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Aqueous humor induces lymphatic regression on the ocular surface

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Abstract

PURPOSE: This study is to investigate the potential effect of aqueous humor on already formed lymphatic vessels of the ocular surface including the conjunctiva and the cornea.

METHODS: Aqueous humor harvested from fresh bovine or murine eyeballs were used in the study. It was injected into the subconjunctival space of Prox-1-GFP (green fluorescent protein) transgenic mice. Pre-existing conjunctival lymphatics were observed in vivo using our advanced live imaging system. Additionally, ex vivo tissue cultures were performed in aqueous humor with normal conjunctival tissues or inflamed corneas with newly formed lymphatic vessels. Time lapse images were taken by an advanced live cell imaging system with an incubator. Moreover, human primary microdermal lymphatic endothelial cell culture system was employed to evaluate the effect of aqueous humor on lymphatic tube regression in vitro.

RESULTS: Aqueous humor induced lymphatic regression in both normal conjunctiva and inflamed corneas. It also led to the regression of formed lymphatic tubes by the lymphatic endothelial cells in vitro.

CONCLUSIONS: This study provides the first direct and real time live imaging evidence showing that aqueous humor induces lymphatic regression. Further investigation promises for divulging new mechanisms and therapeutic strategies to treat lymphatic diseases that occur both inside and outside the eye.

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Keywords

ocular surface; aqueous humor; cornea; conjunctiva; lymphatic vessel; live imaging; endothelial cell function assay

1. Introduction

Lymphatic research represents a field of new discovery in recent years and it has progressed rapidly after centuries of negligence. The lymphatic network penetrates most tissues in the body, and it plays crucial roles including immune surveillance and tissue fluid regulation. Lymphatic dysfunction is associated with many diseases, which include but are not limited to inflammation, infection, transplant rejection, glaucoma, and cancer metastasis[1–4].

The ocular surface is heterogeneous in lymphatic composition. While normal adult cornea is by nature transparent and devoid of any lymphatic vessels, the nearby tissue of conjunctiva is rich in lymphatic supply like many other tissues of the body, such as the skin. It is also known that under a pathological situation, the alymphatic cornea can be invaded by newly formed lymphatic vessels after an inflammatory, infectious, immunogenic, traumatic, or chemical insult, and the lymphatic-invaded cornea is hostile for transplant survival[1, 4]. While the focus of lymphatic research in the field has been on the prevention of lymphatic formation and we have been successful with this effort by targeting on a variety of factors of the VEGF (vascular endothelial growth factor) and integrin families [1, 5, 6], it is much more difficult to induce lymphatic regression once the vessels are formed and established. To date, we have scarce knowledge about lymphatic regression and this research area is still in its infancy. However, it is very important to study lymphatic regression because many patients who suffer from corneal diseases have lymphatic vessels already formed in their corneas, and these patients are poor candidates for vision restoration by transplantation.

The anterior chamber of the eye is filled with aqueous humor (AH). Under normal condition, it is an immune privileged site where corneal transplants enjoy a high rate of survival[1, 4, 7]. AH is a clear colorless liquid produced by ciliary body processes. These processes are delicate finger-like structures that contain stromal capillaries and a bilayer epithelium, the pigmented and non-pigmented epithelium. AH is formed from blood plasma but has lower concentration of proteins and higher concentrations of ascorbate and lactate. It mainly contains water, organic ions, carbohydrates, glucose, and amino acids[8]. Under normal condition, AH exits the anterior chamber through the conventional pathway via trabecular meshwork and Schlemm’s canal, and the nonconventional pathway via uveoscleral outflow[9]. In many ocular surgeries, AH can escape to ocular surface tissues from a surgical incision or opening, whether the procedures are performed to treat corneal opacity, glaucoma, or retinal detachment. Under normal situation, AH functions to provide optical transparency, maintain intraocular pressure, nourish ocular structures, clear waste products, and to facilitate immune quiescence of the anterior segment[10–16]. The potential role of AH on ocular surface lymphatics is largely unknown. Recently, it was reported that AH can prevent the formation of lymphatic vessels in the inflamed cornea[17], however, that study

did not investigate the role of AH on already formed lymphatic vessels in the cornea or the conjunctiva, which are the major focuses of the current study.

In this study, using a unique combination of a panel of in vivo, ex vivo, and in vitro experimental models, the fluorescently labeled transgenic mice of Prox-1 (the master control gene for lymphatic development), human primary microdermal lymphatic endothelial cell (LEC) culture system, and the highly advanced intravital imaging and live cell imaging systems, we provide the first direct and live imaging evidence showing that AH can induce lymphatic regression in vivo, ex vivo, and in vitro. This regression occurs with normal conjunctiva, inflamed cornea, and the lymphatic tubes of the primary endothelial cells as well. The study bears broad implications and offers novel insights into AH and lymphatics. Further investigation promises for divulging new mechanisms and therapeutic strategies to manage lymphatic diseases inside and outside the eye.

