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Lymphatics and the Eye

The lymphatic system, fundamental for body fluid homeostasis and immune system functions, also participates in pathogenic processes including cancer, cardiovascular and neurodegenerative diseases. Many aspects of the lymphatic system are unclear, but great advancements have been made – e.g. the discovery of meningeal lymphatics and the lymphatic-like nature of Schlemm's canal. Lymphatic-like structures were also recently discovered in proliferative diabetic retinopathy, a severe diabetic eye complication, where treatment of pathological neovessel growth is not always effective. Novel findings in the field can help to develop new treatments for lymphatic disorders and for other diseases where lymphatic neovascular growth is involved.

The lymphatic system collects tissue fluid, and participates in the circulation of immune cells and the absorption of long-chain fatty acids from the digestive tract. While the secrets of the circulatory system have been uncovered over the centuries, the lymphatic system has remained enigmatic and riddled with scientific debate. Only recently, answers have been given to questions as simple as *How does the lymphatic system develop?* (1) and *In which organs do lymphatic vessels occur?* (2–5). Despite recent advances, insight into many problems that are important from a patient's point of view have not yet been found. For example, the link between lymphedema and the painful adipose tissue disorder *lipedema* is still unclear. Morover, we still rely almost entirely on symptomatic treatments for diseases of the lymphatics (6).

Since the lymphatic system's function is required to maintain tissue fluid homeostasis and the body's immune defense, it has become a focus of interest in recent years. Unsurprisingly, the lymphatic system has been found to be intricately linked to many chronic diseases that are important from a public health perspective. These diseases include cancer, cardiovascular disease and diabetes, but also neurodegenerative and inflammatory diseases such as the rheumatic disorders (7,8). Advances in basic biomedical research, such as the discovery of lymph vessels in the meninges and advances in understanding the molecular mechanisms of lymphatic neovascularization (*lymphangiogenesis*), have already revealed new promising drug targets for treating neurodegenerative diseases and cancer (2,3,9–11). In addition, Schlemm's canal, a critical part of the ocular outflow system, was recently shown to possess most of the molecular and functional properties of a lymphatic vessel (12), and reduced ocular efflux in glaucoma was shown to cause its degeneration and loss of lymphatic identity (13).

Despite the recent milestones in lymphatic research, our knowledge of the anatomy and function of the lymphatic system is still incomplete and partially controversial, especially with respect to the lymphatic vessels of the eye (5).

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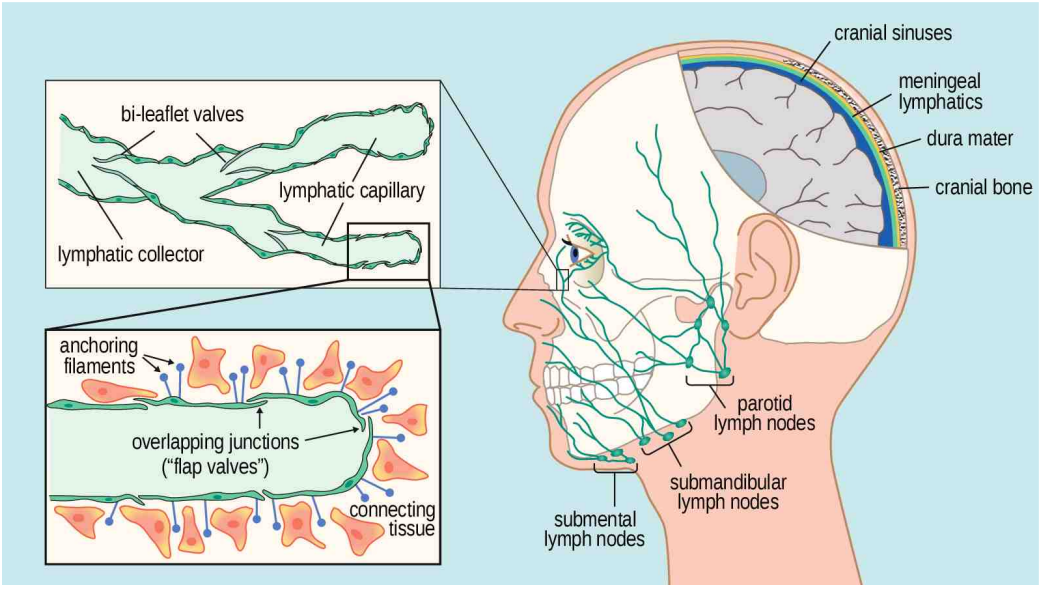

FIGURE 1. The lymphatic system of the head and neck region. Endothelial cells (ECs) are attached to the connective tissue with anchoring filaments. The ECs form thin-walled lymphatic capillaries containing flap valves, which are formed by overlapping cell junctions. The capillaries collect interstitial fluid, which is transported as lymphatic fluid via the collecting lymph vessels. Lymphatic fluid generated around the eyes is transported to the lymph nodes of the head and neck, such as the parotid glands, the anterior (preauricular), and the submandibular lymph nodes. Lymph nodes play a key role in triggering immune responses by acting as meeting points for antigen presenting cells and naive mature but inactive lymphocytes. The lymph eventually returns to the bloodstream at the lymphatico-venous connections, which are located for the lymphatics of the upper left torso and lower body close to the confluence of the left subclavian and internal jugular veins, and for the upper right torso lymphatics close to the confluence of the right subclavian and internal jugular veins.

