Suppression of Inflammatory Corneal Lymphangiogenesis by Application of Topical Corticosteroids

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Objectives: To analyze whether topical application of corticosteroids inhibits inflammatory corneal lymphangiogenesis and to study the potential underlying antilymphangiogenic mechanisms.

Methods: Inflammatory corneal neovascularization was induced by suture placement, and the corneas were then treated with topical fluorometholone, prednisolone acetate, or dexamethasone sodium phosphate. After 1 week, the corneas were stained with lymphatic vessel endothelial hyaluronan receptor 1 for detection of pathological corneal lymphangiogenesis. The effect of these corticosteroids on macrophage recruitment was assessed via fluorescence-activated cell sorting analysis. The effect of these corticosteroids on proinflammatory cytokine expression by peritoneal exudate cells was tested via real-time polymerase chain reaction. Furthermore, the effect of steroid treatment on the proliferation of lymphatic endothelial cells was assessed via enzyme-linked immunosorbent assay.

Results: Treatment with corticosteroids resulted in a significant reduction of inflammatory corneal lymphangiogenesis. The antilymphangiogenic effect of fluorometholone was significantly weaker than that of prednisolone and dexamethasone. Corneal macrophage recruitment was also significantly inhibited by the application of topical steroids. Treatment of peritoneal exudate cells with corticosteroids led to a significant downregulation of the RNA expression levels of tumor necrosis factor and interleukin 1β. Additionally, proliferation of lymphatic endothelial cells was also inhibited.

Conclusions: Corticosteroids are strong inhibitors of inflammatory corneal lymphangiogenesis, with significant differences between various corticosteroids in terms of their antilymphangiogenic potency. The main mechanism of the antilymphangiogenic effect seems to be through the suppression of macrophage infiltration, proinflammatory cytokine expression, and direct inhibition of proliferation of lymphatic endothelial cells.

Clinical Relevance: Steroids block corneal lymphangiogenesis, the main risk factor for immune rejections after corneal transplantation. The different antilymphangiogenic potency of these drugs should be taken into account when using steroids in clinical practice (eg, after keratoplasty).


THE HEALTHY CORNEA IS DEVOID OF BOTH BLOOD AND LYMPHATIC VESSELS AND ACTIVELY MAINTAINS ITS AVASCULARITY.1-3 however, a variety of inflammatory conditions can lead to a breakdown of this “angiogenic privilege.” This leads to the outgrowth of blood as well as lymphatic vessels from the limbus into the avascular cornea, reducing transparency and visual acuity.4,6 Furthermore, corneal neovascularization is also the most important risk factor for graft rejection after corneal transplantation; in particular, lymphangiogenesis has been shown to be essential in mediating immune reactions after corneal grafting.4,5,9 By use of the murine model of corneal transplantation, it has recently been shown that inhibition of corneal neovascularization after low- and high-risk corneal transplantation promotes graft survival.10-13 Thus, antiangiogenic therapy is a very reasonable approach for reducing corneal complications secondary to inflammation and also for preventing immune rejections after corneal transplantation.

Recently, several specific angiogenesis inhibitors have been approved by the US Food and Drug Administration for the treatment of pathologic neovascularization in the eye. Ranibizumab (Lucentis; Genentech, South San Francisco, California) and pegaptanib (Macugen; OSI Pharmaceuticals, Farmingdale, New York) were both approved for the treatment of age-related macular degeneration. Bevacizumab (Avastin; Genentech, South San Francisco, California), approved for co-
lorectal cancer and nonsmall cell lung cancer treatment, is also widely used off-label to treat age-related maculopathy, proliferative retinopathies, and neovascular glaucoma. In contrast, no specific angiogenesis inhibitor against neovascularization at the anterior segment of the eye has been approved by the US Food and Drug Administration so far.

However, many older, established drugs also have antiangiogenic properties in addition to their known activities. For instance thalidomide, spironolactone, non-steroidal anti-inflammatory drugs, and corticosteroids are well-known drugs with known toxicity profiles, and it has been shown that these substances can inhibit angiogenesis in various immune and inflammatory diseases, including those of the eye. Corticosteroids are potent anti-inflammatory drugs widely used for the treatment of macular edema and choroidal and retinal neovascularization. To date, corticosteroid therapy is also the standard anti-inflammatory and antiangiogenic treatment for patients with corneal neovascularization, especially after corneal transplantation.

