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Inhibitory effect of PDGFR-α antisense oligonucleotide on the proliferation of rabbit lens epithelial cells *in vitro*

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PDGFR-α 反义寡核苷酸抑制兔晶状体上皮细胞增殖的实验研究

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摘要

目的:观察血小板源性生长因子受体α反义寡核苷酸(platelet - derived growth factor receptor - α antisense oligonucleotide,PDGFR-α ASODN)对体外培养兔晶状体上皮细胞 N/N1003A 增殖的影响,并探讨其作用机制。

方法:使用阳离子脂质体 Lipofectamine™2000 将 PDGFR-α ASODN 转染入 N/N1003A 细胞,MTT 法分析细胞的增殖活性,RT-PCR 法检测 PDGFR-αmRNA 的表达,透射电镜观察细胞超微结构改变,流式细胞仪检测细胞的周期和凋亡率。

结果:PDGFR-α ASODN 处理后,N/N1003A 细胞增殖明显受到抑制($P<0.05$),PDGFR-α mRNA 表达明显下调($P<0.05$),均呈剂量依赖性;透射电镜观察到细胞出现典型的凋亡特征;细胞凋亡率显著升高($P<0.05$),同时细胞阻滞于 G_1 期。

结论:PDGFR-α ASODN 可沉默兔晶状体上皮细胞中 PDGFR-α 基因表达,抑制细胞增殖,并诱导细胞凋亡,为应用 PDGFR-α ASODN 预防后囊膜混浊的形成提供实验依据。

关键词:血小板源性生长因子受体-α;反义寡核苷酸;晶状体上皮细胞;细胞增殖;凋亡

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Abstract

• AIM: To observe the effect of platelet-derived growth factor receptor-α antisense oligonucleotide (PDGFR-α ASODN) on the proliferation of rabbit lens epithelial cells N/N1003A *in vitro* and investigate its mechanism.

• METHODS: PDGFR-α ASODN was transfected into N/N1003A cells with Lipofectamine™ 2000. The proliferations of the transfected cells were analyzed by MTT assay and the expressions of PDGFR-α mRNA were detected by RT-PCR. Phase microscope and transmission electron microscope were used to observe the changes of cell morphology and ultramicrostructure. Flow cytometry was applied to detect the changes of cell cycle and apoptosis rate.

• RESULTS: After N/N1003A cells were treated by PDGFR-α ASODN, the proliferations of the cells were significantly inhibited ($P<0.05$), and the expression levels of PDGFR-α mRNA were notablely lowered ($P<0.05$) in a dose-dependent manner, respectively. The cells in the experimental group showed typical features of apoptosis under the transmission electron microscope and the rates of cell apoptosis were markedly higher ($P<0.05$). The analysis of cell cycle indicated that the cells were arrested in G1 phase.

• CONCLUSION: PDGFR-α ASODN can silence PDGFR-α gene expression in rabbit lens epithelial cells, inhibit cell proliferation, and induce cell apoptosis, which provides the experimental basis for the application of PDGFR-α ASODN to prevent the formation of posterior capsular opacification.

• KEYWORDS: platelet-derived growth factor receptor-α; antisense oligonucleotide; lens epithelial cell; cell proliferation; apoptosis
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INTRODUCTION

In recent years, with the development of cataract surgery and intraocular lens, the incidence of posterior capsular opacification (PCO) following cataract surgery has decreased than that of the 1990s, but it is still the major complication that causes visual impairment after surgery. It has already been reported that the incidence of PCO after cataract surgery

in adult was up to 14.7%-42.7%[1] and the Nd:YAG laser capsulotomy rate within 5 years after surgery was up to 24%[2]. Studies have confirmed that the principal biological bases of PCO are the residual lens epithelial cells in the peripheral part of the anterior capsule and the equator to undergo proliferation, migration, and metaplasia into fibroblasts[3,4]. Platelet-derived growth factor (PDGF) involved in lens epithelial cell proliferation, migration and differentiation, is one of important mitogenic growth factors, and it plays the roles by activating platelet-derived growth factor receptor (PDGFR) in cell membrane[5,6]. The aim of this study is to construct rabbit lens epithelial cells PDGFR-α gene-specific antisense oligonucleotide (PDGFR-α ASODN), transfect it into the cells, investigate the effects of PDGFR-α ASODN on PDGFR-α gene expression and cell proliferation, and explore its mechanism, which provides an experimental basis for the gene therapy of PCO.

