Minocycline Reduces Proinflammatory Cytokine Expression, Microglial Activation, and Caspase-3 Activation in a Rodent Model of Diabetic Retinopathy

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Diabetes leads to vascular leakage, glial dysfunction, and neuronal apoptosis within the retina. The goal of the studies reported here was to determine the role that retinal microglial cells play in diabetic retinopathy and assess whether minocycline can decrease microglial activation and alleviate retinal complications. Immunohistochemical analyses showed that retinal microglia are activated early in diabetes. Furthermore, mRNAs for interleukin-1β and tumor necrosis factor-α, proinflammatory mediators known to be released from microglia, are also increased in the retina early in the course of diabetes. Using an in vitro bioassay, we demonstrated that cytokine-activated microglia release cytotoxins that kill retinal neurons. Furthermore, we showed that neuronal apoptosis is increased in the diabetic retina, as measured by caspase-3 activity. Minocycline represses diabetes-induced inflammatory cytokine production, reduces the release of cytotoxins from activated microglia, and significantly reduces measurable caspase-3 activity within the retina. These results indicate that inhibiting microglial activity may be an important strategy in the treatment of diabetic retinopathy and that drugs such as minocycline hold promise in delaying or preventing the loss of vision associated with this disease. Diabetes 54:1559–1565, 2005

Diabetic retinopathy is a progressive neurological disease characterized by degeneration of neurons and macroglia and accompanied by profound vascular changes that eventually lead to legal blindness. Despite progress in understanding the pathogenesis of diabetic retinopathy, our knowledge of the mechanisms leading to neuronal cell loss, glial dysfunction, and vascular remodeling is incomplete. Whereas the retina has traditionally been viewed as an immune privileged tissue, evidence is accumulating to support a role for local inflammation in the pathogenesis of diabetic retinopathy. For example, alterations in the level of adhesion molecules, which facilitate the trafficking of leukocytes into the retina, accompany changes in retinal blood vessel permeability (1). Joussen et al. (2) identified tumor necrosis factor-α (TNF-α) and cyclo-oxygenase-2 (COX-2), two proinflammatory mediators, as operative in the early signature pathologies of diabetic retinopathy. In addition, Funatsu et al. (3) documented local production of interleukin (IL)-6 in the vitreous humor of diabetic patients and showed that levels of IL-6 correlate with the severity of retinal pathophysiology.

Elevated levels of cytokines can activate the endogenous immune cells of the central nervous system (CNS), cells known as microglia. Microglia are related to dendritic cells in other tissues and, although similar to macrophages, are a distinct cell type (4). In response to an activating stimulus, quiescent microglia undergo a series of stereotyped morphological, phenotypical, and functional changes (5). In response to signals from dying cells, activated microglia evolve into phagocytes capable of clearing debris (6). It is yet to be determined what signals during diabetes activate the retinal microglia, whether microglial activation precedes the major histopathologic changes that occur in the retina, and what affect these microglia may play in the pathophysiology of the disease.

In this study, we examined the putative role of microglia and inflammation in the progression of early diabetic retinopathy and tested the ability of the drug, minocycline, to abrogate some of the negative consequences of microglial activation. Minocycline is a second-generation, semi-synthetic tetracycline that exerts anti-inflammatory effects that are completely separate from its antimicrobial actions. Studies suggest that it affords neuroprotection because it crosses the blood-brain barrier, inhibits the proliferation and activation of microglia, and inhibits apoptosis (7). It has proven effective in a number of animal models of neurodegeneration in which microglia and inflammation have been implicated, but it has yet to be tested in animal models of diabetes. Because minocycline is well tolerated, it represents a potential new therapeutic...
tool for preventing or controlling the neurological complications of diabetes.

**RESEARCH DESIGN AND METHODS**

After a 12-h fast, SD rats weighing ~200 g received a single 40 mg/kg intraperitoneal streptozotocin (Sigma, St. Louis, MO) and 10 mmol/l sodium citrate buffer (pH 4.5). Control animals were fasted and received the buffer alone. Animals with blood glucose levels >250 mg/dl for 4 days after receiving STZ were considered to be diabetic. Blood glucose levels were checked weekly. All experiments abided by the Association for Research in Vision and Ophthalmology statement on the Use of Animals in Ophthalmology and Vision Research and were approved by the Animal Care and Use Committee of the Pennsylvania State University.

