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Abstract: Diabetic macular edema (DME) is a serious condition that can cause blindness in diabetic patients suffering from diabetic retinopathy (DR). Although vascular endothelial growth factor (VEGF) is known to play a role in the development of DME, the pathological processes leading to the onset of the disease are highly complex and the exact sequence in which they occur is still not completely understood. Angiogenesis and inflammation have been shown to be involved in the pathogenesis of this disease; however, whether angiogenesis following VEGF over-expression is a cause or a consequence of inflammation remains to be clarified. Here, we provide an overview of the current data available in the literature focusing on VEGF, angiogenesis, inflammation, DR and DME. Our analysis suggests that angiogenesis and inflammation act interdependently during the development of DME and that VEGF is a critical player in the molecular crosstalk occurring between these two pathways. Consequently, anti-VEGF therapies hold potential for the treatment of DME.

Angiogenesis and inflammation act interdependently during the development of diabetic macular edema and vascular endothelial growth factor is a critical player in the molecular crosstalk occurring between these two pathways.

Pathophysiology and pharmacological targets of diabetic macular edema: an updated review

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Abstract

Diabetic macular edema (DME) is a serious condition that can cause blindness in diabetic patients suffering from diabetic retinopathy (DR). Although vascular endothelial growth factor (VEGF) is known to play a role in the development of DME, the pathological processes leading to the onset of the disease are highly complex and the exact sequence in which they occur is still not completely understood. Angiogenesis and inflammation have been shown to be involved in the pathogenesis of this disease; however, whether angiogenesis following VEGF over-expression is a cause or a consequence of inflammation remains to be clarified. Here, we provide an overview of the current data available in the literature focusing on VEGF, angiogenesis, inflammation, DR and DME. Our analysis suggests that angiogenesis and inflammation act interdependently during the development of DME and that VEGF is a critical player in the molecular crosstalk occurring between these two pathways. Consequently, anti-VEGF therapies hold potential for the treatment of DME.

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Introduction

Diabetic patients often suffer from diabetic retinopathy (DR) leading to diabetic macular edema (DME), the most common cause of visual loss in this set of patients.[1,2] Pathogenesis of this condition is complex and involves several physiological alterations.

The blood retinal barrier (BRB) plays a key role here, as its disruption leads to several pathological conditions of the eye such as age-related macular degeneration (ARMD), retinal vein occlusions (RVO) and other chronic retinal diseases.[3]

In order to understand how the BRB breakdown is involved we need to take a step back and understand what occurs in diabetes.

In diabetic patients, hyperglycemia is the triggering factor for tissue alterations such as damages to the capillary endothelial cells in the retina.[4] This occurs through several pathways (Figure 1):

- the increased polyol pathway where increased glucose concentration leads to hyperglycemic oxidative stress[5];
- the increased formation of advanced glycation end-products (AGEs), which alters intracellular and transmembrane proteins such as integrins and integrin receptors, thus disturbing crucial interactions with proteins of the basal lamina[6,7];
- the activation of protein kinase C (PKC) isoforms, which leads to an increased production of extracellular matrix and cytokines, enhanced contractility, permeability, and vascular cell proliferation, activation of cytosolic phospholipase A2, inhibition Na+-K+-ATPase, all leading to abnormal retinal hemodynamics.[8]

One hypothesis is that the complex tissue alterations lead to cellular hypoxia.[9] In response to hypoxia, vascular endothelial growth factor (VEGF) production is induced, a key promoter of angiogenesis, abnormal vascular permeability, and, eventually, inflammatory reaction.[10] One of the most important consequences of the formation of AGEs is also the induction of VEGF.[11,13]

Being VEGF a promoter of the inflammatory process, leading to hypoxia, and being hypoxia responsible for the increased expression of VEGF, it becomes clear that this is a self-fueling exponential loop always leading to increased tissue damage.

Negative consequences of increased VEGF expression due to diabetic hyperglycemia are described in several anatomical locations other than the retina, such as the kidney[14,15] or the brain.[16] In the eye, increased VEGF expression leads to pathologic transformation of the retinal vasculature, including permeability, remodeling and neovascularization.[17] Several VEGF isoforms exist, $VEGF_{165}$ being not only the predominant form in the diabetic eye, but, among the VEGF isoforms, also the most potent inducer of leukostasis and BRB breakdown, as shown in an animal model.[18] VEGF binds to its receptors on the vascular endothelium and activates the mitogen-activated protein kinase (MAPK) thus triggering endothelial cell proliferation.[10] VEGF is also a positive regulator of angiogenesis by stimulating endothelial cells to degrade their basement membrane and to migrate by releasing matrix metalloproteinases (MMPs) and plasminogen activators (PAs) and by increasing the expression of integrins.[19] Proliferation and migration of endothelial cells is followed by synthesis of basement membranes for the newly formed capillaries.[10] Not surprisingly, similar mechanisms are found in tumor development.[20]

It therefore becomes clear that VEGF plays a predominant role in the pathogenesis of DME, serving as an attractive target for therapy. Nevertheless, there still is substantial uncertainty on the temporal development of retinal alterations, and the main question that arises from the extensive literature on

this topic, is: what comes first? Is angiogenesis leading to inflammation or the contrary? What is the exact mechanism leading to inflammation and, is it subsequent to angiogenesis? Is it angiogenesis that, due to the production of mediators, such as VEGF, activates the production of nitric oxide and all that could potentially be correlated to tissue damage, increased vascular permeability, endothelial cell proliferation, vascular occlusion ischemic cell death and therefore inflammation, or is it inflammation that triggers the expression of VEGF and subsequently leads to angiogenesis, hyperpermeability and so on?

The purpose of this review is therefore to examine all available literature, which points toward one explanation or the other, to eventually arrive at a conclusion to this highly debated topic. A thorough summary of all studies analyzed in the next sections comparing various aspects of each study is provided (**Supplementary Table 1).**

Studies supporting a predominant role of angiogenesis on the pathogenesis of diabetic retinopathy

There are several studies supporting the idea of angiogenesis being the main reason for DME. An elegant study by Shimada and coworkers evaluated VEGF concentrations from different sites in the vitreous. By taking samples from the pre-macular region, the peripheral cortical vitreous and the mid vitreous they showed that VEGF levels are higher in the pre-macular vitreous compared to the other two sites. Also, VEGF correlated with foveal thickness (FT), and consequently with DME severity. This study demonstrates that there is a diffusion of VEGF from the macular region to the periphery and from the posterior to the anterior globe, forming a concentration gradient. Also, VEGF was associated with the presence and severity of DME. But as the authors state in the discussion, "...while these findings demonstrate that VEGF levels in the vitreous are associated with the presence and severity of DME, they do not prove cause and effect. The role of VEGF in the production of DME can only be proven by interventional approaches, and hopefully a conclusion may be drawn when current clinical trials of anti-VEGF agents for DME are completed...".[21]

The sequence of phenomena has been tried to be verified in a study involving NPDR, PDR and full thickness macular hole (FTMH) patients (as controls). The study shows that, as disease progresses from NPDR to PDR, with capillary loss and retinal ischemia, inflammation increases, since IL-1β concentrations are almost undetectable in NPDR and controls, but raised in PDR patients. Analogously, the interleukin-1 receptor antagonist (IL-1Ra), a member of the IL-1 family that binds to IL-1 receptors but does not induce any intracellular response and prevent IL-1 mediated inflammation, was significantly higher in the control vitreous compared to the diabetic vitreous, meaning that, as disease progresses, proinflammatory cytokines remain unresponsive. Also, retinal microcirculation changes have been noted even before the onset of clinical disease, with endothelin-

1 lower in NPDR compared to PDR, reflecting the high blood flow in NPDR as compared to the lower blood flow in PDR.[22]

Comparing DME patients to ARMD patients and non-diabetic controls, and analyzing their aqueous humor, VEGF and basic fibroblast growth factor (bFGF) levels have been shown to be higher in the first group of patients, with ARMD patients not differing much from controls. These results support the greater involvement of the retina in DR, whereas in ARMD, only a small subfoveal region is affected. Also, in DME, the altered region is the intraretinal space and cytokines penetrate into the anterior chamber more easily than from the subretinal space.[23]

