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LABORATORY INVESTIGATION

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The lens influences aqueous humor levels of transforming growth factor-β2

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Abstract ● Background: Transforming growth factor-beta 2 (TGF-β2) is a pluripotent cytokine which has been suggested to play a number of roles in ocular physiologic and pathologic states. Intraocular fluid (IOF) levels of TGF-β2 are quite high. Although the sources of ocular TGF-β are not completely defined, the retinal pigment epithelium, the epithelium of the ciliary body and trabecular meshwork cells all secrete it. In this study we utilized canine lens and rabbit ciliary pigmented epithelial cell cultures to quantitate the in vitro secretion of TGF-β2. In addition, the effects of aphakia or the presence of cataractous lenses on IOF TGF-β2 levels were determined. ● Methods: Lens and ciliary body epithelial cell culture supernatants and aqueous humors were assayed for total TGF-β2 levels by ELISA and bioassay. ● Results: TGF-β2 accumulated in the media bathing lens epithelial cell cultures (0.7 ± 0.03 ng/ml at day 2) and ciliary pigmented epithelial cell cultures (0.8 ± 0.06 ng/ml at day 2) in a time-dependent manner. Surprisingly, aqueous humor from aphakic rabbit eyes contained significantly higher levels of TGF-β2 than their contralateral phakic controls. Furthermore, aqueous humor from canine eyes with cataracts also contained significantly higher levels of TGF-β2 than normal eyes. ● Conclusions: These results suggest that the lens secretes TGF-β2 and that the presence and status of the lens may influence IOF TGF-β2 levels.

Introduction

The transforming growth factors β (TGF-β) are a family of multifunctional cytokines that modulate many aspects of cellular function, including cell proliferation and inhibition, differentiation, adhesion, and migration [37]. The diversity of these actions allows TGF-β to play a critical role in physiologic and pathophysiologic states. TGF-β exists in at least five isoforms (TGF-β1, -2, and -3 are found in mammals) which share 70–80% homology and is secreted by many cell types [35].

The role of TGF-β2 in the eye has been under investigation for several years. TGF-β2 may influence the ocular immune response and the progression of proliferative diseases. TGF-β2 is a key factor in anterior chamber-associated immune deviation (ACAID) [40], since inhibition of TGF-β2 activity abolishes ACAID [13]. In addition, the concentration of TGF-β2 in the IOFs is altered in various ocular pathologic conditions. TGF-β2 levels are decreased in the aqueous humor during inflammation [1, 36]. However, elevated levels of TGF-β2 are found in the vitreous humor from humans with proliferative vitreoretinopathy [6] and in aqueous humor from eyes with primary open-angle glaucoma [42] or cataracts [10, 19]. TGF-β2 has also been used to treat full-thickness macular holes [15, 41] and endotoxin-induced uveitis in rabbits [1].

The intraocular fluids (IOFs) contain high concentrations of TGF-β2 relative to plasma [7, 16, 19]. It has been demonstrated that the tissues surrounding these fluid compartments make and secrete this cytokine [22, 27, 32, 33, 38]. Immunohistochemical studies show that the different

TGF-β isoforms have distinct and specific distributions in the anterior segment of the eye [31]. TGF-β1 has been found in limbal epithelial cells, in the stroma adjacent to the ciliary processes and in human Tenon's capsule fibroblasts [43]. TGF-β1 and -β2 mRNA and protein are present in corneal epithelial cells and fibroblasts [24]. TGF-β2 has been found in limbal epithelial cells, conjunctival stroma, in the ciliary processes and muscles, and in the stroma adjacent to the pigmented ciliary epithelium. The presence of TGF-β3 in the eye is controversial. TGF-β3 has been reported to be absent in the anterior segment [31] and detected in the ciliary epithelium [32]. De Iongh et al. [11] have reported distinct patterns of the three mammalian isoforms of TGF-β mRNA expression during lens development. TGF-β1 and TGF-β2 mRNA expression was found in lens at all ages studied. In addition, proteins for all mammalian TGF-β isoforms were found in the lens. Knowledge of the tissue distribution of the different isoforms of TGF-β may better define their role in the physiology and pathophysiology of the anterior segment. Purified cultures of ciliary epithelial cells [18], nontransformed retinal pigment epithelial cells [22] and trabecular meshwork cells [2] secrete TGF-β2 into the culture media, and it is therefore likely that they secrete TGF-β2 into the IOFs in vivo.

