Chemotactic and Cytotoxic Effects of Minocycline on Human Retinal Pigment Epithelial Cells

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PURPOSE. To reveal the effects of minocycline, an anti-inflammatory and neuroprotective agent, on the viability and physiological properties of retinal pigment epithelial (RPE) cells and to compare the effects with those of triamcinolone acetonide.

METHODS. The proliferation of human RPE cells in vitro was investigated with a bromodeoxyuridine immunohistoassay; chemotaxis was examined with a Boyden chamber assay. Cell viability was determined by trypan blue exclusion. The gene expression of growth factors and MMP-9 was determined with real-time RT-PCR, and the secretion of VEGF was examined with ELISA. The phosphorylation of p38 MAPK and ERK1/2 proteins was determined with Western blot analysis.

RESULTS. Minocycline at low concentrations (50 nM-20 μM) stimulated chemotaxis and decreased the proliferation of RPE cells. Minocycline at high concentrations (above 5 μM) decreased the viability of RPE cells through the induction of cell necrosis. The chemotactic effect of minocycline was mediated by the stimulation of autocrine PDGF signaling and the activation of p38 MAPK. Minocycline promoted the expression of PDGF-B, HGF, VEGF, and MMP-9 and increased the amounts of phosphorylated p38 and ERK1/2 proteins in RPE cells. Triamcinolone reduced PDGF-evoked chemotaxis and VEGF expression and secretion and had no significant effects on cell viability or proliferation. Triamcinolone did not reverse the effects of minocycline on cell proliferation, chemotaxis, or viability or the expression of VEGF.

CONCLUSIONS. Low-dose minocycline induces the activation of RPE cells, as indicated by the activation of p38 and ERK1/2 and by enhanced chemotaxis mediated by autocrine PDGF signaling. High-dose minocycline induces RPE cell degeneration. (Invest Ophthalmol Vis Sci. 2010;51:2721–2729) DOI:10.1167/iovs.09-4661

Loss of photoreceptor cells is a characteristic of inherited retinal degenerations and other retinopathies, including age-related macular degeneration. The function and metabolism of photoreceptors closely depend on the integrity of the retinal pigment epithelium.1 In addition to the involvement of photopigment recycling, which is crucial for the maintenance of photoreceptor excitability,2 the retinal pigment epithelium digests shed photoreceptor outer segments,3 transports ions, water, and metabolic end products from the subretinal space to the blood,4 and delivers nutrients such as glucose to the photoreceptors.5 Dysfunction or degeneration of the retinal pigment epithelium can lead to photoreceptor degeneration and can contribute to the onset of age-related macular degeneration.6 Mutations in genes that are expressed in the retinal pigment epithelium are the cause of some forms of inherited retinal degenerations.7 Age-related alterations in retinal pigment epithelial (RPE) pigmentation8 may result in increased photo-oxidative stress and subsequent loss of RPE and photoreceptor cells.7 Reduction in the density of RPE cells,9 accumulation of lipofuscin in the retinal pigment epithelium,10 and failure of outer segment phagocytosis10,11 may contribute to inherited retinal degenerations and are suggested to be involved in the pathogenesis of age-related macular degeneration.12 Breakdown of the blood-retina barrier constituted by the retinal pigment epithelium leads to subretinal edema.12 Activated RPE cells are also key players in proliferative diseases such as choroidal neovascularization and proliferative vitreoretinopathy, in which migrating and proliferating RPE cells contribute to the formation of preretinal membranous tissues.13

Minocycline, a semisynthetic, long-acting tetracycline derivative that has good penetration of the blood-brain barrier, is commonly used in humans because of its antimicrobial action. In addition, minocycline has anti-inflammatory and antiapoptotic activities and is effective in delaying disease progression in numerous models of neurodegeneration, including brain ischemia.14 Traumatic brain injury.15 Huntington disease.16 Parkinson disease.17 and multiple sclerosis.18,19

