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Induction of arachidonic acid metabolite release by human fibroblasts in proliferative vitreoretinopathy

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Abstract

Proliferative vitreoretinopathy is a severe ocular disorder characterized by unwanted proliferation of cells and excessive production of fibrous tissue, which leads to the formation of cellular membranes on the surface of the retina and in the vitreous. Proliferative vitreoretinopathy is the most common cause of failure in retinal reattachment surgery, approximately occurring in one out of ten operated eyes. Proliferation of retinal pigment epithelial cells and fibroblasts is a cornerstone in the pathogenesis of proliferative vitreoretinopathy. An in vitro-proliferation assay showed previously that intraocular fluid from patients with proliferative vitreoretinopathy is potently effective in stimulating proliferation of human fibroblasts. Here we show that exposure of human fibroblasts to vitreous fluids from patients with proliferative vitreoretinopathy causes a rapid and sustained increase in arachidonic acid metabolite release as measured by competitive enzyme-immunoassay. The findings implicate prostaglandin E_2 as a contributor to enhanced intraocular fibrosis in proliferative vitreoretinopathy. As prostaglandin E_2 is a mediator of continuous aqueous–blood retinal barrier breakdown in this severe disease, cyclooxygenase inhibitors such as acetylsalicylic acid, which was successfully used in this study for blocking the effect of intraocular fluid, may be useful agents in targeting the progression of intraocular fibrosis. © 1998 Elsevier Science B.V.

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1. Introduction

Proliferative vitreoretinopathy is the most common cause of failure following rhegmatous retinal detachment surgery. This entity is frequently associated with giant retinal tears, posterior segment trauma, and excessive cryotherapy (The Retinal Society Terminology Committee, 1983; Claes et al., 1988). Intraocular disorder is initiated by reparative fibrocellular proliferation and blood–aqueous barrier breakdown and characterized by the formation of contractile fibrous and cellophane membranes which spread into the vitreous on the inner and outer surface of the human retina. Several studies have examined the cellular contents of epiretinal membranes to determine which cells contribute to this process. Immunohistologic together with light and electron microscopic analysis have identified cellular components involved in this innerocular overwhelming fibrosis: macrophages, glial cells, retinal pigment epithelial cells and fibroblasts (Van Horn et al., 1977; Machemer, 1978; Newsome et al., 1981; Hiscott et al., 1983, 1984; Ryan, 1985; Weller et al., 1988; Lopez et al., 1992). Migration, proliferation and production of cytokines and other substances by these cells appear to play important roles in the pathogenesis of this severe disease leading to blindness if not adequately treated. The evolution of proliferative vitreoretinopathy occurs in three consecutive stages (traction, incorporation and proliferation), which include intermediate and transitional stages (Lopez et al., 1992). However, the precise mechanisms by which these cellular functions are affected remain to be elucidated.

Factors that regulate cellular growth and migration appear to act by a variety of mechanisms. In addition to the direct action of growth and chemotactic factors, the regulation of cell functions includes indirect mechanisms, such

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as those mediated by metabolites of the arachidonic acid cascade (Kähler et al., 1993). Arachidonic acid metabolites seem to play a central role in intraocular inflammation (Eakins, 1977). Cyclooxygenase products of arachidonic acid, e.g. prostaglandins, prostacyclin and thromboxane A_2, mediate a wide range of actions. Vasodilatation, increased vascular permeability, platelet aggregation and its inhibition and immunomodulation are some of the important biological actions of cyclooxygenase products. Furthermore, prostaglandins are synthesized by different ocular tissues and some of these endogenous substances serve as mediators of extraocular and innerocular inflammation (Frucht-Pery and Zimmermann, 1992). In the eye prostaglandins cause vasodilatation, increase or decrease in intraocular pressure (Eakins, 1977; Battacherjee, 1980; Bito et al., 1993) and are able to disrupt the blood–aqueous barrier (Neufeld and Sears, 1973; Vegge et al., 1975; Eakins, 1977; Bazan et al., 1985; Csukas et al., 1992; Vinores et al., 1992). Rubin and Rosenbaum (1988) showed that intravitreal administration of recombinant interleukin-1β to the rabbit eye caused infiltration of leucocytes into aqueous humor and disruption of the blood–aqueous barrier. This disruption was significantly inhibited by flurbiprofen, indicating that interleukin-1 induced disruption of blood–aqueous barrier is mediated by prostaglandins. Furthermore, it was shown that intravitreal injection of interleukin-1α significantly increased the release of prostaglandin E_2 into aqueous humor and this release was inhibited by flurbiprofen (Kulkarni and Mancino, 1993). We recently described the enhanced growth-promoting effect of human vitreous from patients suffering from proliferative vitreoretinopathy and the possibility of antagonism by acetylsalicylic acid (Kähler et al., 1994).

