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Inflammation and Angiogenesis in Diabetic Retinopathy

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1. Introduction

Diabetic retinopathy (DR) is a significant cause of global blindness; a major cause of blindness in the world.There is emerging evidence that retinopathy is initiated and propagated by inflammation and angiogenesis. Increased cytokines and growth factors, in conjunction with redox stress, contribute to the development and progression of DR of abnormalities of endothelial cells and pericytes in DR.

The four traditional metabolic pathways involved in the development of DR include: increased polyol pathway flux, advanced glycation end-product formation, activation of protein kinase Cisoforms and hexosamine pathway flux. These pathways individually and synergistically contribute to angiogenic growth factors, anti-angiogenic factors resulting in significant microvascular blood retinal barrier remodeling. The pathways are associated with inflammation and angiogenesis,either. Preventing or delaying the blindness associated with these intersecting abnormal metabolic pathways may be approached through strategies targeted to reduction of tissue inflammation. Understanding these abnormal metabolic pathways and the accompanying inflammation and angiogenesis mayprovide both the clinician and researcher a new concept of approaching this complicated disease process.

2. Diabetic Retinopathy (DR)

DR is associated with the following structural features: basement membrane (BM) thickening, pericyte loss,microaneurysms, intraretinal microvascular abnormalities(IRMA), diabetic macular edema (DME) and pre-retinal neovascularization,processes which can lead to blindness through hemorrhage and tractional retinal detachment[1]. Retinal endothelial cells (EC) are supported and sealed by a nearly equal number of pericytes in the retinal optic nerve fiber, inner and outer plexiform and choroidal layers creating a blood retinal barrier (BRB) of closed capillaries[1, 2].

The vascular disruptions of DR/DME are characterized by abnormal vascular flow, disruptions in permeability,and/or closure or nonperfusion of capillaries.

A hallmark of early DR is the change in the structure and cellular composition of the microvasculature[3].

In early stages of DME, breakdown of the inner blood-retinal barrier may occur, resulting in accumulation of extracellular fluid in the macula[4,5].Pericytes are essential cellular components in the regulation of retinal capillary perfusion, and damage to these cells in diabetes leads to altered retinal hemodynamics, including abnormal autoregulation of retinal blood flow[6]. Loss of retinal pericytes represents another early microaneurysm formation[7-9].

There is evidence that retinal leukostasis may also play an important role in the pathogenesis of DR. Leukocytes possess large cell volume, high cytoplasmic rigidity, a natural tendency to adhere to the vascular endothelium, and a capacity to generate toxic superoxide radicals and proteolytic enzymes[10]. In diabetes,there is increased retinal leukostasis, which affects retinal endothelial function, retinal perfusion, angiogenesis, and vascular permeability. And, leukocytes in diabetes are less deformable, a higher proportion are activated, and they may be involved in capillary nonperfusion, endothelial cell damage, and vascular leakage in the retinal microcirculation[10]. A study showed that diabetic vascular leakage and nonperfusion are temporally and spatially associated with retinal leukostasis in streptozotocin induced diabetic rats[11]. There are many capillary occlusions by leukocytes and capillary dropout or degeneration associated with leukocytes in the diabetic retina[10]. Serial acridine orange leukocyte fluorography and fluorescein angiography(FA) show trapped leukocytes directly associated with areas of downstream nonperfusion in the diabetic retinal microcirculation[10].

A number of proangiogenic, angiogenic and antiangiogenic factors are involved in the pathogenesis and progression of diabetic retinal disease, Vascular Endothelial Growth Factor (VEGF) being one of the most important. Other growth factors, which are known to participate in the pathogenesis of the disease, are: Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF), Transforming Growth Factor (TGF), Placental Endothelial Cell Growth Factor (PlGF), Connective Tissue Growth Factor (CTGF). Other molecules that are involved in the disease mechanisms are: intergrins, angiopoietins, protein kinase C (PKC), ephrins, interleukins, leptin, angiotensin, monocyte chemotactic protein (MCP), vascular cell adhesion molecule (VCAM), tissue plasminogen activator (TPA), and extracellular matrix metalloproteinases (ECM-MMPs).

3. Vascular Endothelial Growth Factor (VEGF)

The VEGFs are a family of proteins that are mitogenic for vascular endothelial cells and increase vascular permeability. VEGF is important in fetal vascular development,with VEGF levels diminishing after birth. VEGF is expressed by retinal glial cells[12] and vascular endothelial cells[13]. VEGF is secreted by numerous ocular cell types[14], and increased levels of VEGF have been detected in ocular fluids of patients with proliferative diabetic retinopathy[15]. In vivo, administration of neutralizing VEGF antibodies to experimental animals reverses high-glucose–induced vascular hyperpermeability[16], which is an early manifestation of endothelial dysfunction in diabetic patients[17].

VEGF expression is regulated largely by hypoxia, but it also accumulates in the retina early in diabetes, before any retinal hypoxia is yet apparent [18-20]. It is produced by multiple cell

types in the retina in diabetes, including ganglion cells, Mueller cells, and pericytes. Repeated injections of high concentrations of VEGF in the eyes of nondiabetic monkeys result in retinal changes which in some ways resemble those in the early stages of diabetic retinopathy, including vascular tortuosity and microaneurysm[21, 22]. Genetic factors are important in the pathogenesis of DR; there is a clear association of increased expression of VEGF with DR as well as numerous VEGF polymorphisms that are linked to increased VEGF levels and DR.[23] Their result has demonstrated that the development of different stages of diabetic retinopathy is closely correlated with an increased VEGF level in the retina[24].Clinical trials using anti-VEGF therapies are showing promising results against stages of diabetic retinopathy[25].

