Cornea

Identification of Novel Endogenous Anti(lymph)angiogenic Factors in the Aqueous Humor

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The cornea is one of the few avascular tissues of the human body.1 The cornea actively maintains its avascular state, a phenomenon that has been described as “corneal angiogenic privilege.”1–3 In addition, the normal cornea also lacks lymphatic vessels.3 Whereas the mechanisms keeping the cornea free of blood vessels have long been studied,3 the precise mechanisms underlying corneal alymphacity are only poorly understood. Since aqueous humor (AqH) is in direct contact with the alymphatic cornea, since its role in maintaining an alymphatic corneal state has not been analyzed, and since there have been reports on a potential antihemangiogenic role of AqH,4 we addressed the question of whether AqH also contributes to corneal lymphangiogenic privilege.

From corneal angiogenic and corneal immune privilege it is known that certain immunomodulatory factors contribute to both privileges.3 Several immunomodulatory factors such as vasoactive intestinal peptide (VIP) and α-melanocyte stimulating hormone (α-MSH) are known to be present in AqH and contribute to anterior chamber-associated immune deviation.5 Therefore, we additionally investigated the role of these factors on lymphatic endothelial cell (LEC) proliferation in vitro.

A potential antilymphangiogenic effect of immunomodulatory factors in AqH becomes even more interesting in light of the recent finding of a lymphatic endothelial nature of Schlemm’s canal and a putative role of lymphangiogenic growth factors in glaucoma pathogenesis.9–12

In summary, our findings suggested AqH exerts significant antilymphangiogenic effects in vivo. This effect is at least partially mediated by known immunomodulatory factors VIP and α-MSH present in the AqH. Therefore, AqH not only contributes to corneal lymphangiogenic privilege and is a new tool to identify novel endogenous regulators of lymphangiogenesis but also may have therapeutic applications.

Keywords: aqueous humor, lymphangiogenesis, immunoregulation, hemangiogenesis

PURPOSE. The avascular cornea is in direct contact with aqueous humor (AqH). Here we investigate whether AqH exerts anti(lymph)angiogenic effects and thereby may contribute to corneal (lymph)angiogenic privilege.

METHODS. Using the murine model of suture-induced inflammatory corneal hem- and lymphangiogenesis, the potential anti(lymph)angiogenic effect of AqH was analyzed by applying murine AqH as eyedrops. Anti(lymph)angiogenic effects were measured using morphometric analysis of flat mounts stained with CD31 as panendothelial and LYVE-1 as specific lymphatic endothelial marker. The potential antilymphangiogenic effect of immunomodulatory factors contained in AqH such as vasoactive intestinal peptide (VIP) and α-melanocyte stimulating hormone (α-MSH) was analyzed in lymphatic and blood vascular endothelial cell proliferation assays in vitro.

RESULTS. Topically applied AqH significantly inhibited corneal hemangiogenesis and even more so lymphangiogenesis in vivo and directly in vitro. The immunoregulatory factors VIP and α-MSH significantly inhibited lymphatic endothelial cell proliferation in vitro. Depletion of VIP or α-MSH from AqH diminished its anti-hem- and lymphangiogenic potential.

CONCLUSIONS. Aqueous humor exerts significant antilymphangiogenic effects in vivo. This is at least partially mediated by the known immunomodulatory factors VIP and α-MSH present in the AqH. Therefore, AqH not only contributes to corneal lymphangiogenic privilege and is a new tool to identify novel endogenous modulators of lymphangiogenesis but also may have therapeutic applications.

Material and Methods

Animal Models

For animal experiments, female BALB/c mice aged 6 to 8 weeks were used. The local animal care committee, in accordance with the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmology and Vision Research, approved all animal protocols.

AqH Collection (Mice)

Prior to surgery, each animal was deeply anesthetized with an intramuscular injection of Ketanest S (8 mg/kg) and Rompun (0.1 mL/kg). Aqueous humor was collected from donor mice.
by exhausting it with a glass capillary injected into the anterior chamber. To inject the capillary a small area of the corneal epithelium was previously scraped off and a small cavity was prepared into the stroma with a Graefe knife. The exhausted AqH was collected in 2-mL tubes and stored at −20°C. Afterward, mice were killed by cervical dislocation. Per eye, approximately 5 μL AqH could be harvested (n = 5 animals).

