



Corneal angiogenesis and lymphangiogenesis

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Purpose of review

The purpose of the present review is to describe new antilymphangiogenic treatment strategies and recent findings on strain-dependency of corneal lymphangiogenesis and the interdependency between blood and lymphatic vessel growth.

Recent findings

Studies on mice have revealed that apart from haemangiogenesis, lymphangiogenesis can also differ markedly between several mouse strains under normal and inflammatory conditions. Although haemangiogenesis and lymphangiogenesis are closely interconnected in their spatial-temporal patterning, recent data suggest that they can also occur independently.

Summary

Understanding the coordinated regulation of blood and lymphatic vessel growth and genetic factors determining lymphangiogenesis in more detail could improve the development of specifically targeted antihaemangiogenic or antilymphangiogenic strategies.

Keywords

angiogenesis, corneal lymphangiogenesis, genetic heterogeneity, inhibitors of lymphangiogenesis

INTRODUCTION

Angiogenesis is commonly defined as the process of new capillary blood vessel growth from the preexisting vasculature.

The established classical mechanisms of new vessel growth are vasculogenesis, sprouting angiogenesis, nonsprouting, intussusceptive angiogenesis and a process described as looping angiogenesis.

New blood vessel formation during embryonic development in vertebrates involves vasculogenesis (formation of the primary vascular plexus via de-novo formation of blood vessels from differentiating progenitor cells) and angiogenesis (formation of blood vessels from preexisting ones) [1]. In adults, angiogenesis is largely downregulated. Exemptions are conditions requiring new vessel growth such as inflammation, wound healing, or in the female reproductive system, wherein cyclic changes are associated with extensive angiogenesis [2].

The guided process of sprouting angiogenesis is characterized by local degradation of the basement membrane, remodelling of the extracellular matrix, increased vascular permeability and proliferation of endothelial cells. Specialized endothelial cells at the tip of the sprout are able to sense stimuli from their environment using long filopodia. Migration of the tip cells strongly depends on vascular endothelial growth factor A (VEGF-A) gradients, as exogenous

administration of VEGF-A leads to a reduced number of filopodia and less migration [3]. VEGF-A signals are transmitted via vascular endothelial growth factor receptor 2 (VEGFR2), which is punctually expressed in the filopodia of the endothelial tip cells. Stalk cells proliferate and establish the vessel lumen depending on local VEGF-A concentrations [3]. Covering the new established microvessels with mural cells like pericytes or smooth muscle cells and establishing a basement membrane follows upon maturation of the vessel.

Intussusceptive angiogenesis was first recognized in the developing lung by Caduff *et al.* [4] and gained wide attention several years later. Intussusception is characterized by the formation of tissue columns (so-called 'pillars') into the vessel lumen of preexisting vessels, which leads to a splitting of the vessel [5]. Three different phases of intussusceptive angiogenesis are known: intussusceptive microvascular growth (IMG), which can lead

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Curr Opin Allergy Clin Immunol 2012, 12:548–554

DOI:10.1097/ACI.0b013e328357b4a2

to a rapid enlargement of the capillary bed without increased vessel permeability and intussusceptive arborisation (IAR) accounting for the expansion of the vascular tree [6]. Intussusceptive branching remodelling (IBR) refers to remodelling at points of bifurcation and to the pruning of vessels, triggered by haemodynamic requirements [6].

Recently, a fourth mechanism described as 'looping angiogenesis' has been proposed by Kilarski and Gerwins [7]. They described the translocation of preexisting vessels with functional circulation during wound healing driven by biomechanical forces and subsequent expansion or remodelling of the new vessels in a chick chorioallantoic membrane (CAM) model and the healing cornea [7]. How and to what degree this mechanism can be applied on tumour angiogenesis or other conditions besides wound healing has yet to be analysed.

A new mechanism contributing to corneal inflammation-induced lymphangiogenesis critically involves macrophages that were suggested to integrate into the newly formed lymphatic structures [8]. Recently vascular adhesion protein-1 (VAP-1) has been shown to be involved in macrophage recruitment during inflammation. Inhibition of VAP-1 led to a decrease in IL-1 β -induced lymphangiogenesis and haemangiogenesis and reduced the number of infiltrating F4/80⁺ macrophages [9].