MATERIALS AND METHODS

Materials Rabbit lens epithelial cell line (N/N1003A) was supplied by the Center for Cell Culture of Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). DMEM medium, trypsin, diethylamine tetra-acetic acid (EDTA), methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were purchased from Gibco Company (Newyork, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Material Co., Ltd (Hangzhou, China). Cationic liposome Lipofectamine™ 2000 was purchased from Invitrogen Company (Invitrogen (San Diego, USA). Ultrapure RNApure kit was purchased from Hunan ProMab Biotechnologies Inc (Changsha, China). One step RNA PCR kit was purchased from Takara Company (Tokyo, Japan). PDGFR-α antisense oligonucleotides sequence, 5′-AACTTCTCCTCCGATGTTA-3′, was modified with phosphorothioate, and synthesized by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd (Shanghai, China).

Methods

Cell culture N/N1003A cells were cultured in DMEM medium supplemented with 10% FBS, and placed in a 5% CO_2 incubator at 37℃. Cell growth was observed under phase microscope every day. When the cells had grown to near confluence, they were digested (0.25% trypsin/0.02% EDTA) and further passaged. Cells were harvested during the logarithmic growth phase and used in the following experiments.

MTT assay Cells were seeded at a density of 1×10^4/well in 96-well plates, incubated for 24 hours, and then transfected with Lipofectamine™ 2000 according to the manufacturer's instructions of cationic liposome. In the experiment, the cells were divided into 4 groups: control group was incubated in DMEM without PDGFR-α ASODN and cationic liposome; liposome group was incubated in DMEM with cationic liposome; 1μmol/L and 5μmol/L ASODN groups were incubated in DMEM containing different concentrations (1μmol/L, 5μmol/L) PDGFR-α ASODN and cationic liposome, respectively. After the cells were cultured for 24, 72 hours respectively, the supernatants were removed, and 20μL MTT solution (5g/L) was added to each well for another 4 hours. The supernatants were drained, and 150μL DMSO was added with 10 minutes shaking to dissolve the crystals completely. The optical density (OD) values of each sample were measured at a wavelength of 570nm by enzyme-linked immunosorbent assay (Awareness Company, USA). The average OD value in each group was used to calculate the cell growth inhibitory rate: the cell growth inhibitory rate (%) = (1-experimental group OD/control group OD)×100%.

RT-PCR Cells were seeded in 6-well plates, transfected with the above steps, and cultured for 72 hours, and then collected for the following process. PDGFR-α primer sequences: 5′-GCTCAAAATGAAGATGCTGTG-3′, 5′-CCTCCACGGTACTCCTGTCT-3′, which was 270 bp. An internal reference GAPDH primer sequences: 5′-CCAATATGATTCCACCCATG-3′, 5′-AGGTCCACCACTGACACGTT-3′, which was 594bp. 25μL of the primers was used during PCR to amplify the reaction. PCR amplification included an initial denaturation at 95℃ for 5 minutes, denaturation at 94℃ for 30 seconds, annealing at 50℃ for 30 seconds, and then extension at 72℃ for 30 seconds followed by 30 cycles of extension at 72℃ for 5 minutes. 5μL PCR products were electrophoresed on 2% agarose gel, and the bands were observed with a UV transilluminator. Then, the relative quantitative values of the bands were calculated by Glyko-Bandscan image analysis system.

Transmission electron microscope observation Cells were seeded at a density of 1×10^5/dish in 50mL dishes, and the cell groups and cell transfection were dealed with the above-mentioned methods. After cultured for 72 hours, the cells were collected, centrifugated at 1000g for 5 minutes, washed 2 times with phosphate buffer solution(PBS), and fixed in 2.5% glutaraldehyde, and then embedded in Epon812 araldite. The ultrathin sections were cut, stained doubly with uranyl acetate and lead nitrate, and observed by using a transmission electron microscope (Hitachi H-2600, Japan).

Flow cytometry The cells in different groups transfected according to the above steps were incubated for 72 hours, digested, centrifugated, washed 2 times with PBS, and fixed in 70% ethanol at 4℃. The samples were stained with propidium iodide (PI) and the cell cycle and apoptosis were detected by flow cytometer (Becton Dickinson Company, USA).

Statistical Analysis Data were expressed as mean±SD, and statistical analysis were performed using SPSS18.0 Statistical software package. Statistical differences among means were determined using one-way ANOVA and SNK-q test. $P<0.05$ was considered statistically significant.

RESULTS

Effect of PDGFR-α ASODN on Cell Proliferation MTT assay Showed that after PDGFR-α ASODN was transfected into cells for 24 and 72 hours, the OD values of 1μmol/L and 5μmol/L ASODN groups were significantly lower than those of control group and liposome group, which were statistically significant respectively ($P<0.05$). The difference of OD

values between the different concentrations was also statistically significant ($P<0.05$), while the OD value of liposome group was not significantly different from that of control group ($P>0.05$) (Table 1). The cell growth inhibitory rates of 1μmol/L and 5μmol/L ASODN groups were 13.6%, 41.0% after the cells were transfected for 24 hours, and were 42.0%, 60.0% after the cells were transfected for 72 hours, respectively.

