

Endogenous Angiogenic Inhibitors in Diabetic Retinopathy

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DIABETIC RETINOPATHY

Introduction

Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion (type 1), insulin action (type 2), or both. Diabetes is a devastating disease as it causes long-term damage, dysfunction, and failure of various organs, such as the eye, kidney, brain, and peripheral nerves. Among these systemic complications, diabetic retinopathy (DR) is one of the most troublesome problems as it is a major cause of blindness. Almost 100% of type 1 diabetic patients and more than 60% of type 2 diabetic patients develop DR during the first two decades of diabetes (1). Great efforts have been made in the past decades to prevent or delay the onset of DR, as well as to prevent vision loss in diabetic patients. Intensive glycemic and blood pressure control, as established by the Diabetes Control and Complications Trial (DCCT) and the Early Treatment Diabetic Retinopathy Study (ETDRS), can decrease the incidence and delay the progression of DR (2–7). Timely laser photocoagulation therapy can prevent the vision loss in a large proportion of patients with severe DR (8–12). However, laser therapy is associated with common adverse effects and high costs. Currently, there is no effective drug treatment for DR and thus, DR remains a leading cause of blindness in the industrialized countries.

There are two common pathological features in DR responsible for vision loss in diabetic patients: diabetic macular edema (DME) and retinal neovascularization (NV). The exact mechanisms underlying the pathogenesis of these changes in DR are largely

unclear. Accumulating evidence has shown that increases of angiogenic stimulators (e.g., vascular endothelial growth factor [VEGF]) and decreases of angiogenic inhibitors (e.g., pigment epithelium-derived factor [PEDF]) under diabetic milieu lead to a disturbed balance of angiogenesis regulation and subsequently result in DME and retinal NV (13–19). In recent years, a number of endogenous angiogenic inhibitors have been identified (19–27). Some of these inhibitors have been implicated in the pathogenesis of DR (14,17,18,28,29). This chapter will review the recent progress in the research of DR, with a focus on the association of endogenous angiogenic inhibitors with DR and their therapeutic potential in the treatment of DME and retinal NV.

Epidemiology

Diabetes is a common disease in the developed countries and is becoming a major problem throughout the world. The latest WHO Global Burden of Disease estimates the worldwide burden of diabetes in adults was around 173 million in the year 2002 (30). The incidence of diabetes has risen dramatically in the past decades, and a twofold or more increase is expected to occur in the next decades (30). The rising prevalence of diabetes causes a consequent increase of long-term diabetic complications, such as retinopathy, nephropathy, and neuropathy, which set considerable impacts on both the patients and society.

According to the American Diabetes Association (ADA) report, DR is the most frequent cause of blindness in American working-age populations (20–70 yr), with 12,000 to 24,000 diabetics losing their sight each year as a result of DR (31). According to the Eye Diseases Prevalence Research Group, the estimated crude prevalence rates for retinopathy and vision-threatening retinopathy are 40.3% and 8.2%, respectively, in US adult diabetic patients (32,33). The estimated US general population prevalence rates for retinopathy and vision-threatening retinopathy are 3.4% (4.1 million) and 0.75% (899,000) (32,33). Apart from the high prevalence of these complications, their severity presents further problems: 50% of all patients with untreated proliferative retinopathy will lose their sight within 5 yr (34). Moreover, 3.6% of patients with type 1 diabetes, and 1.6% of those with type 2 diabetes, are estimated to be blind (35). With the increasing diabetes rate, DR-related blindness will become more common in the future, unless some breakthrough occurs in basic and clinical diabetic research.

Genetic Factors in DR

Although the development of retinopathy in diabetic patients is largely related to the duration of diabetes, the severity of hyperglycemia, and the existence of hypertension and hyperlipidemia, a high incidence of DR occurs in some patient groups with good systemic conditions, whereas some other individuals remain free of retinopathy inspite of poor glycemic control and long duration of diabetes. Epidemiological study has demonstrated ethnic differences in the prevalence of diabetes and DR. In the United States, African Americans and Hispanics have a higher prevalence of diabetes at approx 25%, compared with 6.2% in the general population (36–39). On average, Hispanic/Latino Americans are 1.9 times more likely to have diabetes than non-Hispanic whites of similar age (36,37). As to the incidence of DR, some small sample size studies have shown that African American individuals are significantly more likely to develop retinopathy than Caucasian American

individuals (50% in African Americans vs 19% in Caucasian Americans with type 2 diabetes), and the African American individuals may have higher rates of proliferative diabetic retinopathy (40). In the study of DCCT, familial clustering of severe, vision-threatening forms of DR was observed (41). These data suggest genetic influences are operating in the development and progression of DR as well as in diabetes.