INHIBITORS OF CORNEAL (LYMPH) ANGIOGENESIS

Despite the need of a functional circulatory system, some tissues such as cartilage and cornea remain avascular under normal conditions. The avascular nature of the cornea is maintained by the presence of several antihaemangiogenic factors [such as angiostatin, endostatin and thrombospondin 1 (TSP-1)]. Ectopically expressed VEGFR3 in the corneal epithelium [10] and soluble VEGFR1 [11] contribute to corneal avascularity by entrapping growth factors and acting as decoy receptors.

Under certain circumstances such as inflammation, the tightly regulated balance of proangiogenic and antiangiogenic factors is disturbed and subsequent corneal neovascularization can occur and lead to severe sight-threatening effects. Furthermore, corneal neovascularization increases the risk for immune rejections after normal and high-risk corneal transplantation [12,13]. Recently, it has been shown that blocking lymphangiogenesis as the afferent part of the immune reflex could significantly improve graft survival, although the recipient beds were still haem-vascularized (Fig. 1) [14[■]].

In a mouse model of corneal transplantation, blocking VEGF-C inhibited haemangiogenesis and lymphangiogenesis and further the maturation of antigen presenting cells (APCs) and the migration to the draining lymph nodes, promoting allograft survival [15]. Selectively inhibiting lymphangiogenesis seems to be a promising therapeutic strategy in

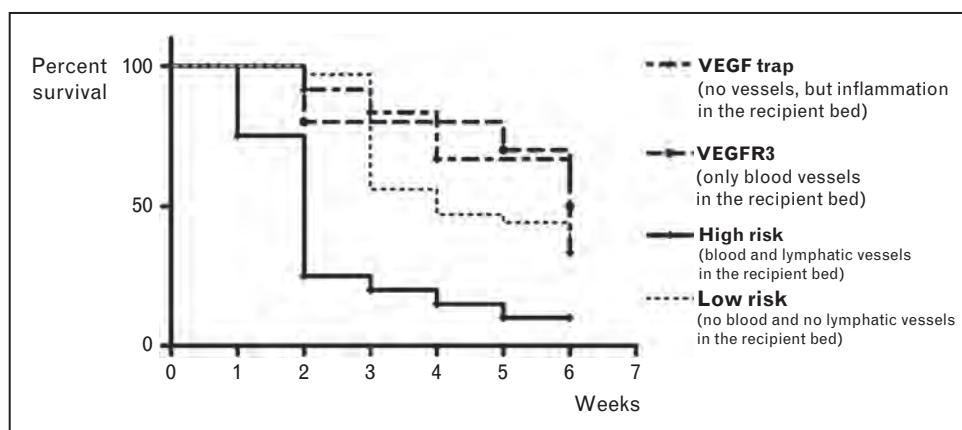


FIGURE 1. Lymphatic vessels in the recipient bed prior to transplantation determine graft survival. In the 2 weeks prior to transplantation (when corneal suture placement was used to induce pathologic corneal neovascularization in the recipient bed), mice were treated with either vascular endothelial growth factor-Trap_{R1R2} (VEGF-Trap_{R1R2}) resulting in no blood or lymphatic vessels, but reduced inflammation in the recipient bed at the time of transplantation, or VEGFR3 Ab mF4-31C1 resulting in the absence of lymphatic vessels, but presence of only blood vessels in the recipient bed at the time of transplantation. Graft survival was compared with prehaem- and prelymphovascularized controls (black line) and avascular recipient controls (dotted line). The graft survival was significantly better when transplants were placed into recipient beds lacking lymphatic vessels compared with beds with lymphatic vessels present at the time of transplantation (VEGF-Trap versus high-risk: *P*, 0.0001; VEGFR3 versus high-risk: *P*, 0.0002; *n* = 10, Kaplan–Meier survival curve). Modified from [14[■]].

corneal transplantation [14^{*}]. The following chapter presents some anti(lymph)angiogenic factors and also recently identified endogenous lymphangiogenesis inhibitors.

Vascular endothelial growth factors

VEGF-A can exert its function via binding of VEGFR1 and VEGFR2, with VEGFR2 thought to be the primarily responsible receptor for proangiogenic signalling in sprouting angiogenesis and endothelial cell survival. The soluble form of VEGFR1 (sVEGFR1) has a potential biological function in inhibiting VEGF-A-dependent signalling [16]. Recently, it has been shown that injection of VEGFR1-specific morpholino increased the production of sFLT1 via alternative splicing leading to a decrease in angiogenesis in a corneal suture model and tumour neovascularization [17,18].

