Microglial Activation in Human Diabetic Retinopathy

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Objective: To investigate microglial activation in human diabetic retinopathy.

Methods: Paraffin sections from 21 eyes of 13 patients with diabetic background, preproliferative, or proliferative retinopathies and 10 normal eyes of 9 individuals were studied with immunolabeling of microglia with antibodies against HLA-DR antigen, CD45, or CD68.

Results: In the healthy human eyes, ramified microglial cells were scattered in the inner retinal layers. In eyes with diabetic retinopathy, the microglia were markedly increased in number and were hypertrophic at different stages of the disease. These cells clustered around the retinal vasculature, especially the dilated veins, microaneurysms, intraretinal hemorrhages, cotton-wool spots, optic nerve, and retinal and vitreal neovascularization. In some retinas with cystoid macular edema, microglia infiltrated the outer retina and subretinal space. Cells in the epiretinal membrane were also labeled with microglial markers.

Conclusions: Microglia were activated at different stages of human diabetic retinopathy and optic neuropathy. Microglial perivasculitis was a prominent feature of the disease process.

Clinical Relevance: Activated microglia and microglial perivasculitis may play a role in vasculopathy and neuropathy in diabetic retinopathy.


Although diabetic retinopathy is a common complication of diabetes mellitus and is a leading cause of adult blindness, the pathogenic process of the retinopathy has not been definitively determined. In previous decades, the investigation was focused on the retinal vasculopathy, initiating breakdown of the blood-retinal barrier, followed by loss of pericytes and development of microaneurysms, exudative retinopathy, and proliferative neovascularization. In more recent years, diabetic neuropathy, affecting neuronal and glial functions of the retina, was studied. However, the relationship between vasculopathy and neuropathy has not been clearly defined.

Retinal glial cells, including macroglia (Müller cells and astrocytes) and microglia, are considered channels of communication between retinal blood vessels and neurons owing to their special spatial arrangement and regulatory functions. Several studies showed that macroglia underwent prominent changes in early diabetic retinopathy with an increase in the Müller cell population and glial fibrillary acidic protein expression and a decrease in the astrocytic population, which might be related to the increased vascular permeability in diabetes. Similarly, the microglial cells are also highly dynamic and capable of assuming different morphologic and functional changes in response to the alterations of the local physiological and cellular environment. In addition to the changes in the cellular morphology, cell number, cell mobility, and up-regulation of surface molecules, the activated microglia have the potential of producing cytotoxic substances, such as cytokine tumor necrosis factor α (TNF-α), reactive oxidative species, proteases, and excitatory amino acids, which may induce neuronal degeneration. Activation of microglia is rapid and often precedes changes in the macroglia. In 3 recent studies of early diabetic retinopathy in animal models, microglial activation was reported, showing that both the number and the activity of microglial cells were increased, as reflected by the increased expression of macrophage marker OX42 or Iba1.

Although the pathogenesis of diabetic retinopathy is not completely defined, accumulating evidence suggests that diabetic retinopathy exhibits many features of chronic inflammation, such as increased nitric oxide production, intracel-
ular adhesion molecule-1 up-regulation, leukostasis, and release of proinflammatory cytokines TNF-α, IL-1β (interleukin 1β), and IL-6.5-7 The microglia are considered one of the principal cells sensing these stimuli and releasing proinflammatory and neurotoxic substances on activation. This was confirmed by a recent study by Krady et al.,5 who suggested a central role of microglia in the inflammatory response in the retinas of diabetic rodents. They also demonstrated that minocycline, an antibiotic that inhibits microglia, decreased diabetes-induced inflammatory cytokine production, reduced the release of cytotoxins from the activated microglia, and significantly reduced caspase-3 activity in the retina.

Despite the progress made in animal models of diabetes, no definitive study of microglial activation in human diabetic retinopathy has been reported. In recent years, a number of human microglial markers, such as HLA-DR (major histocompatibility complex class II antigen), CD68, and CD45,8 have provided opportunities to examine the role of microglia in human diabetic retinopathy. In this study, 21 eyes from 13 patients with different clinical stages of diabetic retinopathy, from background to preproliferative to proliferative, were studied to examine the role of microglia in human diabetic retinopathy.