Suture-Induced, Inflammatory Corneal Neovascularization Assay

Prior to surgery, each animal was deeply anesthetized with an intramuscular injection of Ketanest S (8 mg/kg) and Rompun (0.1 mL/kg). Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were placed intrastromally with two stromal incursions extending over 120° of corneal circumference each. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center equidistant from limbus to obtain standardized angiogenic responses. Sutures were left in place for duration of the experiment. The treatment groups received 5 μL AqH or saline solution on day 0, 1, and 2, once a day. After 7 days, mice were sacrificed.

Corneal Whole Mounts and Morphologic Determination of Hemangiogenesis and Lymphangiogenesis.

The corneas were excised, rinsed in PBS, and fixed in acetone for 30 minutes as described previously. After three additional washing steps in PBS and blocking with 2% BSA in PBS for 2 hours, the corneas were stained overnight at 4°C with rabbit anti-mouse LYVE-1 (1:500; a kind gift of D.G. Jackson, Oxford University, Oxford, United Kingdom) as described previously. On day 2 the tissue was washed, blocked, and stained with FITC-conjugated rat anti-CD-31 (Acris Antibodies GmbH, Hildenhausen, Germany) antibody overnight at 4°C. After a last washing and blocking step on day 3, LYVE-1 was detected with a Cy3-conjugated secondary antibody (rabbit anti-mouse; 1:100; Dianova, Hamburg, Germany). Isotype control was assured with a FITC-conjugated normal rat IgG for CD31-FITC and with a normal rabbit IgG (both Santa Cruz Biotechnology, Santa Cruz, CA, USA) for LYVE-1.

LEC and Blood Endothelial Cell (BEC) Proliferation ELISA.

Human lymphatic or blood microvascular endothelial cells (Cambrex Bio Science) were cultured in EGM2-MV medium (Cambrex Bio Science) following manufacturer’s instructions. For the ELISA, cells were seeded in a 96-well plate in EGM2-MV medium at a density of 2 × 10⁵ cells/well and left overnight to attach. Cells were fixed and stained after 3 days according to manufacturer’s instructions (Cell Proliferation ELISA, BrdU; Roche, Penzberg, Germany). Colorimetric analysis was performed with an ELISA reader (SLT Spectra; SLT Labinstruments [Deutschland] GmbH, Crailsheim, Germany).

Human AqH Samples.

Before cataract surgery, samples of AqH were collected from routine cataract patients. These samples were used in 20% dilution (within EGM2-MV medium) in the above-mentioned ELISA. As control, EGM2-MV medium alone was used. (Institutional review board approval number 14-248; The study was approved by Ethics Commission of Faculty of Medicine, University of Cologne, Cologne, Germany. All subjects were treated in accordance with the Declaration of Helsinki.)

Depletion of VIP and α-MSH From AqH.

To evaluate the effect of endogenous VIP and α-MSH, AqH samples were spun through a 5-kDa cut of filter (Corning Spin-X UF 500-μL concentrator; Corning, Tewksbury, MA, USA) to achieve the low-molecular-weight fraction of the AqH as described. Afterward, the samples were used in a 20% dilution (with EGM-2 MV medium) and incubated on wells either coated with anti-human VIP (Elabscience, WuHan, China) for 90 minutes (following manufacturer’s instructions) or anti-human MSH (Abbeax, Cambridge, UK) for 30 minutes (following manufacturer’s instructions) to bind the target proteins to the well surface and thereby deplete them from the AqH. The VIP- or α-MSH-depleted AqH medium was then used in the cell proliferation ELISA for BECs and LECs as described above. As control, EGM2-MV medium alone was used.

Immunocytochemistry of VIP and α-MSH Receptors on LECs and BECs.

Human lymphatic or blood microvascular endothelial cells (Cambrex Bio Science) were cultured in EGM2-MV medium (Cambrex Bio Science) following manufacturer’s instructions. For immunocytochemistry, cells were seeded on glass slides in 24-well plates (5 × 10⁵ cells per well) and grown overnight in EGM2-MV medium. Cells were washed and fixed in 4% paraformaldehyde. After additional washing, cells were stained with a rabbit anti-human MSHR1 antibody (1:100; MyBioSource, San Diego, CA, USA) or rabbit anti-human VIPR1 antibody (1:100; antikoerper-online GmbH, Aachen, Germany) and a secondary anti-rabbit Alexa 488 antibody (1:2000).

