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Original Article

TGF-βs Synthesized by RPE Cells Have Autocrine Activity on Mesenchymal Transformation and Cell Proliferation

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The present study investigated the effects of transforming growth factor (TGF)-β on retinal pigment epithelial (RPE) transformation in a simplified model and also whether or not TGF-β exhibits similar proliferation effects on transformed RPE cells that it has on primary RPE cells. Furthermore, we examined the cell proliferation effects of RPE-conditioned medium (CM). A vertical wound measuring 2 mm in diameter was made on primary RPE monolayers. The expression of α-smooth muscle actin (SMA) by the cells located at the wound edges was observed using a confocal microscope under immunofluorescent staining. Cell proliferation was measured by incorporating 3H-thymidine into DNA. The presence of α-SMA was observed in the cells within the wound after treatment with TGF-β2, while negative expression was observed in control cells. TGF-βs inhibited the proliferation of the primary cultures of RPE cells in a dose-dependent manner, but the spindle-shaped late-passaged RPE cells were not inhibited by these growth factors. The medium conditioned by RPE cells stimulated the proliferation of subconjunctival fibroblasts and inhibited the proliferation of primary RPE cells, in a manner similar to TGF-β. These findings demonstrate that TGF-β-stimulated RPE cells may evoke proliferative vitreoretinopathy through mesenchymal transformation and cell proliferation.

Key Words: Proliferative vitreoretinopathy, retinal pigment epithelium, transformation, transforming growth factor

INTRODUCTION

The most common cause of failure after retinal detachment surgery is due to proliferative vitreoretinopathy (PVR), an ocular disorder that is characterized by excessive fibrosis on both surfaces of the retina and within the vitreous cavity. Morphological and histochemical studies of proliferative membranes show that these membranes are formed by the migration and proliferation of retinal pigment epithelial (RPE) cells, glial cells, fibroblasts, myofibroblast-like cells and macrophages, as well as by an extensive accumulation of extracellular matrix proteins.[1-2] Several studies have suggested that RPE cells play a central role in the development of PVR.[3-5] In their normal state, RPE cells are mitotically inactive, whereas RPE cells that participate in PVR formation are transformed and proliferate onto the vitreous gel.

There is increasing evidence that cytokines play an important role in the pathogenesis of proliferative ocular disease. A variety of cytokines have been identified, both on epiretinal membranes that have been surgically removed from patients with PVR, as well as those that have been produced experimentally.[6] Furthermore, an animal model of PVR can be created by co-injecting recombinant cytokines and fibronectin.[7] Transforming growth factor (TGF)-β is considered to play a leading role in the process of intraocular proliferation of RPE and other types of cells. Increased levels of TGF-β have been found in the vitreous cavity of patients with PVR,[8] which is characterized by an intraocular wound-healing response. RPE cells are one intraocular source of this increased TGF-β production.[9] The present study investigated the effects of TGF-β on RPE transformation in a simplified in vitro model and the proliferation of transformed RPE cells. In addition, we examined whether RPE-conditioned medium (CM) exhibited any TGF-β-like properties.

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MATERIALS AND METHODS

Cell cultures

Subconjunctival fibroblasts (SCFs): Biopsy specimens of Tenon's capsule, measuring 2 × 2 mm, were taken from the inferior conjunctiva of rabbits. Methods used for securing the animal tissue were humane and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The excised tissue specimens were repeatedly washed in sterile phosphate-buffered saline (PBS) solution. Samples were transferred to 100-mm culture dishes containing 20 % fetal bovine serum (FBS)-Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Grand Island, NY, U.S.A.). The samples were then minced with scissors and forceps and cultured in an incubator for 4-5 days at 37℃ in an atmosphere of 5% CO_2.

RPE cells: Young porcine eyes were obtained from a local abattoir, transported on ice, and processed within several hours after death. After the eyes were washed in PBS containing 500 μg/ml penicillin and 500 μg/ml streptomycin, the muscles of the periorbital tissue were excised on aseptic gauze, and the eyeballs were placed in alcohol and stored in betadine. The equatorial portion of the eye was incised using sharp scissors and the vitreous gel and retina of the posterior cup were carefully removed. Exposed RPE was washed with serum-free DMEM at least 3 times and incubated for 1 hour after the addition of 1 ml 0.25 % trypsin and 5 mM ethylene-diamine-tetraacetic acid (EDTA) solution. After 1 hour, the trypsin solution was gently pipetted to collect the detached RPE. The cells were transferred to a tube containing 3 ml DMEM with 10% FBS (DMEM-10) and centrifuged for 5 min at 800 rpm. The supernatant was removed and a cell suspension was made using DMEM-20. The cells were plated at 2 × 10^5 cells per well in a 6-well plate and incubated in DMEM-20 at 37℃ in an atmosphere of 5% CO_2.