Experimental diabetes studies have also provided evidence supporting the genetic variations in susceptibility to DR. Our group demonstrated that pigmented Brown Norway (BN) rats are more susceptible to hypoxia-induced retinal NV than the albino Sprague-Dawley (SD) rats (42). In the hypoxic retina of BN rats, the level of VEGF, a major angiogenic factor, is significantly increased, whereas the level of PEDF, a potent endogenous angiogenic inhibitor, is substantially decreased, when compared with that in age-matched normal controls. These changes resulted in a significant increase of the VEGF to PEDF ratio. However, these changes are substantially smaller in SD rats after the same treatment. In the streptozotocin (STZ)-induced type 1 diabetes model, diabetic BN rats develop more severe and longer duration of vascular hyperpermeability in the retina, compared with diabetic SD rats with similar hyperglycemic levels (unpublished data). These studies indicate that genetic factors contribute to the different susceptibilities to DR.

A variety of candidate genes have been investigated in diabetic patients as well as in animal models, but few of them have displayed strong associations with DR (43–47). Human lymphocyte antigen (HLA) is one of the earliest genetic factors studied for its association with DR (45–48). However, no consistent result has been obtained so far from large-sized samples of different populations. Recently, HLA-DR7 was reported to be associated with the protection of proliferative retinopathy in type 2 diabetic patients in Mexicans (49). However, another study conducted in Turkey demonstrated that the HLA-DR7 frequency is significantly higher in diabetic patients with proliferative retinopathy than in nonproliferative cases (50). These controversial data suggest that the influence of genetic factors on the development of DR may be complicated, depending on how much influence derives from other risk factors, such as environmental factors and the systemic conditions of the patients examined.

The DR-associated genes identified so far are mainly involved in distinct metabolic and functional pathways known to be affected in diabetes, such as aldose reductase pathway, glucose transporters, cell communication and the extracellular matrix, endothelins, and nitric oxide synthases (51). Different polymorphisms in the same genes can confer either protection against DR or predisposition to the development of DR (52). Recently, two distinct polymorphisms in the genes coding for intracellular adhesion molecule-1 (ICAM-1) and transforming growth factor (TGF)- β have been found as risk factors for retinopathy (53,54). They may be associated with the leukocyte activation and adhesion to the retinal vascular endothelium, which contribute to the development of vascular leakage and capillary closure in DR. However, their genetic effects as risk factors of DR need to be evaluated in large-size α samples. It is also yet to be revealed how the genetic alterations lead to pathological phenotypes of DR.

Pathophysiology and Clinical Features

Prolonged hyperglycemia is the primary and key factor that gives rise to all abnormalities in DR (55). High concentrations of blood glucose lead to changes in cell metabolism,

including the polyol pathway activation, diacylglycerol-protein kinase C pathway activation, stimulation of cell oxidative stress, and changes in macromolecule structure and function via the formation of advanced glycation endproducts (AGE) (55). Further, these biochemical changes result in the dysfunction of vascular cells, including the pericytes and vascular endothelial cells. Activated endothelial cells release proangiogenic growth factors and cytokines, which cause cascade changes of other retinal cell types. The impaired antithrombotic function of endothelial cells, the interactions between leukocytes and endothelial cells, the vasoconstriction caused by overproduced endothelin, and the reduced function of vasodilating factors (prostacyclin, nitric oxide) cause the thrombosis and closure of retinal capillaries, resulting in the failure of retinal vascular function and regional hypoxia in the retina.

There are several common pathological changes in DR: the appearance of microaneurysms, increased vascular permeability, capillary occlusion, and retinal NV (55). The earliest histological change in DR is the loss of pericytes. The loss of pericytes and subsequent dilation of capillaries can cause microaneurysm, which is the earliest visible lesion of DR in clinic. Under ophthalmoscopy, microaneurysm appears as a red dot with various diameters from 15 to 60 μm . Although the pathogenesis of microaneurysm is unclear, the increase of microaneurysms has been shown to associate with the progression of retinopathy. The increased vascular permeability resulting from the breakdown of the blood–retinal barrier (BRB) allows the leakage of plasma macromolecules and the fluid into the retina and results in microexudates, infiltrating protein, lipid exudates and most severely, DME. The appearance of DME represents a more advanced stage of DR and can cause significant impairment of central vision.

The occlusion of capillaries often gives rise to focal retinal ischemia and hypoxia. The local hypoxia then induces the overexpression of angiogenic stimulators and decreases the levels of endogenous angiogenic inhibitors in the retina to stimulate new blood vessel formation to improve oxygenation in the retinal tissue. These new vessels cross over both the normal arteries and the normal veins of the retina, showing a sign of their unregulated growth. At advanced stages, new vessels can grow into the vitreous body, resulting in preretinal NV. The abnormal structure of new blood vessels can lead to leakage of plasma proteins and hemorrhage into the retina or vitreous and consequently compromise vision. Some of the new vessels grow with the fibril tissue to form fibrovascular complexes and cause tractional retinal detachment, further exacerbating vision impairment.

