

# Prevention of Corneal Neovascularization by Adenovirus Encoding Human Vascular Endothelial Growth Factor Soluble Receptor (s-VEGFR1) in Lacrimal Gland

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**PURPOSE.** The aims of this study were (1) to determine the efficacy of adenovirus vector serotype 5 (Ad) encoding human soluble VEGF receptor 1 (*s-VEGFR1*) gene transfer to the lacrimal gland (LG); (2) to investigate whether expression of *s-VEGFR1* prevents corneal neovascularization (CNV) induced by alkali burns; and (3) to evaluate the safety of the procedure.

**METHODS.** AdVEGFR1 vectors (25  $\mu$ L,  $1 \times 10^{10}$  pfu/mL) were injected in the right LGs of rats and were compared with AdNull vector (25  $\mu$ L,  $1 \times 10^{10}$  pfu/mL) or 25  $\mu$ L of saline (Control) before cornea alkali burns with 1 M NaOH. After 7 days, CNV was documented at the slit lamp. Tear secretion was measured with phenol red threads. The animals were tested for *s-VEGFR1* mRNA and protein in the LG by quantitative (q)PCR and immunohistochemistry staining, respectively. qPCR was used to compare the mRNA levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the LG and ipsilateral trigeminal ganglion (TG).

**RESULTS.** Ad-VEGFR1 transfected 83% (10/12) of the rats. VEGFR1 was present in LG acinar cells. CNV was prevented in 9 of 12 animals in the Ad-VEGFR1 group, compared with the Ad-Null (3:10) and Control groups (1:10) ( $P = 0.0317$ ). The tear secretion and cytokine mRNA levels in the LG and TG were similar in all three groups ( $P > 0.05$ ).

**CONCLUSIONS.** Adenoviral vector gene transfer was safe for LG structure and function. The LG as the target tissue showed local expression of human s-VEGFR1, and CNV was prevented in most of the eyes exposed to alkali burns.

Keywords: gene therapy, lacrimal gland, ocular surface, neovascularization

Cornea neovascularization (CNV) is a damaging process caused by angiogenic molecules disrupting the active mechanisms of antiangiogenic protection that result in corneal opacity and visual impairment.<sup>1-3</sup> Neovascularization is part of the corneal wound healing process, which can result in transparency restoration or corneal opacity.<sup>4</sup> Corneal inflammation, trauma, hypoxia, and ischemia can lead to neovascularization, loss of transparency, and visual impairment.<sup>5,6</sup>

In recent years, one of the most used animal models for studying corneal wound healing has been the corneal alkali burn.<sup>7,8</sup> Corneal alkali burns affect the central nervous system by inducing inflammation of the trigeminal ganglion (TG).<sup>9</sup> The explanation for that response is the integrative actions among ocular surface, central nervous system, and lacrimal gland (LG) under physiologic and pathologic conditions in a structure named Lacrimal Functional Unit (LFU).<sup>10</sup> This concept is very important for the corneal avascular structure, because it predicts that the damage to the TG can be caused by a corneal alkali burn and subsequent damage to the neural network can promote or perpetuate the CNV or worse damage.<sup>1,9,11</sup>

VEGF has been shown to be a major angiogenic factor in the cornea, and its inhibition is undertaken by its soluble receptor VEGFR1 (s-VEGFR1). This soluble receptor is produced by the corneal epithelium, and it acts as an antagonist to VEGF action,

reducing the level of the free, active form of VEGF and inactivating the angiogenic properties of VEGF.<sup>2,12-14</sup>

Although many therapies have been proposed for CNV, they have presented limited or transitory results.<sup>5</sup> The standard therapeutic strategy is topical steroid application, with its known demanding compliance and side effects.<sup>6</sup> Our hypothesis predicts that reprogramming the LG to provide a therapeutic protein to the ocular surface is beneficial compared with topical application because the exocrine glands can provide bioactive agents, freshly made, without preservative agents in a constitutive or regulated manner, depending on ocular surface demands, and interactions with other systems, as neural, immune, and endocrine.<sup>15,16</sup> The possibility to reprogram acinar cells for therapeutic purposes were already described in other exocrine glands as salivary glands,<sup>17-19</sup> and the cellular tools, including the cytoplasmic molecules to coordinate the protein secretion has been described in normal and diseased LG.<sup>20-22</sup> This study is justified by the fact that most diseases that induce CNV are chronic and its containment requires continuous treatment.<sup>6,23,24</sup> Therefore, a drug delivery system that does not depend on compliance or interrupting the daily life activities of the patient and does not present discomfort or problems associated with preservatives would improve the success of the treatment.



VEGF-A is a primary and suitable target for the prevention and treatment of ocular neovascularization.<sup>25,26</sup> VEGF-A binds to the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). VEGFR-2 is the major mediator of mitogenic, angiogenic, and permeability-enhancing effects.<sup>26</sup> VEGFR-1 has two isoforms, a full-length, membrane-bound form (m-VEGFR1) and a shorter, nonmembrane-bound, soluble form (s-VEGFR1). The molecular role of s-VEGFR1 is believed to lie in the sequestration of VEGF ligands, resulting in decreased activation of VEGF receptors. Therefore, s-VEGFR1 is essential for preserving corneal avascularity, acting as a decoy receptor of VEGF-A.<sup>2,27</sup> Recombinant adenovirus-mediated expression of s-VEGFR1 has been demonstrated successfully to inhibit the development of corneal angiogenesis when injected into the anterior chambers of rat eyes.<sup>28</sup> These findings support the use of s-VEGFR1 in preventing or treating neovascularization.

