Graefes Arch Clin Exp Ophthalmol (2013) 251:1919–1927
DOI 10.1007/s00417-013-2345-0

BASIC SCIENCE

Short- and long-term corneal vascular effects of tafluprost eye drops

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Received: 27 November 2012 / Revised: 14 March 2013 / Accepted: 2 April 2013 / Published online: 28 April 2013

Abstract
Background Prostaglandin analogs are first line therapy in the treatment of glaucoma, but also display side effects during ocular inflammation. In this context, the potential side effects of prostaglandin analogs on the normally avascular cornea, the main application route for eye drops, are so far not fully defined. Therefore, the aim of this study was to evaluate the vascular effects of the prostaglandin analog tafluprost on the healthy and inflamed cornea.
Methods For in vitro studies, blood and lymphatic endothelial cells were treated with tafluprost; cell proliferation was assessed after 48 h. For long-term in vivo studies under healthy conditions, naïve corneas of BALB/c mice were treated with tafluprost eye drops for 4 weeks. For short-term in vivo studies under inflammatory conditions, corneal inflammation was induced by suture placement; mice then received tafluprost eye drops for 1 week. Afterwards, corneas were stained with CD31 as panendothelial and LYVE-1 as lymphendothelial (and macrophage) marker.
Results In vitro, tafluprost did not alter blood or lymphatic endothelial cell proliferation. In vivo, there was no change in limbal blood or lymphatic vessel anatomy after long-term treatment with tafluprost. Short-term treatment with tafluprost under inflammatory conditions did not influence the recruitment of LYVE-1 positive macrophages into the cornea. Moreover, treatment of inflamed corneas with tafluprost did not significantly influence corneal hem- and lymphangiogenesis.
Conclusions Tafluprost does not affect blood and lymphatic vessel growth, neither under resting nor under inflammatory conditions. These findings suggest a safe vascular profile of tafluprost eye drops at the inflammatory neovascularized cornea.

Keywords Prostaglandin analogs · Corneal neovascularization · Hemangiogenesis · Lymphangiogenesis

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Introduction

Glaucoma is one of the leading causes of blindness in the world. Therapeutic strategies for lowering high intraocular pressure—the most important risk factor for the chronic optic nerve fibre loss occurring in glaucoma—include surgery as well as treatment with antiglaucomatous eye drops. Various substance classes of antiglaucomatous eye drops are in clinical use and usually have well defined pharmacological profiles. In this context, there are also several contraindications for treatment with the different substance classes. For instance, β-adrenergic receptor antagonists should not be used when obstructive pulmonary diseases are manifest [1], and carbonic anhydrase inhibitors show undesirable side effects in patients suffering from corneal endothelial pathologies such as Fuchs' endothelial dystrophy [2]. Prostaglandin analogs are currently first line therapy for the treatment of glaucoma. Approved prostaglandin analogs usually are prostaglandin F2α (PGF2α) analogs and include latanoprost, bimatoprost, travoprost, and tafluprost [3]. Tafluprost differs from the other prostaglandin analogs because it possesses two fluorine atoms at the carbon 15 position instead of the hydroxyl group present in latanoprost, travoprost, and bimatoprost. All prostaglandin analogs are prodrugs and are hydrolyzed by corneal esterases to their active forms [4]. With regards to potential side effects, prostaglandin analogs are discussed to be disadvantageous when ocular inflammatory diseases are present or when ophthalmic surgical procedures have recently been performed. In

this context, there are reports that showed higher incidence rates of Irvine-Gass syndrome after cataract phacoemulsification and intraocular lens implantation and the postoperative use of prostaglandin analogs [5], whereas other groups could not confirm these results [6, 7]. Furthermore, in other inflammatory ocular pathologies such as anterior uveitis, also controversy exists regarding the use of prostaglandin analogs during periods of high inflammatory activity. However, there seems to be no conclusive clinical evidence to support this so far, and several studies suggest a safe use of prostaglandin analogs in uveitic patients [8–10]. Further prostaglandin analog associated ocular side effects include cystoid macular edema and reactivation of herpes simplex keratitis [11].

The cornea, the clear windscreen of the eye, contains no blood or lymphatic vessels. This avascularity is actively maintained by the expression of various antiangiogenic molecules which serve as a sink for angiogenic growth factors and therefore block the growth of new vessels into the cornea [12–14]. Nevertheless, a variety of diseases can overcome this angiogenic privilege and lead to the secondary ingrowths of both blood and lymphatic vessels from the limbal region into the cornea, causing a loss of visual acuity [12]. Furthermore, the presence of blood and especially clinically invisible lymphatic vessels is the most important risk factor for immune rejections after corneal transplantation [12, 15–17]. Pathologic conditions leading to corneal neovascularization are, e.g., chemical and thermal burns, hypoxic diseases, limbal stem cell deficiency, and several infectious and inflammatory diseases.

