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Local Adenoviral Delivery of Vascular Endothelial Growth Factor C Induces Lymphangiogenesis in the Conjunctiva in Rabbits

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Keywords
Vascular endothelial growth factor C · Lymphangiogenesis · Adenoviral gene therapy · Glaucoma surgery

Abstract

Introduction: The purpose of this study was to determine if conjunctival lymphangiogenesis can be induced using adenoviral delivery of vascular endothelial growth factor C (VEGF-C). ***Methods:*** Seventeen New Zealand white rabbits received a subconjunctival injection containing 3.5×10^7 plaque-forming units of an adenoviral vector containing the gene-encoding VEGF-C (Ad-VEGF-C). The contralateral eye was used for control experiment (the same volume of either saline or an empty vector). After 2 weeks, the animals were examined with trypan blue conjunctival lymphangiography, and the eyes were harvested for histology and immunohistochemistry (podoplanin and CD31). ***Results:*** Trypan blue conjunctival lymphangiography revealed significantly more extensive conjunctival vessel network in the Ad-VEGF-C group compared with control: 1.35 ± 0.67 versus 0.28 ± 0.17 vessel length/analysed area (p = <0.0001). This finding was confirmed with immunohistochemistry, where a significant increase in the number of lymphatic vessels was found compared to control; 34 ± 9 per mm^2 versus 13 ± 8 per mm^2 (p = 0.0019). Furthermore, there was a significant increase in lymphatic cross-sectional area; 32,500 ± 7,900 μm^2 per mm^2 versus 17,600 ± 9,700 μm^2 per mm^2 (p = 0.0149). Quantification of blood vessels revealed no significant difference in blood vessel density between Ad-VEGF-C and control; 19 ± 9 per mm^2 versus 14 ± 8 per mm^2 (p = 0.1971). There was no significant difference in total blood vessel area; 13,200 ± 7,600 μm^2 per mm^2 versus 7,100 ± 3,000 μm^2 per mm^2 (p = 0.0715). Eyes treated with an adenoviral vector (VEGF-C or empty vector) responded with a reactive cellular response, predominantly lymphocytes, towards the vector. ***Conclusion:*** The study demonstrates the feasibility of inducing conjunctival lymphangiogenesis with a single subconjunctival injection of Ad-VEGF-C. Future studies will explore how this can be used with a therapeutic purpose.

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Introduction

Throughout the body, lymphatic vessels remove excessive fluid from the tissues and modulate inflammation by regulating immune cell trafficking. The lymphatic vasculature has received little attention in ophthalmology, with most efforts targeted towards the cornea. There is a dense lymphatic network in the conjunctiva [1]. The almost invisible nature of the vessels makes it impossible for the clinician to appreciate this and is contrasted by the clearly visible adjacent blood vessels. The role of the conjunctival lymphatic vessels in normal physiology and in pathology is poorly understood and is to a large extent explained by the difficulties of studying the vessels. The era of molecular medicine with the discovery of lymphatic-specific markers, the ability to develop genetically altered animals, and advances in imaging technology has been a paradigm shift. These advances have made it possible to show that corneal lymphangiogenesis plays an essential part in the rejection of corneal transplants [2], which confirms the importance of the lymphatics in the ocular surface immune system. Lymphatic drainage has been demonstrated in blebs after filtration surgery in both animals and humans [3, 4] and is suggested to be important for the success rate of the procedures.

Lymphangiogenesis is often a normal response after tissue damage and inflammation and can both be transient and permanent, reflecting the great plasticity of the lymphatics. The vascular endothelial growth factor C (VEGF-C) and vascular endothelial growth factor receptor 3 (VEGFR-3) axis has a central role in lymphangiogenesis. VEGFR-3 is expressed by lymphatic endothelial cells, and stimulation results in proliferation and migration of the cells [5]. Targeting lymphangiogenesis has proven to be a successful treatment strategy in conditions where oedema and inflammation play a role [6].

Therapeutical lymphangiogenesis may be beneficial in inflammatory conditions or when increased fluid removal is needed, e.g., glaucoma surgery. To investigate this, the feasibility of inducing conjunctival lymphangiogenesis must first be demonstrated. The hypothesis of this study was that conjunctival lymphangiogenesis can be induced using an adenoviral vector.

Materials and Methods

Experimental Protocol

Seventeen female New Zealand white rabbits (age 12–14 weeks) were treated with 3.5×10^7 plaque-forming units of Ad-VEGF-C (Herantis Pharma Plc, Finland) with the contralateral receiving the same volume of saline or the same dose of an empty vector (Cat. no. 1300, Vector Biolabs, USA). The adenoviral construct encodes the human isoform of VEGF-C. The control group thus consisted of two different cohorts, one receiving an empty vector (n = 14) and one receiving the same volume of saline (n = 3). The volume was 50 µL, which was injected subconjunctivally with a 33 G cannula in the supero-temporal quadrant within 1 mm of the limbus. The procedure was performed with the animal sedated (described below). After 2 weeks, 12 of the animals were sedated and conjunctival trypan blue lymphangiography was performed. Afterwards, the animals were sacrificed and eyes as well as eyelids were harvested en bloc and fixed in 10% neutral-buffered formalin. A 7-0 vicryl marker suture was placed in the cornea limbus in the supero-temporal quadrant. Two animals underwent the same protocol but with the termination date set to 4 weeks. Three animals were sacrificed after 2 weeks with conjunctiva excised and snap frozen in Eppendorf vials in dry ice for later reverse transcriptase PCR (RT-PCR) analysis. Animals were sedated with one of two protocols; protocol 1: medetomidin 0.35 mg/kg, ketamin 20 mg/kg, and diazepam 1 mg/kg; protocol 2: midazolam 6 mg/kg, ketamine 25 mg/kg, and propofol 2.2–10 mg/kg. In both protocols, Metacam 1 mg/kg was administered for post-operative pain management.