Gene transfer to exocrine glands is an interesting strategy due to the potential to treat both the target tissue and the organs served by their secretion.<sup>29,30</sup> These findings suggest that the LG can work as a reservoir for the production of therapeutic factors that can be released in tears, providing treatment for diseases of the cornea. The usefulness of the gene therapy targeting the LG to treat ocular surface diseases compared with topical protein application is 2-fold. First, to deliver a fresh-made active molecule without preservatives, and second to overcome the issues related to compliance in chronic disease that requires constant treatment.

We therefore hypothesized that *s-VEGFR1* gene transfer to LG could be a useful strategy in the prevention of CNV under pathological conditions. In this study, we investigated the therapeutic potential of exogenous recombinant human s-VEGFR1 in inhibiting CNV within alkali-burned mouse corneas.

The aims of the present work were to evaluate (1) the efficacy of AdVEGFR1 transduction to rat LGs, (2) the potential of s-VEGFR1 once expressed in the LG to prevent CNV, and (3) the safety of the procedure.

## MATERIALS AND METHODS

### Animals and Ethics Statement

Adult male Wistar rats (8-weeks-old) bred in the Animal Breeding Center of the Ribeirão Preto Medical School (Ribeirão Preto, São Paulo, Brazil) were used in the experiments. The animals had free access to standard rodent chow and water.

All the experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the committee on animal experimentation of Ribeirão Preto Medical School, University of São Paulo.

### Recombinant Adenovirus Vectors

Recombinant adenovirus 5 Ad-VEGFR1, encoding the gene of human *s-VEGFR1*, and Ad-Null were provided by Vector Biolabs (Malvern, PA, USA) at titers of  $1 \times 10^{10}$  pfu/mL. The transgene expression of s-VEGFR1 was under the control of the immediate early promoter of cytomegalovirus (CMV). Ad-Null has no transgene and was used as a control for Ad-VEGFR1. Previous studies with mice have used similar titers of adenovirus on salivary glands, from  $10^7$  to  $10^9$  particles/mL.<sup>31,32</sup> In a pilot study, we observed that  $10^9$  particles/mL injected into the LG resulted in negative expression of s-VEGFR1 mRNA and no effect in preventing neovascularization.

### Animal Procedures

The rats (32 animals) received intramuscular anesthesia, consisting of a combination of ketamine (5 mg/100 g body weight [b.w.]; União Química Farmacêutica S.A, Embu-Guaçu, SP, Brazil) and xylazine (2 mg/100 g b.w.; Laboratorio Callier S.A., Barcelona, Spain).

### Induction of Alkali Burn Corneal Neovascularization

After ensuring that the corneal and caudal reflexes were abolished, the right cornea was exposed to a 2.5-mm filter paper embedded in 1 M NaOH for 20 seconds and then was irrigated with 60 mL of normal saline according to the method devised by Ormerod and colleagues<sup>33</sup> with modifications.

### In Vivo Gene Transfer

To determine the effects of s-VEGFR1 in preventing CNV, the rats were divided into the following three groups: Ad-VEGFR1 (12 animals), Ad-Null (10 animals), and Control (10 animals). Under the same anesthetic procedure, approximately 25  $\mu$ L of Ad-VEGFR1 ( $1 \times 10^{10}$  pfu/mL, Ad-NULL ( $1 \times 10^{10}$  pfu/mL), or saline were injected into the right LG under direct visualization after an incision in the right temporomandibular area. The incisions were sealed with cyanoacrylate glue (ITW PPF Brasil Adesivos Ltda., Valinhos, SP, Brazil).

Immediately after anesthesia recovery, the animals were housed (4 animals/cage) in a climate- and light-controlled environment and were allowed free access to food and water. The animals were observed on the next day and every other day after the procedure until the 7th day, when they were examined and euthanized for tissue harvesting. An excess (double dose) of anesthesia was used for euthanasia.

### Animal Evaluation

On the 7th day, the animals were anesthetized, and the ocular surface was evaluated at the slit lamp (model125/16; Carl Zeiss, Oberkochen, Germany) and photodocumented with a digital camera (Cyber-Shot DSC-W5; Sony Corporation, Tokyo, Japan).

### Grading of Corneal Neovascularization

The presence of CNV was evaluated in a blinded manner by two independent observers, as previously described<sup>34</sup> and was classified as absent (0) when no new vessels were visible, mild (1) when the growth of new blood vessels extended from the limbus toward the periphery of the chemical burned site and severe (2) when new blood vessels reached the chemical burned site (Supplementary Fig. S1). The categoric data were analyzed among the three groups. Because grades 1 and 2 are abnormal presentations of neovascularization of the cornea, for statistical analysis both categories were merged and compared with grade 0 (absence of neovascularization).

### Tear Secretion Measurement

The modified Schirmer test measured tear secretion from the right eye by placing a phenol red thread (PRT; 1-mm wide and 20-mm long thread, Zone-Quick; Mericon America, Inca., San Mateo, CA, USA) into the conjunctiva fornix of the eyes for 30 seconds.