2. Methods

2.1. Animal

All mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all protocols were approved by the Animal Care and Use Committee of the institute. C57BL/6 mice were purchased from The Jackson Laboratory (Sacramento, CA) and Prox-1 GFP (green fluorescent protein) mice were generated as reported previously[18, 19]. Seven to ten weeks old adult mice of either sex were used in the study. Mice were anesthetized using a mixture of ketamine, xylazine, and acepromazine (50 mg, 10 mg, and 1 mg/kg body weight, respectively) for each surgical procedure. This was complemented with topical anesthesia using 0.5% proparacaine hydrochloride ophthalmic solution (Bausch & Lomb, Rochester, NY). The experiments were repeated 2–3 times with 4–10 mice in each group. A total of 62 mice were used in the study.

2.2. Aqueous humor preparation

AH was collected from fresh bovine or murine eyeballs. Fresh bovine eyeballs were purchased from Sierra for Medical Science (Whittier, CA, USA). Eyeballs were sterilized with 2% iodine solution and washed with sterilized PBS. AH was harvested from the anterior chamber, aliquoted, stored in −80°C, and filtered with 0.45 μm membrane before use. Due to the limited amount of AH that can be collected from murine eyeballs, most data were collected with bovine AH except for Supplementary Figure 1 where murine AH was used to confirm the result.

2.3. Subconjunctival injection

Mice were randomized to receive either 10 μl of AH or control PBS in the subconjunctival space using a Hamilton syringe (Hamilton, Reno, NV, USA). Antibiotic ointment was applied after each injection.

2.4. Cornea suture placement

The experiment was performed as previously reported[18, 20]. Briefly, four 11–0 sutures (AROSurgical, Newprot Beach, CA, USA) were placed into the corneal stroma without

penetrating into the anterior chamber. The sutures were evenly distributed in the nasal and temporal side and at equal distance from the limbus. Antibiotic ointment was applied after each procedure.

2.5. Intravital imaging

Intravital imaging was performed as we previously reported [18, 21]. Briefly, mice were slightly anesthetized with 2% isoflurane supplemented by 0.5% proparacaine hydrochloride ophthalmic solution. Z-stack digital images were captured by an advanced custom-built live imaging system with the Axio Zoom V16 microscope (Carl Zeiss AG, Gorringen, Germany) and processed with Helicon Focus imaging software (Heliconsoft Ltd).

2.6. Ex vivo tissue culture and time lapse imaging

Normal conjunctival or inflamed corneal tissues at day 5 post suturing were harvested for ex vivo culture inside a controlled environmental incubator (Incubator XL, 37°C, humidified 5% CO_2) of an advanced Zeiss live cell imaging system (Axio Observer 7, Carl Zeiss AG). Tissue samples were processed with 2% iodine solution, washed in PBS, placed on a Nylon cell strainer membrane (VWR, Radnor, PA, USA), seeded into a 96-well plate, and cultured with a mixture of 70% AH and 30% DMEM culture medium (Gibco, Life Technologies Corporation, Grand Island, NY, USA) or 100% DMEM as control for 12 h. Time lapse digital images were taken under GFP excitation light for further analysis.

2.7. Lymphatic endothelial cell culture and tube formation assay

The experiment was performed as we reported previously [5, 22]. Briefly, human neonatal microdermal LECs were purchased from AngioBio (AngioBio Co., Del Mar, CA, USA), and maintained in EBM MV2 growth medium (Promocell, Heidelberg, Germany), supplemented with 10% bovine calf serum (HyClone, Logan, Utah) and 1% penicillin/streptomycin. For the tube formation assay, 10,000 cells were seeded onto the 96 well plates coated with Matrigel (Corning, Bedford, MA, USA). Four hours after plating and in the middle of capillary type tube formation, the culture medium was replaced by AH or control medium. Five hours later, phase contrast images were taken using a Zeiss Axio Observer A1 inverted microscope (Carl Zeiss AG). The assays were performed in triplicate and repeated once.

2.8. Immunofluorescent microscopic assay

The experiment was performed as reported previously[18, 20]. Conjunctival tissues at 3 days post subconjunctival injection were harvested from Prox-1 GFP mice, fixed in 4% formaldehyde (PFA), and stained with the anti-LYVE-1 (lymph vessel endothelial hyaluronan receptor-1) primary antibody (Abcam, Cambridge MA, USA), which was recognized by a Cy3 secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Samples were covered with the mounting medium (FluoroshieldTM with DAPI, Sigma-Aldrich, MO, USA) and examined by a Zeiss AxioImager M1 epifluorescence deconvolution microscope (Carl Zeiss AG).