VEGF is a potent vascular permeability factor, and VEGF upregulation has been linked to neovascular eye diseases including diabetic retinopathy[23]. VEGF-induced neovascular changes have previously been demonstrated on animal models based on increasing VEGF levels through implants[26], recombinant adenovirus-mediated VEGF expression[27, 28], or transgenic technologies[29, 30].

In the eye, one of the earliest signs of diabetic retinopathy is retinal capillary occlusion, blocking blood flow and generating capillary-free areas[31]. Hypoxic conditions could develop in these capillary-free areas, and this in turn could induce the upregulation of angiogenic factor production, such as VEGF and intercellular adhesion molecules[32, 33]. The increased concentration of angiogenic factors would then cause vascular changes including vascular dilatations, tortuous blood vessels, microaneurysms, and endothelial cell proliferation. Subsequently,over an extended period of time, these changes could result in the development of poorly matured leaky vessels[34, 35].Previous histological studies have demonstrated a strong correlation between endothelial cell proliferation, pericyte loss, and the development of microaneurysm[36]. Incidentally, VEGF, which is a known factor of endothelial cell proliferation, has also been shown to promote pericyte detachment and loss[37].

Troglitazone and rosiglitazone, another thiazolidinediones (TZD), increase VEGF mRNA levels in 3T3-L1 adipocytes.Although increased VEGF may be beneficial for subjects with macroangiopathy and troglitazone is currently not available for clinical use, vascular complications, especially diabetic retinopathy, must be followed with great caution in subjects treated with TZD [38].

Federico et al[39] tested selective PPARα and PPARγ synthetic agonists potential ability to stimulate neoangiogenesis in well-established in vitro and in vivo assays. They found that specific and selective activation of PPARα and PPARγ leads to increased production of VEGF, a prototypical angiogenic agent, and formation of endothelial tubules when endothelial cells are co-cultured with interstitial cells. In vivo, PPARα and PPARγ synthetic agonists stimulate angiogenesis in the mouse corneal neovascularization assay, whereas fibrates and TZDs are unable to induce angiogenesis in the same experimental setting. PPARα- and PPARγ-angiogenic process is associated with increased expression of VEGF and increased phosphorylation of endothelial nitric oxide (NO) synthase(eNOS) and Akt. Finally, it may be inhibited by blocking VEGF activity. The ability of PPARa and PPARγ agonists to induce neoangiogenesis might have important implications for the clinical and therapeutic management of type 2 diabetes[39].

4. Pigmented Epithelial Derived Factor (PEDF)

PEDF is a member of the serine protease inhibitor (serpin) superfamily with neurotrophic and antiangiogenic properties, and a decreased level of PEDF in the eye is important in the pathogenesis of proliferative DR[40]. In the retina, angiogenesis is regulated by two counterbalancing systems: angiogenic stimulators, such as VEGF, and angiogenic inhibitors, such as angiostatin and pigment epithelium–derived factor (PEDF)[40].

PEDF is a natural extracellular component of the retina and has been found in the vitreous and aqueous humors. Decreased levels of PEDF were reported in the ocular fluids of patients with angiogenic eye diseases[41]. PEDF has potent antiangiogenic activity in retinal EC growth and migration and suppressed ischemia-induced retinal neovascularization[42].

Pericyte loss is one of the earliest hallmarks of DR and an important reason for pericyte loss is reactive oxygen species (ROS)[43]. In DR, PEDF has a novel benefit since PEDF protects retinal pericytes against oxidative stress-induced injury through its anti-oxidative properties, which might slow the development of diabetic retinopathy[43].PEDF protects against high glucose or ROS induced pericyte apoptosis and dysfunction through its anti-oxidative properties via induction of glutathione[44].

Guoquan et al[45] compared susceptibilities of Sprague Dawley(SD) and Brown Norway(BN) rats with ischemia-induced retinal neovascularization. They found that the hyperoxia-treated BN rats showed a significant reduction in retinal PEDF and a substantial increase of VEGF at both the protein and RNA levels, resulting in an increased VEGF-to-PEDF ratio. The results suggested that BN rats developed more severe retinal neovascularization, which correlated with a greater increase of the VEGF-to-PEDF ratio in BN than in SD rats[45].

PEDF, a potent inhibitor of angiogenesis, has been found to be involved in the pathogenesis of PDR[46, 47]. It is well known that there are quite a few stimulators and inhibitors of angiogenesis in the eye; among them, VEGF has been identified as a primary angiogenic stimulator[48] and PEDF as a major angiogenic inhibitor[47]. The time course of the VEGF-to-PEDF ratio change correlated with the development and progression of retinal neovascularization. The VEGF-to-PEDF ratio represented a dynamic balance between angiogenic stimulators and inhibitors; and disturbance of the balance played a key role in the pathogenesis of DR[45, 49, 50]. In vitro study revealed that lowering of the VEGF-to-PEDF mRNA ratio could inhibit the migration of uveal melanoma cells[51].

Additionally, PEDF induces the ERK signal cascade which contributes to retinal pigment epithelial cell cytoprotection against oxidative stress[52]. Thus, retinal cells including the BRB capillaries and their supportive and protective pericytes may possess a system capable of efficiently responding to PEDF[43, 44].

Retinal ischemia induces intraocular neovascularization,presumably by stimulating the expression of angiogenic growth factors and by inhibiting the release of antiangiogenic cytokines[53, 54]. Vitreal levels of angiogenic growth factors have been shown to be directly associated with the degree of retinal angiogenesis[15, 55]. PEDF protects cerebellar granule cells against neurotoxic agents[56] and is also called early population doubling level cDNA-1 (EPC-

1), reflecting its upregulation during cell cycle arrest (G0) in young but not in senescent cultured fibroblasts[57].