### Quantification of Human s-VEGFR1 in Plasma

Systemic (plasma) levels of human s-VEGFR1 were determined by ELISA. Blood was collected by cardiac puncture and was centrifuged. Serum was obtained, and measurements were performed with a human s-VEGFR1 ELISA kit (Quantikine ELISA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions using a spectrophotometer (SpectroMax M3; Molecular Devices, Sunnyvale, CA, USA). According to the manufacturer, the minimal detectable level of VEGFR1 protein is 3.5 pg/mL.

### Morphometric and Immunohistochemical Analyses of the LG and Cornea

After blood collection, the animals were euthanized and tissues from the three groups were harvested for histopathology and biochemical evaluation.

The LG was removed and cut into two pieces. One half of the LG from the right side and the whole corneas of rats from the three groups were fixed in 10% formaldehyde and embedded in paraffin. Sections were cut at a 6- $\mu$ m thickness from the blocks of paraffin-embedded tissues on a rotary microtome (RM 2245; Leica, Bensheim, Germany) and were stained with hematoxylin and eosin (H&E). H&E-stained slides of the three groups were observed under a microscope for glandular structure and leukocyte infiltration.

The assays to locate hVEGFR1 by immunohistochemistry were developed using Reveal HRP (Spring Bioscience, Pleasanton, CA, USA). The LG slides were de-waxed in xylene and rehydrated through a series of graded alcohols. The sections were incubated in 0.1 M citrate buffer (pH = 6) and were subjected to heat retrieval using a water bath at 60°C for 30 minutes. Endogenous peroxidase activity and proteins were blocked according to the manufacturer's protocol. LG slides were incubated at 4°C overnight with anti-VEGF R1/Flt-1 polyclonal goat IgG (R&D Systems, Inc., Minneapolis, MN, USA) at a concentration of 1:200 in a humidified chamber overnight at 4°C. After the incubation with primary antibody, the LG slides were incubated with the visualization system and then with the chromogen substrate, liquid diaminobenzidine (DAB). The slides were then counterstained with hematoxylin, dehydrated in a series of alcohols and permanently mounted with Tissue Tek Glass Mounting Media (Sakura Finetek USA, Inc., Torrance, CA, USA). The same procedure was performed with Hep-2 cells transfected with Ad VEGFR1 (positive control), and LG slides on which the primary antibody was suppressed (negative control). To make the positive control slides, Hep-2 cells cultured with MEM culture media plus 2% fetal calf serum ( $10^6$  cells/mL; Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO<sub>2</sub>, with AdVEGFR1 at 0.1 multiplicity of infection (MOI) for 2 days. After that, the media was removed, cells washed with PBS followed by trypsinization, resuspension in MEM media and centrifugation for 10 minutes, at 112g and 4°C to make a pellet. The pellet was mixed with 100  $\mu$ L of human fresh plasma and coagulation induced by 20  $\mu$ L of thrombin (Dade Behring, Marburg, Germany). The clot was then embedded in paraffin and processed for immunohistochemistry as mentioned above for the other tissues. Photographs were obtained with a microscope (DM4000 B LED; Leica Microsystems, Wetzlar, Germany), and images were obtained with Leica LAS software (Leica, Wetzlar, Germany), version 4.2.

For the cornea immunohistochemistry assay, frozen sections of the rat corneas from naïve rats (Negative control), and from rats from the Control (i.e., right cornea alkali burned), Ad-VEGFR1 (i.e., right cornea alkali burned after right LG injection of Ad-virus with human *VEGFR1* gene), and Ad-Null (i.e., right

cornea alkali burned after LG injection of Ad-virus without therapeutic gene) were transferred to glass slides and submitted to the same staining protocol described above, after peroxidase blockage. The slides ( $n = 3$ /group) were incubated at 4°C overnight with the following primary antibodies: anti-VEGF R1/Flt-1 polyclonal goat IgG (R&D Systems, Inc., Minneapolis, MN, USA) at a concentration of 10  $\mu$ g/mL, IL-1 $\beta$ / IL 1F2 monoclonal mouse IgG (R&D Systems, Inc.) at a concentration of 8  $\mu$ g/mL, or TNF- $\alpha$  monoclonal mouse IgG (R&D Systems, Inc.) at a concentration of 10  $\mu$ g/mL, in a humidified chamber overnight at 4°C. The revealing process was conducted as described above for the LG slides.

Also, for the immunohistochemical reaction to compare the expression of CD 34 in the cornea, organosilane-coated slides (2 of the naïve control, 3 of the AdVEGFR1, and 1 of the Ad Null groups) were hydrated and treated with 3% hydrogen peroxide. For antigen retrieval of CD34 immunomarker, the slides were pretreated by pressure cooker containing 10 mM of sodium citrate buffer (pH = 6.0). Sections were incubated overnight with primary monoclonal antibody CD34 (clone QBEnd/10; Leica Biosystems Nussloch, Germany) at a concentration of 1.0  $\mu$ g/mL, diluted 1:500. After incubation with primary antibody, secondary antibodies conjugated with streptavidin-biotin-peroxidase, developed with diaminobenzidine (DAB) and counterstained with Carazzi hematoxylin, were used.

All photographs were analyzed and described by two independent researchers unaware of the group sampled. We analyzed samples from four LGs from the Control and AdNull groups and five LGs from the AdVEGFR1 group. For histologic and morphometric descriptions of each sample, we used three representative photographs of H&E-stained sections from each group. The areas of five acini of each slide were measured (LAS software, version 4.2; Leica, Wetzlar, Germany) and the values of the acinar areas of each animal were compared among the three groups.