2.9. Lymphatic quantification

Digital images were analyzed using ImageJ software (http://imagej.nih.gov/ij; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). For in vivo conjunctival lymphatic quantification, images taken at the superior quarter from 9 to 3 o'clock were analyzed by segment line length measurement. For ex vivo tissue culture, images were analyzed by lymphatic area measurement. For in vitro tube formation assay, images were analyzed by ImageJ angiogenesis plugin. All data were obtained by normalizing to control condition defined as being 100%, as reported previously [22, 23].

2.10. Statistical analysis

The statistical significance between two groups was assessed by Graphpad Prism 8 (GraphPad Software, La Jolla, CA, USA). Data were reported as mean ± SEM. Data were analyzed using two-way repeated measures ANOVA with a Bonferroni post hoc test to compare between control and experimental groups at multiple time points. The other results were assessed by Student's t-test. The differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Aqueous humor induced the regression of pre-existing conjunctival lymphatic vessels in vivo

We first assessed the effect of AH on pre-existing lymphatic vessels in the conjunctiva by injecting either AH or control PBS into the subconjunctival space of the Prox-1 GFP mice. The dynamic changes of the lymphatic vessels were monitored by our advanced live imaging system. As shown in Figure 1A, our results from this time course in vivo study showed a dramatic regression of conjunctival lymphatic vessels in the AH injected group. Results from repeated experiments were presented in Figure 1B (*$P < 0.05$).

To further confirm the regression phenomenon we observed in vivo is indeed due to the structural disappearance of the lymphatic vessels inside the conjunctival tissue, we also harvested the conjunctival tissues and performed an immunofluorescent microscopic assay with the anti-LYVE-1 antibody. The results were compared with the intravital images. As demonstrated in Figure 2A and 2B, the same vessel that displayed regression in vivo also showed the absence of LYVE-1 staining with the immunostaining assay. Images of the control condition are presented in Supplementary Figure 1. Taken together, these results confirmed that AH can induce lymphatic regression in the conjunctiva in vivo.

3.2. Aqueous humor induced conjunctival lymphatic regression in culture

Next, we harvested conjunctival tissues for ex vivo culture and assessed the effect of AH using the advanced live cell imaging system. In this case, lymphatic regression was observed in both the experimental group with AH and the control group with control medium, as demonstrated in Figure 3A. However, the experimental group with AH showed a significantly greater degree of regression than the control condition. Summarized data from repeated experiments are presented in Figure 3B (*$P < 0.05$).

3.3. Aqueous humor induced lymphatic regression in inflamed cornea

Further, we evaluated whether AH can induce the regression of pathological lymphatic vessels in the cornea. The standard corneal inflammation model induced by suture placement was used in this study. The inflamed corneas invaded by lymphatic vessels were harvested at day 5 post suturing and cultured in the presence of AH or control medium. As shown in Figure 4A, compared with the control condition where only a mild lymphatic regression was observed, the corneas exposed to bovine AH demonstrated a greater scale of lymphatic regression. Summarized data from repeated experiments were presented in Figure 4B (*$P < 0.05$). This effect of lymphatic regression was also observed with murine AH, as presented in Supplementary Figure 2 (*$P < 0.05$).

3.4. Aqueous humor induced the regression of lymphatic tubes formed by human primary endothelial cells in vitro

To further explore the effect of AH on lymphatic regression, we employed another in vitro cell culture system with human primary microdermal LECs. The LEC tube formation assay was performed as we reported previously with some modifications [5, 22]. In this case, in order to study lymphatic regression, we allowed the cells to form capillary type tubes first. Four hours after plating and in the middle of tube formation, the culture medium was replaced by AH or control medium. Five hours later, phase contrast images were taken for comparison. As shown in Figure 5A and 5B, a significant difference was observed with the lymphatic network in the experimental and control groups. In the experimental group with the presence of AH, significantly fewer tubes and looser meshes were noticed. Representative images showing regressed tubes with disrupted ends were presented in panel 5B. Summarized data with repeated experiments are shown in Figure 5C (*$P < 0.05$).

Discussion

In this study, by using a panel of in vivo, ex vivo, and in vitro experimental approaches and several advanced experimental tools for live imaging, we provide the first direct and conclusive evidence showing that AH can induce lymphatic regression in both normal and pathological situations. We have shown that AH can induce lymphatic regression in normal conjunctiva, in inflamed cornea, and with primary human microdermal cells as well.