PEDF has been shown to be a highly effective inhibitor of angiogenesis in animal and cell culture models. The production of PEDF was decreased by hypoxia[47], which is also a central pathogenic stimulus in PDR. Immunoneutralization of PEDF diminished the ability of cadaveric human vitreous to inhibit migration of endothelial cells, thereby demonstrating that a loss of PEDF is functionally important in mediating angiogenic properties of human vitreous ex vivo. Most importantly, systemically administered PEDF prevented aberrant blood vessel growth in a murine model of ischemia-induced retinopathy[58].

PEDF has been shown to be a major antiangiogenic growth factor in the mammalian eye. Joachim[41] et al analyzed the in vivo regulation of PEDF in patients with and without hypoxic eye disease. Their data strongly support the concept that retinal angiogenesis is induced by loss of the major angiogenesis inhibitor in the eye, PEDF, in combination with an increased expression of angiogenic growth factors such as VEGF. These findings suggest that substitution of angiogenesis inhibitors may be an effective approach in the treatment of PDR[41].

In the study of Zhi[59] et al ,diabetic rats and control animals were randomly assigned to receive perindopril or vehicle for 24 weeks, and bovine retinal capillary endothelial cells (BRECs) were incubated with normal or high glucose with or without perindopril. The results showed the VEGF-to-PEDF ratio was increased in the retina of diabetic rats; perindopril lowered the increased VEGF-to-PEDF ratio in diabetic rats and ameliorated the retinal damage. In BRECs, perindopril lowered the hyperglycemia-induced elevation of VEGF-to-PEDF ratio by reducing mitochondrial ROS and the decreased ROS production was a result of perindopril induced upregulation of PPARY and UCP-2 expression[59].

Although VEGF is the major factor in the initiation of advanced stages of diabetic retinopathy, it is increasingly recognized that PlGF is a significant factor in promoting the aberrant angiogenesis characteristic of a variety of pathological states.

5. Adiponectin (ADPN)

The adipocyte derived factor ADPN is an insulin sensitivity activator, and is correlated to retinal redox stress and remodeling in metabolic syndrome and T2DM. Low levels of serum ADPN levels were found to be correlated with the severity of retinopathy[60]. Insulin-sensitizing agents reduce pathological retinal microvessel formation through ADPN mediated modulation of tumor necrosis factor alpha (TNFα) production[61]. ADPN' s effect on diabetic retinopathy is not clear. However, ADPN induces eNO production by stimulating phosphorylation and activation of eNOS. ADPN inhibits specific binding of oxidized LDL and its uptake by macrophages. ADPN possesses anti-inflammatory properties and thus may negatively modulate the process of atherogenesis[62, 63].

In the early phase of diabetic retinopathy, hyperglycemia initiates endothelial cell injury, retinal vessel loss, and ischemia,as well as changes in leukocyte adhesion to the vascular endothelium[64, 65]. These conditions subsequently lead to the overproduction of various proangiogenic factors and proinflammatory cytokines, which, in turn, promotes abnormal

neovascular changes[66].The primary goal for treatment of ischemic retinopathy is to preserve vision through the inhibition of abnormal neovascularization and vascular damage.

Adiponectin is a circulating adipose-derived cytokine with antiinflammatory properties[67, 68].In animal models,adiponectin deficiency is associated with the increased inflammatory responses under conditions of stresses including overnutrition and ischemic insult[69, 70]. In addition, adiponectin has been shown to protect against the development of various diseases including detrimental cardiac and vascular remodeling,ischemic stroke and increased albuminuria[69, 71-74]. In human populations, circulating adiponectin levels inversely correlate with the inflammatory marker C-reactive protein levels in blood stream[67, 75, 76]. Low plasma adiponectin levels are associated with the increased prevalence of type 2 diabetes and its macrovascular complications including ischemic heart disease[63, 67, 77].

Clinical studies regarding the relationship between plasma adiponectin level and retinopathy in diabetes have been inconclusive[78, 79]. Higuchi et al investigated whether adiponectin affects the retinal vascularization and inflammation in a mouse model of ischemia-induced retinopathy.When neonatal mice were subjected to ischemia-induced retinopathy, pathological retinal neovascularization during ischemia was exacerbated in adiponectin-knockout (APN-KO) mice compared with wild-type mice. APN-KO mice also exhibited increased leukocyte adhesion and tumor necrosis factor (TNF)-a expression in hypoxic retina. Adenovirus-mediated overexpression of adiponectin attenuated hypoxia-induced pathological retinal neovascularization by 35% in wild-type mice and by 40% in APN-KO mice and leukostasis by 64% in wild-type mice and by 75% in APN-KO mice, which were associated with reduced TNF-a production. TNF-a blockade diminished the enhanced pathological neovascularization in APN-KO mice, and the inhibitory effects of adiponectin overexpression on retinal neovascularization and leukocyte adhesion were abolished in mice lacking TNF-a. These data provide evidence that adiponectin protects against retinal vessel injury following pathological stimuli through modulation of TNF-a inflammatory responses[80].

ADPN suppresses adverse effects of inflammatory cytokines and reduces oxidative stress induced by oxidized LDL or high glucose in EC[62, 63]. ADPN inhibits VEGF-stimulated human coronary artery EC migration via cAMP/PKA dependent signaling including VEGF-induced generation of ROS, which implicates it as an important role in vascular processes associated with diabetes. Because ADPN is known to act as an antioxidant, anti-inflammatory, antiapoptotic and antifibrotic protein then its low levels may predispose it to a loss of any or all of the above known protective features of ADPN and directly or indirectly affect the capillary BRB including the pericyte. Importantly, ADPN may be used in the future as an early candidate biomarker of DR in CMS and T2DM.