### Quantitative RT-PCR Assay for mRNA in LG and TG Tissue

The other half of the LG from the right side and the ipsilateral trigeminal ganglia (TG) of the three groups were harvested and immediately transferred to microtubes containing RNA. Subsequently, the samples were stored at -80°C until RNA isolation. All the procedures were performed under standard RNase-free conditions to avoid exogenous RNase contamination.

Total RNA was extracted from 50 mg of tissue with a PureLink RNA Mini Kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was quantified by spectrophotometry at an OD of 260/280 (NanoDrop2000c, Wilmington, DE, USA). Genomic DNA was removed using a DNA-free Kit (Ambion). cDNA was synthesized from 1000 ng of total RNA using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ, USA).

Quantitative real-time PCR (qPCR) was performed using a ViiA7 Real-Time PCR System (Applied Biosystems). The following hydrolysis probes (Taqman Gene Expression Assays; Applied Biosystems) were used in this study: Hs01052961\_m1 (Flt-1 human), Rn00580432\_m1 (IL-1 $\beta$  rat), Rn01410330\_m1 (IL-6 rat), Rn99999017\_m1 (TNF- $\alpha$  rat), and Rn00667869\_m1 (beta-actin rat). The cycles of PCR were as follows: one cycle of 95°C for 20 seconds, followed by 50 cycles of amplification (95°C for 1 second, 60°C for 20 seconds). Each PCR assay was performed in triplicate. Beta-actin was used as the reference gene to normalize the reactions.

**TABLE 1.** Frequency of s-VEGFR1 mRNA Expression in Rats From the Groups Submitted to Gene Transfer With AdVEGFR1 ( $n = 12$ ), AdNull ( $n = 10$ ), or Control (saline;  $n = 10$ ) in the LG, Followed by Corneal Alkali Burn With 1 M NaOH and Evaluated 7 Days Later ( $P < 0.0001$ ,  $X^2$  Test)

Groups	Absent	Present
Control (saline)	10 (100%)	0 (0%)
Ad VEGFR1	2 (17%)	10 (83%)
Ad Null	10 (100%)	0 (0%)

### Statistical Analysis

A statistical/analytical software program (Prism 5.0; GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analysis. Continuous data are expressed as the means  $\pm$  SDs of the mean. Comparisons were performed using the Kruskal-Wallis test for continuous data, with Dunn's post-hoc test for paired comparisons, and the  $X^2$  test for categorical data among the three groups. The level of significance used was  $P < 0.05$ .

## RESULTS

### Expression of VEGFR1 mRNA in LGs

qPCR for human s-VEGFR1 was performed to confirm the expression of s-VEGFR1 in the injected LGs. The expression of human s-VEGFR1 mRNA was observed in 10 of 12 animals in the AdVEGFR1 group, whereas the expression of human VEGFR1 mRNA was not detected in the 10 control and 10 Ad-Null groups tested 7 days after the injection, corroborating the finding that gene expression could be achieved by AdVEGFR1 vector injected directly in the LG. The transfection efficiency, measured in terms of frequency of positive samples for s-VEGFR1 mRNA, was 83.3% (Table 1).

### Effects of Recombinant Adenovirus Vector Delivery on LG Function

Tear secretion measured in the right eye by PRT, as described above, showed no differences between the controls and any of the transduced groups. Their values were as follows: Ad-VEGFR1:  $4.5 \pm 1.1$  mm (12 animals); Ad-Null:  $7.7 \pm 1.6$  mm (10 animals); and Control:  $5.8 \pm 1.6$  mm (9 animals) ( $P = 0.225$ , Kruskal-Wallis test).

**TABLE 2.** Distribution of Neovascularization Results by Category (Absent, Mild, or Moderate and Severe) Observed in Rats From the Groups Submitted to Gene Transfer With AdVEGFR1 ( $n = 12$ ), AdNull ( $n = 10$ ), or Control (saline;  $n = 10$ ) in the LG, Followed by Corneal Alkali Burn With 1 M NaOH and Evaluated 7 Days Later ( $P = 0.0336$ ,  $X^2$  Test)

Groups	Absent	Mild	Moderate or Severe
Control (saline)	1 (10%)	6 (60%)	3 (30%)
Ad VEGFR1	9 (75%)	2 (16.7%)	1 (8.3%)
Ad Null	3 (30%)	4 (40%)	3 (30%)