Minocycline was also shown to exert neuroprotective effects in the diseased and injured retina. Minocycline protects retinal ganglion cells and photoreceptors from cell death and reduces retinal microglia activation in vivo and in vitro.19–31 Minocycline reduces the expression and release of proinflammatory cytokines and nitric oxide from activated retinal microglia24,25 and suppresses inflammatory cytokine production and caspase-1 and -3 activities in the retinas of diabetic animals.26–28 Minocycline-induced protection of retinal ganglion cells was described to be mediated by the induction of antiapoptotic intracellular signaling pathways and a decrease in glutamate toxicity, suggesting that it exerts neuroprotective effects also independent of its anti-inflammatory properties.19

Based on its anti-inflammatory and neuroprotective activities, minocycline has been suggested to represent a potential agent for the treatment of age-related macular degeneration and diabetic retinopathy.32,33 However, though minocycline is a neuroprotective agent, it has also been shown to have variable or even contradictory results in different animal models of neurodegeneration35,36; it impairs mitochondrial function37 and has, in higher doses, adverse side effects on the viability of microglial cells.30 Serious adverse effects, including papilledema and visual loss, were described during long-term or high-dose systemic minocycline therapy of acne vulgaris.38–40 Therefore, because minocycline was suggested to be a canti-
date agent for clinical use in the treatment of retinopathies associated with photoreceptor degeneration, it might be helpful to reveal potential adverse effects to the retinal pigment epithelium. The aim of the present study was to determine whether minocycline has effects on the viability and physiological characteristics of human RPE cells in vitro. We compared the effects with those of triamcinolone acetonide (9α-fluoro-16a-hydroxy prednisolone), an anti-inflammatory corticosteroid commonly used to treat retinopathies associated with edema\(^4\) and shown to be neuroprotective in animal models of subretinal hemorrhage.\(^5\)

### MATERIALS AND METHODS

#### Materials

Human recombinant platelet-derived growth factor (PDGF)-BB was purchased from R&D Systems (Minneapolis, MN). PD98059 and AG1296 were obtained from Calbiochem (Bad Soden, Germany). SB203580 was obtained from Tocris (Ellisville, MO). All tissue culture components and solutions were purchased from Gibco BRL (Paisley, UK). All other substances used were from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. Lipophilic substances were dissolved in dimethylsulfoxide; the final dilution of dimethylsulfoxide used in the experiments was 1:1000. The following antibodies were used: rabbit anti-human p44/p42 mitogen-activated protein kinase (MAPK, 1:1000; New England Biolabs, Frankfurt/M., Germany), rabbit anti-human phosphorylated p44/p42 MAPK (1:1000; New England Biolabs), rabbit anti-human p38 MAPK (1:1000; New England Biolabs), rabbit anti-human phosphorylated p38 MAPK (1:750; New England Biolabs), and anti-rabbit IgG conjugated with alkaline phosphatase (1:2000; Chemicon, Hofheim, Germany).

#### Cell Culture

The use of human material was approved by the Ethics Committee of the University of Leipzig, and all procedures were performed in accordance with the Declaration of Helsinki. Human RPE cells were obtained from several donors within 48 hours of death and were prepared and cultured as described. After the vitreous and the retina were removed, the RPE cells were mechanically harvested, separated by digestion with 0.05% trypsin and 0.02% EDTA, and washed two times with phosphate-buffered saline. The cells were suspended in complete medium (Ham F-10 containing 10% fetal bovine serum, Glutamax II, and penicillin/streptomycin; Greiner, Nürtingen, Germany) and were allowed to attach for 48 hours. Thereafter, the cells were growth arrested in medium without serum for 5 hours; subsequently, medium containing 0.5% serum with and without test substances was added for another 24 hours. BrdU incorporation was determined using a cell proliferation ELISA BrdU kit (Roche, Mannheim, Germany). BrdU (10 μM) was added to the culture medium 5 hours before fixation. We tested minocycline (0.005–50 μM) and triamcinolone (0.1–100 μM), respectively, in the absence and presence of PDGF (10 ng/mL).

