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Effect of cytokines on regulation of the production of transforming growth factor beta-1 in cultured human Tenon's capsule fibroblasts

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PURPOSE. *Transforming growth factor-β1 (TGF-β1) is thought to play a pivotal role in the regulation of the wound healing process after glaucoma filtering surgery. The aim of the present study was to investigate whether platelet-derived growth factor isoforms (PDGF-AA, PDGF-BB), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), interleukin-1β (IL-1β) and transforming growth factor-β1 (TGF-β1) modulate the production of latent and/or active TGF-β1 by cultured human Tenon's capsule fibroblasts (HTF).*

METHODS. *Human Tenon's capsule fibroblasts were seeded at two different densities (30 cells/mm² and 150 cells/mm²) and stimulated for five days with PDGF-AA, PDGF-BB, bFGF, EGF, IL-1β and TGF-β1. Control cells were treated with serum-free medium (WM/F12). The concentrations of latent and active TGF-β1 in the medium were determined using an immunoassay before and after activation of TGF-β1 by transient acidification.*

RESULTS. *The concentration of latent TGF-β1 in conditioned media from HTF seeded at high density (150 cells/mm²) significantly increased after stimulation with 5 ng/ml TGF-β1 (151.5 ± 41.7 pg/ml) or 10 ng/ml IL-1β (45.7 ± 8.1 pg/ml). The concentration of active TGF-β1 in conditioned media also significantly increased after stimulation of HTF with 5 ng/ml TGF-β1 (48.4 ± 27.5 pg/ml).*

CONCLUSIONS. *The present results indicate that TGF-β1 is the most potent inducer of its own synthesis in HTF. Activation of an autocrine TGF-β1 loop may play a role in the wound healing response after glaucoma filtering surgery. (Eur J Ophthalmol 2000; 10: 110-5)*

KEY WORDS. *Glaucoma filtering surgery, Wound healing, Transforming growth factor-β1, Cytokines, ELISA*

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INTRODUCTION

Exaggerated wound healing is the main cause of bleb failure after glaucoma filtering surgery. There is now evidence that the activation of Tenon's capsule fibroblasts is the central regulatory element in the wound healing response. The activation of these cells includes mitogenic stimulation and induction of extracellular matrix production, which ultimately leads to the formation of scar tissue (1, 2). Cytokines play a central role in the regulation of these events. Transforming growth factor beta-1 (TGF-β1), a multifunctional signaling molecule with pronounced effects on cell growth, proliferation and extracellular matrix synthesis and

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degradation, regulates fibroblast activation during wound healing (3-6) and inhibition of TGF-β1 by monoclonal antibodies reduces scar formation *in vivo* (7-9).

TGF-β1 is produced and secreted by a variety of circulating and tissue resident cells in a biologically inactive precursor complex. The active, mature TGF-β1 is a 25kD homodimer held together by two disulfide bonds, and originates extracellularly by activation from the large latent TGF-β-complex, which is composed of the latent TGF-β binding protein (LTBP), latency-associated protein (LAP) and TGF-β1 (5, 10, 11). In cultured human Tenon's capsule fibroblasts (HTF) TGF-β1 modulates cell proliferation (12, 13) and stimulates the synthesis of hyaluronic acid and fibronectin (14, 15). Additionally, HTF produce TGF-β1 protein and mRNA *in vitro* (1, 16). However it is still not known how the production of TGF-β1 by HTF is regulated during the wound healing response.

Since platelet derived growth factor isoforms, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) interleukin-1 (IL-1) and TGF-β1 are present in high concentrations in wound fluid (17, 18), in aqueous humour (19, 20) and in the wound region after glaucoma filtration surgery (21), we investigated whether these cytokines were involved in the modulation of TGF-β1 production in cultured HTF.