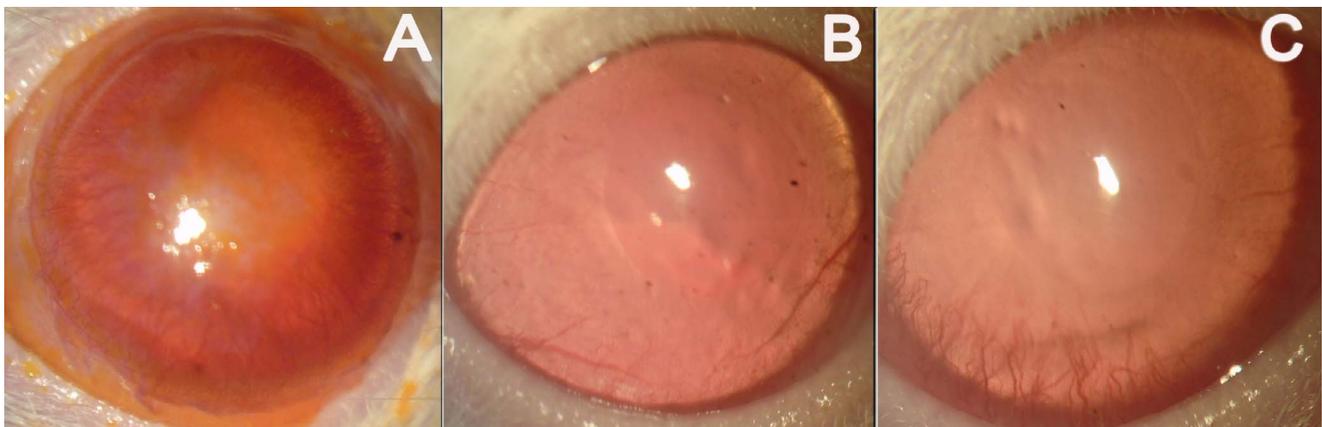
### Comparison of Alkali Burn Outcomes in Cornea Neovascularization

To test whether s-VEGFR1 could protect the cornea against the damage triggered by alkali burns and inhibit the formation of neovessels, the right LG of the rats that received AdVEGFR1 were compared on the 7th day with eyes that received AdNull or saline in the right LG prior to alkali burn of the ipsilateral (right) cornea. The slit-lamp observation showed that all nine animals in the Control group (9:10 or 90%) and seven in the Ad-Null (7:10 or 70%) presented mild, moderate, or severe neovascularization responses on the 7th day postinduced alkali burn. New blood vessels were visible, extending from the limbus and reaching the cauterized area, where they were blocked by the central leucoma (Fig. 1). In contrast, only three animals (3:12 or 25%) in the treated Ad-VEGFR1 group showed mild, moderate, to severe neovascularization, and this proportion was significantly lower, compared with the other groups ( $P = 0.0336$ ,  $X^2$  test) (Table 2).

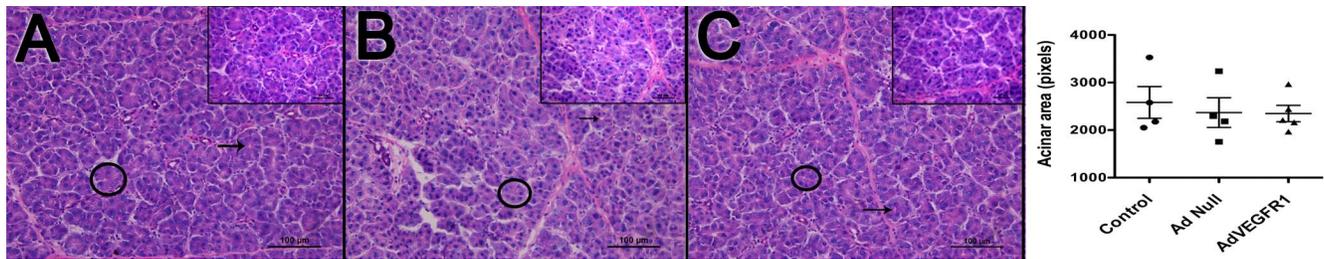
### Effects of Recombinant Adenovirus Vector Delivery on LG Histology

To test the impact of Ad vector injection on LG, studies were conducted, and the repercussions on function and structure were assessed by tear secretion and by LG histology and morphometry.

Seven days after a single dose of Ad-VEGFR1, Ad-Null, or saline (Control) injection, the LGs from the three groups showed no differences and no evidence of inflammation, ductal lumen dilation, acinar area shrinking or fibrosis (Fig. 2). The comparative measurement of the acinar area in series of LG histologic slides was made with the intention to observe



**FIGURE 1.** Cornea image obtained at the slit lamp on day 7, after gene transfection in the LG, followed by alkali burn with 1 N NaOH for 30 seconds. (A) Cornea of a rat of group control (saline), which illustrates a score of 2, where new vessels reaching the burned site; (B) cornea of a rat of the AdVEGFR1 group, score 0, where the vessels observed are only in the iris; and (C) cornea of a rat of the AdNull group, showing new vessels in the periphery of the cornea, therefore score 1.



**FIGURE 2.** LG histology and morphometric analysis: slides were stained with H&E. Five acinar areas of one slide of the right LG (exposed to transfection or saline) of each rat were circled (black perimeter around the acini). The area measured in pixels was obtained by LAS software, version 4.2. The acinar and ductal structures were preserved in the groups, and the absence of inflammatory infiltrates was observed in all slides of the groups: (A) Control ( $n=4$ ); (B) Ad Null ( $n=4$ ); and (C) Ad VEGFR1 ( $n=5$ ) (magnification  $\times 200$  and black arrows show the lumen of the acini). Inserts in the upper right corner show  $\times 400$  magnification. (D) Graphic showing the similarity of the acinar area among the three groups ( $P=0.90$ ).

whether the gene therapy impacted the trophism of the usine of tears secretion. However, the values were similar among the groups ( $P=0.9$ ).

The hVEGFR1 protein was observed as small brown dots in the cytoplasm of several acinar cells, of the LG from group AdVEGFR1 in samples from five of six rats injected and none of the slides from the LGs of the rats in group Ad-Null or Control (Fig. 3).

