CURRENT EYE RESEARCH
https://doi.org/10.1080/02713683.2017.1416146

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Corneal Neovascularization: A Combined Approach of Bevacizumab and Suramin Showed Increased Antiangiogenic Effect Through Downregulation of BFGF and P_2X_2

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ABSTRACT

Purpose: The objective is to analyze the antiangiogenic mechanism of suramab, a pharmaceutical compound of bevacizumab and suramin, in a rabbit model of corneal angiogenesis.

Material and Methods: Corneal neovascularization was induced in four groups of six New Zealand White rabbits by applying a filter paper disk soaked in 1 M Na (OH) on the central cornea. Group one was treated after injury with intravenous suramab at a dose equivalent to 3 mg/kg of bevacizumab and 10 mg/kg of suramin. Group two was treated with intravenous bevacizumab (5 mg/kg). Group three was treated with 10 mg/kg of suramin while the control group received no treatment. Digital photographs were taken at days 9, 15, 21, and 35. Neovessel formation was quantified giving a 0–4 score to each quadrant according to the centripetal growth of the longest vessel (neovessel index, NVI). Animals were sacrificed at day 35. Corneas were processed for histology, immunohistochemistry, and Western-blot using primary antibodies against P_2X_2, basic fibroblast growth factor (bFGF), LYVE-1, PECAM-1, and vascular endothelial growth factor-A (VEGF-A).

Results: Suramab significantly reduced neovessel growth (mean NVI: 4.2) compared to bevacizumab (8.4), suramin (7.22), and control animals (12.2) at 35 days post-injury ($p < 0.01$). A lower protein expression of P_2X_2, bFGF, LYVE-1, PECAM-1, and VEGF-A was found in the cornea of suramab animals than in the other groups of animals.

Conclusions: Joint downregulation of bFGF, P_2X_2, bFGF, and LYVE-1 constitutes a mechanism that induces greater and longer inhibition of corneal angiogenesis. Results might be relevant to ophthalmic care. Ocular administration of suramab is currently being investigated.

ARTICLE HISTORY
Received 23 June 2017
Revised 13 November 2017
Accepted 28 November 2017

KEYWORDS
Corneal Neovascularization; Angiogenesis; Bevacizumab; Suramin; Fibroblast Growth Factor

Introduction

The cornea is normally devoid of both blood and lymphatic vessels. Neovascularization could alter corneal transparency and severely compromise normal vision. Corneal neovascularization is usually associated with inflammatory and infectious diseases. These disorders might disturb the balance between angiogenic and antiangiogenic factors favoring the latter and thus activating neovascularization.[1]

Aflibercept, ranibizumab, and bevacizumab, anti-VEGF drugs, are currently being used in the clinical setting to treat retinal angiogenesis.[2,3] Ranibizumab and aflibercept have been approved by the Food and Drug Administration and the European Medicines Agency for this indication, while bevacizumab is used off-label for the same purpose.[2,3] To treat corneal neovascularization, several attempts have been made using the previously mentioned drugs as well as trastuzumab, lapatinib, and others.[4–8] The drugs were experimentally and clinically tested. However, to our knowledge, there is still no specific and approved drug for treating corneal neovascularization.[5]

We previously showed that suramin, a nonspecific purinergic receptor antagonist, mixed with bevacizumab increased their own effects and probably developed pharmacological synergy. The drug compound called suramab had a greater angiostatic effect on the cornea than bevacizumab alone.[9] The angiostatic effect was even greater when the drug compound was combined with poloxamer407, a copolymer used in pharmaceutical preparations. Poloxamer low toxicity and weak immunogenic properties make it suitable as a vehicle for drug delivery.[10,11] In the current study, we aim to analyze the effect of suramab on target molecules involved in corneal angiogenesis such as VEGF-A (vascular endothelial growth factor-A), b-FGF (basic fibroblast growth factor), and P_2X_2, as well as LYVE-1, which is a marker for lymphangiogenesis.

Material and methods

Animals

Twenty-four New Zealand White rabbits weighing on average 3 kg were included in this study. Three groups of six rabbits each were treated with intravenous suramab (compound of 3 mg/kg of bevacizumab + 10 mg/kg of suramin), bevacizumab (5 mg/kg), and suramin (10 mg/kg) immediately after injury.