Flow Cytometric Analysis

As described above, LECs and BECs were cultured overnight in EBM-2MV medium. Following cell detachment, cells were labeled with a rabbit anti-hMSHR1 antibody (MyBioSource) or rabbit anti-hVIPR1 antibody (antikoerper-online) and a secondary anti-rabbit Alexa 488 antibody. The stained cells were resuspended in 600-μL PBS, 2% FCS, 10 mM HEPES, and 4 μL 7-Aminoactinomycin (7 AAD) (to detect dead cells; Biolegend, Fell, Germany) before analysis in a Guava 12HT (Merck Millipore, Darmstadt, Germany). Data processing was performed using analysis software (FlowJo; Tree Star, Inc., Ashland, OR, USA).

Functional and Statistical Analysis.

Double-stained whole mounts and cryosections were analyzed with a fluorescence microscope (BX51; Olympus Optical Co., Hamburg, Germany), and digital pictures were taken with a 12-bit monochrome charged-coupled device camera (F-View II; Soft Imaging System, Münster, Germany). Each whole-mount picture was assembled out of nine pictures taken at 100× magnification. The areas covered with blood or lymphatic vessels were detected with an algorithm established in an image-analyzing program (analySIS^B; Soft Imaging System, Münster, Germany). Prior to analysis gray value images of the whole-mount pictures were modified by several filters. Blood and lymphatic vessels were detected by the threshold setting including the bright vessels and excluding the dark background. Statistical analysis was done with statistical analysis software (InStat 3, version 3.06; GraphPad Software, Inc., San Diego, California, USA), and graphs were drawn using graphing software (Prism4, version 4.05; GraphPad Software, Inc.).

RESULTS

AqH Exerts Anti–hem- and Antilymphangiogenic Effects in Vivo and in Vitro

To elucidate the potential anti(lymph)angiogenic properties of AqH, AqH was collected from healthy eyes of 6-week-old mice and used as eyecrops in the suture-induced model of combined
pathologic corneal hem- and lymphangiogenesis. The mice received one time daily 3 μL AqH on the day of the surgery and on postoperative day 2 and 3 as eyedrops. After 7 days, sutures were removed and corneas were collected for morphologic analysis. Hemangiogenesis \((P < 0.001)\) and lymphangiogenesis \((P < 0.0001)\) were significantly inhibited in the AqH-treated group in comparison to the control group, which received saline eye drops \((n = 16; \text{Fig. 1})\).

To analyze if these in vivo findings are due to a direct effect of the AqH on the endothelial cells of blood and lymphatic vessels, we performed cell proliferation ELISAs with human LECs, BECs, and AqH. We could show that both BEC and LEC proliferation were directly inhibited significantly by 20% AqH, whereas the proliferation of LECs was even more hampered than the proliferation of BECs (Fig. 2).

We next investigated whether this anti(lymph)angiogenic effect of AqH could be related to known anti-inflammatory factors in AqH that also contribute to corneal immune privilege, that is, α-MSH and VIP, and whether the effect is at least partially mediated via a direct effect on lymphatic endothelium.

**The Endogenous Immune Modulating Factor α-MSH Inhibits Lymphangiogenesis in Vitro**

Due to its known immune-modulatory effect\(^{17,18}\) and the presumed antiangiogenic effect,\(^{1-21}\) the effect of α-MSH on human BECs and LECs in vitro was tested. The cells were incubated with 20 pM (physiological concentration\(^{22}\)) or 200 pM α-MSH. The proliferation of LECs was inhibited significantly at both concentrations \((20 \text{ pM}: P < 0.001; \text{200 pM}: P < 0.0001)\). In contrast, α-MSH had no effect on BECs \((20 \text{ pM}: P > 0.05; \text{200 pM}: P > 0.5 \text{ pM}; \text{Fig. 3})\).

**Neuropeptide VIP Inhibits Both Hem- as Well As Lymphangiogenesis in Vitro**

The vasoactive intestinal peptide is a neuropeptide that suppresses IFN-γ production by antigen-stimulated primed T cells in vitro.\(^{16}\) Again, the inhibitory effect on the proliferation of LECs and BECs was tested (Fig. 4).

The blood and lymphatic cells were treated with 12 nM as physiological concentration\(^{16}\) and the 10-fold higher concentration \((120 \text{ nM})\) of VIP, but not at higher concentrations \((\text{LEC 12 nM}: P < 0.001; \text{BEC 12 nM}: P < 0.008; \text{LEC 120 nM}: P > 0.05, \text{BEC 120 nM}: P > 0.40)\).