#### DNA Synthesis Rate

The proliferation rate was determined by measuring the incorporation of bromodeoxyuridine (BrDU) into the genomic DNA. The cells were seeded at 3 × 10^5 cells/well in 96-well microtiter plates (Greiner), and were allowed to attach for 48 hours. Thereafter, the cells were grown in medium without serum for 5 hours; subsequently, medium containing 0.5% serum with and without test substances was added for another 24 hours. BrDU incorporation was determined using a cell proliferation ELISA BrdU kit (Roche, Mannheim, Germany). BrDU (10 μM) was added to the culture medium 5 hours before fixation. We tested minocycline (0.005–50 μM) and triamcinolone (0.1–100 μM), respectively, in the absence and presence of PDGF (10 ng/mL).

#### Chemotaxis

Chemotaxis was determined with a modified Boyden chamber assay. RPE cell suspension cultures (100 μL; 5 × 10^5 cells/mL serum-free medium) were seeded onto polyethylene terephthalate filters (diameter, 6.4 mm; pore size, 8 μm; Becton Dickinson, Heidelberg, Germany) coated with fibronectin (50 μg/mL) and gelatin (0.5 mg/mL). Within 16 hours after seeding, the cells attached to the filter and formed a semiconfluent monolayer. The cells were pretreated with blocking substances for 30 minutes; thereafter, the medium was changed to medium without additives in the upper well and medium containing test substances in the lower well. After incubation for 6 hours, the inserts were washed with buffered saline, fixed with Karnovsky’s reagent, and stained with hematoxylin. Nonmigrated cells were removed from the filters by gentle scrubbing with a cotton swab. The migrated cells were counted, and the results were expressed relative to the cell migration rate in the absence of test substances. The following test substances were applied: minocycline (0.05–50 μM) and triamcinolone (0.01–100 μM), respectively, in the absence and presence of PDGF (10 ng/mL); minocycline (20 μM) in the absence and presence of AG1296 (10 μM; an inhibitor of the PDGF receptor tyrosine kinase), SB203580 (10 μM; an inhibitor of p38 MAPK activation), and PD98059 (20 μM; an inhibitor of MEK1 activation).

#### Cell Viability

Cell viability was determined by trypan blue exclusion. The cells were seeded at a density of 5 × 10^4 cells/well in six-well plates. After reaching approximately 90% confluence, the cells were cultured in serum-free medium for 16 hours and then stimulated with test substances for 24 hours. After trypan blue staining, the cells were stained with trypan blue (0.4%), and the numbers of viable (nonstained) and dead (stained) cells were determined with a hemocytometer. Test substances applied were minocycline (0.05 to 50 μM) and triamcinolone (0.01 to 100 μM), respectively.

#### DNA Fragmentation

ELISA (Cell DNA Fragmentation ELISA; Roche) was used to determine whether the RPE cells were to undergo apoptosis or necrosis in the absence and presence of Triton X-100 (1%), minocycline (0.5–50 μM), or triamcinolone (0.1–100 μM). Cells were seeded at 5 × 10^4 cells/well in six-well plates and were cultured until confluence was reached. After the culture media were changed, the cells were prelabeled with BrdU for 16 hours and then incubated in the absence or presence of the test substances in F-10/0.5% fetal calf serum for 6 and 24 hours, respectively. Necrosis was determined by analysis of BrdU-labeled DNA fragments in the cell-free culture supernatants, and apoptosis was determined with the cytoplasmic lysates of the cells. The absence of a minocycline-induced change in the DNA fragmentation rate of cell lysates was confirmed by electrophoretic separation of the DNA samples on a 1.2% agarose gel (Apoptotic DNA Ladder Detection Kit I; Promokine, Heidelberg, Germany).