The control group included six rabbits and received no treatment. Animals were kept in individual cages with free access to food and water under controlled cycles (12 h light,

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12 h dark). All animals were handled according to ARVO Statement for the Use of Animals in Ophthalmic Research.

Alkali burn model

The animal was anesthetized with 70 mg/kg intramuscular (IM) ketamine, 1 mg/kg IM midazolam, and topical anesthesia using 0.5% proparacaine (Alcon, Brazil). Corneal neovascularization was then induced by applying a Whatman filter paper disk (7 mm of diameter) soaked in 1 M Na (OH) in the central cornea of the left eye for 1 min. The eye was immediately washed with 10 ml of 0.9% NaCl.

Quantification of corneal vessels

Digital photographs were taken at days 9, 15, 21, 28, and 35 using a Canon 5D Camera (Farmington Hills, MI, USA). Neovessel index (NVI) was calculated using the ImageJ program. Neovessel formation was quantified giving a 0–4 score to each quadrant according to the centripetal growth of the longest vessel in increments of 1 mm. Scores from the four quadrants were summed up to obtain the NVI. Besides, the area of corneal neovascularization was measured and expressed as a percentage of the total corneal area.

Histology

Animals were euthanized at day 35 and corneas were removed, cut into halves, and fixed in 4% paraformaldehyde. The 16-µm-thick cryosections were stained with hematoxylin and eosin and examined in a Nikon Eclipse Microscope (Tokyo, Japan). Photographs from central and peripheral cornea were taken (Figure 2). We also quantified endothelial cells. Vascular endothelial cells were manually counted in the corneal epithelium and stroma. Cell count was performed in 10 fields (40× magnification) of the periphery and in 10 fields of the center of each histological section for stroma and epithelium. Mean and standard deviation were obtained from eight sections for each animal examined under a Nikon Eclipse Microscope.

Immunofluorescence

Rabbits were euthanized and corneas were excised and fixed in 4% paraformaldehyde for 6 h. The 14-µm-thick cryosections were incubated overnight at 4°C with anti-Von Willebrand factor (vWF) (1:500; Sigma-Aldrich, St Louis, MO: f3520) to assess neovascularization. Sections were revealed using the secondary goat anti-mouse antibody with fluorescein. Slides were analyzed using a Nikon Eclipse Microscope.

Immunohistochemistry

Rabbits were euthanized 35 days post-injury, and the corneas were excised and fixed in 4% paraformaldehyde for 6 h. The 16-µm-thick cryosections were incubated overnight at 4°C with rabbit anti-mouse LYVE-1 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) to assess lymphangiogenesis. Cornea sections were also incubated with primary antibody guinea pig anti-receptor P_2X_2 (1:600; Neuromics, Minneapolis, USA). Sections were first incubated in biotinylated goat–anti-mouse IgG, then in avidin–biotin peroxidase complex kit, and finally in 3,3′-diaminobenzidine/nickel solution. Slides were analyzed using a Nikon Eclipse Microscope (Tokyo, Japan).

Western blot

Corneas were extracted in a buffer containing 5 mM Tris–HCl with 2 mM $MgCl_2$, 2 mM EDTA, 65 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). After measuring protein concentration with the Bradford protein assay (Sigma-Aldrich), samples (30 µg protein per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (BioRAD, Canton, MA, USA). They were transferred to nitrocellulose filters (Hybon-P; Amersham Pharmacia Biotech, Piscataway, NJ, USA) using standard techniques. Membranes were incubated with primary antibodies against VEGF-A (Santa Cruz Biotechnology, Santa Cruz, CA), LYVE-1 (Santa Cruz Biotechnology, Santa Cruz, CA), PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA), bFGF (Santa Cruz Biotechnology, Santa Cruz, CA), and P_2X_2 (Neuromics, Minneapolis, USA) followed by appropriate biotinylated secondary antibodies and extravidin peroxidase (Sigma-Aldrich). Immunoreactive bands were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). Outcomes were pixels per band measured with the ImageJ program.

Corneal protein was isolated in pools from five control, suramab, suramin, and bevacizumab animals. At least three independent experiments were performed for each of the four groups studied.

Statistical analysis

The one-way analysis of variance was used to compare the results between groups. The $p < 0.05$ was determined to be statistically significant.

