Expressions of TGF-β2, bFGF and ICAM-1 in lens epithelial cells of complicated cataract with silicone oil tamponade

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Abstract

● **AIM:** To investigate the expression differences of transforming growth factor-β2 (TGF-β2), basic fibroblast growth factor (bFGF) and intercellular cell-adhesion molecule-1 (ICAM-1) in lens epithelial cells (LECs) of complicated cataract with silicone oil tamponade and age-related cataract.

● **METHODS:** Totally 150 eyes of 150 patients (aged 35 to 77y) were investigated, including 75 patients with complicated cataract after silicone oil tamponade and 75 patients with age-related cataract. The central piece of anterior capsules was collected during cataract surgery. TGF-β2, bFGF and ICAM-1 were detected in the 60 specimens of the two groups by immunohistochemistry. The expression levels of the three kinds of messenger ribonucleic acid (mRNA) were determined by real-time quantitative reverse transcription-polymerase chain reaction in the 90 specimens of the two groups.

● **RESULTS:** TGF-β2 was detected in the cytomembrane and cytoplasm of the LECs and bFGF was detected in the nucleus. ICAM-1 was positive in the cytomembrane of the LECs and the distribution of positive cells was uneven. The mRNA genes expression of the TGF-β2, bFGF and ICAM-1 was significant differences between the two groups and markedly increased in complicated cataract group ($P<0.05$).

● **CONCLUSION:** The up-regulated TGF-β2, bFGF and ICAM-1 maybe associate with the occurrence and development of complicated cataract with silicone oil tamponade.

● **KEYWORDS:** transforming growth factor-β2; basic fibroblast growth factor; intercellular cell-adhesion molecule-1; lens epithelial cell; complicated cataract; age-related cataract; silicone oil

INTRODUCTON

Vitrectomy combined with silicone oil tamponade as effective treatment for ocular fundus diseases is widely used. The most important complication of intraocular silicone oil is cataract formation. This leads not only to deterioration of the patients' vision, but also to impairment of fundus visualization[1]. Previous study has shown epithelial-mesenchymal transition (EMT) of lens epithelial cells (LECs) was the major pathological mechanism in anterior subcapsular cataract (ASC) and posterior capsule opacification (PCO)[2]. Cytokines play an important role in this process. Therefore, exploring the expression of cytokines in cataract formation and identifying potential mechanisms are important to reduce or prevent cataract formation.

Transforming growth factor-β2 (TGF-β2) inducted EMT and extracellular matrix (ECM) synthesis in LECs *via* activation of Smad signaling pathway[3]. In addition, other signaling pathways were involved in the proliferation and migration of LECs[4-6]. The basic fibroblast growth factor (bFGF) related to PCO and stimulating proliferation of the LECs *in vitro* had been confirmed[7-9]. The roles of TGF-β2 and bFGF were not exactly the same. TGF-β2 increased collagen gel contraction and alpha-SMA expression in bovine LECs, whereas bFGF decreased these parameters[10-11]. The intercellular cell-adhesion molecule-1 (ICAM-1) are highly expressed in inflammatory conditions, chronic diseases and a number of malignancies. It was involved in adhesion of LECs to ECM components of the lens capsule[12-13].

In the present study, we immunohistochemically located TGF-β2, bFGF and ICAM-1 in human lens capsules and tested the differences of their messenger ribonucleic acid (mRNA) expression in the LECs of complicated cataract patients with silicone oil tamponade and age-related cataract patients.

SUBJECTS AND METHODS

Surgical Procedure The protocol for research involving human tissue was approved by the Xi'an Jiaotong University Ethics Committee, and complied with the guidelines set forth by the Declaration of Helsinki. The study was conducted on 150 eyes of 150 patients (aged 35 to 77y), recruited at the Department of Ophthalmology, Affiliated Guangren Hospital, School of Medicine, Xi'an Jiaotong University, from June 2013 to December 2015. The required phacoemulsification for treatment was carried out for a total of 150 cases, including 75 patients with complicated cataract after silicone oil tamponade and 75 patients with age-related cataract (Table 1). Retinal detachment associated with proliferative vitreoretinopathy was the primary disease of the patients who underwent vitrectomy. The course of silicone oil tamponade in eyes with complicated cataract ranged from 6 to 18mo. The diameter of lens anterior capsule were 5-6 mm[14] obtained by the continuous circular capsulorhexis technique.

