REVIEW

Glycobiology of ocular angiogenesis

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Ocular neovascularization can affect almost all the tissues of the eye: the cornea, the iris, the retina, and the choroid. Pathological neovascularization is the underlying cause of vision loss in common ocular conditions such as diabetic retinopathy, retinopathy of prematurity and age-related macular neovascularization. Glycosylation is the most common covalent posttranslational modification of proteins in mammalian cells. A growing body of evidence demonstrates that glycosylation influences the process of angiogenesis and impacts activation, proliferation, and migration of endothelial cells as well as the interaction of angiogenic endothelial cells with other cell types necessary to form blood vessels. Recent studies have provided evidence that members of the galectin class of β-galactoside-binding proteins modulate angiogenesis by novel carbohydrate-based recognition systems involving interactions between glycans of angiogenic cell surface receptors and galectins. This review discusses the significance of glycosylation and the role of galectins in the pathogenesis of ocular neovascularization.

Keywords: angiogenesis / choroidal neovascularization / corneal neovascularization / galectin-3 / glycans / glycosylation / integrins / neovascularization / retinal neovascularization

Introduction

Ocular angiogenesis is a major cause of blindness and visual impairment. Angiogenesis or neovascularization affects almost all tissues of the eye: the cornea, the iris, the retina, and the choroid (Adamis et al. 1999). Epiretinal neovascularization in patients with proliferative diabetic retinopathy and choroidal neovascularization (CNV) in patients with neovascular age-related macular degeneration (AMD) are the two most common causes of catastrophic vision loss. Much like tumor-induced vessels, the ocular angiogenic vessels are leaky and lack structural integrity (Dorrell et al. 2007). The resultant hemorrhage and fibrosis cause severe damage to the ocular tissues and frequently leads to vision loss or impairment (Dorrell et al. 2007).