Results

Clinical neovascularization

Suramab

NVI was remarkably lower in the animal group treated with suramab than in controls after 35 days of follow-up. Nine days after injury, the NVI was much higher in the control group than in the treated group (Figure 1). In these animals, the centripetal growth of neovessels was fivefold diminished ($p < 0.01$). No serious ocular or systemic adverse event was detected.

Bevacizumab

Nine days after injury, NVI was lower in the treated group compared to controls. Bevacizumab significantly reduced neovessel formation compared to control animals at 21 days. However, the difference began to narrow from day 22 onward (Figure 1).

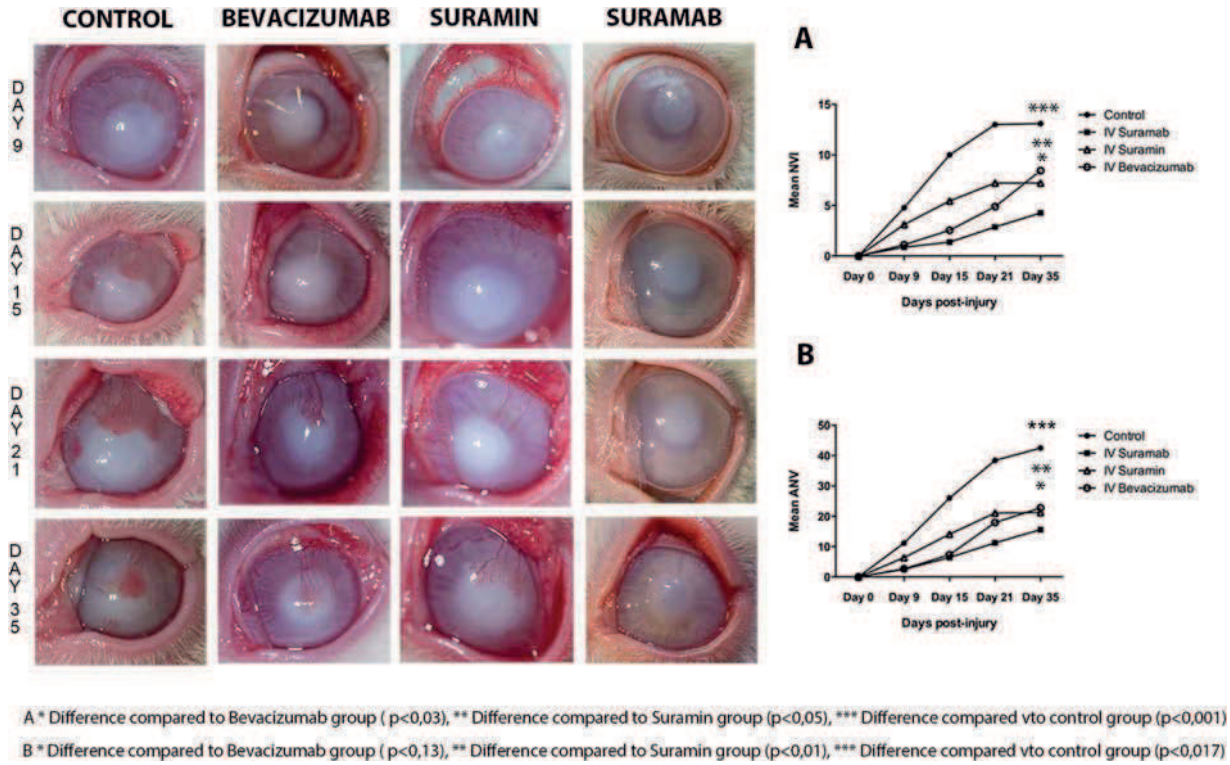

Figure 1. Clinical progress of corneal neovascularization.
Representative photographs of the four groups of animals: suramab, bevacizumab, suramin, and controls. Graph showing the mean corneal neovessel index (A) and area (B) in the animal groups, through 35 days of follow-up.

Suramin

Nine days after injury, NVI was lower in the treated group compared to controls. Suramin reduced neovessel formation compared to control animals at 21 days. The difference, however, became smaller afterward (Figure 1).

Suramab compared to bevacizumab and suramin

A greater inhibitory effect was obtained with suramab compared with bevacizumab and suramin. The difference was observed along the 35-day follow-up considering the corneal neovascularization area as well (Figure 1).