α-SMA staining for confocal laser microscopy

The presence or absence of α-SMA synthesis in the cells located at the wound edges was observed by confocal microscopy (Hitachi, Tokyo, Japan) after immunofluorescent staining. A vertical wound, 2 mm in diameter, was made on primary RPE monolayers using a silicone-tipped instrument to create a denuded area. The cultures were then incubated in the presence of TGF-β2 (10 ng/ml) or media alone after 24 hours. Cell culture medium was removed and the cells were washed 3 times with PBS. The cells were then fixed with 3% paraformaldehyde in PBS solution at 4℃ for 15 minutes and permeabilized with 0.5% Triton X-100 in PBS at 4℃ for 15 minutes. After the cells were rinsed with PBS again, they were incubated in PBS and 2% bovine serum albumin (BSA) for 1hour in a moist chamber at room temperature. The BSA solution was removed, and a 1 : 150 dilution of mouse anti-α-SMA antibody (R&D systems, Minneapolis, MN, USA) in PBS was added and the mixture was incubated for 1 hour at room temperature. The first antibody was removed and the cells were washed 3 times with PBS for 5 minutes. A 1:300 dilution of biotinylated goat anti-mouse IgG (Sigma, St.Louis, MO, USA) in PBS was subsequently prepared and reacted with the cells [M7] for 1 hour at room temperature. It was washed 3 times with PBS and treated with avidin-fluorescein isothiocyanate (FITC) conjugate solution (Vector Laboratories, Burlingame, CA, USA) that had been diluted 1:200 in PBS. The reaction was performed for 30 min at room temperature before being thoroughly washed with PBS. The water on the surface of the cells was removed by incubating the cells at room temperature for 5 min. The cover slips were finally mounted onto slides using Vector-seal solution for observation with a confocal microscope.

DNA synthesis assay

Primary and sixth-passaged porcine RPE cells were used for the proliferation assay. Cell proliferation was measured by incorporating ^{3}H-thymidine (90 Ci/mole; Amersham Life Science, Buckinghamshire, England) into DNA. We confirmed that DNA synthesis correlated with changes in cell number by counting cells using a hemocytometer. Briefly, the primary and sixth passaged RPE cells were starved of serum for 24 hours, prior to the addition of TGF-βs (in concent-

rations ranging from 1 ng/ml to 10 ng/ml) and 3 μCi of ^{3}H-thymidine. In some experiments, the medium conditioned by RPE cells at a 25% concentration was added to the primary RPE cells and SCFs in the presence of radioactive thymidine to measure the effect of CM on cell proliferation. In other experiments, 1 μg/ml of anti-TGF-β was added to block the activity of RPE-CM. The labeled DNA was precipitated with trichloroacetic acid, and the resultant pellet was dissolved in 200 μl of 0.2 N NaOH before the cells were counted.

Production of conditioned medium

Confluent primary RPE cells in a 100-mm culture dish were washed twice in serum-free DMEM and maintained in serum-free DMEM for 1 day. The medium was removed and additional serum-free medium was added to the cells and incubated for either 24 or 48 hours. The medium was removed once more and clarified by centrifugation at 3,000 rpm for 10 minutes. It was sterilized, diluted to a concentration of 25% (vol/vol) with serum-free DMEM and assayed to assess its proliferative effect.

RESULTS

Effect of TGF-β2 on epitheliomesenchymal transformation

After 24 hours, the wound had been partially closed by a variety of polymorphic that included polygonal, spindle, and elongated shapes. Given that mesenchymal cells are known to produce α-SMA when activated by outside factors, we examined α-SMA expression by the outgrowing cells within the wound. The presence of this protein was observed after treatment with TGF-β2. The cells were prepared with α-SMA antibody before the cells formed a membrane and were analyzed by a confocal laser microscope. Cells treated with TGF-β2 presented characteristic cytoskeletal staining and showed processes located throughout the cell surfaces. Conversely, control cells stained diffusely within the cells (Fig. 1). Wound healing in RPE may be initiated by epitheliomesenchymal transformation, which is subsequently followed by cell proliferation.