The combination of the highly advanced live imaging system and the Prox-1 GFP transgenic mice gives us a unique opportunity to catch the novel phenomenon that pre-existing conjunctival lymphatics can regress upon the exposure to AH in vivo. This phenomenon bears great clinical significance since many ocular surgeries are associated with AH leakage to the conjunctiva. Since the functions of the conjunctival lymphatics are yet to be fully explored, we remain interested to further explore this phenomenon in future studies, whether it relates to a positive or negative effect for ocular disease management. In glaucoma filtration surgery where AH is intentionally introduced to the subconjunctival space to treat intraocular hypertension, it would be interesting and important to know how the procedure affects conjunctival lymphatics and what would be the overall effects on the outflow facility and pressure control.

The effect of AH on inflamed corneal lymphatic regression is novel and exciting. Further investigation on this finding may offer new therapeutic strategies to treat pathological lymphangiogenesis and related diseases such as inflammation, infection and transplant rejection. In this case, as a natural product of the eye, AH should have no or fewer issues of toxicity or side effects. It is yet to be determined what specific factor(s) of the AH attribute to the effect of lymphatic regression. Various immuno-regulatory factors have been reported in AH, such as alpha-melanocyte stimulating hormone (a-MSH), vasoactive intestinal peptide (VIP), thrombospondin-1 (TSP-1), transforming growth factor beta (TGF-β), and Fas ligand (FasL) [24–27]. However, none of the previous studies explored the topic of lymphatic regression, which demands further assessment. It is also possible that this phenomenon of lymphatic regression is mediated by some unknown factor(s) of the AH, which merits further investigation as well.

In conclusion, this study offers novel insights into AH, ocular surface, and lymphatics. This study combining intravital imaging in live subjects and human primary cell work should provide highly translatable information for patient care in the future. Given the broad distribution of lymphatic vessels in the body and their involvement in various diseases, it is hopeful that this study will facilitate lymphatic research and related disease management in multiple disciplines inside and outside the eye.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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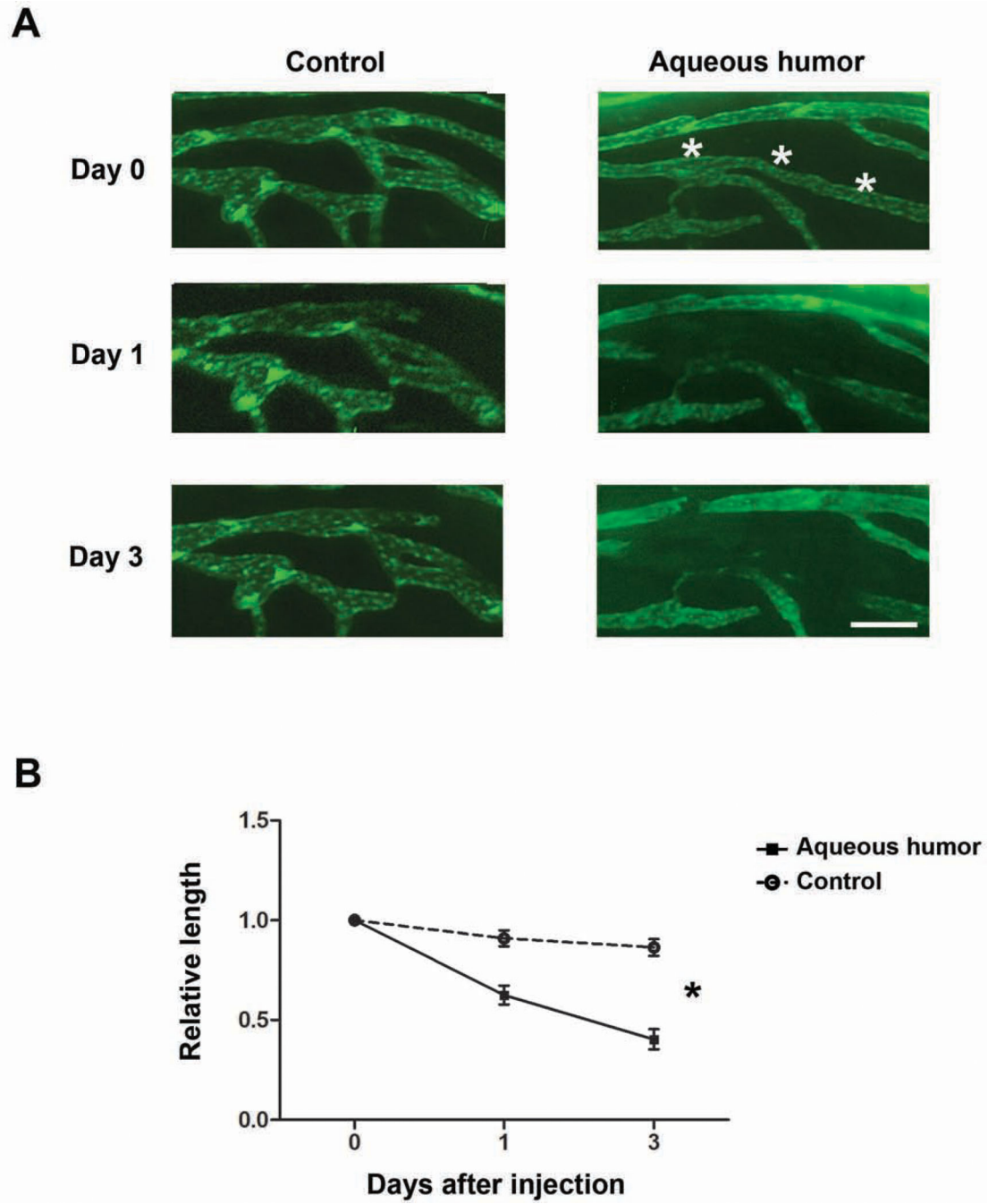