Although it has been established that corticosteroids are able to inhibit corneal hemangiogenesis, little is known about their ability to suppress corneal lymphangiogenesis. Nakao and colleagues showed that systemic application of dexamethasone sodium phosphate was able to inhibit interleukin 1β (IL-1β)–induced corneal hemangiogenesis. However, to our knowledge, it has not been shown whether corticosteroids are also able to inhibit corneal lymphangiogenesis. Therefore, the aims of our study were to assess whether topical application of corticosteroids is able to inhibit inflammatory corneal lymphangiogenesis and to compare the antilymphangiogenic potential of various clinically used corticosteroids (fluorometholone, prednisolone and dexamethasone). Furthermore, we analyzed the effect of steroids on inflammatory cell recruitment and proinflammatory cytokine expression by macrophages, which are known to be crucial mediators of inflammatory corneal lymphangiogenesis. In addition, we investigated whether steroids are also able to directly suppress the proliferation of lymphatic endothelial cells (LEC).

**METHODS**

**ANIMALS AND ANESTHESIA**

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. Mice were anesthetized with an intraperitoneal injection of a combination of 8 mg/kg of ketamine hydrochloride and 0.1 mg/kg of xylazine hydrochloride. All mice were female BALB/c mice, aged 6 to 8 weeks (purchased from Charles River Laboratories, Sulzfeld, Germany).

**SUTURE-INDUCED, INFLAMMATORY CORNEAL NEOVASCULARIZATION ASSAY**

The mouse model of suture-induced inflammatory corneal neovascularization was used as previously described. Prior to corneal neovascularization, each animal was deeply anesthe-

**FLOW CYTOMETRIC ANALYSIS OF INFLAMMATORY CORNEAL CELL RECRUITMENT**

Corneal inflammation was induced by suture placement, and corneas were then treated with topical fluorometholone, pred-

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nisolone, dexamethasone (each at a dosage of 1 mg/mL, 3 eye drops daily, 5 µL per drop), or saline solution (control). On days 2 and 5 after suture placement, corneas (3 per group) were harvested and pooled. Single-cell suspensions were prepared from corneal samples using collagenase digestion, as previously described.33 Briefly, corneal buttons were removed and minced into small fragments, followed by digestion with 2 mg/mL of type IV collagenase (Sigma-Aldrich, St Louis, Missouri) and 0.05% mg/mL of deoxyribonuclease I (Roche, Basel, Switzerland) for 1 hour at 37°C with agitation. The suspension was then triturated through a 10-ml syringe to homogenize the remaining tissue and filtered through a 70-µm cell strainer. Once in single-cell suspension, all samples underwent Fc receptor blockade via incubation with α-CD16/CD32 (BD Pharmingen, San Diego, California) at 4°C in 0.5% bovine serum albumin (Sigma-Aldrich). Subsequent antibody labeling included α-CD11b (BD Pharmingen) and α-F4/80 (BD Pharmingen). All antibodies were analyzed with the appropriate isotype controls.

**COLLECTION, CULTURE, AND TREATMENT OF PERITONEAL MACROPHAGES**

Thioglycollate-induced macrophages were collected from the peritoneal cavity (peritoneal exudate cells) of 8-week-old female mice as described previously.33 Peritoneal exudate cells were washed, resuspended, and cultured at 37°C in RPMI-1640 medium containing 10% fetal calf serum, 10 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 100 U/mL penicillin, and 100-µg/mL streptomycin. After adhesion, nonadherent cells were removed by washing with culture medium, and adherent cells were then used as macrophages. Cells collected by this method are F4/80+ (>90%) and CD11b+ (>99%).33,34 Cells were incubated in RPMI-1640 medium containing 25 nmol/L of fluorometholone, prednisolone, or dexamethasone for 24 hours, followed by RNA isolation. To analyze RNA expression under higher inflammatory conditions, 50 ng/mL of tumor necrosis factor (TNF; Biomol GmbH, Hamburg, Germany) was added in some experiments, and cells were then incubated for 24 hours with or without 25 nmol/L of dexamethasone.

