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Corneal Lymphangiogenesis in Herpetic Stromal Keratitis

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Abstract

Corneal lymphangiogenesis is the extension of lymphatic vessels into the normally alymphatic cornea, a process that compromises the cornea's immune privileged state and facilitates herpetic stromal keratitis (HSK). HSK results most commonly from infection by herpes simplex virus-1 (HSV-1) and is characterized by immune- and inflammation-mediated damage to the deep layers of the cornea. Current research demonstrates the potential of anti-lymphangiogenic therapy to decrease and prevent herpes-induced lymphangiogenesis.

Keywords

Herpes simplex virus-1; cornea; lymphangiogenesis; vascular endothelial growth factor (VEGF); neovascularization

I. Introduction

Herpes simplex virus 1 (HSV-1) is one of the most prevalent human infectious pathogens, with an estimated global seroprevalence rate between 50% and 90%.¹³¹ Corneal infection by HSV-1 can lead to herpetic stromal keratitis (HSK), the primary cause of infectious blindness in the developed world.⁸⁹ HSK is characterized by immuno-inflammatory corneal lesions and neovascularization of the normally avascular cornea, which together create clouding and compromise visual acuity.^{4; 6; 8} The resulting angiogenesis also compromises corneal angiogenic privilege, i.e., the cornea's ability to halt the growth of new blood vessels

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after injury.^{4; 23} Corneal angiogenic privilege is maintained by a delicate balance involving upregulation of anti-angiogenic factors, including angiostatin, endostatin, and soluble vascular endothelial growth factor receptor 1 (sVEGFR1), and downregulation of angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2).⁴² HSV-1 infection disrupts this equilibrium between angiogenic and anti-angiogenic factors, leading to an angiogenic switch.⁶⁸

Corneal lymphangiogenesis, the extension of lymphatic vessels into the normally alymphatic cornea, is a recently identified significant contributor to HSK.¹⁵⁵ Lymphangiogenesis involves the infiltration of inflammatory mediators and host immune cells, which cause tissue damage with potentially blinding effects. We provide a brief overview of the pathogenesis of HSK and corneal immune privilege and focus on HSK to highlight the lymphangiogenesis that occurs in response to HSV-1 corneal infection, considering both the viral and host immune contributors to this process. In addition to the possible mechanisms leading to HSV-1-induced lymphangiogenesis, we discuss how key insights into this process may aid the discovery and development of potential therapeutics and prophylactics for the treatment and prevention of this vision-threatening disease.

II. HSV-1 Background and Corneal Infection, HSV-1 Cell Entry, and Subsequent Pathogenesis of HSK

A. HSV-1 Background and Corneal Infection

Herpes simplex virus is an enveloped, double-stranded DNA virus and a member of *Alphaherpesvirinae*, a subfamily of *Herpesviridae*. Of the three members of this subfamily (HSV-1, HSV-2, and varicella zoster virus), HSV-1 has the greatest association with ocular infection.⁴⁴ HSV-1 is a regular and spherical-shaped enveloped virion. Apart from the envelope, the other two main structural elements are tegument, which is a proteinaceous region, and the icosahedral-shaped nucleocapsid that houses its genome, which is composed of linear double-stranded DNA. HSV-1 contains 11 viral glycoproteins, four of which are essential for viral entry (gD, gH, gL, and gB). The proteinaceous tegument is also required in the early stages of infection for delivery of viral proteins into the host cell.³

HSV-1 is usually acquired from individuals with clinical or subclinical facial, oral mucosal, or genital mucosal manifestations.⁸⁸ Less often, HSV-1 is transmitted via corneal transplantation.¹¹⁷ Primary infection of corneal epithelial cells by HSV-1 rarely leads to HSK. Instead, HSK is more often initiated upon reactivation of HSV-1 virions with life-long latency that lie dormant in the trigeminal ganglion.¹¹² During reactivation, newly synthesized virion particles migrate along the sensory fibers of the trigeminal nerve and often give rise to orolabial lesions; however, because the sensory fibers from the trigeminal nerve also supply the cornea, occasional reactivation of HSV from its latent state results in the delivery of virion particles to the cornea.¹⁵⁵ The manifestations of this disease in the cornea demonstrate that, although the corneal epithelium may serve as a physical barrier to other infectious microorganisms,⁶⁸ viruses such as HSV-1 can still infect the corneal epithelium.⁴⁴ Repeated reactivation of dormant HSV-I virions, a process induced by a wide

array of stressors, may ultimately lead to chronic inflammation in the corneal stroma, with clinical or subclinical manifestations.¹²³