Figure 1.

Aqueous humor induced conjunctival lymphatic regression in vivo. (A) Intravital images taken at different time points before and after the subconjunctival injections of AH or control PBS, respectively. White asterisks indicate the vessel that regressed with time. Green, Prox-1; Scale bar: 100 μm. (B) Summarized data assessed in lymphatic length measurement. *$p < 0.05$.

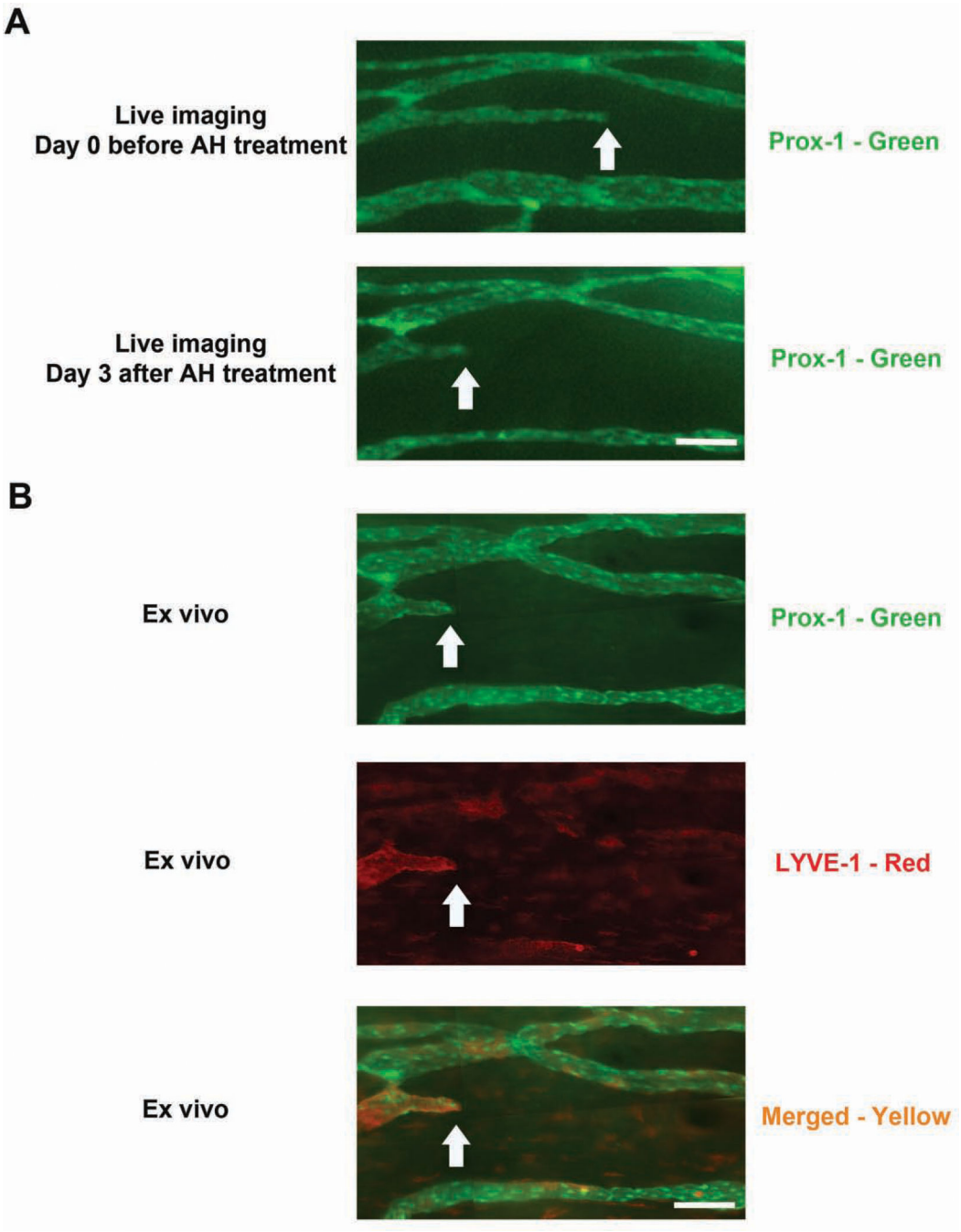

Figure 2.