Elevated levels of VEGF also predict for the risk of post-operative exacerbations of ME in NPDR patients after cataract surgery, together with hypertension and IL-6 levels.[24]

Patients undergoing cataract surgery also show a post-operative rise in angiogenic factors. In the post-operative period, both VEGF and hepatocyte growth factor (HGF) (both angiogenic) increased and clinical outcomes of angiographic macular hyperfluorescence were shown, as well as clinically significant ME (CSME). This suggested that the increase in these factors (which can damage the BRB) are able to induce the clinical and angiographic changes seen 1 month after surgery.[25] As expected after a surgical procedure, IL-1β levels were also higher, which, in the opinion of the authors, may also contribute to the increase in VEGF and HGF. On top of these changes, a decrease in the anti-angiogenic pigment epithelial derived growth factor (PEDF) levels was also observed, further explaining the post-operative macular changes.[25]

The same group showed that, in NPDR patients with CSME undergoing PPV, there was an upregulation of VEGF in the vitreous, and a reciprocal decrease in PEDF, compared to FTMH. Although VEGF levels were slightly higher in NPDR patients compared to PDR, there was not a reciprocal decrease in PEDF, compared to PDR patients. Also, in the diabetic environment, the

soluble VEGF receptor (sVEGFR)-1, an anti-angiogenic growth factor, was less concentrated. The authors suggested that in PDR VEGF, though lower than in NPDR, is still capable of producing the angiogenesis observed in PDR since both sVEGFR-1 and PEDF levels are low. The full angiogenic potential in NPDR is limited by the sufficiently high levels of PEDF.[26] The authors also propose that structural and molecular optical coherence tomography (OCT) macular profiles may explain different responses to PPV in DME: when a posterior hyaloid traction is present, macular volume decreases after PPV independent of VEGF concentration, suggesting that raised TGF-β1 stimulates a fibrotic response in the posterior hyaloid providing the mechanism for generating tractional forces which cause DME. When a combined diffuse macular thickening and an elevated VEGF level is present, both decrease after PPV indicating that VEGF may be important in the etiology in this group.[26]

Similar results were later obtained by Javanmard and coworkers, who demonstrated that, although no difference was detectable in aqueous VEGF levels between NPDR patients and normoglycemic controls, sVEGFR-1 levels were significantly decreased in the test subjects versus control.[27] The ratio VEGF/sVEGFR-1 was positively correlated with FT. They suggested that the decreased chelating effects of sVEGFR-1 could allow VEGF to induce permeability, so it is the imbalance between VEGF and sVEGFR-1 that determines the fate of DME.[27]

Asato and coworkers, on the other hand, did not find any difference in sVEGFR-1 levels among different eye diseases, including idiopatic macular hole (MH), branched RVO (BRVO), central RVO (CRVO), DME and PDR patients. However, they did note that sVEGFR-1 correlated with age and that in active PDR sVEGFR-1 levels were lower compared to quiescent PDR, suggesting that this might be the reason why PDR tends to be more aggressive in youth.[28]

Anti-permeability factors have also been involved in this pathological mechanism, which does not seem to have a simple explanation. Angiopoietin-1 (ANG-1) may act as a anti-permeability factor and ANG-2 antagonizes ANG-1.[29] The predominance of ANG-2 in NPDR with CSME could promote an increased permeability combined with the elevated levels of VEGF, facilitating the BRB breakdown.[30]

Angiotensin II (AII) is yet another factor related to the increase in vascular permeability in DME, together with VEGF.[31] Vitreous concentrations of AII were increased in patients with DME compared to non-diabetic patients and VEGF was increased also compared to diabetics without retinopathy. Also, AII and VEGF correlated with each other and were higher in hyperfluorescent DME compared to hypofluorescent, hinting towards a correlation with disease severity.

Figure 2 shows a schematic representation of angiogenesis events followed by inflammation.

Studies supporting predominant role of inflammation on the pathogenesis of diabetic retinopathy

A recent study by Umazume and coworkers demonstrated that soluble CD14 (sCD14) may act as a key regulator of DME, since this mediator has been found to be elevated in DME patients compared to controls.[32] A correlation between sCD14 and interleukin-8 (IL-8) or monocytochemotactic protein-1 (MCP-1) had also been found in the vitreous fluid of patients with proliferative DR (PDR).[33] It is therefore possible that sCD14 is involved in the upregulated expression of IL-8 and MCP-1 in DME patients.[32]

Cytokines that repeatedly have been found elevated in DR/DME are interleukin-6 (IL-6) and IL-8. Sonoda and coworkers found that, in patients with type 2 diabetes mellitus and DR undergoing pars plana vitrectomy (PPV), IL-6 was the factor most significantly associated with the presence of a serous retinal detachment (SRD). They therefore suggested that VEGF cannot be the only factor responsible for the pathogenesis of DR and speculated that the presence of IL-6 increases the inflammatory reaction in the outer retina resulting in a further disruption of the external limiting membrane.[34] It is therefore not surprising that the condition of SRD responds well to corticosteroid therapy[35], but the fact that there is a strong correlation between SRD and IL-6 levels only means that inflammation facilitates retinal detachment. Furthermore, it does not necessarily mean that inflammation is involved in the pathogenesis of DME, but it may be an effect secondary to the underlying angiogenetic process.

The possibility that VEGF can promote inflammation-induced damage in DR triggered by the two most fount cytokines, IL-6 and IL-8, is supported by several studies. Koskela et al showed that the increased cytokine concentrations in the vitreous of PDR patients were due to intra-ocular changes rather than to BRB breakdown,which had been damaged by DR.[36] The origin of the vitreous

inflammatory factors IL-6 and IL-8 was the retina or other ocular tissues. A theory that emerges is that there might be a common pathway involved in the inflammation process in vitreoretinal diseases. In DME, indeed, again IL-6 and IL-8, together with MCP-1, have been suggested to be promoting vascular permeability causing the pathology, and that ischemia and VEGF further promote DME to develop into PDR.[37] Although they argue that the concentrations of inflammatory soluble factors might not necessarily reflect a pathogenic process, in this study it is strongly believed that the high correlation between the three factors indicates that common pathways are involved in the pathogenesis of various vitreoretinal disorders.[37] But how can they exclude that VEGF was not the initial promoter of inflammation following angiogenesis? Based on this study, this cannot be excluded; indeed, one of the doubts that the authors have is that a substantial amount of VEGF can be initially produced by the sudden profound retinal ischemia, which in turn induces the major three factors, i.e. IL-6, IL-8 and MCP-1, afterward.[37]

These same 3 cytokines, in addition to induced protein-10 (IP-10), were found to be elevated in a subsequent study including non-PDR (NPDR) and PDR patients.[38] The study suggested that the simultaneous measurement of several factors in the same study samples may reveal the relative contribution of each factor to the pathogenesis of DR. VEGF had a major role in PDR mediating ocular angiogenesis. But, as for many similar studies, the authors eventually concluded that further investigations are required to define the precise roles of these factors in the pathogenesis of DR and DME.[38]

Funk and coworkers observed slightly different results, finding, in the aqueous humor of DME patients at an advanced stage of disease, only MCP-1 and IL-8 significantly elevated compared to controls, and IL-6 and VEGF only slightly, but not significantly, higher. Their conclusion was that the inflammatory markers MCP-1 and IL-8 might have a role in the pathogenesis of DME.[39]

Bevacizumab therapy, in these patients, was not correlated with changes in clinical disease activity, and other growth factors or inflammatory cytokines did not change over time, explaining the negative biologic response. One major limitation of the study, though, was the small sample size (10 DME patients and 10 controls undergoing cataract surgery).[39]

