·Basic Research·

Experimental study of TGF-β2 antisense oligodeoxynucleotide as an anti-scarring agent in glaucoma surgery

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Abstract

• AIM: To investigate the effect of TGF-β 2 antisense oligodeoxynucleotide on differentiation, proliferation of subconjunctival fibroblast following glaucoma filtration surgery.

• METHODS: Glaucoma filtration surgery was performed on both eyes of 28 rabbits. TGF-β 2 antisense oligodeoxynucleotide was subconjunctivally injected in the right eyes (group A),and TGF-β 2 missense oligodeoxynucleotide (group B) or PBS (group C) was used at the same method in the left eyes as controls. Rabbits were killed at 4, 7, 14 and 28 days after surgery. Intraocular pressure (IOP), bleb characteristics were recorded at different time point. Subconjunctival fibroblasts were examined by immunohistochemistry and electron microscopy.

• RESULTS: The IOP of rabbits in group A was significantly lower at 14 days (6.74±1.18mmHg) and 21 days (8.15±1.97mmHg) after operation than the IOP in group B (8.53±1.04, 9.72±1.09mmHg; $P<0.01$) and group C (8.79±1.21, 9.43±1.27mmHg; $P<0.05$). The mean bleb survival time was longer (17.2 days) in group A than that of group B (14.5 days) and group C (13.5 days) ($P<0.05$). The population of the cells expressing α -smooth muscle actin (α -SMA) and proliferating cell nuclear antigen (PCNA) was significantly reduced in group A compared with the group B and C. The ultrastructure of fibroblast was not altered by TGF-β 2 antisense oligodeoxynucleotide.

• CONCLUSION: TGF-β 2 antisense oligodeoxynucleotide can prevent the scar formation after glaucoma surgery by inhibiting the differentiation and proliferation of subconjunctival fibroblast. It could be a potentially useful anti-scarring alternative for the prevention of late surgical failure.

• KEYWORDS: TGF-β 2; antisense therapy; glaucoma surgery; scarring

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INTRODUCTION

Glaucoma is one of the major causes of irreversible blindness in both developed and developing countries. Now in clinical practice, the treatment of glaucoma is focusing on the reduction of intraocular pressure (IOP) through surgeries, medication and laser treatments. Of all the therapies, surgery has been shown to be the most effective, achieving lower IOP and preventing progressive vision loss[1,2]. The determinant factor of the long-term outcome of glaucoma surgery is the subconjuntival scar formation derived from wound-healing response, excessive postoperative scarring is associated with poor postoperative IOP control[3]. Although intraoperative use of the anti-proliferation agents has increased the success rate of glaucoma filtration surgery, unfortunately, anti-proliferation agents can cause widespread cell death and apoptosis and can result in corneal erosions and cystic avascular blebs [4] with severe and potentially blinding complications. Better anti-scarring agents are therefore needed for postoperative prevention of bleb failure. Of all the growth factors involved in wound-healing cascade, TGF-β has been shown to be one of the most potent stimulators of scarring in eye [5]. TGF-β2 is the most potent growth factor in the aqueous stimulating conjunctival fibroblast function. Mead *et al* [6] had demonstrated that neutralization of TGF-β2 activity with repeated application of its antibody during the first week after glaucoma surgery significantly improved surgical results. Antisense oligonucleotide is a promising therapeutic strategy that has been successfully applied in inflammatory and viral infective disease [7,8]. The current study was intended to reveal the anti-scarring aspects of TGF-β2 which was subconjuctivally injected following anti-glaucoma filtration surgery.