Conjunctival Trypan Blue Lymphangiography

A 7-0 vicryl corneal traction suture was placed at 12 o'clock and the eye was rotated downwards. Trypan blue (0.055%) was injected subconjunctivally at the limbus with a 33 G needle at three positions: the supero-temporal and supero-nasal quadrants as well as over the superior rectus muscle. The dye was slowly injected while uptake in lymphatic vessels was noted. No additional dye was injected once a reasonably sized bleb was formed (<50 µL injection volume). Photos were exported to ImageJ for analysis. The lymphatic vessels were manually mapped, and total length was calculated and divided by the total area. This was repeated for all three injection sites, and an average was calculated for each eye.

Histology

Eyes were harvested and fixed in 10% neutral buffered formalin solution for at least 48 h at room temperature. The whole globes were processed and embedded in paraffin tissue blocks using standard techniques. The globes were serially sectioned (3-µm sections) in the sagittal plane. The treated eye region was identified by the presence of a marker suture. The resulting sections were then de-paraffinized and rehydrated in xylene and graded ethanol and stained using standard haematoxylin and eosin for general tissue and cellular morphology. Collagen changes were assessed with both haematoxylin and eosin and Picrosirius red. Image analysis was performed in QuPath v.0.2.3 (Queen's University, Belfast, Northern Ireland). Fibrosis and collagen deposition were assessed semiquantitatively (zero to three) by two masked and independent pathologists based on five representative slides from each eye. Cellular infiltration was quantified on a zero-to-three scale (no cells, <10 cells, 10–40 cells, and >40 cells per high-power field). An average score for each eye was calculated based on the two pathologists grading.

Immunohistochemistry

Myofibroblasts were assessed by immunohistochemistry using a primary mouse anti-α-smooth muscle actin (α-SMA) antibody (clone 1A4Dako, Agilent). Mouse anti-rabbit podoplanin (clone PMab-32, Wako Pure Chemicals) and mouse anti-human CD31

(clone JC/70A, Dako, Agilent) were used for detection of lymphatic vessels and all vessels. Sections were dewaxed and then rehydrated in graded alcohol. Endogenous peroxidase activity was quenched by immersion in 1.5% hydrogen peroxide followed by heat-induced epitope retrieval (HIER) in T-EG (9.0 pH for 15 min) for α-SMA, citrate buffer (6.0 pH for 15 min) for podoplanin, and CC1 antigen retrieval buffer (48 min at 100°C, Roche) for CD31. Antibodies were detected using EnVision+ detection system (EnVision™+ System, HRP-labelled polymer; K4001; Agilent Technologies) and mouse DISCO-HQ-HRP_Ms (Roche) and visualized with diaminobenzidine (DAB) as chromogen. Finally, the sections were counterstained using Mayer's haematoxylin. For control experiments, rabbit liver, kidney, and eye tissues were used. Primary antibody omission was used as a negative control. Blood vessel and lymphatic vessel density as well as combined cross-sectional area were analysed in five adjacent regions of interest each measuring 400 × 400 μm on the two best preserved sections (0.8 mm^2) with the highest number of vessels. The final assessed area corresponded to 1.6 mm^2 per eye. Podoplanin staining was used to detect lymphatic vessels. Blood vessels were detected by comparing adjacent (3 μm) sections stained with panendothelial marker CD31 and podoplanin. Vessels positive for CD31 and negative for podoplanin were classified as blood vessels. Staining of α-SMA was also used to distinguish blood and lymphatic vessels. Vessel count and cross-sectional area were quantified using available QuPath tools. The mean density of vessels in each eye was calculated by dividing the number of vessels by the total analysed area (1.6 mm^2). The cross-sectional area of all vessels was calculated in a corresponding manner and the mean vessel area was calculated by dividing it with the vessel density.

VEGF-C RT-PCR Analysis

Total RNA was isolated from 20 to 40 mg frozen conjunctival tissue per eye using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany) as previously described [7]. In brief, the tissue was lysed in RLT+ lysis buffer, and QIAshredders (Qiagen) were used for homogenization of tissue lysate. The gDNA eliminator column was omitted, and DNase treatment was not performed on the column. The 14-μL RNA eluate was treated with DNA-free (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. NanoDrop One was used to determine the RNA concentration, and cDNA was synthesized from 0.1 μg of RNA in a total reaction volume of 10 μL using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

For the detection of human VEGF-C (*hVEGF-C*) and the housekeeping gene *GAPDH*, reverse-transcribed total cDNA was used for PCRs with gene-specific forward (f) and reverse (r) primers, which have been previously validated [8, 9]: hVEGF-C (f) 5′-CACGAGCTACCTCAGCAAGA-3′, hVEGF-C (r) 5′-GCT GCCTGACACTGTGGTA-3′, GAPDH (f) 5′-ATGGTGAAG GTCGGAGTGAA-3′, and GAPDH (r) 5′-GGGTGGAATCAT ACTGGAACA-3′. For each synthesis reaction, 1 μL cDNA was used as template and the primer concentration was 5 μM. Cycling conditions were set according to the manufacturer's protocol for the use of the DreamTaq DNA polymerase (Thermo Fisher Scientific) with 30 s of denaturation, 30 s of annealing, and 60 s of elongation for 35 cycles and an annealing temperature of 58°C. For all samples, a DNA contamination control without reverse transcriptase was included. The NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) was used to purify DNA from Ad-VEGF-C particles according to manufacturer's protocol for purification of genomic DNA from blood. Purified Ad-VEGF-C DNA was used as positive PCR control for detection of *hVEGF-C*. Gel electrophoresis analysis was used to determine PCR product length. To verify detection of *hVEGF-C*, the PCR product was purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced (Mix2Seq; Eurofins Genomics, Ebersberg, Germany).

Statistical Analysis

For quantification of blood and lymphatic vessels, all the animals were pooled, so that the control group consisted of both eyes receiving an empty viral vector and saline injection as control experiment. The control group was only separated into two when cellular response was assessed, since these analyses were performed to distinguish between the effects of injecting a vector versus saline. All data are presented as mean ± standard deviation. Data were analysed with paired Student's *t* test with a significance level set to 0.05.