A different approach was adopted by Shimura and coworkers where patients with bilateral PDR requiring PPV were treated with pan-retinal photocoagulation (PRP) in one eye and not in the other. PRP induced the worsening of macular edema (ME) and this was linked with pro-inflammatory cytokines such as IL-6 and RANTES (Regulated upon Activation Normal T-cell Expressed and Secreted), but not with VEGF and stromal derived factor-1 (SDF-1).[40] They speculated that a possible reason was that the vitreous level of VEGF in PDR had been saturated before PRP, so no increase could have been measured. Interestingly, in eyes not undergoing PRP (controls), "spontaneous" ME appeared, and therefore the status of ME in the control eyes was correlated with vitreous levels of VEGF. Another possible explanation the authors gave was that inflammatory cytokines, compared with VEGF, had a major role in the pathogenesis of DME. For sure, the pathogenesis of PRP-induced ME was likely to be different from that of "spontaneous" DME.[40]

In PDR patients who had ME, IL-6 was found to be higher compared to the same type of patients without ME.[41] An interesting result of this study was also that IL-6 was not correlated with age, duration of diabetes, vitreous hemorrhage, PRP, type of therapy, hyperglycemia or renal function. This is another study supporting a major role of IL-6 in the development of ME in DR patients. An interesting approach that the authors suggested for further studies is the determination of mRNA levels of both IL-6 and IL-6 receptors in the vitreous, which may be important in understanding the temporal association of stimuli such as hypoxia, hyperglycemia, and growth factors (e.g., VEGF) with the induction of IL-6 synthesis, as well as in analyzing molecular responses to potential anti-inflammatory treatment strategies.[41]

To our knowledge, the only study that found elevated IL-6 levels in plasma, compared to others that consistently detected this increase in the vitreous or aqueous humor in the eye, was performed by Shimizu and coworkers.[12] They observed that plasma levels of IL-6 correlated with the severity of ME, along with the presence of posterior vitreous detachment, while plasma levels of VEGF, transforming growth factor-β1 (TGF-β1), and tumor necrosis factor-α (TNF-α) did not correlate with ME.[12]

Figure 3 shows a schematic representation of inflammatory events followed by angiogenesis.

Studies supporting both angiogenesis and inflammation as causative of diabetic retinopathy

VEGF contribution to the pathogenesis of PDR has been confirmed by the "ex-adiuvantibus" results that anti-VEGF therapy is efficacious in the treatment of DR. A recent study by Costagliola and coworkers showed that, together with VEGF, also adiponectin (APN) is upregulated in PDR compared to non-diabetic controls. They also showed that the anti-VEGF molecule bevacizumab induced a decrease in both VEGF and APN, decreasing FT and improving best-corrected visual acuity (BCVA). But they argued that, since anti-VEGF treatment is not associated with total regression of retinal neovascularization secondary to PDR, it might not neutralize other inflammatory molecules involved in the cascade of the BRB breakdown, such as insulin-like growth factor-1 (IGF-1), ANG, SDF-1, bFGF-2, HGF, TNF, IL-6, erythropoietin (EPO) and Pigment epithelium-derived factor (PEDF), which are those identified as novel factors in the DR pathogenesis.[42]

Two studies published in 2012, one by Lee et al. and another by Jonas et al., and one study from 2011 by Suzuki et al., can perhaps be considered the most comprehensive analyses of inflammatory and angiogenic factors in DME.[43–45] The first study analyzed the aqueous humor of DME patients and compared it to that of BRVO-ME and of normal controls. A total of fourteen different inflammatory and angiogenic cytokines were analyzed and among these, they showed that DME, IL-8, MCP-1, platelet derived growth factor (PDGF)-AA and VEGF levels were higher and IL-13 lower compared to controls. Compared to BRVO-ME, DME patients had higher IL-6 and MCP-1 levels. IL-8 correlated positively and interferon (IFN)-γ negatively with DME severity. In BRVO-ME, IL-8 positively correlated with ME severity, as for DME, and with retinal ischemia. The authors concluded that the inflammatory reaction in DME is very active, certainly to a greater extent than in BRVO-ME. Also, the relatively gradual course of the disease could result in slow upregulation of VEGF and vascular remodeling, or a fibrotic process could continuously occur

through expression of MCP-1. The authors eloquently describe the same concerns we share: first of all, they point out that it is not appropriate to assume that a particular cytokine plays a role in pathogenesis based simply upon measurement of elevated levels in the aqueous. The particular cytokine is released as a result of the disease process, and it could not be the cause of the disease process. Second, since they did not compare those concentrations between DR or BRVO with and without ME, it seems to be difficult to consider that the cytokines which had aqueous concentrations significantly higher than those in controls, may play a role in the development of ME. Third, they could not control all possible confounding variables, such as time from onset, which can affect cytokine levels in the eye.[45]

The second study compared aqueous humor levels of 34 different molecules among cytokines in patients with diffuse DME and controls undergoing cataract surgery. On top of elevated levels of VEGF, DME eyes showed an increase in many different cytokines including epidermal growth factor (EGF), HGF, IL-1a2, IL-6, IL-8, IFN-γ-IP10, MCP-1, vascular cell adhesion molecule (VCAM), monokine induced by IFN-γ (MIG), MMP-1, MMP-9, PA inhibitor (PAI)-1, placenta growth factor (PlGF), and TGF-β, most of which were associated with retinal macula thickness (RMT). Intracellular adhesion molecule (ICAM)-1 was the cytokine most associated to DME and its severity and VEGF levels correlated with many other cytokine levels. Caution in concluding that "elevated concentrations of molecules in the eyes with DME were causally related to DR and may thus be therapeutic targets" has also been proposed by the authors. They propose that an explanation could also be given by retinal leakage due to an insufficient BRB, leakage from the ciliary body directly into the aqueous humor in the case that the concentrations of these cytokines were systemically elevated in the blood, or a local production or hyperproduction of the cytokines in the diseased retina.[44]

The third study was performed on patients with DR, with CRVO and controls with idiopatic epiretinal membrane and MH. In this study the authors presented results from the simultaneous identification of 27 different cytokines and chemokines. The elevated molecules in DME in this study were IL-1Ra, IL-6, IL-8, IL-10, IL-13, IP-10, MCP-1, macrophage inflammatory protein-1β (MIP-1β), PDGF and VEGF. So, on top of VEGF, the authors suggest that other cytokines and chemokines may be involved in the pathogenesis of DR and CRVO, and that they are correlated with VEGF levels in the vitreous. The most significantly correlated cytokines to VEGF were IL-10, IL-13 and PDGF, suggesting that not only inflammation as well as ischemia is active in the vitreous body of the retina of DR patients, but also that inflammation may activate an intrinsic defense mechanism. The logical conclusion drawn by these authors is that treatment options should simultaneously target inflammation and ischemia, according to the stage of the disease.[43]

In a study performed on bilateral DME patients and cataract surgery controls, the DME patients being treated with an intravitreal corticosteroid (triamcinolone acetonide, IVTA) in one eye and an intravitreal anti-VEGF molecule (bevacizumab, IVBe) in the other, the authors show that the pathogenesis of DME is not only related to VEGF, but many cytokines may be involved. IL-8, IP-10, MCP-1 and VEGF were significantly higher in DME patients versus controls, and IVTA significantly reduced IL-6, IP-10, MCP-1 PDGF-AA and VEGF, more than IVBe, which only reduced VEGF, but to a larger extent than IVTA. Interestingly, no significant difference in IL-6 aqueous levels was observed between the DME and control groups prior to drug administration.[46]

Another factor that might be associated with DR, and could be used as a biomarker, is soluble IL-6 receptor (sIL-6R) which was found to be elevated in the vitreous of both proliferative and pre-proliferative DR, compared to non diabetic controls. Its levels also correlated with levels of VEGF.[47]

Two studies from the same group, slightly differing from each other, showed that both VEGF and IL-6 are elevated in DME and correlated with disease severity. Sampling was performed in aqueous humor and plasma in one study [48], and in vitreous humor and plasma in the other.[49] IL-6 and VEGF correlated with each other. The suggestion therefore was that both VEGF and IL-6 are produced together in the intraocular tissues and are involved in the pathogenesis of DME, and it could be either in concert or IL-6 production via VEGF.[48,49] They actually proposed 3 possibilities: both VEGF and IL-6 may indirectly cause an increase of vascular permeability; IL-6 may indirectly cause an increase of vascular permeability via upregulation of VEGF; VEGF alone may cause vascular permeability to increase, with the elevated vitreous level of IL-6 being related to hyperglycemia and not having an influence on vascular permeability.[49]

Figure 4 shows a schematic representation of how angiogenesis and inflammation are part of a network of events ultimately leading to tissue damage.