Especially in the context of inflammatory and vascular diseases, prostaglandins are known to exert wide ranging effects and are able to promote leukocyte migration and vascular leakage. A recent report by Schoenberger et al. showed increased prostaglandin levels in diabetic retinopathy and its correlation with inflammatory cytokine and vascular endothelial growth factor (VEGF) expression in the vitreous [18]. Furthermore, it has previously been shown that prostaglandins facilitate VEGF expression and subsequent neovascularization at the posterior pole of the eye [19].

Although prostaglandin analogs are widely used as eye drops for the treatment of high intraocular pressure, their potential side effects on the normally avascular cornea, the main administration route of eye drops, are so far only partially characterized. Therefore, the aim of this study was to analyze potential corneal vascular alterations which might occur during treatment with prostaglandin analogs. Among the clinically used prostaglandin analogs, tafluprost was the first topical PGF2α analog approved by the Food and Drug Administration for treatment of glaucoma that was available in a preservative-free formulation without benzalkonium chloride. Therefore, in order to exclude the effects of benzalkonium chloride, we chose this preservative-free prostaglandin analog to study its effect on vascular endothelial cells in vitro and on the corneal vasculature in vivo. For in vivo studies, we both analyzed possible vascular changes in healthy, long-term treated corneas and in inflamed, short-term treated corneas.

Materials and methods

Blood and lymphatic endothelial cell culture and proliferation ELISA

Human blood and lymphatic microvascular endothelial cells (BEC and LEC, respectively; purchased from Cambrex Bio Science, Walkersville, MD) were cultured in EGM2-MV medium. For the assessment of cell proliferation, a BrdU-based proliferation ELISA was used as previously described [20]. Briefly, cells were seeded in a 96-well plate at a density of 4×10^3 cells per well (n=6 per group). Six hours after seeding, medium was replaced with fresh medium, BrdU and tafluprost were added. A 1:100 dilution of the standard commercially available preservative-free Taflupost formulation without benzalkonium chloride (Taflotan Sine Single Doses, Santen) was used, according to 150 ng/ml (0.3315 nM) tafluprost. For the analysis of proliferation under high stimulatory conditions, the culture medium was additionally supplemented with 100 ng/ml VEGF-A (BEC) or VEGF-C (LEC). After 48 h, cells were fixed and stained according to manufacturer's instructions. Colorimetric analysis was performed with an ELISA reader (Epoch Microplate Spectrophotometer, BioTek, Bad Friedrichshall, Germany). The mean extinction of the non-VEGF stimulated control wells was defined as 100 %, the extinction of all wells was then related to this value (cell proliferation ratio).

Animals and long-term treatment with tafluprost eye drops

All animal protocols were approved by the local animal care committee and were in accordance to the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmology and Vision Research. All mice were 8-week old female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany).

To analyze the effects of long term treatment with tafluprost, the standard commercially available preservative-free tafluprost eye drop formulation without benzalkonium chloride (Taflotan Sine Single Doses, 15 μg/ml, Santen) was used. Five μl of tafluprost was applied once daily to the healthy right eye of previously untreated mice, control mice received equal amounts of saline solution (n=5 per group). Although tafluprost in its commercially available formulation is solubilized in a mixture of glycerol, sodium dihydrogen phosphate dihydrate, disodium edetate, polysorbate 80, hydrochloric acid and/or sodium hydroxide to adjust the pH and water for injections, we used saline as control because the

vehicle in its exact composition was temporarily not available. After 4 weeks of treatment, corneal wholemounts were prepared and analyzed.

Suture-induced corneal inflammation/inflammatory corneal neovascularization and short-term treatment of inflamed corneas

The mouse model of corneal suture placement is known to lead to a strong inflammatory response with high amounts of recruited inflammatory cells and inflammation associated corneal neovascularization [16, 21–24]. This model was performed as described previously [16, 22, 25]. Prior to suturing, each animal was anesthetized with an intraperitoneal injection of a combination of ketamine hydrochloride (8 mg/kg) and xylazine hydrochloride (0.1 ml/kg). Three 11–0 nylon sutures (Serag Wiessner, Naila, Germany) were then placed intrastromally into the right cornea, with two incursions each extending over 120° of the corneal circumference. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center. Mice received the standard commercially available preservative-free tafluprost eye drop formulation without benzalkonium chloride (Taflotan Sine Single Doses, 15 μg/ml, Santen, once daily, 5 μl per eye drop), control mice received equal amounts of saline solution (n=5 per group). After 1 week of treatment, corneal wholemounts were prepared and analyzed. We decided to determine corneal suture induced inflammatory cell counts and vascularized areas in the inflamed setting already after 1 week of treatment (in contrast to 4 weeks of treatment in the uninflamed setting), while a strong inflammatory and vascular response was still present [22–24].