The lymphatic structures of the eye

The lymphatic capillaries around the eye are connected to the lymphatic vessels and nodes of the head and neck (**FIG. 1**). An official consensus about recommendations for lymphatic markers (VEGFR-3, Lyve1, Prox1, PDPN; see **TABLE**) for the identification of the ocular lymphatics was only recently reached (14).

In the adult healthy eye, lymphatic vessels have been found in the conjunctiva, the corneal limbus, the iris, the ciliary body and in the periorbit (**FIG. 2**). Under normal conditions, vessels positive for lymphatic markers are not found in the retina, sclera or cornea, but the sclera and cornea do contain Lyve1-positive macrophages. Lyve1-positive macrophages have also been found in the choroid, but the presence of lymph vessels in the choroid is still controversial (15).

The endothelium-lined Schlemm's canal surrounds the cornea at the base of the iris, providing a conduit for aqueous fluid flow away from the anterior chamber. The endothelial cells of the Schlemm's canal express Prox1, a molecule crucial for the determination of lymphatic identity, and the Schlemm's canal is considered to be a lymphatic-like vessel.

Lymphatics in eye diseases

Pathological lymphatic formation in the human eye has been previously described for many diseases and injuries (**FIG. 3**; 16–18,20). In open globe injuries, lymphatic vessels may form in parts of the eye which are normally alymphatic, such as the sclera and cornea (16). Corneal lymph vessel formation may also occur associated with corneal graft rejection if the transplant recipient suffered from pre-existing corneal inflammation and vascularization (17,18). Herpes simplex virus (HSV-1) infec-

TABLE. Markers and factors of pathological angiogenesis and lymphangiogenesis (14,15,23,27).

					Increased in PDR vitreous samples	
	Abbrev.	Name	Lymphatic marker (14)	Lymphangio-genesis-promoting in vivo (15)	Angiogenic factors (15,23,27)	Inflam-matory factors (15)
Receptors	Lyve-1	lymphatic vessel endothelial hyaluronan receptor-1	√			
	PDPN	podoplanin	√			
	VEGFR-3	vascular endothelial growth factor receptor-3	√	√		
	VEGFR-2	vascular endothelial growth factor receptor-2		√		
Transcription factors	Prox1	prospero homeobox-protein-1	√			
	HIF-1α	hypoxia-inducible factor 1α			√	
Growth factors	VEGF-A	vascular endothelial growth factor-A		√	√	
	VEGF-C	vascular endothelial growth factor-C		√		
	VEGF-D	vascular endothelial growth factor-D		√		
	Ang2	angiopoietin 2		√	√	
	PDGF	platelet derived growth factor			√	
	TGF-β	transforming growth factor β			√	
	bFGF	basic fibroblast growth factor		√	√	
	EPO	erythropoietin			√	
ECM proteins	OPN	osteopontin			√	
	CYR61	cysteine-rich angiogenic inducer 61			√	
Proteases	MMP2	matrix metalloproteinase-2		√	√	
	MMP9	matrix metalloproteinase-9		√	√	
Cytokines	SDF1	stromal cell-derived factor 1			√	
	TNFα	tumor necrosis factor α		√		√
	IL-1β	Interleukine 1β		√		√
	IL-6	Interleukine 6				√
	IL-8	Interleukine 8				√
	IL-10	Interleukine 10				√
	IL-18	Interleukine 18				√
	MCP1	monocyte chemoattractant protein 1				√
	MIF	macrophage migration inhibitory factor				√
	M-CSF	macrophage colony stimulating factor				√
Other factors	HMGB1 protein	high mobility group box 1-protein				√
	NLRP3 inflamma-some	nucleotide-binding oligomeri-zation domain-, leucine-rich re-peat and pyrin domain-contain-ing protein 3 inflammasome		√		√

PDR = proliferative diabetic retinopathy

tion can cause corneal inflammation (herpetic keratitis), which in severe cases can lead to the formation of blood and lymph vessels in the cornea. Such lymphatic structures may remain in the cornea even after the infection has ended (17,18). Also dry eye disease (DED), a mild inflammation of the cornea, caused by insufficient or too rapidly evaporating tear fluid, can, in severe cases, lead to the formation of lymphatic vessels without the formation of blood vessels. However, the underlying mechanisms are not yet known (17,18). In addition, different tumors of the eye can be associated with pathologic lymphangiogenesis depending on their location and cell type. Lymphatic vessel formation has been found in both conjunctival squamous cell carcinoma, conjunctival melanoma, and ciliary body melanoma with extraocular extensions (15,17,18).