B. HSV-1 Cell Entry

HSV-1 infection at a cellular level begins with interactions between viral envelope glycoproteins and their respective host cell receptors.¹³² The initial attachment step involves glycoprotein B (gB) and glycoprotein C (gC) interactions with the host cell surface heparan sulfate proteoglycans.^{58; 124} HSV-1 glycoprotein D (gD) then undergoes conformational changes that allow it to bind to any of its host cell receptors, including nectin-1, herpes virus entry mediator (HVEM),⁷⁷ or 3-O-sulfated heparan sulfate (3-OS-HS).¹³⁰ These conformational changes of gD represent a critical event that permits the gD-host-cell-receptor complex to associate with the heterodimer complex of glycoprotein H/glycoprotein L (gH/gL). Next, gH/gL binds with gB, allowing viral fusion with the host cell membrane.^{41; 130} The capsid is then transported to the nuclear pores, DNA is released to the nucleus, and viral replication and transcription begins. Synthesis of proteins from the HSV virion progresses in three rounds, designated as immediate early (IE), early (E), and late (L). The IE products regulate gene expression, the E products are involved in viral DNA synthesis, and the L products include virion structural proteins and viral glycoproteins.^{68; 73}

C. HSK Pathogenesis

If HSV-1 infection is confined to the corneal epithelium, the resulting pathology is referred to as infectious epithelial keratitis.³⁶ HSK can either result from a progressive infectious epithelial keratitis or present as a principle manifestation of keratitis.⁷³ In fact, epithelial keratitis progresses to involve the stroma in approximately 20% of cases.¹¹² Pathologic findings of HSK include corneal stromal¹⁴⁴ and endothelial damage resulting from both direct viral effects and host immune responses. Two main HSK subtypes exist: non-necrotizing HSK and necrotizing HSK. Non-necrotizing HSK does not include an epithelial defect¹⁵² and does not require active viral replication, whereas necrotizing HSK involves an epithelial defect and active viral replication. Necrotizing HSK induces an immune response from the host, resulting in corneal tissue damage.^{59; 87}

HSK pathogenesis is frequently performed in the primary disease mouse model. Although the murine corneal model produces herpetic stromal lesions by a different mechanism than occurs in humans,^{127; 135} the lesions themselves are similar.¹⁵ HSK in the mouse cornea requires viral replication in the corneal tissue as well as chronic inflammation driven by the host immune response, mediated by CD4⁺ T-cell infiltration and lymph node drainage in the sclera and cornea.^{13; 22; 151} Stromal lesions are absent in animal without T cells, but way from upon introduction of transferred CD4⁺ T cells in these animals.^{96; 121} Other important players in the host immune response are macrophages,¹¹ natural killer (NK) cells,¹³⁹ corneal Langerhans cells⁹⁷, and polymorphonuclear neutrophils (PMNs), which are the predominant cell type in early primary HSV-1 infection (Table 1).^{11; 17; 28; 51; 64; 97; 99; 138-140; 145; 149; 155} The significance contributions of the host response is apparent given the development of HSK even with minimum to no viral replication.¹⁵

Murine models demonstrated that HSK pathology is primarily due to CD4⁺ T cells and the immunoinflammatory reactions they mediate.^{103; 142} Specifically, Th1 and Th17 cells, which are subsets of CD4⁺ T cells, secrete proinflammatory cytokines that induce an increase in leukocytes in the cornea and the resulting lesions. IL-17-secreting CD4⁺ T cells (Th17 cells) contribute to HSK pathogenesis especially in the later clinical phases.⁹⁹ One possible link between HSV-1 and this observed immunopathology is that HSV-1 infection of corneal epithelial cells can elicit an innate immune response via the toll-like receptor (TLR) family. Studies show that HSV-1 DNA can directly ligate TLR-9 and generate a potent receptor response.^{76; 92} Additionally, HSV-1 can trigger two other TLR ligands for TLR-2 and -3, both of which activate the innate immune system^{78; 122; 157}

HSK is characterized by a breakdown of the functional barrier between blood vessels, lymph vessels, and corneal tissue. This compromised barrier results in an increased presence of mature professional antigen-presenting cells (APCs) and other leukocytic infiltrates, including the predominant neutrophils.⁵⁶ Once HSV-1 has attached to and successfully entered corneal epithelial cells, neovascularization of the normally avascular cornea is critical to the pathogenesis of stromal keratitis. The new vessels formed from the nearby limbus are not structurally sound, allowing for the extravasation of inflammatory mediators into the corneal stroma and ultimately leading to scarring and vision impairment.^{13; 22; 68; 125} Thus, a prerequisite for corneal neovascularization seems to be an intact adaptive immune response that includes an appropriate degree of CD4⁺ T-cell infiltration into the cornea.⁷