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a tightly regulated process that begins when the endothelial cells of a mature blood vessel wall are activated by angiogenic factors that include, but are not limited to, the vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) families of cytokines (Cross and Claesson-Welsh 2001). Activation promotes the loosening of endothelial cells from their basement membrane and the supporting periendothelial cells, thereby allowing them to migrate, proliferate, and ultimately form a capillary lumen, which is stabilized by pericytes and smooth muscle cells. Glycosylation, the most common covalent posttranslational modification of proteins, profoundly influences the process of angiogenesis. A growing body of evidence demonstrates that glycosylation can impact activation, proliferation, and migration of endothelial cells as well as the interaction of angiogenic endothelial cells with other cell types necessary to form blood vessels. Early studies have shown that N-linked glycosylation is critical for angiogenesis, as inhibition of enzymes early in the glycosylation pathway block vessel growth. The inhibition of N-acetylgalactosamine-1-phosphotransferase, which catalyzes the first step of glycoprotein biosynthesis, blocks endothelial cell proliferation and alters endothelial cell–extracellular matrix (ECM) interactions (Taggins et al. 1992). Similarly, inhibition of glucosidase I and glucosidase II, which sequentially remove terminal glucose residues from the N-acetylgalactosamine-1-phosphotransferase product by castranospermidine and N-methyl-1-deoxynojirimycin, reduce endothelial cell migration in vitro and FGF-induced angiogenesis in vivo (Pili et al. 1995). Likewise, inhibition by 1-deoxynojirimycin of Golgi-α-mannosidase, which acts on the glucosidase I/II product, blocks angiogenesis in vitro (Nguyen et al. 1992). Treatment with swainsonine, an inhibitor of Golgi-α-mannosidase II, also markedly reduces vessel density and distorts the placentangiosis in vivo (Hafez et al. 2007). O-Linked N-acetylgalactosamine (O-GlcNAc) modifications also play a role in angiogenesis. Recent studies have shown that increased expression of O-GlcNAc transferase, which catalyzes the transfer of N-acetylgalactosamine from UDP-N-acetylgalactosamine (UDP-GlcNAc) to serine and threonine, enhances the angiogenic potential of prostate cancer cells in part by modulating the function of FOXM1 (Lynch et al. 2012). However, in a different study, increased expression of O-GlcNAc glycans was shown to reduce vascular sprouting from aortic rings, as well as migration and capillary tube formation of endothelial cells (Luo et al. 2008). Accordingly, removal of
Gal-3 is a member of the galectin family of mammalian lectins characterized by a conserved sequence within the carbohydrate recognition domain (CRD) that has affinity for \( \beta\)-galactoside structures. Extracellularly, the lectin is assumed to mediate cell–cell and cell–matrix interactions by binding to lactosamine-containing cell surface glycoconjugates via the CRD. That Gal-3 is a novel proangiogenic molecule was first suggested by Nangia-Makker et al. (2000) who reported that tumor angiogenesis induced by subcutaneous injections of breast carcinoma cells in an animal model is significantly greater when the carcinoma cells express Gal-3 as compared with Gal-3-null controls, and that exogenous Gal-3 promotes endothelial cell migration and capillary tubule formation in vitro. In addition, it was reported that modified citrus pectin, a galactose-rich polysaccharide that binds to Gal-3, and possibly also to other members of the galectin family, reduces bFGF-mediated migration of endothelial cells, suggesting that one or more members of the galectin family may participate in bFGF-mediated angiogenesis (Nangia-Makker et al. 2002). More recent studies in our laboratory aimed at characterization of the mechanism by which Gal-3 promotes angiogenesis revealed that Gal-3 is a mediator of VEGF- and bFGF-mediated angiogenic response (Markowska et al. 2010). In these studies, we demonstrated that Gal-3 inhibitors, \( \beta\)-lactose and dominant negative Gal-3, reduce VEGF- and bFGF-mediated angiogenesis in vitro, and that VEGF- and bFGF-mediated angiogenic response is reduced in Gal-3 knockdown cells and Gal-3\(^{−/−}\) animals (Markowska et al. 2010). A well-known proangiogenic integrin, \( \alpha\beta_3 \), was identified as a Gal-3-binding protein. Anti-\( \alpha\beta_3 \) integrin function-blocking antibodies significantly inhibited the Gal-3-induced angiogenesis in vitro. Furthermore, Gal-3 promoted the clustering of integrin \( \alpha\beta_3 \) and activated focal adhesion kinase (FAK). The knockdown of GnTV, an enzyme that synthesizes high-affinity glycan ligands for Gal-3, reduced: (i) complex N-glycans on \( \alpha\beta_3 \) integrins and (ii) VEGF- and bFGF-mediated angiogenesis. Taken together, these data suggest that Gal-3 modulates VEGF- and bFGF-mediated angiogenesis, at least in part, by binding via its CRD to the GnTV synthesized N-glycans of integrin \( \alpha\beta_3 \), and subsequently activating the signaling pathways that promote the growth of new blood vessels. Additional studies in our laboratory demonstrated that Gal-3 also modulates cell surface expression and activation of VEGF-R2 in human endothelial cells (Markowska et al. 2011). In this study, we found that Gal-3 interacts with VEGF-R2 in a carbohydrate-dependent manner, Gal-3 promotes VEGF-R2 phosphorylation in time- and dose-dependent manner, VEGF-R2 bears GnTV-modified \( \alpha\)-glycans, and knockdown of GnTV or Gal-3 reduces the cell surface expression of VEGF-R2 (Markowska et al. 2011). These data led us to propose that Gal-3 oligomers cross-link VEGF receptors into a lattice formation on the cell surface and thereby delay their removal by endocytosis and enhance VEGF-R2 signaling and angiogenesis (Markowska et al. 2011).

Gal-3 may also modulate angiogenesis by inducing the expression of MMPs. In a recent study, Argueso and colleagues (Mauris et al. 2014) have demonstrated that Gal-3 plays a key role in destabilizing cell–cell interactions by interacting with and clustering CD147 on the epithelial cell surface. In this study, the authors identified CD147 as a membrane receptor for Gal-3 in human keratinocytes and demonstrated that Gal-3 initiates keratinocyte cell–cell disassembly by inducing MMP expression in a CD147-dependent manner. These findings are relevant to angiogenesis because endothelial cells express CD147 and disruption of cell–cell assembly and the degradation of the ECM to mitigate the physical constraint to cell movement is the first step in the onset of angiogenesis. Interestingly, MMPs have been shown to cleave Gal-3 to release a highly proangiogenic fragment that shows diminished self-association and ability to hemagglutinate red blood cells, but drastically improved: (i) binding affinity to laminin and endothelial cells, (ii) chemotactic properties toward endothelial cells, (iii) ability to upregulate pFAK in migrating endothelial cells and promote angiogenesis (Nangia-Makker et al. 2010). Thus, it is reasonable to speculate that Gal-3 may also modulate angiogenesis by inducing the expression of MMPs which, in turn, cleave Gal-3 itself to promote angiogenesis. Studies of tumor-associated macrophages have shown that Gal-3 also promotes angiogenesis by accelerating M2 macrophage infiltration into tumors (Jia et al. 2013) and by enhancing the VEGF secretion from macrophages (Machado et al. 2014).