### Expression of Proinflammatory Cytokines mRNA in LGs and TG

To evaluate the inflammatory response after the injection of the recombinant adenovirus vector in the LG and TG, we performed qPCR for the proinflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . The mRNA values of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  did not show different expression in the ipsilateral LG among the AdVEGFR1 ( $n=12$ ), Ad-Null ( $n=10$ ), and Control ( $n=10$ ) groups ( $P=0.74$ , 0.90, and 0.97, respectively). In the same manner, in the ipsilateral TG, the mRNA values of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  did not show different expression among the AdVEGFR1 ( $n=7$ ), Ad-Null ( $n=6$ ), and control groups ( $n=5$ ) ( $P=0.80$ , 0.59, and 0.60, respectively).

### Expression of s-VEGFR1, Proinflammatory Cytokines, and CD34 in Corneas

The s-VEGFR1 and proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  proteins expression were investigated in corneas from naïve rats, and rats submitted to corneal alkali burn after saline, AdVEGFR1 or Ad-Null injection in the ipsilateral LG (Fig. 4). The results revealed that naïve corneas have thicker stroma and epithelia. The corneas from the AdVEGFR1 group show stromal staining for VEGFR1 (Fig. 4, upper lane). The corneas from Ad Null group presented more intense staining for IL-1 $\beta$ , in the epithelia and in the anterior stroma, compared with the

other groups (Fig. 4, middle lane). The TNF- $\alpha$  was similarly higher expressed in the cornea of the three groups submitted to alkali burn, in the epithelia and in the stroma, regardless the intervention, compared with the naïve control corneas (Fig. 4, lower lane).

The phosphoglycoprotein CD34 expression was investigated in the cornea of naïve, Ad-VEGFR1, and Ad-Null groups ( $n=2$ , 3, and 1, respectively), as a marker of neovessels and stem cells, in response to alkali burn or not and the preventive action of the s-VEGFR1. The expression of CD34 was more intense in the Ad-Null sample than in all samples of Ad-VEGFR1 and, it was very mild in the naïve control corneas (Fig. 5).

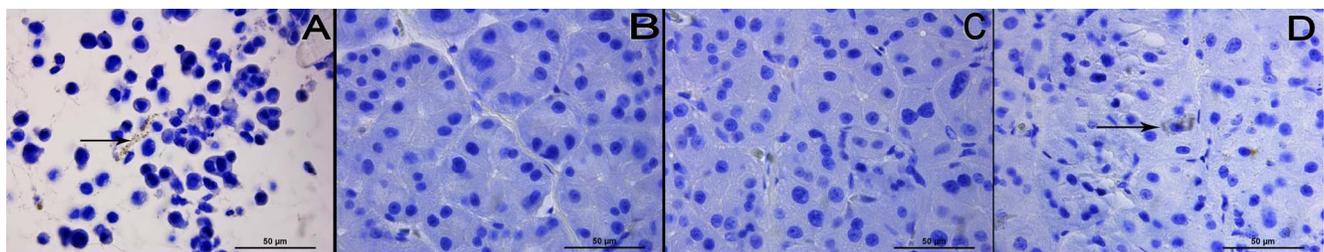
### Absence of Human VEGFR1 in Peripheral Blood

To investigate whether transfection of AdVEGFR1 to the LG could cause systemic spread, the levels of VEGFR1 was investigated in blood harvested from animals in the three groups ( $n=5$ /group) by ELISA. None of the samples showed detectable levels (data not shown).

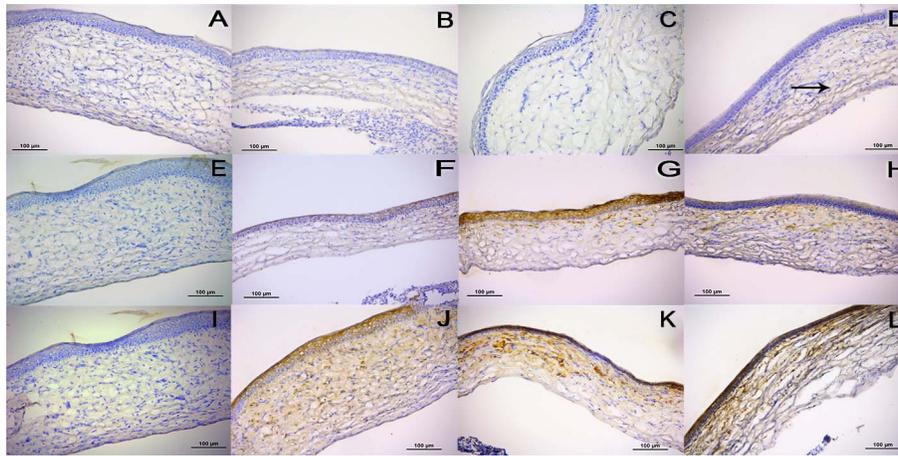
## DISCUSSION

The present work revealed that AdVEGFR1 therapy in the LG to prevent CNV caused by alkali burns is feasible and safe.