Results

In vivo Examination

No difference in animal behaviour or welfare was observed in any animal. The eyes were examined under a surgical microscope 2 weeks after the injection. The eyes appeared normal with no signs of hyperaemia and the conjunctiva felt normal when investigated with a surgical spear or forceps. Trypan blue lymphangiography resulted in instant uptake in the lymphatic vessels (shown in Fig. 1). The lymphatic vessel network appeared more extensive in eyes treated with Ad-VEGF-C compared to control. Quantified in ImageJ, the total vessel length, normalized to total area, was significantly higher in the Ad-VEGF-C group compared with the control group: 1.28 ± 0.74 versus 0.31 ± 0.18 total vessel length/analysed area (p = 0.0022; paired Student's *t* test).

Immunohistochemistry

Panendothelial marker CD31 could successfully visualize all vessels in the conjunctiva (shown in Fig. 2). Podoplanin staining on the other hand resulted only in positive staining in lymphatic vessels.

Comparison of adjacent sections stained with the two antibodies was successfully used to distinguish blood vessels from lymphatic vessels. Blood vessel identity was also confirmed by positive α-SMA staining, whereas lymphatic vessels were negative or with only a focal and incomplete staining pattern in larger vessels. Lymphatic vessels were localized subepithelially (shown in Fig. 3). Ad-VEGF-C resulted in a significant

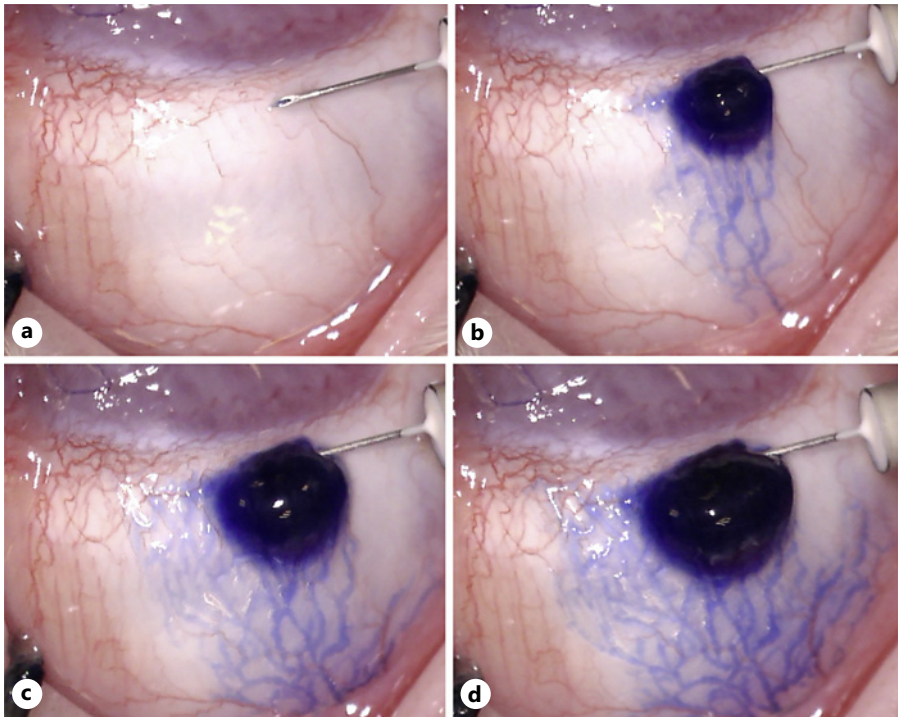

Fig. 1. Trypan blue conjunctival lymphangiography. **a** Time series over approximately 10 s demonstrating subconjunctival trypan blue injection with a 33 G needle in an eye treated with Ad-VEGF-C. **b–d** Rapid uptake of the dye in the conjunctival lymphatic vessels is seen and the otherwise invisible conjunctival lymphatic network is visualized.

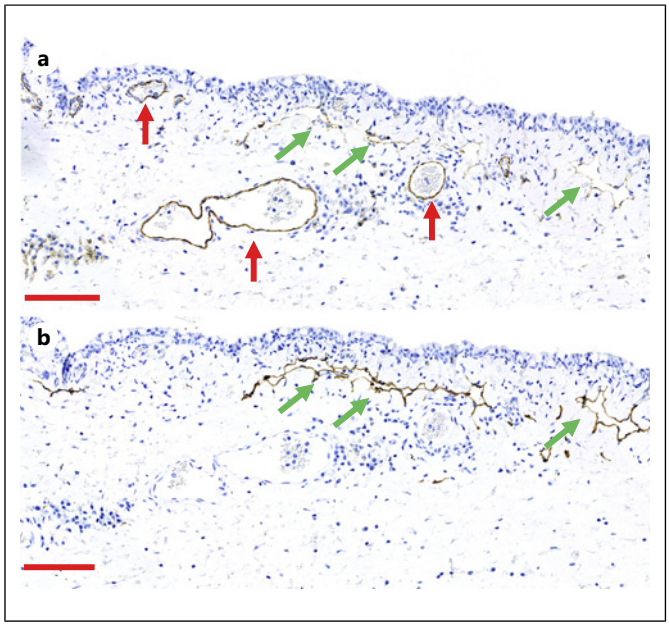

Fig. 2. Panendothelial marker CD31 and lymphatic endothelial cell marker podoplanin can be used to detect blood and lymphatic vessels in the conjunctiva. **a** Staining with CD31 resulted in positive reaction in both blood (red arrows) and lymphatic vessels (green arrows), but with a stronger intensity in the blood vessels. **b** Staining of the same area as in **a** with podoplanin resulted in positive reaction in only lymphatic vessels. Scale bar, 100 μm.

increase in lymphatic vessels compared to control (shown in Fig. 4), 34 ± 9 per mm^2 versus 13 ± 8 per mm^2 ($p = 0.0019$). There was also a significant increase in lymphatic cross-sectional area; 32,500 ± 7,900 μm^2 per mm^2 versus 17,600 ± 9,700 μm^2 per mm^2 ($p = 0.0149$). The mean vessel area was not different between Ad-VEGF-C and control group, 1,160 ± 660 μm^2 versus 1,614 ± 1270 μm^2 ($p = 0.4328$). There was no difference in any of the three parameters in the control group when the group was divided into saline versus empty vector. To test if there was a global conjunctival lymphangiogenic response lymphatic vessel density was quantified in the infero-nasal quadrant, which was the quadrant furthest away from the injection site. In Ad-VEGF-C-treated eyes, there was a significant lower lymphatic vessel density in the infero-nasal quadrant compared to the supero-temporal, 3.43 ± 1.9 per mm^2 versus 29.4 ± 8.6 per mm^2 ($p = 0.0035$). In the control group, there was no significant difference in vessel density between infero-nasal and supero-temporal quadrants, 6.25 ± 2.55 per mm^2 versus 8.44 ± 1.2 per mm^2, respectively ($p = 0.3141$). There was no significant difference in vessel density in the infero-nasal quadrant between Ad-VEGF-C and control ($p = 0.1265$), which suggests that Ad-VEGF-C does not induce global lymphangiogenesis. Two animals were treated and sacrificed after 4 weeks and confirmed that