Potts et al. [34] demonstrated that the embryonic avian lens contains TGF-β2 mRNA and protein. In a previous study, we demonstrated that the lens can secrete another growth factor, transferrin [28]. In a preliminary study we found that lens epithelial cells secrete TGF-β2 [29]. It was the purpose of the present study to further define lens secretion of TGF-β2 and to determine whether the lens contributes to this cytokine's high intraocular concentration. Secretion of TGF-β2 by lens epithelial cells was compared to secretion by pigmented ciliary epithelial cell cultures. In order to determine whether the lens influences the IOF concentration of this cytokine in vivo, the concentration of TGF-β2 in aqueous humor from canine cataractous versus normal eyes and in aqueous humor of normal and aphakic rabbit eyes was compared.

Methods

Experimental animals

New Zealand white rabbits (weight 2.5 kg; Daly Rabbitry, Rocky Mount, N.C.) were used in these studies. Rabbits were killed by exposure to a saturated CO_2 atmosphere. These animals received no topical or systemic therapy prior to anesthesia. Rabbit eyes were used as a source of ciliary body pigmented epithelial cells and whole lenses. In addition, some rabbits (n=7) underwent cataract surgery to determine the effects of aphakia on aqueous humor TGF-β2 content. The surgical methods are described below. Aqueous humor samples were also obtained from normal eyes of healthy dogs killed at a local animal shelter, from normal eyes of anesthetized dogs used in a teaching laboratory in the North Carolina State University (NCSU) College of Veterinary Medicine (n=13) and from dogs undergoing cataract surgery in the Veterinary Teaching Hospital, NCSU (n=28) [9]. The animals were used in accordance with the ARVO *Statement on the use of animals in ophthalmic and vision research,* and all protocols were approved by the Institutional Animal Care and Use Committee of NCSU.

Lens and pigmented ciliary epithelial cell cultures

Canine lens epithelial cells from anterior capsules of normal eyes from dogs of both sexes and generally less than 6 years old were grown from capsule explants in Dulbecco's minimal essential medium (DMEM; Gibco Laboratories, Grand Island, N.Y.) + 10% fetal calf serum (FCS; Hyclone, Logan, Utah) and 1% antibiotic-antimycotic (Gibco) as previously described [28]. After some outgrowth from the capsule, the cells were dispersed by trypsinization and grown to confluence. At this point the cells were removed from the plates and replated in six-well plates, and again grown to confluence. This generally took 4 days. The secretion studies were then initiated in serum-free medium. The six-well plates contained 3 ml of medium and 150 000–180 000 cells/well. Conditioned media was serially collected from the same well on a daily basis (10% of total volume); dilution resulting from the sampling was considered in the final calculations.

Primary cultures of pigmented ciliary body epithelial cells were obtained from rabbits according to previously described methods [12, 14]. The cells were grown to confluence (approximately 180 000 cells/well) in minimal essential medium (MEM; Gibco) + 15% FCS in six-well plates (Falcon). A sample of the medium was removed at the 2-day time point for measurement of TGF-β2.

Aqueous humor collection from control and cataractous dog eyes

A 0.25-ml aliquot of aqueous humor was collected by limbal paracentesis with a 27-gauge needle from the eyes of pet dogs immediately prior to cataract extraction. The cataracts were graded as immature, mature or hypermature according to a previous schema [9]. Control aqueous humor was collected in a similar fashion from young, normal dogs used in a teaching laboratory while the animals were under general anesthesia and immediately prior to their death. Both groups of animals received a preoperative topical therapy regimen consisting of one drop of 1% atropine solution, 10% phenylephrine, 0.1% flurbiprofen, 1% prednisolone acetate, and a topical antibiotic consisting of neomycin, bacitracin, and polymixin B every 30 min for 2 h prior to aqueous humor collection. The 28 aqueous humor samples were from dogs aged 2–12 years. Half were from dogs with genetic cataracts, the other 14 from dogs with diabetic cataracts. The degree of cataract formation or absence of cataract, age or sex of the dog, and the length of time that the cataract had been present were statistically analyzed for possible correlation with TGF-β2 levels using regression analysis.