III. Corneal Immune Privilege

Corneal immune privilege, which fosters optimal visual acuity, is compromised in HSK. In a typical cornea, the absence of blood and lymphatic vessels restricts the cornea from interacting with the immune system³⁰, and the lack of blood vessels affords the cornea a transparency precluding unwanted diffraction of light.³⁰ One mechanism by which the cornea maintains its alymphatic status is through the corneal epithelial cells' secretion of soluble vascular endothelial growth factor receptor 2 (sVEGFR2), which restricts physiologic lymphatic growth.¹⁵³ Because the cornea does not normally contain lymphatic vessels, transport of APCs to lymph nodes¹⁴⁸ is largely precluded, affording the corneal immune privilege.^{30; 46; 80; 133} Sequestration of antigens from the body's lymphoid tissues was previously thought to create immune privilege;⁹⁵ however, allogeneic cells introduced into the anterior chamber leave the eye and elicit antibody responses.⁶⁶ These studies, however, did not reveal cell-mediated responses that would result in allograft rejections.⁶⁶ Thus, the absence of lymphatic vessels as well as the production of various inhibitory factors help maintain corneal immune privilege.^{30; 104; 133}

Corneal immune privilege is maintained by production of inhibitory factors that prevent the delivery of antigen from the cornea to the lymphoid organs and that counteract T cells that enter the cornea.⁵⁶ These inhibitory factors include transforming growth factor β (TGF- β), alpha-melanocyte stimulating hormone (alpha-MSH), and Fas ligand (CD95L).¹³⁴ VEGF receptor-3 (VEGFR3), which is ectopically expressed throughout the corneal epithelium,³⁴ and soluble VEGF receptor-1 (sVEGFR-1)², also sequestering growth factors.¹¹⁵ T-cell

energy is also inhibited during early development; eye antigens are not normally presented during this critical time.⁸⁰ Furthermore, reduced expression of major histocompatibility complex (MHC) class Ia molecules on ocular corneal endothelial cells may prevent corneal damage from cytotoxic T lymphocytes (CTLs) in cases of viral infection, thereby supporting immune privilege in the eye.¹⁰⁵ Additionally, protective expression of two nonclassical MHC class Ib molecules^{63; 75; 82; 106; 118; 119} of human leukocyte antigen (HLA)-G and HLA-E inhibits NK cell-mediated cell lysis.^{75; 83; 118} Inhibition of cell mediated lysis occurs despite the absence of MHC Ia molecules on corneal endothelial cells, which would normally elicit targeting by NK cells.

The role of innate immune system factors in maintaining immune privilege is unclear. A recent study using a murine model indicates that leukocytes are present throughout the cornea.⁵³ Since this study, it has become clear that the normal mouse cornea possesses several innate immune system factors. Specifically, dendritic cells are present throughout the epithelial basal layer with some evidence of their presence in the corneal stroma as well.⁵⁴ The corneal stroma is also thought to contain macrophages; however, studies to date have not shown the involvement of these resident leukocytes in the response to primary HSV-1 infection of the cornea.¹²⁰ More research is needed to uncover the role of resident immunity factors in maintaining corneal immune privilege.

IV. Initial Reports of HSV-1–induced Corneal Lymphangiogenesis

The lymphatic vascular system acts as a draining apparatus for extracellular matrix fluid by lowering tissue pressure and providing a route for antigen and cytokine passage from inflammatory sites to lymph nodes.^{29; 110} The conjunctiva was thought to be the only anatomical region of the eye that contained lymphatics. Although the cornea is alymphatic, histological evidence exists for lymphatic vasculature in other tissues of the eye.⁴⁹ Recent studies reveal lymphatics present in the orbital meninges, limbus, lacrimal gland, ciliary body, and extraocular muscles, and potentially the choroid.¹⁰¹

New developments have allowed for a greater focus on the role of lymphangiogenesis in HSK pathogenesis. Corneal lymphangiogenesis has not been studied extensively. One reason for this was a lack of specific markers targeting lymphatic vessels;¹⁰¹ however, recently identified lymphatic endothelial markers enable the tracking and characterization of new vessels.^{9; 18} Wuest et al., using a mouse model, presented the first visualization of limbal lymphatic vessel ingrowth into corneal tissue upon HSV-1 infection via confocal microscopy using a lymphatic vessel-specific antigen, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1).¹⁵⁵ Small lymphatic vessel development was noted as early as day 1 post infection (p.i.) with vessel growth continuing until at least day 7 p.i. The total number of lymphatic vessels increased, indicating that the ingrowth was in fact new and not simply a remodeling of lymphatic vasculature from the limbus into the cornea (Figure 1).¹⁵⁵ With increased ability to identify new lymphatic growth, greater attention to the process of lymphangiogenesis within HSK pathogenesis is possible.