Table 1 IOP comparison between experimental group and control groups pre-and post- operation ($\bar{x}\pm s$,mmHg)

Group	Pre-op	IOP in post-op			
		4d	7d	14d	21d
A	9.8 ± 1.52	5.98 ± 1.24	5.75 ± 1.23	6.74 ± 1.18[a,c]	8.15 ± 1.97 [a,c]
B	9.29 ± 1.28	6.14 ± 1.43	5.16 ± 1.27	8.53 ± 1.04	9.72 ± 1.09
C	9.46 ± 1.31	6.02 ± 0.96	5.41 ± 1.51	8.79 ± 1.21	9.43 ± 1.27
F	0.35	0.799	0.418	23.053	7.26
P	0.706	0.455	0.661	0.00	0.043

[a]$P<0.05$, [c]$P<0.05$ *vs* respective B and C group(univariate ANOVA,SNK-*q* test)

MATERIALS AND METHODS

Design of TGFβ2 Anti –sense Oligodeoxynucleotide (ASON) According to the nucleotide sequences of the cDNA coding regions for rabbit TGFβ2 (Genebank AY429466), we selected the most active ASON targeting rabbit TGFβ2, its sequences is 3'AGAAGTTGGCATTA TACCCTT 5', it contains both a phosphorothioate backbone and 2'-methoxyethyl sugar modification which increase nuclease stability and antisense potency. The universal missense control oligonucleotide is a random mixture of 21-mer nucleotide sequences with the same chemical modification as TGF-β2 ASON.

Rabbit Model of Glaucoma Filtration Surgery Twenty-eight New Zealand White rabbits weighing 1.5 to 2.3kg were used in this prospective randomized, controlled, observer-masked study. Glaucoma filtration surgery was performed on both eyes of rabbits under general anesthesia (intramuscular injection of ketamine 50mg/kg), TGF-β2 ASON (100μL, 100g/L) was subconjunctivally injected in the right eyes (group A), and TGF-β2 MSON (100μL, 100g/L, group B) or PBS (100μL, group C) was used at the same method in the left eyes. They were injected at Tenon's sa (3mm behind the limbus) on days 0 and 2 post-operation under topical anesthesia (Oxybuprocaine Hydrochloride, 4g/L eye drops, 1 drop per eye). No other treatment was given after surgery. The glaucoma filtration surgery was performed by the same experienced ophthalmologist under microscope.

Clinical Evaluation of TGF −β2 ASON Effect Post – operation All animals were examined by a masked observer. IOP was measured with Schiotz Tonometer after topical anesthesia, a mean reading was arrived from three recordings. Assessment of both eyes was made daily from days 0 to 5 and thereafter twice a week until the end of the experiment. Bleb failure was defined as the appearance of a flat, vascularized, and scarred bleb in the presence of a deep anterior chamber.

Analysis of Subconjunctival Fibroblasts by Immunoh – istochemistry and Electron Microscope On days 4, 7, 14, 28 after surgery, animals were killed with air intravenous injection, eye globes were enucleated and fixed in 100mL/L buffered formalin saline for 72 hours, before the graded dehydration and paraffin embedding. Sequential 5-μm sections of the operative wound site were prepared, and immunohistochemistry staining was performed to demonstrate cellularity, including α-SMA (myofibroblast phenotype identification) and PCNA (recent cell division). We also compared the electron microscopic graph characteristics of 6 rabbit eyes treated with TGF-β2 ASON and TGF-β2 MSON, PBS. When they were killed on 7th day, operative wound sites were processed for transmission electron microscope.

Statistical Analysis All experimental data were analyzed with Statistical Package for Social Science (SPSS, 12.0). IOP and population of cells expressing α-SMA and PCNA were analyzed with the multivariate analysis of variance (*F* test). Bleb survival rate was analyzed with log rank statistics. The level of significance applied to the statistical analysis was $P<0.05$.

RESULTS

IOP Analysis of mean IOP in the surgical eyes showed no significant difference between experimental group and control groups before operation. IOP of rabbits decreased after operation ($P>0.05$). Within 7 days, there was no significant difference among three groups. The IOP of rabbits increased as bleb was scarring. At 14 and 21 days, the IOP of group A was lower than that of group B and C. There was no significant difference between control groups (B and C, Table 1).