Clinically, DR is classified into two stages: nonproliferative DR (NPDR) and proliferative DR (PDR). At the stage of NPDR, the lesions are within the retina and include microaneurysms, small “dot and blot” hemorrhages, “splinter” hemorrhages, intraretinal microvascular abnormalities (IRMAs), and “cotton wool” spots. At the stage of PDR, in addition to the changes in NPDR, NV develops along the surface of the retina or extends into the vitreous cavity.

DME, RETINAL NV, AND VEGF

Breakdown of the Blood–Retinal Barrier and DME

DME can occur at any stage of DR. However, the incidence of DME is closely correlated with the severity of DR. The incidences of DME are 40% and 71% for patients

with NPDR and PDR, respectively. As DME directly affects the function of the macula, it often results in significant vision impairment. DME is the single greatest cause of vision loss in diabetic patients (56–59). Approximately 20% of DME patients with type 1 diabetes and 50% of those with type 2 diabetes have visual acuity worse than 20/40. This level of vision loss limits or prevents daily activities such as driving and reading.

Diabetic patients also have significantly higher incidence of cystoid macular edema (CME) secondary to cataract surgery (60). As diabetic patients have increased risks of developing cataract, and many need cataract surgery, CME is a common clinical complication in diabetic patients.

The current treatments for DME are far from satisfactory. Intensive glucose control, as demonstrated by the DCCT, decreased the incidence of DME by 23%, when compared with standard, conventional glucose control (6,61). The ETDRS demonstrated that treatment of DME by focal laser photocoagulation is beneficial for reducing the rate of moderate visual loss by only 50%, and the rate of visual improvement is low (62,63). Furthermore, the laser burns that result from such focal laser treatment in patients treated in the ETDRS have been shown to increase the atrophy of the retinal pigment epithelium (RPE) with the progressive enlargement of the initial focal scars of laser photocoagulation (10–12,64). This may lead to visual loss with central scotomas and a decrease in color vision.

The breakdown of the BRB and subsequent increase in vascular permeability are believed to play a major role in the development of DME. Vascular leakage caused by the breakdown of the BRB is an early and common pathological change in DR and some other ocular disorders (65–67). At early stages of DR, it is found that the increase of retinal vascular permeability precedes the appearance of clinical retinopathy (68,69).

The BRB plays an important role in maintaining normal physiological functions of the retina. The BRB is composed of two spatially distinct barriers limiting the flow of macromolecules and fluid into the retina: the inner barrier is the vascular endothelium, mainly residing at the tight junction between adjacent endothelial cells; the outer barrier is the tight junction between the RPE cells (60). The tight junction between endothelial cells contains an assembly of unique proteins such as occludin, claudins, and zonula occludens (ZO)-1, ZO-2, and ZO-3. The structural interactions between these proteins constitute the tight junctions and limit the fluid flow (70).

Impaired inner BRB has been found to play a major role in the evolution of DME and DR (71,72). In the diabetic animal model, the early BRB breakdown is localized to the retinal venules and capillaries of the superficial retinal vasculature (73). Later, the BRB interruption progresses from the superficial layer to the deep capillary bed (74). The decreased expression, redistribution, and changed phosphorylation of some of the tight junction proteins, such as occludin, can result in disorganization of the tight junction proteins in the vascular endothelium which is considered responsible for the breakdown of the inner BRB (74–76).

Recent studies have shown that unbalanced expression of angiogenic factors and angiogenic inhibitors plays an important role in the development of DME (77,78), and thus represents a new target for pharmacological intervention of DME.

Retinal NV and PDR

Retinal NV is another central feature of DR and a major cause of blindness in diabetic patients. The appearance of NV represents the progression of the disease from NPDR to the advanced stage—PDR. In severe NPDR, the extensive area of capillary closure caused by the dropout of pericytes and the loss of endothelial cells result in local retinal hypoxia, which in turn stimulates the release of angiogenic factors and cause retinal NV to alleviate local ischemia. However, these newly formed blood vessels are malformed with fragile basement membrane, deficient tight junction between endothelial cells, and lack of pericytes. The walls of the new vessels are weak and may break, resulting in hemorrhage into the vitreous and compromised vision.

In the more advanced stages of PDR, the new vessels accompanied by fibrous tissue grow from the anterior retinal surface into the vitreous cavity, forming the fibrovascular membrane, which can pull the retina away from the underlying choroid. This can cause tractional retinal detachment and result in blindness if untreated. In many cases, retinal NV coexists with DME or macular ischemia caused by capillary nonperfusion, leading to more severe condition of vision impairment.