Lymphatic regression confirmed by both intravital imaging and ex vivo immunofluorescent microscopic analysis. (A) Intravital images before and after the subconjunctival injections of AH, respectively. Scale bar: 50 μm. (B) Ex vivo anti-LYVE-1 staining showing the disappearance of LYVE-1 signal in the regressed lymphatic vessel. White arrows indicate the end of the regressed vessel. Green, Prox-1; Red: LYVE-1; Scale bar: 100 μm.

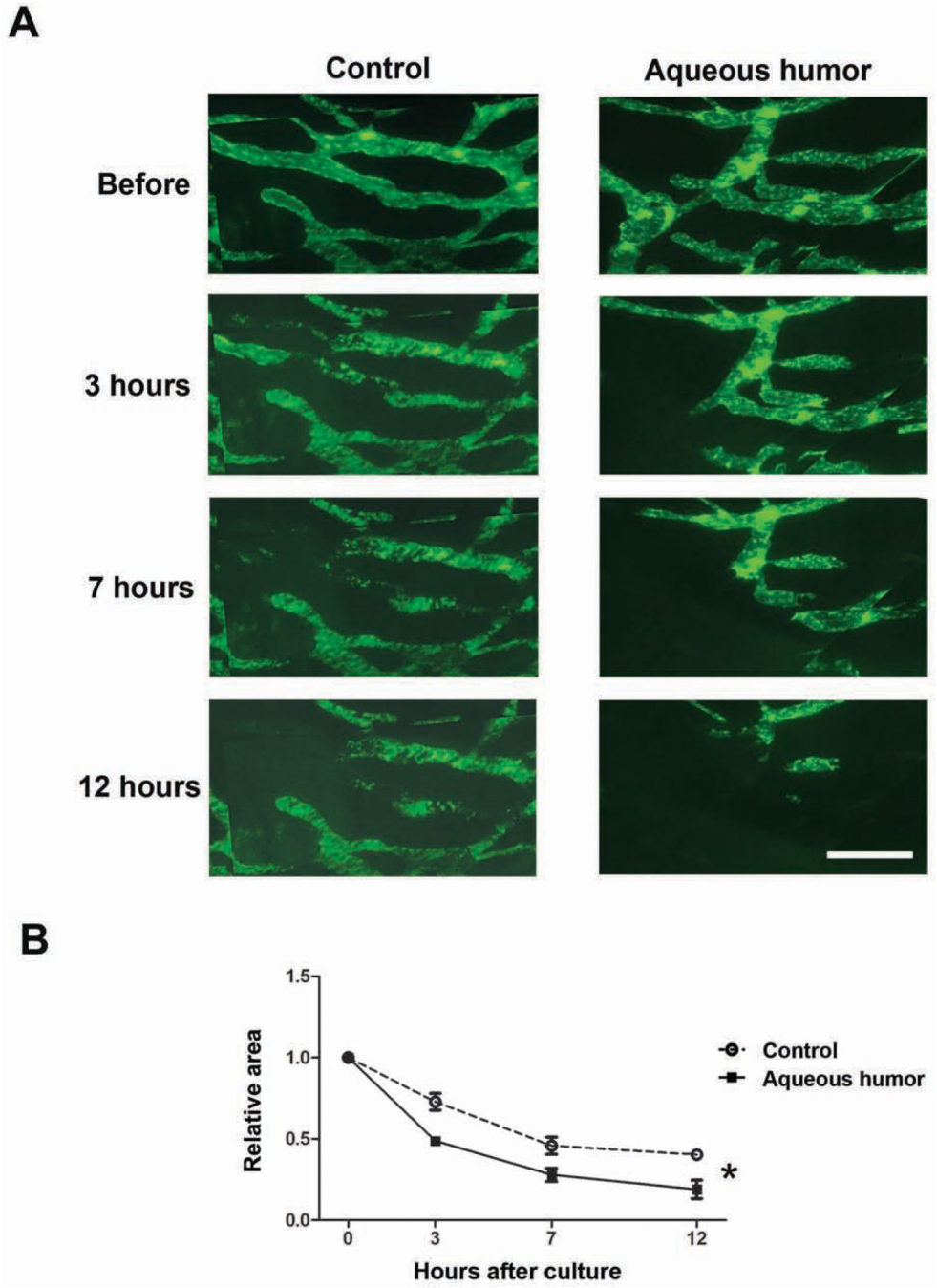

Figure 3.