As prostaglandins of the E series and to a certain extent prostaglandin $F_{2\alpha}$ elicit ocular responses ranging from vasodilatation to breakdown of the blood–aqueous barrier and to reduction of intraocular pressure in vivo, we examined whether intraocular fluid of patients suffering from proliferative vitreoretinopathy, was able to induce the production and release of arachidonic acid metabolites, which may underhold the inflammatory process and might cause blood–aqueous barrier breakdown in proliferative vitreoretinopathy.

2. Materials and methods

2.1. Establishment and conditions of human fibroblasts in culture

Human skin fibroblasts were isolated from human skin fragments (kindly provided by Dr. N. Romani, Department of Dermatology, Faculty of Medicine, University of Innsbruck) as described previously (Kähler et al., 1993). Fibroblasts were grown in enriched medium (M199 in Earle's salts base with L-glutamine (Biological Industries, Beth Haemek, Israel), 10% foetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin) in 175 cm^2 plastic tissue culture flasks and kept in a humidified incubator at 5% CO_2 and 37°C. After reaching confluence the cells were subcultured and reseeded at a ratio of 1:3.

2.2. Measurement of prostaglandins

Cells to be used for prostanoid measurement were grown to confluency, washed, trypsinized and seeded in M199 containing 10% foetal calf serum into 24 well culture dishes at 3×10^4 cells/well. The next day the medium was aspirated, the attached cells were washed with HEPES/EDTA and then the medium was changed to M199 supplemented with 0.5% foetal calf serum. The cells were thus maintained in a growth-arrested state for 48 h preceding the assay. Following this procedure the medium was removed and replaced by test substances and intraocular fluid dissolved in M199 containing 0.5% foetal calf serum. After incubation for various time periods supernatants were removed and immediately stored at −80°C.

Prostaglandins in the supernatants of fibroblast cultures were measured without further extraction by competitive enzymo-immunoassay. Assays for prostaglandin E_2, 6-keto prostaglandin $F_{2\alpha}$, thromboxane B_2 and leukotriene B_4 were performed using commercial kits according to the manufacturer's recommendations. The antibody used to detect prostaglandin E_2 crossreacts with 15-keto prostaglandin E_2 by 9.2% and with prostaglandin E_1 by 5% but not with other prostaglandins of the A, B, D and F series or thromboxane B_2. The antibody used for the 6-keto prostaglandin $F_{1\alpha}$ assay crossreacts by 8.7% with 2,3 dinor-6-keto prostaglandin $F_{1\alpha}$, by 2.1% with prostaglandin $F_{2\alpha}$, by 0.9% with prostaglandin E_2 and by 0.8% with prostaglandin $F_{1\alpha}$. Crossreactivities of the thromboxane B_2 antibody are 8.2% with 2,3-dinor thromboxane B_2, 0.2% with 11-dehydrothromboxane B_2 and 0.44% with prostaglandin D_2. The leukotriene B_4 antibody crossreacts by 0.35% with 5(*S*),12(*S*)di-hydroxy-eicosatetraenoic acid (DiHETE) but not with other HETEs and leukotrienes tested. Acetylsalicylic acid (Aspisol®, Bayer, Leverkusen, Germany) blocked cyclooxygenase activity for the duration of the experiments as already described (Kähler et al., 1993, 1994).