Removal of lenses from rabbit eyes

Unilateral, intracapsular cryoextraction was performed in adult rabbits using conventional techniques. Rabbits were premedicated with four doses of topical 1% atropine, 10% phenylephrine and 0.1% flurbiprofen at 15-min intervals to achieve and maintain mydriasis; subconjunctival injection of 1.25 mg triamcinolone (Vetalog, Solvay) was used to control postoperative uveitis. Anesthesia was induced by intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). With aid of an operating microscope (OPMI 6, Zeiss) a 3/4 depth 160° clear-corneal incision was made. The anterior chamber was entered with a no. 64 Beaver blade, and 0.2–0.3 ml of 1:5000 alpha chymotrypsin (Zolyce, Alcon) was irrigated into the posterior chamber. After 2–3 min of incubation, the corneal incision

was completed with section scissors and the lens extracted with a 2-mm nitrous oxide cryoprobe (Frigitronics, Coopervision). When necessary, intact zonular fibers were gently broken with a blunt instrument. Post-operative inflammation was monitored by slit-lamp examination. Ten to 30 weeks later, the rabbits were killed and the aqueous humor was removed from both the control eye and the contralateral aphakic eye. Both the white blood cell content and protein concentration were determined in order to have a quantitative assessment of the extent of inflammation and barrier function at the end point of the experiment.

TGF-β2 ELISA

Pooled aqueous humor or cell-free supernatants were assayed for total TGF-β2 using a commercially available ELISA kit (R & D Systems, Minneapolis, Minn.; detection limit of 62.5 pg/ml) or a specific-capture ELISA [8] (detection limit of 300 pg/ml). Aliquots of each sample were activated for detection of total TGF-β2 [23] by acidifying to pH 1.5 (1 N HCl). We were limited by sample volume to measurement of total TGF-β2. Samples were kept at 4°C for 60 min and then neutralized with 1 N NaOH. TGF-β2 concentration was determined by comparing the absorption of samples with that of serial dilutions of the human recombinant TGF-β2 standard in the kit. There was no cross-reactivity with TGF-β1. Samples assayed in the two different ELISAs gave similar results.

TGF-β bioassay

The activity of total TGF-β in pooled aqueous humors or cell-free supernatants was measured by inhibition of the growth of mink lung epithelial cell line, CCL64 (American Type Culture Collection, Rockville, Md.), which are maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics [3]. Duplicate samples of twofold dilutions of samples or TGF-β2 standard were added to CCL-64 cells, and incorporation of tritiated thymidine was determined [26]. TGF-β concentrations were determined by comparison to the TGF-β standard curve ($ED_{50} \sim 40$ pg/ml).

Whole lens incubations

In order to determine whether the whole lens can secrete or take up TGF-β2, lenses were incubated with or without excess TGF-β2. Briefly, after rabbits were killed, the eyes were enucleated and the lenses were carefully dissected and then incubated in DMEM in 24-well culture dishes with up to 10 ng/ml TGF-β2 for up to 24 h. Contralateral control lenses were incubated without TGF-β2. At the end of the incubation period, the incubation medium was analyzed for TGF-β2 and the lenses were homogenized in 1 ml PBS and the homogenate centrifuged at 13 000 *g* for 30 min. After acid activation, the supernatants were analyzed by ELISA for TGF-β2.

Statistical analysis

The results of the time-course experiments and the canine aqueous humor TGF-β2 levels in the cataract study were analyzed by ANOVA coupled with Tukey's test for multiple comparisons. The null hypothesis was rejected at $P<0.05$. TGF-β2 in aqueous humor from aphakic eyes was compared to the contralateral control aqueous using Student's *t*-test. Results are presented as mean ± SEM.