Effect of TGF-βs on the proliferation of RPE cells

The inhibitory effect of individual TGF-βs (TGF-β1, TGF-β2 and TGF-β3) on the prolifera-

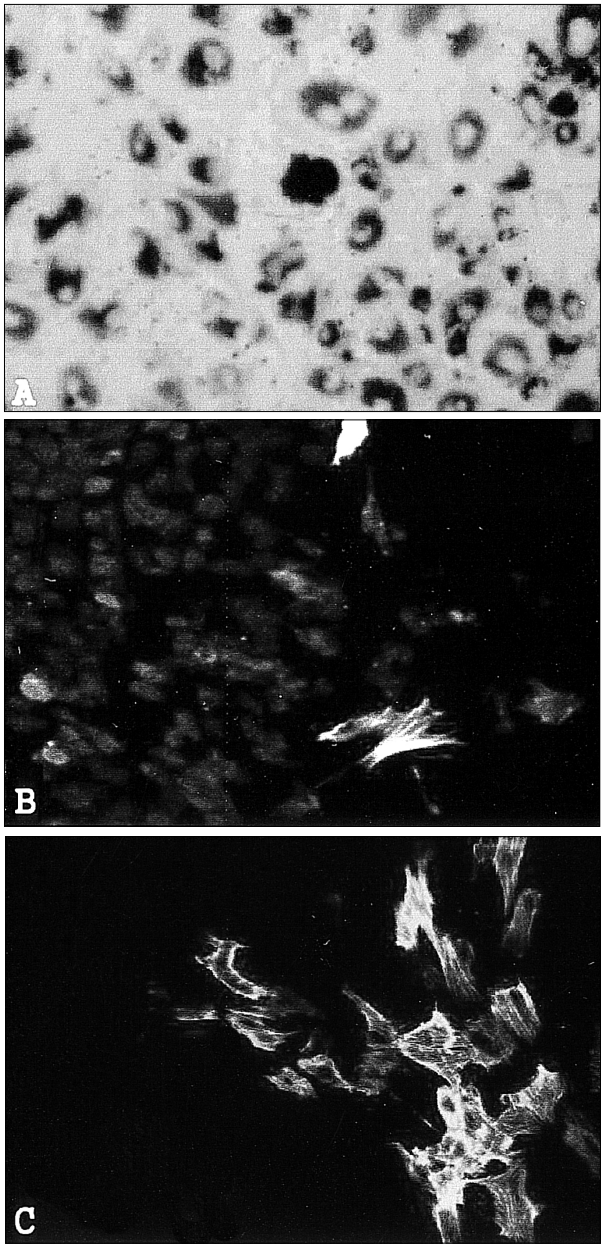

Fig. 1. Morphology of RPE cells responding to "wounding" in culture. Phase-contrast photomicrograph of primary RPE cells (A) and immunofluorescence confocal laser photomicrographs treated with media alone (B) or TGF-β2 10 ng/ml (C) (original magnification, × 200). RPE cells probed with anti-α-SMA antibody.

tion of primary RPE cells was investigated. TGF-β1, TGF-β2 and TGF-β3 inhibited primary RPE cell proliferation in a dose-dependent manner as determined by DNA synthesis. At 1.0 ng/ml, TGF-β1 was slightly inhibitory, while TGF-β2 and TGF-β3 moderately inhibited RPE cell proliferation. At 10 ng/ml, TGF-β2 and TGF-β3 inhibited cellular proliferation by more than 50%, while the inhibitory activity exhibited by TGF-β1 was less (Fig. 2). During routine culture on tissue culture plastic, porcine RPE cells lose their epithelial characteristics and acquire a mesenchymal cell-like phenotype as the cells are subcultured. Primary RPE cells maintained their polygonal shape, while the sixth-passaged RPE cells acquired an elongated morphology. We wanted to know whether or not the cell proliferation differed under the effects of TGF-β2 when RPE cells transformed into fibroblast-like cells. The effects of TGF-β2 were also analyzed in the sixth-passaged RPE cells. Interestingly, TGF-β2 had no effect on late-passaged RPE cells; this contrasted with its effect on primary cultures (Fig. 3).

