly prevented the expression of α-SMA and the lens epithelial cell growth in capsular bags to suppress PCO [15]. After cataract surgery the residual lens epithelial cells can produce α-SMA. The expression of α-SMA was the differentiation of the residual lens epithelial cells and the sign of PCO[9]. TGF-β was considered to be the most important cytokines in this procedure [16]. TGF-β2 was mainly present in the eye and could not be found in other

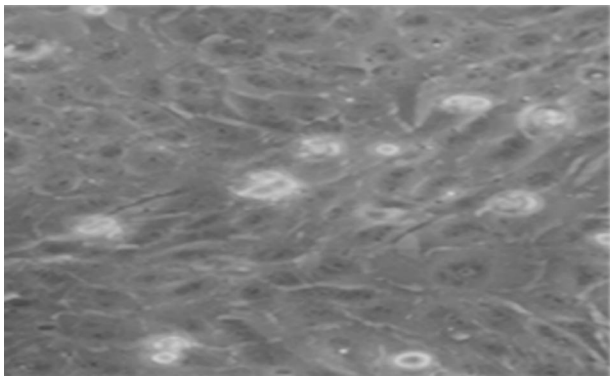

Figure 1 HLE B-3 cells cultivated with the 10 ng/mL TGF-β2 for 12h.

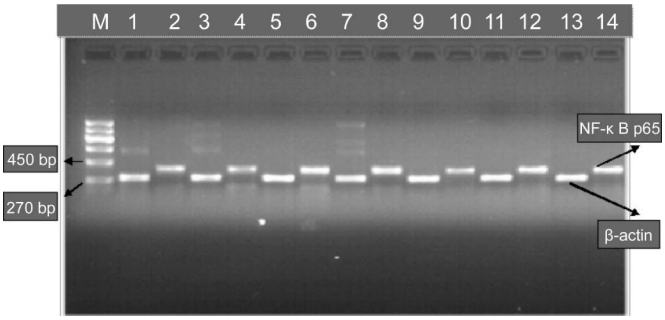

Figure 2 The agarose gel electrophoresis result of PCR.

Table 1 The content of NF-κ B p65 mRNA and α -SMA protein

Group	NF-κ B p65 mRNA	α -SMA protein
Control group	0.173±0.028	0.491±0.102
T1	0.459±0.092[b]	0.884±0.122[b]
T2	0.865±0.106[b]	1.393±0.116[b]
T3	1.308±0.138[b]	1.810±0.190[b]
A+T	0.255±0.056[a]	0.729±0.097[a]
M+T	1.366±0.125[b,c]	1.979±0.175[b,c]
Negative control group	1.371±0.153[b,c]	1.971±0.151[b,c]

[a]*P*>0.05 *vs* control group. [b]*P*<0.05 *vs* control group, A+T group. [c]*P*>0.05 *vs* T3 group (Student's *t*-test, Excel, Microsoft 2007).

organization [17]. Li *et al* [18] found that the expression of TGF-β2 in residual lens epithelial cells was significantly increased after cataract surgery, which can lead to lesion of the lens epithelial cells *in vitro* and result in PCO. The differentiation of the lens epithelial cells could be induced by TGF-β2 to form the PCO model *in vitro*. We observed that with the TGF-β2 stimulation prolongation, HLE B-3 cells morphology changed significantly to form similar fibroblasts, cells spacing increased, the expression of α-SMA also increased, and the PCO model was found successfully *in vitro*. NF-κB in almost all cells was expressed. NF-κB played an important role in the regulation of the immune response, inflammation, cell growth, differentiation and apoptosis. When the cell was not subjected to any stimulation, the NF-κB p65 subunit was "imprisoned" by IκB protein in the cytoplasm and NF-κB was in a non-activated state. When the cells receive various stimuli, the IκB proteins degradated, allowing nuclear translocation of NF-κB p65[19]. This process is called activation of NF-κB. In this experiment, the content

of NF-κB mRNA was observed significantly increased with the TGF-β2 stimulation prolongation, indicating that TGF-β2 stimulation could activate NF-κB. NF-κB had been found to be related to the proliferation, migration of lens epithelial cells [2]. NF-κB might be activated by TGF-β2 to participate into the expression of α-SMA and the differentiation of the lens epithelial cells.

In recent years, with the development of antisense technology, ASODN had been provided to people as a more direct and effective method to artificially control gene expression. Neuropeptide Y ASODN had been testified a useful therapeutic approach to the treatment of ulcerative colitis [20]. The key to the specific action of antisense technology was the selected target sequence of the ASODN and its stability in the cell. In this study the ASODN was a 21 bp sequence complemented to the selected 77-97 bp nucleotide sequence after NF-κB p65 mRNA gene initiation codon. After phosphorothioate modification, the ASODN had been enhanced in terms of stability of ribozyme resistance, good solubility, hybridization, and improved transfection efficiency. ASODN inhibited the expression of NF-κB p65 mRNA, and the following production, activation, and other signal transduction, translation and expression of NF-κB.

In this experiment, when the HLE B-3 cells were successfully transfected by NF-κB p65 ASODN, the expression of NF-κB p65 mRNA was significantly down regulated *in vitro*. In the meanwhile, the expression of α-SMA protein induced by TGF-β2 was decreased, which might be related to the decreased expression of NF-κB p65. We speculated that NF-κB as a triggering factor of the expression of α-SMA protein induced by TGF-β2, NF-κB p65 ASODN inhibited the transdifferentiation of lens epithelial cells induced by TGF-β2 *in vitro* and the cataract formation. NF-κB p65 ASODN could be used as a new biological therapeutic target of PCO.

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