Aqueous humor induced conjunctival lymphatic regression in culture. (A) Time lapse images taken at different time points before and after tissue culturing with AH or control medium, respectively. Green: Prox-1; Scale bar: 200 μm. (B): Summarized data assessed in lymphatic area measurement. *$p < 0.05$.

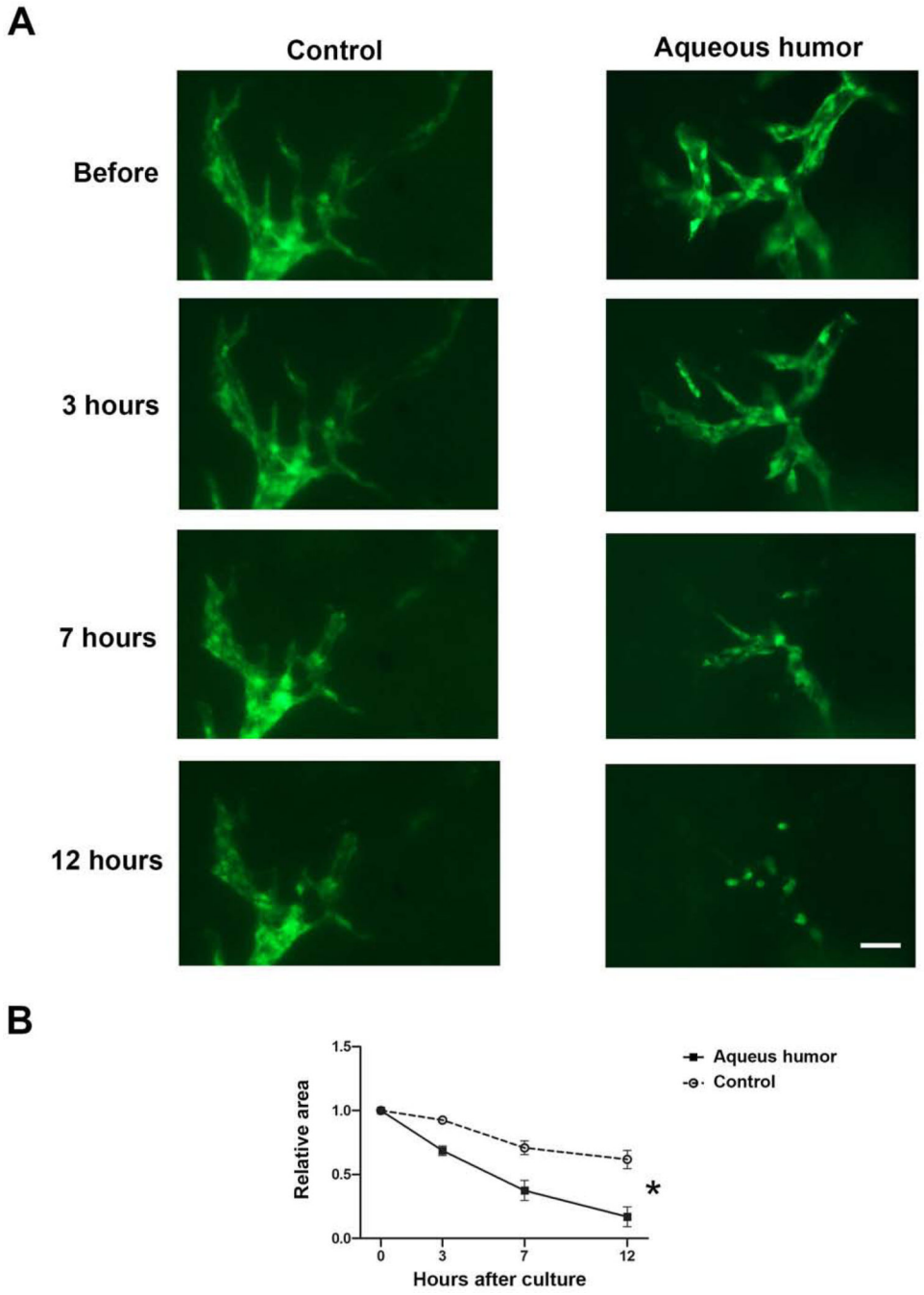

Figure 4.