PDGFR-α mRNA Expression After PDGFR-α ASODN was transfected into cells for 72 hours, the PDGFR-α mRNA relative expression levels in 1μmol/L and 5μmol/L ASODN groups (0.287±0.051, 0.086±0.019) decreased in comparison with control group (0.527±0.063) and liposome group (0.520±0.009), and the differences were statistically significant ($P<0.05$). The difference of mRNA relative expression levels between the different concentrations was also statistically significant ($P<0.05$), while the relative level in liposome group was not significantly different from that of control group ($P>0.05$) (Figure 1).

Ultrastrucral Changes The cells in control group showed multiple microvillus, loose chromatin uniformly distributed, and the structures of endoplasmic reticulum and mitochondria were clear (Figure 2A). The cells in 1μmol/L ASODN group displayed microvillus reduced, chromatin slightly concentrated, swelling of the cytoplasm, and the structures of organelles were not distinct (Figure 2B). The classic features of apoptosis, including condensation and margination of nuclear chromatin and fragmentation of nucleus, were observed in 5μmol/L ASODN group (Figure 2C). The ultrastructure of cells in liposome group had no significant difference compared with control group (data not shown).

Effect of PDGFR-α ASODN on Cell Cycle and Apoptosis Flow cytometry results showed that after PDGFR-α ASODN was transfected into cells for 72 hours, the proportions of cell cycle phases happened obvious changes, and an evident apoptotic peak (sub-G1 phase) appeared before G1 phase. The apoptosis rates and percentages of each phase in 1μmol/L and 5μmol/L ASODN groups were significantly different from those of control group and liposome group, respectively ($P<0.05$). The differences of apoptosis rates and percentages of each phase between the different concentrations were also statistically significant ($P<0.05$), while the apoptosis rate and percentages of each phase in liposome group were not significantly different from those of control group ($P>0.05$) (Table 2).

Table 1 Effect of different concentrations of PDGFR-α ASODN on cell proliferation ($\bar{x}\pm s, n=5$)

Groups	24 hours OD value	72 hours OD value
Control	0.641±0.022	0.964±0.022
Liposome	0.633±0.018[e]	0.948±0.032[e]
1μmol/L ASODN	0.554±0.021[a]	0.558±0.020[a]
5μmol/L ASODN	0.378±0.012[a,c]	0.382±0.023[a,c]

[a]$P<0.05$ *vs* control group and liposome group; [c]$P<0.05$ *vs* 1μmol/L ASODN group; [e]$P>0.05$ *vs* control group.

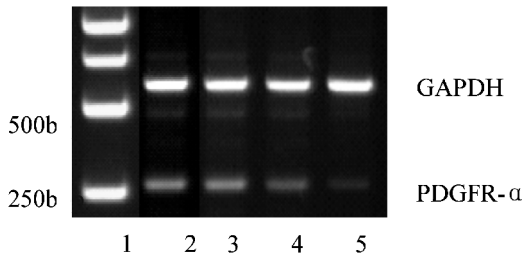

Figure 1 PDGFR-αmRNA expression of different groups Lines 1, 2, 3, 4 and 5 corresponded to marker, control group, liposome group, 1μmol/L ASODN group, 5μmol/L ASODN, respectively.

DISCUSSION

The levels of multiple cell growth factors in aqueous humor of the eye following cataract surgery, such as transforminggrowth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and PDGF, were altered due to surgical stimulation and postoperative inflammatory reaction[7,8]. These cytokines formed a complex network and regulated the pathophysiological process of lens epithelial cells, which played an important role in the occurrence and development of PCO. The specific binding of PDGF to PDGFR in lens epithelial cells and subsequent activation of downstream signaling pathways is one of the key factors to maintain the normal development of lens, promote the processes of lens epithelial cell proliferation and migration[9].

PDGFR family includes both αand β receptors, and PDGFR-α has high affinity to four PDGF dimmers as following PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC. So PDGFR-α may be more closely related to PCO. Researchers[10] used the PDGF-specific antibody to block PDGF signaling pathway, and discovered that the proliferation of lens epithelial cells was inhibited significantly, which suggested that it was possible to prevent the formation of PCO by antibodies or inhibitory polypeptides to block PDGF pathway. To date, however, there has not been any reports on the application of PDGFR-α antisense oligonucleotide to affect the lens epithelial cell biological characteristics.