Bleb Survival Rate TGF-β2 ASON significantly improved glaucoma filtration surgery outcome in this animal model of aggressive postoperative scarring. It significantly prolonged bleb survival compared with control groups, as shown in bleb survival curve in Table 2 (Log-rank test, $P<0.01$). Treatment with TGF-β2 ASON was associated with elevated, diffuse, fleshy looking blebs compared with the flat, scarred blebs in control groups. The shortest time of functioning bleb in group A was 13 days, the longest time was 21 days. The median survival rates were 17.2, 14.5, 13.9 days in group A, B, C, respectively (Log-rank test $\chi^2=9.47$, $0.005<P<0.01$, Figures 1 and 2).

Table 2 Number of fibroblasts expressing α-SMA and PCNA of three groups in post-operation

($\bar{x}\pm s$,cell/μm^2)

Post-op time(d)	α -SMA			PCNA		
	A	B	C	A	B	C
4	13.9 ± 2.0[a,c]	23.4 ± 2.6	25.4 ± 3.7	14.0 ± 2.4[a,c]	17.8 ± 2.1	19.63 ± 1.95
7	14.6 ± 2.4[a,c]	17.5 ± 1.8	17.79 ± 2.4	8.1 ± 1.3[a,c]	12.6 ± 1.5	13.2 ± 1.66
14	0	3.3 ± 1.8	3.1 ± 1.4	0	3.5 ± 0.9	4.2 ± 1.3
28	0	0	0	0	0	0

[a]P<0.05, [c]P<0.05 *vs* respective B and C group(SNK-q test)

Histologic Effects TGF-β2 ASON significantly reduced the population of cells expressing α-SMA and PCNA. We selected 3 visual field randomly under light microscope (×200) and calculated the total number of positive fibroblasts, mean number of 3 visual field was recorded. As shown in Table 2, the positive fibroblasts expressing α-SMA and PCNA of experimental and control groups were appeared at 4 to 14 days after operation and disappeared around 28 days. The population of expressing α-SMA and PCNA in experimental group was fewer than control groups within 14 days, after 14 days, positive fibroblasts in group A disappeared, but group B, C had a few (Figures 3-5). In group A, bleb architecture was looser and the orientation of collagen fibril was more regular, and fewer inflammatory cells. Some fibroblasts became enlarged and had many mitochondrions, endoplasmic reticulums, indicating that their metabolization was active, but the ultrastructure of fibroblast had no significant difference in three groups (Figure 6).

DISCUSSION

Despite current anti-scarring strategies, continued scarring leading to late failure of glaucoma filtration surgery remains a major barrier to long-term IOP control and arrest of disease progression [9]. The anti-metabolites 5-FU and MMC are currently the backbone of anti-scarring treatments. The intraoperative regimen of MMC has gained popularity due to the convenience of a single treatment and the delivery of lower IOP in certain eyes[10]. However, even short exposure to MMC results in local irreversible tissue destruction. The advantage of TGF-β2 ASON over anti-metabolites lies in its