METHODS

Cell culture

Fibroblasts were grown from human Tenon's capsule biopsies as previously described (22). In brief, 5 x 5 mm pieces of human Tenon's capsule explants were placed in 10 cm^2 six-well plates (Falcon, Germany) in a mixture of Waymouth's MB 752/1 and Ham's F12 nutrient mixture (1:1, v/v) supplemented with 10% fetal calf serum (FCS). Cells were grown to confluency in a humidified atmosphere of 7% CO_2 and 93% air at 37°C in an incubator. Medium was changed three times a week. For subculture, confluent cultures were detached from culture flasks by trypsin/EDTA treatment (0.05%/0.02 %; pH 7.2). Only second-passage HTF were used for the experiments.

To check for differences between confluent and non-confluent cell layers, experiments were carried out at two different cell densities. For the *low density* assay, cells were seeded at 30 cells/mm^2 in 24-well culture plates in WM/F12 containing 1% FCS. Before stimulation with cytokines, cells were allowed to attach for 24 hours. For the *high density assay* (approximately 150 cells/mm^2), cells were grown to confluency in culture medium containing serum (FCS 10%) for five days before stimulation with cytokines.

In order to investigate the cytokines' role in the regulation of TGF-β1 production by HTF, we investigated the effect of six representative cytokines that all have the potential to modulate wound healing and are present at high concentrations in the wound region after glaucoma filtration surgery fluid (17-21). Each cytokine was tested at concentrations shown previously to induce proliferation of HTF *in vitro* (except EGF, which does not induce proliferation of these cells): PDGF-AA (10 or 100 ng/ml), PDGF-BB (10 or 100 ng/ml), EGF (10 or 100 ng/ml), bFGF (10 or 100 ng/ml), IL-1β (10 or 1 ng/ml), TGF-β1 (5 or 0.5 ng/ml) (23).

The medium was removed 24 hours after seeding and the cells were washed three times with PBS. After washing, the cells were stimulated for five days with the cytokines, diluted to the appropiate concentrations in WM/F12 serum-free medium. Controls were treated with serum-free medium only.

Quantitative ELISA of TGF-β1

Conditioned medium was obtained from cells pretreated with cytokines or control medium as described above, centrifuged at 10,000 g for 5 min to remove cell debris, and immediately stored at -20°C. The concentrations of active TGF-β1 and native TGF-β1 in the medium were determined using a new, sensitive immunoassay (10). In brief, assay plates (MaxiSorp Immuno modules; Nunc, Wiesbaden, Germany) were washed three times with a solution of 9 g/L NaCl and 0.5 g/L Tween 20 by a mechanized washer. Microwells were then coated with chicken anti-TGF-β1 (2 mg/L, R&D Systems, Wiesbaden, Germany) at 4°C overnight with shaking and were then blocked for 2h at room temperature with the assay buffer (5 g/L BSA, 0.5 g/L Tween 20, 0.05 mol/L Tris, pH 7.7), 250 µl/well.

Calibrators (recombinant human TGF-β1 at 667, 222, 74, 25, and 8.2 ng/L dissolved in assay buffer) and samples were incubated in the microwells in duplicate for 2h at room temperature. The secondary an-

tibody was monoclonal panspecific mouse anti TGF-β1,2,3 (5 mg/L, 2h at room temperature with shaking). Subsequently, biotinylated anti-mouse IgG antibody (0.5 mg/L in assay buffer) was added and incubated for 45 min at room temperature with shaking. Incubation for 30 min at room temperature with streptavidin-peroxidase (50 µg/L) followed. Finally, BM blue POD substrate was added to each well. Color development was stopped after 4-5 min by addition of 50 µl of 1 mol/L H_2SO_4, and the absorbance was measured at 450 nm.

For measurement of active TGF-β1, media were used directly for the immunoassay. Total TGF-β1 was determined after transient acidification of the media, by addition of 20 µl of 1 mol/L HCl to 200 µl media (pH 2.3). After thorough mixing and 10 min incubation at room temperature, 11 volumes of samples were neutralized with 1 volume of 1 mol/L NaOH (pH 7.8) then directly added to the coated microwell plate. The concentration of latent TGF-β1 was determined by subtracting the concentration of active TGF-β1 from the concentration of total TGF-β1.