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Vision requires that ocular tissues remain transparent such that light is able to reach photoreceptors undistorted. Complex mechanisms are in place to ensure transparency and provide a route for cells to obtain metabolites and oxygen (Figure 1A and B). Resourceful arrangement of the vasculature and partial avascularity combine to complete this task. As described earlier, ocular neovascularization nearly always impairs vision. Corneal, retinal and CNV are among serious clinical conditions encountered in tertiary-care ophthalmology clinics around the world.

Corneal neovascularization

Corneal neovascularization (Figure 1C and D) is a vision-threatening condition affecting ~1.4 million individuals each year in the United States alone (Chang et al. 2001; Shakiba et al. 2009). It is associated with a wide range of ocular disorders...
Fig. 1. Schematic and photographic representation of the eye and corneal, retinal, and CNV. (A) Schematic depiction of the eye. (B) The fundus, i.e., the inner lining of a normal eye. (C and D) Normal cornea is transparent and avascular; in response to trauma, graft rejection or infection, blood vessels from the limbus (region where transparent cornea meets the opaque sclera) invade the cornea. (E and F) The retina is a highly ordered, multilayered structure that is richly vascularized. Diabetic retinopathy can lead to ischemia and neovascularization on the surface of the retina. (G and H) AMD can be associated with subretinal neovascularization originating from the choriocapillaris, and this can lead to subretinal hemorrhage. Credit: (A), (E) and (G) downloaded from National Eye Institute, NIH website with permission (ref.: NEA04, EDA01, EDA 24); (B) provided by J. S. Duker; (C) provided by Sugaya Satoshi and Tohru Sakimoto; (F) and (H) from Friedlander (2007) with permission.
including viral, bacterial and parasitic infections, inflammatory disorders of the ocular surface and trauma. The complications of corneal neovascularization include corneal scarring, edema, lipiddic deposition and increased risk of graft rejection. The frequency of rejection of corneal grafts placed in vascularized high-risk host beds can reach as high as 90%. This is in sharp contrast to the >90% acceptance rate of corneal grafts placed in avascular host beds. Thus, the development of effective strategies to prevent the growth of blood vessels in the cornea is a high priority, not only to prevent corneal graft rejection but also to treat numerous other inflammatory disorders of the ocular surface.

Increased expression of VEGF is a common trigger for loss of corneal angiogenic privilege. Vascularized corneas express significantly greater amounts of VEGF and its receptors compared with normal corneas (Philipp et al. 2000). VEGF is secreted by the corneal epithelial cells and corneal fibroblasts upon injury and by macrophages subsequently recruited to the site (Cursiefen et al. 2004; Nakao et al. 2007; Sivak et al. 2011). Activation of conjunctival blood vessels by interaction of VEGF with VEGF-R2 promotes vessel growth (Mimura et al. 2001). Multiple other factors such as bFGF (Knighton et al. 1990; Gaudric et al. 1992), angiopoietin-1 (Asahara et al. 1998) and PDGF (Cao et al. 2002) are also involved in corneal neovascularization. Integrins also play a critical role in corneal neovascularization. Integrins αvβ3 and αvβ1 are preferentially expressed on the neovascularature in the corneal alkaline burn model (Zhang et al. 2002). Moreover, inhibition of integrin αvβ1 by a small molecule inhibitor, JSM5562, significantly reduces corneal neovascularization in a mouse scrape model (Muether et al. 2007). Interestingly, one study has shown that while αv integrin antagonist inhibits FGF pellet-induced corneal neovascularization, it did not inhibit inflammatory corneal angiogenesis induced by chemical burns (Klotz et al. 2000). The authors concluded that angiogenic pathways independent of αv integrin are involved in corneal inflammatory angiogenesis. In contrast, retinal (Luna et al. 1996; Riecke et al. 2001; Economopoulou et al. 2005; Yoshida et al. 2012), choroidal (Honda et al. 2009) and tumor angiogenesis is inhibited by αv integrin antagonists. Thus, it appears that there are context- and tissue-dependent differences in angiogenic pathways.