Bevacizumab, a monoclonal antibody against VEGF-A inhibited inflammation-induced angiogenesis and lymphangiogenesis in the murine cornea [19]. The penetration of bevacizumab after topical application was reported to be limited for corneas with healthy epithelium, but reached the corneal stroma in neovascularized corneas in a murine model [20]. In patients with corneal neovascularization, it was shown that blocking VEGF-A with bevacizumab reduced the vascularized area and the vessel diameter after topical application [21]. Recently, a meta-analysis including clinical studies and animal studies suggested that a reduction in the corneal neovascularization area can be achieved with both topical and subconjunctival application of bevacizumab [22]. In a mouse model of epithelial erosion, no significant side effects concerning wound closure were found and normal corneas with intact epithelium seem not to be negatively affected in the resting corneal nerve density by application of bevacizumab [23]. However, decreased corneal wound healing in rats after application of bevacizumab as eye drops [24] and an impaired regeneration of the corneal subbasal nerve plexus following corneal wounding were reported [25]. Due to the potential side effects of bevacizumab, the anti-VEGF therapy should be carefully monitored in patients with neurotrophic keratopathy or epithelial defects [21].

A recent study [26] on ranibizumab and bevacizumab as topical treatment options for corneal neovascularization suggested both anti-VEGF drugs as well tolerated and effective therapeutic treatment options.

Steroids

Topical application of anti-inflammatory corticosteroids is commonly used after corneal transplantation.

Recently, corticosteroids have been shown to inhibit the outgrowth of corneal lymphatic vessels and macrophage recruitment into the cornea, with significant differences in the antilymphangiogenic potency among the analysed steroids fluorometholone, prednisolone acetate and dexamethasone sodium phosphate [27,28].

Antisense oligonucleotides against insulin receptor substrate-1

Blocking insulin receptor substrate (IRS-1), an intracellular protein implicated in the angiogenic signalling cascade exerts antiangiogenic effects *in vivo* [29,30]. GS-101 (aganirsen), an antisense oligonucleotide against IRS-1, was analysed in a phase II study [31], which suggested aganirsen to be a well tolerated and effective treatment strategy for excessive vascular growth in the cornea. Currently, aganirsen as eye drops is being tested in an ongoing phase III study. Topical application of aganirsen in the cornea seems to exert also antiangiogenic activity in an animal model of retinal neovascularization [30]. Recently, it has been shown that blocking IRS-1 by GS-101 also inhibits murine corneal lymphangiogenesis [32].

Endogenous inhibitors

Far less information is available about endogenous inhibitors contributing to the alymphatic status of the normal cornea up to now. Soluble VEGFR2, a splice variant of VEGFR2, which is present in the corneal epithelium and corneal stroma specifically inhibited lymph but not blood vessel growth in the cornea [33].

TSP-1, already known as an antihaemangiogenic factor [34], additionally exerts antilymphangiogenic properties in the cornea via monocytic cells. TSP-1 downregulated the expression of VEGF-C mRNA in macrophages via ligation of its receptor CD36 present on corneal macrophages but not on corneal lymphatic vessels, thereby indirectly inhibiting lymphangiogenesis [35^{*}].

Vasohibin (VASH1), an intrinsic inhibitor of angiogenesis expressed in endothelial cells, suppressed VEGF-A, VEGF-C, fibroblast growth factor 2 (FGF2) and platelet-derived growth factor BB (PDGF-BB) induced lymphangiogenesis [36] in a corneal micropocket assay.

Identifying further endogenous inhibitors of lymphangiogenesis using, for example, genetic strategies could provide new treatment options for diseases connected with lymphatic vessel growth. In the next part, the genetic heterogeneity in angiogenesis and lymphangiogenesis as a source for identifying such candidates will be discussed.

GENETIC HETEROGENEITY OF ANGIOGENESIS AND LYMPHANGIOGENESIS

Genetic heterogeneity of angiogenesis had been intensively studied for several years. Based on up to 10-fold differences in blood vessel growth in response to bFGF and VEGF [37], further analysis of angiogenic responsiveness led to the identification of several quantitative trait loci (QTL). QTL analysis is used to combine phenotypic information (e.g. vessel growth) and genotypic data (e.g. molecular markers) to reveal possible candidate genes contributing to the observed variation in the

analysed trait. Thereby VEGF responsiveness was linked to two regions on chromosome 10 and 2 (designated AngVq1 and AngVq2) [38] and bFGF responsiveness to regions on chromosomes 4, 13, 15 and 18, designated AngFq1–AngFq4, respectively [39]. Nowadays, about 10 years later, research in genetic heterogeneity of lymphangiogenesis experiences more attention, as specific markers for lymphatics (LYVE-1, Prox-1, VEGFR-3) are now available. Phenotypic differences in the surface area of corneal lymphatic vessels were demonstrated for growth factor (VEGF-C) (Fig. 2) and inflammation-induced lymphangiogenesis and for the preexisting