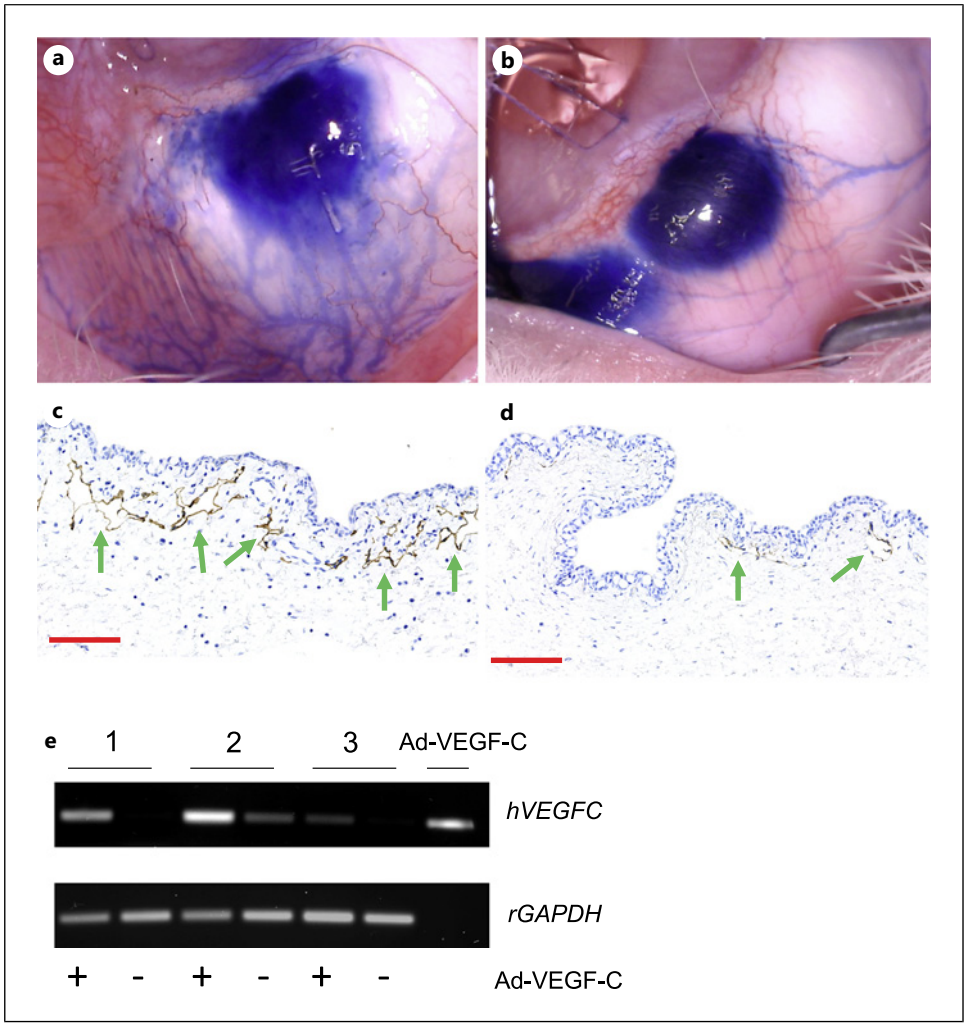

Fig. 3. Ad-VEGF-C treatment results in conjunctival lymphangiogenesis. **a**, **b** Trypan blue lymphangiography showing a denser lymphatic network after Ad-VEGF-C treatment (**a**) compared with an empty vector (**b**). Podoplanin staining in an Ad-VEGF-C-treated eye (**c**) showing more subepithelial lymphatic vessels (green arrows) compared to the contralateral eye (**d**) treated with an empty vector from the same animal as in **b**. **e** PCR-based analysis of six eyes from three rabbits demonstrating the expression of *hVEGF-C* in all three eyes treated with Ad-VEGF-C (+), confirming successful transduction of the adenoviral construct. The contralateral eye was treated with an empty vector (–). Purified Ad-VEGF-C DNA and housekeeping gene (*rGAPDH*) were used as positive controls. Scale bar, 100 μm.

the lymphangiogenic response persisted at this timepoint: a 2.5-fold higher density of lymphatic vessels compared with the control group and a 3-fold increase in total vessel area compared with the control group. Both numbers are similar to the cohort sacrificed after 2 weeks.

Quantification of blood vessels using CD31, α-SMA, and podoplanin stainings revealed no significant difference in blood vessel density between Ad-VEGF-C and control (shown in Fig. 5), 19 ± 9 per mm^2 versus 14 ± 8 per mm^2 (p = 0.1971). There was no significant difference in total blood vessel area, although there was a tendency towards a larger area in the Ad-VEGF-C group: 13,200 ± 7,600 μm^2 per mm^2 versus 7,100 ± 3,000 μm^2 per mm^2 (p = 0.0715; paired Student's t test). The mean vessel area was not different between treated and control eyes, 709 ± 270 μm^2 and 697 ± 590 μm^2 (p = 0.9510).