Normally avascular cornea has been extensively used as the in vivo model to investigate the molecular mechanism of angiogenesis and to examine the efficacy of the inhibitors and activators of the growth of new blood vessels. In these assays, known as corneal micropocket assays, standardized slow-release pellets containing test substances are implanted into the corneal stroma. The vessel area representing the extent of angiogenesis is calculated 5–8 days after pellets are implanted in the corneas (Rogers et al. 2007). Using mouse corneal micropocket assays, we have shown that Gal-3 directly promotes corneal angiogenesis in vivo. In this study, the vessel area representing the extent of angiogenesis was calculated 5 days after pellets containing various concentrations of Gal-3 were implanted in the mouse corneas. In the concentration range tested (20–160 ng Gal-3/pellet), the extent of angiogenesis increased in a dose-dependent manner (Markowska et al. 2010). A dominant negative inhibitor of Gal-3 that competes with the CRD, but is unable to oligomerize, effectively inhibited angiogenesis advanced by full-length Gal-3, suggesting that Gal-3 promotes angiogenesis in the cornea in a carbohydrate-dependent manner (Markowska et al. 2010).

Alterations in the N-glycosylation pathway markedly influence the progression of corneal neovascularization. VEGF-A-induced as well as suture-induced inflammatory corneal neovascularization is significantly reduced in the knockout mice deficient in GnTV and Gal-3 (Markowska et al. 2010, 2011). GnTV synthesizes N-glycan intermediates, the β1,6GlcNAc-branched glycans, that are elongated with N-acetyllactosamines to create high-affinity ligands for Gal-3 (Denis et al. 2002). Its disruption prevents interaction of Gal-3 with N-glycan moieties of angiogenic cell surface receptors VEGF-R2 and αvβ3 integrins and, thereby, results in reduced neovascularization (Markowska et al. 2010, 2011). Gal-3 also mediates the interaction of integrin αvβ1 and NG2 proteoglycan to promote cell–cell communication between pericytes and endothelial cells during the early stages of corneal angiogenesis (Fukushi et al. 2004). In the corneal micropocket assays, Gal-1 and Gal-8 also promote angiogenesis (Markowska and Panjwani unpublished). Currently, we are characterizing the mechanism by which these galectins promote corneal angiogenesis.

In conclusion, carbohydrate-mediated recognition plays a critical role in corneal neovascularization. In contrast, much less is known about the glycobiology of retinal and CNV.