Preparation and staining of corneal wholemounts

Excised corneas were rinsed in PBS and fixed in acetone for 20 min. After blocking with 2 % bovine serum albumin in PBS for 2 h, corneas were stained overnight at 4 °C with a rabbit anti-mouse LYVE-1 antibody (AngioBio, Del Mar, CA) and FITC-conjugated rat anti-mouse CD31 antibody (Acris Antibodies, Herford, Germany). On the next day, LYVE-1 was then detected with a Cy3-conjugated secondary goat anti-rabbit antibody (Dianova, Barcelona, Spain). All corneas were moved to slides (Superfrost; Thermo Scientific), covered with fluorescent mounting medium (Dako, Carpinteria, CA) and stored at 4 °C in the dark.

Analysis of lymphatic vascular extensions, analysis of macrophage numbers, analysis of hem- and lymphangiogenesis

Stained wholemounts were analyzed with a fluorescence microscope (Olympus BX53, Olympus, Germany) and digital pictures were taken with a digital monochrome CCD camera (Olympus XM10, Olympus, Germany). Each wholemount picture was assembled automatically from 9 to 12 pictures taken at 100× magnification.

In uninflamed, long-term treated corneas, centrally oriented vascular extensions ("sprouts") from the main limbal lymphatic vessel were counted like previously described [26, 27]. Furthermore, LYVE-1 positive cells were semiautomatically counted in 3 representative peripheral sections per cornea, cell counts were then related to the mean cell count of the untreated control corneas (LYVE-1 positive cell ratio).

For the analysis of macrophage recruitment under inflammatory conditions after suture placement, LYVE-1 positive cells were semiautomatically counted in 3 representative peripheral sections per cornea, cell counts were then related to the mean cell count of the untreated control corneas (LYVE-1 positive cell ratio).

For analysis of corneal hem- and lymphangiogenesis under inflammatory conditions after suture placement, the area covered with blood and lymphatic vessels was detected with an algorithm established in the image analyzing program CellF (Soft Imaging System) as previously described [28]. Briefly, before analysis, gray value images of the wholemount pictures were modified by several filters, vessels were then detected by threshold setting including the bright vessels and excluding the dark background. The mean vascularized area of the untreated control wholemounts was defined as 100 %, vascularized areas were then related to this value (vessel ratio).

Statistical analyses

Statistical analyses were performed with Microsoft Excel (Microsoft Corp, Redmond, Washington) and InStat Version 3.06 (GraphPad Software Inc., San Diego, California). Statistical significance was determined using the Student's t-test. P values<0.05 were considered statistically significant. Graphs were drawn using Prism4 version 4.03 (GraphPad Software Inc., San Diego, California).

Results

Vascular endothelial cells show no significant change in proliferation after tafluprost treatment in vitro

Blood and lymphatic endothelial cells were treated with tafluprost, proliferation was then assessed using ELISA. The addition of tafluprost to unstimulated, low proliferating blood endothelial cells showed no significant change in cell proliferation (mean m=+8.6 % in comparison to controls, p=0.53). VEGF-A stimulated blood endothelial cells showed significantly higher proliferation rates than unstimulated cells (m=+82.4 %, p<0.001). There was no difference in proliferation

detectable when tafluprost was additionally supplied to VEGF-A stimulated cells (VEGF-A treated: m=+82.4 % vs. VEGF-A and tafluprost treated: m=+80.5 %, p=0.86; Fig. 1a).

Regarding the proliferation of lymphatic endothelial cells, there was no significant change between unstimulated controls and tafluprost treated cells detectable (m=+7.9 % in comparison to controls, p=0.46). Under VEGF-C stimulated, high proliferatory conditions, the addition of tafluprost led to no significant change of lymphatic endothelial cell proliferation (VEGF-C treated: m=+41.6 % vs. VEGF-C and tafluprost treated: m=+30.1 %, p=0.40; Fig. 1b).