Histological findings

Histological findings were consistent with clinical evaluation. Treated eyes had less neovascularization and cellular infiltration than controls both in central and peripheral cornea. Suramab animals did not show neovessels in corneal periphery and showed much less formation in the central cornea compared to the other treated groups. The cellular infiltration reveals the presence of inflammatory cells such as granulocytes and monocytes. The infiltrate extension was clearly greater in controls than in treated animals (Figure 2).

Neoendothelial cells

The quantification of neoendothelial cells was remarkably lower in the suramab group (mean 2 ± 1.2) compared to the bevacizumab (mean 5 ± 1.5), suramin (mean 6 ± 1.7), and control groups (mean 8 ± 2.4).

Immunofluorescence

Immunoreactivity of vWF disclosed vascular neoendothelial cells in control, suramin, and bevacizumab animals. In suramab animals, immunoreactivity was slightly seen (Figure 3).

Immunohistochemistry

Immunoreactivity of P_2X_2 was seen much more extended in controls than in treated animals. The pattern was similar in control, bevacizumab, and suramin animals. Staining was found in the apical and basal layers of epithelium and in stromal neoendothelial cells. However, among suramab animals, staining was much weaker in the epithelium. P_2X_2 was downregulated in treated animals compared to controls. In addition, P_2X_2 was found to be downregulated in suramab animals compared to bevacizumab and suramin groups (Figure 4).

Immunoreactivity of LYVE-1 was observed in all animal groups. The pattern was similar among control, bevacizumab, and suramin animals. Staining was seen in the corneal epithelium and stroma. However, suramab animals disclosed positive immunoreactivity only in the epithelium. LYVE-1 was found to be downregulated in suramab-treated animals compared to other groups of animals (Figure 5).

Western blot

The protein expression of VEGF-A, bFGF, LYVE-1, P_2X_2, and PECAM was found to be significantly lower in animals treated with suramab than in controls, bevacizumab-treated, and