Inclusion and Exclusion Criteria Eligible patients met the following criteria: 1) vitrectomy and phacoemulsification were completed by the same operator respectively; 2) refered to emergy-little nuclear hardness classification standard, the degree of cataractous opacity was less than or equal to the first level before vitrectomy and equal to the third level when performing phacoemulsification. The patients having the following criteria were excluded: 1) the patients could not tolerate surgery; 2) ocular complications caused by diabetes and severe systemic diseases; 3) with a history of ocular trauma or other ocular diseases complicated with cataract; 4) mechanical injury of lens happened in the vitrectomy; 5) no adherence to treatment and failed to keep follow-up according to plan.

Immunohistochemistry Sixty lens anterior capsules were fixed with 4% paraformaldehyde for 24h, then whole specimens were incubated in 30% sucrose overnight at 4℃. All lens anterior capsules rinsed in phosphate buffer saline (PBS) (pH 7.4) three times for 10min. After this procedure, they were randomly divided into three groups. Each group included 10 lens anterior capsules obtained from complicated cataract patients and the same number obtained from age-related cataract patients. The three groups were incubated overnight at 4℃ with the primary antibodys specific for TGF-β2 (ab36495) (1:1000; Abcam, Cambridge, UK), bFGF (ab181) (1:250; Abcam, Cambridge, UK) and ICAM-1 (ab53013) (1:50; Abcam, Cambridge, UK) respectively. Following application of secondary antibodies: biotin donkey-anti-mouse antibody (AP192B) (1:500 for TGF-β2, bFGF; Millipore, Billerica, MA, USA) and biotin donkey-anti-rabbit antibody (AP182B) (1:500 for ICAM-1; Millipore, Billerica, MA, USA) for 4h at room temperature. The primary and secondary antibodys were diluted by mixed liquid (0.01% PBS, 0.3% Triton X-100,

Table 1 The general information for the patients with complicated cataract after silicone oil tamponade and with age-related cataract *n* (%)

Items	Complicated cataract	Age-related cataract
No. of cases	75	75
Age (a)		
35-40	19 (25.3)	0 (0.0)
41-50	36 (48.0)	0 (0.0)
51-60	20 (26.7)	27 (36.0)
61-70	0 (0.0)	32 (42.7)
71-77	0 (0.0)	16 (21.3)
Gender		
M	31 (41.3)	33 (44.0)
F	44 (58.7)	42 (56.0)
Systemic disease types	No	No
Drug intervention	No	No
Course of disease (course of silicone oil tamponade) (mo)		
6-12	32 (42.7)	0 (0.0)
13-18	43 (57.3)	7 (9.3)
19-24	0 (0.0)	35 (46.7)
≥25	0 (0.0)	33 (44)
Cataract types (phacoemulsification)		
Posterior subcapsular cataract	31 (41.3)	19 (25.3)
Nuclear cataract	19 (25.3)	27 (36.0)
Cortex cataract	25 (33.3)	29 (38.7)

0.03% NaN$_3$, 0.1% carrageenin and 5% normal goat serum). After regularly rinsed in PBS (pH 7.4), whole specimens were labeled using AB reagent (1:200, abcam, Cambridge, UK) for 2h to couple with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma) and immediately washed under PBS (pH 7.4) after color development. The three different specimens were mounted on slides and dry overnight at room temperature, after dehydration in graded ethanol and being transparent in xylene, slides were mounted with dibutyl phthalate xylene (DPX) and then were observed under a light microscope (Olympus P70).