Limbal blood and lymphatic vessel anatomy shows no alteration after long-term treatment with tafluprost

Naïve murine corneas were treated with tafluprost for 4 weeks, the limbal blood and lymphatic vessel anatomy was then analyzed immunohistochemically in corneal wholemounts. For both blood as well as lymphatic vessels, no significant change in the resting limbal vessel vasculature was observed after tafluprost treatment: tafluprost led to no change in the anatomy of limbal blood vessel networks (Fig. 2, upper panels). Furthermore, there was no significant alteration in limbal lymphatic vessel configuration (Fig. 2, middle panels): the limbal area covered by a main circumferential lymphatic vessel did not vary significantly between tafluprost treated corneas and untreated controls. Additionally, no significant change in the number of centrally orientated lymphatic vascular extensions ("sprouts") could be observed between tafluprost treated corneas and untreated control corneas (controls: m=8.2 "sprouts" per cornea; tafluprost: m=8.6 "sprouts" per cornea, p=0.84; Fig. 2, middle panels). Furthermore, no significant change in the number of resident LYVE-1 positive macrophages could be observed in uninflamed, long-term treated corneas (tafluprost treated: m=−21.0 % in comparison to controls, p=0.31; Fig. 2f).

Fig. 2 Effect of long-term tafluprost treatment on resting limbal blood and lymphatic vessel anatomy. Naïve corneas of BALB/c mice were treated with tafluprost eye drops (15 μg/ml, 5 μl, once daily for 4 weeks). Afterwards, corneal wholemounts were prepared and analyzed immunohistochemically using CD31 as marker for blood vessels (**a** and **b**) and LYVE-1 as marker for lymphatic vessels (**c** and **d**). **a** blood vessel anatomy of untreated corneas; **b** blood vessel anatomy of tafluprost treated corneas; **c** lymphatic vessel anatomy of untreated corneas; **d** lymphatic vessel anatomy of tafluprost treated corneas; **c** and **d**) arrowheads indicate centrally oriented vascular extensions from the main limbal lymphatic vessel ("sprouts"); **e** quantification of centrally oriented vascular extensions ("sprouts") from the main limbal lymphatic vessel, data are expressed as mean + SD; **f** quantification of resident LYVE-1 positive macrophage counts; data are expressed as mean + SD

Short-term tafluprost treatment does not alter the recruitment of macrophages into the cornea under inflammatory conditions

Macrophages are essential mediators of inflammatory corneal hem- and lymphangiogenesis [21, 29]. After recruitment into the inflamed tissue, macrophages secrete a multitude of proangiogenic growth factors and lead to a strong amplification of both hem- and lymphangiogenesis [21]. Additionally, macrophages are able to integrate into preexisting lymphatic vessels and are also capable of generating vessel-like structures de novo [29]. Therefore, we further extended the vascular analysis of this study and also investigated whether treatment of inflamed corneas with tafluprost leads to a modification of LYVE-1 positive macrophage counts in the inflamed cornea. As shown in Fig. 3, treatment with tafluprost eye drops did not significantly alter