**RNA ISOLATION AND REAL-TIME POLYMERASE CHAIN REACTION**

RNA from cultured peritoneal macrophages was isolated with the RNeasy Micro kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen, Darmstadt, Germany) according to the manufacturer’s recommendations. Real-time polymerase chain reaction (PCR) was performed using TaqMan Universal PCR Mastermix and preformulated primers for TNF (assay Mm00434228_m1), IL-1β (assay Mm00465512_m1), and GAPDH (assay Mm99999915_g1); FAM-MGB dye-labeled predesigned primers were used for TNF, IL-1β, and GAPDH (Applied Biosystem, Foster City, California). The results were analyzed by the comparative threshold cycle method and normalized by GAPDH as an internal control. The relative messenger RNA (mRNA) level in the untreated group was used as the normalized control for the treatment groups. All assays were performed in duplicate; a non-template control was included in all of the experiments to evaluate DNA contamination of the reagents used. Experiments were conducted twice.

**LEC PROLIFERATION ENZYME-LINKED IMMUNOSORBENT ASSAY**

The LEC proliferation enzyme-linked immunosorbent assay (ELISA) was used, with slight modifications, as previously described.13 Human lymphatic microvascular endothelial cells (Cambrex Bio Science, Walkersville, Maryland) were cultured in EGM2-MV full medium (Cambrex Bio Science) according to the manufacturer's instructions; EGM2-MV full medium contains endothelial cell growth factors such as vascular endothelial growth factor and basic fibroblast growth factor. For this ELISA, cells were seeded in a 96-well plate in EGM2-MV medium at a density of 4 × 10^4 cells per well. After 6 hours, the medium was replaced with a serum-free medium. 10 µL/mL of 5-bromodeoxyuridine (BrdU; Cell Proliferation ELISA, BrdU, Roche, Penzberg, Germany), and the corticosteroids (25 mmol/L of fluorometholone, prednisolone, or dexamethasone) were added. Cells were fixed and stained after 48 hours according to the manufacturer's instructions. Colorimetric analysis was performed with the ELISA reader SLT Spectra (SLT Labinstrumenten Deutschland GmbH, Crailsheim, Germany). The mean extinction of the control wells was defined as being 100%, and extinction of all wells were then related to this value (LEC proliferation ratio).

**STATISTICAL ANALYSIS**

Statistical analyses were performed using by Microsoft Excel 2000 (Microsoft Corp, Redmond, Washington) and InStat version 3.06 (GraphPad Software Inc, San Diego, California). Statistical significance was determined using the 1-way analysis of variance test. P < .05 was considered statistically significant. Graphs were drawn using Prism4 version 4.03 (GraphPad Software Inc).

### RESULTS

**INHIBITORY EFFECT OF TOPICAL TREATMENT WITH CORTICOSTEROIDS ON CORNEAL LYMPHANGIOGENESIS AND HEMANGIOGENESIS IN VIVO**

Treatment with corticosteroids resulted in a significant reduction of inflammatory corneal lymphangiogenesis in vivo. In comparison with controls, lymphangiogenesis was inhibited by 33% via topical fluorometholone treatment (mean [SD], 67% [19%]; P < .001), by 53% via topical prednisolone treatment (mean [SD], 47% [17%]; P < .001), and by 55% via topical dexamethasone treatment (mean [SD], 45% [13%]; P < .001). The inhibitory effect of fluorometholone was significantly weaker than the inhibitory effect of prednisolone or dexamethasone (P < .01). Every tested steroid showed very similar effects on corneal hemangiogenesis: In comparison with controls, blood vessel growth was inhibited by 30% via fluorometholone treatment (mean [SD], 70% [9%]; P < .001), by 50% via topical prednisolone treatment (mean [SD], 50% [12%]; P < .001), and by 57% via topical dexamethasone treatment (mean [SD], 43% [7%]; P < .001). The inhibitory effect of fluorometholone was again significantly weaker than the inhibitory effect of prednisolone or dexamethasone (P < .01) (Figure 1).
INHIBITORY EFFECT OF TOPICAL TREATMENT WITH CORTICOSTEROIDS ON INFLAMMATORY CELL RECRUITMENT IN VIVO