6. Leptin

Not all patients with poor control of diabetes over long periods of time, develop retinopathy, suggesting the involvement of other mechanisms. The adipose tissue is an important endocrine organ that secretes many biologically active substances such as free fatty acids, adiponectin, and interleukin (IL)-6. They are collectively termed adipocytokines[81]. Leptin is one of adipocytokines, acting directly on the hypothalamus, thereby regulating food intake and energy expenditure[82]. The leptin receptor (Ob-R) is a

single transmembrane protein that belongs to the gp130 family of cytokine receptor superfamily. The leptin receptor has several alternatively spliced isoforms, one of which, a biologically active Ob-Rb isoform, is expressed not only in the hypothalamus but also in a variety of peripheral tissues, suggesting the direct action of leptin in the periphery. The peripheral actions of leptin include the activation of platelet aggregation , the modulation of immune function[83], and the stimulation of vascular endothelial cell proliferation and angiogenesis[84, 85]. Upon binding to Ob-Rb, leptin has been shown to activate signal transducers and activators of transcription (STAT).

A study has revealed that plasma leptin concentrations are elevated significantly in patients with proliferative diabetic retinopathy relative to those with nonproliferative retinopathy[86]. Furthermore, vitreous leptin concentrations are higher in patients with proliferative diabetic retinopathy or retinal detachment[87].

Using the retinopathy of prematurity model, a mouse model of ischemia-induced retinal neovascularization, Eri Suganami [88]et al have demonstrated more pronounced retinal neovascularization in 17-day-old transgenic mice overexpressing leptin than in age-matched wild-type littermates. Leptin receptor expression was also detected in primary cultures of porcine retinal endothelial cells, where it upregulated VEGF mRNA expression. This effect was thought to be mediated at least partly through the activation of signal transducers and activators of transcription(STAT)3, because adenoviral transfection of the dominant negative form of STAT3 abolished the leptin-induced upregulation of VEGF mRNA expression in retinal endothelial cells. This study provides evidence that leptin stimulates the ischemia-induced retinal neovasucularization possibly through the upregulation of endothelial VEGF[88].

7. Insulin-like Growth Factor-1 (IGF-1)

Similar to VEGF, the activation of IGF-1 also increases PKC activation, so IGF-1 may be regulated by oxidative stress via the PKC pathway[89]. Retinal IGF-1 mRNA levels are lower in the human and diabetic rat when compared to age matched non-diabetic controls[90] and IGF-1 can have direct mitogenic effects on retinal EC[60].IGF-1 can stimulate glucose transport into retinal microvascular EC via activation of PKC and can modulate the expression and activity of VEGF[91].

Growth hormone and IGF-I have been suspected of playing a role in the progression of diabetic retinopathy. In a previous era, hypophysectomy was shown to lead to regression of proliferative retinopathy in a study of 100 patients[92]. Similarly, diabetic dwarfs with low systemic IGF-I levels due to growth hormone deficiency have a reduced incidence of proliferative DR compared with age- and sex matched diabetic patients. Such observations have raised interest in the use of growth hormone–inhibitory and antiproliferative somatostatin analogs to treat severe proliferative DR, however, a growth hormone receptor antagonist, pegvisomant, failed to induce regression of neovascularization [93]. This negative result may have occurred because the treatment was initiated too late; treatment may need to have started prior to the development of proliferative DR. In another small-scale trial (23 patients), octreotide (a somatostatin analog) treatment reduced the requirement for laser photocoagulation compared with conventional treatment in patients with either severe NPDR or early proliferative DR [94]. Over the 15-month study, only1 of 22 octreotide-treated patients required photocoagulation compared with 9 of 24 conventionally treated patients.

8. Interleukin-1 Beta (IL-1β)

Levels of the proinflammatory cytokine, IL-1β , are known to be increased in retinas from diabetic rats[95-97]. Intravitreal injection of IL-1β or exposure of retinal endothelial cells to the cytokine in vitro was shown to be capable of causing degeneration of retinal capillary endothelial cells[98], but the relevance of these findings to capillary degeneration in vivo is not clear because the levels of IL-1β likely were pharmacologically high. The role of IL-1β in the pathogenesis of diabetic retinopathy recently has been more directly studied using diabetic mice in whom the enzyme responsible for IL-1β production was inhibited or in whom the IL-1β receptor was deleted. IL-1β is the predominant product of caspase-1, and the biological activity of IL-1β is mediated by binding to the cell surface receptor, IL-1R1. Activity of caspase-1 is increased in retinas of diabetic mice, galactosefed mice, and diabetic humans, and in retinal Müller cells incubated in elevated glucose concentration[99]. Inhibition of caspase-1 using minocycline inhibited the diabetes induced increase in IL-1β and decreased degeneration of retinal capillaries in those animals[95]. Likewise, inhibition of IL-1β signaling using IL-1β receptor knock-out mice protected the animals from diabetes-induced retinal pathology at 7 months duration of diabetes[95]. The results indicate that activation of caspase-1 and subsequent production of IL-1β play an important role in the development of diabetes induced retinal pathology. One known action of IL-1β is to activate NF-κ B.

IL-1β gene expression is known to reside in EC and glial cells and its expression is significantly upregulated in high glucose conditions allowing for BRB allowing inflammatory cells to increase their migration across the BRB[98].

