Recovery of Radiation-Induced Dry Eye and Corneal Damage by Pretreatment with Adenoviral Vector-Mediated Transfer of Erythropoietin to the Salivary Glands in Mice

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Abstract

Therapeutic doses of radiation (RTx) causes dry eye syndrome (DES), dry mouth, and as in other sicca syndromes, they are incurable. The aims of this work are as follows: (a) to evaluate a mouse model of DES induced by clinically relevant doses of radiation, and (b) to evaluate the protective effect of erythropoietin (Epo) in preventing DES. C3H female mice were subjected to five sessions of RTx, with or without pre-RTx retroductal administration of the AdLTR2EF1a-hEPO (AdEpo) vector in the salivary glands (SG), and compared with naïve controls at Day 10 (10d) (8 Gy fractions) and 56 days (56d) (6 Gy fractions) after RTx treatment. Mice were tested for changes in lacrimal glands (LG), tear secretion (phenol red thread), weight, hematocrit (Hct), and markers of inflammation, as well as microvessels and oxidative damage. Tear secretion was reduced in both RTx groups, compared to controls, by 10d. This was also seen at 56d in RTx but not AdEpo + RTx group. Hct was significantly higher in all AdEpo + RTx mice at 10d and 56d. Corneal epithelium was significantly thinner at 10d in the RTx group compared with AdEpo + RTx or the control mice. There was a significant reduction at 10d in vascular endothelial growth factor (VEGF)-R2 in LG in the RTx group that was prevented in the AdEpo + RTx group. In conclusion, RTx is able to induce DES in mice. AdEpo administration protected corneal epithelia and resulted in some recovery of LG function, supporting the value of further studies using gene therapy for extraglandular diseases.

Introduction

DRY EYE SYNDROME (DES) is a common and globally distributed disease caused by different conditions (Epidemiology Subcommittee, 2007). Among these causes of DES are radiation (RTx) treatment for head and neck cancers (Bessell *et al.*, 1987; Parsons *et al.*, 1994; Jeganathan *et al.*, 2011). Moreover, RTx is an adjuvant treatment for ocular diseases like Graves' ophthalmopathy and pterigium (Bradley *et al.*, 2008; Murube, 2009).

The mechanism of therapeutic action of RTx is attributed to oxidative stress (Nishi *et al.*, 1986a; Yamada *et al.*, 2010). Oxidative stress is also an important factor in the pathophysiologies of inflammatory and endocrine diseases, some of which, such as Sjögren's syndrome (SS) and Diabetes Mellitus (DM), affect the lacrimal and salivary glands (Halliwell 1995; Nogueira *et al.*, 2005; Ryo *et al.*, 2006; Cejkova

et al., 2008). Hydrogen peroxide (H₂O₂), a known reactive oxygen species (ROS), induces SS-A/Ro protein (which is a marker of SS) to translocate to the cell nucleus. This translocation is reported to induce inflammation through the mitogen-activated protein (MAP) kinase pathway (Nobuhara *et al.*, 2007). Therefore, administering agents (e.g., Epo, vitamin C, or vitamin E) that mitigate oxidative stress may protect the lacrimal glands (LG) and the ocular surface, as previously reported (Blades *et al.*, 2001; Peponis *et al.*, 2004; Drouault-Holowacz *et al.*, 2009).

Despite its clinical relevance, no models related to RTx and DES or its mechanisms in LG are published. On the other hand, Epo has been ascribed as having anabolic, antioxidant, and anti-inflammatory effects against injuries, including RTx, and may have local effects in salivary glands (SG), LG, and retinas (Gerina, 1980; Brines and Cerami, 2006; Arcasoy, 2008a; Wang *et al.*, 2009). Previous studies have

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suggested that SG are useful tissues for sustained expression of recombinant proteins, as the glands are easily accessible via ductal opening in the mouth (avoiding invasive procedure) and act as bioreactor and endocrine organs that can release proteins into the blood stream and/or the saliva, allowing strategies for local and systemic treatments (Voutetakis *et al.*, 2007, 2010; Zheng *et al.*, 2009).

The aims of the present work are (a) to report the features of a rodent model of DES related to RTx, (b) to investigate the mechanisms of this condition, and (c) to test the protective effect of Epo expression on LG secretion and ocular surface integrity.

Material and Methods

Vector preparation

A modified E1-, replication-deficient Ad5 vector was generated as previously described (Zheng *et al.*, 2008). This modified vector has transgene expression in the SG and can persist for 2–3 months at least, which is longer than expected for conventional Ad5 vectors.