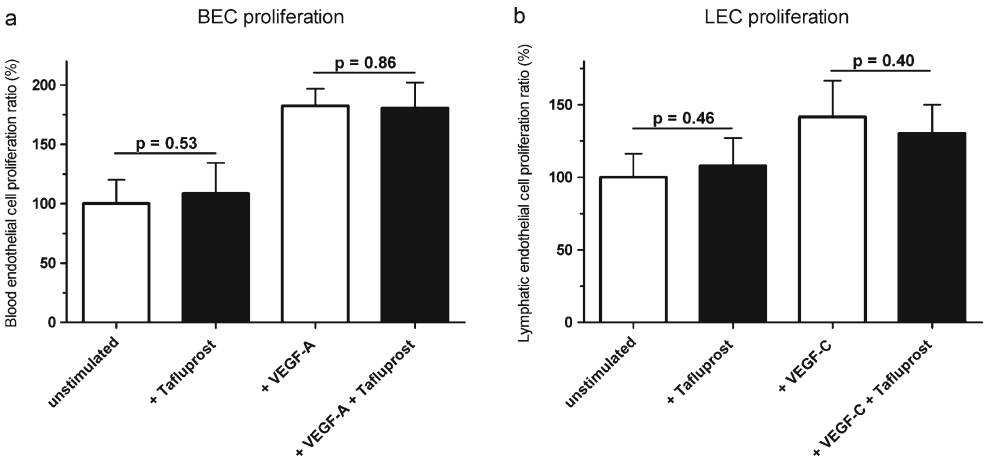
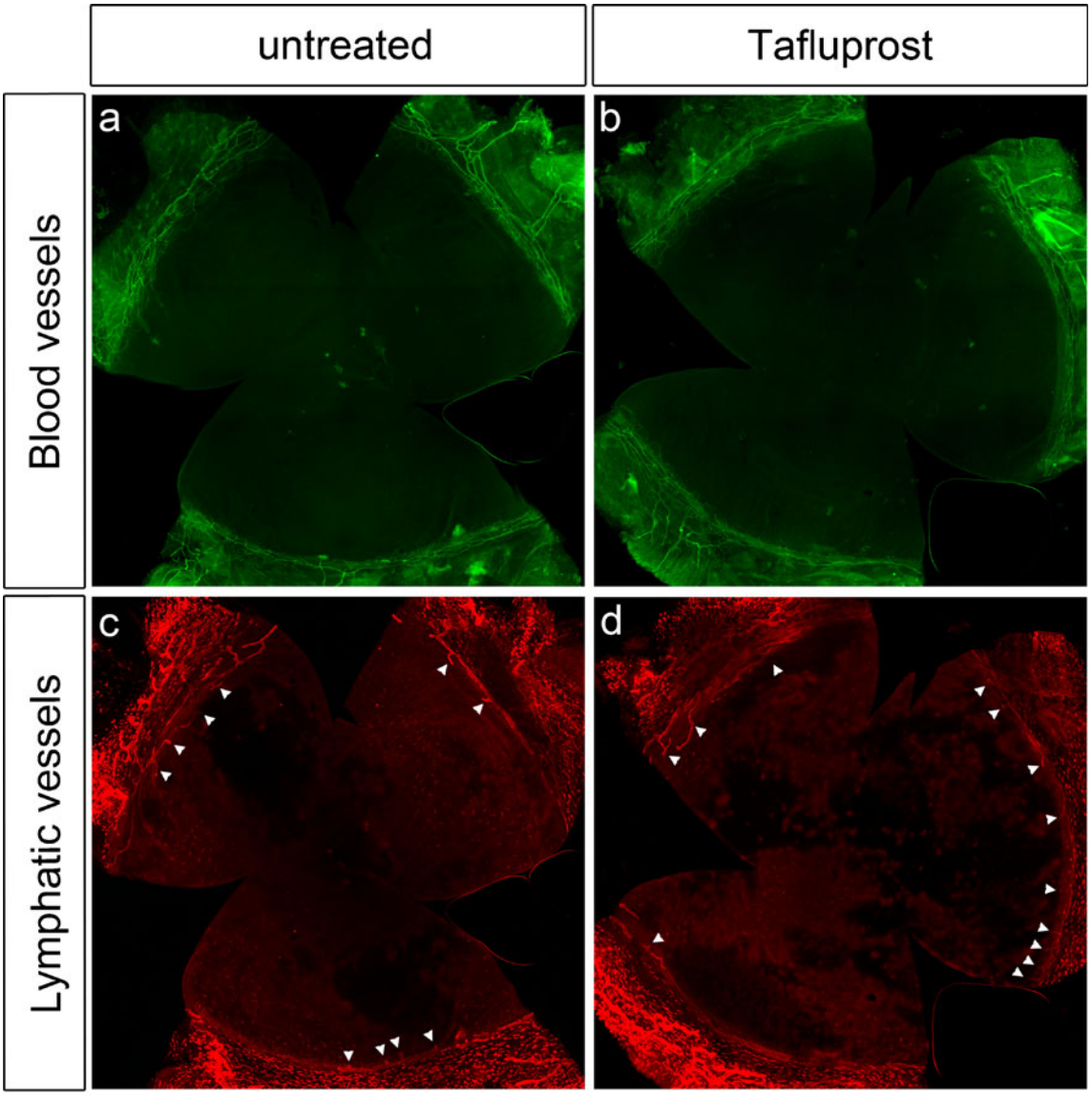
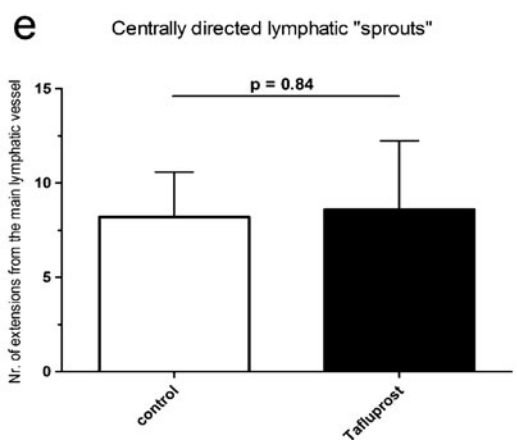
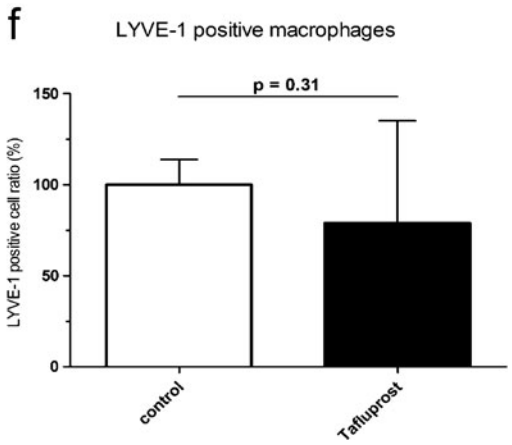