#### Total RNA Isolation

Total RNA was extracted from cultured cells (RNeasy Mini Kit; Qiagen, Hilden, Germany). RNA quality was analyzed by agarose gel electrophoresis. The A₂₆₀/A₂₈₀ ratio of optical density was measured (GeneQuantpro device; Pharmacia, Uppsala, Sweden) and was between 1.9 and 2.1 for all RNA samples, indicating sufficient quality.

#### Real-Time RT-PCR

After treatment with DNase I (Roche), cDNA was synthesized from 1 μg total RNA (RevertAid H Minus First-Strand cDNA Synthesis Kit; Fermentas, St. Leon-Roth, Germany). For subsequent PCR amplification, the cDNA was diluted by the addition of 20 μL RNase-free water. Semiquantitative real-time RT-PCR was performed with a PCR detection system (Single-Color Real-Time; Bio-Rad, Munich, Germany) using the primer pairs described in Table 1. The PCR solution contained 1 μL cDNA, specific primer set (0.25 μM each), and 10 μL SYBR green PCR kit (QuantiTeq; Qiagen) in a final volume of 20 μL. The following conditions were used: initial denaturation and enzyme activation (1
cycle at 95°C for 15 minutes); denaturation, amplification, and quantification, 45 cycles at 95°C for 30 seconds, 58°C for 20 seconds, and 72°C for 45 seconds; melting curve, 55°C with the temperature gradually increased (0.5°C) up to 95°C. Amplified samples were analyzed by standard agarose gel electrophoresis. mRNA expression was normalized to the levels of ACTB mRNA. Changes in mRNA expression were calculated according to the 2^{-ΔΔCT} method (cycle threshold [CT]), with ΔCT = CTtarget gene − CTACTB and ΔΔCT = ΔCTtreatment − ΔCTcontrol.

ELISA

Cells were cultured at 3 × 10^5 cells/well in 96-well plates (100 μL culture medium/well). At a confluence of approximately 80%, the cells were cultured in serum-free medium for 16 hours. Subsequently, the culture medium was changed, and the cells were stimulated with minocycline (0.005–100 μM) and triamcinolone (0.001–100 μM), respectively, in the absence and presence of PDGF (10 ng/mL). The supernatants were collected after 6 hours, and the level of vascular endothelial growth factor (VEGF)-A140 in the cultured media (200 μL) was determined by ELISA (R&D Systems).

Western Blot Analysis

To reveal whether minocycline alters the phosphorylation state of p38 MAPK and of the extracellular signal-regulated kinases (ERK)-1/2 (p44/p42 MAPK), we performed Western blot analysis. Cells were seeded at 5 × 10^5 cells/well in six-well plates in 1.5 mL complete medium and were allowed to grow up to a confluence of approximately 80%. After growth arrest for 16 hours, the cells were pretreated with blocking substances for 30 minutes and thereafter with test substances for 10 or 30 minutes. Then the medium was removed, the cells were washed twice with prechilled phosphate-buffered saline (pH 7.4; Invitrogen, Paisley, UK), and the monolayer was scraped into 150 L lysis buffer (Mammalian Cell Lysis-1 Kit; Sigma). Total cell lysates were centrifuged at 10,000 g for 10 minutes, and supernatants were analyzed by immunoblots. Equal amounts of protein (30 μg) were separated by 10% SDS-PAGE. Immunoblots were probed with primary and secondary antibodies, and immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. We tested minocycline (5 μM) in the absence and presence of SB203580 (10 μM) and PD98059 (20 μM), respectively. PDGF (10 ng/mL) was used as positive control.

Statistical Analysis

With the exception of PCR data, all data are expressed as a percentage of untreated control (100%). For each test, at least three independent experiments were carried out in triplicate. Data are expressed as mean ± SEM; statistical significance (Mann Whitney U test) was accepted at P < 0.05.