Briefly, the E1 deletion was achieved by recombination of the pACCMV-pLpA shuttle plasmid with pJM17. A DNA fragment from the Moloney murine leukemia virus (MoMLV) of the envelope p15E protein coding sequence, the 3' end of the envelope p15E protein coding sequence, and a long terminal repeat (LTR) sequence were combined to create plasmid pACLTR1. The LTR sequence was ligated into pACLTR1 to create the plasmid pACLTR2. The EF1 alpha promoter was excised from pEF-BOS and ligated into the pACLTR2 to create plasmid pACLTR2EF1 alpha. Human Epo cDNA and Simian virus 40 (SV40) polyA sequence were excised from pEAK8-hEpo and ligated into pACLTR2EF1 alpha to create plasmid pACLTR2EF1 alpha hEpo. Ad-LTR2EF1a-hEPO (AdEpo) was generated by homologous recombination of pACLTR2EF1 alpha hEpo with pJM17 in C7 cells using a calcium phosphate transfection system (Life Technologies, Gaithersburg, MD). Recombinant vector was plaque screened, propagated in C7 cells, and purified by CsCl gradient centrifugation as described (He *et al.*, 1998; Baum and O'Connell, 1999). After purification, vector was dialyzed and stored in aliquots at -80°C for later use. Vector titers in particles/ml were determined by real-time quantitative polymerase chain reaction (qPCR) using primers from E2 region (ABI Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA).

In vivo gene transfer

Eight-week-old female C3H mice bred in the National Cancer Institute (NCI) Animal Production Area (Frederick, MD) were used in this study, after approval by the NCI Animal Care and Use Committee and NIH Biosafety Committee. All experimental procedures adhered to the "Principles of laboratory animal care" (NIH publication no. 85-23) and the statement for the use of animals in ophthalmic and vision research (ARVO).

Animals had free access to standard rodent chow and water. Intramuscular anaesthesia, consisting of a combination of ketamine (KetaVed Vedco Inc., St. Joseph, MO) at 100 mg/kg of body weight; and xylazine (Lloyd Inc., Shennandoah, IA) at 14 mg/kg of body weight, were administered

to allow for surgical procedures and later for tissue collection. All procedures and manipulations were conducted immediately following confirmed abolition of the caudal and corneal reflex.

After anesthesia, each mouse received 10^9 particles of AdEpo in 50 μl aliquots by submandibular duct cannulation as previously described (Zheng *et al.*, 2008).

Animal RTx

One day after gene transfer, the AdEpo was treated and an untreated group of mice were subjected to head and neck RTx. The RTx field included all LG and SG and was accomplished by placing each animal into a specially built Lucite jig in such a way that the animal could be immobilized without the use of anesthetics (Cotrim *et al.*, 2007a, 2007b). Additionally, the apparatus was fitted with a Lucite cone that surrounded the head and prevented head movement during the radiation exposure. Serial RTx doses at 8 Gy or 6 Gy (for the 10-day and 8-week studies, respectively) were delivered for five consecutive days by a Therapax DXT300 X-ray irradiator (Pantak, Inc., East Haven, CT) using 2.0-mm Al filtration (300 kVp) at a dose rate of 1.9 Gy/min. These RTx doses lead to significant (60%) loss of salivary flow (Cotrim *et al.*, 2005). The reason for using 8 Gy dose for mice being observed after 10 days and 6 Gy dose for mice being observed after 8 weeks was to attenuate systemic side effects in the latter group, which otherwise do not tolerate persistent oral mucositis, alimentary reduction, and complications of severe and prolonged weight loss.

Immediately after irradiation, animals were removed from the Lucite apparatus and housed (four animals/cage) in a climate- and light-controlled environment and allowed free access to food and water.

Animal evaluation

After being anesthetized as described above, the mice from control, RTx, and AdEpo treatment prior to radiation (AdEpo + RTx) were evaluated ($n=3-18$ /group; see legends). Mice were weighed and the ocular surface was evaluated by direct observation. The modified Schirmer test measured tear secretion from the right eye by placing a phenol red thread (Zone-Quick, Mericon America, Inc., San Mateo, CA), (1-mm width and 20-mm long thread) into the conjunctival fornix of the eyes for 30 sec. Blood was collected by cardiac puncture and used to measure hEpo serum levels by enzyme-linked immunosorbent assay (ELISA) and hematocrit (Hct) (Fig. 1).