Fig. 1 Effect of tafluprost on blood and lymphatic endothelial cell proliferation in vitro. Blood and lymphatic endothelial cells were treated with 150 ng/ml tafluprost, 100 ng/ml VEGF-A (blood endothelial cells), 100 ng/ml VEGF-C (lymphatic endothelial cells), or a combination of VEGF (−A and −C, respectively) and tafluprost for 48 h; cell proliferation was then assessed via BrdU-based ELISA. **a** proliferation of blood endothelial cells; **b** proliferation of lymphatic endothelial cells; data are expressed as mean + SD


untreated
Tafluprost
Blood vessels
Lymphatic vessels
a
b
c
d


e
Centrally directed lymphatic "sprouts"
p = 0.84
Nr. of extensions from the main lymphatic vessel
15
10
5
0
control
Tafluprost


f
LYVE-1 positive macrophages
p = 0.31
LYVE-1 positive cell ratio (%)
150
100
50
0
control
Tafluprost

the amount of LYVE-1 positive macrophages in the cornea. Although tafluprost treated corneas contained 17.6 % more LYVE-1 positive macrophages in comparison to untreated control corneas, this effect was not statistically significant (p=0.15; Fig. 3).

Tafluprost treatment does not influence inflammatory corneal hem- and lymphangiogenesis

Inflammatory corneal hem- and lymphangiogenesis was induced by suture placement; corneas were then treated with tafluprost eye drops for 1 week, followed by immunohistochemical analysis of hem- and lymphangiogenesis.

In control eyes, 23.17 % of the cornea was covered with newly developed blood vessels whereas 6.65 % of the cornea was covered with newly developed lymphatic vessels. Tafluprost treated corneas showed no significant alteration of blood vessel growth (m=23.61 %, p=0.83; Fig. 4c). Furthermore, the amount of corneal lymphatic vessels also showed no significant change (m=6.94 %, p=0.83; Fig. 4f).

Discussion

Prostaglandin analogs are widely used to reduce intraocular pressure in the treatment of glaucoma. The main mechanism of pressure reduction seems to be through the facilitation of (mainly uveoscleral) aqueous humor outflow [30]. Being generally well tolerated, however, there are differing opinions regarding the use of prostaglandin analogs under various, mostly inflammatory conditions like anterior uveitis or keratitis [6]. One of the main arguments against the use of prostaglandin analogs is the possible aggravation of these inflammatory responses [10, 31]. At the posterior pole of the eye, for example, prostaglandin analogs are discussed to disrupt the blood–retinal barrier along with development of cystoid macular edema [5, 7]. However, most of the literature regarding the topical use of prostaglandin analogs and the development of cystoid macular edema consists of clinical reports. There is so far no definitive mechanistic evidence for proinflammatory effects of prostaglandin analogs in humans [7, 11].

In contrast to the retina, the cornea is usually avascular but can secondarily be invaded by both blood and lymphatic vessels as response to severe inflammation [12]. Whereas it has recently been shown that endogenous prostaglandins may play a role in facilitating ocular surface pathologies like dry eye disease [32], the potential proinflammatory and pro(lymph)angiogenic effects of prostaglandin analogs at the cornea are so far only poorly characterized. To our knowledge, only one study focused on the hemangiogenic impact of several topical antiglaucomatous drugs, including the prostaglandin analog latanoprost. In a rat corneal pocket assay, the commercially available eye drop formulation of latanoprost seemed to further stimulate the growth of preexisting corneal blood vessels [33]. However, this observation still needs further detailed analysis, as this prostaglandin analog formulation also contained the preservative benzalkonium chloride, proven to be proinflammatory and proangiogenic itself [34]. Furthermore, there was so far no single report on the impact of prostaglandin analogs on corneal lymphangiogenesis. Therefore, the aim of this study was to provide a comprehensive characterization of the corneal vascular effects of prostaglandin analogs. We analyzed the direct impact of the preservative-free prostaglandin analog tafluprost on blood and lymphatic endothelial cell proliferation in vitro. Additionally, we investigated whether tafluprost eye drops affect the corneal blood and lymphatic vasculature under resting and inflammatory conditions in vivo.