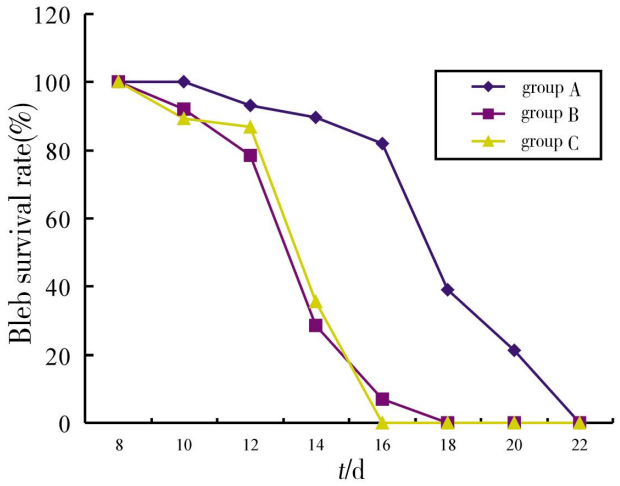

Figure 1 Curve of bleb survival rate

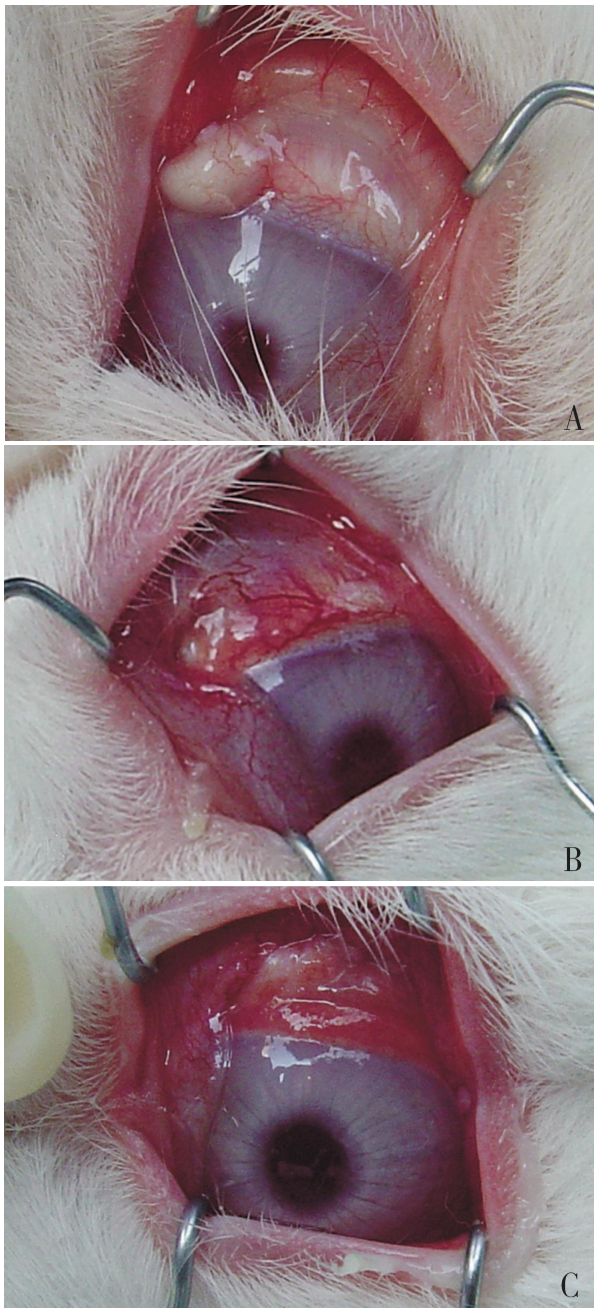

Figure 2 Bleb morphology at 14 days after glaucoma surgery treatment with TGF-β2 ASON (A) was associated with elevated, diffuse, fleshly looking blebs compared with the flat, scarred blebs in the TGF-β2 MSON group and PBS group (B,C)

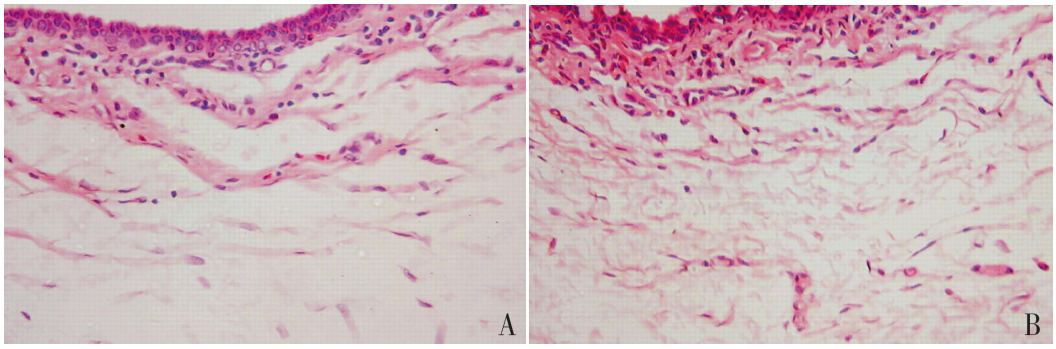

Figure 3 Histologic characteristics of filtration blebs on day 7 after glaucoma surgery ˙ 200. Hema-toxylin and erosinstained sections were shown that bleb architecture was looser and the orientation of collagen fibril was more regular and fewer inflammatory cells in TGF-β2 ASON group (A) than TGF-β2 MSON group(B)