Results

Detection of TGF-β2 secreted by the whole lens and by lens and pigmented ciliary epithelial cells in culture

Increased amounts of TGF-β2 were detected in the culture medium of canine lens epithelial cells (LEC) over a 7-day period, reaching a plateau between day 5 and day 7 (Fig. 1). There was no signifcant difference in the amount of TGF-β2 found in the media at day 5 and 7. A comparison of the amount of TGF-β2 present in the medium on day 2 reveals that the cultured rabbit pigmented ciliary epithelial cells secreted 0.8 ± 0.06 ng/ml, while the LEC secreted 0.7 ± 0.03 ng/ml at the same day 2 time point. Whole lenses were cultured for 6 h in DMEM without serum. After 6 h the medium contained 1.7 ± 0.3 ng/ml TGF-β2 (n=12), indicating that whole lenses were capable of making and secreting this cytokine.

Effect of removal of the lens on aqueous humor TGF-β2 levels

Slit-lamp assessment of the rabbit eyes from which the lens had been removed indicated the absence of clinically detectable inflammation by 10 weeks after surgery. In addition, analysis of the aqueous humor revealed the absence of leukocytes. However, blood-aqueous barrier function was still disrupted to a variable degree (Table 1), as indicated by aqueous humor protein levels, which averaged 7.2 ± 3.7 mg/ml, compared to normal levels of less than 1 mg/ml. The TGF-β2 concentration, determined by ELISA, was significantly higher in the aqueous

Table 1 Protein and TGF-β2 levels in rabbit aqueous humors. The results are presented as the mean ± SEM of the number of samples in parentheses

	Protein (mg/ml)	TGF-β2 (ng/ml)
Aphakic (7)	7.2 ± 3.7*	3.1 ± 0.6*
Sham (7)	0.6 ± 0.1	0.9 ± 0.1

* $P<0.01$, significantly different from the sham-operated eyes as determined by a *t*-test

Table 2 TGF-β2 levels in aqueous humor from cataractous dog eyes. The results are presented as the mean ± SEM of the number of samples in parenthesis

Cataract type	TGF-β2 (ng/ml)
None	0.97 ± 0.05 (13)
Genetic	1.40 ± 0.16* (14)
Diabetic	0.89 ± 0.12 (14)

* $P<0.01$, significantly different from the other two groups as determined by ANOVA and Tukey's HSD test for multiple comparisons

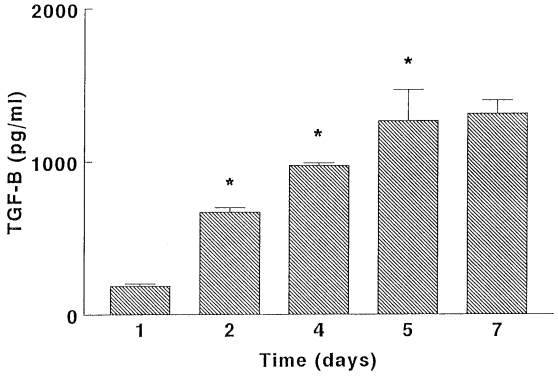

Fig. 1 Quantitation of TGF-β2 secretion by lens epithelial cells (LEC) during 7 days of culture. Cell-free culture supernatants from dog LEC were acidified and TGF-β2 levels were measured using an ELISA (see Methods). Data represent the mean ± SEM of at least six samples. $*P<0.05$, significantly different from the previous time point, ANOVA and Tukey's HSD test for multiple means

humor from the aphakic eyes at this 10-week time point than in their contralateral controls (3.1 ± 0.6 vs 0.9 ± 0.1 ng/ml, $P<0.01$). There was no statistical correlation between TGF-β2 levels and either the time since surgery or the amount of protein present in the aqueous humor.

The possibility that lenses can take up TGF-β2 from the media was explored. After incubation with up to 10 ng/ml TGF-β2 for 3–24 h, none of the lenses contained detectable TGF-β2. If TGF-β2 is expressed only by the epithelial cells and perhaps some outer cortical fiber cells, it is possible that by homogenizing the whole lens, TGF-β2 present in the ourter regions was diluted to undetectable levels. Although we were unable to detect TGF-β2 in our cultured lenses, some TGF-β2 could be bound to the capsule in vivo, since TGF-β2 binds to collagen IV and that extracellular matrix may function as an affinity matrix for binding and immobilization of cytokines of the TGF family [30].