Disturbances of the Schlemm's canal function may prevent normal ocular fluid outflow and thus heighten the risk of increased intraocular pressure and glaucoma development (19). Lymphatic structures have also been found in the optic nerve area of eyes removed for glaucoma. Recently, the formation of lymphatic-like vessel formation has been discovered in two neovascular diseases of the posterior eye: in PDR and in hemi-central retinal vein occlusion (hemi-CRVO; 15,20-22). When edema and chronic inflammation develop in normally alymphatic tissues of the posterior eye, the formation of lymphatic-like structures may potentially facilitate fluid removal and the trafficking of immune cells.

Formation of lymphatic-like vessels in severe proliferative diabetic retinopathy

Diabetic retinopathy is the 6th most common cause of visual impairment in Finland's working age (18–64 years) population, typically in its proliferative form. Proliferative diabetic retinopathy (PDR) is a multifactorial eye dis-

Abbreviations

ADAMTS3 = a disintegrin and metalloproteinase with thrombospondin motifs 3
Ang1,2,4 = angiopoietin 1, 2, 4
CYR61 = cysteine-rich angiogenic inducer 61
DED = dry eye disease
bFGF = basic fibroblast growth factor
CRVO = central retinal vein occlusion
EPC = endothelial progenitor cell
EPO = erythropoietin
HIF-1α = hypoxia-inducible factor 1α
HMGB1 = high mobility group box 1-protein
HSV-1 = Herpes simplex virus 1
IL = interleukine
LEC = lymphatic endothelial cell
Lyve1 = lymphatic vessel endothelial hyaluronan receptor-1
MCP1 = monocyte chemoattractant protein 1
MIF = macrophage migration inhibitory factor
M-CSF = macrophage colony stimulating factor
M-LECP = macrophage-derived LEC precursor
MMP = matrix metalloproteinase
MRI = magnetic resonance imaging
NLRP3 = nucleotide-binding oligomerization domain-, leucine-rich repeat and pyrin domain-containing protein 3
NRP1,2 = neuropilin-1, -2
OPN = osteopontin
PDR = proliferative diabetic retinopathy
PDGF = platelet derived growth factor
PDPN = podoplanin
PlGF = placental growth factor
Prox1 = prospero homeobox-protein-1
PSA = prostate-specific antigen
RNA = ribonucleic acid
SDF1 = stromal cell-derived factor 1
Sema3A = semaphorin 3A
TIE1,2 = tyrosine kinase receptor with immunoglobulin and epidermal growth factor homology domains 1, 2
TGF-β = transforming growth factor β
TNFα = tumor necrosis factor α
VEGF-A, -B, -C, -D = vascular endothelial growth factor A, B, C, D
VEGFR-1, -2, -3 = vascular endothelial growth factor receptor 1, 2, 3
VESC = vascular endothelial stem cell