Other studies

Besides the clinical studies performed on various types of patient with various degrees of retinopathy, in vitro studies provide useful information in order to elucidate the sequentiality of phenomena.

Cohen and coworkers showed that treatment of various cell lines with IL-6 for 6–48 h results in a significant induction of VEGF mRNA. The induced transcription is mediated by specific DNA motifs located on the putative promoter region of VEGF as well as by specific elements located in the 5'-UTR (untranslated region). IL-6 exerts its biological effects through association with specific cell surface receptors resulting in the activation of specific transcription factors that interact with two types of cis-acting DNA control elements mediating IL-6 response. So they conclude that induction of IL-6 by hypoxia may promote the expression of VEGF that eventually leads to angiogenesis.[50] They also showed that other cytokines like IFN-β and TNF-α can induce the transcription of VEGF mRNA. Being IFN-β expressed during inflammation, rheumatoid arthritis, and wound healing, the authors think it is probable that expression of IFN-β in response to these disorders might be one of the signals that triggers the angiogenic process through the induction of VEGF expression.[50]

The first direct demonstration that VEGF can increase vascular permeability in the eye at clinically relevant concentrations and activate PKC isoforms in the retina was provided by Aiello and coworkers in 1997.[51] In this study, intravitreal VEGF administration to adult rats rapidly increased retinal vascular permeability. PKC mediation of VEGF-stimulated retinal vascular permeability in vivo was supported by multiple findings, including >98% suppression of the vasopermeability response using PKC inhibitors, mimicking of the vasopermeability response using PKC agonists, and direct activation of retinal PKC activity by intravitreal injection of VEGF.[51]

Another ex vivo study was performed on primary porcine retinal pigment epithelium (RPE) cells and the human RPE cell line ARPE-19, in order to study the mechanisms responsible for VEGF-mediated changes in RPE permeability. The administration of VEGF to both cell types resulted in a 30-50% reduction in trans-epithelial resistance (TER) within 5 h of treatment, and this was only measurable following apical administration. They showed that VEGF-R2 receptors were responsible for the mediation of the VEGF effect, and that these receptors were localized to the apical surface. So they conclude that VEGF initiates RPE permeability.[52]

We have seen that ANG combined with VEGF is implicated in the increased permeability in the eye of NPRD patients with CSME.[30] Confirmation comes from an *ex vivo* study performed on porcine retinal endothelial cells (PREC). This study shows that ANG-2 and VEGF have synergistic effects on the increase of permeability, the combination of both having 5 times more inducing potential than VEGF alone, which is more potent than ANG-2 in inducing permeability. They were also able to show that the increase in permeability goes along with changes in tight junction integrity.[53]

We have already mentioned that PPV performed on NPDR patients with ME reduces ME severity.[26] In a rabbit model, PPV increased VEGF clearance by 400%, after injection of human $VEGF_{165}$ in the rabbits' eyes. The authors suggest that ME improvements after PPV could be explained by the decrease in vitreous VEGF levels.[54]

In two studies by Deissler *et al.* (2011 and 2013), immortalized bovine REC (iBREC) cells have been used.[55,56] The first study wanted to test the hypothesis of whether bFGF and IGF-1 as single factors or in combination with $VEGF_{165}$ influence permeability and tight junctions and if these effects could be restored by inhibition of VEGF. An interesting result of the study was that bFGF and IGF-1 alone did not influence cell permeability measured by TER, but they had a synergistic effect with VEGF, most likely to be caused by an enhanced secretion of VEGF. The inhibition of

VEGF by ranibizumab could completely reverse the decrease in TER. So, these results support the major contribution of VEGF to the change in permeability.[55]

The second study investigated the effects of other members of the VEGF family, such as $VEGF_{121}$, PlGF and viral VEGF-E, which activate different sets of VEGF receptors, on barrier function. They strongly supported the role of VEGF-A isoforms since even in the presence of all growth factors, TER and tight junction composition could be restored to normal in the presence of ranibizumab, which only targets $VEGF_{165}$. They also showed that VEGFR-2, probably together with NRP-1, is involved in the process of REC barrier impairment. Nevertheless the authors conclude that the involvement of other factors should not be ruled out.[56]

Conclusion

Data derived from the literature support the notion that angiogenesis and inflammation are interdependent processes that may interact synergistically to create the pathogenetic framework of DME. Pre-clinical and translational clinical studies performed by using a combination of high-throughput gene expression and proteomic technologies are needed to provide new insights into the multilevel highly-regulated signaling network involved in this type of disease.

Clinical implications

In the present Review, we suggest the presence of a molecular crosstalk between pro-angiogenic and pro-inflammatory pathways that occurs through the production of growth factors, chemokines/cytokines, proteolytic enzymes, prostaglandins, and nitric oxide. It is worth highlighting that among the different molecules investigated, VEGF may act as an angiogenic stimulator and as a pro-inflammatory mediator and it is therefore an important link between angiogenesis and the inflammatory process in this type of disease.

Based on these pieces of evidence, anti-VEGF therapies can selectively ameliorate DME symptoms and, at least partially, reverse its fundamental pathology.

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Figure legends

Figure 1. Physiopathological mechanisms of diabetic retinopathy

Figure 2. Angiogenesis events followed by inflammation

Figure 3. Inflammatory events followed by angiogenesis

Figure 4. Combined mechanisms of angiogenesis and inflammation

Table 1

Tables

Supplementary Table 1. Summary overview of studies evaluating levels of inflammation and angiogenesis mediators in diabetic retinopathy patients

Study (first name and year)	Type of patients (n)	Disease stages/severity evaluated	Molecule(s) investigated	Sampling site	Main results	Conclusions of authors
Clinical studies supporting predominant role of angiogenesis on the pathogenesis of diabetic retinopathy						
Javanmard 2012[27]	• NPDR (n=27) • Normoglycemic controls (n=33)	FT	• VEGF • sVEGFR-1	Aqueous humor	• No difference in VEGF aqueous levels between subjects and controls • NPDR had lower sVEGFR-1 vs controls • Positive correlation between VEGF/sVEGFR-1 concentration and FT	• Decreased chelating effects of sVEGFR-1 may allow VEGF to activate the proangiogenic endothelial cell state and induce permeability • Imbalance of VEGF and sVEGFR-1 may determine the fate of DME
Asato 2011[28]	• MH (n=30) • BRVO (n=37) • CRVO (n=27) • DME (n=42) • PDR (n=51) • All treated by vitrectomy	Yes	sVEGFR-1	Vitreous humor	• sVEGFR-1 not significantly different among different eye diseases • SVEGFR-1 correlated with age • In active PDR, sVEGFR-1 lower vs quiescent PDR	• High sVEGFR-1 decreases risk of angiogenesis • The increase in sVEGFR-1 concentration with age might explain why PDR in youth tends to be more aggressive
Shimada 2009[21]	• DME w/o PVD nor treated by PRP (n=71) • MH (n=10)	FT	VEGF	Vitreous humor	• VEGF higher in premacular vitreous vs peripheral cortical vitreous and mid vitreous • FT correlated with VEGF • In controls VEGF was below detection limit	• Diffusion of VEGF from macular region to periphery and from posterior to anterior globe • VEGF is associated with presence and