Effect of RPE-CM on cell proliferation

To investigate whether there were TGF-β-like activities in the media conditioned by primary RPE cells, samples of media conditioned by RPE were assayed for their stimulatory effects on SCF proliferation and their inhibitory activity in primary RPE cells. The RPE-CM exhibited stimulatory effects on the proliferation of SCFs, regardless of cell stage. At the subconfluent stage, there was twice as much cell proliferation in the treated group as in the control cells. When SCFs reached confluency, the stimulatory effect of RPE-CM decreased compared with the proliferation at the subconfluent stage. In contrast to this stimulatory effect of RPE-CM on fibroblasts, the RPE-CM had an inhibitory effect on the proliferation of primary RPE cells (Table 1). To assess whether TGF-β is the active component for cell proliferation, we used anti- TGF-β antibody to render TGF-β inactive. When RPE-CM was simultaneously added to the SCFs with the anti-TGF-β antibodies, SCF proliferation was inhibited by 30% for anti-TGF-β1, 37% for anti-TGF-β3, and 62% for combined treatment with anti-TGF-β-antibodies. In the same manner, anti-TGF-β antibodies were found to block the inhibitory activity of RPE-CM on the cellular proliferation of RPE cells (Table 2).

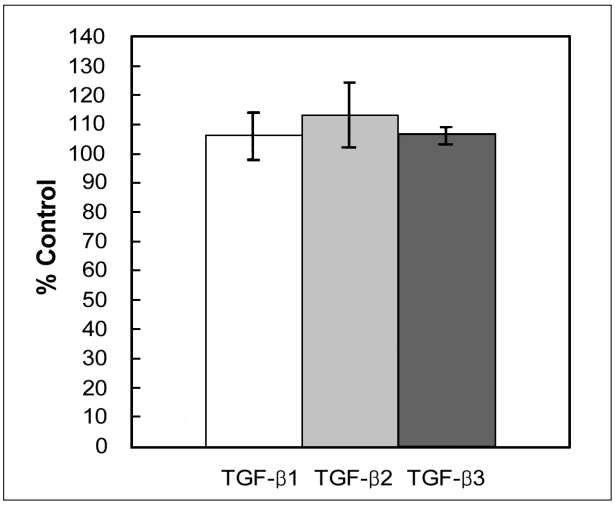

Fig. 2. Effects of each TGF-βs on primary RPE cell proliferation. Cells at 80% confluence were washed and incubated with serum-free media for 24 h. They were then treated with TGF-βs (β1 or 2 or 3) in the presence of 3 μCi/ml ^{3}H-thymidine for 24 h. Serum free medium without TGF-βs was used as the control. Each bar represents the mean ± SEM from four experiments performed in triplicate.

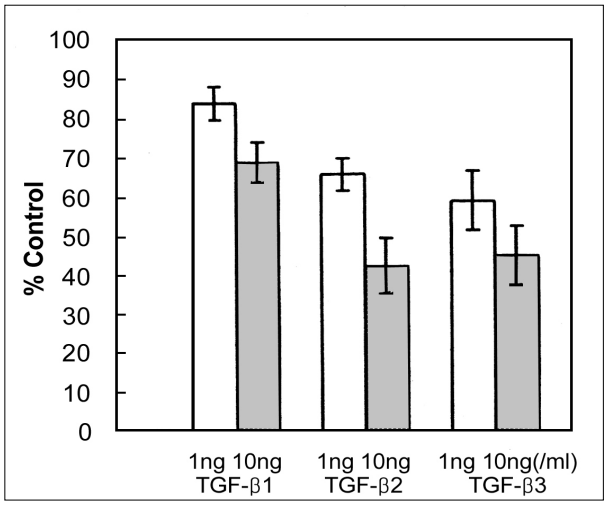

Fig. 3. Effects of TGF-β, TGF-β2, and TGF-β3 on sixth passaged RPE cell proliferation. The sixth passaged RPE cells were plated at 1 × 10^5 cells per 35mm dish. When the cells reached approximately 80% confluence, they were starved of serum for 24hours. Cells were then treated with or without 10 ng/ml of TGF-βs with 3 μCi/ml of ^{3}H-thymidine for 24 h. Each bar represents the mean ± SEM from four experiments performed in triplicate.

Table 1. Effect of RPE-CM on Cell Proliferation

	Target cells		
	SCF (cpm) (subconfluent)	SCF (cpm) (confluent)	RPE (cpm)
control	126655 ± 8206	132048 ± 4204	131825 ± 5434
RPE-CM	251134 ± 14214	147811 ± 3278	109087 ± 6270

*$p<0.05$ by Student paired t-test.