Total Ribonucleic Acid Extraction and Complementary Deoxyribonucleic Acid Generation Ninety lens anterior capsules were fixed with liquid nitrogen immediately when they removed from the patient's eye, then whole specimens were stored at -80℃. They were randomly assigned (Table 2). Total RNA was subsequently extracted from LECs using an RNeasy kit (RNeasymicrokits; Qiagen) in accordance with the manufacturer's instructions. RNA was quantified with a spectrophotometer (ND-1000; NanoDrop, Wilmington, DE, USA), the ratio of absorbance at 260 and 280 nm was measured, this ranged from 1.8 to 2.2 (mean 2.0), which is indicative of a pure (uncontaminated) RNA sample. Where possible, total RNA was immediately used for cDNA generation or was briefly stored at -80℃. Single-strand
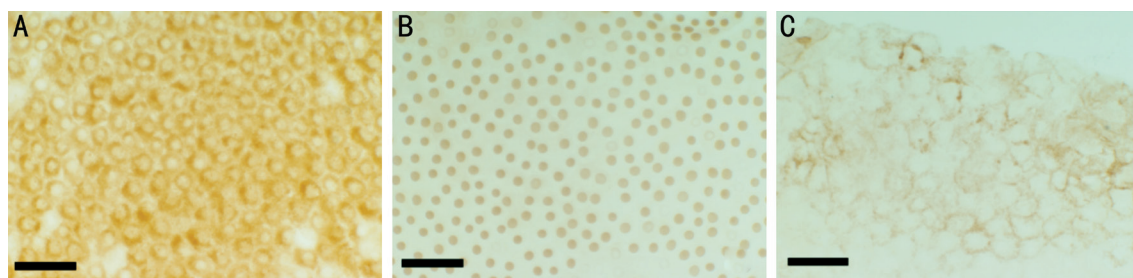
Figure 1 Morphologic characterization of TGF-β2, bFGF and ICAM-1 on the LECs and positive results were brown A: TGF-β2 was detected in the cytomembrane and cytoplasm of LECs, deeper positive signal was presented in cytomembrane area; B: Positive stain of bFGF was detected diffusely in the nucleus of LECs; C: ICAM-1 was detected in the cytomembrane LECs, the immunoreactive positive cell shows uneven distribution. Morphology at a 40 magnification. Bars: 50 μm.

Table 2 The group assignments for lens anterior capsules of the complicated cataract after silicone oil tamponade and the age-related cataract

Items	Complicated cataract		Age-related cataract
Group	I	II	III
Course of disease (mo) (silicone oil tamponade time)	≥6	≥12	>12
TGF-β2	7	8	15
bFGF	7	7	15
ICAM-1	8	8	15

cDNA was synthesized from 1 μg of total RNA by reverse transcription according to the manufacturer's instructions (Toyobo, Japan).

Real-time Quantitative Reverse Transcription-polymerase Chain Reaction for Messenger Ribonucleic Acid Expression Analysis Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to analyze mRNA expression for TGF-β2, bFGF and ICAM-1 genes in the LECs and performed on an ABI-7500 sequence detector (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified using specific primers respectively. The specific primers used were as follows: TGF-β2 (F: 5′-TGGATGCG GCCTATTGCTTTA-3′; R: 5′-CCAGCACAGAAGTTGGCATTGTA-3′), bFGF (F: 5′-CTGTACTGCAAAAACGGGG-3′; R: 5′-TAGCTTGATGTGAGGGTCGC-3′) ICAM-1 (F: 5′-TTGAGGGCACCTACCTCTGT-3′; R: 5′-GATAG GTTCAGGGAGGCGTG-3′). The product sizes were 133 bp for TGF-β2, 94 bp for bFGF and 255 bp for ICAM-1. Conditions for the PCR amplification were 3min at 95℃, 5s at 95℃, 20s at 60℃ , 5s at 78℃ , 5s at 80℃ and then 39 cycles, each consisting of 5s at 95℃, 20s at 60℃, 5s at 78℃ and 5s at 80℃. Melting curve was 70℃ to 95℃ and increment was 0.5℃ for 5s. Expression levels relative to the control condition were calculated using the ΔΔCt method.