For the in vivo minocycline (Sigma) treatment, rats were given the drug at a dosage of 45 mg/kg every 12 h the 1st day and 22.5 mg/kg every subsequent 12 h. Vehicle was injected every 12 h for the length of the experiment.

**Cell culture.** Primary microglial cultures were obtained from SD rats on postnatal day 0–2 (8). Microglial cultures were maintained in Eagle’s minimum essential medium (MEM) supplemented with 0.66 mg/ml BSA, 10 ng/ml n-biotin, 5 ng/ml insulin, 5 ng/ml selenium, 5 μg/ml iron-poor transferrin, 2 μg/ml glutamine, 15 nmol/l HEPES buffer, and 100 units · 100 μg · ml−1 penicillin/streptomycin (MCDM). Microglia were maintained in MEM with 0.5% FCS for 24 h then placed in serum-free MCDM for 16 h. The retinal neuronal R28 cell line was maintained in defined medium containing MEM 0.5% FCS for 24 h then placed in serum-free MCDM for 16 h. The retinal neuronal R28 cell line was maintained in defined medium containing MEM 0.5% FCS for 24 h then placed in serum-free MCDM for 16 h. The retinal neuronal R28 cell line was maintained in defined medium containing MEM 0.5% FCS for 24 h then placed in serum-free MCDM for 16 h.

**Immunofluorescence.** Diabetic and matched control rats (n = 4 each group) were anesthetized with 75 mg/kg ketaset and 15 mg/kg xylazine 4 weeks after diabetes was induced, after which transcardial perfusion with heparinized saline was begun. The rats’ eyes were removed, punctured 1 mm posterior to the limbus with a 30-gauge needle, and immersed for 1 h at room temperature in 4% buffered formaldehyde. The eyes were washed in three changes of PBS, cryoprotected in 2.5 mol/l sucrose, and frozen. Cryostat sections (12 μm) were thaw mounted onto Superfrost Plus glass slides and stored at −20°C until use. Sections were blocked in 10% BSA, 10% normal goat serum, 0.05% Triton X-100, and 0.3% hydrogen peroxide in Tris-buffered saline with 0.05% Triton X-100, incubated for 2 h with primary antibodies, washed extensively with PBS, cryoprotected in 2.5 mol/l sucrose, and frozen. Cryostat sections were floated onto Superfrost Plus glass slides and stored at −20°C until use. Sections were blocked in 10% BSA, 10% normal goat serum in Tris buffer for 1 h, then incubated overnight at 4°C with rabbit anti–Iba-1 (Wako Pure Chemical, Wako, TX, 1:500) diluted in 1/5 blocking solution supplemented with 0.2% Triton X-100. Sections were rinsed extensively in Tris-buffered saline with 0.05% Triton X-100, incubated for 2 h with biotinylated secondary antibody, then rinsed and incubated in streptavidin–lissamine rhodamine B sulfonyl chloride and 4′,6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml) diluted in 10% BSA. After being washed, sections were coverslipped with aqueous mountant, dried, and viewed using a Leica SP-2 confocal microscope.

**RESULTS**

Microglia became activated early in the course of diabetic retinopathy. It has been postulated that inflammation plays an integral role in the etiology of diabetic retinopathy. Microglia, as the immune-effector cells within both the CNS and retina, might be expected to have an important role in initiating this inflammatory response. To determine whether microglia become activated early in the course of diabetic retinopathy, we used immunofluorescence to characterize the microglia in the retina of rats with STZ-induced diabetes. Diabetic rats and age-matched controls were killed 4 weeks after receiving a single intraperitoneal dose of STZ, and their eyes were isolated and stained for Iba-1, a calcium-binding protein expressed by microglial cells. In control retinas, the microglia possessed thin processes that branched several times as they extended 10–30 μm from the cell soma (Fig. 1). This morphology is characteristic of resting microglia. In contrast, microglia in the retinas of the diabetic rats appeared activated. Their cell bodies were hypertrophied, they had fewer processes, and those processes were thicker and shorter. In some cases, microglia in the diabetic retinas were amoeboid with few or no processes, consistent with differentiation toward a phagocyte. At this early stage, only a subpopulation of the microglia in the diabetic retinas appeared activated. Microglia in both control and diabetic retinas appeared primarily in the granular cell layer, and
no gross differences in distribution or cell number were observed between the two conditions. Our results indicated that diabetes leads to an early activation of microglia within the inner retina and that activation occurs locally in response to diabetes and not globally throughout the retina.