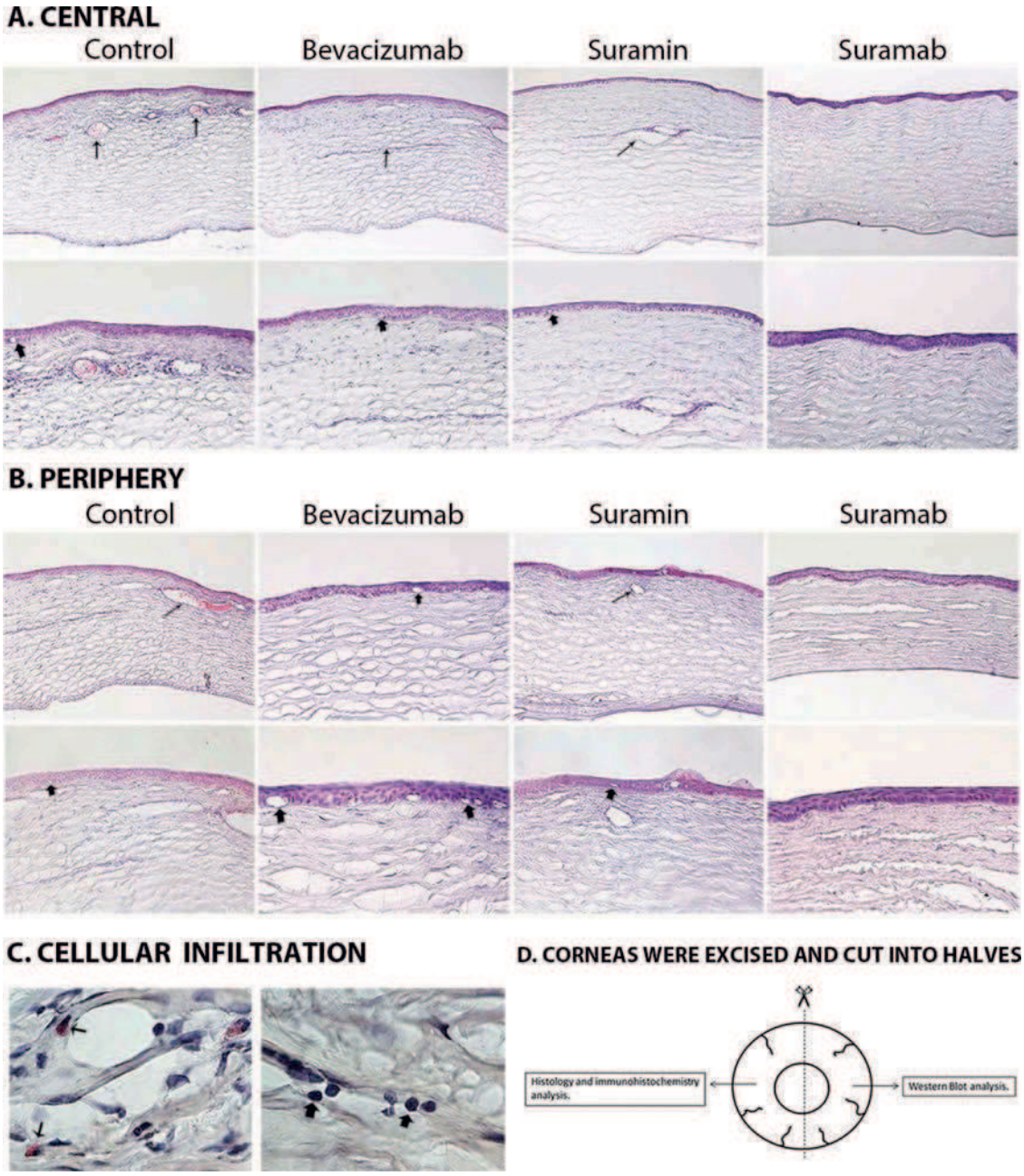

Figure 2. Histological findings.

Central cornea (A) and peripheral cornea (B), 20× magnification. Stromal and superficial neovessels (long arrows and arrowheads) and cellular infiltration are clearly seen in controls (A, B). Less infiltration and smaller neovessels are observed in bevacizumab, suramin, and suramab-treated animals (A, B). Granulocytes (long arrows) and monocytes (arrowheads) are found in the cellular infiltration; 100× magnification. Drawing for corneal cut (D).

suramin-treated animals. However P_2X_2 expression was similar among suramab and suramin animals (Figure 6).

Discussion

We carried out a study in a rabbit model of corneal angiogenesis in which a combination of bevacizumab and suramin (suramab) reduced corneal neovascularization. The angiostatic effect achieved by downregulation of bFGF, P_2X_2, VEGF-A, and LYVE-1 was histologically and clinically assessed. Suramab efficacy was greater and longer than that observed in animals treated with bevacizumab or suramin alone at 35 days of follow-up.

In ophthalmology, bevacizumab was first used intravenously to treat exudative macular degeneration in patients. In the first prospective study, researchers used a dose of 5 mg/kg with good results.[12,13] We used the same dose in the group of bevacizumab-treated animals. However, a lower dose of bevacizumab (3 mg/kg) was combined with suramin in order to show how the combination of these drugs, called suramab, significantly increased the pharmacological effects even when compared with higher doses of bevacizumab.

Suramab inhibits VEGF-A and b-FGF, which are known factors that play major roles in ocular angiogenesis.[14–17] In addition, we found LYVE-1 to be downregulated. This is a lymphatic-specific marker. The hyaluronic acid is an