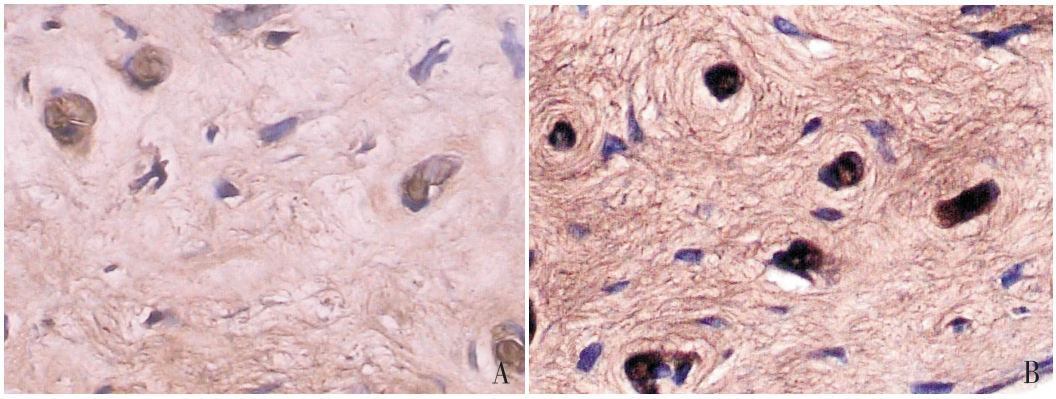

Figure 4 The subconjunctival positive fibroblast expressing α-SMA at 7 days after glaucoma surgery×400. The population of positive fibroblast in TGF-β2 ASON group (A) is fewer than TGF-β2 MSON group (B)

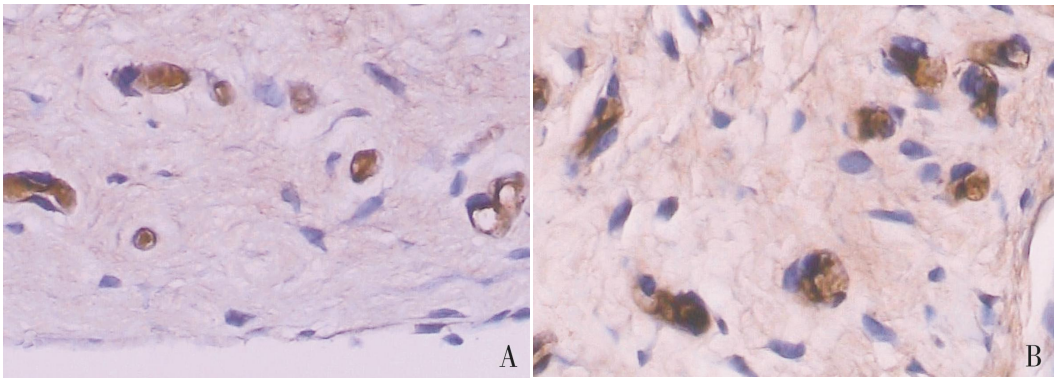

Figure 5 The subconjunctival positive fibroblast expressing PCNA at 7 days after glaucoma surgery× 400. The population of positive fibroblast in TGF-β2ASON group (A) is fewer than TGF-β2MSON group (B)

more physiological method of action, providing long-term IOP control, while maintaining normal tissue architecture in the absence of side effects.