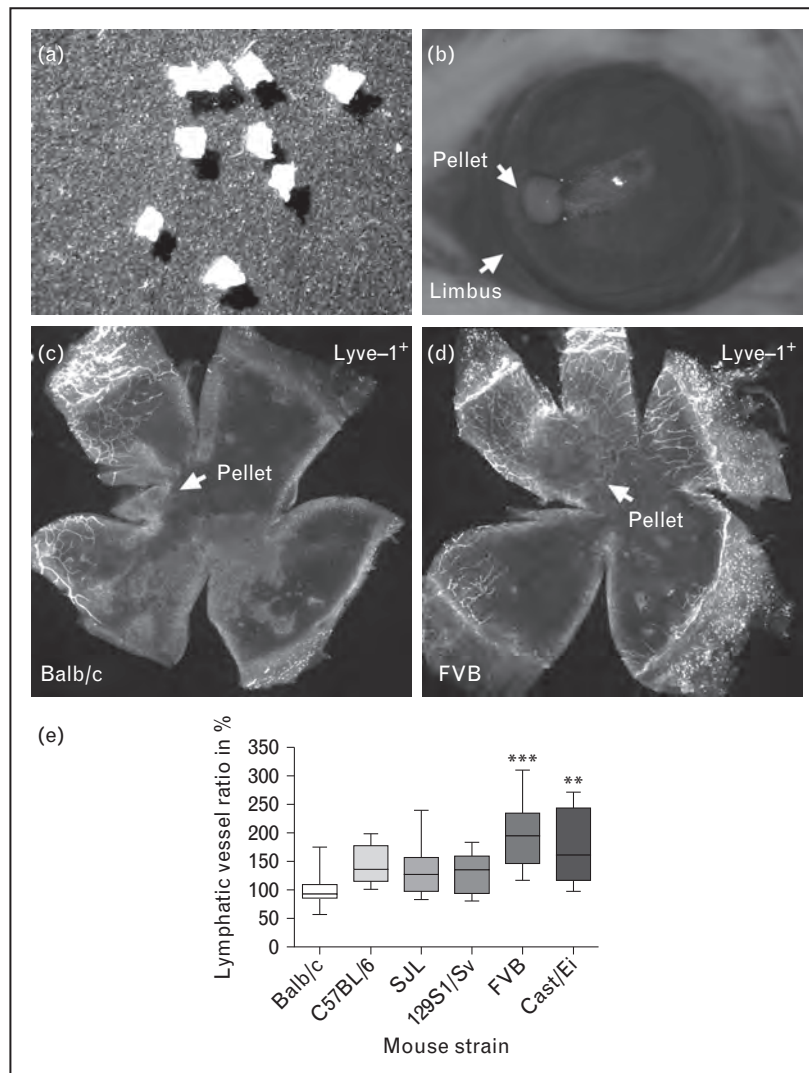


FIGURE 2. Interindividual differences on vascular endothelial growth factor C-induced lymphangiogenesis. (a) Pellets with similar dimensions containing 200 ng vascular endothelial growth factor C (VEGF-C). (b) Micropocket in the corneal stroma near the limbus containing a pellet. (c) VEGF-C induced lymphangiogenic response in corneal wholemounts of BALB/c mice with LYVE-1⁺ lymphatic vessels. (d) VEGF-C induced lymphangiogenic response in corneal wholemounts of FVB mice with LYVE-1⁺ lymphatic vessels. (e) The lowest relative vascularization area (lymphatic vessel area versus corneal surface area) was induced by VEGF-C in BALB/c mice (c), the highest in FVB mice (d), resulting in a 1.9-fold difference between the 'high-responder' and 'low-responder' strain ($P < 0.001$). Modified from [41].