A major obstacle in studying PDR is the lack of ideal animal models. All diabetic rodent models examined so far do not develop typical NV identical to that in PDR patients (79). The STZ-diabetic rat model is a commonly used type 1 diabetes model. This model develops some NPDR features such as pericyte loss, increased retinal vascular permeability, and so on, but does not develop retinal NV, even after long durations of severe hyperglycemia (80). Transgenic mice overexpressing VEGF in the retina have displayed intraretinal NV, but lack preretinal NV (81,82). Galactose-fed dogs can develop some retinal vascular changes similar to human DR, including appearance of microaneurysms and acellular capillary beds associated with nonperfusion areas. Some galactose-fed dogs even develop some PDR-like features, such as appearance of fibrovascular membrane on the retinal surface and on the posterior hyaloid membrane, after several years of galactose diet (79,83–85). However, this model is not practical for the large-scale research, as it is associated with high costs and long experimental durations. Most commonly used model for PDR is oxygen-induced retinopathy (OIR) in newborn rats or mice. This model has also been established in cats and dogs (86,87). Although this is not a diabetic model, the OIR model indeed develops most of the human features of PDR, such as increased vascular permeability, microaneurysm, nonperfusion area, preretinal and intraretinal NV, and hemorrhage. Therefore, OIR is commonly accepted as a model for PDR studies. It is noteworthy that this model has species difference and strain difference in terms of severity of NV and vascular hyperpermeability, even in the same species (42). As mentioned above, BN rats have shown significantly more severe and longer duration of retinal NV and vascular leakage than SD rats after the same high oxygen exposure. Therefore, the strain of rat or mouse used for this model should be brought into the consideration, when results from different groups are compared.

VEGF in DME and Retinal NV

In the past two decades, extensive studies have been conducted to understand the role of growth factors in the development of DR. VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1, endothelin (ET), and a number of other

angiogenic factors have been implicated in DR (88–91). Among these angiogenic factors, VEGF is believed to play a key role in the development of DME and retinal NV (71,88,92). VEGF is a homodimeric glycoprotein composed of four isoforms resulting from alternative RNA splicing (93). It is a potent angiogenic stimulator with endothelial cell-specific mitogenic activity and plays a crucial role in both normal and pathological angiogenesis (88,94). VEGF is also referred to as vascular permeability factor (VPF), based on its ability to induce vascular hyperpermeability (94–96). It has a potent activity in increasing vascular permeability, with an efficacy 5000-fold higher than that of histamine (97). VEGF is produced by multiple cell types in the retina, including the RPE, pericytes, endothelial cells, glial cells, Müller cells, and ganglion cells (98–100). Among them, Müller cells and RPE are believed to be the major source of VEGF in the retina, and endothelial cells to be the primary target of VEGF (99,101). VEGF exerts its bioactivities through two known VEGF receptors, Flt-1 and Flk-1/KDR, which are expressed predominantly in endothelial cells, and to a lesser extent on monocytes and macrophages (102,103). The binding of VEGF to its receptors initiates a signal transduction cascade mediating vascular permeability and endothelial cell proliferation and migration.

It has become evident from both diabetic patients and diabetic animal models that VEGF levels are increased in the retina with DR. The earlier studies demonstrated that VEGF levels are significantly elevated in the vitreous and the retina from patients with PDR, compared with those with NPDR, and are correlated with the severity of DR (98,104–108). Laser photocoagulation decreased vitreous VEGF levels by 75% in patients with PDR (109), suggesting that the development and regression of retinal NV is closely associated with VEGF levels in the retina. In addition, significantly elevated VEGF levels in the aqueous humor were also reported in diabetic patients with macular edema and correlated with the severity of DME (108).

VEGF overexpression was also confirmed in animal models of DR. In early stages of STZ-diabetic rats, significant increases of retinal VEGF mRNA levels have been found to correlate with retinal vascular permeability (110). This early BRB breakdown can be successfully prevented by VEGF TrapA₄₀, a soluble VEGF receptor Flt/F_c chimera (73). These coincided increases of retinal VEGF level and the BRB breakdown were also observed in the relative long-term diabetic animal model (71).

In the OIR model, retinal VEGF levels are significantly elevated, which correlate with the retinal NV progression. The VEGF levels decline to the normal level when the regression of NV occurs (14). The contribution of VEGF to the formation of retinal NV is also supported by observation that intravitreal injection of VEGF successfully induces iris NV in monkey eyes (111,112). Repeated injections of VEGF can cause severe iris NV and neovascular glaucoma, mimicking the condition of neovascular glaucoma that occurs in the very advanced stage of PDR (111–113).

These previous observations all support that overproduction of VEGF is the major cause of DME as well as retinal NV in diabetes (88–114).