After glaucoma filtration surgery, aqueous flow bathes the wound and provides a unique and changeable environment that influences postoperative healing. Of all the growth factors in the aqueous, TGF-β is the most potent stimulator of human Tenon's fibroblast activity [11]. Latent TGF-β2 is produced by tissues within the eye (iris, ciliary body and trabecular meshwork) before activation by plasmin and thrombospondin released from blood components [12]. Aqueous humour in glaucomatous eyes contains increased level of TGF-β2 [13]. After glaucoma filtration surgery, elevated level of activated TGF-β2 at the wound site is therefore likely to be related to aqueous concentration, the flow of aqueous, and breakdown of the blood aqueous barrier. In addition, TGF-β also displays the ability to autoinduce its own production, thereby initiating a perpetuating cascade of activation [14]. In our study, TGF-β2 ASON through subconjunctival injection can inhibit TGF-β2

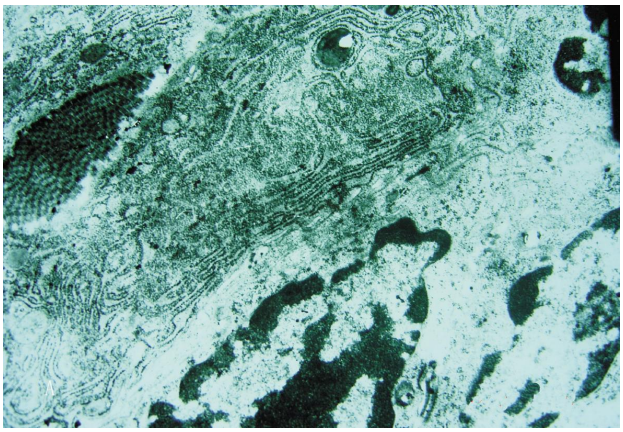

Figure 6 In experimental group at 7 days after glaucoma surgery, the ultrastructure of bleb revealed that collagen architecture was looser and the orientation of collagen fibril was more regular, some fibroblasts became bigger and had many mitochondrions, endoplasmic reticulums×6 000

biological synthesis in molecule level and inhibit its function. In our study, the IOP of rabbits was lower after operation, at 14 days after operation, the IOP of experimental group was lower than control groups, indicates that IOP is a reliable indicator of filtration in this model of glaucoma surgery. As the bleb is scarring, the IOP is increase. So, IOP and bleb are correlated with failure of surgery or not. TGF-β2 ASON significantly prolonged bleb survival rate, this can explain with histological changes. Histological analysis of the wound sites showed that TGF-β2 ASON significantly reduced the population of cells expressing α-SMA and PCNA, indicated that TGF-β2 ASON can inhibit differentiation and proliferation of subconjunctival fibroblast. Myofibroblasts are specialized fibroblast that play an important role in wound healing, they are present transiently during tissue repair and are thought to generate the contractile force that is integral to normal wound closure, excessive or abnormal contraction of granulation tissue leads to pathologic scarring. Fibroblast differentiation into the myofibroblast phenotype, characterized by the expression and assembly of α-SMA into stress fibers, is modulated by cytokines [15]. PCNA represents fibroblast proliferation, decrease of the positive fibroblast expressing PCNA indicates reduction of the collagen production by myofibroblast. Our observations suggest that the beneficial effects of TGF-β2 ASON in glaucoma surgery may be mediated by a reduction in TGF-β2 induced collagen production and contraction.

The positive fibroblasts expressing α-SMA and PCNA in experimental and control groups were appeared on 4 to 14 days after operation and disappeared on 28 days, this indicates that the most active period of fibroblast is 4 to 14 days after glaucoma surgery and return to baseline on 28 days. So, it is very important to control the activity of fibroblast before 14 days after glaucoma surgery, this is benefit to form an efficacy bleb and to maintain maximal IOP control in the longer term.

TGF-β2 ASON treatment is an effective and safe anti-scarring therapeutic agent, furthermore, it may potentially have widespread applications anywhere in the body where modulation of the wound-healing response is important.

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