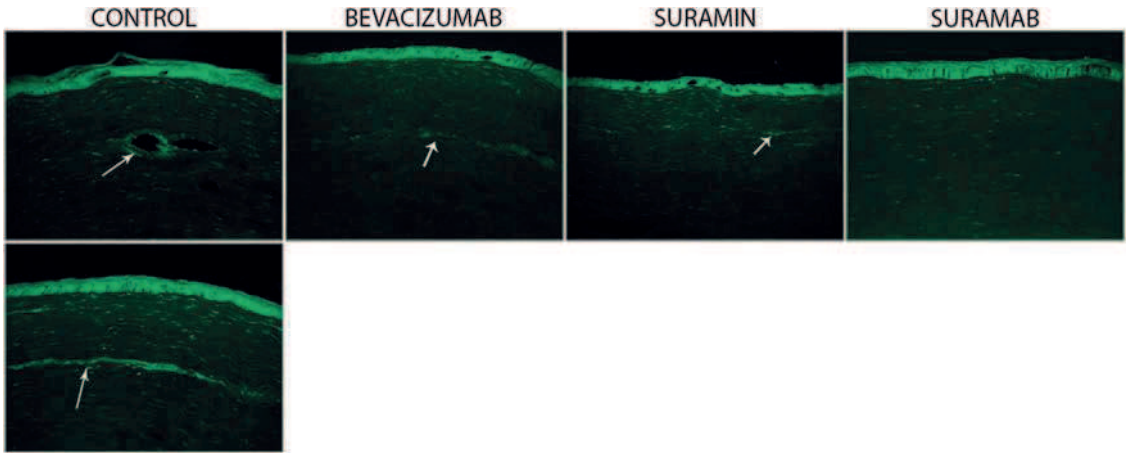

Figure 3. Immunofluorescence analysis of Von Willebrand factor.
Arrows highlight vascular endothelial cells in corneal stroma of control (A), bevacizumab (B), and suramin (C) animals. No vessels are shown in the suramab animals (D).

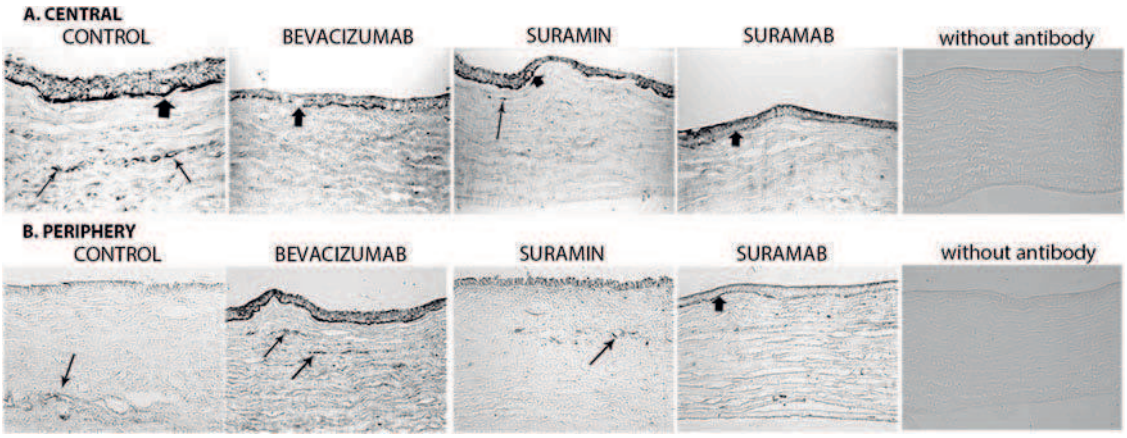

Figure 4. Immunohistochemical analysis of P_2X_2.
Central cornea (A) and peripheral cornea (B). P_2X_2 staining is more extended in controls than in treated animals. Staining was found in the apical and basal layers of epithelium and in corneal stroma. However, staining in suramab animals was weaker in the epithelium. P_2X_2 was found to be downregulated in suramab animals compared to bevacizumab, suramin, and control groups. Cornea without primary antibody for P_2X_2 is included.

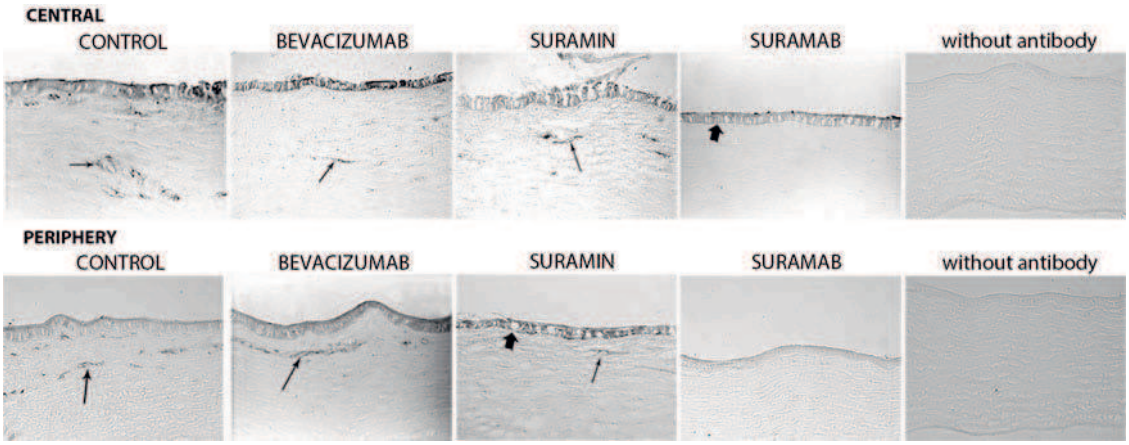

Figure 5. Immunohistochemical analysis of LYVE-1.
Central cornea (A) and peripheral cornea (B). LYVE-1 staining is larger in controls than in treated animals. Immunoreactivity is observed in the corneal stroma and epithelium. Suramab animals only showed slight staining in the epithelium. Cornea without primary antibody for LYVE-1 is included.