Statistical Analysis The statistical analyses were performed using SPSS version 19.0. The incidence of gender between the two groups was compared with the Chi-square test. The age, course of disease and cataract types between the two groups were analyzed using the Wilcoxon rank sum test. Student's *t*-test was used to compare the differences in cytokine levels between the two groups. A *P*-value of <0.05 was considered statistically significant in all tests.

RESULTS

General Information Age, course of disease and cataract types were significant differences between the two groups (*P*<0.05). The complicated cataract patients were younger and had shorter course of disease. The incidence of posterior subcapsular cataract was higher in complicated cataract patients. There were no obvious differences in gender between the two groups (*P*>0.05).

Characterization of Transforming Growth Factor-β2, Basic Fibroblast Growth Factor and Intercellular Cell-adhesion Molecule-1 in the Lens Epithelial Cells TGF-β2, bFGF and ICAM-1 positive signals were brown in colour and all detected in the LECs, which obtained from complicated cataract and age-related cataract (Figure 1). TGF-β2 was detected in the cytomembrane and cytoplasm of LECs and bFGF was detected diffusely in the nucleus of LECs. As for the deposition of ICAM-1, it was positive in the cytomembrane of LECs in all the samples. Expression pattern of ICAM-1 in LECs showed regional heterogeneity. The distrlbution of ICAM-1 positive cells in the LECs were uneven.

Transforming Growth Factor-β2, Basic Fibroblast Growth Factor and Intercellular Cell-adhesion Molecule-1 Expression in the Lens Epithelial Cells We examined the TGF-β2, bFGF and ICAM-1 gene expression differences on the LECs between complicated cataract and age-related cataract. Cells were collected and total cellular RNA was extracted. TGF-β2, bFGF and ICAM-1 mRNA expression was studied by real-time qRT-PCR. Their mRNA expression showed significant differences between the groups I and III (*P*<0.05). The same results were found between the groups II and III (*P*<0.05) (Figures 2-4). The mRNA expression of TGF-β2, bFGF and ICAM-1 in complicated cataract was markedly increased, which demonstrated that silicone oil tamponade was an effective way to modulate the TGF-β2, bFGF and ICAM-1 mRNA expression in LECs.
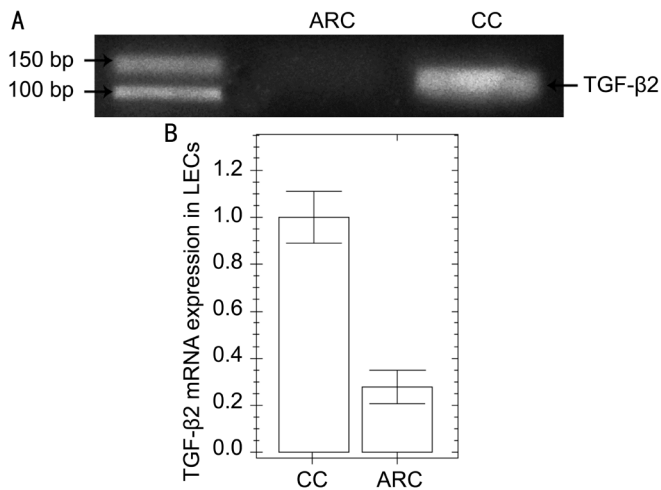
Figure 2 The qRT-PCR result of TGF-β2 A: The qRT-PCR result of TGF-β2 between group II and group III; B: TGF-β2 mRNA expression was significant difference in LECs between group II and group III (*P*<0.05). CC: Complicated cataract; ARC: Age-related cataract.
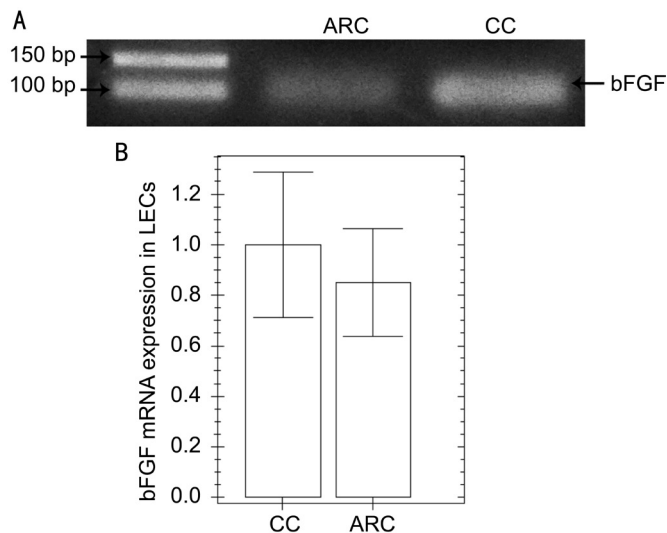

