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Review article

The importance of glial cells in the homeostasis of the retinal microenvironment and their pivotal role in the course of diabetic retinopathy

Francesco Saverio Sorrentino [a,*], Michael Allkabes [b], Giulia Salsini [b], Claudio Bonifazzi [c], Paolo Perri [b]

[a] Department of Surgical Sciences, Unit of Ophthalmology, Ospedale Maggiore, Bologna, Italy
[b] Department of Biomedical and Surgical Sciences, Division of Ophthalmology, University of Ferrara, Ferrara, Italy
[c] Department of Biomedical and Surgical Sciences, Section of Human Physiology, University of Ferrara, Ferrara, Italy

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ABSTRACT

Diabetic retinopathy (DR) is a remarkable microvascular complication of diabetes and it has been considered the leading cause of legal blindness in working-age adults in the world. Several overlapping and interrelated molecular pathways are involved in the development of this disease. DR is staged into different levels of severity, from the nonproliferative to the advanced proliferative form.

Over the years the progression of DR evolves through a series of changes involving distinct types of specialized cells: neural, vascular and glial. Prior to the clinically observable vascular complications, hyperglycemia and inflammation affect retinal glial cells which undergo a wide range of structural and functional alterations. In this review, we provide an overview of the status of macroglia and microglia in the course of DR, trying to briefly take into account the complex biochemical mechanisms that affect the intimate relationship among neuroretina, vessels and glial cells.

Contents

1. Introduction

Diabetic retinopathy (DR) is the primary cause of visual impairment in the working-age population of the Western world as a recent report has indicated [1]. Among microvascular complications related to diabetes mellitus such as nephropathy and neuropathy, DR is the most common. Several complex inflammatory mechanisms are involved in the pathogenesis of DR. A systemic low/high grade of inflammation mediates structural and molecular changes in the neuro-vascular network. Still too little is known about the subtle mechanisms underlying the inflammatory pathways associated with DR [2]. However, the following four distinct biochemical pathways have been assumed to be associated with the development of DR: increasing polyol pathway flux, increasing hexosamine pathway flux, accumulation of advanced glycation end products (AGEs), and activation of protein kinase C isoforms [3]. As a consequence of the above-mentioned molecular dysregulation, several biochemical mechanisms are activated to counterbalance the abnormalities of the microenvironment of diabetic tissues. The oxidative stress as well as the production of free radicals in mitochondria markedly increase. Plus, the abnormal rheology and the activation of the renin-angiotensin system contribute to upregulate the release of inflammatory

* Corresponding author at: Via Tagliapietre, 10, Bologna, 40123, Italy.
E-mail address: dr.fsorrentino@gmail.com (F.S. Sorrentino).

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molecules and growth factors [2–4]. As a result, the vascular wall integrity diminishes, the vascular permeability goes up, and the increasing leukostasis is responsible for the lumen occlusion and the resulting ischemia [5,6].

The constriction of major arteries and arterioles observed in DR diminishes the retinal blood supply provoking several biochemical and metabolic alterations. The loss of capillary pericytes causes endothelial cell degeneration and unstable retinal perfusion [7]. The resulting retinal ischemia and hypoxia are strong stimulus to enhance the expression of VEGF (vascular endothelial growth factor) and other proinflammatory cytokines (tumor necrosis factor alpha - TNF-α, interleukin 6 - IL-6, and interleukin 1β – IL-1β) [8,9]. In addition, hyperglycemia can stimulate the production of VEGF and some cytokines implicated in insulin resistance such as TNF-α and IL-6 [10]. Ischemia also provokes the expression of chemokines such as monocyte chemotactic protein-1 (MCP-1) that acts making macrophages to be attracted into less perfused areas. Hypoxia-activated macrophages and microglia produce TNF-α, which in turn stimulates the release of IL-6, MCP-1, and VEGF in endothelial or in retinal microglial cells [11].