Samples collection and analysis

After tear and blood collection, mice were euthanized and tissues from the three groups, at both time-points, were harvested for morphological and biochemical evaluation. LG and eye globes from the right side were embedded in 4% paraformaldehyde overnight, washed three times in tap water, and fixed in 70% ethanol. After paraffin block embedding and section cutting (5 μm), part of the samples were stained with hematoxylin and eosin (H&E). In addition, in order to detect oxidative damage, indicated by lipofuscin deposits, LG slides were stained with Kinyoun's fuchsin carbol solution for 1 hr, washed in tap water and differentiated in an acid alcohol solution (3% hydrochloric acid and

FIG. 1. Study design: The figure presents a schematic representation of the timeline of experiments conducted with mice herein. Shorter lines represent experiments with the groups that received five sessions of 8 Gy or their controls and were evaluated at Day 10 (10d). Longer lines represent experiments with groups that received five sessions of 6 Gy and were evaluated at Day 56 (56d).

95% ethanol), prior to counterstaining with hematoxylin (Electronic Microscopy Sciences, Hatfield, PA).

Slides were observed and images obtained with a light microscope (Leica Leitz DM IRB, Leica Microsystems GmbH, Wetzlar, Germany), connected to an Optiplex 960 Desktop Personal Computer (Dell, Round Rock, TX) by a Q color 5 Olympus camera (Olympus, Center Valley, PA) and MetaMorph MAG Biosystems software version 7.5.6 (MDS Analytical Technologies, Downingtown, PA). The number of focal infiltrates (defined as contiguous accumulation of 50 or more leukocytes) in tissue sections of all the three groups were counted in a blind fashion.

Corneal epithelia and whole corneal thickness were measured in histological samples converted to digital images, with Image J software (NIH, Bethesda, MD), and the data obtained from all the groups at both time-points were compared.

LG from the left side were homogenized in a solution containing 10 mM Tris Buffer, pH 7.4, 150 mM, 1% Triton and Complete Protease Inhibitor cocktail tablet (Roche Diagnostic Co, Indianapolis, IN), prepared as indicated by the provider, with Omni TH homogenizer (Omni International, Kennesaw, GA) and stored at -20°C until evaluation.

Hcts were determined in blood samples from all groups using micro hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) that collected blood samples and were centrifuged for 3 min at 12000 rpm in an LW scientific M24 centrifuge. The red blood cell fraction column length was then measured, compared with total blood column length, and expressed as a percentage.

Biochemical analysis

Measurement of human Epo in LG was performed with homogenates of the three groups, prepared as above, with a human erythropoietin ELISA kit (StemCell Technologies, Inc, Tukwila, WA) according to manufacturer's instructions, with a spectrophotometer (SpectroMax M2; Molecular Devices,

Sunnyvale, CA). In addition, Epo measurements in serum were performed in control and AdEpo+RTx-treated mice. Serum was obtained by centrifugation, and analysis was performed with the same ELISA kit.

The antioxidant enzyme glutathione peroxidase-3 was measured with ELISA kit according to manufacturer instructions (AdipoGen Inc., Incheon, Korea). The LG-secreted protein lactoperoxidase (LPO) was measured in homogenized LG samples by ELISA following manufacturer instructions (USCN Lifescience & Technology Co., Ltd, Wuhan, China). Assays to measure inflammatory cytokines (i.e., IL-1 β and TNF- α) and cell endothelial markers (i.e., vascular endothelial growth factor receptor 2 and ICAM-1) were performed using homogenized samples by multiplexed immunoassay (Aushon BioSystems, Inc, Billerica, MA).

Quantitative PCR assay for Epo DNA in LG

Quantitative PCR was performed using genomic DNA extracted from (i) the LG of mice ($n=2$) administered AdEpo in their salivary duct, (ii) the SG of the same mice (as positive controls, $n=2$), (iii) and the SG ($n=1$) and LG ($n=2$) from naive control mice (negative controls), using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). The qPCR assays were performed using above DNA samples and primers LTRTaql (5'-GTCCAGCCCTCAGCAGTTTCTA-3'), LTRTaql2 (5'-GCAGCTGCATGTGGATAAAGC-3'), and LTRTaqlprobe (5'-/56-FAM/CCATCAGATGTTTCCAGGGTG CCC/36-TSMTSp/-3') plus TaqMan Universal and PCR Master Mix (Applied Biosystems, Carlsbad, CA). The qPCR was amplified using an Applied Biosystems thermocycler. A standard curve was generated using pLTR₂EF1 α -hEPO plasmid, which was diluted in a range from 10^8 to 10^1 .