Histology

Histological examination revealed a cellular response, predominantly lymphocytes, in all eyes treated with Ad-VEGF-C, on average, 2.4 ± 0.23 (shown in Fig. 6). This corresponds to at least moderate inflammation (grade 2: 10–40 cells per high power field) in all eyes. This was also present in the eyes in the control group that were treated with an empty vector but in the eyes receiving saline. Subgroup analysis revealed a statistically higher score in Ad-VEGF-C eyes compared with control (saline): 2.5 ± 0.27 versus 1.0 ± 0.0 (p = 0.0020; paired Student's t test). There was no difference between Ad-VEGF-C and control (empty vector): 2.3 ± 0.14 versus 2.7 ± 0.57 (p = 0.3828). The immune response was thus a response to the vector and not related to VEGF-C. The dominant cell type was lymphocytes followed by macrophages. Although limited to only two animals, which is insufficient for statistical analysis, the two blinded observers agreed

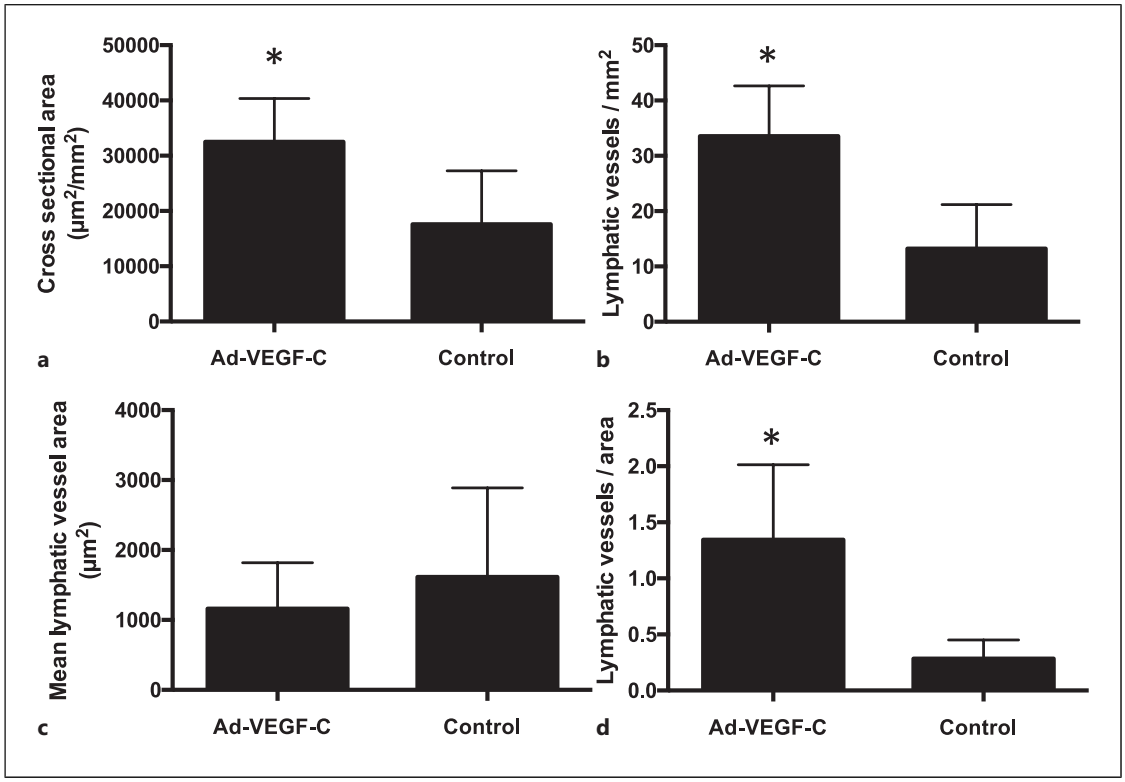

Fig. 4. Ad-VEGF-C induces conjunctival lymphangiogenesis. There was a significant increased total cross-sectional area of lymphatic vessels and lymphatic vessel density (**a**, **b**), as assessed with immunohistochemistry, after Ad-VEGF-C treatment. **c** Mean vessel area per lymphatic vessel was not affected. With trypan blue lymphangiography an increased total vessel length was seen in the Ad-VEGF-C group (**d**), consistent with the immunohistochemistry data.

that cellularity was lower in the specimens from the animals sacrificed after 4 weeks compared with those sacrificed after 2 weeks. Subepithelial collagen bundles was an inconsistent finding in eyes treated with Ad-VEGF-C. There was agreement between both graders that there were subepithelial collagen bundles in three out of seven eyes treated with Ad-VEGF-C and one additional eye where one pathologist noted subepithelial collagen (shown in Fig. 7). Subepithelial collagen was not found in the control group. Myofibroblasts or fibroses were not seen.

VEGF-C RT-PCR Analysis

The Ad-VEGF-C construct used in this study encodes the human isoform of VEGF-C. Successful transduction of the eyes with the adenoviral construct was demonstrated by RT-PCR analysis for detection of human *VEGF-C* (*hVEGF-C*) transcripts. Expression of *hVEGF-C* was successfully demonstrated in the three eyes that were treated with Ad-VEGF-C (Fig. 3). The molecular identity was confirmed by sequencing of the PCR products. The contralateral eye that was treated with an empty vector did not show expression of *hVEGF-C* in two out of three eyes. In the third eye, a weak band consistent with *VEGF-C* was detected. Sequencing of the PCR product could not confidently determine whether the product was *hVEGF-C* or endogenous rabbit *VEGF-C* because of the low amount of RNA and the high homology between the *VEGF-C* isoforms in the two species. No amplicons were detected in samples without reverse transcriptase.

Discussion

The main finding in our study is that a single injection of Ad-VEGF-C induces a robust lymphangiogenic response in all treated eyes. The treatment resulted in a higher density of vessels but did not change the diameter, suggesting that the mechanism is sprouting of new small vessels. The effect was limited to the upper hemisphere