To examine the effect of topical corticosteroid treatment on suture-induced macrophage infiltration, we quantified the number of F4/80$^+$CD11b$^+$ cells using flow cytometry. Fluorescence-activated cell sorting analysis showed that topical treatment with corticosteroids resulted in a significant reduction of inflammatory cell recruitment in vivo. Although, in control corneas, the percentage of F4/80$^+$CD11b$^+$ macrophages per cornea was 23.58% at 2 days after corneal suture placement and 27.14% at 5 days after corneal suture placement, treatment with fluorometholone reduced the amount of F4/80$^+$CD11b$^+$ cells to 2.07% at day 2 and 13.57% at day 5. Treatment with prednisolone reduced the amount of F4/80$^+$CD11b$^+$ cells to 4.51% at day 2 and 10.44% at day 5, and treatment with dexamethasone reduced the amount of F4/80$^+$CD11b$^+$ cells to 1.88% at day 2 and 11.86% at day 5. All of the steroids used led to a stronger inhibitory effect on macrophage recruitment on day 2 than on day 5 (Figure 2).

SUPPRESSIVE EFFECT OF CORTICOSTEROIDS ON PROINFLAMMATORY CYTOKINE EXPRESSION BY MACROPHAGES IN VITRO

Peritoneal exudate cells were incubated with 25-nmol/L fluorometholone, prednisolone, or dexamethasone for 24 hours, and mRNA expression levels of the proinflammatory cytokines TNF and IL-1$^\beta$ were then measured via real-time PCR. In vitro treatment of resting peritoneal exudate cells with corticosteroids led to a significant inhibition of mRNA expression levels of TNF and IL-1$^\beta$. Fluorometholone treatment inhibited mRNA expression levels by 83% (TNF) and 68% (IL-1$^\beta$), whereas prednisolone treatment suppressed mRNA expression levels by 79% (TNF) and 50% (IL-1$^\beta$). Dexamethasone was the strongest inhibitor of proinflammatory cytokine expression: TNF expression was reduced by 88%, and IL-1$^\beta$ expression by 73%. With respect to TNF expression levels, there were no significant differences detectable between fluorometholone treatment and prednisolone treatment ($P > .05$) or between fluorometholone treatment and dexamethasone treatment ($P > .05$). However, treatment with
dexamethasone showed a statistically stronger inhibition of TNF mRNA expression than did treatment with prednisolone (P < .05). The assessment of IL-1β mRNA levels revealed that both fluorometholone and dexamethasone had a stronger inhibitory effect on IL-1β expression than did prednisolone (P < .01), with the effect of fluorometholone and dexamethasone being comparable (P > .05).

To analyze whether steroids can also inhibit TNF and IL-1β expression under higher inflammatory conditions, peritoneal exudate cells were incubated with 50 ng/mL of TNF. The addition of this inflammatory stimulus led to a strong upregulation of proinflammatory cytokine expression (a 4.6-fold increase in TNF expression and a 4.4-fold increase in IL-1β expression). However, further treatment with dexamethasone could still suppress proinflammatory cytokine expression, even under this inflammatory stimulation (a 0.3-fold decrease TNF expression and a 0.4-fold decrease IL-1β expression) (Figure 3).

**SUPPRESSIVE EFFECT OF CORTICOSTEROIDS ON LEC PROLIFERATION IN VITRO**

To assess whether corticosteroids have a direct effect on LECs as well, we studied their effect on LEC proliferation in vitro. Treatment with corticosteroids (dose, 25 mmol/L) significantly suppressed the proliferation of LECs. Compared with controls, application of fluorometholone resulted in an inhibition of LEC proliferation by 42% (P < .001), application of prednisolone resulted in an inhibition of LEC proliferation by 30% (P < .001), and application of dexamethasone resulted in an inhibition of LEC proliferation by 51% (P < .001). Between the analyzed steroids, prednisolone had the weakest effect on LEC proliferation (prednisolone vs fluorometholone [P < .01]; prednisolone vs dexamethasone [P < .001]). Dexamethasone was the strongest inhibitor of LEC proliferation (dexamethasone vs fluorometholone; P < .05) (Figure 4).