V. Direct Contribution of HSV-1 to Lymphangiogenesis in HSK

The vascular endothelial growth factor (VEGF) family is widely recognized for its role in lymphangiogenesis. The mammalian VEGF family is known to consist of five members: VEGF-A, -B, -C, -D, and placenta growth factor (PlGF).¹⁰¹ VEGF-C and VEGF-D mediate lymphangiogenesis during bacterial infection and wound healing.^{32; 33; 67; 149} VEGFA, another VEGF isoform, also has shown prolymphangiogenic effects in vivo during inflammatory lymphangiogenesis, though appears less potent than VEGF-C and -D.^{29; 32} Studies suggest that activated macrophages are recruited to the inflammatory site, prompting the synthesis of pro-lymphangiogenic factors.^{32; 33; 67; 149} These factors can act as ligands for the lymphatic endothelial cell-expressed receptor VEGFR-3, promoting mitosis and the development of lymphatic vessels.^{60; 109; 113} Inhibiting interactions between VEGF-C/D and VEGFR-3, and depleting macrophages,^{29; 32; 67} decreases inflammatory lymphangiogenesis.

Initial studies suggested that, in contrast to other types of inflammatory lymphangiogenesis, neither VEGF-C nor -D played a role in HSV-1-induced lymphangiogenesis.¹⁵⁵ Wuest et al. isolated VEGF-A as the only ligand among VEGF-A, -C, and -D that is able to induce lymphangiogenesis upon HSV-1 infection of the cornea.¹⁵⁵ Particularly, VEGF-A binding to its receptor VEGFR-2 produces lymphatic vessels that are more prone to leakage and more dilated than those induced by interactions between VEGF-C/D and VEGFR-3.^{60; 65; 100} In addition, although vessels arising from HSV-1-induced lymphangiogenesis carry antigens from the cornea to the draining lymph nodes, they are not as effective transporter as the vessels elicited by either VEGF-C or -D.¹⁵⁵ VEGF-A levels in the cornea reach their peak at 1 day p.i. before leukocytes are able to infiltrate the cornea during HSV-1 infection;^{13; 72} therefore, HSV-1-infected cells may be the predominant source of VEGF-A.¹⁵⁵ Some hold that infected cells are unable to become the predominant source of VEGF because viral infections shut down host mRNA machinery.⁷⁹ In contrast this theory proposes that that neighboring uninfected cells are the predominant source of VEGF.¹³ Other potential sources of VEGF-A exist. Leukocytes and macrophages contribute to the production of VEGF in certain animals infected with HSV.⁸⁴ HSV-1 infection increases VEGF-A levels by suppressing expression of sVR-1 mRNA (a VEGF-A trap) while degrading intact sVR-1 proteins.⁹⁹ Specifically, matrix metalloproteinase-7 (MMP-7) degrades human sVEGFR-1, increasing the bioavailability of VEGF near the surrounding endothelial cells.¹ After day 2 p.i., when inflammatory cells begin infiltrating the corneal stroma, macrophages and neutrophils likely contribute as additional sources of VEGF-A. Moreover, treatment of murine corneas with neutrophil-depleting antibody at day 7 p.i. results in a significantly decreased angiogenic response.¹ Evidence also suggests that upregulation of VEGF is not simply a host response to any viral infection. For example, vesicular stomatitis virus infection does not stimulate a similar increase in VEGF-A-infected cells¹⁵⁵, suggesting that HSV-1 has a unique ability to mediate this increase.

Closer inspection of lymphangiogenesis within the cornea after HSV-1 infection reveals that type 1 interferon and its intact receptor system help stop viral replication and keep lymphatic vessels intact. Bryant-Hudson et al. produced a transgenic mouse deficient in the type 1 interferon receptor and referred to it as CD118^{-/-}. Compared to wild-type mice, HSV-1-infected CD118^{-/-} mice present robust growth of lymphatic vessels until day 5 p.i. when

lymphatic vessels disappear. Wild-type mice with a functioning type 1 interferon system exhibit intact lymphatic vessels. Further inspection revealed that CD118^{-/-} mice have more robust VEGF-A protein levels than wild-type mice, but with no changes in VEGF-C or VEGFR-2 levels to explain the loss of lymphatic vessels. Type 1 interferon receptor however, is associated with the influx of NK cells, monocytes, and macrophages, and indeed, levels of these leukocytes were greater in wild-type mice than in CD118^{-/-} mice post HSV-1 infection. The lack of leukocytes in CD118^{-/-} mouse corneas leads to unhindered viral growth believed to cause the loss of lymphatic vessels in the cornea day 5 p.i.¹⁹