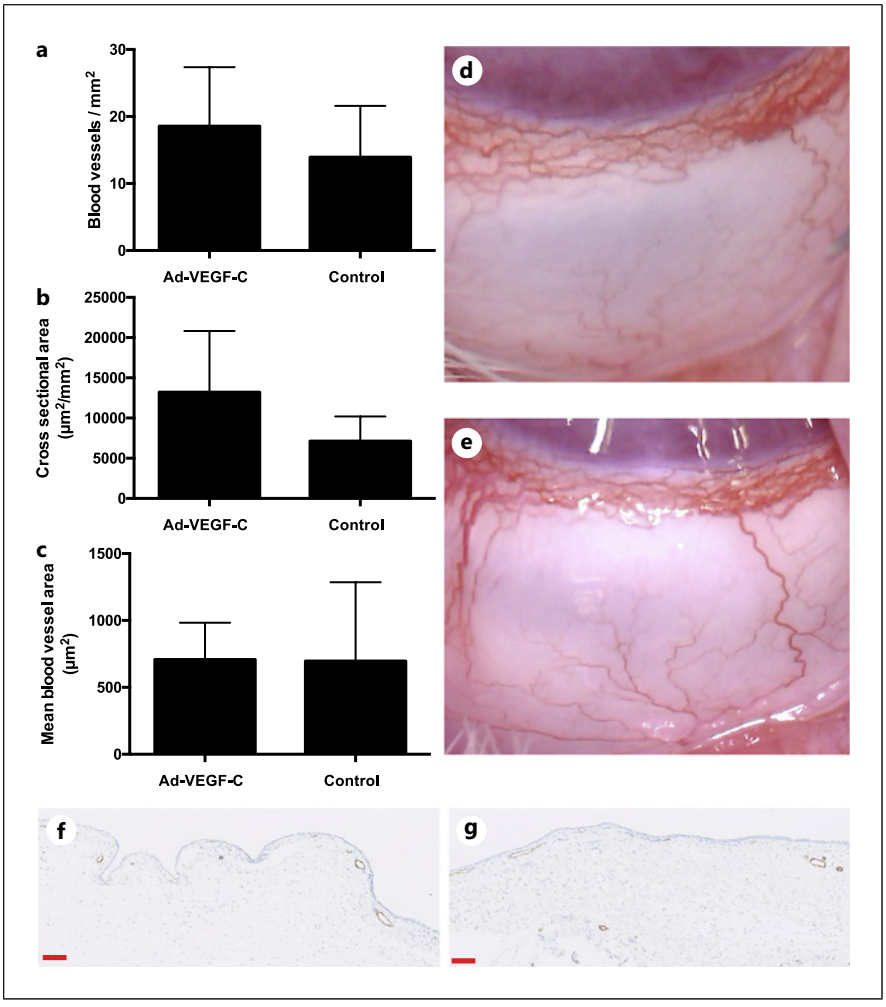

Fig. 5. Ad-VEGF-C has no significant effect on angiogenesis. **a–c** Blood vessel density, cross-sectional area, and mean blood vessel area was quantified using immunohistochemistry, with no significant difference between Ad-VEGF-C and control. **d**, **e** Photograph of an eye before treatment with Ad-VEGF-C (**d**) and 2 weeks later (**e**). No hyperaemia or angiogenesis can be seen. **f**, **g** Identification of blood vessels with alpha smooth muscle actin staining. Blood vessels appear to be of similar size and density in the Ad-VEGF-C-treated eye (**f**) compared with the control eye (**g**). Scale bar, 100 µm.

and did not affect the lower hemisphere, making it possible to induce local lymphangiogenesis. The immunohistochemistry data are consistent with the in vivo data from trypan blue conjunctival lymphangiography, where a more extensive conjunctival lymphatic network was seen in the Ad-VEGF-C treated eyes.

The dose Ad-VEGF-C (3.5×10^7 PFU) used in this study resulted in a robust lymphangiogenic response. Our findings are similar to those from Visuri et al. [10] and Tammela et al. [11] who used Ad-VEGF-C to induce lymphangiogenesis in conjunction with axillary lymph node transplantation in pigs and mice, respectively. The dose in pigs and mice was 100 and 10 times higher, respectively, than that used in the current study. That a higher dose is required in pigs is not surprising given the larger treated volume of tissue. However, it is interesting that we could use a 10-fold lower dose than in mice since the treated tissue volume is quite small in mice. Intracameral Ad-VEGF-C injection was used by Aspelund et al. [12] with the purpose of stimulating Schlemm's canal at a dose that was almost twice as high as in this study. In comparison with other tissues and species, it appears that a relatively small dose Ad-VEGF-C is required to induce lymphangiogenesis in the conjunctiva in rabbits.

There was no significant effect on blood vessels, but if more animals had been included a minor effect on blood vessel may have appeared. VEGF-C is a VEGFR-3 agonist, which is not expressed by mature blood vessels but extensively expressed by lymphatic vessels. Upon proteolytic cleavage in the C-terminal, VEGF-C acquires affinity for VEGFR-2. Therefore, in total, VEGF-C is predominantly a VEGFR-3 agonist, but can act as a weak VEGFR-2 agonist and have a slight effect on blood vessels in addition to its major effect on lymphatic vessels. In tumour biology, VEGF-C can have a marked effect on

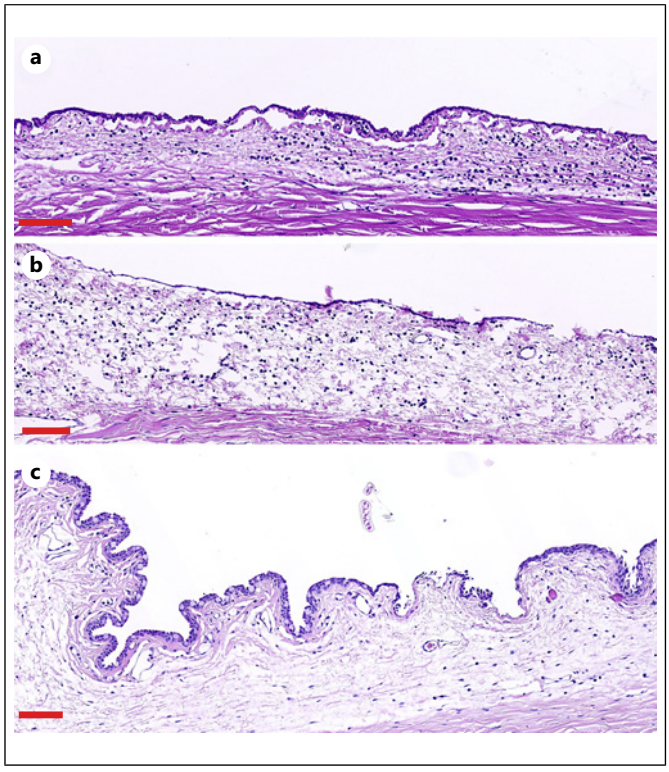

Fig. 6. Adenoviral vectors induce an immune cell response in the conjunctiva. **a**, **b** Representative images showing inflammatory (>40 cells/high power field) cellular infiltration in the conjunctiva in an eye treated with Ad-VEGF-C (top) as well as the contralateral eye treated with an empty viral vector (middle). **c** Normal (<10 cells/high power field) cellularity in an eye treated with a saline injection. Scale bar, 100 μm.

blood vessels since proliferative blood vessels from tumours express VEGFR-2. Interestingly, proliferative blood vessels in wound healing do not express VEGFR-2 [13].