**Retinal and choroidal neovascularization**

Epiretinal neovascularization (Figure 1E and F) is characteristic of proliferative diabetic retinopathy, retinopathy of prematurity and retinal vein occlusion. Proliferative diabetic retinopathy is a leading cause of blindness in working age population affecting ~20.8 million people worldwide (Afzal et al. 2007; Morello et al. 2007). Oxidative injury from diabetic hyperglycemia and the resulting hypoxia activates retinal vasculature in diabetic retinopathy (Qazi et al. 2009). Activated retinal vessels grow into the vitreous causing hemorrhage. This leads to degeneration and eventual collapse of the vitreous that pulls the retina and results in retinal detachment, and consequently impairment of vision. VEGF is the primary mediator of retinal neovascularization. Vitreous VEGF levels are significantly greater in patients with diabetic retinopathy than controls (Adamis et al. 1994). Moreover, the severity of retinopathy is closely associated with VEGF expression (Funatsu et al. 2002). Expression of VEGF receptors is also increased in retinal neovascularule. Cadavers with a history of diabetes mellitus have higher levels of vitreal VEGF-R1 and VEGF-R2 than controls. Integrins also play a key role in retinal neovascularization. Increased expression of integrin αvβ3 and αvβ5 was observed in tissues from patients with diabetic retinopathy (Friedlander et al. 1996; Ljubimov et al. 1998). Inhibition of integrin α4 reduced expression of VEGF, as well as vascular hemorrhage in vivo (Iliaki et al. 2009). Moreover, inhibitors of various integrins including αvβ3, αvβ5 and αvβ1 have been shown to inhibit retinal neovascularization in animal models of proliferative retinopathy (Riecke et al. 2001; Iliaki et al. 2009; Yoshida et al. 2012) and retinopathy of prematurity (Luna et al. 1996; Witter et al. 2002; Economopoulou et al. 2005; Wilkinson-Berka et al. 2006). Other factors thought to play a role in retinal neovascularization include stromal-derived growth factor 1 and its receptor CXCR4 (Lima e Silva et al.
Carbohydrate recognition is a largely underappreciated regulatory mechanism in the pathogenesis of ocular angiogenesis that warrants investigation in ocular disease as it may provide valuable mechanistic insight as well as potential therapeutic targets. Considering that Gal-3 is a mediator of VEGF-mediated angiogenic response and VEGF is a primary mediator of retinal and CNV, it is very likely that carbohydrate-mediated recognition plays a prominent role in the mechanisms modulating the growth of abnormal blood vessels in the retina and choroid. It is our hope that this review will provide impetus for future studies to characterize the role of carbohydrate-based, galectin-mediated angiogenic pathways in the pathogenesis of retinal and CNV. Also, future studies targeting galectins to develop novel therapeutic strategies to control ocular angiogenesis are likely to prove rewarding. Therapeutic strategies to prevent abnormal angiogenesis have, thus far, largely targeted VEGF since it plays a central role in the pathogenesis of ocular neovascularization. A major limitation of VEGF targeting therapies is the adverse effect of sustained VEGF inhibition on the choriocapillaris. Long-term inhibition of VEGF leads to chorioretinal atrophy (Yamazaki et al. 2012; Rofagha et al. 2013; Fernandez-Robredo et al. 2014). Intravitreal injections of bevacizumab (Genentech), a full-length recombinant humanized antibody that binds to all isoforms of VEGF, have been shown to cause a significant reduction of choriocapillaris endothelial cell fenestrations in primate eyes (Peters et al. 2007; Schraermeyer and Julien 2012). Despite advances in anti-VEGF therapies designed to combat choroidal and retinal neovascularization, many patients do not significantly improve (Mitchell 2011; Patel et al. 2011). Why some patients with CNV and proliferative diabetic retinopathy do not respond to anti-VEGF therapy is a very important and a clinically relevant question. In this respect, Croci et al. have identified a glycosylation-dependent pathway that supports angiogenesis in a VEGF-independent manner (Croci et al. 2014). This study revealed that anti-VEGF refractory tumors exhibit a distinct glycosylation signature that interferes with Gal-1-induced angiogenesis. Specifically, it was demonstrated that vessels within anti-VEGF-sensitive tumors exhibit high levels of alpha2-6-linked sialic acid, which prevent Gal-1–endothelial cell interactions. In contrast, vessels with the anti-VEGF refractory tumors display glycosylation patterns that facilitate Gal-1–endothelial cell interactions. It was further demonstrated that silencing of Gal-1 itself or a glycosyltransferase that synthesizes Gal-1 ligands in endothelial cells converted refractory tumors into anti-VEGF-sensitive tumors. These findings provide impetus to investigate whether vitreous and retina of patients who do not respond to anti-VEGF therapy have distinct glycosylation signature compared with those who respond. Unfortunately, such samples are not readily available for analyses. Regardless, it is logical to assume that galectin-based strategies hold the potential to develop drugs to enhance the efficacy of anti-VEGF treatment. Last but not least, current anti-VEGF-based therapies are associated with a high rate of retinal fibrosis and geographic atrophy (Kuiper et al. 2008; Van Geest et al. 2012). Therefore, there is a major unmet need for developing dual target drugs for inhibition of both angiogenesis and fibrosis. In this respect targeting galectins, particularly Gal-3 may prove to be beneficial. As described above in the section on Gal-3 and angiogenesis, Gal-3 is an important mediator of VEGF-mediated angiogenic response. These findings in conjunction with reports showing that Gal-3 is also a profibrotic protein that modulates TGF-β-driven fibrosis (Henderson et al. 2006, 2008, 2012; MacKinnon et al. 2008, 2012) suggest that inhibiting carbohydrate-mediated Gal-3 function is likely to inhibit both angiogenesis and fibrosis.

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Conflict of interest statement

None declared.
Abbreviations
AGEs, advanced glycation end products; AMD, age-related macular degeneration; bFGF, basic fibroblast growth factor; CNN, choroidal neovascularization; CRD, carbohydrate recognition domain; ECM, extracellular matrix; FAK, focal adhesion kinase; Gal-3, galectin-3; O-GlcNAc, O-linked N-acetylglucosamine; RPE, retinal pigment epithelium; UDP-GlcNAc, UDP-N-acetylglucosamine; VEGF, vascular endothelial cell growth factor.

References
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