RESULTS

RPE Cell Proliferation

To determine whether minocycline alters the physiological characteristics of RPE cells, we measured the proliferation and chemotaxis of cultured cells in the absence and presence of PDGF, a known mitogen and motogen of RPE cells.43 As shown in Figure 1A, minocycline induced a concentration-dependent decrease in the proliferation rate of RPE cells, both in the absence and in the presence of PDGF. Significant proliferation-decreasing effects were observed at concentrations above 50 nM minocycline. At concentrations above 25 μM, minocycline nearly fully inhibited the proliferation of RPE cells. In contrast to minocycline, triamcinolone did not significantly change the proliferation rate of the cells, either in the absence or in the presence of PDGF (Fig. 1B). Triamcinolone did not alter the proliferation-inhibitory effect of minocycline (Fig. 1C).

Chemotaxis

Minocycline between concentrations of 50 nM and 20 μM induced a concentration-dependent increase in the chemotaxis of RPE cells (Fig. 2A). The stimulatory effect on RPE cell migration was apparently decreased at 50 μM minocycline. Minocycline did not change the chemotaxis evoked by PDGF (Fig. 2A). Triamcinolone alone did not alter the chemotaxis in RPE cells, but it significantly decreased the chemotaxis evoked by PDGF (Fig. 2B). The stimulatory effect of minocycline (20 μM) on chemotaxis was not inhibited by the coadministration of triamcinolone (100 μM; not shown).

Figure 1. Concentration-dependent effects of minocycline (A) and triamcinolone acetamide (B) on the proliferation of RPE cells. The effects were determined in the absence (control) and presence of PDGF (10 ng/mL). (C) Triamcinolone (Triam; 50 μM) did not alter the proliferation-inhibitory effect of minocycline (Mino; 20 μM). The rate of BrdU incorporation was measured after 24-hour incubation with the agents. Data are mean ± SEM of four or five independent experiments using cells from different donors and are expressed as percentage of untreated control (100%). Significant difference versus control: *P < 0.05; **P < 0.01.

Table 1. Primer Pairs Used for PCR Experiments

<table>
<thead>
<tr>
<th>Gene Accession No.</th>
<th>Primer Sequence (5′→3′)</th>
<th>AmpliCON (bp)</th>
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<tr>
<td>ACTB</td>
<td>s: ATGGCCGAGGTTGCCTTCGACG</td>
<td>237</td>
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<tr>
<td>NM_0011101</td>
<td>a.s: CATGTTGTCGCGGCGGCGAAGACC</td>
<td>275</td>
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<tr>
<td>VEGFA</td>
<td>s: CCGTGTGCACTTCTCAAGGACTA</td>
<td>407, 347</td>
</tr>
<tr>
<td>NM_001171630</td>
<td>a.s: CTGACGCCGTCGGGGCTGGTCACA</td>
<td>190</td>
</tr>
<tr>
<td>HGF</td>
<td>s: GGTGCGGCGTCTCACTGGATTTG</td>
<td>179</td>
</tr>
<tr>
<td>NM_000601</td>
<td>a.s: CACACCATATCCCGCTACAT</td>
<td>190</td>
</tr>
<tr>
<td>PDGFA</td>
<td>s: GAAGACCCAGGAGCTCACTTTTTT</td>
<td>235</td>
</tr>
<tr>
<td>NM_035023</td>
<td>a.s: CCTGACGTATTCCACCGGTTG</td>
<td>190</td>
</tr>
<tr>
<td>PDGFB</td>
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<td>179</td>
</tr>
<tr>
<td>NM_035016</td>
<td>a.s: CTGACGCTCAAGGGGCTTCCT</td>
<td>179</td>
</tr>
<tr>
<td>MMP9</td>
<td>s: CGCAGCCGTCAAGGAGTGG</td>
<td>179</td>
</tr>
<tr>
<td>NM_004994</td>
<td>a.s: GCCATTCACGTGGTCTTTAT</td>
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s, sense; as, antisense.
Although both minocycline and PDGF stimulated the chemotaxis of RPE cells, coadministration of both agents did not result in an additive effect (Fig. 2A). This suggests that the effect of minocycline could be mediated by the stimulation of autocrine release of PDGF. To prove this assumption, we tested the selective inhibitor of the PDGF receptor tyrosine kinase AG1296 and found that this tyrphostin fully prevented the stimulatory effect of minocycline on the chemotaxis of RPE cells (Fig. 3A).