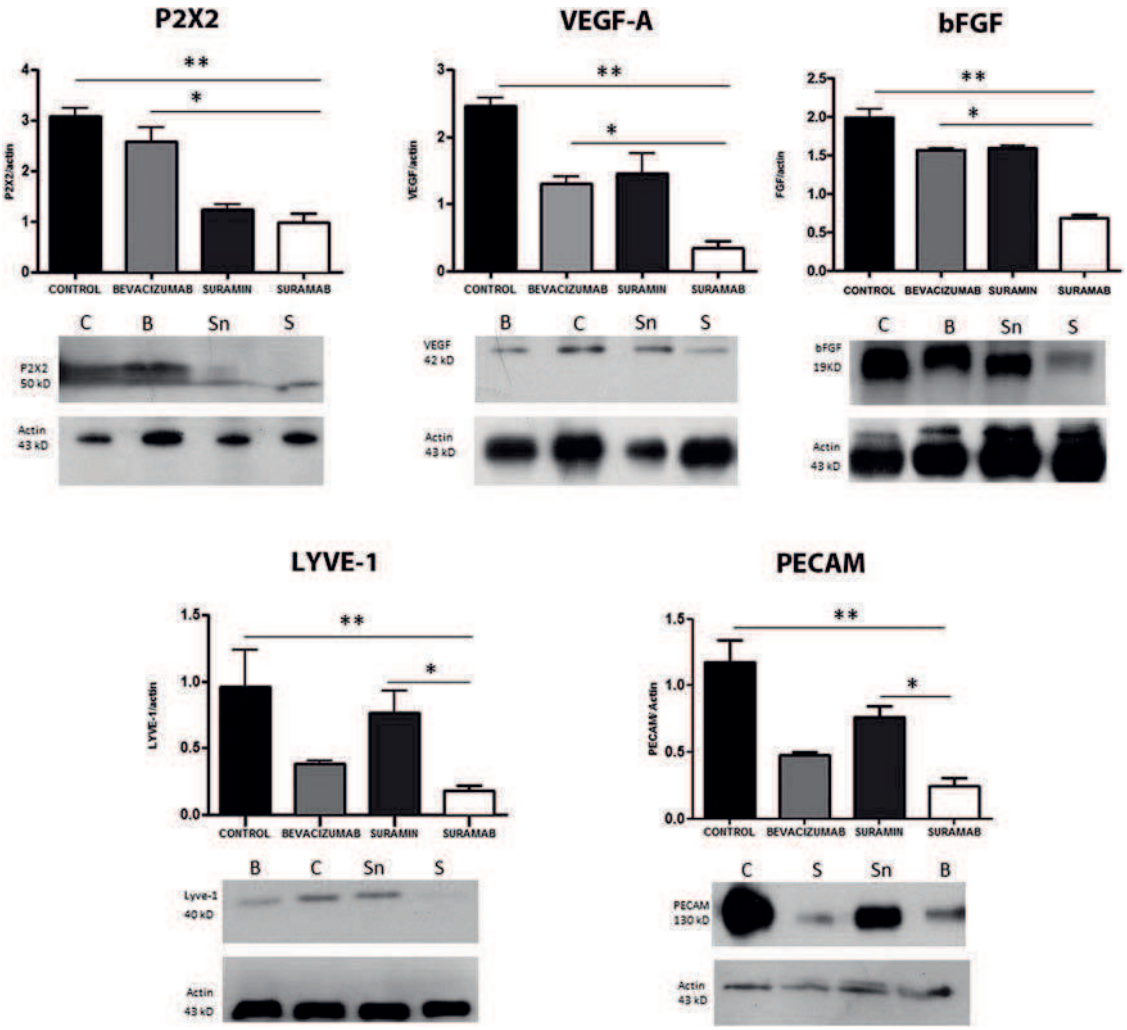

Figure 6. Western blot of P_2X_2, VEGF-A, LYVE-1, bFGF, and PECAM.