A prior study used adenovirus transfection of PKC- $\alpha$  in rat LG cells in culture.<sup>35</sup> The transfection of  $10^7$  pfu/150  $\mu$ g cell protein, although hardly comparable, was capable of transfection 84% of cells and promoted the enhancement of peroxidase secretion by 1% to 2% compared with basal conditions, whether stimulated or not with phenylephrine.<sup>35</sup> It is difficult to compare the efficacy of transfection between a monolayer cell culture incubated with vector overnight and an injection of a much smaller volume inside an organ in vivo. However, it was clear that, even with an increase in PKC- $\alpha$  of approximately 176 times, the number of cells transfected and the increase in



**FIGURE 3.** Immunohistochemistry showing hVEGFR1 expression in the (A) control Hep-2 cells transfected with AdVEGFR1, LG of the Control (B), Ad Null (C), and AdVEGFR1 group (black arrow showing the VEGFR1 in the cytoplasm of acinar cells) (D). The rats were treated in parallel and the LG were harvested at 7 days but did not show any staining for VEGFR1. The slides were counterstained with Hematoxylin. Magnification  $\times 630$ .

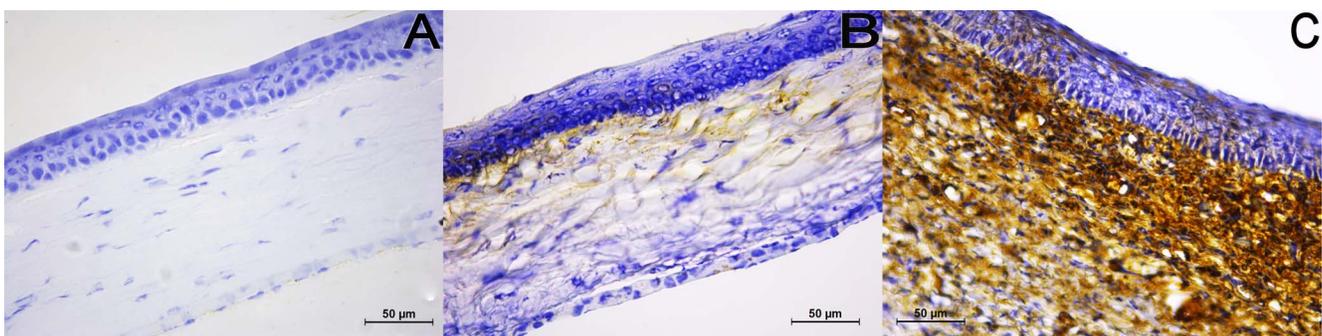


**FIGURE 4.** Corneal immunohistochemistry for VEGFR1, IL-1 $\beta$ , and TNF- $\alpha$ , counterstained with H&E. (A) Naïve control cornea, (B) LG saline-treated followed by corneal alkali burn, (C) LG injected with Ad-Null, followed by corneal alkali burn, (D) LG-injected AdVEGFR1 followed by corneal alkali burn: VEGFR1/F1 was detected in slides using anti-VEGFR1/F1-1 polyclonal antibody (R&D Systems). *Middle lane:* (E) naïve control cornea, (F) LG saline-treated followed by corneal alkali burn, (G) LG injected with Ad-Null, followed by corneal alkali burn, and (H) LG-injected AdVEGFR1 followed by corneal alkali burn: IL-1 $\beta$  was detected in slides using anti-IL-1 $\beta$  monoclonal mouse IgG antibody (R&D Systems). *Lower lane:* (I) naïve control cornea, (J) LG saline-treated followed by corneal alkali burn, (K) LG injected with Ad-Null, followed by corneal alkali burn, and (L) LG-injected AdVEGFR1 followed by corneal alkali burn: TNF- $\alpha$  was detected in frozen sections using anti-TNF- $\alpha$  monoclonal mouse IgG antibody (R&D Systems). All tissue sections were counterstained with Hematoxylin. Original magnification  $\times 200$ .

the secretion were less expressive.<sup>35</sup> In vivo, a gene capable of inhibiting TNF- $\alpha$ , once transfected into the LGs of rabbits using adenovirus as the vector, showed that it was able to reverse LG inflammation and signs of dry eye in those rabbits.<sup>36</sup> The authors applied  $10^8$  pfu and a volume of 200  $\mu$ L. The extension of transduction or rate of injection failure among the animals treated was not mentioned. The limit of successful transfection in the present work (83% or 10/12 animals) could perhaps be attributed to the delicate procedure related to vector injection into the rat LG. Using 25  $\mu$ L of volume might have resulted in reflux or failure to target a significant region of acinar cells.

There have been several interesting potential approaches described to prevent or reverse CNV.<sup>6,23,37</sup> Several studies have shown that it is possible to inhibit CNV by blocking or suppressing VEGF.<sup>24,37</sup> Typically, the delivered method used has been topical therapy, but these studies have revealed that the actual therapeutic benefit of this method is modest in efficacy and duration.<sup>38</sup> The strategy of applying viral vectors to the cornea directly has also been described.<sup>39-41</sup> Our impression is that external damage to the cornea, in addition to the different responses and turnover of the corneal tissues, can limit these strategies targeting the cornea for clinical application.