Statistical analysis

Data were expressed as means $\pm$ SD. Comparisons were made using Kruskal-Wallis test for continuous data comparison among the three groups for each time-point (i.e., 10 days or 8 weeks) and Mann-Whitney U for comparison between control and AdEpo+RTx groups (Graphpad 5.0 software, Prism, San Diego, CA). The level of significance used was $p < 0.05$.

Results

RTx-induced dry eye model

To test the ability of RTx to induce a DES condition in mice, animals were treated with five sessions of 6–8 Gy that was localized to the head and neck. The mice subjected to RTx exhibited significantly reduced body and LG weights by 10d (8 Gy) after initiation of the RTx and 56d (6 Gy) compared with controls (Table 1). Direct examination of the eyes did not reveal obvious changes; however, at 10d, tear secretion was significantly lower in the 10d (8 Gy) RTx. This was also observed at 56d time-point after 6 Gy RTx (Fig. 2). Mild hair loss in the back of the head and neck region was observed in the RTx groups (data not shown).

Histological examination at 10d of the eyes subjected to RTx revealed a reduction in the thickness of epithelial layers of the cornea. There was also an absence of cells with characteristics of basal epithelia and thicker stroma in corneas of

TABLE 1. CHARACTERISTICS (BODY WEIGHT AND LG WEIGHT) OF MICE IN CONTROL, RTx, AND AdEpo + RTx GROUPS

	Control	RTx	AdEpo + RTx
Day 10			
Body weight (g)	23.5 ± 2.1 ^{a,b,c}	17.4 ± 1.5 ^a	17.3 ± 1.3 ^b
LG weight (mg)	9.4 ± 2.4 ^d	5.6 ± 3.0 ^d	6.0 ± 2.0
Week 8			
Body Weight (g)	28.3 ± 2.3 ^e	25.2 ± 2.4	26.9 ± 2.2
LG weight (mg)	8.1 ± 1.6 ^f	5.0 ± 2.5	7.2 ± 1.6

Kruskal-Wallis test was used for multiple comparison and *post hoc* Dunn's test for comparison between pairs of all groups.

^aP = 0.0001 and ^bp < 0.05 for *post hoc* between control and RTx groups, and ^cp < 0.05 for *post hoc* test between control and AdEpo + RTx groups. There was no significant difference between AdEpo + RTx and RTx groups.

^dP = 0.02 and p < 0.05 for *post hoc* comparison between control and RTx groups. There was no significant difference between AdEpo + RTx and control and between AdEpo + RTx and RTx groups.

^eP = 0.022 and p < 0.05 for *post hoc* comparison between control and RTx groups. There was no significant difference between AdEpo + RTx and control and between AdEpo + RTx and RTx groups.

^fP = 0.018 and p < 0.05 for *post hoc* comparison between control and RTx groups. There was no significant difference between AdEpo + RTx and control and between AdEpo + RTx and RTx groups.

LG, lacrimal glands; RTx, therapeutic doses of radiation.

mice from the RTx group compared with controls (Fig. 3). However, by 56d no difference was apparent (Fig. 3E).

These results suggest that fractionated (5 days) RTx treatment was able to induce DES in both the short term (8 Gy) and long term (6 Gy). Intriguingly, the gross histology of LG was not obviously changed by RTx (Fig. 4).

Effect of AdEpo administration

In order to test if circulating Epo protein expression could protect against the damage triggered by RTx, mouse salivary ducts were cannulated and AdEpo was instilled in their SG. Human Epo presence was confirmed by qPCR of DNA isolated from SG tissue at the time of euthanasia (not shown). Serum levels of hEpo in the AdEpo + RTx mice were

FIG. 2. Tear secretion measured by phenol red thread in mice of control, therapeutic doses of radiation (RTx), and AdEpo + RTx groups at 10d (A) (n = 8–22/group), p < 0.0001 Kruskal-Wallis and p < 0.05 *post hoc* Dunn's for control vs. RTx (*) and control vs. AdEpo + RTx (#); and 56d (B) (n = 10–12/group), p = 0.0003 Kruskal-Wallis test and p < 0.05 *post hoc* Dunn's test for control vs. RTx (f) and AdEpo + RTx vs RTx (Σ).