It has been shown that activation of the p38 MAPK is involved in the mediation of PDGF-evoked RPE cell migration. To prove whether activation of p38 MAPK and ERK1/2 is involved in mediating the chemotactic effect of minocycline, we tested minocycline in the presence of pharmacologic blockers. As shown in Figure 3B, the chemotactic effect of minocycline was fully prevented in the presence of the inhibitor of p38 activation, SB203580, whereas the inhibitor of ERK1/2 activation, PD98059, did not alter the effect of minocycline.

It has been shown that PDGF evokes the phosphorylation of p38 and ERK1/2 proteins in RPE cells (Fig. 3C). To determine whether minocycline treatment results in the activation of p38 and ERK1/2, we carried out Western blot analysis. As shown in Figure 3C, effects of minocycline (Mino; 5 μM) and PDGF (10 ng/mL) on the cellular amount of phosphorylated p38 and ERK1/2 proteins. Amounts of phosphorylated proteins are shown above; amounts of total proteins are shown below.

Cells were preincubated with the blocking substances for 30 minutes and, subsequently, stimulated with minocycline and PDGF, respectively, for 30 minutes. Similar results were obtained after 10-minute stimulation with minocycline and PDGF (not shown). (A, B) Data are mean ± SEM of five or six independent experiments using cells from different donors and are expressed as percentage of untreated control (100%). Significant difference versus untreated control: *P < 0.05; **P < 0.01; ***P < 0.001. Significant difference versus PDGF: *P < 0.05.
shown in Figure 3C, minocycline treatment resulted in increased amounts of phosphorylated p38 and ERK1/2 proteins in RPE cells. The amount of phosphorylated p38 protein was decreased by SB203580 (under both control and minocycline-stimulated conditions), whereas the amount of phosphorylated ERK1/2 was decreased by PD98059 (Fig. 3C). Western blot analysis also revealed that the inhibition of ERK1/2 activation by PD98059 resulted in increased phosphorylation of p38, whereas the inhibition of p38 activation by SB203580 resulted in increased phosphorylation of ERK1/2 (Fig. 3C). These results confirm the previous finding of the presence of an inhibitory cross-talk between p38 and ERK1/2 in RPE cells. This cross-talk is assumed to be the reason for the chemotaxis-stimulatory effect of PD98059 (Fig. 3B) and of the proliferation-enhancing effect of SB203580 (not shown), respectively.

**Cell Viability**

To examine whether minocycline has an effect on the viability of RPE cells, we stained the cells with trypan blue and counted the living and dying cells. As shown in Figure 4A, minocycline at high concentrations (20 and 50 μM) dose dependently decreased the viability of RPE cells. This suggests that minocycline at high concentrations is toxic to RPE cells. Although triamcinolone also decreased slightly the viability of RPE cells at higher concentrations, this effect was not significant (Fig. 4B). Triamcinolone did not significantly alter the effect of minocycline on cell viability (Fig. 4C).