### METHODS

#### PARTICIPANTS

Ten healthy eyes from 9 individuals aged 49 to 89 years (mean age, 71 years) served as controls. Twenty-one eyes from 13 patients with diabetic background, preproliferative, or proliferative retinopathy aged 32 to 80 years (mean age, 62 years) were retrieved from the W. Richard Green Ophthalmic Pathology Laboratory at the Wilmer Eye Institute, Johns Hopkins University School of Medicine. Of the 21 eyes with diabetic retinopathy, 17 were obtained at autopsy and 4 were obtained from surgical enucleation. Fifteen eyes had background retinopathy, 3 had preproliferative retinopathy, and 3 had proliferative retinopathy. Diagnosis and staging were established by reviewing the clinical history and histopathologic sections. Participants had diabetes from 2 to 16 years with a mean duration of 9 years. Two participants had type 1 (insulin-dependent) and 4 had type II (non-insulin dependent) diabetes; the insulin requirement of the 7 other cases was not known.

#### TISSUE PREPARATION

The eyes were fixed in buffered formaldehyde, 4%, soon after surgical enucleation or within 24 hours after death. The fixed tissues were washed in phosphate-buffered saline (pH 7.4), embedded in paraffin, and sectioned. The use of human tissue material was done in accordance with the Declaration of Helsinki.

#### IMMUNOHISTOCHEMICAL STUDY

The tissue sections were deparaffined with xylene and a series of graded ethanol steps and rehydrated in phosphate-buffered saline. The tissues were heated at 95°C to 99°C in citrate acid (pH 6.0) for 20 minutes for antigen retrieval. The sections were blocked with normal goat serum, 5%, for 20 minutes and incubated at 4°C overnight with a first primary antibody, a mouse anti–HLA-DR antigen (1:100; DAKO Corp, Carpinteria, California), mouse anti-human CD45 (1:100, DAKO Corp), or mouse anti-human CD68 (1:200, DAKO Corp). Primary antibody was omitted in the negative control. After being washed in phosphate-buffered saline, the sections were further incubated with secondary antibody biotin-conjugated goat anti-mouse IgG (1:250; Vector Laboratories, Burlingame, California) and streptavidin peroxidase (ABC Kits; Vector Laboratories) for 30 minutes at room temperature. Peroxidase activity was visualized using 3,3-diaminobenzidine. After being counterstained with hematoxylin, the sections were dehydrated in ascending ethanol series, immersed in xylene, and coverslipped. The sections were observed by light microscope and images were captured by digital photography.

#### RESULTS

### MICROGLIA IN THE HEALTHY HUMAN RETINA

In healthy human eyes, a few ramified HLA-DR–positive microglial cells were seen in the retinal parenchyma or in the perivascular space in the inner retinal layers (Figure 1A). These microglial cells had oval cell bodies with long, slender processes. The CD45-positive cells (not shown) displayed similar distribution and cellular morphologic features as the HLA-DR–positive cells in the retina. In contrast, the CD68-positive microglial cells (not shown) were much fewer in number, but were frequently observed in the perivascular area.

### ACTIVATION OF MICROGLIA IN BACKGROUND DIABETIC RETINOPATHY

The remarkable pathologic features in background diabetic retinopathy (BDR) were as follows: (1) A moderate increase of microglial cells labeled by all 3 markers was mostly seen in the inner retinal layers extending from internal to middle limiting membranes. The activated microglia appeared hypertrophic or amoeboid and frequently clustered around the perivascular region involving medium-sized arterioles, venules, and capillaries. The HLA-DR–positive perivascular microglia showed numerous nodular processes reaching out to the retinal parenchyma (Figure 1B and C). Occasionally, some endothelial cells of blood vessels were also labeled with the HLA-DR antibody. (2) The HLA-DR–positive microglia clustered around fresh hemorrhages in 8 of 10 microaneurysms seen. In the eye with exudative retinopathy, cell number and labeling intensity of the microglia were markedly increased. Hypertrophic HLA-DR–positive microglia disrupted the architecture of the inner nuclear layer and reached out to the outer plexiform layer and even the outer nuclear layer. However, the proteinaceous exudation that accumulated in the outer plexiform layer had no excessive infiltration of microglia (Figure 1D). (3) In cases with diabetic macular cystoid edema (Figure 1E), there was a markedly increased number of HLA-DR–positive microglia. The microglia infiltrated the inner retinal layers, photoreceptor cell layer, and subretinal space. The cells in epiptelial membranes were also heavily labeled with all 3 microglial markers (Figure 1E, showing HLA-DR labeling). In BDR eyes, both CD45- and CD68-positive microglial cells showed similar changes as those of HLA-DR–
positive cells, but their cell number and labeling intensity were less when compared with HLA-DR–positive microglial cells. CD68-positive microglial cells exhibited hypertrophic cell bodies with few cellular processes (Figure 1F).