FIG. 3. Representative images of cornea of control (A), RTx (B), and AdEpo + RTx (C) (arrows indicate epithelial basal cells, and the bar indicates the center of the cornea where the epithelial layer thickness was measured). Magnification is 400× for the figures and 100× for inserts (right lower corner). The graphs show the comparative thickness of corneal epithelial layer at 10d (D) (n = 8–18/group) and 56d (E) (n = 3–4/group) after RTx; * and # p < 0.0001, Kruskal-Wallis test, followed by Dunn's *post hoc* test.

73.2 ± 52.4 mU/ml by 10d and at 12.5 ± 6.6 mU/ml by 8 weeks (n = 4/group). In agreement with past reports (Zheng *et al.*, 2008), the AdEpo vector DNA was not detected outside SG (i.e., LG; data not shown). Consistent with the elevated Epo expression, Hct levels were significantly higher in the AdEpo + RTx mice at 10d and 56d compared to controls (Fig. 5), suggesting the Epo produced by the vector in the salivary gland was biologically active.

Importantly, there was a statistically significant increase in tear secretion by 56d (Fig. 2B) (p = 0.0003), but not at 10d (Fig. 2A). This latter finding suggests Epo expression from transduced SG could be therapeutic to LG.

Mechanism of RTx damage and AdEpo protection

To examine a possible mechanism associated with LG gland protection, tissue sections were stained with H&E. Microvessels were apparent at both time-points in the AdLTR2EF1a-hEPO RTx and control groups compared with the RTx group (Fig. 4A–C).

In corneas of the AdEpo + RTx group, there were a higher number of epithelial layers and cells with large round nuclei, which was more similar to morphology observed in the corneas of the control group than with the RTx group (Fig. 3).

To test for oxidative damage, LG sections were stained for cytoplasmic lipofuscin. However, no change was observed compared to age-matched controls (Rios *et al.*, 2005) (Fig. 4D–

FIG. 4. Upper lane: Representative histological images of the lacrimal glands (LG) of mice from control (**A**), RTx (**B**), and AdEpo+RTx (**C**) groups stained with H&E (arrows: microvessels). Lower lane: LG of aging (26 months old) female mouse (**D**), mouse submitted to RTx (**E**), and AdEpo+RTx (**F**) stained with Kinyoun's fuchsin carbol and counterstained with hematoxylin (arrow: lipofuscin staining). Magnification 100 $\times$ for the figures and 400 $\times$ for inserts that show details of acinar cells structure (right lower corner).

F). Also, to test for antioxidant response to RTx, changes in the level of the antioxidant enzyme glutathione peroxidase-3 were also compared in LG homogenates of the different groups. Glutathione peroxidase-3 levels were reduced in the LG of AdEpo+RTx mice at 10d compared with either control or RTx-only groups; however, glutathione peroxidase-3 levels were unchanged at 56d (Table 2). The LG-secreted protein LPO, which is related to innate defense, remained unchanged at both 10d and 56d, and the inflammatory markers IL1 β , TNF- α , and ICAM-1 also remained unchanged among the three groups at either time-point (Table 2).

The levels of vascular endothelial growth factor (VEGF) receptors, a marker of microvessel endothelial cells, were compared in the LG of the three groups. VEGF receptor levels were lower at 10d in the LG of the RTx group compared with control and AdEpo+RTx mice ($p=0.03$) (Fig. 6A), consistent with a reduction in microvessel endothelia in the LG of RTx

mice as previously described regarding SG (Cotrim *et al.*, 2007b). At 56d, the levels of VEGF receptors were not significantly different among the three groups, despite a trend toward lower levels in the RTx group (Fig. 6B).

Discussion

This study represents a detailed evaluation of ocular surface changes and tear production rate decreases in mice that previously have been studied as a model of RTx-induced salivary dysfunction. This model develops both DES and dry mouth, with a deficiency in 60–70% of saliva volume, as previously reported (Cotrim *et al.*, 2007a), and a ~50% reduction in tear secretion (herein). Moreover, we observed that neither the body nor the LG weight recovered to control levels in the RTx groups at 56d. Similarly, tear flow remained low, suggesting the lower 6Gy RTx dose employed was sufficient to induce long-term damage and serve as a model for DES. An animal model that presents with a combination of DES and dry mouth caused by RTx, as shown here and in previous publications, will be useful for future physiopathological and therapeutic studies (Cotrim *et al.*, 2007b; Vouretakis *et al.*, 2007; Jeganathan *et al.*, 2011).