TGF-β2 levels in aqueous humor from normal and cataractous dog eyes

TGF-β2 levels were initially determined by bioassay. However, since the levels of TGF-β2 were so high (5–10 ng/ml), it was decided to confirm them by ELISA. The ELISA results were substantially lower than those of the bioassay for the same samples. In separate experiments, we demonstrated that dexamethasone at 5 nM has the same effect as approximately 0.8 pM TGF-β2 in the bioassay. Therefore, TGF-β2 levels measured by bioassay may have been influenced by corticosteroids that entered the aqueous humor following their topical administration. Therefore, ELISA was used to analyze TGF-β2 levels in all of the aqueous humor samples.

Aqueous humor from canine eyes with either genetic or diabetic cataract was analyzed. The aqueous humor from the genetic cataract eyes had significantly higher TGF-β2 levels than the control aqueous humor (Table 2). On the other hand, aqueous humor from the diabetic cataract eyes had significantly lower TGF-β2 levels than the genetic group. However, TGF-β2 levels in the aqueous humor from diabetic eyes were not different from the controls. In both the genetic and diabetic groups there was no correlation between the amount of TGF-β2 and the degree of cataract formation, the age or sex of the dog, or the length of time that the cataract had been present.

Discussion

The normal canine aqueous humor contains about 1 ng/ml TGF-β2. This is comparable to the concentrations of this cytokine in the IOFs of other species. Other investigators have determined that pure cultures of many different intraocular cell types secrete TGF-β2 into the culture media. For example, nontransformed retinal pigment epithelial (RPE) cells secreted 0.5–2.0 ng TGF-β2/10^6 cells in 24 h [22]. Interestingly, virally transformed RPE cells secrete TGF-β1, but not -β2. In another study, media from ciliary body epithelial cell cultures contained 1.5–3.7 ng/ml TGF-β2 after 3 days in culture. It was estimated that 2.5–6.2 pg TGF-β2/h/cm^2 was produced. If this rate of release occurred in vivo, it would contribute substantially to aqueous humor TGF-β2 concentration [18]. Assuming that a 25-cm^2 plate contains approximately 0.5 × 10^6 cells, these cells were producing 125–310 pg TGF-β2/10^6 cells/h. Trabecular meshwork cells produced a much lower amount of TGF-β2 (7 pg/ml in 24 h) [2]. In the present study, we have shown that cultured canine LEC secreted 243 pg/10^6 cells/h (using the 48 h secretion value). In order to compare our results to those found by other investigators for TGF-β2 secretion by other ocular tissues, we examined secretion of this cytokine by cultured rabbit pigmented ciliary epithelial cells. These cells secreted 278 pg/10^6 cells/h. This is very similar to the values found in the above-mentioned studies of other ocular tissues. In addition, whole lenses secreted 1.7 ng/ml TGF-β2 in 6 h. Therefore, the lens, as well as pigmented ciliary epithelial cells, may be an important source of IOF TGF-β2. However, the in vivo situation may be quite different, since other growth factors, a different extracellular matrix and even differences in pO_2 probably alter growth factor production and secretion.

Surprisingly, removal of the lens from rabbit eyes resulted in significantly higher aqueous humor TGF-β2 levels than in control eyes. The factors responsible for this difference are unclear. Intraocular inflammation caused by the trauma of removing the whole lens in its

capsule by enzymatic and mechanical disruption of the zonular fibers results in disruption of the blood-ocular barrier and dramatically changes IOF composition. However, TGF-β2 levels are generally decreased during intraocular inflammation [1, 10]. Since the plasma contains no detectable TGF-β2 [4], blood-ocular barrier disruption would not be expected to increase levels of this cytokine. Furthermore, the lens did not take up TGF-β2 when incubated with exogenously added TGF-β2. Therefore, the lens probably does not serve as a sink for TGF-β2.