**Depletion of VIP or α-MSH From AqH Significantly Diminishes the Antilymphangiogenic Effect of AqH**

To confirm that the observed antilymphangiogenic effect of AqH is at least partially mediated by VIP and α-MSH in AqH, we performed depletion experiments of VIP and α-MSH in human AqH. The low-molecular-weight fraction in which Taylor et al.\(^{16,22}\) identified VIP and α-MSH as immune regulatory proteins in the AqH was used: As recombinant VIP had only a slight impact on LEC proliferation (Fig. 4a), the depletion of VIP from AqH samples did not influence the inhibitory effect of AqH on lymphatic cell proliferation. In comparison to the lack of VIP, depletion of α-MSH significantly decreased the inhibitory potency of AqH (Fig. 5a), supporting its antilymphangiogenic properties as shown above (Fig. 3a). When performing the same experiments with BECs, the depletion of VIP abolished the inhibitory effect of AqH on BEC proliferation. In contrast, α-MSH–depleted AqH still had an inhibitory effect on BECs (Fig. 5b).
5b). Again, these data support the findings that recombinant α-MSH had no effect (Fig. 3b), whereas VIP had an inhibitory effect on BEC proliferation (Fig. 4b).

The differential effects of VIP and α-MSH seen in the in vitro assays can partially be explained by the differential expression of the VIP receptor 1 (VIPR1) as well as the melanocyte stimulating hormone receptor 1 (MSHR1). When performing immunocytochemistry, we found that LECs and BECs stained positive for both receptors (Figs. 6b, 6d, 6f, 6h). The flow cytometric analysis revealed that LECs have robust MSHR1 expression (measured by mean fluorescence intensity [MFI]: MSHR1, 13,203; IgG, 2831), whereas the VIPR1 expression was very weak (MFI: VIPR, 3649; IgG, 2831), supporting the strong inhibitory effect of α-MSH and the more moderate effect of VIP on the proliferation of LECs, respectively (Fig 6c, 6d). In addition, BECs strongly expressed VIPR (MFI: VIPR, 15,794; IgG, 2687), supporting its inhibitory effect on BECs, whereas MSHR1 was only weakly expressed (MFI: MSHR1, 3441; IgG, 2687), fitting to the findings that α-MSH had no effect on BEC proliferation (Fig. 6a, 6e).

**DISCUSSION**

The main finding of this study is that AqH significantly inhibits angiogenesis and, even more so, lymphangiogenesis, in a murine model of inflammatory corneal neovascularization in vivo. This is the case even when AqH is administrated as eyedrops at the low dosage of 3 µL per drop once daily. In addition, in vitro proliferation of BECs and LECs was inhibited by human AqH obtained from routine cataract patients. The in vitro findings show that there is at least partially a direct effect of AqH on BECs and LECs. These findings support the hypothesis that AqH contributes to corneal lymphangiogenic privilege.

The molecular players mediating this novel antilymphangiogenic effect of AqH are yet unknown. In fact, since this effect is most likely redundantly organized, AqH may act as a model to identify novel endogenous regulators of lymphangiogenesis. Here, as a pilot study, we tested the hypothesis that anti-inflammatory factors from AqH may also exert antilymphangiogenic effects.

Aqueous humor contains many factors contributing to the immune-privileged state of the anterior chamber. Taylor et al. mentioned that only a few have been studied for their role in suppressing induction of DTH. He defined the criteria for such an immunosuppressive factor as follows: (1) Such a factor has to be physically present in the normal AqH, (2) the AqH concentration of such a factor has to have an in vitro immunosuppressive activity similar to whole AqH, and (3) neutralization of such a factor also leads to a neutralization of some aspects of AqH immunosuppressive activity. According to these criteria, only TGF-β2, the calcitonin gene-related peptide, fits these criteria.
peptide, a-MSH, and VIP were identified so far as ocular immunesuppressors of DTH. 16–18,22

The a-MSH receptor MSHR1 has been shown to be the only melanocortin receptor expressed on human dermal microvascular endothelial cells. 21,23 Via this receptor, various matrix metalloproteinases (MMPs) are expressed that in turn activate several antiangiogenic factors such as angiostatin, endostatin, or tumstatin.20