The human retina works through fine-tuning interactions among neurons, glia and blood vessels. As described by Cajal more than one hundred years ago, three main types of glial cells are found in the retina: astrocytes, Müller cells and resident microglia. They not only provide structural support, but they are also involved in maintaining the complex homeostasis of the retina by regulating the metabolism, the phagocytosis of neuronal debris, and the release of neurotransmitters and trophic factors [12] (Table 1).

Neuroinflammation is one of the aspect that plays a remarkable role in the pathogenesis of DR [13]. A recent report has proposed the phrase "microglial activation" to better describe the microenvironment where microglia cells become activated and start to produce proinflammatory mediators in response to retinal nervous tissue perturbations [14]. The diabetic retina always presents a low chronic level of inflammation due to different factors such as hyperglycemia, dyslipidemia and oxidative stress [15,16]. This particular context induces the activation of the retinal resident innate immune system, which is primarily composed of tissue-resident macrophage-like cells called microglia [17]. Since microglial cells are supposedly hypersensitive to early tissue damage, they generally give birth to neuroinflammation. Afterwards, other glial cells such as astrocytes are activated and amplify the inflammatory response [18]. As Graeber and coauthors claimed, microglial activation is the main mechanism by which neuroinflammation is induced in response to nervous tissue perturbations [14].

DR is characterized by pathologic microvascular abnormalities, including dilated veins, microaneurysms, hemorrhages, cotton-wool spots, as well as vitreoretinal neovascularization. Many studies have highlighted a large prevalence of cells staining with monocytic markers near the retinal vessels [19,20]. Monocytes may originate from the retina (microglia), the vitreous (hyalocytes), or the circulation (macrophages). These cells might play an important role in the origin and development of DR. Reacting microglia cells are usually located in the periphery of leaky vessels in order to try to clear away hemorrhage or exudates deposits. When the phagocytic clearance of extravasated plasma proteins no longer suffices, neuroinflammation rapidly increases. Furthermore, activated glial cells contribute to expand the inflammatory response featured by diapedesis through activated endothelial cells and leukostasis in the vascular lumen [19].

With this review, we would like to overview the dysfunction of retinal glial cells in the course of DR, where glial cells play a critical role along with neuronal sufferance and vascular abnormalities (Table 2).

2. Astrocytes

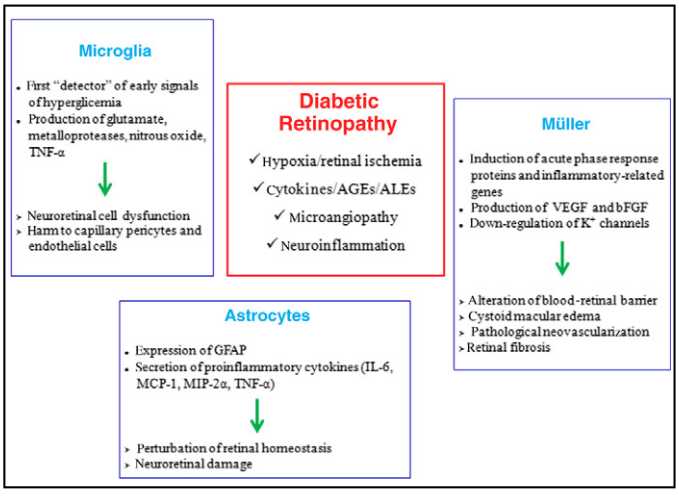
Derived from the Greek, the term astrocyte was first used in 1893 by Michael von Lenhossek to describe the stellate morphology of those cells which have been previously observed by Camillo Golgi in the nervous system and regarded as the "glue" of the brain [21]. Astrocytes do not originate from the retinal embryonic epithelium but seem to originate in the optic nerve head. It is believed that the mitotic precursors of the oligodendrocytes and astrocytes migrate into the optic nerve from the sub-ependymal germinal layer of the brain [22]. After local mitoses in the optic nerve, astrocytes enter the retina along with the blood vessels migrating to the innermost retinal layers [23,24]. Thus, they are almost exclusively located in the inner nuclear and plexiform layers and their processes cover the blood vessels forming the inner retinal blood barrier. Astrocytes appear to play a pivotal role in ion homeostasis, in neuronal signaling and in making up retinal endothelial barrier properties [25].