Aqueous humor induced the regression of formed lymphatic vessels in the inflamed cornea. (A) Time lapse images taken at different time points before and after culturing with bovine AH or control medium. Green, Prox-1; Scale bar: 50 μm. (B) Summarized data assessed in lymphatic area measurement. *$p < 0.05$.

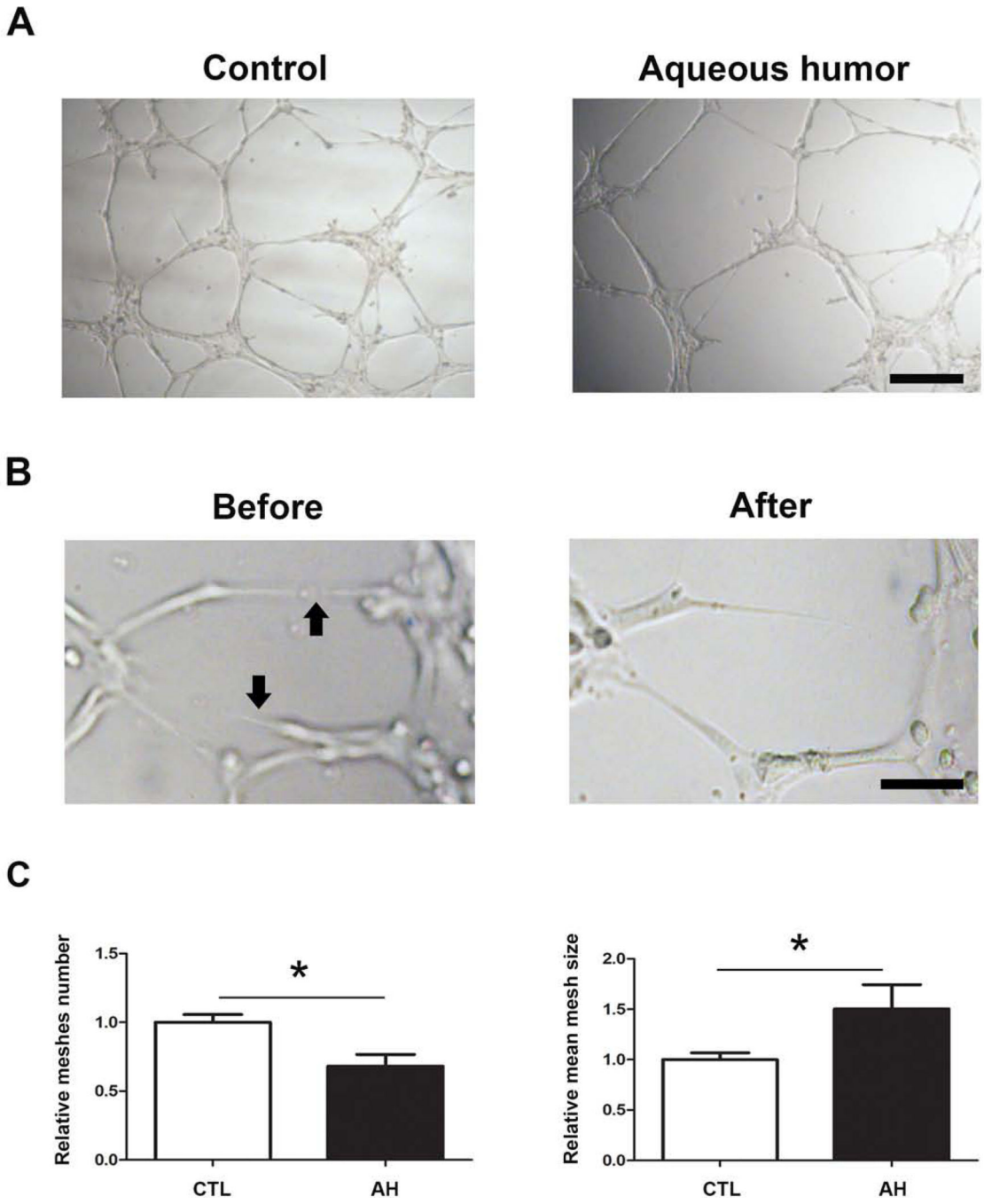

Figure 5.

Aqueous humor induced the regression of lymphatic tubes formed by human microdermal lymphatic endothelial cells in vitro. (A) Representative images showing lymphatic meshes 5 hours after culturing with AH or control medium, respectively. Scale bar: 100 μm. Images showing the sites of regression, as indicated by the arrows, are presented in (B). Scale bar: 20 μm. (C) Summarized data assessed in the number and mean size of the meshes, respectively. Assays were performed in triplicates and repeated once. *$p < 0.05$.