**DNA Fragmentation**

By measurement of the internucleosomal DNA fragmentation, we determined whether the decrease in cell viability was mediated by evoking apoptosis or necrosis in RPE cells. An increase in the DNA fragmentation rate in the cell lysate reflects a higher apoptosis rate, whereas an increase in the DNA fragmentation rate in the cultured media indicates an increase in cell necrosis. Triton X-100 was used as positive control. As shown in Figure 5A, Triton induced an increase in the DNA fragmentation rate in the RPE cell lysate after 6 and 24 hours of stimulation, whereas the DNA fragmentation rate in the cultured media remained unchanged after 6 hours and increased significantly after 24 hours of stimulation. The data suggest that Triton evoked early apoptosis and delayed necrosis of RPE cells. Minocycline did not change the DNA fragmentation rate in the RPE cell lysates, even at the highest concentration tested (50 μM; Fig. 5B). The absence of minocycline (0.5, 5, 50 μM)-induced change in the DNA fragmentation rate of RPE cell lysates was confirmed by the absence of DNA laddering in electrophoretically separated DNA samples (data not shown). However, minocycline at 50 μM evoked a time-dependent increase in the DNA fragmentation rate in the cultured media (Fig. 5B). The data suggest that minocycline at high concentrations induces the necrosis of RPE cells. Treatment of the cultures with triamcinolone did not result in changes of the DNA fragmentation rate in the cell lysates or cultured media (Fig. 5C).

**Expression of Growth Factors and MMP-9**

We found that minocycline at low concentrations promotes the chemokinesis of RPE cells through stimulation of autocrine PDGF signaling (Fig. 3A). Cell scattering induced by the hepatocyte growth factor (HGF) is a precondition of RPE cell migration.44–46 In addition, matrix metalloproteinases (MMPs) such as MMP-9 stimulate the chemotaxis of RPE cells.47 To examine whether minocycline stimulates the expression of growth factors and MMP-9, we carried out semiquantitative real-time RT-PCR. As shown in Figures 6A and 6B, stimulation of the cultures with minocycline for 2 hours resulted in a significant increase in the expression of HGF, PDGF-B, MMP-9, and VEGF. In contrast, triamcinolone did not promote the expression of these factors (Figs. 6A, 6B). PDGF is known to upregulate the expression of VEGF in RPE cells.45 We found that the PDGF-evoked increase in VEGF expression was prevented by triamcinolone but not by minocycline (Fig. 6B). Triamcinolone did not inhibit the stimulatory effect of minocycline on the expression of VEGF (Fig. 6C). The additive effect of PDGF and minocycline on the expression of VEGF (Fig. 6B) suggests that the effects of both agents are mediated by different intracellular signaling mechanisms.

**Secretion of VEGF**

Because minocycline stimulated the expression of VEGF (Fig. 6B), we determined whether this agent has an effect on the secretion of VEGF from RPE cells. We found that minocycline in concentrations between 5 nM and 5 μM did not alter the VEGF content of the cultured media (Fig. 7A). At higher concentrations (50 and 100 μM), minocycline significantly reduced the secretion of VEGF from the cells (Fig. 7A). Minocycline in concentrations between 5 nM and 5 μM slightly, but nonsignificantly, decreased the PDGF-evoked secretion of VEGF (Fig. 7A). At higher concentrations, it fully prevented the effect of PDGF (Fig. 7A). In contrast to minocycline, triamcinolone at all concentrations tested reduced significantly the secretion of VEGF from RPE cells, under both control and PDGF-stimulated conditions (Fig. 7B). The absence of an effect of triamcinolone on the expression of VEGF (Fig. 6B) and the depressing effect of triamcinolone on the secretion of VEGF (Fig. 7B) are in agreement with the previous finding that this steroid acts at the posttranscriptional level.48

**Discussion**

The anti-inflammatory and neuroprotective effects of minocycline observed in models of retinal degeneration recommend...
this substance as a candidate agent for further consideration as a therapeutic drug for reducing retinal complications of diabetes and for treating age-related macular degeneration. In the present study, we determined the effects of minocycline on the viability and physiological properties of cultured human RPE cells. We found that minocycline at low concentrations stimulated the chemotaxis and inhibited the proliferation of RPE cells. The chemotactic effect of minocycline was mediated by the stimulation of autocrine PDGF signaling, the activation of p38 MAPK, and an increase in the expression of other factors involved in cell scattering and cell migration, such as HGF and MMP-9. Minocycline at higher concentrations decreased the viability of RPE cells through the induction of cell necrosis. Coadministration of triamcinolone did not reverse the adverse effects of minocycline on RPE cells.