The distribution of retinal astrocytes is strikingly correlated with the presence and distribution of retina blood vessels, so vascularized areas of the retina are rich in astrocytes, while avascular zones contain no astrocytes. This peculiar distribution might confirm that this population of macroglia enters the retina along with its vasculature. During both normal and pathological vessel formation, astrocytes are the main producers of VEGF [26,27]. In different circumstances, the role of astroglial cells is of paramount importance because of their strong relationship with the retinal vasculature. Astrocytes provide neurotrophic and mechanical support for both healthy and degenerating axons, but their main activity is the maintenance of the inner blood-retina barrier. In response to injury or disease, they are able to upregulate the expression of various genes encoding cytokines, chemokines and elements of the complement cascade, compromising the integrity of the blood-retina barrier and promoting retinal degeneration [28].

Table 1
Physiological role of glial cells in the human retina.

Physiological role of retinal glial cells	**Astrocytes** ▪ Ion homeostasis ▪ Neuronal signaling ▪ Making the inner blood retinal barrier
	Müller ▪ Supporting neuroretinal architecture ▪ Main nutritional support of the retina ▪ Redistributing the metabolic waste of the photoreceptor neural activity
	Microglia ▪ Resident inflammatory cells ▪ Neurogenesis and synaptic pruning

Table 2
Activation of glial cells in the course of diabetic retinopathy.



In both forms of DR, proliferative and non-proliferative, dysregulated metabolites (glucose, amino acids, lipids, amines, vitamins, minerals) disturb the homeostasis of growth factors, neurotrophic factors, cytokines and adhesion molecules, consequently affecting capillary permeability and cell turnover [29]. Consistent high blood glucose, AGEs accumulation, dyslipemia and the complex inflammatory systemic status lead to the rapid rise of reactive oxygen species. As a result, the retinal microvasculature and the glial cells are affected and, somehow, damaged [30,31]. Astrocytes react becoming activated [32,33] and undergo a series of changes: proliferation, migration, hypertrophy, glial fibrillary acid protein (GFAP) expression, secretion of pro-inflammatory signals such as IL-6, MCP-1 and macrophage inflammatory protein 2 alpha (MIP-2α). Nagayach and coworkers carried out several experiments on developing diabetes in rats. They have seen phenotypic changes of astrocytes characterized by a stellate morphology due to elevated level of S100β. In the meantime, they have observed an increase in the number of these macroglial cells along with the overexpression of GFAP [34].

Astrocytes try to face the insult of the disease and they need to cooperate with other glial cells [35]. High glucose blood levels alter inflammatory cytokine expression of astroglial cells, stimulating the TNF-α/TNFR signaling, activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and increasing the oxidative stress [36]. Therefore, the natural history of DR is characterized by high level of signaling among retinal neurons, endothelial cells and astrocytes with subsequent alteration of the retinal homeostasis and the upcoming death of neuroretinal cells.

3. Müller cells

Müller cells are a subset of the retinal macroglia. They form the supporting architectural structure radially stretching across the entire thickness of the neuroretina and take part in making the outer and inner limiting membrane, which are respectively the limits of the neuroretina. Müller cells can be regarded as the core of a columnar 'micro-unit' in the neurosensory retina since they constitute the anatomical link between retinal neurons, blood vessels and vitreous body, where nourishing exchanges of molecules and oxygen occur. Müller cells establish a strong relationship with the large retinal blood vessels by means of their little conical terminal buttons featured by no specialized junctions forming the inner limiting membrane on the innermost surface of the retina. Also, Müller cells strongly link with the retinal pigment epithelium, establishing connections with the subretinal space and choroidal vasculature [37].