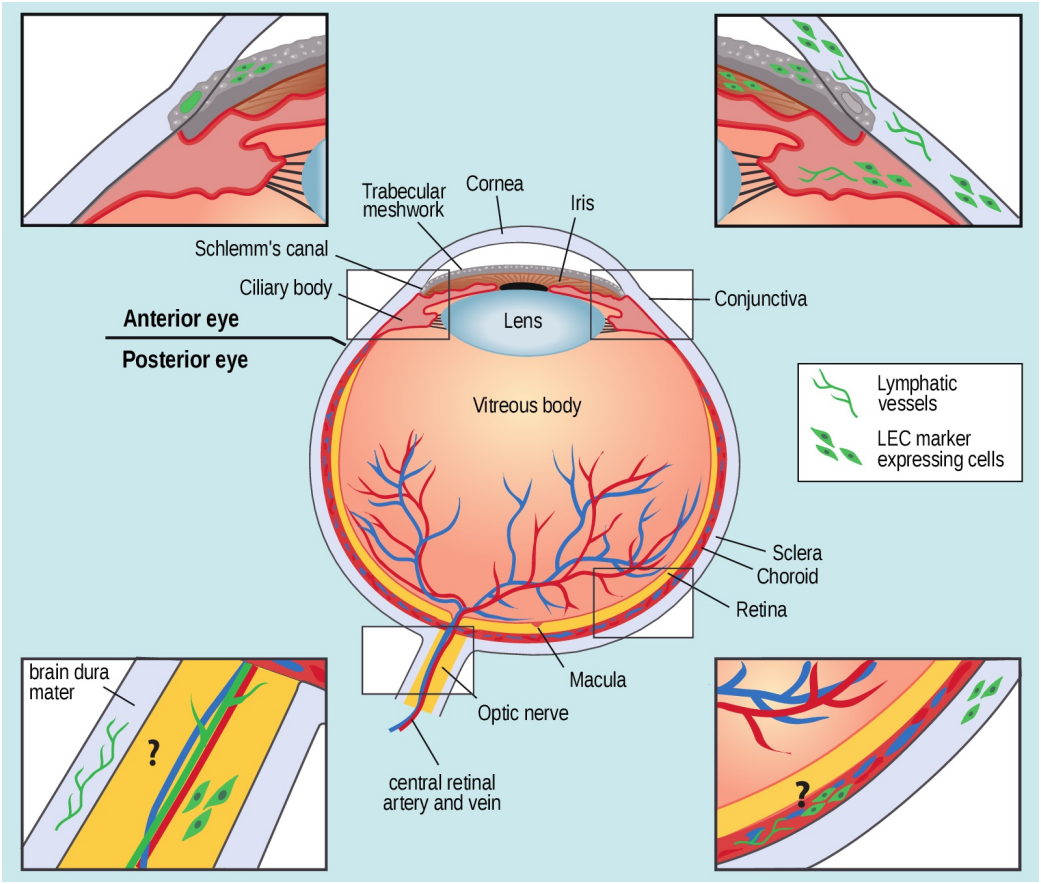

FIGURE 2. Lymphatic vessels in normal eye structures. In the adult healthy eye, lymphatic vessels have been consistently found in the conjunctiva, the iris, the corneal-conjunctival border (i.e., the limbus region), the ciliary body, and the periorbit. Based on the absence of LEC markers, the retina, the sclera and the cornea are considered to be normally alymphatic. While typical lymphatic vessels are absent, the sclera and cornea nevertheless contain macrophages which are positive for the lymphatic marker Lyve1. Under normal conditions, the adult choroid membrane is not considered to contain lymphatics, but it can feature Lyve1-positive macrophages. On the other hand, the circular, endothelial-lined Schlemm's canal has recently been identified as a lymphatic-like vessel based on its functions and the expression of lymphatic markers such as Prox1 (12,15).

ease that involves, among others, microvascular, neurodegenerative, metabolic, genetic/epigenetic, and inflammatory mechanisms.

In the diabetic eye, chronic oxygen deficiency (hypoxia/ischemia) and low-grade inflammation lead to the development of neovascularization (angiogenesis) in the retina, the optic disc and the iridocorneal angle (neovascular glaucoma; 23,24). For the treatment of severe diabetic retinopathy, first-line treatments include laser photocoagulation, cortisone and/or vascular endothelial growth factor-A (VEGF-A) inhibitor injections into the vitreous humor of the eye, or a combination thereof. The most severe forms, typically associated with vitreous haemorrhage from retinal neovascularization, connective tissue scarring, fibrosis and retinal detachment, have to be operated using modern vitreoretinal surgery. The development of this most severe form of PDR appears to be associated with the activation of endothelial stem cells or endothelial progenitor cells during neovascularization and, in some cases, the formation of lymphatic-like structures (20,22).