2.3. Patients

Intraocular fluid specimens were obtained from the eyes of 20 patients with clinically and intraoperatively established diagnosis of proliferative vitreoretinopathy aged 27 to 66 years (mean = 56.6 years). Control intraocular fluid specimens were collected during cataract operations which were performed on 21 patients, aged 59 to 92 years (mean = 77.8 years). For experiments pairs of samples were pooled in order to give sufficient sample volumes. All patients suffered from moderate to severe grades of

proliferative vitreoretinopathy, according to the system proposed (The Retinal Society Terminology Committee, 1983).

2.4. Sampling

In all patients operated on for proliferative vitreoretinopathy, about 100–500 μl of aspirate (intraocular fluid) was carefully withdrawn from the operated eyes at the beginning of vitrectomy via the pars plana, before any intraocular infusion was instilled. In all cases the aspirates were obtained by using a 30-gauge needle on a 1 ml syringe and were transferred to a sterile tube and immediately stored at −80°C. In patients operated on for cataract, intraocular fluid was aspirated from the anterior chamber before opening of the anterior capsule for extracapsular cataract surgery. Only serum-free, clear intraocular fluid samples were used for investigation. Operations were performed under local anaesthesia using mepivacaine hydrochloride and patients were not preoperatively treated with anti-inflammatory drugs.

2.5. Statistical analysis

Data are expressed as the mean and standard errors of the mean (S.E.M.). Means were compared by Mann–Whit-