Figure 3 The qRT-PCR result of bFGF A: The qRT-PCR result of bFGF between group II and group III; B: bFGF mRNA expression was significant difference between group II and group III (*P*<0.05).
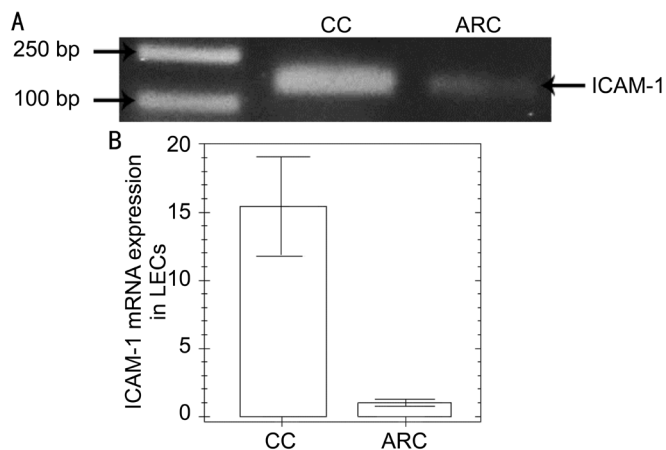

Figure 4 The qRT-PCR result of ICAM-1 A: The qRT-PCR result of ICAM-1 between group II and group III; B: ICAM-1 mRNA expression was significant difference between group II and group III (*P*<0.05).

DISCUSSION

TGF-β2, bFGF and ICAM-1 have been putatively identified as involved in the development of cataract processes. Aims of our work were to identify these cytokines played some important roles and accelerated cataract formation in complicated cataract. The primary disease of patients was retinal detachment associated with proliferative vitreous retinopathy in complicated cataract group. According to the inclusion criteria and exclusion criteria, we chose the patients who met the requests. By comparing the general information of the patients with complicated cataract after silicone oil tamponade and with age-related cataract, age, course of disease and cataract types were significant differences, gender was no significant difference. The younger age of the complicated cataract patients with silicone oil tamponade should be related to selection criteria and the shorter course of disease suggested that the complicated cataract developed faster. The contact between silicone oil and lens posterior capsule might be the cause of the higher incidence of posterior subcapsular cataract. Our study showed TGF-β2, bFGF and ICAM-1 were detected in the LECs of complicated cataract and age-related cataract. Previous researches showed that the TGF-β2, bFGF in lens capsules could be immunohistochemically located before and after cataract. *In vitro*, these cytokines also could be detected and take part in promoting LECs growth and differentiation[15-18]. The total amount of active TGF-β2 and bFGF increased in the aqueous humor and lens in a new animal model of ASC formation[19]. In our study, TGF-β2 was detected in the cytomembrane and cytoplasm of LECs and bFGF in the nucleus. The characterization could be found in all areas of the specimens and existed simultaneously. Fan *et al*[20] founded ICAM-1 was immunolocalized on the surface, side, and basement of LECs in the cataract patients with and without type 2 diabetes. Consequently, we also demonstrated the immunolocalization of ICAM-1 in the cytomembrane of LECs. In this study, we found the distribution of ICAM-1 positive cells in the LECs were uneven and showed regional heterogeneity. These findings suggested that TGF-β2, bFGF and ICAM-1 existed in LECs.