ACTIVATION OF MICROGLIA IN PREPROLIFERATIVE DIABETIC RETINOPATHY

In preproliferative diabetic retinopathy, retinal ischemic changes, such as cotton-wool spots, intraretinal microangiopathy, venous dilation, and venous beading, were superimposed on BDR. There was a dramatic increase of microglial cells labeled with 3 markers in the inner retinal layers. Hypertrophic microglial cells clustered around the peripheral region of cotton-wool spots (Figure 2A) and around dilated venules (Figure 2B). In cases of proliferative diabetic retinopathy, the pial septa of the optic nerve were also markedly infiltrated with hypertrophic or amoeboid microglia (Figure 2C).

ACTIVATION OF MICROGLIA IN PROLIFERATIVE RETINOPATHY

In proliferative retinopathy, retinal neovascularization broke through the internal limiting membrane and extended into the vitreous cavity. This proliferative process was most prominent in the region of the optic nerve head. Along the central Kuhnt meniscus of the optic nerve head, a marked increase in the number of microglia was observed (Figure 3A). In proliferative retinopathy, the microglial cells clustered around the new blood vessels in the nerve fiber layer at the retina where funnel-shaped detachment
was observed (Figure 3B). As the retinal neovascularization broke through the internal limiting membrane and grew into the vitreous cavity, the dilated new vessels were heavily surrounded by labeled microglial cells (Figure 3C and D).

The Table summarizes the semiquantitative results in different stages of diabetic retinopathy and diabetic optic neuropathy for each of the markers used and the number of eyes exhibiting the activated microglial phenotype in each category. The number of HLA-DR–, CD45–, and CD68-positive cells were remarkably increased in 3 stages of diabetic retinopathy and diabetic optic neuropathy.

**COMMENT**

In this study, we used 3 markers (HLA-DR, CD45, and CD68) to label human microglial cells and observed that these microglial cells were markedly activated in different stages of human diabetic retinopathy. The microglia were increased in number and exhibited hypertrophic features and up-regulation of HLA-DR, CD45, and CD68 expression. Furthermore, microglial perivasculitis, characterized by marked activation of perivascular microglia, was a striking feature of the disease process.

The activated microglia observed in diabetic retinopathy could not be differentiated from hematogenous macrophages by morphology and/or antigen expression. Microglia are resident macrophages in the central nervous system and retina. When activated, microglia become plump, showing few slender processes and morphologically resemble hematogenous macrophages invading the retina. There are no specific markers available to definitely distinguish hematogenous macrophages from activated microglial cells. However, CD68 is generally considered a macrophage marker that labels only some of the microglial cells more frequently labeled by HLA-DR and CD45 antibodies. In our study, there were remarkably more HLA-DR–positive cells than CD68-positive cells when the adjacent retinal sections of the same eye were compared. This observation suggests that most of the labeled cells in the diabetic eye were activated resident microglia with a few scattered hematogenous macrophages.

In BDR eyes, activated microglial cells surrounded most of the microaneurysms, and the labeling intensity of these cells were exaggerated in BDR with exudative and hemorrhagic lesions. In cases with diabetic cystoid macular degeneration, activated microglia infiltrated all layers of the retina and extended into the subretinal space. These observations were consistent with the animal studies that reported that the number and activity of microglia were dramatically increased early in experimental diabetes.

The signals for microglial activation might be of various natures and origins. Proinflammatory chemokines and cytokines are likely candidates. Meleth et al9 reported that the levels of serum chemokines RANTES and SDF-1 were significantly elevated in patients with nonproliferative diabetic retinopathy and also noted positive immunostaining for MCP-1 and RANTES in the inner retina of diabetic patients. Elevated proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, were identified in the retina of experimental diabetic animals3 and in the vitreous of diabetic patients.10 These chemokines and cytokines were considered the strong activators of microglia.11 One of the striking features of diabetic retinopathy in our study was activated microglia surrounding the retinal vasculature. This pathologic feature may be labeled microglial perivasculitis. Retinal perivasculitis, as seen in sarcoidosis, tuberculosis, and
certain forms of uveitis, exhibits cuffing of retinal vessels by lymphocytes and mononuclear cells. The cuffing of retinal vasculature by activated microglia has not been previously described. Cunha-Vaz described leakage of fluorescein from retinal vasculature in the earliest stage of diabetic retinopathy before structural changes took place in