ENDOGENOUS ANGIOGENIC INHIBITORS AND THEIR IMPLICATION IN DR

Angiogenesis is normally regulated by two counterbalancing systems: angiogenic stimulators (e.g., VEGF) and angiogenic inhibitors (e.g., PEDF) (Fig. 1). It is the delicate

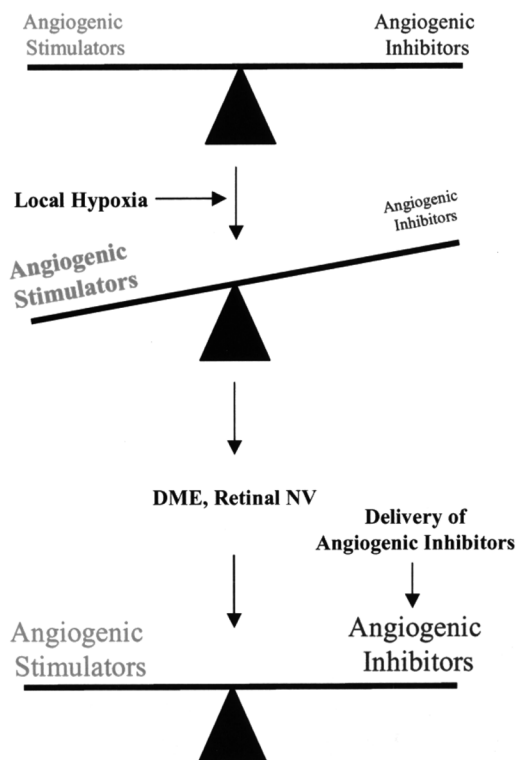


Fig. 1. Significance of the balance between angiogenic stimulators and inhibitors in diabetic retinopathy. Angiogenesis and vascular permeability are normally regulated by the balance between angiogenic stimulators such as vascular endothelial growth factor and angiogenic inhibitors such as pigment epithelium-derived factor. Under diabetic conditions, the retina overproduces angiogenic stimulators and decreases the expression of angiogenic inhibitors. The disturbed balance results in vascular hyperpermeability and diabetic macular edema (DME) and retinal neovascularization (NV). Administration of angiogenic inhibitors can restore the balance and has therapeutic potential in the treatment of diabetic retinopathy. *See* color version on companion CD.

balance between angiogenic stimulators and inhibitors that determine where and when new blood vessels are formed. In the adult retina, the angiogenic inhibitors are predominant in the balance to maintain the quiescent status of retinal vasculature. Our recent studies demonstrate that the disruption of the balance plays an essential role in the development of a variety of neovascular diseases, such as cancer and PDR (14,115–117). In these pathological conditions, the ratio of angiogenic stimulators to inhibitors increases, which breaks the dormancy of angiogenesis and consequently, results in abnormal retinal NV (Fig. 1). Therefore, restoration of the balance by either increase of angiogenic inhibitors or decrease of angiogenic stimulators, or both, should lead to the quiescence of angiogenesis, which may become an important strategy in the prevention and treatment of PDR and other neovascular diseases.

The hypothesis that naturally occurring inhibitors of angiogenesis exist and play important roles in the regulation of angiogenesis was initially proposed by Judah Folkman (115). As early as 1977, evidence has been documented that inhibitors of

angiogenesis exist in the vitreous fluid (119). These inhibitors may be responsible for maintaining the avascular status of the vitreous body (120,121). The first endogenous angiogenic inhibitor was isolated in 1994 and named angiostatin, which was later identified in the human vitreous (25). In recent years, nearly 30 angiogenic inhibitors have been identified in a variety of tissues (20). They are classified into five major groups in a recent review (20): (1) endothelial cell specific inhibitors such as angiostatin, endostatin, antithrombin III, plasminogen kringle 5 (K5) and plasminogen kringle 1–5 (K1–5); (2) avascular tissue-derived inhibitors such as kallistatin or kallikrein-binding protein (KBP) and PEDF; (3) antiangiogenic cytokines such as interferon- α , interleukin (IL)-12, interferon- γ , and IL-18; (4) angiogenic factor antagonists, including soluble fibroblast growth factor receptor (FGFR)-1, soluble VEGF receptor (VEGFR)-1 and angiopoietin-2; and (5) the other angiogenic inhibitors such as thrombospondin (TSP)-1, tissue inhibitors of metalloproteinases (TIMPs), maspin, canstatin, and tumstatin (20). Among these inhibitors, 16 have been identified in the eye, and nine of them have been shown as active antiangiogenic factors in the retina, including angiostatin, endostatin, K5, kallistatin, PEDF, interferon- α , interferon- γ , soluble VEGFR-1, and angiopoietin-2.

In contrast to the extensive studies of angiogenic inhibitors in cancer research since 1994, the implication of angiogenic inhibitors in DR was not established until 2001. Our observation that retinal PEDF levels correlate negatively with retinal NV in the OIR model first demonstrated that the VEGF–PEDF ratio in the retina is correlated with the progression of retinal NV (14). Therefore, we proposed that the disturbed balance between angiogenic stimulators and inhibitors is responsible for the development and progression of PDR. Since then, several other angiogenic inhibitors have been implicated in PDR in animal models and in diabetic patients and have displayed therapeutic potential for the treatment of PDR (122,123). Here, we will briefly summarize recent progresses about the implication of angiogenic inhibitors in DR.