An interesting finding in the current study was the immune cell response elicited towards the viral vector. The predominant cell type was lymphocytes, consistent with an anti-viral response. It is well known that the viral vectors used in gene therapy can induce a host response towards the vector. However, there are limited reports of this in terms of VEGF-C-induced lymphangiogenesis. One explanation may be the treated tissue in this study. The conjunctiva has its own immune system, conjunctiva-associated lymphoid tissue, and the physiological function of the conjunctiva is to act as a barrier against pathogens. Rabbits furthermore have an aggressive immune system. It is therefore unclear how these findings translate to humans. The inflammation is probably related to the dose, with more viral particles leading to more inflammation. Given the robust lymphangiogenic effect of the current dose, it is possible that the dose can be reduced and still yield a relevant lymphangiogenesis but with less inflammation. The inflammation seems to be transient as the cellular infiltration was considered lower at 4 weeks compared to 2 weeks. This is similar to an adenoviral conjunctivitis, which is also transient and has a spontaneous resolution.

Subepithelial collagen was noted in almost half of the Ad-VEGF-C-treated eyes but not in any eye in the control group (neither empty vector nor saline). Whether this is an effect of VEGF-C, which the results imply, is not clear given the small size of the sample. There is no obvious correlation between VEGF-C and collagen accumulation. It is possible that with longer follow-up, fibrosis may develop, but the absence of myofibroblasts does not indicate it and the animals sacrificed later did not show signs of fibrosis.

In the current study, an adenoviral vector was used to induce lymphangiogenesis. A recent publication [14] used a subconjunctival injection of recombinant VEGF-C to induce conjunctival lymphangiogenesis in mice. The same approach was tested in 3 rabbits, but instead of imaging and quantifying lymphatic vessel, Western blot was used to demonstrate an increased protein expression of a lymphatic endothelial marker at the time of sacrifice (10 days). In contrast to the study by Lee et al. [14], we provide structural and functional evidence that our approach with gene therapy results in lymphangiogenesis for at least 4 weeks. Treatment with the pure VEGF-C protein is limited by rapid turnover of the protein. To overcome this, gene therapy with viral vectors is often used and was the reason we also chose this approach. With a viral vector, the gene-encoding VEGF-C can be transferred to host cells, which leads to endogenous VEGF-C production for weeks to months. Both adenoviral vectors and adeno-associated viral vectors are used for this purpose. Gene therapy has successfully been used in many animal models and can lead to sustained lymphangiogenesis for several weeks. Adenoviral vectors are characterized by a high transfection efficiency of a broad range of cell types with a fast onset (<2 days) but may be accompanied by an immune response. Adeno-associated viral vectors have on the other hand a slower onset with minimal host response [15]. Not all serotypes are equally efficient in all cell types, and it is necessary to identify the best serotype for the tissue of interest. Adeno-associated viral vectors are in recent times often preferred to adenoviral vectors [16]. Adeno-associated viral vectors have

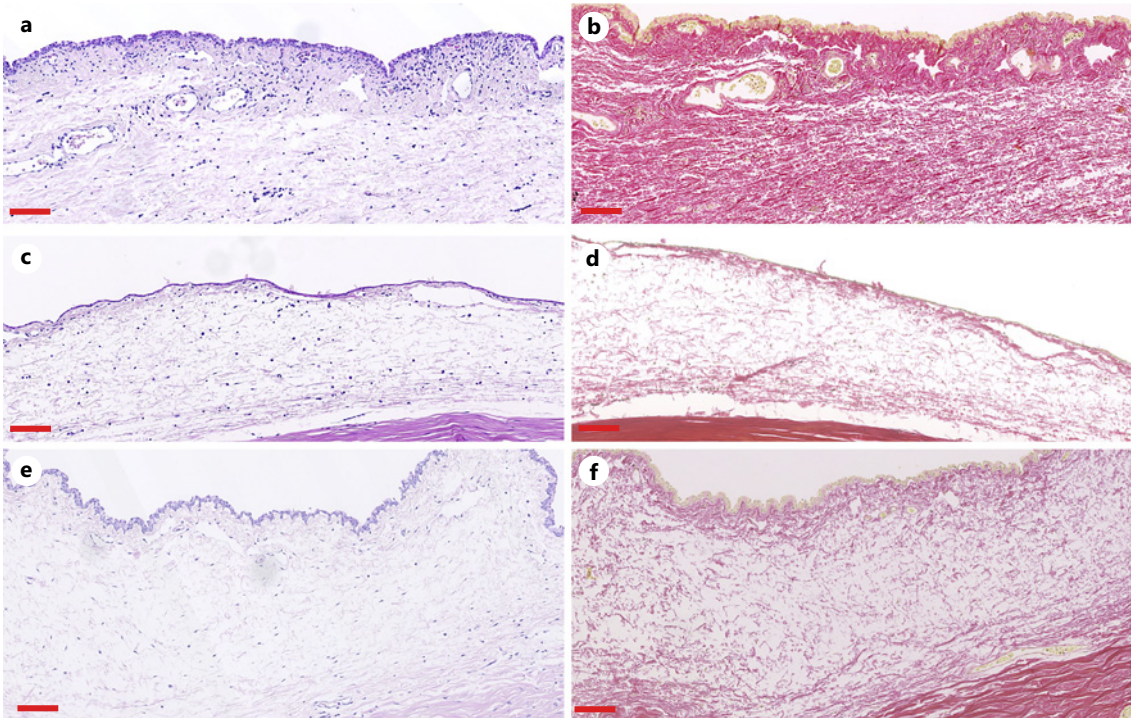

Fig. 7. Subepithelial collagen accumulation can be seen as a response to adenoviral vectors. As exemplified in this figure, some eyes that were treated with Ad-VEGF-C developed some subepithelial collagen accumulation (**a**, **b**), whereas some did not (**c**, **d**). **e**, **f** No subepithelial collagen accumulation was seen in eyes receiving a saline injection. **a**, **c**, and **e** are HE stainings and **b**, **d**, and **f** are Picrosirius red stainings. Scale bar, 100 μm.

not been used in the conjunctiva in rabbits but in the cornea as well as in the conjunctiva of mice [17, 18]. It is therefore likely that lymphangiogenesis can be induced with adeno-associated viral vectors and if so, presumably with less inflammation.