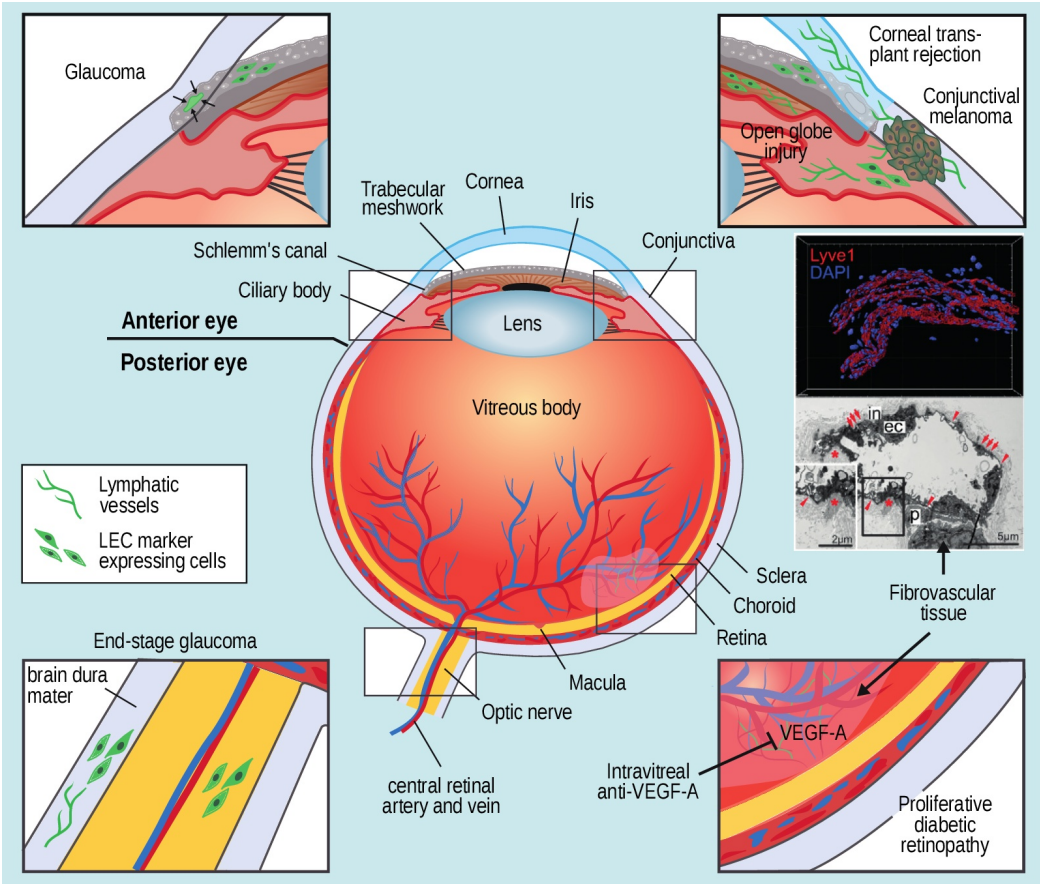

FIGURE 3. Lymphatic vessels of the eye in pathological conditions (15,20). Pathological neovascularization occurs in several diseases of the anterior eye. Neovascularization occurs in corneal transplant rejection, in conjunctival melanoma at the border of the uveoscleral outflow system, and in both cornea and sclera in the context of wound healing and penetrating ocular injuries. PDPN-positive vascular structures have also been found in eyes surgically removed due to end-stage glaucoma. The development of lymphatic vessels in posterior human eye pathologies (PDR, hemi-CRVO) has only recently been reported (20–22). The findings were based on immunohistochemical stainings, electron microscopy and a 3D PDR ex-vivo tissue model.
To the right, the 3D immunofluorescence staining of PDR tissue depicts Lyve1-positive vascular structures. The transmission electron microscope image below shows a cross-section of PDR tissue with electron-dense interstitial matrix. The vessels consist of flat or outward-bulging endothelial cells connected by either tight junctions or interdigitations (finger-shaped membrane protrusions). The endothelial cells display fenestations (arrowheads) and several basal invaginations and luminal protrusions. The neovasculature features lymphatic characteristics, such as thin walls, an absence of pericytes, a discontinuous basal membrane (arrows), overlapping junctions (in), and anchoring filaments (*). Original magnification x1200. Higher magnification in the box.

Growth factors and cytokines in the patophysiology of PDR

Long-term inflammation in the diabetic eye and prolonged elevated blood glucose levels (hyperglycemia) result in oxidative stress, oxidation of lipoproteins, and production of advanced glycosylation end products and free oxygen radicals, all of which contribute to the development and progression of PDR (15). In PDR, both cell-autonomous and intercellular signal transduction mechanisms can simultaneously promote and maintain angiogenesis, inflammation, and lymphangiogenesis (15,23–25; **FIG. 4**). Many inflammatory agents, growth factors, inflammatory cytokines, chemokines, and cell adhesion molecules (see **TA-**