The detection limit of the ELISA was 1.9 pg/ml. All experiments were repeated at least four times. Statistical analysis of the data was done using the two-tailed Student's t-test.

RESULTS

Latent TGF-β1

Stimulation of *low density* cultures by the different cytokines did not result in measurable TGF-β1 concentrations in the medium, except for HTF treated with 5 ng/ml TGF-β1 (16.8 ± 18.7 pg/ml). In the high density cultures (150 cells/mm^2) the TGF-β1 concentration in conditioned media from HTF significantly increased after stimulation with 5 ng/ml TGF-β1 (151.5 ± 41.7 pg/ml; p=0.007) and 10 ng/ml IL-1β (45.7 ± 8.1 pg/ml; p=0.004) when compared to control media (10.0 ± 12.0 pg/ml) (Tab. I).

Active TGF-β1

Stimulation of *low density* cultures by the different cytokines did not result in measurable TGF-β1 concentrations in the medium, except for HTF treated with 5 ng/ml TGF-β1 (17.6 ± 12 pg/ml). In the *high-density* cultures (150 cells/mm^2) the TGF-β1 concentration in conditioned media from HTF significantly increased only after stimulation with 5 ng/ml TGF-β1 (48.4 ± 27.6 pg/ml; p=0.03). No TGF-β1 was detectable in control media (Tab. II).

TABLE I - CYTOKINE EFFECTS ON PRODUCTION OF LATENT TGF-β1 BY HUMAN TENON'S CAPSULE FIBROBLASTS (HTF) SEEDED AT A DENSITY OF 150 CELLS/mm^2

Cytokines	Concentration of cytokines	Concentration of latent TGF-β1 (mean ± SD, pg/ml)	p value
Control		10.0 ± 12.0	
PDGF-AA	10	12.1 ± 18.0	NS
	100	58.2 ± 69.9	NS
PDGF-BB	10	22.1 ± 26.2	NS
	100	18.8 ± 22.0	NS
bFGF	10	12.5 ± 14.4	NS
	100	6.0 ± 12.0	
EGF	10	20.6 ± 24.0	NS
	100	11.1 ± 22.2	NS
IL-1β	1	23.4 ± 29.1	NS
	10	45.7 ± 8.1	0.004
TGF-β1	0.5	13.6 ± 32.4	NS
	5	151.5 ± 41.7	0.007

NS: not significant

p<0.05 was accepted as statistically significant

DISCUSSION

During the wound healing reaction after glaucoma filtering surgery, activation of Tenon's capsule fibroblasts is held responsible for scar formation. Stimulation of mitosis, myofibroblast transformation and matrix synthesis is a complex process, which seems to be controlled by cytokines. TGF-β1 is considered to be a central regulator of matrix synthesis and myofibroblast transformation during this process (24).

Previous studies provided evidence that TGF-β1 is secreted not only by inflammatory cells but also by the tissue-resident HTF (17). Since cytokines are produced at high concentrations in the wound region by circulating and tissue-resident cells (17, 18) and are also elevated in the aqueous humor due to breakdown of the blood-aqueous barrier, we decided to investigate now they affect the production of TGF-β1 by HTF.