Protein expression is significantly lower in suramab-treated animals than in bevacizumab-treated, suramin-treated, and control animals (*$p < 0.0001$; **$p < 0.05$, ANOVA test). P_2X_2 expression is similar in suramin and suramab-treated animals.

important component of the extracellular matrix and a key mediator of cell migration in tissues during inflammation, wound healing, and neoplasia. LYVE-1 is expressed in lymphatic endothelial cells, leukocytes, dendritic cells, and tumor cells. Its participation in ocular neovascularization has been reported.[18,19]

The purinergic pathway may be involved in the pathogenesis of corneal neovascularization. Previous investigations identified the role of the purinergic pathway through P2 receptors promoting angiogenesis. Blockade of these receptors diminished neovascularization.[20] Suramin is a nonspecific, non-competitive antagonist of P2 purinergic receptors, with antiangiogenic effect on several tissues. Suramin is capable of inhibiting the binding of growth factors, the GTPase activity of certain G-proteins, the ecto-nucleotidase, and DNA and RNA polymerases. Besides, it can interfere with mural cell (pericytes) and endothelial cell interaction.[21–23] The antiangiogenic effect of suramin seems to be partially mediated by inhibition of VEGF.[24] P_2X_2 is an ion channel purinergic receptor that is expressed in vascular endothelial cells[25] and was found to be upregulated in retinal neovessels of animals exposed to oxygen. Blockade of P_2X_2 diminished neovessel growth.[26] Subconjunctival suramin inhibited corneal neovascularization in an experimental animal study. Its effect was lower compared to bevacizumab but lasted longer than bevacizumab.[8]

Bevacizumab, a recombinant humanized monoclonal antibody that binds to soluble VEGF, prevents the binding of VEGF to VEGF receptors inhibiting neovessel formation. Topical and subconjunctival administration of bevacizumab have been used in animals and humans with corneal neovascularization.[4,27–29] Bevacizumab inhibited corneal lymphangiogenesis through LYVE-1 downregulation.[29] Its antiangiogenic effect has been frequently evaluated by the expression of PECAM-1 (CD 31) (blood vessel marker platelet/endothelial cell adhesion molecule)[30,31] and vWF that demonstrates the presence of vascular endothelial cells.

It is noteworthy to mention that systemic administration of bevacizumab has been tolerated and effective in patients with neovascular age-related macular degeneration.[12,13] In the current study, we obtained similar results for diminishing corneal neovascularization.

We have not previously investigated if suramab passes the blood ocular barrier. However, it is known that inflammation

causes the breakdown of the blood ocular barrier. In our study, we observed inflammatory cell infiltrates in the injured corneas. Suramab clearly reduced the infiltration and angiogenesis compared to controls and other treated animals. So, based on the result of the suramab therapy, it seems reasonable to assume that the drug passes the blood ocular barrier.

Side effects of bevacizumab and suramin have been reported in patients. Nevertheless, the concentration and doses used in suramab are much lower than those considered to be toxic for suramin and bevacizumab.[12,13,32,33] No significant side effects were seen in our study.

Overall, the simultaneous inhibition of P_2X_2, bFGF, VEGF-A, and LYVE-1 by suramab resulted in a longer and greater inhibition of corneal angiogenesis. Similar results were seen in a recently published report using the periocular route.[34] Combining P_2X_2 and b-FGF blocking with VEGF-A and LYVE-1 inhibition seems to be the target used by suramab to enhance antiangiogenesis. To maintain the corneal angiostatic effect of anti-VEGF agents in the clinical setting is a challenging task.[1,5,28] We believe that this makes the pharmacological combination of bevacizumab and suramin given either systemically, topically, or subconjunctivally a promising tool for the treatment of corneal neovascularization.

Acknowledgments

We are grateful to Guillermo Gaston, Soledad Arregui, German Ruffolo, and Norma Montalbetti for their skillful technical assistance.

Declaration of interest

The authors alone are responsible for the content and writing of the paper. Dr Lopez, Dr Croxatto, Mr. Ortiz, and Ms. Potilinski report no conflicts of interest. Dr Gallo is the inventor of the suramab patent (EP 2186529; US 9,023,350).

Funding

Part of the work was funded by a Research Grant from Universidad Austral.

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