IL-1β is known to increase the expression of VEGF in retinal EC, and induces the expression of various genes whose promoters are regulated through complex interactions with NFκB[100]. IL-1β has been found to be increased in streptozotocin diabetic rat models[98] and IL-1β accelerates apoptosis in retinal capillary cells, specifically pericytes,through activation of NFκB, which is exacerbated by high glucose conditions[101]. NFκβ is a key regulator of antioxidant enzymes and can initiate transcription of genes involved in apoptosis and additionally increases downstream inflammatory cytokines[101]. Importantly, IL-1β activation—stimulation results in the translocation of NFκB from its cytosolic compartment to the nucleus where it initiates apoptotic genes and downstream inflammatory cytokines[101].

Additionally, IL-1β is considered as one of the most potent stimuli for inducible NOS (iNOS), contributing to ongoing inflammation via induction of iNOS protein and augmentation of its activity[98]. IL-1β receptor antagonism (IL-1 βra) in the retina and IL-1βhave been shown to interfere with the development of not only diabetic retinopathy but also pancreatic islet inflammation and beta cell apoptosis in humans with T2DM[102].

9. Interleukin-6 (IL-6)

The IL-6 cytokine shares common characteristics with VEGF, in that both are induced by hypoxia and hyperglycemia, and both play a role in vascular inflammation, permeability and angiogenesis[103]. Human studies have demonstrated that both VEGF and IL-6 were elevated in aqueous humor of patients with DR and even higher in those with proliferative DR indicating that VEGF and IL-6 play important roles in the development of DR[104]. Even

peripheral blood levels of IL-6 and TNF α were elevated in humans with DR with the highest elevations found in those with proliferative DR[105]. It has been shown that the AngII-induced vascular alterations involved activation of NAD(P)H oxidase, IL-6, and increases in VEGF expression and further,that deletion of IL-6 prevented these effects of vascular inflammation in DR[106].

10. Monocyte Chemoattractant Protein (MCP-1)

MCP-1 contributes to the recruitment of inflammatory cells (monocytes/monocyte derived macrophage/microglia) in injured tissue and ROS injury may play a role in DR and retinal detachment[107]. MCP-1 is a potential angiogenic factor in the proliferative phase of DR and is associated with proliferation DR[107].Hyperglycemia increases the expression of MCP-1 in vascular EC[63] and AGE-induced ROS generation induced the MCP-1 gene and mRNA expression[63]. Recently, aqueous samples in humans with DR have revealed higher levels of MCP-1 and VEGF when compared to nondiabetic subjects and authors further state that inflammatory changes may precede the development of neovascularization in proliferative DR[108].

11. Vascular Cell Adhesion Molecule (VCAM)

Many specific growth factors mediate angiogenic process of diabetic retinopathy. VCAM-1, a member of the immunoglobulin supergene family of cellular adhesion molecules, is involved in the recruitment of leukocytes, their adhesion to vascular endothelium, and their subsequent migration into surrounding tissue. Interestingly, the expression of VCAM-1 has been found in epiretinal membranes from diabetic patients with PDR[109, 110]. In addition, it has been demonstrated that VCAM-1 promotes angiogenesis both in vitro and in vivo[111, 112].Olson et al. detected increased serum levels of VCAM-1 in diabetic patients with PDR[113]. Moreover, circulating levels of various adhesion molecules increase in patients with progressively worsening retinopathy, presumably as a result of shedding from both activated leukocytes and injured epithelium. However, systemic levels of VCAM-1 do not reflect the local production of VCAM-1 by the retina. Vitrectomy fluid samples obtained from diabetic patients with PDR are currently being used to explore indirectly

the retinal synthesis of several proteins, including growth factors, cytokines, and adhesion molecules. Two previous studies demonstrated that soluble VCAM-1 is increased in the vitreous cavity of diabetic patients with PDR compared with the vitreous of patients undergoing macular hole repair[114]or from cadaveric eyes[15].

12. Connective Tissue Growth Factor (CTGF)

The tissue repair process is regulated by a number of polypeptides including cytokines and growth factors. CTGF is a 38-kDa cysteinerich polypeptide that was originally identified from conditioned medium of human umbilical vein endothelial cells (HUVECs)[115]. CTGF, considered to be a downstream mediator of transforming growth factor-ß(TGF- ß)[116, 117], is indicated to induce the production of extracellular matrix,such as collagen and fibronectin, and to cause fibrosis[118]. One study have shown that CTGF is overexpressed in the

membranes of eyes with PDR[119], suggesting that CTGF might be involved in the pathogenesis of PVR and PDR. In addition, A study revealed that CTGF is overexpressed also in the vitreous with PVR and PDR and additionally demonstrated that various types of vitreoretinal cells could be the sources of CTGF[120].

Furthermore, CTGF has been recently indicated to be one of the regulators of angiogenesis. In vitro, CTGF has been demonstrated to have proangiogenic effects on Human umbilical vein endothelial cell[121] and bovine aortic endothelial cells (BAECs)[122], and in vivo, CTGF has been indicated to induce angiogenesis in rat corneal pocket implants[123] and to be involved in tumor angiogenesis[124] and choroidal neovascularization[125, 126]

In the study of Takeshi et al, they demonstrated CTGF also stimulated the synthesis of fibronectin by hyalocytes and BRPEs without significant effect on collagen gel contraction by these cells. And CTGF promoted VEGF gene expression by hyalocytes and BRPEs. There was no significant correlation between the concentrations of CTGF and VEGF. These findings indicate that CTGF appears to be involved in the formation of proliferative membranes without direct regulation of their cicatricial contraction in the pathogenesis of proliferative vitreoretinal diseases. It is possible that CTGF has indirect effects by modulating the expression of VEGF[127].