Angiostatin

Angiostatin is a proteolytic fragment (kringle 1–4) of plasminogen (25). It exists naturally in significant amounts in the circulation of patients with primary tumors (20). Angiostatin was shown to be a potent angiogenic inhibitor, which blocks NV and suppresses tumor growth and metastases (25). It specifically inhibits proliferation and induces apoptosis in vascular endothelial cells (124). Later evidence has suggested that decreased angiostatin levels in the vitreous may play a role in the development of PDR (29). Moreover, recombinant angiostatin has been shown to block retinal NV in the OIR model (125). Delivery of a recombinant virus expressing angiostatin has been found to suppress laser-induced choroidal neovascularization (126). Systemic and intravitreal injections of angiostatin before the appearance of retinal NV resulted in significantly fewer preretinal vascular cells in the OIR model, suggesting a preventive effect (127). In normal neonatal mice, however, angiostatin does not affect any physiological development of retinal vasculature or the normal development of animals, suggesting no or low toxicities to normal vasculature at the dose and duration of angiostatin administration (127). Recently, we have shown that angiostatin also reduces vascular leakage in the retina of both the OIR and STZ-diabetic rat models, suggesting that decreased angiostatin in the vitreous and retina may also contribute to the development of DME (128).

PEDF

PEDF is a 50-kDa glycoprotein originally identified in conditioned media of cultured fetal human retinal RPE cells by Tombran-Tink and Johnson (129). In 1999, Dawson et al. first reported that PEDF is a potent inhibitor of endothelial cell proliferation and migration, even more potent than the well-studied antiangiogenic factor angiostatin (19). This finding suggests that PEDF is a bifunctional protein and thus has opened a new era for PEDF study.

PEDF is believed to be the major endogenous angiogenic inhibitor in the eye. A number of studies have been documented in the past few years to reveal the role of PEDF in retinal angiogenic diseases. Our group reported that PEDF levels in the retina are significantly decreased in OIR rats, and the decrease is correlated with the progression of retinal NV (14). Laser treatment, which is known as the only effective therapy for retinal NV in PDR, increases the PEDF level in the rat retina and also in cultured RPE cells (130). The PEDF gene knockout results in abnormal vessel density in the retina and prostate (131). The correlation between decreased PEDF levels and DR was later confirmed in human patients (132,133). PEDF levels in the vitreous and aqueous humor have been found significantly lower in patients with PDR than those from nondiabetic eyes (28,132). Furthermore, in diabetic patients with no or very mild retinopathy, the decreased PEDF level in the aqueous humor predicts the progression of DR (134).

The implication of PEDF levels in PDR is also supported by several therapeutic approaches using PEDF. Systemic delivery of a low dose of PEDF successfully inhibited retinal NV in OIR mice by inducing endothelial cell apoptosis (135). Intracocular delivery of PEDF by a viral vector caused regression of retinal NV in VEGF transgenic and OIR mouse models (136,137). Recently, PEDF has also been shown to reduce VEGF-induced vascular leakage, implying its involvement in the regulation of vascular permeability and DME (138). All these studies suggest that PEDF is a crucial inhibitor of retinal NV and DME and has therapeutic potential in the treatment of PDR.

Endostatin

Endostatin is a 20-kDa C-terminal fragment of collagen XVIII, initially purified from conditioned media of murine hemangioendothelioma cells as an angiogenic inhibitor based on its ability to inhibit the proliferation of bovine vascular endothelial cells in vitro and potently inhibit angiogenesis and tumor growth in vivo (26). Although the function of endostatin in the eye and in the retina has not been well studied as that in tumors, solid evidence indicates that endostatin has an important function in the ocular system. Deficient endostatin production in the collagen XVIII gene knockout mouse causes delayed regression of blood vessels in the vitreous and abnormal outgrowth of retinal vessels, suggesting that collagen XVIII/endostatin is important for normal ocular blood vessel formation (123).

Endostatin levels in the vitreous and aqueous humor are decreased in patients with DR and negatively correlated with the severity of retinopathy and the VEGF levels (122,139). Funatsu et al. (13) demonstrated that the diabetic patients with low endostatin levels and high VEGF levels in the vitreous have a significantly higher risk of progression of PDR after vitreous surgery than those with high endostatin levels and

low VEGF levels. These studies suggest that endostatin may be used as a marker to predict the outcome of surgery treatment in diabetic patients.

Endostatin has also been shown to be a promising antiangiogenic agent in the treatment of ocular neovascularization and DR. Intravenous injection of adenoviral vectors containing sig-mEndo transgene increased the serum level of endostatin and inhibited laser-induced choroidal neovascularization (140). The effect of endostatin on retinal NV was demonstrated by adeno-associated virus (AAV)-mediated delivery of endostatin to the eye in the OIR mouse model (141). Recently, delivery of endostatin into the eyes of VEGF transgenic mice using two different viral systems demonstrated that endostatin not only significantly reduced VEGF-induced retinal vascular hyperpermeability, but also inhibited retinal NV and retinal detachment (142).