To carry out their noteworthy tasks, Müller cells are endowed with several ion channels, ligand receptors, transmembraneous transporters, and numerous enzymes. Müller are supposed to be the main nutritional supporting cells of the retina. They have an elevated rate of glycolysis and present, under normal conditions, the transporter isoform glucose transporter-1 (GLUT1), typically expressed by tissues with a barrier function. For instance, in the neovascular tissue of proliferative diabetic retinopathy the lack of expression of this glucose transporter means the loss of selective permeability of retinal capillary network [38,39].

Photoreceptors have the highest rate of oxidative metabolism in respect to all other cells in the body, so they most require oxygen and glucose. Glucose metabolism generates a lot of carbon dioxide and water. Thus, another important function of Müller cells is to redistribute, through their long processes, the metabolic waste (not only carbon dioxide and water, but also potassium and neurotrasmitters released) of the intense neural activity of photoreceptors and the other neuronal cells into the blood and vitreous [40]. So this important subset of macroglia are not only essential for structural reasons but also for the role they have in the complex molecular network of activation/response of the sensory neuroretina under stressful conditions as it occurs in traumas or diseases characterized by the cascade of inflammation [41]. In the early diabetes with just a mild elevated blood glucose concentration, the leakage of retinal vessels and the accumulation of leukocytes in retinal capillaries contribute to the activation of Müller cells which play a key role in the development of neovascularization and fibrosis during the late stages of DR [42].

Hyperglycemia increases insulin-like growth factor 1 and hypoxia-inducible factor-1 alpha in both serum and vitreous, contributing to stir up hypoxia and inflammation locally and systemically. The upregulation of these factors activates Müller cells and raises the production of VEGF and basic fibroblast growth factor, which in turn trigger pathological neovascularization and retinal fibrosis [43]. The combined epiphenomena of new abnormal blood vessel growth and epiretinal fibrosis bring about tractional forces on the surface of the retina, leading to retinal detachment with subsequent severe vision loss.

In diabetic eyes, there is also the upregulation of heme oxygenase-1 that is a well-known oxidative stress marker supposedly involved in the formation of advanced lipoxidation end-products and AGEs, both remarkably involved in macroglial dysfunction [44].

Actually, in DR Müller cells acquire a complex and specific reactive phenotype characterized by the induction of acute-phase response proteins and other inflammation-related genes [45]. Beyond the overexpression of interleukin 1 as the possible main mediator of local inflammation, recent studies have investigated the upregulation of the following genes in DR: alpha-2 macroglobulin, angiotensinogen, ceruloplasmin, complement components C1, C3, C2, and C1 inhibitor, lipocalin 2, metallothionein, serine protease inhibitor 2/antichymotrypsin, transferring, tissue inhibitor of metalloprotease 1, transcription factor C/EBPδ [46]. Other investigations studied several molecules involved in the inflammatory response, such as major histocompatibility complex, intercellular adhesion molecule-1, nuclear factor kappa-light-chain-enhancer of activated B cells, osteopontin, scavenger receptor B1, galectin-3, and annexin 1 [47].

The intimate connection between macroglia and retinal blood vessels is definitely affected during the natural history of DR and the development of late complications. A large number of neurotrophic factors are produced from both Müller and endothelial cells contributing to the alteration of the integrity of the blood-retina barrier. Some factors (glial cell line-derived neurotrophic factor, neurturin, pigment epithelium-derived growth factor) increase the tightness of this efficient barrier, whereas others (TNF-α, VEGF) make it weaker and leaky [48,49].

In proliferative DR Müller cell are deeply involved in the process of retinal fibrosis. They strongly react to the chronic inflammatory stimulus with gliosis, that is a non-specific reactive answer of glial cells in response to damage and involves the production of a dense fibrous network underneath or inside the neuroretina [50]. Gliotic Müller changes also comprise a sort of downregulation of the K^+ channels since the K^+ conductance remarkably decreases in the neuroretinal tissue in the course of diabetes [51].