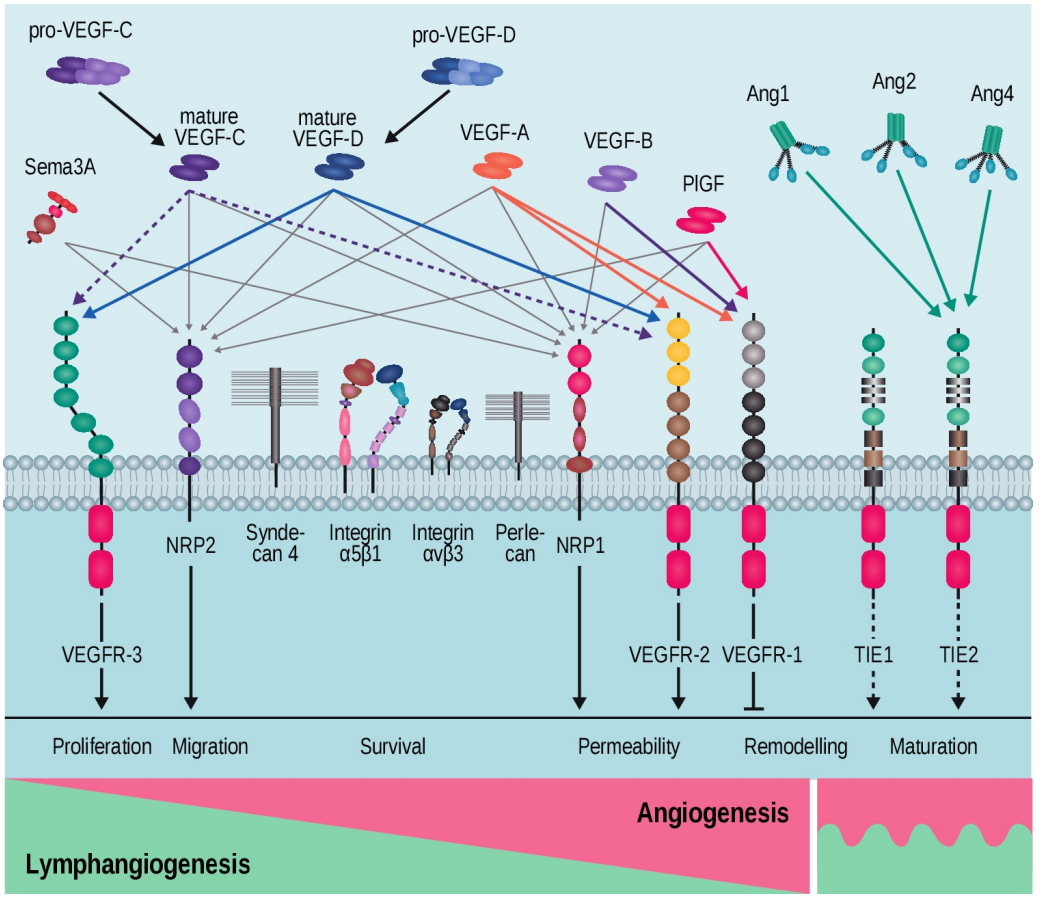

FIGURE 4. The growth of blood and lymphatic vessels and their functions are regulated primarily by vascular endothelial growth factors (VEGF) and angiopoietins (ANG) and their corresponding receptors, which are largely specific for endothelial cells. VEGF-A and VEGF-C are the major growth factors for blood vessels and lymphatic vessels, respectively. VEGF-A recognizes VEGF receptor-1 (VEGFR-1) and VEGFR-2, while VEGF-C and VEGF-D recognize VEGFR-3 (and, under certain circumstances, also VEGFR-2). As a rule, VEGFR-1 is only present on vascular endothelial cells, and VEGFR-3 is only present on lymphatic endothelial cells. In contrast, VEGFR-2 is found on both types of vessels. Co-receptors support the VEGF receptors: Neuropilins (NRPs), integrins and heparan sulfate proteoglycans stabilize the interaction between the VEGF receptors and the VEGFs and thus enhance the signaling. Unlike VEGF-A, VEGF-B and PlGF, the lymphangiogenic VEGF-C and VEGF-D are produced as inactive proproteins that must be activated through cleavage by specific proteolytic enzymes before they can stimulate lymphatic growth. ADAMTS3 functions as an activating protease during embryonic development, but in wound healing and pathological processes such as cancer, several other proteases such as PSA and cathepsin D are believed to be responsible for activation (10,25).

BLE) are contributing to the development of PDR (26–28). Activated vascular endothelial cells are one of the major sources of these factors. Many factors that are implicated in PDR-associated angiogenesis can also promote lymphangiogenesis (see TABLE). Inflammation is a major driver in the upregulation of VEGF-C expression (29,30), and local inflammation-activated macrophages could be one of the major producers of VEGF-A and VEGF-C, thus contributing to increased angiogenesis and lymphangiogenesis (31,32).

Mechanisms of neovascularization

In diseases of the posterior eye, such as PDR and hemi-CRVO, the neovascular (Lyve1$^+$, Prox1$^+$, VEGFR-3$^+$) lymphatic network is typically connected to the pathological