Table 2. Effect of Neutralizing TGF-β Antibodies on RPE-CM Mediated Cell Proliferation

	Target cells	
	SCF	RPE
RPE-CM	265826 ± 11366	9358 ± 574
RPE-CM + anti-TGFβ1	206080 ± 9640	9924 ± 594
RPE-CM + anti-TGFβ3	168645 ± 9910	11950 ± 723
RPE-CM + anti-TGFβs	100911 ± 6474	13272 ± 521

*$p<0.05$ for difference between parallel wells with and without anti-TGF-β by Student t-test.

DISCUSSION

RPE cells do not proliferate at their origin, but they do transform themselves and proliferate as they migrate onto the vitreous gel. RPE cells in culture transdifferentiate, and this transition is accompanied by a shift in their biological activities.[10] As RPE cells leave their origin, they produce growth factors that have autocrine activities and these factors may play a key role in the pathogenesis of proliferative ocular disease.[11] TGF-β levels are elevated in the vitreous of PVR patients.[8] However, the source of this cytokine has not yet clearly been elucidated. In RPE cells, the expression of α-SMA was considered as the reflection of mesenchymal transdifferentiation.[10] The proliferation of cells in the preretinal membrane and hyperplasia of the RPE observed in this study, meant that α-SMA was being expressed.[12,13] α-SMA has been implicated as being partially responsible for cell contraction in fibrocontractive tissue.[14,15] In this study, we verified that RPE cells expressed α-SMA under the influence of TGF-β during wound healing. This result suggests that TGF-β initiates wound healing by changing the characteristics of the RPE cells.

Not only does TGF-β induce morphological transformation in fibroblast cells, but it also stimulates their proliferation.[16] Conversely however, epithelial-cell proliferation has been demonstrated to be either inhibited, or unaffected, by TGF-β.[17] While the mechanisms of TGF-β inhibition in epithelial cell proliferation have not yet been completely defined, the mitogenic effect of TGF-β on fibroblast cells is reported to be an indirect effect of TGF-β caused by the release of PDGF-like growth factors.[18] Collagen contraction, in the presence of TGF-β and interleukin-1-β, is also mediated by the production of PDGF or a PDGF-like molecule.[19] We have demonstrated that TGF-β stimulates cell proliferation via autocrine production of FGF-2 in corneal stromal fibroblasts.[20] SCFs produce both TGF-βs and FGF-2, moreover, FGF-2 seems to stimulate TGF-β-mediated cell proliferation directly.[21] Since the pathogenesis of PVR is based on the proliferation of RPE cells and the formation of the extracellular matrix, it is somewhat strange that TGF-β can inhibit primary RPE cell proliferation. The pathogenesis of PVR therefore, may be explained on the basis of the mesenchymal transformation of PRE cells. It is not clear how TGF-β affects the proliferation of transformed RPE cells on vitreous gel. However, in late-passaged RPE cells, all types of TGF-β are unable to inhibit cell proliferation.

It may therefore be possible that the decreased

inhibitory effects of TGF-βs on transformed epithelial cells may increase their proliferative potential and accelerate phenotypic changes. This hypothesis is supported by the fact that TGF-β does not exert significant effects on the proliferation of transformed RPE cells. It can be speculated that other growth factors may induce the proliferation of RPE cells. Functionally transformed RPE cells release cytokines into the vitreous cavity, and the indirect effects of these cytokines may be the key to explaining the development of PVR.

RPE cells produce both inhibitory and stimulatory cytokines, and the interaction between these is complex. The net effect of the cytokine mixture released by RPE cells is unknown, and the relative predominance of stimulatory and inhibitory signals may determine the final effect.[22] The present study examined the paracrine activities of cytokines synthesized by RPE cells. The results showed that RPE-CM inhibited the proliferation of primary RPE cells while stimulating the growth of SCFs. This effect was especially evident for SCFs when they were actively proliferating. In light of this fact, it may be speculated that PRE-CM induces activities similar to those of TGF-β, and that RPE may in fact, generate TGF-β. The present study confirms the presence of TGF-β in RPE-CM, showing that RPE-CM could neither stimulate the proliferation of SCFs, nor could it inhibit the proliferation of primary RPE cells in the presence of anti-TGF-β antibodies. Interestingly, RPE cells in culture seem to activate the latent form of TGF-βs, suggesting the presence of the same activation mechanism in RPE cultures.

Various studies show that TGF-β is a key cytokine, that both initiates and terminates tissue repair, and that the continuous formation of this substance may result in tissue fibrosis. It is reasonable to speculate that TGF-β may even be involved in the pathogenesis of PVR. RPE cells synthesize TGF-β. At the beginning of development of PVR, TGF-β may promote epitheliomesenchymal transformation and then enhance proliferation of transformed RPE cells. Our results suggest the presence of a RPE autocrine system in the development of PVR.