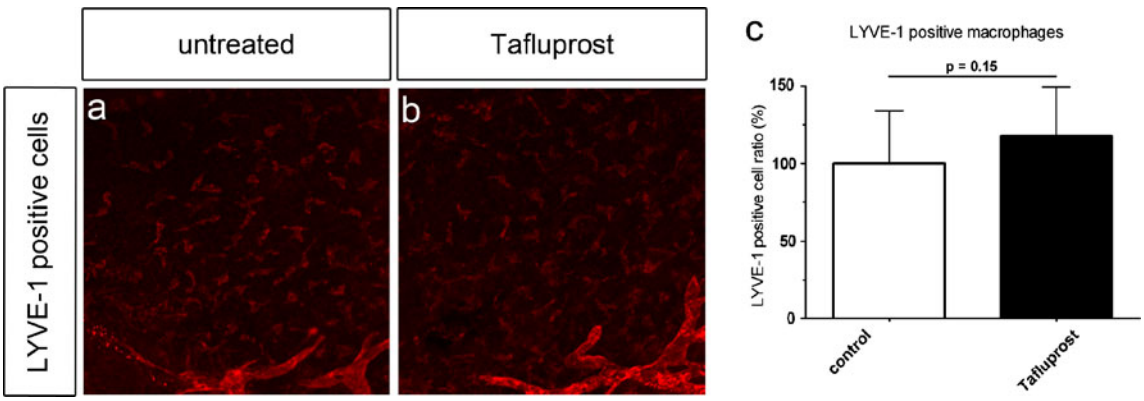


Fig. 3 Effect of short-term tafluprost treatment on the recruitment of macrophages into the inflamed cornea. Corneal inflammation was induced by suture placement in BALB/c mice, corneas were then treated with tafluprost eye drops (15 μg/ml, 5 μl, once daily for 1 week). Afterwards, corneal wholemounts were prepared and analyzed immunohistochemically using LYVE-1 as marker for (lymph)angiogenic macrophages. Representative corneal sections of **a**) untreated and **b**) tafluprost treated corneas; **c** quantification of LYVE-1 positive cell counts; data are expressed as mean + SD