The mechanism leading to RTx structural and secretory damage was investigated and, although still unclear, may be related to a transient loss in vascularization of the tissue. At the time-points measured, neither oxidative stress nor inflammatory markers showed altered expression in the RTx group compared with controls. One possible reason for the lack of correlation with oxidative stress and inflammatory markers could be that the biomarkers selected to identify these phenomena (i.e., antioxidant enzymes for oxidative stress and cytokines for inflammation) do not persist after RTx treatment. This was observed previously in RTx studies, where lipid peroxidation and 8-hydroxydeoxyguanosine (8-OHdG) were restored to control

FIG. 5. Systemic effects of AdLTR2EF1a-hEPO administration to salivary glands. Hematocrit of mice from control and AdEpo+RTx groups at 10d (**A**) ($n=4-8$ /group, $p=0.0079$, Mann-Whitney U) and 56d (**B**) ($n=4-8$ /group, $p=0.0084$, Mann-Whitney U) after treatment.

TABLE 2. BIOMARKERS OF SECRETORY FUNCTION, ANTIOXIDANT REACTIONS, AND INFLAMMATORY ACTIVITY IN THE LG OF MICE FROM CONTROL, RTx, AND AdEpo+RTx GROUPS AT DAY 10 AND WEEK 8 OF RTx

	Control		RTx		AdEpo+RTx	
	Day 10	Week 8	Day 10	Week 8	Day 10	Week 8
Lactoperoxidase (Umol/l)	0.59±0.03	0.54±0.01	0.54±0.02	0.53±0.02	0.58±0.04	0.56±0.03
Glutathione peroxidase-3 (ng/ml)	17.8±3.6	11.0±2.7	14.2±2.1	11.4±4.3	8.3±1.7*	13.5±2.3
Erythropoietin (mU/ml)	1.8±1.1	N/P	2.3±0.9	N/P	2.7±1.2	N/P
Interleukin-1 β (pg/ml)	52.0±13.7	82.3±14.0	36.3±28.4	77.1±33.3	76.7±36.9	91.1±13.1
TNF- α (pg/ml)	10.8±2.2	11.7±2.8	8.5±1.8	21.7±17.1	10.8±3.6	21.3±11.0
ICAM-1 (ng/ml)	8.4±5.2	6.9±2.5	6.1±2.8	5.2±2.2	5.5±1.5	7.0±2.1

*P=0.0096 for GPx-3 in LG of AdEpo+R_xT, compared to control and RTx groups at Day 10 (Kruskal-Wallis). All other comparisons among the three groups at the same time-point were nonsignificant (p>0.05). N/P means analysis not performed.

levels after radiation, and represents a distinct observation not seen in chronic and persistent challenges to the LG, as with diabetes mellitus or during normal aging (Kasai *et al.*, 1986; Nishi *et al.*, 1986b; Alves *et al.*, 2005; Jorge *et al.*, 2009).

The possibility that a regenerative process occurs in the LG after the RTx damage, and it is enhanced by AdEpo treatment, must also be considered. The complexity of this response has recently been reviewed and detailed and reported in mice after interleukin-1 injection in the LG (Zoukhri *et al.*, 2007; Zoukhri, 2010).

Importantly, we found that transgenic Epo expression post RTx provided significant protection against loss of tear secretion. The anabolic effect of Epo is now well recognized to extend beyond the red blood cell lineage, for example, in epithelial cells (Brines and Cerami, 2006; Arcasoy, 2008b). Indeed, it has been demonstrated that Epo can have a role in retinal protection against lipopolysaccharide injury in sheep, and it has been suggested that it plays a role in age-related macular disease and pterygium due to its vasculo-proliferative characteristics (Kase *et al.*, 2007; Loeliger *et al.*, 2011). In the present report, Epo clearly mitigates acute corneal, LG epithelial, and also vascular damage related to RTx.

Although it is unknown whether Epo overexpression could jeopardize the outcome of patients with neoplasia, the strategy of SG- or LG-targeted expression may be useful to facilitate wound healing in several diseases that cause neurotrophic or inflammatory keratopathy (e.g., facial palsy,

diabetic neuropathy, Hansen disease, SS, herpes, etc.). Similarly, additional studies are needed to clarify whether the epithelial protection offered by Epo observed here would have additional clinical benefits (e.g., diminish pain, photophobia, and blurred vision).

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Author Disclosure Statement

No competing financial interests exist.

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FIG. 6. Vascular endothelial growth factor (VEGF) receptor expression in LG of control, radiation, and AdEpo+RTx groups at 10d (A) and 56d (B); *p=0.03, Kruskal-Wallis test, followed by Dunn's *post hoc* test, p<0.05 for RTx versus control group.

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