At present, the cause of cataract formation was studied by various aspects. The evidence showed that EMT of LECs were the major pathologic changes in development of ASC and PCO. The proliferation of LECs closely related to this processes[19,21-23]. TGF-β2 was known to stimulate the cell proliferation, migration and EMT of LECs and promoted the production of ECM components in the cell culture media[24-25]. Some signaling pathways involved in this pathological process. At the other side, TGF-β2-induced proliferation, migration and EMT of human LECs could be inhibited by drug[24,26-28]. In lens development, bFGF stimulated cell proliferation and cell migration and was founded in gene and protein levels[29-31].

Proliferation of LECs was dose dependently induced by bFGF and TGF-β2. They were strong mitogens for LECs and contributed to the progression of the cataract, but their mechanism of action was not the same[32-34]. ICAM-1 usually was considered to be an inflammatory molecule and related to many ocular pathological processes, such as cell adhesion, migration, proliferation, apoptosis and cell signal transmission. ICAM-1 might serve in the attachment process of cataractous LECs to ECM and be involved in the formation and disruption of cell-to-cell and cell-to-posterior capsule interactions when LECs migrated onto the posterior capsule after surgery[13,35-36]. Based on the previous studies, the three cytokines had different acting pathways on the cataract and existed in the process of the cataract development.

In our study, the course of silicone oil tamponade in eyes with complicated cataract was 6 to 18mo. We divided the lens anterior capsules of the complicated cataract into two groups according to this time. TGF-β2, bFGF and ICAM-1 mRNA genes expression showed significant differences between the complicated cataract and age-related cataract and a higher level in the state of silicone oil tamponade. We could not consider the magnitude of gene expression was proportional to the role of each cytokine. However, our results supported the TGF-β2, bFGF and ICAM-1 could be associated to cataract formation and high expression of these cytokines mRNA genes suggested they should play a role in promoting the development of complicated cataract after silicone oil tamponade.

Previous clinical and experimental researches indicated that cataract formation was one of the most common complications after vitrectomy, a progressive nuclear opacification might occur after any type of vitrectomy, but showed a faster progression. The main cause for nuclear cataracts most probably was oxidative stress. Other reason of the complication arising from vitrectomy was a tamponade of the vitreous space with silicone oil[37-39]. Our results revealed that TGF-β2, bFGF and ICAM-1 could be located in LECs of the two groups and had up-regulated gene expression in the LECs of the complicated cataract with silicone oil tamponade. Except a high oxygen pressure and direct damage, the high expression of these cytokines should be one important reason in occurrence of complicated cataract with silicone oil tamponade and maybe could promote the cataract accelerated development.

In summary, our study showed that TGF-β2, bFGF and ICAM-1 were existed in the LECs of the complicated cataract with silicone oil tamponade and age-related cataract. The distrlbution of ICAM-1 positive cells in the LECs were uneven. The mRNA genes expression of TGF-β2, bFGF and ICAM-1 was significant differences between the two groups and had higher level in the state of silicone oil tamponade. The TGF-β2, bFGF and ICAM-1 are related to the cataract formation and the up-regulated TGF-β2, bFGF and ICAM-1

maybe accelerate the occurrence and development of complicated cataract with silicone oil tamponade. Due to the limitations of human eyes, we don't know the levels of cytokines at all stages, future work will assess the different expression levels of cytokines associated with complicated cataract with silicone oil tamponade at different stages of disease in animal experiments. The changes under the drug's influence will be observed.

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