K5

K5 is a proteolytic fragment of plasminogen, consisting of 80 amino acids (143,144). Based on in vitro assays, K5 has a more potent antiangiogenic activity than angiostatin (145). Although K5 levels in the retina and vitreous have not been examined in DR patients or animals models, a single intravitreal injection of K5 has been shown to prevent the formation of retinal NV in the OIR rat model (24). Moreover, injection of K5 after the partial formation of retinal NV has been shown to stop the progression of retinal NV (24). However, the injection of K5 does not decrease preexisting preretinal vessels or retinal vasculature in normal retina (24). These results, in consistence with the in vitro studies, suggest that K5 is angiostatic. Recently, we have shown that K5 also reduces vascular leakage in the retina of the OIR and STZ-diabetic models (146). This effect is independent of the K5-induced inhibition of retinal NV. More importantly, the effect of K5 on vascular permeability can be achieved at doses substantially lower than that required for its antiangiogenic activity (146).

Kallistatin

Kallistatin was originally identified from rat serum as a specific inhibitor of tissue kallikrein, a serine proteinase that cleaves kininogen to generate bioactive kinins. Kallistatin is a glycoprotein of 425 amino acids and 58 kDa in the human (147,148). Kallistatin specifically binds to tissue kallikrein, forming a SDS-stable complex (149,150), and thus, is also named KBP. It inhibits kallikrein activity in vitro and in transgenic mice overexpressing kallikrein (147,151).

Kallistatin shares significant sequence homology with other serine proteinase inhibitors (serpins) such as α 1-antitrypsin, suggesting that it belongs to the serpin superfamily (148). It also shares significant sequence homology with antithrombin III and PEDF, which are both potent angiogenic inhibitors. Our earlier studies showed that kallistatin levels are significantly reduced in the vitreous from patients with PDR and in the retina of STZ-diabetic rats, suggesting that it is implicated in DR (152,153). Lately, we have shown that kallistatin is a specific inhibitor of endothelial cells and VEGF (23); it inhibits cell proliferation and induces apoptosis in endothelial cells. Moreover, kallistatin inhibits retinal NV and reduces vascular leakage in the retina of the OIR model (23). These vascular activities of kallistatin are independent of its interactions with the kallikrein-kinin system (23). As kallistatin is an angiogenic inhibitor present in

the retina and vitreous at high levels, decreased kallistatin levels in the vitreous of patients with PDR may contribute to the development of DME and retinal NV.

Mechanism for Vascular Activity of Angiogenic Inhibitors

In contrast to the significance of these angiogenic inhibitors in DR and their therapeutic potential, little is clear about the mechanisms underlying their vascular activities. Angiostatin is among the most studied angiogenic inhibitors in terms of the mechanism of action. An earlier study reported that angiostatin binds to ATP synthase on the surface of human umbilical vein endothelial cells (HUVEC). This binding was speculated to mediate the inhibitory effect of angiostatin on endothelial cell proliferation and migration (154). However, the ATP synthase-binding mechanism has not been confirmed by other groups. Angiostatin has also been found to inhibit the VEGF- and bFGF-induced activation of the p42/p44 MAP kinase (155). As VEGF- and bFGF-induced angiogenesis is mediated, in part, through the MAP kinase pathway, blocking the activation of MAP kinase has been suggested to be a possible mechanism responsible for the antiangiogenic activity of angiostatin (156,157).

Recent evidence has shown that angiostatin binds to integrins on the surface of endothelial cells. Using blocking antibodies, Tarui and coworkers demonstrated that $\alpha_v\beta_3$ is a predominant receptor for angiostatin on endothelial cells (158). The binding of angiostatin with integrins on the surface of endothelial cells does not induce stress fiber formation, implying that the antiangiogenic activity of angiostatin may be through interfering with the $\alpha_v\beta_3$ -mediated signaling in endothelial cells (158). Similarly, endostatin and tumstatin have also been shown to bind to integrins (159). Tumstatin binds with $\alpha_v\beta_3$ integrin in a vitronectin/fibronectin/RGD cyclic peptide-independent manner. This binding may mediate the inhibition of endothelial cells proliferation and induction of apoptosis. Endostatin competes with fibronectin/RGD cyclic peptide for binding with $\alpha_5\beta_3$ integrin, and this interaction with integrin has been suggested to mediate the endostatin-induced inhibition of endothelial cell migration (159).