VI. Contribution of Host Immune Response to HSV-1-induced Lymphangiogenesis

A significant presence of neutrophils in infected corneas has made them a popular subject of research in HSK pathogenesis.¹⁵⁵ The host's immune cells do not appear to play an important role in early lymphangiogenesis following HSV-1 infection; however, the host response seems to be a significant factor in later stages.⁴⁷ CD4⁺ T cells, Th1 cells, Th17 cells, and intact lymph tissue that drains the sclera and cornea appear to be necessary for HSK induction,^{13; 22; 99; 151} with tissue damage resulting from immune mediation.⁵⁶ As detailed before, much of the immunopathology in HSK is from infiltrating neutrophils^{40; 156} and CD4⁺ T cells.^{103; 142} Despite this, the role of neutrophils in the progression and severity of HSK is still contested.

West et al. investigated the role of a potent neutrophil chemokine, CXCL1, a pro-inflammatory factor that recruits neutrophils to the cornea following reactivation of disease. West et al. treated corneas with either anti-CXCL1 or control Ig and then scored mice with recurring HSK based on the resulting levels of neovascularization and corneal opacity. The anti-CXCL1 group demonstrated significantly lower levels of both neovascularization and corneal opacity compared to the control. To further investigate the role of this chemokine, CXCL1 receptor knockout, b6-CXCR2, transgenic mice were created, and recurrent HSK occurred significantly less often in these mice than in wild-type b6 mice.^{139; 150} West et al.'s results indicate that neutrophils aid the host response in HSK.

Shen et al., however, suggest a different function of neutrophils. They studied a chemokine ligand, CXCL10, that has primary functions of attracting T cells, NK cells, and neutrophils. CXCL10 also serves as an angiostatic factor by inhibiting VEGF-A expression. In an HSK mouse model, high quantities of CXCL10 were observed at days 2 and 6 p.i. with almost no CXCL10 present on days 14 to 18 p.i. Compared to those in the control mock-infected mouse, CXCL10 levels were higher in the HSK-infected mouse eye from days 1–8 p.i. Also, VEGF-A levels were significantly higher in CXCL10^{-/-} mice at day 14 p.i. than in wild-type mice. CXCL10^{-/-} mice displayed more severe HSK features, including neovascularization and opacity, compared to wild-type mice.¹²⁶

The central role of CXCL10 in HSK is suggested by its ability to aid the clearance of viral titers from the trigeminal ganglia. From days 1–7 p.i., mean viral titers in CXCL10^{-/-} mice were higher than those in wild-type infected mice with significant differences at days 3 and

5 p.i 1. The absence of CXCL10 has a substantial effect on the presence of neutrophils postinfection. Compared to wild-type mice, CXCL10^{-/-} mice showed a decrease in the primary neutrophil influx immediately postinfection, but an increase in the secondary neutrophil influx with distinct symptoms of HSK.¹²⁶

Shen et al. and West et al. present data indicating contradictory roles of neutrophils and CXCL1. West et al. found that upon reactivation of HSV, CXCL1^{-/-} mice exhibit less corneal opacity and neovascularization than wild-type mice. Although the manifestation of HSK was much less severe in the CXCL1^{-/-} mice, CXCL1^{-/-} mice carried greater levels of virus postinfection, a phenomenon known as viral shedding, than wild-type mice.¹⁵⁰ West et al. argued that although there are greater amounts of virus, the absence of neutrophils in the cornea will lead to less disease. This finding is incongruent with Shen et al.'s conclusion that the severity of HSK is a product of the mouse's inability to clear the virus from the cornea.^{126; 150} Other studies also indicate that T and NK cells are primarily responsible for removal of virus from the cornea, rather than neutrophils.^{17; 140; 145}

The role of CD8⁺ T cells in HSK, and particularly in HSV-1-induced lymphangiogenesis, is also uncertain. Conrady et al. first investigated the role of CD8⁺ T cells in lymphangiogenesis in HSV-1 corneal infection using a murine model. CD8⁺ T cells were once thought to reduce ocular pathology in HSV infection by clearing the virus and limiting VEGF-A increase and lymphatic growth.^{154; 155} More recently, they were identified as contributors to lymphangiogenesis, aiding production of VEGF isoforms that support the migration and proliferation of endothelial cells.¹¹⁰ CD8⁺ T cells also induce VEGF-C expression in the cornea, which promotes lymphangiogenesis. Whereas VEGF-A is produced by infected and uninfected epithelial cells during the initial days after HSV infection^{51; 155} and thought to initiate lymphangiogenesis, VEGF-C produced by CD8⁺ T cells is believed to facilitate growth of existent lymphatic vessels. CD8⁺ T cells are a potential source of secreted VEGF-C in the later stages of HSK, given that transgenic mice with >98% CD8⁺ T cells specific for HSV-1 demonstrate lymphatic vessel growth only in response to VEGF-C.³⁸ VEGF-C neutralization leads to shorter lymphatic vessels compared to those seen in IgG-neutralized controls, indicating a temporal difference between the onset of actions by VEGF-A and -C isoforms.²⁸ For example, mice void of CD8⁺ T cells have shorter lymphatic vessels than control mice.²⁸ Whereas the initial increase in VEGF-A expression seems to be induced by HSV-1 virions in the local corneal environment, increased VEGF-C expression occurs following infiltration by host immune cell mediators.