						severity of DME but this does not imply a cause/effect relationship
Patel 2008[22]	• NPDR (n=15) • PDR (n=5) • FTMH (n=5)	Yes	• ET-1 • Prostacyclin • NO • IL-1β • IL-1 Ra	Vitreous humor	• No difference of NO and prostacyclin in different groups • ET-1 lower in NPDR vs PDR and FTMH • ET-1 correlated with FT and macular volume in NPDR with ME • IL-1β detected in PDR • Diabetics had lower IL-1 Ra	• As disease progresses with capillary loss and retinal ischemia (PDR) inflammation increases • Retinal microcirculation undergoes changes even before the onset of clinical disease • ET-1 inversely correlates with blood flow, high in NPDR and low in PDR
Jonas 2007[23]	• ARMD (n=35) • DME (n=21) • Controls (n=24)		• VEGF • bFGF	Aqueous humor	• VEGF and bFGF higher in diabetics vs ARMD and controls • Controls and ARMD did not differ much • More marked differences for VEGF	• In DR more retina is involved and more tissue is affected • In ARMD, only a small subfoveal region is affected
Patel 2006[25]	• PDR/NPDR (n=7) undergoing uneventful phacoemulsification with intraocular lens implant (cataract surgery)	Yes	• VEGF • HGF • IL-1β • PEDF	Aqueous humor	• VEGF(165) increased from 68pg/ml to 723pg/ml 1 day after surgery and decreased to 179pg/ml at 1 month • HGF steadily increased over the month • IL-1β and PEDF had acute rise on day 1 and decreased again	• Cataract surgery causes altered concentrations of angiogenic and antiangiogenic growth factors worsening diabetic maculopathy
Patel 2006[26]	• NPDR with CSME undergoing PPV (n=20) • FTMH (n=8) • PDR (n=22)	Clinical assessment including OCT	• VEGF-A • PEDF • HGF • MMP-9 • sVEGFR-1 • TGF-β1	• Baseline vitreous humor • Baseline aqueous humor • Post operative aqueous humor	• VEGF-A higher in NPDR vs FTMH and PDR • PEDF higher in FTMH vs NPDR and PDR • PEDF in NPDR higher vs PDR • HGF, sVEGFR-1 and TGF-β1 differed in NPDR vs PDR and controls	• Upregulation of VEGF in the vitreous of diabetics with a reciprocal decrease in PEDF • Structural and molecular OCT macular profiles may explain

						varying response to PPV in diffuse CSME
Patel 2005[30]	• NPDR and CSME (n=17) • PDR (n=10) • MH (n=5) • All undergoing PPV		ANG-1 and -2	Vitreous humor	• Median ANG-1 was low in MH (17pg/ml), very high in NPDR with CSME (2002pg/ml) and 186pg/ml in PDR • Median ANG-2 was very high in NPDR with CSME (4000pg/ml) and undetectable in MH and PDR	• ANG-2 is an antagonist of ANG-1 which may act as anti-permeability factor • Predominance of ANG-2 may facilitate VEGF induced retinal vascular permeability in CSME
Funatsu 2002[24]	NPDR undergoing cataract surgery (n=104)	Postoperative exacerbation of ME	• VEGF • IL-6 • proteins	Aqueous humor	• Hypertension, VEGF, IL-6 and protein correlated with exacerbation of ME • Increase in VEGF increased ME after surgery	• VEGF predicts risk for postoperative exacerbation of ME
Funatsu 2002[31]	• DME (n=20) • Diabetics w/o retinopathy (n=6) • Non diabetics (n=14)	Severity assessed by fluorescence	• VEGF • AII	Vitreous humor	• VEGF higher in DME vs others • AII higher in DME vs non diabetics • AII correlated with VEGF • AII and VEGF higher in hyperfluorescent DME vs hypofluorescent	AII and VEGF are related to the increase of vascular permeability in DME
Clinical studies supporting predominant role of inflammation on the pathogenesis of diabetic retinopathy						
Umazume 2013[32]	• DME (n=14) • PPV non diabetic controls (n=24) • All undergoing cataract surgery	No	• sCD14 • IL-8 • IFN-IP-10 • MCP-1 • MIG • VEGF	• Vitreous humor • Aqueous humor • Serum	• All factors elevated in vitreous of DME eyes • sCD14 and VEGF in vitreous and aqueous fluids higher in DME vs controls • Vitreous and aqueous levels of sCD14 correlated in DME eyes • Vitreous sCD14 correlated with VEGF, IL-8, MCP-1, and preoperative visual acuity	sCD14 may act as a key regulator of VEGF production and have a role in DR pathology
Sonoda 2014[34]	T2DM with DR and undergoing PPV (n=52)	• SRD • retinal cystic changes • retinal swelling	• VEGF • IL-6, -8	Vitreous humor	• IL-6 associated to SRD • retinal cystic changes and retinal swellings not associated with the concentrations of intravitreal cytokines	• IL-6 can increase the inflammatory reaction in the outer retina resulting in a further disruption of the ELM • VEGF is not the only factor
Koskela 2013[36]	• PDR (n=38) • Non diabetic controls with macular hole or	All patients had advanced disease	• sE-selectin • sICAM-1, -3 • sPECAM-3	• Vitreous humor • Plasma	• IL-6 and IL-8 higher in vitreous vs plasma • Vitreous IL-10, sPECAM-1, sE-selectin, sICAM-1 and sVCAM-1 higher in PDR vs	• Local inflammation in PDR triggered by IL-6 and IL-8

	idiopathic epiretinal membrane (n=16) • All undergoing PPV		• sP-selectin • sVCAM-1 • IL-1β, -2, -4, -5, -6, -8, -10, -12p70 • TNF-α, -β • IFN-γ		controls • Adhesion molecule concentrations in vitreous in PDR were less than 10% of those in plasma • Il-10 lower in vitreous vs plasma • vitreous IL-10/IL-8 ratio was lower in PDR vs controls	• VEGF might promote inflammation-induced damage in DR triggered by IL-6 and IL-8
Oh 2010[38]	• DR (n=50) • Non diabetics (n=28)	Severity of diabetic retinopathy assessed by OCT	• IL-1β • TNF-α • MCP-1 • IP-10 • IL-6, -8 • VEGF	Aqueous humor	• MCP-1, IP-10, IL-8, and VEGF higher in DR vs non diabetics • MCP-1 and IP-10 correlated with severity of DR • IL-6 correlates with macular thickness	• Chemokines may play a role in pathogenesis of DR • VEGF mediates mainly angiogenesis in PDR
Funk 2010[39]	• DME (n=10) • cataract surgery controls (n=10)	All patients had an advanced stage of DR	• IL-4, -6, -8, -10 • ICAM-1 • IFN-γ • MCP-1 • TNF-α • EGF • FGF-2 • PDGF-AB, -BB • VEGF	Aqueous humor	• MCP-1 and IL-8 higher in DME vs controls • IL-6 and VEGF higher in DME but not significant	• MCP-1 and IL-8 might have a role in pathogenesis of DME
Yoshimura 2009[37]	• DME (n=92) • PDR (n=147) • BRVO (n=30) • CRVO (n=13) • RRD (n=63) • MH or ERM as controls (n=83)	Yes	20 soluble factors (9 cytokines, 6 chemokines, and 5 growth factors)	Vitreous humor	• IL-6, -8 and MCP-1 elevated in all groups of vitreoretinal diseases vs control • VEGF elevated in PDR and CRVO	• There may be a common pathway involved in the inflammation process in vitreoretinal diseases • IL-6, IL-8 and MCP-1 promote vascular permeability causing DME • Ischemia and VEGF further promote DME development to PDR
Shimura 2009[40]	Bilateral PDR requiring PPV (n=14)	FT	• VEGF • SDF-1 • IL-6	Vitreous humor	• IL-6 and RANTES in PRP pretreated eyes were higher vs controls • Macular thickness correlated with VEGF	• PRP induced macular edema was caused by inflammation