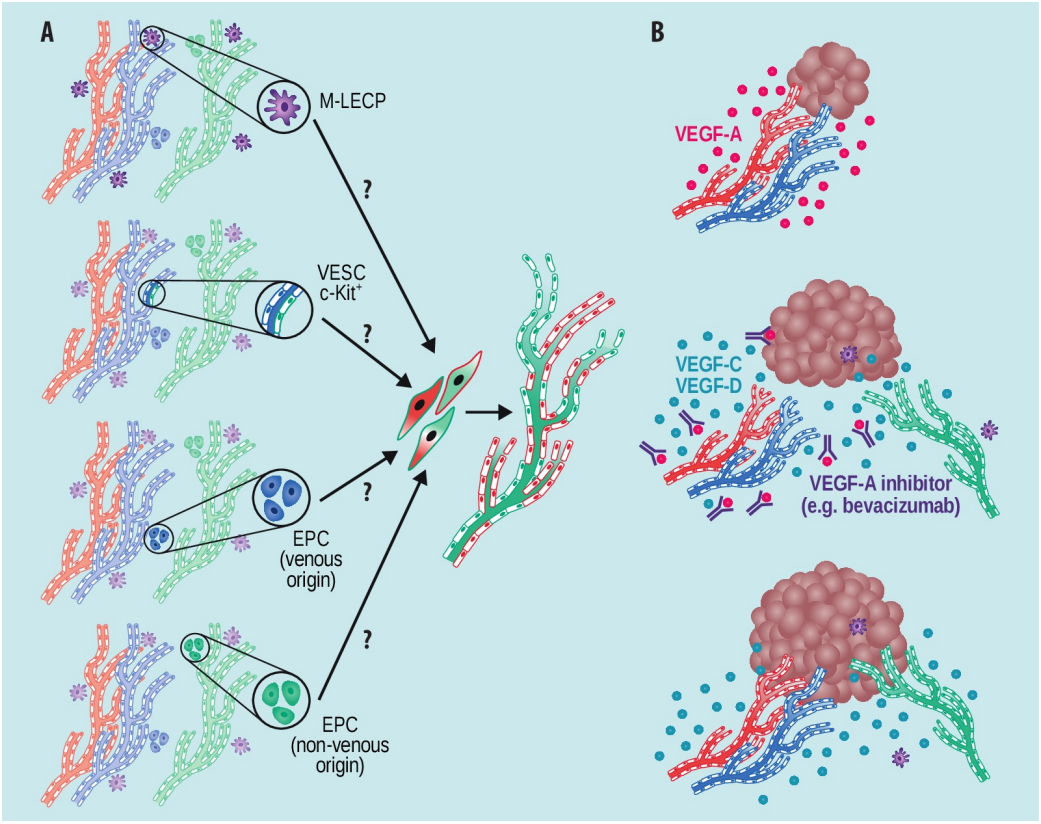

FIGURE 5. A) Possible mechanisms of pathological lymphangiogenesis. The pathological lymphatic structures either grow from existing lymphatic vessels (lymphangiogenesis) and/or by a de-novo mechanism (lymphvasculogenesis). Macrophage-derived cells (*M-LECP*) have been proposed to contribute to de-novo neovascularization. Alternatively, neovascularization may proceed via the transdifferentiation of vascular wall-resident vascular endothelial stem cells (VESCs) or via LEC cell fate induction/determination from endothelial precursor cells (EPCs) of venous, mobilized bone marrow or non-venous origin. **B**) Similar to angiogenesis, lymphangiogenesis is involved in the pathogenesis of several diseases. The vascular growth factors secreted by cancer cells and the tumor-associated inflammatory cells promote lymphangiogenesis. Anti-angiogenic drugs (such as the VEGF-A-blocking bevacizumab) are ineffective against lymphangiogenesis. The expanded lymphatic network acts as a pathway for the spread of cancer cells to the draining lymph nodes, which may allow for later metastasis to distant organs.

vasculature at the optic nerve entry region (20–22). Thus, it is possible and quite likely that this vasculature could connect to the dura mater of the brain (2,33). Macrophages, which are central to the inflammatory process, may also be involved in the formation of pathologic lymphatic vessels by another mechanism, namely by transforming into lymphatic endothelial cell precursors (*M-LECPs*). Alternatively, c-Kit+ endothelial stem cells could contribute to the formation of lymphatic-like vascular structures in PDR eyes (**FIG. 5A**; 15,20,22,34).

Emerging research on diabetic eye pathological neovascularization

Before the introduction of insulin treatment in 1921, the life expectancy of diabetic patients was very short, and therefore diabetic eye neovascularization and PDR formation were not medical concerns. Additionally, the presently used vitreous and retinal surgical techniques are still relatively new, with the first vitrectomy surgery on the human eye performed in 1970. While angiogenesis research has rapidly advanced over the recent decades, lymphangiogenesis research has pro-

gressed significantly slower, because lymphatic research has been more challenging.

Just in the last decade, advanced ophthalmic surgical techniques and associated translational research have enabled systematic blood and lymphatic vascular biology studies of posterior eye segment neovascular diseases (PDR and hemi-CRVO). Only relatively recently, lymphatic-like structures have been searched for and successfully detected by electron microscopy and, after specific immunohistochemical stainings, by light microscopy (20,21).

Current animal models do not adequately replicate the human disease

Diabetes-related intraocular neovascularization progresses very slowly. Typically, intraocular neovascular changes require 15 to 20 years to develop in a type 1 diabetic patient. Although examination of vascular structures, e.g. in mice, has been facilitated by transgenic technology, there is currently no single experimental animal model that truly combines the multifactorial disease mechanisms involved in neoangiogenesis and neolymphangiogenesis in the human eye (35,36). Contrary to this, a mouse model has been generated to study the neovascular mechanisms of another eye disease: wet age-related macular degeneration (AMD). Understanding the pathogenesis of early vascular neovascular changes and subsequent fibrovascular phases of AMD might be helpful in developing future therapies (37).

Various anti-VEGF-A therapies are used to inhibit ocular neovascularization: the humanized monoclonal antibody bevacizumab (*Avastin*™), its binding moiety derivative ranibizumab (*Lucentis*™), aflibercept and conbercept (both *VEGF traps* consisting of extracellular domains from human VEGFR-1 and -2 fused to the Fc portion of immunoglobulin G1, *Eylea*™/*Lumitin*™). In order to develop more effective combination therapies, a comprehensive and profound understanding of the disease mechanisms involved in the formation of blood and lymph vessels is a prerequisite.