Recently, interactions between angiogenic stimulators and angiogenic inhibitors have been revealed and may represent a mechanism for the vascular activities of angiogenic inhibitors. Gao and Ma (117) demonstrated that K5 downregulates the expression of endogenous VEGF while up-regulating endogenous PEDF in vascular cells and in the retina of OIR rats, suggesting autocrine or paracrine regulations of VEGF and PEDF expression. These regulations can restore the balance between endogenous angiogenic stimulators and angiogenic inhibitors and thus may contribute to the vascular activity of K5. Later, kallistatin and angiostatin were found to block the overexpression of VEGF in the retina under ischemia and diabetic conditions, but do not affect VEGF levels in normal retinas. These angiogenic inhibitor-induced downregulations of VEGF correlate with their antiangiogenic activities (23,128). Although it is not certain how these angiogenic inhibitors regulate VEGF expression, K5 has been shown to block the nuclear translocation of HIF-1 α and thus inhibit the activation of HIF-1. Angiostatin has also been shown to diminish the activation of MAP kinase ERK1 and ERK2 in endothelial cells (155). As HIF-1 and MAP kinase are both known to play roles in the regulation of VEGF, the blockade of the HIF-1 and MAP kinase activation may contribute to the K5-induced downregulation of VEGF expression. Recently, K5 was found

to bind with voltage-dependent anion channel (VDAC1) on the membrane of endothelial cells and thus, VDAC1 was proposed to serve as the K5 receptor on endothelial cells (160). It is unknown, however, how this receptor mediates the K5-induced regulation of VEGF expression.

In addition, endostatin has been shown to downregulate many other proangiogenic genes and pathways but upregulate many antiangiogenic genes (161). Unlike K5, however, the antiangiogenic activity of endostatin has been shown to be HIF-1-independent. Therefore, it is likely that different angiogenic inhibitors may interact with VEGF via distinct mechanisms.

The interactions between angiogenic inhibitors with angiogenic factors are not limited at the regulation of gene expression. Recent studies have shown that some angiogenic inhibitors also block VEGF signaling. Kim et al. (162) showed that endostatin blocks VEGF signaling via direct interactions with VEGF receptor KDR on HUVEC. Binding of endostatin with KDR can block VEGF binding to its receptor and thus block the function of VEGF in endothelial cells. Recently, kallistatin has also been shown to compete with VEGF for binding to its receptors on endothelial cells (23). Under the same conditions, however, K5 does not compete with VEGF for receptor binding. These findings further confirm that different angiogenic inhibitors may have distinct mechanisms of action or molecular targets. Combinations of two or more angiogenic inhibitors with different mechanisms or targets may achieve synergistic effects on vascular leakage and retinal NV.

In summary, our understanding about molecular mechanisms underlying the vascular activities of the angiogenic inhibitors and the regulation of their expression are very limited, compared with those of angiogenic factors such as VEGF signaling. Normal angiogenic regulation and the development of retinal NV are complicated processes and involve multiple, interacting factors.

FUTURE PERSPECTIVE

The disturbed balance between angiogenic stimulators and inhibitors represents a new pathogenic mechanism for DR. Identification of the involvement of angiogenic inhibitors in DR has not only opened a new field for investigation of the pathogenesis of diabetes, but also has revealed a new target for pharmacological interventions. However, there are many unknown features about the implication of angiogenic inhibitors in DR. First, it remains to be investigated how these inhibitors are decreased in DR. Second, their molecular targets and signaling pathways need to be identified. Third, as evidence has shown that there are interactions between different angiogenic inhibitors and between the inhibitors and angiogenic stimulators, it is important to examine these interactions and to study how these interactions are achieved.

The therapeutic approaches using peptide angiogenic inhibitors for the treatment of DR raise both hopes and challenges. Possible therapies using endogenous angiogenic inhibitors for the treatment of DME and retinal NV should offer some advantages over the current treatments. In general, the treatment of DME using angiogenic inhibitors is more promising than the treatment of retinal NV, as the doses required are substantially lower for the DME than that needed for antiangiogenic activity. These approaches may

lead to the development of noninvasive, effective, economic, and safe treatments to prevent vision loss from DR.

Despite the encouraging results from animal models, the therapeutic application of these angiogenic inhibitors is still facing many challenges. First, diabetic retinopathy, unlike cancer, requires a local antiangiogenic treatment. The reason is that diabetic patients, although developing abnormal NV in the retina, have common wound-healing problems in peripheral tissues. This can result in foot ulcer, which represents a major challenge in diabetes care. Therefore, systemic administration of angiogenic inhibitors may exacerbate wound-healing problems in diabetic patients. Efficient local drug administration is desirable and needs to be developed. Second, diabetic retinopathy is a chronic disorder and requires a long-term administration of drugs. A sustained, long-term ocular drug delivery system needs to be developed. Third, most of the existing angiogenic inhibitors are large proteins or peptides. Efforts are needed to improve their delivery into the retina and prolong their bioavailability in the retina. Fourth, the costs of production of these large peptides are high. The minimal functional domains responsible for the vascular activities of these inhibitors need to be defined, as the production of small peptides is more economic and less problematic in general.

Taken together, more intensive, multidisciplinary research efforts are needed to reveal the pathogenesis of DR and to develop new, noninvasive therapies to prevent vision loss from this diabetic complication.

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