			• RANTES		and IL-6	• VEGF levels could have been saturated before PRP • Status of macular edema in control eyes correlated with VEGF • Inflammatory cytokines may be more responsible than VEGF in causing DME
Mocan 2006[41]	• PDR (n=8) • Non diabetics undergoing vitrectomy (n=8)	With or without ME	IL-6	Vitreous humor Serum	• IL-6 higher in PDR vs controls (vitreous) • Serum IL-6 undetectable • PDR with ME had higher IL-6 vs non ME • No correlation between IL-6 and age, duration of DM or vitreous hemorrage, PRP, type of therapy, hyperglycemia, renal function	IL-6 may have a role in PDR and is produced intraocularly
Shimizu 2002[12]	• Mild DR (n=159)	• no macular edema • focal edema • diffuse edema • cystoid edema.	• VEGF • IL-6 • TGF-β_1 • TNF-α • Lipoprotein(a) - plasma • VonWillebrand factor - serum • Thrombomodulin - serum	Plasma Serum	• Plasma IL-6 and PVD correlated with severity of ME	IL-6 in plasma and PVD can be predictors of ME
Clinical studies supporting both angiogenesis and inflammation causative of diabetic retinopathy						
Costagliola 2013[42]	• PDR with ME receiving bevacizumab (n=20) • Non diabetics undergoing cataract surgery (n=20)	BCVA and FT	• VEGF • APN	Aqueous humor	• APN and VEGF higher in PDR vs controls • After IVBe APN and VEGF decreased significantly • IVBe decreased FT and improved BCVA	• Efficacy of anti-VEGF treatment indicates that VEGF contributes to pathogenesis of PDR • anti-VEGF may not achieve neutralization of other inflammatory molecules involved in the cascade of the breakdown of BRB
Lee 2012[45]	• DME (n=18)	• CSMT	• IL-2, -5, -6, -8, -12p70, -	Aqueous	• IL-8, MCP-1, PDF-AA and VEGF higher and	• Role of inflammation

	• BRVO-ME (n=12) • Normal controls (n=16)	• TMV	13 • MCP-1 • MIP-1α • PDGF-AA • TGF-α • IFN-γ • EGF • FGF2 • VEGF	humor	IL-13 lower in DME vs control • IL-8 and VEGF higher in BRVO-ME vs control • IL-6 and MCP-1 higher in DME vs BRVO-ME • IL-8 positively and IFN-γ negatively correlated to DME severity • In BRVO-ME, IL-8 positively correlated with ME severity and retinal ischemia	in BRVO-ME less influential than in DME • Ischemic insult may be central in BRVO-ME
Jonas 2012[44]	• Diffuse DME (n=23) • Controls undergoing cataract surgery (n=22)	RMT	• TGF-α, -β • EGF • FGF-β • HGF • IFN-α, -β, -γ • IL-1a2, -1b, -2, -3, -4, -5, -6, -8, -10, -12p40, -12p70 • IFN-γ-IP10 • ICAM-1 • MCP-1, -3 • MMIF • MIG • MMP-1, -9 • PAI-1 • PlGF • PDGF-BB • SCDF-1 • TRAIL • VCAM • VEGF	Aqueous humor	• DME patients vs controls had higher EGF, HGF, ICAM-1, IL-1a2, -6, -8, IFN-γ-IP10, MCP-1, MIG, MMP-1, -9, PAI-1,PlGF, TGF-β, VCAM, VEGF • RMT was associated with concentrations of EGF, ICAM-1, IL-3, -6, -8, MCP-1, MIG, MMP-9, TGF-β, PlGF, VCAM, VEGF • VEGF correlated with PlGF, PAI-1, ICAM-1, MIG, MCP-1, VCAM, IL-6, -8, EGF, MMIF	• Many cytokines correlate with DME and its severity • ICAM-1 was the most associated • No causality can be inferred
Suzuki 2011[43]	• DR (n=76) • CRVO (n=10) • ERM/MH (n=23)	No	27 different cytokines and chemokines	Vitreous humor	• In DR, IL-6, -8, -10, -13, IP-10, MCP-1, MIP-1β, PDGF and VEGF were higher than in controls • In CRVO, IL-1β, -2, -5, -8, -9, -10, -12, -13, eotaxin, G-CSF, IFN-γ, IP-10, MCP-1, MIP-1β, TNF-α and VEGF were higher than in controls • IL-2, -9, -12, MCP-1 and IFN-γ higher in	• On top of inflammatory cytokines and neutrotrophic factors like VEGF, IL-10 and -13 may be involved in pathogenesis of DR and CRVO

					CRVO vs DR • IL-10 and -13 correlated to VEGF in DR • PDGF was inversely correlated to VEGF	• Cytokines and chemokines may be correlated to VEGF in vitreous • Inflammatory reaction may be more active in CRVO vs DR
Sohn 2011[46]	• Bilateral DME (n=11, 22 eyes) • Cataract surgery (n=6) • DME patients received triamcinolone in 1 eye and bevacizumab in the other	No	• IL-6, -8 • IP-10 • MCP-1 • PDGF-AA • VEGF	Aqueous humor	• IL-8, IP-10, MCP-1, VEGF higher in DME vs control • IVTA reduced FT more than IVBe • IVTA reduced IL-6, IP-10, MCP-1, PDFG-AA, VEGF • IVBe reduced VEGF more than IVTA	• Pathogenesis of DME not only related to VEGF • Many cytokines may be involved in DME pathogenesis
Kawashima 2007[47]	• PDR (n=28) • PPDR/DME (n=7) • MH/ERM (n=10)	Yes	• sIL-6R • VEGF	• Vitreous humor • Serum	• sIL-6R higher in PDR and PPDR vs non diabetics (vitreous) • sIL-6R higher in PDR vs control (serum) • sIL-6R correlated with VEGF in vitreous in PDR	• sIL-6R can be a biomarker of PDR
Funatsu 2003[49]	• DME w/o PVD (n=26) • Non diabetic ocular disease (n=12)	Hyperfluorescent DME (more severe)	• IL-6 • VEGF	• Vitreous humor • Plasma	• Vitreous IL-6 and VEGF higher in DME vs control • IL-6 correlated with VEGF • IL-6 and VEGF higher in hyperfluorescent DME vs minimally fluorescent DME	IL-6 together and/or via VEGF may promote an increase of vascular permeability in DME subjects w/o PVD
Funatsu 2002[48]	• DME (n=54)	ETDRS scale	• VEGF • IL-6	• Aqueous humor • Plasma	• Aqueous VEGF and IL-6 correlated with severity of DME and with aqueous protein concentration • Aqueous levels of VEGF and IL-6 higher than plasma levels • VEGF levels correlated with IL-6 levels • Status of the posterior vitreous correlated with severity of ME	Both VEGF and IL-6 are produced together in the intraocular tissues and are involved in the pathogenesis of ME

AII, angiotensin II; **ANG**, angiopoietin; **APN**, adiponectin; **ARMD**, age related macular degeneration; **b**, basic**; BCVA,** best-corrected visual acuity; **BRB**, blood retinal barrier; **BRVO**, branch retinal vein occlusion; **CRVO**, central retinal vein occlusion; **CSME**, clinically significant macular edema; **CSMT**, central subfield macular thickness; **DM**, diabetes mellitus; **DME**, diabetic macular edema; **DR**, diabetic retinopathy; **EGF**, epidermal growth factor; **ELM**; external limiting membrane; **ERM**, idiopatic epiretinal membrane; **ET**, endothelin; **ETDRS**, Early Treatment Diabetic Retinopathy Study; **FGF**, fibroblast growth factor; **FT**, foveal