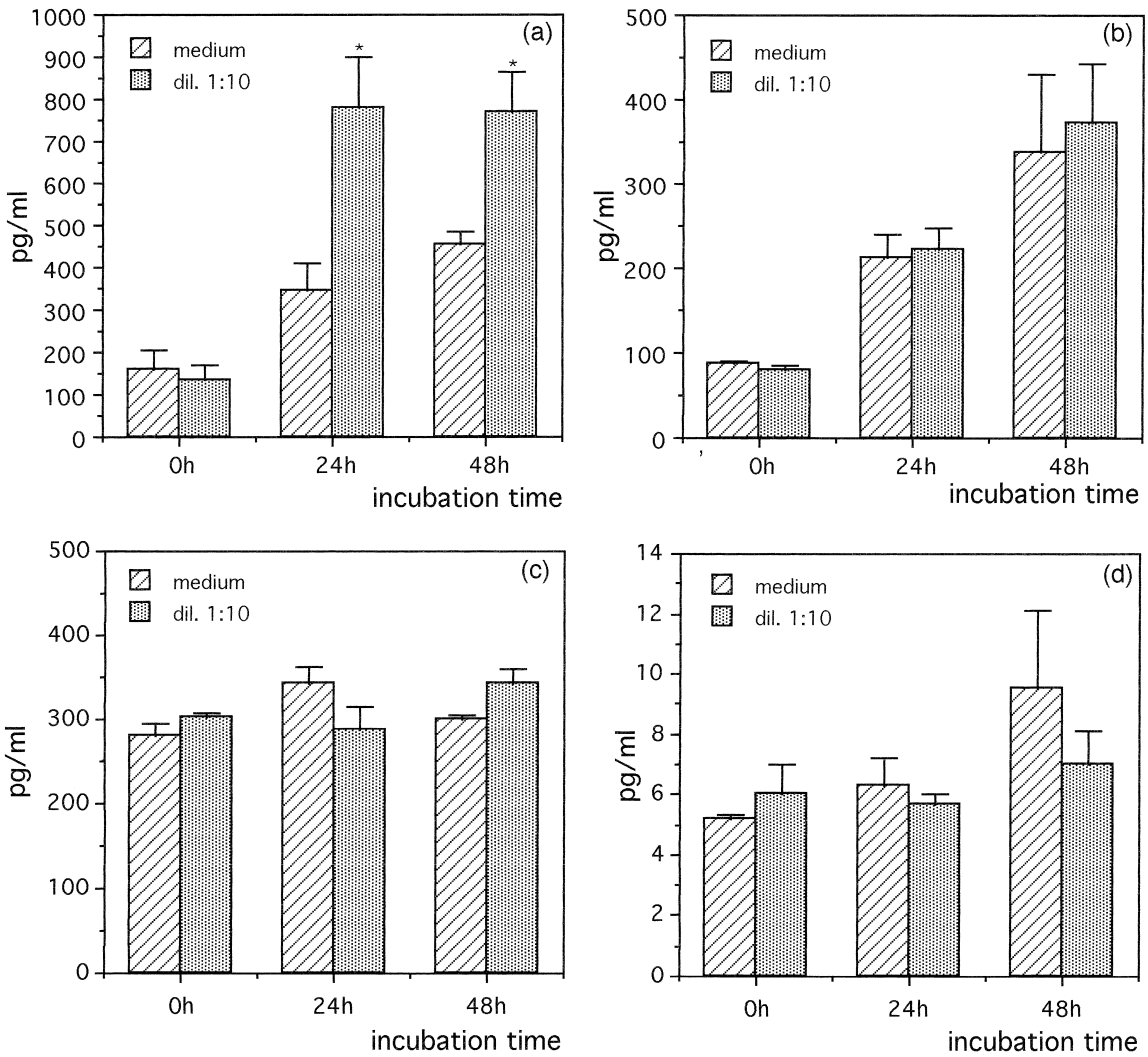

Fig. 1. (a) Stimulation of PGE_2 release from cultured human skin fibroblasts by intraocular fluid of proliferative vitreoretinopathy patients. Growth arrested cells were incubated for 0, 24 or 48 h in medium with 0.5% foetal calf serum containing a dilution of intraocular fluid of 1:10 ($n = 3$). Mean + S.E.M. Mann–Whitney *U*-test; $p^* < 0.05$. (b) Stimulation of 6-keto PGF_{1a} release from cultured human skin fibroblasts by intraocular fluid of proliferative vitreoretinopathy patients. Growth arrested cells were incubated for 0, 24 or 48 h in medium with 0.5% foetal calf serum containing a dilution of intraocular fluid of 1:10 ($n = 3$). Mean ± S.E.M. (c) Stimulation of thromboxane B_2 release from cultured human skin fibroblasts by intraocular fluid from proliferative vitreoretinopathy patients. Growth arrested cells were incubated for 0, 24 or 48 h in medium with 0.5% foetal calf serum containing a dilution of intraocular fluid of 1:10 ($n = 3$). Mean ± S.E.M. (d) Stimulation of leukotriene B_4 release from cultured human skin fibroblasts by intraocular fluid of proliferative vitreoretinopathy patients. Growth arrested cells were incubated for 0, 24 or 48 h in medium with 0.5% foetal calf serum containing a dilution of intraocular fluid of 1:10 ($n = 3$). Mean ± S.E.M.

ney's *U* test or the Kruskal–Wallis analysis of variance. Statistics were performed using the StatView512 + software package (Abacus Concepts, Berkeley, CA).

2.6. Chemicals

The materials used included acetylsalicylic acid (Aspisol) which was purchased from Bayer (Leverkusen, Germany).

3. Results

3.1. Intraocular fluid of patients suffering from proliferative vitreoretinopathy stimulates prostanoid synthesis by human fibroblasts in vitro

Recent findings implied the role of prostanoids in blood–aqueous barrier breakdown. Thus, fibroblasts were incubated in 24-well plates in M199 containing 0.5% foetal calf serum and prostaglandins were measured in the supernatants after 0, 24 and 48 h, respectively. Under these basal conditions, fibroblasts produced negligible amounts of leukotriene B_4, but significant amounts of prostaglandin E_2, prostacyclin and thromboxane B_2.