13. Retinal Intercellular Adhesion Molecule-1 (ICAM-1) and CD18

The retinal vasculature of diabetic humans contains increased numbers of leukocytes, a finding that coincides with the increased expression of ICAM-1 in retinal vasculature[128]. The phenomenon is also present in diabetic animal models and occurs whether the diabetes is spontaneous in nature or is induced[11, 129, 130]. The increased density of leukocytes in the retinal vasculature begins as early as 1 week following the onset of experimental diabetes and results in injury to the endothelium via a FasL-mediated mechanism; a process that leads to breakdown of the BRB [131, 132]. Retinal ischemia is a second sight-threatening diabetic complication. Histolopathological analyses have shown that areas of angiographic non-perfusion in vivo frequently co-localize to regions full of acellular capillaries, that is, basement membrane tubes devoid any viable endothelial cells or pericytes[133].

The leukocytes that adhere to the diabetic retinal vasculature use specific adhesion molecules such as the integrin ligand CD18, which forms the invariable portion of the heterodimers Mac-1(CD11a/CD18) and LFA-1 (CD11b/CD18)[134]. Leukocytes use CD18 to tether themselves to intercellular adhesion molecule-1 (ICAM-1) on the surface of diabetic retinal vasculature.

A work has established the role of CD18/ICAM-1 leukocyte adhesion in the pathogenesis of early diabetes-induced leukostasis and blood-retinal barrier breakdown[131]. The study of Antonia et al also showed that retinal leukostasis increased within days of developing diabetes and correlated with the increased expression of retinal intercellular adhesion molecule-1 (ICAM-1) and CD18[135]. Mice deficient in the genes encoding for the leukocyte adhesion molecules CD18 and ICAM-1 were studied in two models of diabetic retinopathy with respect to the long-term development of retinal vascular lesions. CD18−/− and ICAM-1−/− mice demonstrated significantly fewer adherent leukocytes in the

retinal vasculature at 11 and 15 months after induction of diabetes with STZ. And this condition is associated with fewer damaged endothelial cells and lesser vascular leakage.

Galactosemia of up to 24 months causes pericyte and endothelial cell loss and formation of acellular capillaries. However, these changes are significantly reduced in CD18- and ICAM-1-deficient mice. Basement membrane thickening of the retinal vessels is increased in long-term galactosemic animals independent of the genetic strain. Thus,the chronic, low-grade subclinical inflammation is responsible for many of the signature vascular lesions of diabetic retinopathy.These data highlight the central and causal role of adherent leukocytes in the pathogenesis of diabetic retinopathy[135].

Attraction and adhesion of leukocytes to the vascular wall are important components of inflammatory processes. This leukostasis has been found to be significantly increased in retinas of diabetic animals, and might contribute to the capillary nonperfusion in diabetic retinopathy. Leukocyte stiffness has been reported to be increased in diabetes (decreased filterability) and to contribute to the development of capillary nonperfusion in retinal vessels [136, 137]. Diabetes increases expression of ICAM-1 in retinas of animals [11]and interaction of this adhesion molecule on retinal endothelia with the CD18 adhesion molecule on monocytes and neutrophils contributes to the diabetes-induced increase in leukostasis within retinal vessels[11]. Leukostasis has been postulated to be a factor in death of retinal endothelial cells in diabetes[131].

White blood cells bind to ICAM-1 on the surface of endothelial cells as a component of a multistep process leading to adherence of the white blood cell to the endothelial wall[11].This leukostasis is known to be increased in retinal blood vessels in diabetes, and this process is mediated via ICAM-1[11]. ICAM-1 is upregulated by several stimuli, including VEGF, PARP activation, oxidative stress, and dylipidemia[138-141], at least in part by NF-κ B.Genetically modified C57B1/6J mice have been used to explore the roles of ICAM-1 and its ligand on white blood cells (CD18) in the pathogenesis of diabetes-induced retinal vascular disease[135].

14. NF-κB

NF-κ B is a widely expressed inducible transcription factor that is an important regulator of many genes involved in mammalian inflammatory and immune responses, proliferation and apoptosis. NF-κ B is composed of homodimers and heterodimers, the most abundant and best-studied form in mammalian cells consisting of the p65 and p50 subunits. Diabetes has been found to cause migration of the p65 subunit into the nucleus of retinal pericytes[101], and of the p50 subunit into nuclei of retinal endothelial cells, pericytes, ganglion cells, and cells of the inner nuclear layer[142].

Evidence in support of an important role of NF-κ B in the pathogenesis of early stages of diabetic retinopathy is twofold. First, inhibition of proteins whose expression is regulated by NF-κ B (such as iNOS and ICAM) inhibit diabetes induced degeneration of retinal capillaries (described below).Second, compounds known to inhibit NF-κ B likewise inhibit the development of the retinopathy. For example, several different antioxidants which inhibit the development of capillary degeneration and pericyte loss in retinas of diabetic rats[143] also inhibit the diabetes-induced activation of retinal NF-κ B[138].Likewise, low-intermediate doses of salicylates (aspirin, sodium salicylate, and sulfasalazine) which inhibited NF-κ B activation in retinas of diabetic rats, also inhibited expression of inflammatory mediators like iNOS and ICAM-1, and capillary degeneration and pericyte loss in those animals[143, 144]. Aspirin is known to inhibit also production of prostaglandins, but salicylate and sulfasalazine have much less of

this activity, suggesting that the common action of these salicylates to inhibit retinopathy in diabetes was not primarily mediated by inhibition of prostaglandins.

15. *iNOS*

Inducible isoform of nitric oxide synthase(iNOS) expression is regulated at least in part by NF-κ B. Interestingly, experimental sympathectomy itself increases gene and protein expression of iNOS in retinas of nondiabetic rats[145], suggesting that loss of sympathetic activity, such as which occurs in diabetes, might contribute to the upregulation of this inflammatory protein in the retina.