In this work the different in vitro effects of a-MSH were demonstrated. For example, a-MSH significantly inhibited the proliferation of LECs at physiological concentrations and at 10-fold higher concentrations but had no effect on BEC proliferation. Depleting a-MSH from the low molecular fraction of the AqH could confirm these findings. In addition, we could show that the receptor MSHR1 is only weakly expressed on BECs, whereas LECs show a quite robust expression of this receptor. These different expression patterns partly explain the differential anti-hem- and antilymphangiogenic effects of a-MSH. Furthermore it was shown that proangiogenic effects can also be provoked by this hormone: a-MSH also leads to the release of interleukin-8 (IL-8; CXCL 8).19 This chemokine was shown to have a paracrine and autocrine angiogenic effect via NF-kB.24 Therefore, the antiangiogenic effect on BECs via different MMPs might be outbalanced by the autocrine proliferative effect of IL-8 on endothelial cells, whereas this balance might be shifted in favor of the antiangiogenic effect in LECs. Vasoactive intestinal peptide is known to inhibit in vitro invasion and migration of murine Colon 26-L5 carcinoma cells without affecting their growth25 and the establishment of their liver metastases in mice, probably by suppressing their arrest

Figure 5. Depletion of VIP or a-MSH from AqH alters the anti(lymph)angiogenic properties of AqH. (a) Aqueous humor samples were depleted of either VIP or a-MSH or left untreated, and LEC proliferation was measured in comparison to control medium. (b) Aqueous humor samples were depleted of either VIP or a-MSH or left untreated, and BEC proliferation was measured in comparison to control medium. Control medium is set to 0% inhibition (not shown).

Figure 6. Vasoactive intestinal peptide receptor 1 and MSHR1 are differently expressed on BECs and LECs. (a, c) Flow cytometry on BECs and LECs for MSHR1; (b, d) immunocytochemistry on BECs and LECs for MSHR1; (e, g) flow cytometry on BECs and LECs for VIPR; (f, h) immunocytochemistry on BECs and LECs for VIPR; (i–j) IgG controls for immunocytochemistry. Mean fluorescence intensities (MFI) equals (a) MSHR1: 3441; IgG: 2687; (c) MSHR1: 13,203; IgG: 2831; (e) VIPR: 15,794; IgG: 2687; (g) VIPR: 3649; IgG: 2831.
in the liver. The process of angiogenesis by endothelial cells is considered to be functionally similar to that of invasion by tumor cells, so VIP may affect angiogenic responses of endothelial cells by regulating their invasive and motile activities. It was demonstrated that VIP inhibited the morphogenesis of hepatic sinusoidal epithelial cells into capillary-like structures on Matrigel-coated wells.

We could demonstrate that VIP has a significant, but only modest, effect on the growth of both BECs and LECs. The expression of its receptor VIPR1 could be found on both cell types, whereas LECs showed only a very weak expression. This also explains the finding that the depletion of VIP from AQH did not alter its antilymphangiogenic effect.

Here, we believe for the first time, we show that both immunomodulatory factors VIP and α-MSH exert antilymphangiogenic effects by inhibiting LEC proliferation in vitro. This also suggests that AQH—alogous to avascular cornea—may be a useful new model system to identify novel endogenous regulators of lymphangiogenesis. These have significant therapeutic implications beyond ophthalmology, for example, in cancer metastasis and blood pressure regulation. Whether VIP and α-MSH or AQH itself can be used to inhibit corneal (lymph)angiogenesis in patients remains to be studied.

Given the recent interest in the novel presumed role of lymphangiogenic growth factors such as VEGF-C and its receptor VEGFR3 in potentially regulating Schlemm’s canal lymphatic endothelial lining and thereby glaucoma development, the question of antilymphangiogenic factors in AQH counterregulating VEGF-C also becomes relevant and needs further studies. Both VIP and MSH at least inhibit LEC proliferation. Whether they affect Schlemm’s canal lymphatic endothelial lining remains to be studied.

In summary, AQH displays significant antilymphangiogenic effects in vivo and directly inhibits blood and lymphatic vascular endothelial proliferation in vitro. This effect is at least partially mediated by known immunomodulatory factors VIP and α-MSH present in the AQH. Therefore, AQH not only contributes to corneal lymphangiogenic privilege and is a new tool to identify novel endogenous regulators of lymphangiogenesis, but also it may have therapeutic applications.

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References