Diabetes increases the expression of mRNAs for IL-1β and TNF-α in the retina. Because microglia can produce cytokines as a consequence of activation, we measured changes in the relative levels of two proinflammatory cytokine mRNAs in the retinas of rats during STZ-induced diabetes. Total RNA was extracted from isolated retinas and used as template for real-time PCR analysis; the real-time PCR analysis was performed for IL-1β and TNF-α. As a reference, primers to 18S RNA were used. Real-time PCR was performed on six retinas per group in duplicate. Results were analyzed using REST software, which calculates the confidence levels of real-time data. Our analysis revealed increased expression of both TNF-α and IL-1β mRNAs in retinas from diabetic rats compared with retinas of controls (Fig. 2). TNF-α mRNA levels were increased 6-fold over those of controls, whereas IL-1β mRNA levels increased 4.5-fold.

Next, diabetic rats were treated with minocycline (22.5 mg/kg, i.p.) twice daily beginning 3 days after STZ injection. mRNA levels of TNF-α and IL-1β were measured 2 weeks later by real-time PCR. Minocycline treatment abrogated the increases in mRNA expression seen as a result of diabetes for both cytokines. Minocycline-treated animals had 50% lower mRNA levels of TNF-α and 40% lower levels of IL-1β. These results confirmed that diabetes causes an early increase in the expression of inflammatory mediators within the retina and showed that minocycline treatment reduces this inflammatory component.

Minocycline prevents TNF-α–induced COX-2 expression in microglia. To further characterize minocycline’s effect on inflammation, we designed experiments to assess minocycline’s ability to affect the production of other proinflammatory stimuli. For these experiments, microglial cultures with proinflammatory cytokines were pretreated with or without 20 nmol/l minocycline. Previous dosage-response experiments showed that this dosage of minocycline provides maximal protection to neuronal cultures treated with 500 μmol/l glutamate and significantly reduces proliferation and nitric oxide (NO) and IL-1β release from similarly treated purified microglial cultures (7). Minocycline was added 1 h before treatment with cytokines. Then 24 h after cytokine treatment, cul-

![Figure 1](image1.png)  
**FIG. 1.** Microglia in the retina become activated in STZ-induced diabetes. Retinas from control and diabetic rats were isolated, sectioned, and processed for Iba-1 (red), an antigen specific for microglia, and counterstained with DAPI (blue) 4 weeks after diabetes was induced. Microglia in the control retinas possessed long, thin processes characteristic of ramified microglia (A and B). Microglia in the retinas of diabetic rats displayed shorter, thicker processes indicative of an activated phenotype (C and D). B and D represent fivefold enlargements of sections of A and C.

![Figure 2](image2.png)  
**FIG. 2.** Minocycline abrogates the increased retinal expression of TNF-α and IL-1β mRNAs as a consequence of diabetes. Rats received minocycline (mino; 22.5 mg/kg, i.p.) twice daily for 2 weeks beginning 3 days after STZ injection. RNA was isolated from the retinas and analyzed by real-time PCR. Primers for 18S RNA were used to calibrate changes in mRNA expression. REST software was used for statistical analysis. *P < 0.001 for diabetic vs. control animals; **P < 0.05 for diabetic vs. minocycline-treated diabetic animals.
containing activated microglia were then incubated with minocycline. Cytokine treatment lasted for 6 h, after which TNF-α-activated microglia were grown on transwell filters and activated by toxicity, we cocultured cytokine-activated microglia with whether minocycline could prevent this microglial cytotoxicity. Pretreatment with minocycline completely prevented the increase in cell death. Similar results were seen when TUNEL was used as an index of apoptotic cell death. R28 cells exhibited ninefold higher TUNEL staining when they were cocultured with TNF-α-activated microglia as opposed to untreated microglia. Again, pretreatment with minocycline significantly blocked the effects of TNF-α-induced cytotoxicity of the microglial cells. TNF-α alone did not induce the death of R28 cells. These results provide further evidence that minocycline can abrogate the negative aspects of microglial activation.