This study was the first that used the LG as a bioreactor to produce a factor capable of scavenging VEGF, without exogenous or repeatedly medication. A similar approach, using an Ad virus carrying the gene of erythropoietin and targeting the submandibular glands of mice exposed to radiotherapy of the head and neck, was able to prevent signs of dry eye (i.e., reduction in tear secretion and cornea epithelial damage), possibly due to the systemic effects of this hormone.<sup>42</sup> In fact, the concept of using an exocrine gland as a bioreactor to produce factors capable of treating diseases of tissues supported by the gland's secretion was originally conceived for salivary and mouth or systemic diseases.<sup>43,44</sup> The present strategy reduced the CNV in vivo, after 7 days. Also, CD34, a marker of vascular endothelial cells,<sup>45,46</sup> was less expressed in the corneas of the Ad-VEGFR1 than the Ad-Null group. Keratocytes and other stem cells can also express CD34 in a wound healing process, overlapping the observations regarding neovascularization and corneal healing<sup>46-48</sup>; however, the distinction among the samples of the groups, in terms of neovascularization and CD34 expression reinforce the hypothesis favorable to the therapy based on the s-VEGFR1 transduction in the LG. Future studies on therapeutic strategies for CNV must include more specific markers of blood vessels



**FIGURE 5.** Corneal immunohistochemistry for CD34, counterstained with H&E. (A) Naïve control cornea; (B) LG-injected AdVEGFR1 followed by corneal alkali burn; (C) LG injected with Ad-Null, followed by corneal alkali burn: CD34 was detected in slides using CD34 primary monoclonal antibody (clone QBEnd/10; Leica Biosystems). Original magnification  $\times 400$ .

endothelial cells, like CD31, and of lymphatic vessels, as LYVE1, due to the more specific vessels staining and less interference from the staining of other cells involved in the tissue repair (Bignami F, et al. *IOVS*. 2015;56:ARVO E-Abstract 4497).<sup>49</sup> Immunofluorescence can be measured allowing the comparison of the corneal neovascularogenesis and neolymphangiogenesis responses, using CD31 and LYVE1 as biomarkers with a clear demonstration of the vessels structure, as recently demonstrated in studies showing the role of Angiopoietin 2 as a mediator of angiogenesis, and the benefit of neurokinin-1 receptor antagonist topical ocular application after alkali burn in mice to revert the neovascularogenesis.<sup>50,51</sup>

The safety of the gene therapy procedure was demonstrated by the following observations: preserved structure and tear flow in the LG transfected either with Ad VEGFR1 and Ad-Null. Moreover, the levels of a panel of cytokines, known to identify inflammatory disturbances in the LG and TG ipsilateral to corneal alkali burns,<sup>9</sup> were not different among the three groups compared here. Also, the VEGFR1 gene transfer to the LG did not prevent the presence of proinflammatory cytokines in the cornea, in response to corneal alkali burn. The present work was the first to demonstrate that Ad virus transfection of rat LGs in vivo did not affect the LG parenchyma or its tear secretion capacity. Similar observations on the safety to the LG were obtained with a series of serotypes of adeno-associated virus (AAV) carrying the luciferase gene to transfect mice.<sup>52</sup>

The limitations of the present study were associated with the chosen virus. Ad virus as a proof of concept is an extremely useful gene vector; however, its transfection is limited in time without genetic adaptations due to immunoreaction.<sup>53</sup> Therefore, it is unable to prevent the long-lasting effects of certain ocular surface diseases, including the model applied here, neovascularization or dry eye syndrome.<sup>28,54-56</sup> In the salivary glands of mice, the amount of the relative expression of  $\beta$ -Gal was reduced by seven times by the fourth day after injection; however, in the lacrimal gland cells in primary culture, the peak of  $\beta$ -Gal expression, also transfected by adenovirus, occurred on day 7.<sup>32,57</sup>

Another issue is the tropism of adenovirus to the acinar cells. While some studies could achieve successful transduction in LGs in culture, rabbit LGs or mouse salivary glands in vivo, there is only limited information regarding whether this vector tropism to rat LG acinar cells depends on other conditions, such as species, serotype, and/or the carried gene.<sup>35,57,58</sup> In this work, we were able to confirm the transfection by qPCR and the protein expression in the LG and in the cornea stroma of the LG-transfected rats. Both levels were modest, which might reflect the low capacity of the target cells, low transduction, or low capacity to express the protein s-VEGFR1 in vivo. The study confirmed that human s-VEGFR1 was not present in the blood circulation. However, the attempts to detect s-VEGFR1 in the tear secretion was not reliable due to the limited volume of tears and need to dilution to follow the technical recommendations of the ELISA kit provider.

Previous authors, addressing cornea gene therapy, chose to target different sites, such as the subconjunctival tissue and the anterior chamber.<sup>28,59</sup> Both sites proved to be easily transfected, persisted for more than 2 months for both AdVEGFR1 and AAV-endostatin and were effective in preventing CNV.

However, there were concerns that the vector tropism and its gene product were not limited to the ocular surface, as observed in rats in a recent study, and that adenovirus might lead to extensive inflammation, once in the conjunctival area, as recently reported by MRI imaging in an individual with adenoviral conjunctivitis, indicating that the LG, as a postmitotic, encapsulated organ, might be a preferential tissue for gene therapy therapeutic purposes.<sup>60,61</sup>

In conclusion, the present work reported proof of concept for using gene therapy with Ad-VEGFR1, targeting the LG to prevent the CNV caused by alkali burns. To improve the efficacy of this strategy, we might need to investigate other vectors capable of carrying s-VEGFR1 and alternatives to block other angiogenic signaling pathways in the cornea.

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