The experiments performed in our study demonstrate the following: (1) Corticosteroids are potent inhibitors of inflammation-induced lymphangiogenesis and hemangiogenesis in vivo. (2) Significant differences exist between different corticosteroids with respect to their antilymphangiogenic and antihemangiogenic properties. In general, the stronger the anti-inflammatory effect, the stronger the in vivo antilymphangiogenic and antihemangiogenic effect. This fits well with the known close interrelation between inflammation and angiogenesis. (3) Corticosteroids strongly block inflammatory cell recruitment into the inflamed cornea. It has already been shown that systemic application of dexamethasone was able to inhibit the recruitment of CD11b single positive cells after IL-1β pellet implantation. We analyzed the effect of topical steroid treatment on F4/80+CD11b+ double-positive cells after corneal suture placement in order to primarily focus on the role of macrophages, because various leukocyte populations (e.g., granulocytes and natural killer cells) also express CD11b, whereas F4/80 seems to be mainly expressed by macrophages. Furthermore, compared with pellet implantation, the suture-induced neovascularization model used in our study is known to induce a strong inflammatory response. The inhibition of F4/80+CD11b+ macrophage infiltration by steroids af-

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**Figure 2.** Inhibitory effect of topical treatment with corticosteroids on inflammatory cell recruitment in vivo. Fluorescence-activated cell sorting analysis of F4/80+CD11b+ cells obtained from corneas after suture placement. Cell recruitment was strongly inhibited by topical fluorometholone, prednisolone acetate, or dexamethasone sodium phosphate. All of the steroids used led to a stronger inhibition of cell recruitment on day 2 than on day 5. FITC indicates fluorescein isothiocyanate; and PE, phycoerythrin.

**Figure 3.** Inhibitory effect of topical corticosteroids on inflammatory cell recruitment in vivo. Fluorescence-activated cell sorting analysis of F4/80+CD11b+ cells obtained from corneas after suture placement. Cell recruitment was strongly inhibited by topical fluorometholone, prednisolone acetate, or dexamethasone sodium phosphate. All of the steroids used led to a stronger inhibition of cell recruitment on day 2 than on day 5. FITC indicates fluorescein isothiocyanate; and PE, phycoerythrin.
ter suture placement allowed us to conclude that, even under these very high inflammatory conditions, corticosteroids strongly block macrophage recruitment after topical application. (4) In vitro, corticosteroids significantly inhibit proinflammatory cytokine expression by macrophages. It is well established that both TNF and IL-1β (IL-1β), both under resting (A) and inflammatory conditions (B).

Figure 3. Suppressive effect of corticosteroids on proinflammatory cytokine expression by macrophages in vitro. Treatment of peritoneal exudate cells with corticosteroids led to a significant inhibition of mRNA expression levels of tumor necrosis factor (TNF) and interleukin 1β (IL-1β), both under resting (A) and inflammatory conditions (B).

Figure 4. Inhibitory effect of corticosteroids on proliferation of lymphatic endothelial cells (LECs) in vitro. Proliferation was measured by a cell proliferation enzyme-linked immunosorbent assay with 5-bromodeoxyuridine.
antiangiogenic properties surely has to be weighed against its increasing risk of adverse effects in patients. A plethora of clinical indications exist for antiangiogenic treatment of the cornea (eg, to stop sight-threatening neovascularization after inflammation or to improve graft survival after corneal transplantation).\textsuperscript{4,41-45} To date, steroid therapy is the standard anti-inflammatory and antiangiogenic treatment for patients with corneal neovascularization, especially after corneal transplantation.\textsuperscript{15} Furthermore, it is widely accepted that topical steroid treatment protects against immunologic graft rejections after corneal transplantation.\textsuperscript{2,21,22} This protection may largely be attributable to the fact that corticosteroids are very potent anti-inflammatory substances. The fact that these substances are also able to inhibit lymphangiogenesis, as shown in our study, and that lymphangiogenesis has been shown to be an important risk factor regarding graft rejection after corneal transplantation\textsuperscript{17} may lead one to conclude that both the anti-inflammatory effects and the antilymphangiogenic properties of these substances can result in a better outcome for patients after corneal transplantation.

However, the use of steroid therapy in the management of eye diseases remains controversial because of the adverse effects associated with this type of therapy.\textsuperscript{23,46} Alternative therapeutic approaches are necessary; for example, specifically blocking angiogenesis by targeting vascular endothelial growth factor appears to be a very reasonable and promising approach with less adverse effects.\textsuperscript{2,47-49} In fact, initial successful results were obtained using specific antiangiogenic drugs at the cornea (eg, bevacizumab eye drops and GS-101 antisense oligonucleotide eye drops).\textsuperscript{45,49}

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