**Minocycline can prevent diabetes-induced apoptosis in the retina.** To further characterize minocycline’s effects on apoptosis, we assessed the ability of systemic minocycline treatment to decrease the activation of caspase-3 within the retina in adult SD rats with STZ-induced diabetes. In these studies, 3-month, STZ-induced diabetic animals were injected with 22 mg/kg of minocycline twice daily for 10 days. Protein lysates from the retinas of treated or untreated diabetic rats or treated or untreated euglycemic rats were assayed for caspase-3 activity as a measure of apoptosis. Caspase-3 activity increased 50% within the retina of diabetic compared with control animals (Fig. 5). Minocycline treatment for 10 days significantly abrogated the increase in caspase-3 activity ($P < 0.004$). There was no statistical difference between the levels of caspase-3 activity in control animals administered vehicle and diabetic animals administered minocycline. Minocycline had no effect on caspase-3 activity in the retinas of euglycemic rats. The blood glucose of control rats given vehicle and those given minocycline was 7.1 ± 0.3 and 7.1 ± 0.2 mmol/l, respectively; the levels of diabetic rats given vehicle and those given minocycline were 24.7 ± 0.6 and 24.3 ± 0.9 mmol/l, respectively. These experiments demonstrated that short-term treatment with minocycline late in diabetes can significantly reduce apoptosis within the retina.

**DISCUSSION**

The goal of this study was to determine whether retinal microglia become reactive as a consequence of diabetes, leading to the release of soluble cytotoxins that contribute to the progression of diabetic retinopathy. We also evaluated the efficacy of minocycline in abrogating the retinal cell death that occurs as a consequence of diabetes. We showed that 1) microglia become activated before the onset of neuronal cell death; 2) diabetic conditions lead to an elevation of proinflammatory cytokine expression within the retina; 3) proinflammatory cytokines such as TNF-α can directly activate microglia, leading to their producing cytotoxins that will kill retinal neurons; 4) minocycline decreases the release of these cytokines, resulting in neu-
5) minocycline decreases the expression of proinflammatory cytokines in an animal model of diabetes and decreases the levels of active caspase-3, thus affording neuroprotection in vivo.

**Microglia are sentinels within the central nervous system.** Our analysis of retinal microglial activity in STZ-induced diabetes confirms observations by others (11,12). Microglia clearly exhibited a more reactive phenotype 4 weeks after diabetes was induced with STZ. However, we did not observe global microglial reactivity within the retina. Instead, we observed patches of locally activated microglia, suggesting that there are hot spots in the retina that experience elevated stress as a consequence of diabetes. Although resting microglia promote and maintain homeostasis in the normal CNS, reactive microglia have been implicated in the progression of many neurological diseases, including Alzheimer disease, multiple sclerosis, HIV-related dementia, and stroke (13–18). Microglial-mediated cytotoxicity appears to be involved in the tissue damage and functional loss seen in these diseases by the release of reactive oxygen and reactive nitrogen species, cytokines, excitotoxins, and proteases.

Although few studies have associated microglial activation with the pathogenesis of diabetic retinopathy, a strong case can be made for inflammation. Elevated IL-6 and IL-8 have been documented in the vitreous fluid of patients with diabetic retinopathy (19), and TNF-α has been shown to be elevated in the serum (19) and epiretinal membranes (20) of patients with diabetic retinopathy. In addition, platelets from patients with active proliferative diabetic retinopathy express higher than normal levels of TNF-α and its receptors, TNF-RI and TNF-RII (21). The expression of intracellular adhesion molecule (ICAM)-1 is increased early in diabetic retinopathy and has been associated with increased TNF-α, leukostasis, and subsequent endothelial cell death characteristic of the disease (2,21,22). Increased levels of IL-1β have also been measured in the retinas of STZ-induced diabetic rats and correlate with elevated levels of the inducible isoform of NO synthase (23). In addition, nonsteroidal anti-inflammatory...
tory drugs have been shown to prevent the changes in ICAM-1 and integrin expression associated with diabetic retinopathy via TNF-α suppression and to decrease vascular leukostasis and blood-retinal barrier breakdown (24). These study findings suggest that cytokines and the resulting neuroinflammation cause several of the hallmark features of diabetic retinopathy.