In a recent study, the maximum protection of cultured photoreceptors from light-evoked apoptosis was observed with minocycline at concentrations between 20 nM and 1.25 μM. Although this dose was below the concentration of minocycline that decreased the viability of RPE cells (above 5 μM; Fig. 4A), we found that minocycline stimulated the chemotaxis of RPE cells at low concentrations (50 nM-20 μM; Fig. 2A), and decreased the proliferation of RPE cells at low concentrations (above 50 nM; Fig. 1A). Because the cell viability was depressed only at higher concentrations, we assume that the decrease in RPE cell proliferation at concentrations between 0.5 and 5 μM minocycline was not caused by inducing cell necrosis. At higher concentrations (above 5 μM), the necrosis-inducing effect of minocycline contributed to the decreased proliferation rate (Fig. 1A), the lower stimulatory effect of minocycline on chemotaxis (Fig. 2A), and the decreased secretion of VEGF (Fig. 7A).

RPE cells are key players in proliferative retinopathies and subretinal edema. Based on data obtained in animal models, HGF and PDGF have been particularly implicated in the development of proliferative retinal diseases. HGF and MMPs were shown to induce the first steps of RPE cell activation, including an epithelial-to-mesenchymal shift in the cell shape, cell scattering, and cell migration. HGF-evoked cell scattering and MMP-induced degradation of the tight junction protein occludin may contribute to the development of subretinal edema. We found that minocycline increased the expression of HGF, PDGF-B, and MMP-9 in the retinal pigment epithelium. VEGF is the major angiogenic factor in the retina. We found that minocycline increased the expression but not the secretion of VEGF. Although minocycline and PDGF increased the expression of VEGF in an additive fashion (Fig. 6B), the PDGF-evoked secretion of VEGF was slightly reduced in the presence of minocycline (Fig. 7A), suggesting that minocycline exerts multiple effects in RPE cells at transcriptional and posttranscriptional levels.

How minocycline induces chemotaxis and necrosis of RPE cells remains to be determined in future experiments. It has been shown that minocycline reduces mitochondrial calcium retention capacity, resulting in enhanced sensitivity toward mitochondrial permeability transition. Mitochondrial dysfunction evoked by minocycline may contribute to the induction of RPE cell necrosis at high dosing. Imbalances in cellular calcium metabolism may also induce RPE cell activation, including chemokinesis and enhanced expression of growth factors and MMP-9 at low dosing.

The dose of minocycline commonly used in humans to treat chronic infections is 3 mg/kg/d, which corresponds to peak concentrations below 10 μM in the cerebrospinal fluid.
though the most deleterious effects of minocycline on RPE cells were observed at concentrations greater than 5 μM (Fig. 4A), activation of RPE cells resulting in cellular migration was observed at the lowest concentration of minocycline tested (50 nM; Fig. 2A). On the other hand, the decrease in PDGF-evoked VEGF release (Fig. 7A) might have contributed to the protective effect of minocycline on the retina. The relevance of the present in vitro results for the understanding of the in vivo situation remains to be determined. It has been described that high-dose or long-term treatment of acne vulgaris with minocycline may be associated with adverse visual effects, including papilledema.38–40 No adverse effects of minocycline on the retinal function and integrity have been described until now in animal models of retinopathies. Therefore, further research is required to reveal the protective and adverse effects of minocycline on retinal cells in vivo and to determine a concentration window with maximal protective and minimal adverse effects.

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References