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REFERENCES

1. Kampik A, Kenyon KR, Michels RG. Epiretinal and vitreous membranes. Comparative study of 56 cases. Arch Ophthalmol 1981;99:1445-54.
2. Radtke ND, Tano Y, Chandler D, Machemer R. Simulation of massive periretinal proliferation by autotransplantation of retinal pigment epithelial cells in rabbits. Am J Ophthalmol 1981;91:76-87.
3. Hiscott PS, Grierson I, McLeod D. Retinal pigment epithelial cells in epiretinal membranes: an immunohistochemical study. Br J Ophthalmol 1984;68:708-15.
4. Mandelcorn MS, Machemer R, Fineberg E, Hersch SB. Proliferation and metaplasia of intravitreal retinal pigment epithelium cell autotransplants. Am J Ophthalmol 1975;80:227-37.
5. Newsome DA, Rodrigues MM, Machemer R. Human massive periretinal proliferation. In vitro characteristics of cellular components. Arch Ophthalmol 1981;99:873-80.
6. Malecaze F, Mathis A, Arne JL, Raulais D, Courtois Y, Hicks D. Localization of acidic fibroblast growth factor in proliferative vitreoretinopathy membranes. Curr Eye Res 1991;10:719-29.
7. Yeo JH, Sadeghi J, Campochiaro PA, Green WR, Glaser BM. Intravitreous fibronectin and platelet-derived growth factor. New model for traction retinal detachment. Arch Ophthalmol 1986;104:417-21.
8. Connor TB Jr, Roberts AB, Sporn MB, Danielpour D, Dart LL, Michels RG, et al. Correlation of fibrosis and transforming growth factor-type 2 levels in the eye. J Clin Invest 1989;83:1661-6.
9. Tanihara H, Yoshida M, Matsumoto M, Yoshimura N. Identification of transforming growth factor-beta-expressed in cultured human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 1993;34:413-9.
10. Grisanti S, Guidry C. Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. Invest Ophthalmol Vis Sci 1995;36:391-405.
11. Bryan JA, Campochiaro PA. A retinal pigment epithelial cell-derived growth factors. Arch Ophthalmol 1986;104:422-5.
12. Fuchs U, Kivela T, Tarkkanen A. Cytoskeleton in normal and reactive human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 1991;32:3178-86.
13. Walshe R, Esser P, Wiedemann P, Heimann K. Proliferative retinal diseases: myofibroblasts cause chronic vitreoretinal traction. Br J Ophthalmol 1992;76:550-2.

14. Darby I, Skalli O, Gabbiani G. α-Smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. Lab Invest 1990;63:21-32.
15. Hunt RC, Davis AA. Altered expression of keratin and vimentin in human retinal pigment epithelial cells in vivo and in vitro. J Cell Physiol 1990;145:187-99.
16. Goustin AS, Leof EB, Shipley GD, Moses HL. Growth factors and cancer. Cancer Res 1986;46:1015-29.
17. Tezel TH, Del Priore LV. TGF-bata secretion modulates the density-dependent growth of pig retinal pigment epithelium in vitro. Ophthalmic Res 1999;31:192-202.
18. Moses HL, Coffey RJ Jr, Leof EB, Lyons RM, Keski-Oja J. Transforming growth factor-regulation of cell proliferation. J Cell Physiol 1987;5 Suppl:1-7.
19. Choudhury P, Chen W, Hunt RC. Production of platelet-derived growth factor by interleukin-1 beta and transforming growth factor-beta-stimulated retinal pigment epithelial cells leads to contraction of collagen gels. Invest Ophthalmol Vis Sci 1997;38:824-33.
20. Kay EP, Lee HK, Park KS, Lee SC. Indirect mitogenic effect of transforming factor on cell proliferation of subconjunctival fibroblasts. Invest Ophthalmol Vis Sci 1998;39:481-6.
21. Kay EP, Lee MS, Seong GJ, Lee YG. TGF-βs stimulate cell proliferation via an autocrine production of FGF-2 in corneal stromal fibroblasts. Curr Eye Res 1998;17: 286-93.
22. Kishi S, Mishima HK, Yamashita U. Growth regulation of RPE cells *in vitro*. Curr Eye Res 1994;13:661-8.