The addition of intraocular fluid from patients suffering from proliferative vitreoretinopathy enhanced the release of prostaglandin E_2 manifold, both after 24 and 48 h of incubation (Fig. 1a). In contrast, no significant effect on leukotriene B_4, 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2 release was observed (Fig. 1b–d) after 24 or 48 h of incubation. Prostaglandin E_2 was found as the main product of eicosanoid synthesis in intraocular fluid-stimulated quiescent fibroblasts. In time-kinetic experiments we observed a maximum release of prostaglandin E_2 after 24 h, whereas no further increase in prostaglandin E_2 release was observed after 48 h of incubation. In corresponding experiments we investigated the effect of intraocular fluid from non-inflammatory eyes (cataract) on eicosanoid synthesis. As described in Table 1 no stimulation of prostaglandin E_2, 6-keto prostaglandin F_{1a}, leukotriene B_4 and thromboxane B_2 release from human fibroblasts above baseline levels (medium alone) was observed after 24 h by stimulation with intraocular fluid of cataract patients.

Table 1
Eicosanoid release by human fibroblasts stimulated with human vitreous of patients suffering from cataract. Growth arrested cells were incubated for 24 h in medium containing 0.5% foetal calf serum with or without a dilution of intraocular fluid of 1:10 ($n = 3$). Mean ± S.E.M.

Eicosanoids	Cataract (pg/ml)	Control (pg/ml)
PGE_2	47.0 ± 4.6	99.7 ± 16.4
6-Keto PGF_{1a}	43.7 ± 5.5	47.5 ± 3.6
Thromboxane B_2	289.7 ± 9.5	225.0 ± 7.1
Leukotriene B_4	5.5 ± 0.3	5.8 ± 0.6

Table 2
Effect of the anti-inflammatory drug acetylsalicylic acid on eicosanoid release by human fibroblasts stimulated with a 1:10 dilution of human vitreous of patients suffering from proliferative vitreoretinopathy. Growth arrested cells were incubated for 24 h in medium with 0.5% foetal calf serum containing a dilution of intraocular fluid of 1:10 ($n = 3$). Mean ± S.E.M.

Eicosanoids	+ Acetylsalicylic acid (pg/ml)	− Acetylsalicylic acid (pg/ml)
PGE_2	671.5 ± 53.8	64.0 ± 18.0
6-Keto PGF_{1a}	221.5 ± 44.5	70.5 ± 04.5
Thromboxane B_2	287.6 ± 26.8	297.0 ± 11.7
Leukotriene B_4	5.6 ± 0.3	7.3 ± 2.3

3.2. Inhibition of intraocular fluid-stimulated prostanoid synthesis in proliferative vitreoretinopathy by cyclooxygenase inhibition

To elucidate whether a cyclooxygenase inhibitor affects the intraocular fluid-evoked release of arachidonic acid metabolites from fibroblasts, we investigated the activity of intraocular fluid of patients suffering from proliferative vitreoretinopathy in the presence of acetylsalicylic acid. To this aim, serum-starved human fibroblasts were incubated with a 1:10 dilution of intraocular fluid and acetylsalicylic acid, at a concentration (100 mM) which had been shown to block the cyclooxygenase pathway of arachidonic acid metabolism (Kähler et al., 1994). Under these conditions, acetylsalicylic acid significantly antagonizes the stimulatory effect on prostanoid release of intraocular fluid from proliferative vitreoretinopathy patients on quiescent human fibroblasts (Table 2). Acetylsalicylic acid (100 mM) inhibited intraocular fluid-induced augmentation of prostaglandin E_2 production. Leukotriene B_4 release remained unchanged in the presence of acetylsaliylic acid. As reported previously, a maximum inhibitory effect of acetylsalicylic acid on intraocular fluid-stimulated fibroblast proliferation occurred at a concentration of 10^{-4} M, which was shown to be non-toxic on human fibroblasts in vitro (Kähler et al., 1994).