Although previous studies demonstrated the importance of macrophage infiltration in initial lymphangiogenesis in HSV-1 infection^{32; 33; 67; 149}, macrophages and other bone marrow-derived cells including leukocytes do not contribute significantly to the infection. For inflammatory episodes in chronic bacterial infections, lymphangiogenesis relies upon macrophage recruitment and VEGF-C and -D expression; however, these elements of the host response do not play a role in lymphangiogenesis following acute HSV-1 infection.¹⁵⁵ Other studies, however, have indicated a role for macrophages in interleukin-1 β (IL-1 β)-induced corneal lymphangiogenesis.¹⁴⁹ Additionally, macrophages and possibly other cells originating from the bone marrow can transdifferentiate and display the phenotype of endothelial cells. These cells secrete factors to promote lymphatic vessel formation as well

as themselves become a direct part of lymphatic vessel structures.⁹³ Wuest et al. , however, found that during HSV-1 infection of the cornea, this structural contribution does not occur.¹⁵⁵

VII. Clinical Significance of HSV-1–induced Lymphangiogenesis

The formation of new lymphatic vessels disrupts visual acuity by allowing drainage into local lymph nodes. Activation of the immune system leads to an influx of destructive inflammatory mediators including those involved in chronic infections.^{6; 111} Therapeutic molecules directed at the lymphangiogenic cascade may help to preserve vision in HSV-1–infected patients. As noted above, VEGF-A appears to be a primary molecule that stimulates lymphangiogenesis in early HSV infection, whereas VEGF-C appears to come into play after the influx of immune mediators. Therefore, direct inhibition of VEGF-A and -C in HSV-infected patients may dampen the lymphangiogenic process and prevent related immune-mediated damage. Further investigation is needed.

Another important clinical consideration is corneal graft rejection. Lymphatic vessels confer an increased risk for graft rejection, with significantly increased corneal transplant rejection rates among patients with HSK.^{31; 33; 81; 116} The graft survival rates in patients without previous HSK is greater than 90%,⁹⁰ compared to 50% in patients with HSK.⁴³ As noted previously, lymphangiogenesis compromises the cornea's normal immunological privilege by transporting corneal antigens to draining lymph nodes.¹⁵⁵ The 5-year acceptance rates for corneal grafts represent a therapeutic manifestation of this immune privilege. Even without tissue typing or systemic immunosuppressants, these rates are comparable to those of other transplanted tissues with the use of these additional factors,⁶¹ presumably due to the absence of lymphatic vessels in the cornea to drain APCs. Whereas angiogenesis diminishes visual acuity simply by its presence, lymphangiogenesis compromises vision by facilitating immune-mediated graft rejection.³⁸

VIII. Potential Anti-lymphangiogenic Therapies

Understanding the mechanisms of lymphangiogenesis in chronic viral infection will aid development of novel treatments against HSK. Because the development of lymphatic vessels is largely dependent on VEGF-A, targeting this molecule will likely have profound consequences. Antagonists of VEGF-A, including bevacizumab, ranibizumab, pegaptanib, and VEGF-trap, have already shown positive effects in combating corneal neovascularization and other ocular diseases.^{24; 143} Many of the mediators of proangiogenic and prolymphangiogenic factors are similar in the context of HSK (Table 2).^{7; 12; 15; 23; 28; 30; 35; 56; 69; 70; 74; 84-86; 91; 98; 102; 107; 129; 136; 138; 141; 146; 155; 156.}., therefore, it is necessary to study angiogenesis and lymphangiogenesis in concert as well as individually.