lymphatic vessels in the limbal area [40,41]. The amount of preexisting lymphatics was demonstrated to be significantly more in C57BL/6 mice compared with BALB/c mice [40–42]. This was accompanied with an increase of macrophages in the untreated cornea, which were shown to be involved in lymphangiogenesis under inflammatory conditions by releasing prolymphangiogenic growth factors and by integrating in lymphatic structures [8]. Differences in cell numbers, cell recruitment and in lymphangiogenic vessel growth may also be linked to different corneal graft acceptance rates observed between BALB/c and C57BL/6 [43]. Future studies on genetic variability in lymphangiogenesis and QTL analysis will help to elucidate the molecular basis and the effects of genetic variants in lymphangiogenesis. This is especially relevant as genetic variability was shown to influence expression of angiogenic factors or disease susceptibility/progression and can also affect the outcome of therapies [44–47]. Conventional cytotoxic drugs and antiangiogenic agents such as bevacizumab, an anti-VEGF humanized monoclonal antibody, have interindividual heterogeneity regarding their efficacy and toxicity in patients, which can lead to different treatment outcomes [44,45,48,49]. Therefore, pharmacogenetic studies are designed to identify genetic variations (including e.g. mutations, copy number alterations and SNPs, the most common type of polymorphisms) affecting, for example activity of drug-metabolizing enzymes, target proteins or factors involved in the disease pathway that could alter the pharmacological response. Several studies have been carried out to identify and characterize the effect of polymorphisms in proangiogenic and antiangiogenic factors such as VEGF [50–53], angiopoietins [54] and TSPs [55,56] in order to reveal their effect on disease progression or disease risk. It was demonstrated that VEGF polymorphisms are linked to the severity or susceptibility to cancer, outcome of several treatments and to other diseases such as arthritis, cardiovascular disease and ocular diseases like age-related macular degeneration [51,53,57] and diabetic retinopathy [52,58,59]. Identifying underlying genetic factors of different angiogenic and lymphangiogenic phenotypes could help to improve treatment strategies. Furthermore, this information could provide new insights into the interdependence of blood and lymphatic vessel growth and the spatial-temporal regulation.

INTERDEPENDENCE OF ANGIOGENESIS AND LYMPHANGIOGENESIS

VEGFR2 expression on angiogenic tips was suggested as a mechanism for blood vessels influencing their

environment and the growth pattern of blood and lymphatic vessels by trapping VEGF-C, causing delayed lymphangiogenesis in response to VEGF-A [60]. Predominantly, lymphatic vessel growth without angiogenesis was demonstrated using low-dose bFGF treatment and inhibiting angiogenesis with sVEGFR-1 showed no effect on lymphangiogenesis, suggesting that lymphatic vessels can also grow independently of preexisting blood vessels [61]. Data from Hajrasouliha *et al.* [62] demonstrated a spatially distinct outgrowth pattern of blood and lymphatic vessels induced via bFGF-pellets. Whereas lymphatic vessels grew predominantly from the opposite site of the pellet, blood vessels seem to grow from the prepellet area into the centre of the cornea. Distinct changes for lymph vessels but not blood vessels were also found between 3-week-old and 8-week-old mice: whereas lymphatic vessels of C57BL/6 mice exhibited an increase in the vessel calibre during ageing, the blood vessels retained their diameter [63]. Differences in the distribution pattern of blood and lymphatic vessels were further shown during inflammation. In the limbal area of BALB/c and C57BL/6 corneas, blood and lymphatic vessels were predominantly located in the nasal side of the cornea. Suture-induced inflammation changed the nasally dominant pattern of blood vessels to an even distribution on both sides, whereas lymphatic vessels retained their nasal predominance [64]. In summary, the data suggest that although angiogenesis and lymphangiogenesis are closely interconnected and lymphangiogenesis often accompanies angiogenesis, both processes may also occur independently of each other.

CONCLUSION

Corneal lymphangiogenesis and antilymphangiogenic treatment options in view of inflammation, tumour biology and transplant rejection are an emerging field in ophthalmologic research. Identifying novel, specific antilymphangiogenic factors to temporarily inhibit lymphangiogenesis may lead to novel treatment options for diseases of the cornea and the ocular surface connected with neovascularization. Nevertheless, identifying the underlying molecular details and mechanisms of coordinated regulation of blood and lymphatic vessel growth will be needed to understand disease pathways in more detail.

Acknowledgements

The study was supported by the German Research Foundation (DFG: Sonderforschungsbereich SFB 643: B10; DFG Cu 47/4–1).

Conflicts of interest

C.C. has been consultant for Gene Signal.

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- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 573).

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