4. Discussion

The clinico–pathologic findings suggest that proliferative vitreoretinopathy is an extension of the physiologic wound-healing response to rhegmatous retinal detachment. Progressive structural damage to the ciliary body by contraction of the membranes in combination with other damaging factors may result in permanent impairment of aqueous secretion and chronic intractable hypotony. Breakdown of the blood–aqueous barrier from traction on the ciliary epithelium or, in advanced stages, from neovascularization of the vitreous base, adds a vascular component to this wound-healing response (Lopez et al., 1992). However, the precise pathogenic mechanisms responsible for prolifera-

tion and blood–aqueous barrier breakdown remain poorly understood.

Observations provide a circumstantial link between prostaglandins and cellular growth and blood–aqueous barrier disruption. Fibroblasts, besides other cells such as (corneal) endothelial cells, possess the ability to synthesize eicosanoids, e.g. prostaglandin E_2 and prostaglandin F_2 (Mayer et al., 1984). The release of various proinflammatory chemical mediators such as prostaglandins, leukotrienes and cytokines not only plays a direct role in inflammation, but also modulates inflammatory responses by interacting with one another (Battacherjee, 1980). Some of these prostanoids act as mediators in intraocular inflammation and may disrupt the blood–aqueous barrier (Neufeld and Sears, 1973; Vegge et al., 1975; Eakins, 1977; Csukas et al., 1992). Prostanoids produced by human fibroblasts are mainly prostaglandin D_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$ and 6-keto prostaglandin $F_{2\alpha}$, the stable metabolite of prostacyclin, with prostaglandin E_2 being the major product of the cyclooxygenase pathway in human fibroblasts.

Weinreb et al. (1985) described a 60-fold increase of prostaglandin E_2 levels in aqueous humor, which can be found normally in the anterior and posterior eye, after irridation of the iris by laser photocoagulation. In contrast, to our knowledge prostaglandin $F_{2\alpha}$ has not yet been detected in the human posterior vitreous. Furthermore, prostaglandin $F_{2\alpha}$ receptors in the monkey eye are predominantly localized in the anterior eye, while retina and choroidal melanocytes express low levels of messenger RNA and protein as done by in situ hybridization and immunohistochemistry (Ocklind et al., 1996), thus indicating a predominant role of prostaglandin E_2 in the vitreous in inflammatory responses.

Prostaglandin E_2 was shown to be the most potent prostanoid in the disruption of the blood–aqueous barrier, whereas other prostaglandins are less effective in this context (Crawford et al., 1987). On the other hand, the lipid soluble prostaglandin $F_{2\alpha}$ mainly causes a potent dose-dependent vasoconstriction, but exerts only weak blood–aqueous barrier disrupting effects (Crawford et al., 1987). Earlier studies demonstrated that exposure to a sufficient quantity of this prostanoid resulted in iridial vasodilatation, hyperemia, ciliary process edema and alteration of the junctional complexes of the ciliary epithelia. It is not known whether the primary event triggering blood–aqueous barrier breakdown is altered iridial blood flow, direct action at the ciliary epithelia, or some other, as yet undescribed event. A recent study by Protzmann and Woodward (1990) demonstrated that rabbit blood–aqueous barrier breakdown was mediated by E prostanoid EP_2 receptor agonist but not prostanoid EP_1 or prostanoid EP_3 selective receptor agonists. In contrast, FP, IP and TP receptor agonists were ineffective in disrupting the blood–aqueous barrier. Additional studies show that topical administration of prostaglandin E_2 to rabbit eyes causes breakdown of the blood–aqueous barrier by opening tight junctions between nonpigmented ciliary epithelium. Thus, the opening of tight junctions may be a mechanism by which prostaglandins cause barrier leakage at various sites in the posterior eye (Vinores et al., 1992).