Several molecules show potential in arresting HSV-induced lymphangiogenesis. Bevacizumab, a monoclonal antibody targeting VEGF-A, inhibits both hemangiogenesis and lymphangiogenesis in corneal inflammatory conditions in mice.¹⁶ Subconjunctival bevacizumab shown a greater ability than topically administered bevacizumab to penetrate the cornea stroma.³⁷ Recent evidence suggests that IRS (insulin receptor substrate)-1, an

intracellular protein involved in angiogenic signaling, contributes to lymphangiogenesis, as IRS-1 blockade via GS-101 (antisense oligonucleotide) significantly inhibits corneal lymphangiogenesis in mice.⁶² Other therapies may be derived from endogenous sources. sVEGFR1 inhibits VEGF-A-induced prolymphangiogenic signaling.¹⁵³ Soluble VEGFR2, a splice variant of VEGFR2 normally found in the corneal stroma, inhibits corneal lymphangiogenesis without inhibiting corneal hemangiogenesis.¹ Use of this molecule for therapeutic purposes would allow for selective inhibition of lymphangiogenesis, if necessary.

Inhibiting lymphangiogenesis and thus host immune cell infiltration into the cornea could have significant implications for allograft survival.³⁸ Encouraging evidence in a mouse model of corneal transplantation demonstrates that blockade of VEGF-C-induced lymphangiogenesis improves allograft survival. Notably, this effect may be mediated not only via inhibition of hemangiogenesis and lymphangiogenesis, but also by inhibition of APC maturation and migration to draining lymph nodes⁵², an effect also observed with blockade of VEGFR-3.²⁵ In addition, studies on human and experimental models indicate that neutralizing VEGF-A in subjects with corneal neovascularization significantly improves allograft survival.^{5; 31; 50; 147} Most of these studies, however, assessed only hemangiogenesis and not lymphangiogenesis. Understanding the importance of VEGF-A in HSV-induced lymphangiogenesis will have important implications for characterizing the ability of anti-VEGF-A therapeutic agents (bevacizumab, VEGF trap, etc.) to improve allograft survival rates in patients with HSK.

IX. Future Direction

Despite progress in identifying specific anti-lymphangiogenic molecules, a greater understanding of how lymphangiogenesis contributes to the pathogenesis of HSK is needed. Topical antivirals targeting viral replication and corticosteroids targeting immune-mediated mechanisms are standard treatments for HSK,^{10; 26; 27; 114} but have associated risks. For example, corticosteroid use is associated with increased risks of developing glaucoma and cataracts.^{20; 48; 94} New therapies specifically targeting the lymphangiogenic process are needed. Further research is needed to determine the interrelation of angiogenic and lymphangiogenic treatments. Studies indicate that lymphangiogenesis precedes the onset of angiogenesis¹⁵⁵, but the precise mechanisms of both processes as well as their degree of interdependence remain poorly understood.¹¹⁵ A topic of clinical interest is determining whether a therapy targeting one process is also effective for the other. Current anti-angiogenic treatments for HSK, which focus on early intervention following viral infection, may not be clinically relevant, as HSK is not always detected early.⁵¹ This caveat may be even more pertinent when considering anti-lymphangiogenic treatments, because lymphangiogenesis may precede angiogenesis. It is also unclear whether active lymphangiogenesis terminates following HSV-1 infection of the cornea or whether the lymphatic vessels maintain other functions and thus remain present following the clearance of lymphangiogenic stimuli.¹⁵⁵ Elucidating these mechanistic details will be crucial for therapy development.

X. Method of Literature Search

In preparing this review, we conducted a MEDLINE and PubMed search of the medical literature for the period between 1985 and 2013 using the following key words in various combinations: herpes, HSV-1, VEGF, cornea, neovascularization, lymphangiogenesis, hemangiogenesis, corneal lymphangiogenesis, and *herpes stromal keratitis*. Additional articles not included in the electronic databases were identified in the reference lists of the selected articles. Articles were critically appraised, and pertinent information was included in this review and cited accordingly.

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Figure 1. Induction of corneal lymphangiogenesis after HSV-1 infection

Either saline (mock) or 10^5 PFU HSV-1 was applied to corneas of wild-type mice after sacrifice. Corneas were harvested on the indicated days postinfection (PI) and stained for the lymphatic vessel antigen LYVE-1 (red). In uninfected mouse corneas, lymphatic vessels were present in the limbus but did not extend into the cornea (indicated by dashed line). After HSV-1 infection, lymphatic sprouts and vessels in the limbus could be seen extending into the cornea. Scale bars, 100 μ m. (Reproduced with permission)¹⁵⁵

Figure 2. Schematic of the mechanisms underlying corneal angiogenesis resulting from HSV-1 infection

Left panel: Normal uninfected corneal cells secrete large amounts of sVR-1 but only small amounts of VEGF-A. sVR-1 inhibits VEGF-A by binding to it with very high affinity. Center panel: Soon after HSV-1 infection of the cornea, a sudden increase in VEGF-A production occurs primarily by infected or nearby uninfected corneal epithelial cells. By contrast, sVR-1 levels decrease primarily due to decreased sVR-1 production by corneal epithelial cells and also due to degradation by MMP-2, -7, and -9. The decrease in sVR-1 further increases the amount of physiologically active VEGF-A, which potentiates the initial angiogenic sprouting response to HSV infection. Right panel: In chronic HSK, when HSV-1 is no longer present in the cornea, inflammatory cells, particularly neutrophils, produce VEGF-A and various MMPs, which continue to promote angiogenesis. The new, leaky blood vessels continuously introduce additional plasma and inflammatory cells into the cornea, leading to chronic SK and eventually blindness. (Reproduced with permission) ¹³⁷

Table 1

Host cell mediators induced by HSV-1 corneal infection and their involvement in corneal angiogenesis and lymphangiogenesis.