It is possible that the surgery could result in alterations in cytokine production of other ocular tissues, such as retinal pigmented epithelium and ciliary epithelium, which can secrete this cytokine. In fact, pathologic changes in the posterior segment of the eye such as proliferative vitreoretinopathy are associated with an increase in TGF-β2 [6]. If, in the present study, the trauma of removal of the lens by intracapsular extraction caused posterior segment tissues to increase secretion of this cytokine, loss of the vitreous-aqueous barrier after complete removal of the lens could further contribute to the higher levels of TGF-β2 found in aqueous humor from these aphakic eyes.

TGF-β2 levels have been reported to be elevated in the aqueous humor from human eyes containing cataracts [19]. Using a bioassay, the investigators found very high levels (2.3–8.1 ng/ml). They suggest that the composition of the aqueous humor samples may have been influenced by topical pharmaceutical agents that these patients received preoperatively, as a portion of the TGF-β2 activity was not neutralized by antibodies to either β1 or β2 isoforms. In the present study we also found extremely high TGF-β2 levels in the aqueous humor from eyes with genetic cataracts (5–10 ng/ml) using the same bioassay. However, measurement of the same samples by ELISA resulted in much lower values (0.7–2.9 ng/ml). We subsequently determined that dexamethasone, which had been administered preoperatively (topically), mimics the effects of TGF-β2 in this bioassay. Therefore, the TGF-β bioassay may be inappropriate for analysis of aqueous humor samples which contain corticosteriods. However, steroids had no effect on the ELISA. Using this method to measure TGF-β2, the original observation that aqueous humor from eyes with cataracts contain higher levels of TGF-β2 than control eyes was confirmed in aqueous humor from canine eyes with genetic cataracts (Table 1). However, the absolute amount was substantially less than previously reported for human eyes [19]. Interestingly, there was no relationship between the stage of cataract development (immature, mature or hypermature), age, sex or the length of time the cataract had been present and the concentration of TGF-β2 in the aqueous humor. However, there was a significant difference between TGF-β2 levels in aqueous humor from genetic cataract eyes and from diabetic cataract eyes (genetic > diabetic). This can be viewed in either of two ways; either TGF-β2 production is down-regulated in the cataractous eye by the diabetic condition, or TGF-β2 levels in aqueous humor from the diabetic cataractous eyes are unchanged compared to the control eyes. In this regard, Gregor et al. [17] have shown a decrease in TGF-β2 levels in vitreous humor from humans with insulin-dependent diabetes. The reasons for the elevation of TGF-β2 levels in aqueous humor from genetic cataract eyes in comparison to eyes of control and diabetic dogs need further elucidation.

It is important to note that TGF-β2, at levels similar to those found in normal and pathologic IOFs, induces cataractous changes in lens cell explants [21, 25, 39]. Of course, under normal circumstances most IOF TGF-β2 is in the latent form [5, 7, 16]. However, in some pathological conditions, such as proliferative vitreoretinopathy, a substantial amount of active TGF-β2 is present [1, 10] and could cause damage to the lens resulting in pathological conditions such as posterior subcapsular opacification. It is difficult to explain why the lens would secrete, in such large quantities, a cytokine which has potentially deleterious effects. It is possible that secretion of TGF-β2 has an autocrine function which may include decreasing the rate of epithelial cell division, since this cytokine has anti-proliferative potential [20]. Just as the nature of its response on a particular cell is dependent on many parameters, such as cell type and state of differentiation, concentration, and the presence of other growth factors, the influence of the lens on IOF TGF-β2 may be the result of a balance between several factors. In this regard, Wallentin et al. [44] have recently reported findings suggesting that the proliferation of residual lens epithelial cells following extracapsular lens extraction may be due to an imbalance between growth-promoting growth factors, such as basic fibroblast growth factor, and anti-proliferative growth factors, such as TGF-β.

In conclusion, the lens secretes significant quantities of TGF-β2 and probably contributes to the content of this cytokine in IOF. A clearer definition of the role of the lens in intraocular TGF-β2 processing will improve our understanding of the physiological and pathological influences of this important cytokine.

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