The present study demonstrates for the first time that TGF-β1 production can be upregulated significantly by IL-β1 and by TGF-β1 itself in cultured HTF. In confluent high-density cultures and in non-confluent low-density cultures TGF-β1 was the most potent stimulator of its own synthesis when compared with PDGF-AA, PDGF-BB, bFGF, EGF and IL-1β. Although small amounts of exogenous TGF-β1 may still be present after five days of incubation of HTF in the culture medium, its concentration must be lower than the TGF-β1 concentrations measured in the medium of low-density cells. Therefore we can conclude that the dramatic increase in both active and latent TGF-β1 must be due to endogenous TGF-β1 production by HTF. Our results confirm previous findings with other cell types, that the expression of TGF-β1 is differentially regulated by cytokines. TGF-β1 interacts with its own promotor to upregulate steady-state levels of TGF-β1 mRNA in several non-transformed and transformed cell lines *in vitro* (6, 25, 26). These events represent an autoinductive process which may amplify the effects mediated by TGF-β1 in the wound region (8). TGF-β1 mRNA was shown to be upregulated after TGF-β1 treatment of HTF (1). IL-1β increased the production of TGF-β1 in astrocytes (27) and endothelial cells (28) in a dose-dependent manner. This is of particular interest since we know the concentration of IL-1β is very high in human wound fluid (approx. 5 ng/ml) (18). Since IL-1 induces secretion of TGF-β1 with no change in steady-state mRNA, IL-1 stimulated TGF-β1 secretion is presumably accelerated by the presence of TGF-β1 mRNA in the cells eliminating the time needed for transcription of the TGF-β1 gene (29).

TABLE II - CYTOKINE EFFECTS ON PRODUCTION OF ACTIVE TGF-β1 BY HUMAN TENON'S CAPSULE FIBROBLASTS (HTF) SEEDED AT A DENSITY OF 150 CELLS/mm^2

Cytokines	**Concentration of cytokines**	**Concentration of active TGF-β1 (mean ± SD, pg/ml)**	**p value**
Control		ND	
PDGF-AA	10	10.2 ± 12.8	NS
	100	13.9 ± 16.6	NS
PDGF-BB	10	ND	
	100	3.5 ± 7	NS
bFGF	10	ND	
	100	ND	
EGF	10	ND	
	100	ND	
IL-1β	1	8.5 ± 10.0	NS
	10	ND	
TGF-β1	0.5	5.0 ± 10.0	NS
	5	48.4 ± 27.5	0.04

ND: not detected; NS: not significant

$p < 0.05$ was accepted as statistically significant

Our results confirm the report by Da Cunha et al that IL-1 induces mainly production of latent TGF-β1 (27), and we were able to show that PDGF-AA and TGF-β1 also mainly stimulated production of the latent form, the increase in active TGF-β1 being small. Other substances must therefore be present in the wound postoperatively to activate TGF-β1 which is secreted in response to cytokine stimulation. Several factors regulate the bioavailability of TGF-β1 during wound healing. Current concepts suggest that latent TGF-β1 may be targeted to the extracellular matrix by LTBP and that a truncated form of latent TGF-β1 can be cleaved from the matrix by the action of proteases. After association on the cell surface, latent TGF-β1 may be activated by a cell-associated protease like plasmin (26). Finally, the bioavailability can be regulated by soluble or matrix-associated molecules [e.g., alpha-2-macroglobulin, decorin, betablycan, heparin and fucoiden] which inactivate free TGF-β1 (26). Multiple mechanisms of activation are therefore possible *in vivo*.

In conclusion, we found that TGF-β1 production by HTF after glaucoma filtering surgery is cytokine-controlled. The exact nechanisms of activation of TGF-β1 in the wound region *in vivo* remain largely unknown. Our observation that TGF-β1 induces its own production is of major importance since excessive skin scarring may be associated with a failure to eliminate TGF-β receptor upregulation on fibroblasts during the late healing phase (remodeling) (30). Such a situation would result in a persistent, autocrine, positive feedback loop, leading to overproduction of scar tissue. Even after cessation of the acute inflammatory phase with high concentrations of cytokines, a TGF-β1 autocrine loop could still self-perpetuate fibrogenesis. This is of particular interest for understanding wound healing after glaucoma filtering surgery. Studies of the regulation of TGF-β receptor expression on HTF are therefore needed in the future.

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