Finally, in DR Müller cells, characterized by their unique geographic arrangement spanning the whole thickness of retina, play an active role in inducing chronic inflammation, neovascularization and vascular leakage. In the meantime, part of these complex cells go through apoptosis after the activation of specific signaling biochemical cascades [48,52]. Also, it has been suggested that the cysts featuring the cystoid macular edema are formed by swollen and dying Müller cells, which undergo several morphological and functional changes because of hypoxia and breakdown of the blood-retinal barrier [53].

4. Microglia

Microglia are the resident inflammatory cells of the CNS (central nervous system) and they are regarded as the innate neural immune system. Like dendritic cells, they have a small round cell body with many branching projections or processes. In the adult retina, under normal conditions, microglia cells are localized in both inner and outer plexiform layer. During the early childhood these cells play a crucial role for neurogenesis and synaptic pruning [54,55], but they also take

on great importance during the development of the retina. At any time the communication among microglia, Müller cells and neurons is fine-tuned due to a delicate balance between excitatory and inhibitory neurotransmission. The survival or apoptosis of photoreceptors are finely regulated by a molecular network made up of several neurotrophic factors (brain-derived neurotrophic factor, ciliary neurotrophic factor, glial cell-derived neurotrophic factor, nerve growth factor, neurotrophin-3 and basic fibroblast growth factor) which are consistently released into the retina. Therefore, the final role of microglia is not only to monitor the retinal microenvironment but also to respond to potential abnormalities maintaining the tissue homeostasis and modulating the inflammatory processes. Microglia are equipped with an array of molecular pattern recognition receptors and scavenger receptors that enable them to recognize and react to the presence of "nonself," such as pathogens, and to "altered self," such as damaged and/or apoptotic neurons [56].

Microglia cells, approximately 5% to 12% of cells of CNS, have been first described by Pio del Rio-Hortega in 1932 [57]. They origin from mesoderma/mesenchima deriving from myeloid progenitors that migrate from periphery during the late embryogenic and postnatal life [58–60]. They are bone marrow-derived mononuclear phagocytes. For long they have been considered similar to peripheral macrophages, but recent profile gene expression studies have detected a unique molecular signature [61,62]. Several studies have shown that microglia cells survived by continuously extending and retracting their highly motile processes in order to promptly react to any subtle or big alteration of their microenvironment [63,64]. These cells can act as either neuroprotective or toxic elements [65–67]. In response to neuroinflammation or any neural damage, the activation of microglia is featured by proliferative, morphological, immunoreactive and migratory changes [68,69]. In non proliferative DR, perivascular microglia settled into the retinal plexiform layers are moderately hypertrophic and slightly increased in number. In proliferative DR, cluster of microglial cells surround ischemic areas, whereas a significant rise in their number with enhanced level of ionized calcium binding adaptor molecule 1 has been observed around new dilated vessels [70]. Like macrophages in the rest of the body, microglia use phagocytic and cytotoxic mechanisms to destroy foreign materials. Furthermore, as peripheral macrophages are able to present distinct phenotypes in different situation, microglia cells can convert from their surveilling phenotype into a spectrum of alternative activation states, depending on the type and extent of tissue dysfunction, damage, or infection [71].

Concerning the morphological appearance, microglia cells are distinguished in "surveillant" phenotype with highly ramified units and "activated" (or ameboid) state with larger cell bodies and thicker processes. The activated phenotype represents the first reply to threatens against the CNS such as autoimmune inflammation, neuronal injury, cancer, infection and hyperglycemia [72].

During the average development of CNS, microglia are first characterized by an ameboid outfit and then change to the mature highly ramified form. Microglia may be activated in acute inflammation or infections to protect the CNS, but they can also have a remarkable role with deleterious effects on the CNS during chronic inflammation in diseases such as HIV, multiple sclerosis, Alzheimer and diabetes [73].