thickness; **FTMH**, full thickness macular hole; **G-CSF**, granulocyte colony-stimulating factor; **HGF**, human growth factor; **ICAM**, intercellular adhesion molecule; **IFN**, interferon; **IL**, interleukin; **IP**, induced protein; **IVBe**, intravitreal bevacizumab; **IVTA**, intravitreal triamcinolone acetonide; **MCP**, monocytochemotactic protein; **ME**, macular edema; **MH**, idiopatic macular hole; **MIG**, monokine induced by IFN-γ; **MIP**, macrophage inflammatory protein; **MMIF**, macrophage migration inhibitory factor; **MMP**, matrix metalloproteinase; **n**, number of subjects; **NO**, nitric oxide; **NPDR**, non proliferative diabetic retinopathy; **OCT**, optical coherence tomography; **PAI**, plasminogen activator inhibitor; **PDGF**, platelet-derived growth factor; **PDR**, proliferative diabetic retinopathy; **PECAM**, platelet-endothelial cell adhesion molecule; **PEDF**, pigment epithelial derived growth factor; **PlGF**, placenta growth factor; **PPDR**, pre-proliferative diabetic retinopathy; **PPV**, pars plana vitrectomy; **s**, soluble; **PRP**, pan-retinal photocoagulation; **PVD**, posterior vitreous detachment; **R**, receptor; **Ra**, receptor antagonist; **RANTES**, regulated upon activation normal T-cell expressed and secreted; **RMT**, retinal macula thickness; **RRD**, rhegmatogenous retinal detachment; **s**, soluble; **SCDF**, stromal cell-derived factor; **SDF**, stromal derived factor; **SRD**, serous retinal detachments; **T2DM**, type 2 diabetes mellitus; **TGF**, transforming growth factor; **TMV**, total macular volume; **TNF**, tumor necrosis factor; **TRAIL**, TNF-α-related apoptosis inducing ligand; **VCAM**, vascular cell adhesion molecule; **VEGF**, vascular endothelial growth factor; **vs**, versus; **w/o**, without.

Figure 1

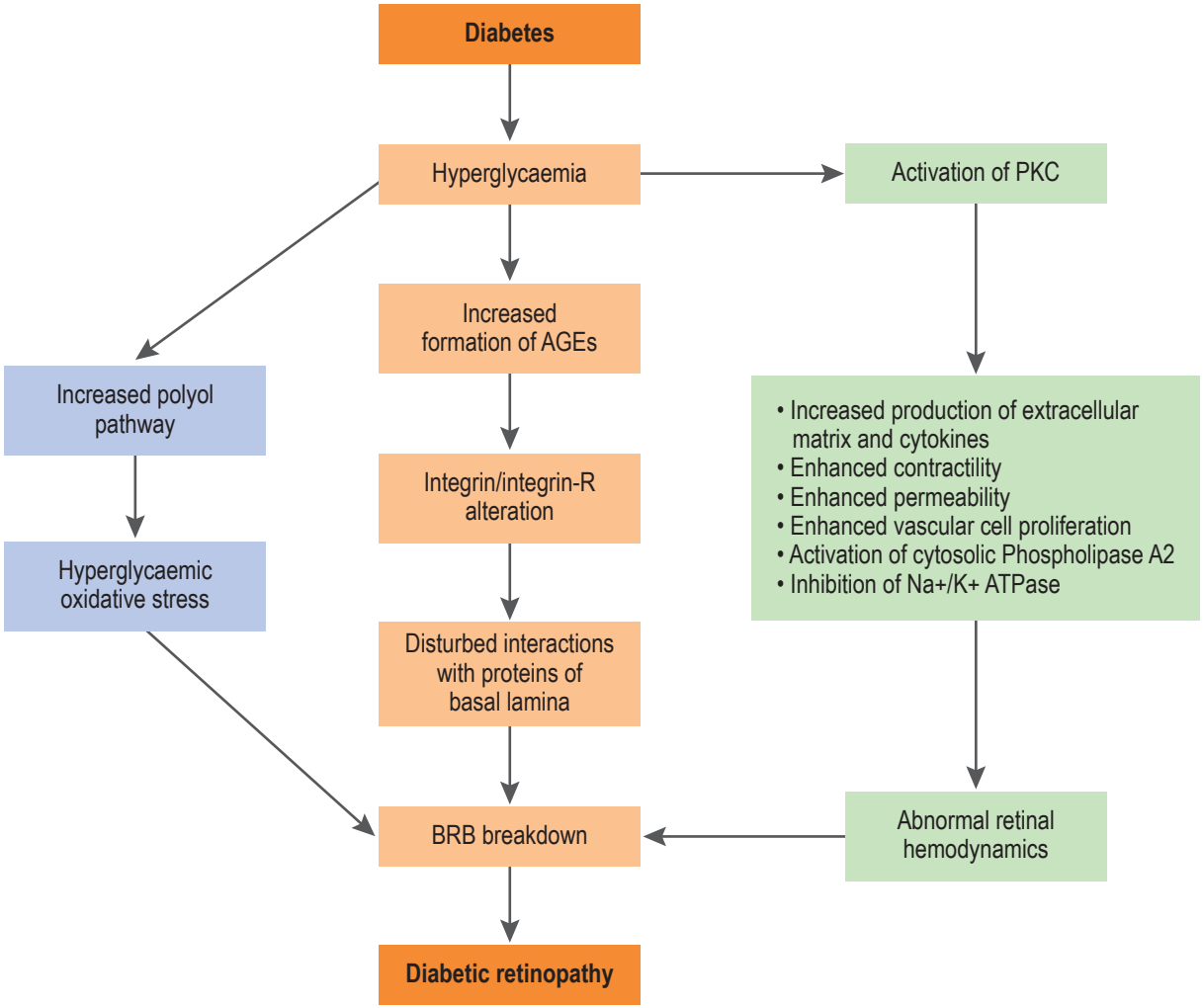
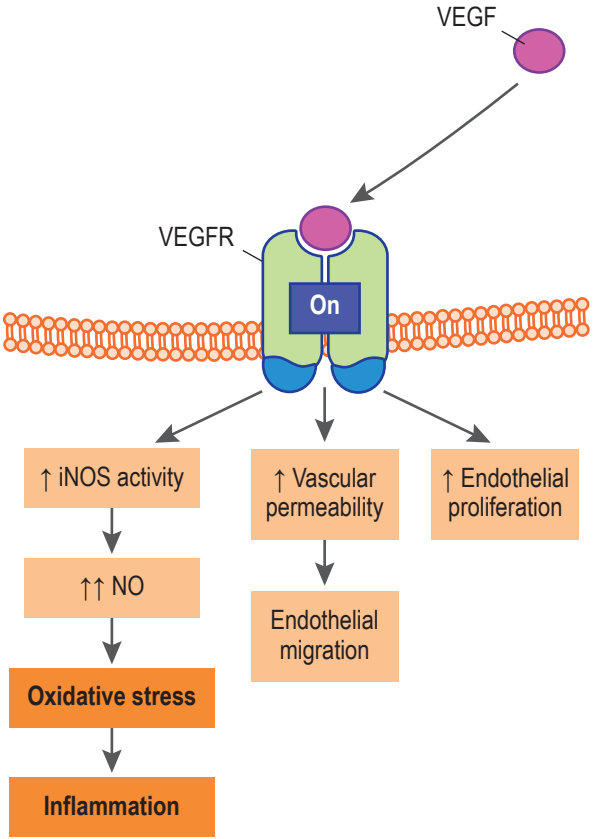

Diabetes
Hyperglycaemia
Activation of PKC
Increased formation of AGEs
Increased polyol pathway
• Increased production of extracellular matrix and cytokines
• Enhanced contractility
• Enhanced permeability
• Enhanced vascular cell proliferation
• Activation of cytosolic Phospholipase A2
• Inhibition of Na+/K+ ATPase
Integrin/integrin-R alteration
Hyperglycaemic oxidative stress
Disturbed interactions with proteins of basal lamina
BRB breakdown
Abnormal retinal hemodynamics
Diabetic retinopathy

Figure 2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Oxidative stress
Inflammation
↑ Vascular permeability
Endothelial migration
↑ Endothelial proliferation