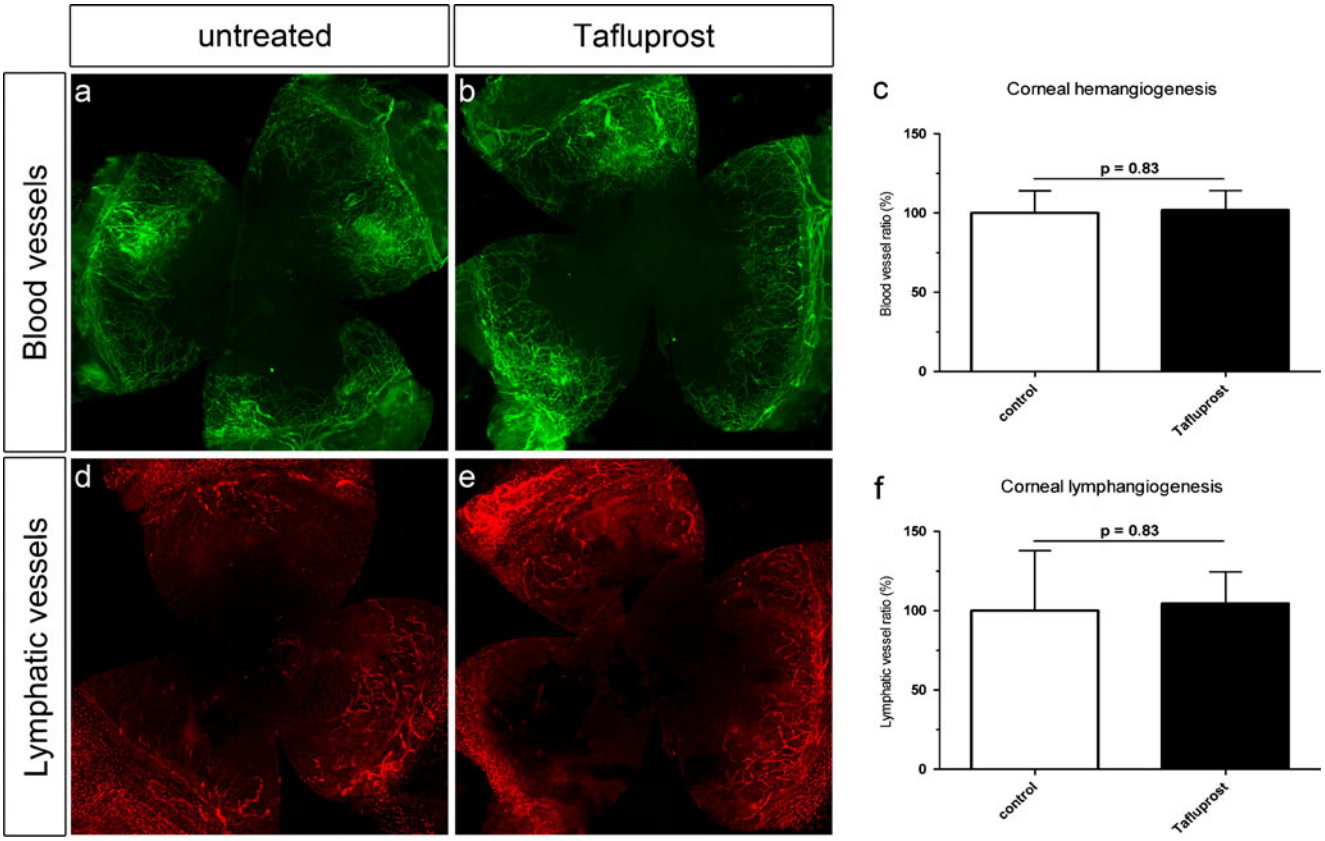


Fig. 4 Effect of short-term tafluprost treatment on inflammatory corneal hem- and lymphangiogenesis. Inflammatory corneal hem- and lymphangiogenesis was induced by suture placement in BALB/c mice, corneas were then treated with tafluprost eye drops (15 μg/ml, 5 μl, once daily for 1 week). Afterwards, corneal wholemounts were prepared and analyzed immunohistochemically using CD31 as marker for blood vessels (**a** and **b**) and LYVE-1 as marker for lymphatic vessels (**d** and **e**). **a** hemangiogenesis in untreated corneas; **b** hemangiogenesis in tafluprost treated corneas; **c** quantification of hemangiogenesis; data are expressed as mean + SD; **d** lymphangiogenesis in untreated corneas; **e** lymphangiogenesis in tafluprost treated corneas; **f** quantification of lymphangiogenesis; data are expressed as mean + SD

The key findings of this study are: 1) tafluprost does not directly affect blood and lymphatic endothelial cell proliferation, neither under low nor under high stimulatory conditions; 2) long-term treatment of healthy, not inflamed corneas with tafluprost eye drops does not lead to changes in the resting limbal blood and lymphatic vasculature or resident macrophage counts; 3) tafluprost eye drops do not significantly modify the number of macrophages recruited into the cornea under inflammatory conditions; and 4) treatment of inflamed corneas with tafluprost eye drops does not alter the ingrowths of pathological corneal blood or lymphatic vessels.

Our in vitro results do not show a direct proliferatory effect of tafluprost on vascular endothelial cells under the analyzed conditions. In line with these findings, we could not observe changes in the resting corneal vasculature after tafluprost treatment in vivo. The results of these experiments imply that vascular alterations which might occur during corneal inflammation could only be indirect, e.g., due to changes in the amount of recruited macrophages. Macrophages are well known mediators of inflammatory hem- and lymphangiogenesis, mainly by the secretion of various pro(lymph)angiogenic growth factors or by the integration into preexisting (lymphatic) vessels [21, 29]. Especially LYVE-1 positive macrophages seem to be a (lymph)angiogenic macrophage subtype. The analysis of LYVE-1 positive macrophage counts showed no significant difference between untreated and tafluprost treated corneas, indicating also no alteration of the indirect inflammatory angiogenesis pathway. Likewise, our experiments showed no significant effect of tafluprost on the amount of corneal blood and lymphatic vessels developing after an inflammatory stimulus.

Taken together, our results indicate that tafluprost treatment does not affect (murine) corneal hem- and lymphangiogenesis. This finding suggests a safe vascular profile of tafluprost eye drops at the inflammatory neovascularized cornea. Our results warrant further clinical studies on the use of prostaglandin analogs in glaucoma patients with inflammatory corneal (vascular) diseases.

Acknowledgments The authors thank Frank Lacina for expert help at the animal facility.

Funding/Support This work was supported by the German Research Foundation, Sonderforschungsbereich SFB 643 (B10), DFG Cu 47/4-1, DFG HE 6743/2-1 and by the GEROK-Programme, University Hospital of Cologne (to DH and LMH).

Financial disclosures All authors declare no financial disclosures.

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