Pivotal role for the new PDR ex-vivo models in future research

Our research team has published a new ex-vivo model of PDR which can be used to study both angiogenesis and lymphangiogenesis (22,38). Using this new model, we were able to detect Lyve1$^+$, Prox1$^+$, and VEGFR-3$^+$ structures in cultured tissues removed from PDR eyes. These results are also supported by separate RNA sequencing data. Some abnormal vascular structures resembled blood vessels while others were more reminiscent of lymphatic structures. We believe that this PDR ex-vivo model could be useful e.g. in the search for targeted therapeutic interventions. Inhibition of the angiogenesis- and lymphangiogenesis-associated fibrosis is also important to improve the prognosis for severe PDR (22). Although an ex-vivo tissue model is a valuable new research tool, it cannot, for example, be used to test and optimize in-vivo drug delivery, apart from some exceptional cases.

In the absence of other structurally and functionally appropriate alternatives, further investigation of the three-dimensional PDR ex-vivo model appears mandatory. Current commercially available angiography systems capable of retinal imaging don't provide sufficient resolution for the study of pathological neovascular and/or potential lymphatic structures (39). Future magnetic resonance imaging (MRI) at 7–10 tesla might not only provide additional possibilities for neural imaging, but also enable the detection of very small pathological changes to blood and lymphatic vessels, notably also in posterior eye diseases (40).

Future therapies

VEGF-A plays undisputedly a central role in the development of intraocular neovascularization and has therefore been extensively studied. Current VEGF-A-blocking treatments administered by vitreous injection have been a major step forward. However, they only help in about 30–60% of cases, and they need to be given repeatedly, which places a strain on ophthalmic units. Also for this reason, worldwide drug discovery and development efforts conti-

Core concepts

- Translational cell and tissue research is needed to develop more effective treatments for lymphatic system disorders.
- Proliferative diabetic retinal disease is the most severe ocular complication of diabetes and can lead to loss of vision.
- According to current research, pathological angiogenesis of the eye may also be associated with the development of lymphatic vessels.
- New findings from the lymphatic system may help develop more effective therapies for VEGF-A inhibitory therapies.
- The pathological angiogenesis of the eye and its associated lymphangiogenesis are some of the challenges that modern medicine can solve.

nue to find more effective agents than the current VEGF-A inhibitors (41). Among others, targets of recent interest include tyrosine kinase signaling, integrins, Ang/Tie2 signaling, combined anti-VEGF-A/ anti-Ang2 therapy, the plasma kallikrein system, the Sema3A system, and inhibition of MMP2 and MMP9. Anti-inflammatory drugs (statins) and stem cells are also being actively studied in the treatment of eye diseases. Inhibition of VEGF-C may be a novel therapeutic option in the near future for the treatment or prevention of severe diabetic eye disease (42). Personalized medicine and *omics* research (e.g. proteomics, transcriptomics and lipidomics) have the potential to contribute to an improved understanding of the pathogenesis of PDR and to the development and introduction of more effective therapies. Potential future therapies include notably also gene therapy, which can provide long-term (> 1 year) therapeutic effects with a single intraocular injection. Such gene therapy approaches are also actively researched in Finland (43), and research results may prove to be particularly valuable because neovascular growth inhibitors are not only promising for the treatment of PDR, but as such are also potential new anticancer drugs (**FIG. 5B**).

Conclusions

Despite the achievements of recent years, the lymphatic research community cannot complain about a lack of genuine scientific controversies. Many of these issues will probably require accurate post-mortem human tissue studies and the use of animal or ex-vivo models that better reflect human anatomy. Particularly in diseases such as PDR, the formation of which involves retinal edema and chronic inflammation, the lymphatic system is a potentially prolific subject of research. Given the large projected increase in the numbers of diabetics worldwide, it is becoming increasingly important to focus resources on basic research into severe PDR. The goal should be to progressively resolve the pathological mechanisms involved in its development in order to be able to prevent or at least delay PDR development and related vision loss. ■

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ZUSAMMENFASSUNG

Die Lymphgefäße des Auges

Das Lymphsystem bildet die Grundlage für die Homöostase der Körperflüssigkeiten und für die Funktionen des Immunsystems. Es ist allerdings auch an vielen pathologischen Prozessen beteiligt wie z.B. an Krebs, Herz-Kreislauf- und neurodegenerativen Erkrankungen. Trotz großer Fortschritte (z.B. der Entdeckung der meningealen Lymphgefäße und der lymphatischen Eigenschaften des Schlemm-Kanals) sind viele Fragen noch ungeklärt. Lymphgefäßähnliche Strukturen wurden kürzlich auch in normalerweise avaskulären Bereichen von Augen entdeckt, die an proliferativer Retinopathie erkrankt waren, einer Diabetes-Komplikation, bei der die medikamentöse Unterdrückung des pathologischen Gefäßwachstums nicht immer erfolgreich ist. Fortschritte in diesem Forschungsbereich könnten dazu beitragen, neue Therapien für Krankheiten des Lymphgefäßsystems zu entwickeln und auch für andere Krankheiten, in denen Lymphangiogenese eine Rolle spielt.

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