In retinas of diabetic animals, increased levels of nitric oxide products (nitrotyrosine, nitrite, nitrate) have been reported[144-146]. Upregulation of iNOS has been found in retinas of experimental diabetic rodents and patients in most studies[145-152]. Diabetes-induced alterations in expression of other isoforms of nitric oxide synthase also have been reported[153, 154]. A possible role of iNOS in the pathogenesis of diabetic retinopathy is suggested by the studies of aminoguanidine. Aminoguanidine is a relatively selective inhibitor of iNOS[155-158], and has been found to inhibit the diabetes-induced increase nitric oxide production and iNOS expression in retina[145].

Aminoguanidine also has been found to inhibit the development of the microvascular lesions of diabetic retinopathy in diabetic dogs[159]and rats[160].The role of iNOS in the development of the early stages of diabetic retinopathy recently has been investigated directly using mice genetically deficient in iNOS[161]. In that study, wildtype diabetic mice developed the expected degeneration of retinal capillaries, as well as increase in leukostasis and superoxide generation. In contrast, diabetic mice deficient in iNOS did not develop these structural or functional abnormalities.

16. Fas

Fas levels are increased in retinas of diabetic rats[132, 162]. Blocking FasL in vivo has been shown to prevent endothelial cell damage, vascular leakage, and platelet accumulation in diabetes, suggesting that the Fas/FasL system might contribute to the diabetes-induced damage that contributes to the development of the retinopathy[132], but its role in the development of retinal histopathology has not been assessed.

17. Angiopoietin-1

Angiopoietin-1 has been found to have anti-inflammatory actions, including inhibition of vascular permeability and adhesion protein expression[163]. When administered intravitreally to diabetic rats, angiopoietin-1 normalized blood-retinal barrier function, leukostasis and endothelial injury, and inhibited upregulation of retinal VEGF and ICAM-1mRNA and protein[164].

18. Hepatocyte Growth Factor (HGF)

HGF in the etiopathogenesis of PDR remains to be elucidated. A lot of studies[165-169] have found high intravitreous concentrations of HGF in patients with PDR. In the present study,

we consider all these confounding factors in order to evaluate the vitreous levels of HGF in patients with PDR and to investigate its relationship with VEGF and retinopathy activity. A total of 28 diabetic patients with PDR, in whom a vitrectomy was performed, were included in the study. Thirty nondiabetic patients with other conditions requiring vitrectomy but in whom the retina was not directly affected by neovascularization served as a control group. Patients in whom intravitreous hemoglobin was detectable by spectophotometry were excluded. HGF and VEGF were determined by enzyme-linked immunosorbent assay. Vitreal levels of both VEGF and HGF were higher in diabetic patients with PDR than in the control group. These differences remained highly significant after adjusting for serum levels. To explore the influence of the breakdown of the blood-retinal barrier and, in consequence, the increased serum diffusion that occurs in PDR patients, the levels of both HGF and VEGF were normalized for total vitreal protein concentration. After correcting for total vitreous protein concentration, the ratio of VEGF to vitreal proteins remained significantly higher in diabetic patients with PDR than in the control group, respectively. However, the ratio of HGF to vitreal proteins was lower in diabetic patients than in nondiabetic control subjects. The lower intravitreous levels of HGF obtained after correcting for intravitreal proteins in patients with PDR in comparison with nondiabetic control subjects suggest that serum diffusion largely explains the differences detected in the intravitreous HGF levels between these groups. The vitreous concentrations of VEGF were higher in patients with active PDR than in patients with quiescent PDR. By contrast, vitreous HGF was not related to PDR activity[170].

19. Angiotensin II

Angiogenesis, the growth of new vessels, is a physiologic process that occurs under normal conditions. During these processes, angiogenesis is well regulated by a balance of positive and negative factors. However, in various disease states, such as tumor progression, inflammation, and diabetic retinopathy, deregulated overactive angiogenesis contributes to disease progression[171].Recent reports suggest that receptor tyrosine kinases (RTKs) of endothelial cells play a major role in both physiological and pathological angiogenesis[171, 172]. Two distinct RTK subfamilies are characterized by their abundant expression of endothelium. One subfamily consists of VEGF receptors Flt-1/VEGF-R1, Flk-1/VEGF-R2, and Flt-4/VEGF-R3[173-175]. VEGF, also known as vascular permeability factor, is an endothelial cell-specific mitogen that induces angiogenesis and increases vasopermeability[171].

The other endothelium-specific RTK subfamily is the Tie receptor family, consisting of Tie1 and Tie2[176]. Tie1-null mice die in utero with defects that may implicate the hemodynamics of transcapillary fluid exchange[177, 178]. Similarly, Tie2-knockout mice die from day 9.5 to 10.5, because of immature vessels and lack of microvessel formation[178, 179]. Unlike the VEGF receptor–knockout mouse[180], the number of endothelial cells was normal, and tubular formation was detected in Tie2-knockout mice. A mutation in Tie2 in humans was reported to cause venous malformations, which are typically an imbalance of endothelial cells and smooth muscle cells[181]. These findings suggest that the Tie2 system has a role in endothelialstromal cell communication and in maturation and stabilization of vascular structures.