Antisense oligodeoxynucleotide (ASODN) technology is a new method for gene therapy that the synthetic small molecule DNA sequence introduced into cells can bind with the target gene mRNA in a specific complementary way, interfere with replication, transcription and translation of the target gene, thereby block the expressions of proteins. In recent years, ASODN technology has been widely used in the studies of eye diseases, which shows a broad prospect[11]. In this experiment, it was the fist time that PDGFR-α ASODN was transfected into rabbit lens epithelial cells N/N1003A, RT-PCR detection showed that the expression levels of PDGFR-α mRNA were significantly lowered, and MTT assay showed that the proliferations of cells were significantly reduced in a dose-dependent manner, respectively. The binding of PDGF in fetal bovine serum to PDGFR in cells can activate receptor tyrosine protein kinase, and the Phosphorylated receptor subsequently activates signaling molecules as following MAPK/ERK,

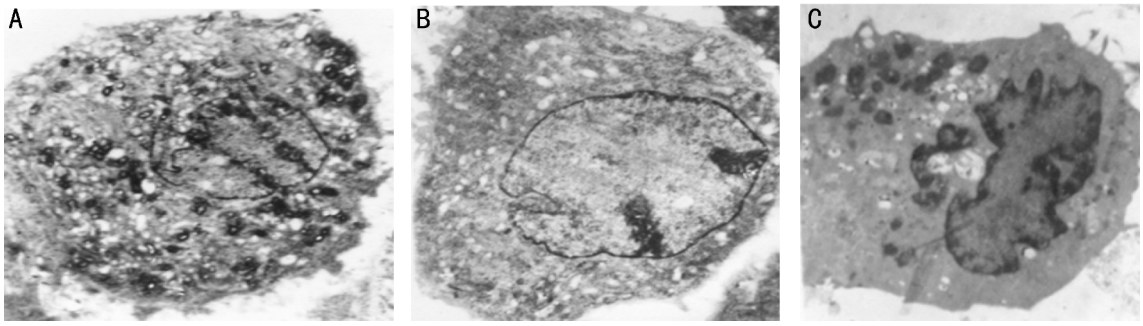

Figure 2 Ultrastrucral changes under transmission electron microscope (magnification 60000) A: Control group; B: 1μmol/L ASODN group; C: 5μmol/L ASODN group.

Table 2 Effect of PDGFR-α ASODN on cell cycle and apoptosis ($\bar{x}\pm s$, $n=5$)

Groups	Sub-G1 phase (%)	Cell cycle distribution (%)		
		G_0/G_1 phase	S phase	G_2/M phase
Control	2.40±0.47	68.45±1.00	22.75±0.94	8.79±0.35
Liposome	2.38±0.38[e]	68.39±1.22[e]	23.03±0.70[e]	8.59±0.79[e]
1μmol/L ASODN	7.48±0.81[a]	82.55±1.45[a]	13.58±0.78[a]	3.88±1.27[a]
5μmol/L ASODN	15.94±0.95[a,c]	92.06±1.18[a,c]	5.67±1.49[a,c]	2.33±0.80[a,c]

[a]$P<0.05$ *vs* control group and liposome group; [c]$P<0.05$ *vs* 1μmol/L ASODN group; [e]$P>0.05$ *vs* control group.

ERK1/2, JNK and Akt[12,13]. These molecules get into the cellular nucleus and stimulate the expressions of early response genes, which can regulate cell cycle and promote cell proliferation. In this experiment, the expression of PDGFR-α gene in lens epithelial cells was silenced by antisense technology, and the activations of a series of downstream signaling molecules were blocked, and then cell proliferation was significantly decreased.

Research has already shown that mouse lens expressed PDGFR-α for life, and cataract was observed in mouse and the proportion of S phase in lens epithelial cells was significantly increased in transgenic mouse containing the over-expression of PDGF-AA in lens[14]. In this experiment, when PDGFR-α gene expression in rabbit lens epithelial cells was silenced by PDGFR-α ASODN, cell cycle was altered. The proportions of S phase and G2/M phase reduced significantly, but the proportion of G0/G1 phase increased significantly, which indicated that cell cycle was arrested in G1 phase. These results correspond to the above study. Furthermore, the typical apoptotic changes in cells were observed by transmission electron microscopy and flow cytometry in the study. It is to suppose that the silence of PDGFR-α gene expression blocks the activations of downstream cell signaling molecules, and simultaneously initiates apoptotic program that induced cell apoptosis, but further research is needed for the exact mechanism.

In summary, the present study found that synthetic PDGFR-α antisense oligonucleotide could effectively inhibit PDGFR-α gene expression in rabbit lens epithelial cells, inhibit cell proliferation and promote cell apoptosis, which provided an experimental basis for the further application of PDGFR-α antisense oligonucleotide to prevent the formation of PCO.

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