Figure 3

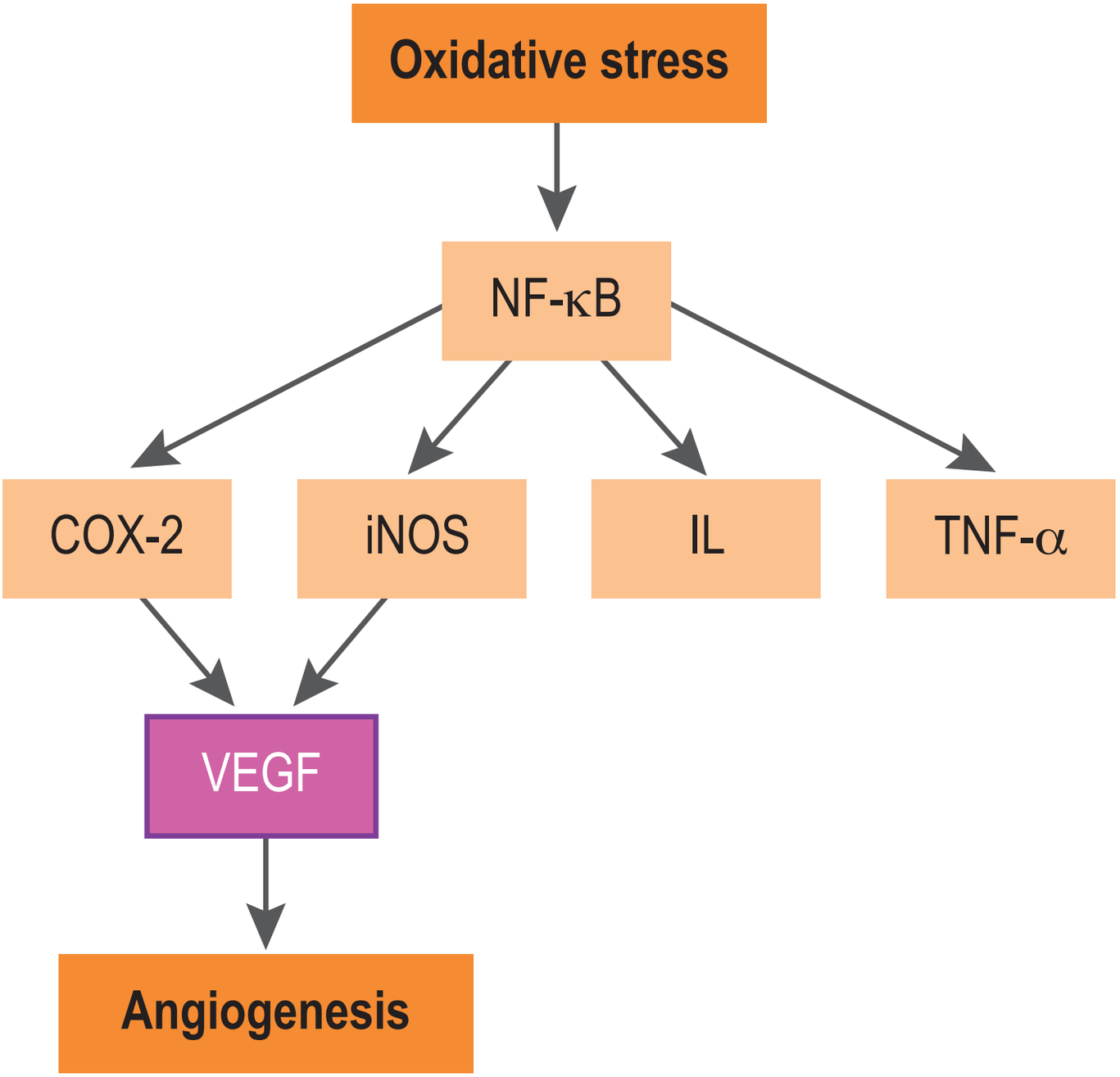
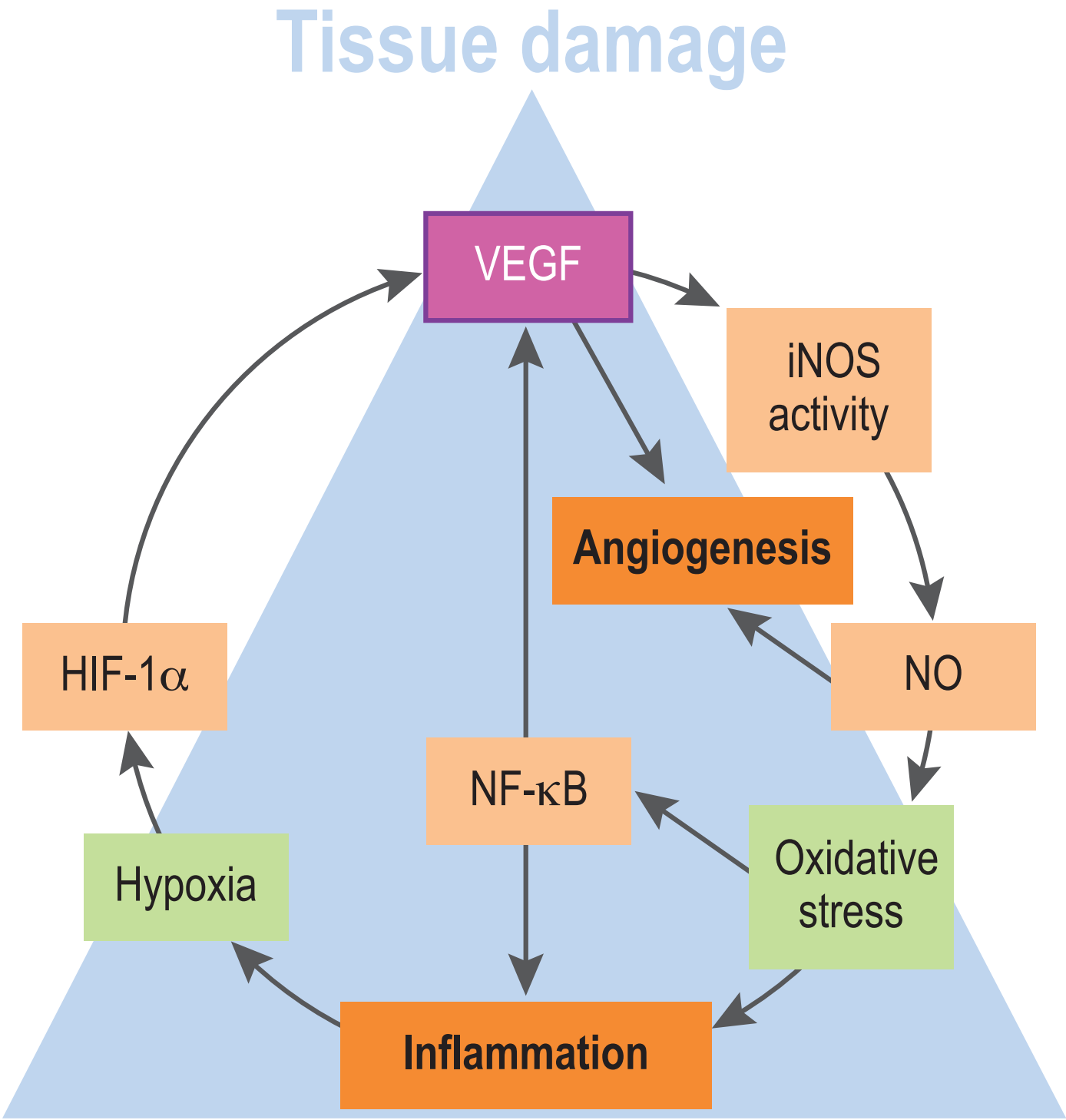


Figure 4


Tissue damage
VEGF
iNOS activity
Angiogenesis
HIF-1α
NO
NF-κB
Hypoxia
Oxidative stress
Inflammation

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(L. R. T. 24 febbraio 2005, n. 40)

Comitato etico di Area Vasta Nord-Ovest per la Sperimentazione Clinica

Regione 090 - Azienda 901

Pisa, 12/6/2014

To whom it may concern,

The Ethics Committee for Clinical Trials does not require submission for approval of review manuscripts, provided they do not report on original, unpublished research data on humans.

Romano Danesi
Chairman, Ethics Committee

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