Ligands for the Tie2 receptor have been identified as angiopoietin (Ang)-1 and Ang2 [182, 183] and, more recently,Ang3 and Ang4[184]. Ang1 phosphorylates Tie2 in cultured endothelial

cells[182], whereas Ang2 does not induce phosphorylation of Tie2, but rather inhibits the Ang1-induced phosphorylation of Tie2 in vascular endothelial cells[183].Ang2-overexpressing transgenic mice die with vascular defects similar to Tie2- or Ang1-knockout mice[178, 185]. These observations suggest that Ang2 acts as a natural antagonist of Tie2 by blocking receptor activation by Ang1[183]. Recently, wide expression of Tie2 in the quiescent vasculature of adult tissues was reported[186]. A study using a corneal angiogenesis model revealed that Ang1 and Ang2 facilitates VEGF-induced neovascularization; Ang1 promotes vascular network maturation, whereas Ang2 initiates neovascularization[187].These data support the idea that angiopoietins/Tie2 may have a role not only in embryonic angiogenesis, but also in postnatal angiogenesis.

The renin-angiotensin system (RAS) is known to be a key factor in the cardiovascular homeostasis that regulates blood pressure and fluid electrolyte balance[188]. RAS abnormalities have also been reported to play a role in the progression of diabetic retinopathy[189]. Angiotensin II has been reported to regulate cell growth by inducing several growth factors[190-192]. In 1998 and 2000, Atsushi Otani et al reported that Angitensin II potentiates VEGF-mediated angiogenic activities through upregulation of VEGF-R2 expression in bovine retinal endothelial cells (BRECs) and upregulation of VEGF in bovine retinal pericytes (BRPs) [193, 194]. As RAS played a major role in the retinal angiogenic abnormalities associated with diabetes, Atsushi Otani et al investigated the effect of angiotensin II (AII) on Ang1 and Ang2 expression in cultured bovine retinal endothelial cells(BRECs). Their results showed that AII stimulated Ang2 but not Ang1 mRNA expression in a dose- and time-dependent manner. This response was inhibited completely by angiotensin type 1 receptor (AT1) antagonist. AII increased the transcription of Ang2 mRNA, but did not change the half-life. Protein kinase C (PKC) inhibitor completely inhibited AIIinduced Ang2 expression, and the mitogen-activated protein kinase (MAPK) inhibitor also inhibited it. In addition, the upregulation of Ang2 in an AII-induced in vivo rat corneal neovascularization model was also confirmed. These data suggest that AII stimulates Ang2 expression through AT1 receptor-mediated PKC and MAPK pathways in BREC, and AII may play a novel role in retinal neovascularization[195].

20. Glial cell-derived cytokines

BRB is a biological unit of retinal vessels with a well-differentiated network, including glial cells such as astrocytes and Müller cells, maintaining the retinal microenvironment and low permeability. The substantial apparatus of the BRB is a barrier comprised of tight junctions between the capillary endothelial cells that strictly regulate the paracellular pathways between the cells[196]. BRB breakdown is closely associated with a number of retinal diseases such as diabetic retinopathy, which is characterized by vascular leakage due to increased vascular permeability in its early pathogenesis[197].

Hich is believed to be a critical factor in the development of diabetic retinopathy[12, 15, 48]. However, the molecular pharmacology that directly inhibits activated VEGF has not been proven to satisfactorily block microangiopathy in diabetic retinopathy[198, 199]. Glial cell line–derived neurotrophic factor (GDNF) was originally identified as a neurotrophic differentiation factor for dopaminergic neurons in the central nervous system and retina.The certain advanced glycation end products could increase the vascular permeability of the BRB in vitro by the induction of VEGF and reduction of GDNF expression from glial cells

have been demonstrated, suggesting that phenotypic alteration of glial cells in diabetes is responsible for the BRB breakdown[200-202].

The vitamin A metabolite all-*trans* retinoic acid (ATRA) is a potent regulator of cell differentiation and an essential signaling molecule in embryonic development and throughout life. A study has shown that ATRA can differentiate pluripotent embryonal carcinoma cells into neuronal and glial tissues and that it plays an important role in the induction of GDNF responsiveness in these cells[203]. Nami Nishikiori et al demonstrated that retinoic acid receptor (RAR)a stimulants preferentially act on glial cells, resulting in the enhanced expression of glial cell line–derived neurotrophic factor (GDNF) through recruitment of the RARa-driven trans-acting coactivator to the 5´-flanking region of the gene promoter. Conversely, RARa decreases expression of VEGF/vascular permeability factor. These gene expression alterations causally limit vascular permeability by modulating the tight junction function of capillary endothelium in a paracrine manner in vitro. The phenotypic transformation of glial cells mediated by RARa is sufficient for significant reductions of vascular leakage in the diabetic retina, suggesting that RARa antagonizes the loss of tight junction integrity induced by diabetes. These findings reveal that glial cell–derived cytokines such as GDNF and VEGF regulate BRB function, implying that the glial cell can be a possible therapeutic target in diabetic retinopathy[204].

21. References

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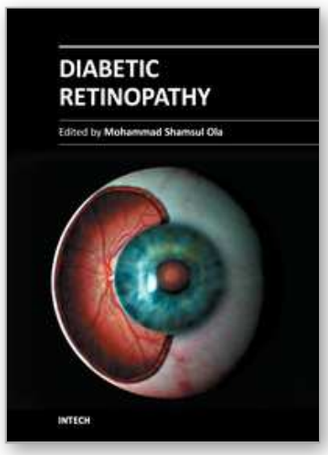

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The aim of this book is to provide a comprehensive overview of current concepts in pathogenesis, diagnosis and treatments of diabetic retinopathy. It provides a collection of topics written by excellent authors, covering discussions on advances in understanding of pathophysiology, immunological factors and emerging concepts, relating to clinical aspects and treatment strategies. The contents of the book will not only provide a resource for our knowledge but also improve diagnosis and treatment options for those patients who suffer vision loss due to diabetic retinopathy.

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