Consequently, we were primarily interested in prostaglandin E_2 in this context. Our findings indicate that the prostanoids measured in the supernatants are produced and released from human fibroblasts in response to intraocular fluid of patients suffering from proliferative vitreoretinopathy and are not originally present in the evaluated ocular fluids. In conclusion, we showed that pathologic proliferative vitreoretinopathy vitreous stimulated the production of measurable quantities of arachidonic acid metabolites.

Tractional structural damage to the ciliary epithelium by advanced anterior proliferative vitreoretinopathy may not only result in impaired aqueous secretion and chronic hypotony in some eyes, but may also result in breakdown of the ciliary blood–aqueous barrier and the occurrence of postvitrectomy fibrin syndrome in some eyes. Consequently, the approach of this study was to explore whether arachidonic acid metabolites are involved in the pathogenesis of proliferative vitreoretinopathy. For this reason, we investigated the effect of intraocular fluid of patients suffering from proliferative vitreoretinopathy on arachidonic acid metabolite release from human fibroblasts, which represent the major cellular component of the intraocular membranes. The data of our experiments showed a significant increase in prostaglandin E_2 release from vitreous-stimulated fibroblasts as compared to control conditions, whereas leukotriene B_4 release, prostaglandin I_2 and thromboxane A_2 release were not significantly affected in our experiments.

Therapeutical prevention of proliferative vitreoretinopathy attempts to intervene at an early stage, i.e. to block the proliferation and migration of contractile cells and consequently inhibit tractional retinal detachment. Although antiproliferative agents such as the antracyclines adriamycin (*N,N*-dimethyadriamycin, doxorubicin) (Sunalp et al., 1985; Steinhorst et al., 1993) and daunomycin (Wiedemann et al., 1983) or minoxidil (Handa et al., 1993) can be used to prevent cell proliferation and consequently blood–aqueous barrier breakdown in proliferative vitreoretinopathy. Retinal toxicity and the inherent problems of drug delivery have limited the feasibility of this drug therapy. Acetylsalicylic acid was shown to significantly suppress the ability of intraocular fluid in proliferative vitreoretinopathy to stimulate mitogenesis of human fibroblasts (Kähler et al., 1993).

Anti-inflammatory medications are traditionally administered to the eye only postoperatively to control inflammation. The principal action of nonsteroidal anti-inflammatory drugs (NSAIDs) is to inhibit cyclooxygenase activity and consequently the formation of prostaglandin mediators of inflammation. Anti-inflammatory medications are traditionally used to control ocular inflammation. Recently,

Roberts (1996) showed that pretreatment with an NSAID before cataract surgery can reduce the amount of initial postoperative inflammation. Here, we demonstrate that acetylsalicylic acid inhibited intraocular fluid-stimulated arachidonic acid metabolite release. These findings indicate, that as compared to cataract experiments, the enhanced prostanoid release from fibroblasts in proliferative vitreoretinopathy may be induced by humoral factors of intraocular fluid. However, further studies have to be done evaluating the role of other prostanoids, especially prostaglandin $F_{2\alpha}$ in the pathogenesis of proliferative vitreoretinopathy, which does not seem to be intensively involved in blood–aqueous barrier breakdown, but might influence proliferative responses of involved cell types.

In case arachidonic acid metabolites participate in the pathogenesis of proliferative vitreoretinopathy, their overproduction in this disorder may be amenable to specific pharmacotherapy, as suggested by the observed in vitro inhibition of intraocular fluid-induced prostanoid production by acetylsalicylic acid or other NSAIDs. Furthermore, recent data imply the involvement of two cyclooxygenase isozymes that may catalyze the formation of prostanoids. Cyclooxygenase exists as a constitutive (cyclooxygenase-1) and a mitogen-inducible (cyclooxygenase-2) isoform. Appleton et al. (1995) showed that cyclooxygenase-2 is the predominant isoform in all stages of the inflammatory response. Selective inhibition of cyclooxygenase-2 may prove more beneficial and highly relevant in the future therapeutic therapy of proliferative vitreoretinopathy.

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