The most reliable characteristics differentiating microglia from invading monocytes are a low cluster of differentiation 45 (CD45) expression and a highly ramified, dendritic morphology. Nevertheless, the activated phenotype can sometimes have high expression of CD45 being less ramified at the same time [74]. In addition, circulating macrophages may infiltrate damaged nervous tissue, adopting a microglial phenotype and replacing resident microglial populations [75,76]. However, further studies are needed to differentiate the activation and proliferation of resident microglia from the infiltration of the retina by circulating macrophages and conversion into microglial cells. Yang and coauthors defined the DR as a chronic low-inflammatory disease of the retina. The activation of microglia is due to the occurrence of the following vicious circle triggered by the strong stimulus of chronic hyperglycemia: recruitment of leukocytes, vascular breakdown, release of cytotoxic substances, glial dysfunction and neuronal cell death [77].

To date little is known about the exact way of activation of microglial cells in the course of diabetes. Normally standing in the capillary wall or in the inner retina, microglia are assumed to be the first detector of early signals of hyperglycemia. The chronic low-inflammatory state in DR causes an increase of the capillary permeability with a subsequent remarkable rise of cytokines, advanced glycation end-products and oxidative stress. As a result, activated microglia start to produce glutamate, metalloproteases, and nitrous oxide. All of these molecules are extremely toxic to retinal ganglion cells, provoking neuronal cell dysfunction and deeply damaging capillary pericytes and endothelial cells. These cells, partially injured, contribute to maintain the chronic inflammatory state of the diabetic retina by expressing several molecules such as intercellular adhesion molecule 1, vascular cell adhesion protein 1 and cyclooxygenase-2.

At early stages of DR, perivascular microglial cells moderately grow in number and gradually become hypertrophic in the innermost retinal layers. In the pre-proliferative form, hypertrophic microglia pool surrounding cotton-wool spots and then migrate into the optic nerve region. In the proliferative DR, several microglial cells have been detected around dilated new vessels [78]. Although the precise mechanisms of glial activation are not yet fully understood, it can be said that both genotypic variations and genetic susceptibility might regulate individual microglial response in the course of DR. However, some molecular pathways have been described. For instance, there has been observed accumulation of Amadori-glycated albumin, an AGE product which stimulates microglia to produce TNF-α in early stages of DR [79]. Plus, some specific enzymes are activated during the inflammatory cascades featuring diabetes. For example, aldose reductase, an enzyme that catalyzes the synthesis of polyols, is activated already during the early stages of DR inducing the activation of retinal microglia [80]. It is interesting to see that the inhibition of this enzyme or its genetic deficiency significantly reduce the inflammatory changes, ameliorating the degeneration of capillaries and diminishing the production of superoxide anions and other reactive oxygen species found in DR [81,82].

5. Conclusion

Many studies carried out in diabetic patients and diabetic animal models have shown that tissue hypoxia and immuno-dysregulation might provoke the growing expression of intravitreal inflammatory molecules, such as cytokines, chemokines, and other growing factors responsible for the development of DR.

Glial cells are critically located between vasculature and neurons of the retina, having a key role in finely regulating the molecular composition of the retinal microenvironment which is often responsible for early harm of neuroretina in the beginning stages of DR.

As research carries on, there is evidence that neuroinflammation and neurodegeneration play a significant role in the pathophysiology of early DR. Thus, changes in the metabolism of glia cells and subsequent damages of retinal neurons contribute to increase the microvascular impairment. New technologies able to detect early alterations in astrocytes and Müller cells or changes in the amount of microglial cells might be an interesting support for interventions in advance just trying to slow down the progression of DR. We believe that neuroprotection is a valid option to prevent chronic neurodegeneration, but supplements acting on vessel walls and improving the microvascular blood flow are worth to be administered in early phases of DR. In fact, alterations in the neurovascular coupling, that is the strong physiological link between neurosensory retina and retinal vasculature, are crucial in the course of DR. Glial cells are the main actors that help maintain this coupling. In diabetes, there is a progressive harm in both macroglia and microglia, resulting in neuroretinal damage and visual impairment. The

improvement of microcirculation in both retina and choroid could be an important therapeutic target if associated to neuroprotection.

Further studies are needed to better understand the deepest links and the complex molecular networks underlying the background of ocular inflammation in patients with DR.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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The authors alone are responsible for the content and writing of this paper.

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