<table>
<thead>
<tr>
<th>Disease Stage</th>
<th>Marker</th>
<th>Microglial Cell Density</th>
<th>Eyes Showing Active Phenotype/Total Eyes, No.</th>
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<tr>
<td>Retina</td>
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<tr>
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<td>CD45-Positive Cells</td>
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<td>10/10</td>
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<td>BDR</td>
<td>CD68-Positive Cells</td>
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<tr>
<td>PDR</td>
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<td>3/3</td>
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<td>Optic nerve</td>
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<tr>
<td>Diabetic</td>
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</table>

Abbreviations: BDR, background diabetic retinopathy; PDR, proliferative diabetic retinopathy; PPDR, preproliferative diabetic retinopathy; +, scattered, 5 to 20 cells/section; + +, moderate, less than or equal to 10 cells/high power field; + + +, abundant, more than 10 cells/high power field.
the retina vessels. We speculate that serum chemokines and cytokines might leak from the retinal vasculature, activating perivascular microglial cells. On the other hand, microglia perivasculitis might also be a primary pathologic feature resulting from metabolic stress in the diabetic retina. In addition to the close topological association with blood vessels, perivascular microglia were also closely apposed to ganglion cell bodies and their axons as well as the neurons in the inner nuclear layers. This association could lead to an initial microglial activation by compromised neurons with a release of factors such as free fatty acids owing to metabolic stress in the early diabetes. Activated microglia are considered a major source of proinflammatory and neurotrophic cytokines and substances, such as vascular endothelial growth factor, TNF-α, IL-1β, and nitric oxide. On their release, these cytokines may propagate the inflammatory response within the retina, further exacerbating the vascular permeability and neuronal damage in eyes with diabetic retinopathy, leading to a vicious cycle.

The prominent labeling of the cells in the epiretinal membrane overlying the inner limiting membrane of the retina by microglial markers HLA-DR, CD68, and CD45 was observed in all eyes in our study. The epiretinal membrane, which consists of fibrocellular proliferations on the inner surface of the retina, is a prominent feature of diabetic retinopathy. The epiretinal membrane tends to contract, resulting in distortion or detachment of the underlying retina. It is generally believed that the glial components of the epiretinal membrane come from Müller cells and astrocytes. However, our study showed that the microglia is also an important component of epiretinal membrane.

Activation of microglia has been seen in the ischemia-reperfusion lesions of both the brain and retina. Similar activation of microglia was also prominent in preproliferative ischemic diabetic retina in our study, which was characterized by cotton-wool spots. In one study, del Zoppo et al. reported that activation of microglia following cerebral ischemia led to production of cytokines TNF-α, IL-1β, and reactive oxidative species, neurotoxic agents that induced neuron death.

In proliferative diabetic retinopathy, ischemia of the inner retinal layers secondary to capillary bed closure leads to the production of vascular growth factors such as vascular endothelial growth factor, which induces new vascular growth in the ischemic retina, extending into the vitreous cavity. Vascular endothelial growth factor is highly expressed in Müller cells and astrocytes in the ischemic retina, neovascularized membranes, and vitreous of patients with proliferative diabetic retinopathy. Several studies reported that activated microglia also produced vascular endothelial growth factor. Our study showed exuberant activation of microglia in the neovascularized membrane of eyes with proliferative diabetic retinopathy, suggesting that microglia may indeed play a significant role in the angiogenesis of proliferative vitreoretinopathy.

Significant microglial activation was observed in the optic nerves of participants with BDR but was more prominent in participants with ischemic retinopathy. It is generally believed that diabetes may affect the optic nerve by inflicting nonarteritic ischemic optic neuropathy secondary to microvascular and autonomic abnormalities. The causal relationship between diabetic retinopathy and nonarteritic ischemic optic neuropathy is still disputed. Microglial activation in the optic nerve in diabetic retinopathy might be an additional pathogenic factor in diabetic optic neuropathy.

In summary, our study showed significant microglial activation in human diabetic retinopathy and optic neuropathy. Microglial perivasculitis may play a connecting role between vasculopathy and neuropathy in diabetic retinopathy.

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REFERENCES