Host cell mediators induced by HSV-1 corneal infection	Involved in corneal angiogenesis	Involved in corneal lymphangiogenesis	Reference(s)
CD4 ⁺ T cells	<i>Yes</i>	Yes	Conrady et al. ²⁸ , Gimenez et al. ⁵¹
CD8 ⁺ T cells	<i>Yes</i>	Yes	Conrady et al. ²⁸
Polymorphonuclear neutrophils	<i>Yes</i>	Yes	Suryawanshi et al. ⁹⁹
Macrophages	<i>Yes</i>	Yes	Watari et al. ¹⁴⁹ , Wuest et al. ¹⁵⁵
Natural killer cells	<i>Yes</i>	ND	Bouley et al. ¹⁷ , Tamesis et al. ¹³⁹
Dendritic cells	<i>ND</i>	ND	Jager et al. ⁶⁴
$\gamma\delta$ T cells	<i>Yes</i>	Yes	Suryawanshi et al. ¹³⁸
Corneal Langerhans cells	<i>ND</i>	ND	Miller et al. ⁹⁷

ND, Not determined

Table 2

Chemokines/cytokines/growth factors identified in HSV-1 corneal infection and their involvement in corneal angiogenesis and lymphangiogenesis.

Chemokines/cytokines/growth factors induced by HSV-1 corneal infection	Involved in corneal angiogenesis	Involved in corneal lymphangiogenesis	Reference(s)
IL-1	<i>Yes</i>	Yes	Biwas et al. ¹⁴ , Biswas et al. ¹²
IL-1 β	<i>Yes</i>	ND	Cursiefen et al. ³⁰ , Hayashi et al. ⁵⁵
IL-6	<i>Yes</i>	Yes	Banerjee et al. ⁷ , Biswas et al. ¹³
CXCL8/IL-8	<i>Yes</i>	ND	Oakes et al. ¹⁰⁸
IL-10	<i>No</i>	ND	Keadle et al. ⁶⁹
IL-12	<i>Inhibitory</i>	ND	Lee et al. ⁸⁴
IL-17	<i>Yes</i>	Yes	Biswas et al. ¹⁵
IL-17A	<i>Yes</i>	ND	Suryawanshi et al. ¹³⁸
IL-18	<i>Inhibitory</i>	ND	Kim et al. ⁷¹
IFN- δ	<i>Yes</i>	ND	Cursiefen et al. ³⁰ , He et al. ⁵⁷
TNF- α	<i>ND</i>	ND	Minagawa et al. ⁹⁸
CXCL1	<i>ND</i>	ND	Suryawanshi et al. ¹³⁸
CXCL2/MIP-2	<i>ND</i>	ND	Yan ¹⁵⁶
CXCL3/KC	<i>Yes</i>	ND	Frank et al. ⁴⁵
CXCL10/IP-10	<i>Inhibitory</i>	ND	Carr et al. ²¹
CCL2/MCP-1	<i>ND</i>	ND	Divito et al. ³⁹
CCL3/MIP-1 α	<i>ND</i>	ND	Biswas et al. ¹⁵
CCL5/RANTES	<i>ND</i>	ND	Biswas et al. ¹⁵
CCL20/MIP-3 α	ND	ND	Shirane et al. ¹²⁸
VEGF-A	Yes	Yes	Wuest et al. ¹⁵⁴ Wuest et al. ¹⁵⁵
VEGF-C	Yes	Yes	Conrady et al. ²⁸
bFGF	Yes	ND	Biswas et al. ¹⁵
MMP-9	Yes	ND	Lee et al. ⁸⁵
XCL1	ND	ND	Thomas et al. ¹⁴¹

CXCL, chemokine (C-X-C motif) ligand; ND, not determined; IFN, interferon; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; IP, induced protein; CCL, chemokines (C-C motif) ligand; MCP, monocyte chemotactic protein; RANTES, regulated on activation, normal T cell expressed and secreted; bFGF, basic fibroblast growth factor; MMP, matrix metalloproteinase.