Angiogenesis has been the focus of much research in ophthalmology, and targeting the VEGF-A/VEGFR-2 axis has been a paradigm shift in the clinical care of patients with, e.g., neovascular age-related macular degeneration and proliferative diabetic retinopathy. The current study expands previous studies where inhibition of corneal lymphangiogenesis has been successfully targeted [19] to now include inducing lymphangiogenesis as a potential new treatment target in ophthalmology. There are more than 20 approved drugs targeting angiogenesis but no approved drugs targeting lymphangiogenesis [20]. Currently, Ad-VEGF-C has completed phase 1 and 2 clinical trials as an adjuvant treatment with lymph node transplantation to prevent breast cancer-related lymphoedema [21].

The findings in the current study have implication for inflammatory ocular surface conditions as well as, e.g., glaucoma surgery, where aqueous fluid is diverted to the subconjunctival space and a filtration bleb is formed. Lymphatic drainage has been demonstrated in blebs from humans with well-regulated IOP (12.6 ± 3.2 mm Hg), whereas no lymphatic drainage was found in patients with not well-regulated IOP (23.6 ± 8.8 mm Hg) [3]. Failed glaucoma drainage devices have been shown to lack lymphatic vessels in the bleb capsule [22]. The exact relationship between lymphatic drainage, IOP, and fibrosis is yet to be elucidated. But it is well known that fibrosis is the main reason that glaucoma operations cease to function and inflammation is the single most important risk factor for fibrosis [23]. This is evident in patients with uveitis, where the success rate of glaucoma surgery is lower and an increased number of lymphocytes, fibroblasts, and macrophages in conjunctiva have been demonstrated [24]. It is possible that stimulation of lymphangiogenesis in conjunction with glaucoma surgery may improve outcomes in two ways that actually overlap; removal of fluid and reduction of the inflammatory response. Fluid accumulation can per se be inflammatory. Inflammation is a cardinal feature of chronic lymphoedema secondary to lymphatic insufficiency. Oedema in other tissues such as the myocardium has also been shown to be an inflammatory stimulus [25, 26]. Increasing fluid removal from the tissues

is therefore a way to prevent inflammation from developing. The second reason that lymphangiogenesis may be beneficial is that the lymphatic vessels actively regulate the immune cell trafficking [27] and therefore modulate any inflammatory response. Stimulation with VEGF-C has been shown to induce lymphangiogenesis and, as a consequence, reduces leucocyte numbers, including CD4+ and γδ T-cells in a mouse model of chronic skin inflammation [28, 29]. Immune cells play a key role in wound healing and ultimately the development of fibrosis. Dampening the immune response can lead to less aggressive wound healing with less fibrosis. The fact that lymphangiogenesis, as demonstrated in other tissues as well as in the cornea [30], can alleviate inflammation has implications beyond glaucoma surgery. If these findings can be transferred to the conjunctiva, then therapeutic lymphangiogenesis can play a role in the treatment of different inflammatory ocular surface disorders. However, ocular surface disorders such as pterygium and conjunctivochalasis may be promoted by VEGF-C, which is important to consider as an adverse advent [31, 32], although the dilated lymphatics seen in conjunctivochalasis is likely secondary to the conjunctival changes [28]. Lastly, conjunctival lymphangiogenesis plays a role in tumour biology. Conjunctival malignant melanomas and squamous cell carcinoma seem to use lymphangiogenesis to spread and metastasize [33, 34], and stimulating lymphangiogenesis in this context is likely detrimental. These malignancies are fortunately rare and can be discovered with a careful clinical examination in the slit lamp. While stimulating, lymphangiogenesis should probably be avoided in the presence of malignancies; they may actually play a role in the treatment of malignancies. Immunotherapy with checkpoint inhibitors has changed the treatment of, e.g., skin melanoma and lung cancer. With this type of treatments, it is important that the lymphatic vessels transport dendritic cells to the lymph nodes for priming of T-lymphocytes [35] so that an immune response is generated towards the tumour cells. In this context, it could be beneficial to stimulate lymphangiogenesis to boost the immune response.

Conclusion

The current study demonstrates the feasibility of inducing conjunctival lymphangiogenesis. Additional studies are needed to determine the optimal way of inducing lymphangiogenesis, both in terms of dose and choice of vector. The lymphatic vessels and lymphangiogenesis have a complex role in many different situations, ranging from normal physiology to pathophysiology. Future studies are needed to explore in which conditions and how the lymphatics can be used as a treatment pathway.

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Statement of Ethics

The study was approved by the Danish Animal Experiments Inspectorate (reference number 2020-15-0201-00601).

Conflict of Interest Statement

Niklas Telinius has received honoraria from Thea and Santen, none with any relation to this study. The other authors have no conflicts of interest.

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Author Contributions

The study was conceived and designed by Niklas Telinius (N.T.). Animal experiments were performed by N.T. Lymphangiography data were analysed by N.T. Histology and immunohistochemistry protocols were developed by Dusan Rasic (D.R.) and Martin Wirenfeldt (M.W.), with the experimental work conducted by D.R. Interpretation and analysis were done by D.R. and M.W. PCR experiments were conceived by Thomas J. Corydon (T.J.C.) and Anne Louise Askou (A.L.A.). Interpretation and analysis were done by T.J.C. and A.L.A. The first manuscript draft was written by N.T. and subsequently revised by D.R. and M.W. N.T. revised the resubmitted manuscript. All authors approved the final version.

Data Availability Statement

Data are not publicly available due to ethical reasons. Further enquiries can be directed to the corresponding author.

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