Microglia release numerous cytotoxic molecules in response to cytokines. There are strong data implicating cytokines in microglial activation. Cytokines such as IL-1β, γ-interferon, TNF-α, and IL-6 have been shown to directly activate microglia (25–27). For example, TNF-α decreases cAMP levels within microglia, leading to a more destructive phenotype (28). Our results confirm the induction of proinflammatory cytokines TNF-α and IL-1β within the retina as a result of STZ-induced diabetes. The most likely source of these cytokines are retinal microglia; however, other retinal cell types (e.g., pericytes) release proinflammatory drugs have been shown to prevent the changes in ICAM-1 and integrin expression associated with diabetic retinopathy via TNF-α suppression and to decrease vascular leukostasis and blood-retinal barrier breakdown (24). These study findings suggest that cytokines and the resulting neuroinflammation cause several of the hallmark features of diabetic retinopathy.

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Activated microglia release soluble effectors that can directly or indirectly cause damage to neural cells. Microglia release IL-1β, IL-3, IL-6, TNF-α, vascular endothelial growth factor (VEGF), lymphotoxin, macrophage inflammatory protein-1α, matrix metalloproteins (MMPs), NO, and reactive oxygen species (ROS) (30–32). The cytokines IL-1β, IL-6, TNF-α, and lymphotoxin alter expression of vascular cell adhesion molecules to recruit lymphocytes and macrophages to injury sites (33). In addition, lymphotoxin, TNF-α, NO, and ROS can directly kill cells. TNF-α has been shown to induce the death of neurons via a direct apoptotic effect after receptor binding and by directly interfering with intracellular substrates used by growth factors such as IGF-1 and the insulin receptor (34,35). VEGF, NO, and MMPs can weaken the blood-retinal barrier, thus enhancing the infiltration of leukocytes into the CNS. Minocycline as a potential new therapeutic tool to prevent diabetic retinopathy. Recent reports have shown that diabetic retinopathy involves a chronic, low-grade inflammatory component (3,36–38). Elevated cytokines activate microglia, thereby stimulating a cycle of inflammation that recruits leukocytes, causes vascular breakdown, and directly induces cell death through the release of cytotoxic substances. Our in vitro bioassay data clearly demonstrated the ability of activated microglia to release soluble factors that can cause neuronal death. Moreover, the microglia appear to become activated coincident with the increase in apoptosis documented within the diabetic retina (39). Microglia, therefore, appear to be implicated in a number of the pathologies associated with diabetic retinopathy, including vascular complications, neuronal death, and glial dysfunction. Drugs designed to counter these negative aspects of microglial activation should, therefore, have numerous benefits in diseases such as diabetic retinopathy.

Minocycline is effective in a number of animal models of neurodegeneration in which microglia and inflammation have been implicated. Minocycline dramatically reduces inflammation and protects against excitotoxic neuronal death after cerebral ischemia (40,41) and photoreceptor degeneration (42). It provides this neuroprotection in part by inhibiting the proliferation and activation of microglia (7). Results from our in vivo experiments indicate that minocycline reduces the expression of inflammatory mediators and the incidence of apoptosis within the retina as a consequence of diabetes. In addition to direct actions on microglia, minocycline also has been shown to exert antiapoptotic effects by inhibiting caspases-1 and -3 and the release of cytochrome c from mitochondria (43–45). These actions likely contributed to our in vivo finding of reduced caspase-3 activity. However, it is unlikely that they explain all of the beneficial effects of minocycline, as we demonstrated decreased proinflammatory gene expression with minocycline treatment at 2 weeks of STZ-induced diabetes. Because minocycline’s anti-inflammatory and antiapoptotic effects will be beneficial in reducing the severity of retinal changes induced by diabetes, there is evidence to support the administration of minocycline for treating diabetic retinopathy. Early inhibition of microglial activity would attenuate inflammation, slowing the progression of leukostasis and vascular damage, whereas minocycline’s antiapoptotic effects, compounded with a decrease in the release of cytotoxic compounds by activated microglia, would prevent the neuronal death seen later as retinopathy progresses. Although further studies are required to test the prediction that minocycline will reduce vascular permeability in the retina, the studies presented here recommend minocycline as a strong candidate for further consideration as a therapeutic drug in reducing the retinal complications of diabetes.

ACKNOWLEDGMENTS
This work was supported by a program project grant from the Juvenile Diabetes Research Foundation (#4-2002-455, project 5).

The authors express their thanks to Alistair Barber and Rhona Ellis for assistance with the confocal microscopy.

Parts of this study were presented at the annual meeting of the Association for Research in Vision and Ophthalmology, Ft. Lauderdale, Florida, 25–29 April 2004 (Krady 2004 poster session 375;3224 [available at www.arvo.org]).

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