Diabetic retinopathy (DR) is a retinal microvascular complication of diabetes with multifactorial pathogenesis and is still a leading cause of visual impairment in the developed countries. In its advanced stage, proliferative DR (PDR), retinal ischemia due to vasculature obliteration, triggers aberrant angiogenesis at the vitreoretinal interface, leading to the formation of fibrovascular tissues. The contraction of fibrovascular tissues causes subsequent tractional retinal detachment and vitreous hemorrhage, both of which eventually result in severe visual dysfunction in patients with DR.

Previous studies revealed that Müller glial cells, the principal glia of the retina, participate in the fibrovascular tissue formation as cellular components in eyes affected by PDR. Furthermore, accumulating experimental evidence has demonstrated that Müller cell–derived fibroblasts are one of the cell populations capable of generating tractional force against fibrovascular tissue in PDR eyes. In addition, it is well known that migrated glial cells are the source of VEGF, a key mediator of PDR pathogenesis. Therefore, the knowledge of how glial cell migration is enhanced in eyes affected by PDR is of great practical interest; however, the conclusive details remain elusive.

Acrolein is a highly reactive unsaturated aldehyde that causes dysfunction of multiple proteins through the preferential conjugation to cysteine (Cys), lysine (Lys), and histidine (His) residues. Acrolein was previously considered an exogenous pollutant that plays a causative role in pulmonary disorders. However, recently, it has been elucidated that acrolein is produced endogenously as a consequence of peroxidation of unsaturated fatty acids and polyamine metabolism, and much attention has been paid to the role of this toxic aldehyde in other systemic disorders. We recently reported that the level of the acrolein-conjugated protein, Nε-(3-formyl-3,4-dehydropipеридино) lysine adduct (FDP-Lys) was elevated in the vitreous fluid of the patients with PDR. In addition, we also found that FDP-Lys largely accumulated in glial cells of fibrovascular tissues obtained from the patients with PDR. Therefore, our previous data indicate that acrolein and/or the conjugated protein FDP-Lys play a crucial role in the pathogenesis of DR, possibly through the modulation of cellular characteristics of glial cells.

In the current study, we explored the impact of acrolein on retinal glial cell migration.

**Purpose.** To investigate the effect of the unsaturated aldehyde acrolein on retinal glial cell migration.

**Methods.** Müller glial cell markers expression in TR-MUL5 were confirmed by RT-PCR and immunostaining. Cell viability and migration rate of TR-MUL5 cells were assessed after the stimulation with acrolein. DNA microarray analysis was performed to analyze changes in the expression levels of migration-related genes in Müller glial cells stimulated with acrolein. Real-time PCR and ELISA were performed to validate DNA microarray analysis results. Inhibitors of C-X-C motif chemokine ligand 1 (CXCL1), one of the genes highly upregulated after the exposure to acrolein, and blockers of its receptor, CXCR2, were used to investigate the role of the CXCL1-CXCR2 axis on glial cell migration. CXCL1 concentration was measured in vitreous fluid samples obtained from proliferative diabetic retinopathy (PDR) and non-diabetic control eyes. CXCL1 and CXCR2 expression in glial cells of fibrovascular tissues obtained from PDR patients was examined by immunostaining.

**Results.** At a high concentration, acrolein (100 μM) significantly decreased cell viability. However, in moderate, sublethal concentrations (25–50 μM), acrolein induced cell migration and substantially increased the production of CXCL1 in TR-MUL5 cells. CXCL1 concentration was significantly elevated in vitreous fluids of PDR patients, and CXCL1 and CXCR2 were present in glial cells in fibrovascular tissues of PDR patients. CXCL1 stimulation increased glial cell migration in a dose-dependent manner, which was abrogated by the neutralization of the CXCL1-CXCR2 axis.

**Conclusions.** Our data demonstrate that acrolein promotes retinal Müller glial cell migration by enhancing CXCL1 production.

Keywords: Acrolein, CXCL1, oxidative stress, Müller cell
**Materials and Methods**

**Cell Culture**

Conditionally immortalized retinal Müller cell line TR-MUL5 from transgenic rats harboring the temperature-sensitive SV40 large T antigen gene was provided by Fact Inc. (Sendai, Japan). TR-MUL5 cells were cultured at 33°C in Dulbecco’s modified Eagle’s medium (Fuji Film Wako Pure Chemicals, Osaka, Japan), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) in the atmosphere of 95% air and 5% CO₂.

**Cell Viability Assay**

Cell viability was assessed by using RealTime-Glo MT Cell Viability Assay (Promega, Fitchburg, WI, USA). TR-MUL5 cells were seeded into a 96-well plate at a density of 2 × 10⁴ cells per well and incubated for 24 hours at 33°C in the atmosphere of 95% air and 5% CO₂. The cells were starved and incubated with RealTime-Glo reagent for 17 hours and stimulated with acrolein (2 to 200 µM; AccuStandard, New Haven, CT, USA). Luminescence was measured by an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland).

**Migration Assay**

Migration capacity was assessed by using an Oris cell migration assay kit (Platypus Technologies, Madison, WI, USA) according to the manufacturer’s protocol. Briefly, TR-MUL5 cells were seeded into a collagen I-coated 96-well Oris plate at a density of 5 × 10³ per well and incubated for 24 hours at 33°C in the atmosphere of 95% air and 5% CO₂. The cells were starved for 17 hours and stimulated with 10 to 50 µM acrolein with or without a CXCL1-neutralizing antibody (1–10 µg/mL, MAB515; R&D Systems, Minneapolis, MN, USA) or the CXCR2 inhibitor SB225002 (20–200 nM; Cayman Chemical, Ann Arbor, MI, USA) for 24 hours. Silicone stoppers were then removed to allow cell migration to the central detection zone for 6 hours at 37°C.

**DNA Microarray Analysis**

TR-MUL5 cells were stimulated with or without 25 µM acrolein for 6 hours. Total RNA was collected using NucleoSpin RNA Plus (MACHEREY-NAGEL, Düren, Germany) and subjected to microarray analysis (SurePrint G3 Rat Gene Expression 8 × 60K v2 Microarray; Agilent, Santa Clara, CA, USA), which was outsourced to Takara Bio Inc. (Shiga, Japan). The quality of total RNA was analyzed by a 2100 Bioanalyzer (Agilent). Total RNA was fragmented at 60°C, denatured with denatured salmon sperm DNA, and hybridized to SurePrint G3 Rat Gene Expression 8 × 60K Microarray (Agilent). Slides were scanned immediately after washing on the Agilent SureScan Microarray Scanner (G2600D) using one color scan setting for 8 × 60 k array slides (Scan Area 61 × 21.6 mm, scan resolution 3 µm, dye channel is set to green, PMT is set to 100%). The scanned images were analyzed with Feature Extraction Software 12.0.3.1 (Agilent) using default parameters to obtain background subtracted and spatially detrended processed signal intensities. Processed signal intensities were normalized by the global scaling method. Trimmed mean probe intensity was determined by removing 2% of the lower and the higher end of the probe intensities to calculate the scaling factor. Normalized signal intensities were then calculated from the target intensity on each array using the scaling factor, so that the trimmed mean target intensity of each array was arbitrarily set to 2500.

**Quantitative Real-Time PCR**

Expression level of Cxcl1 mRNA was examined by quantitative real-time PCR. Total RNA was extracted from cells using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) and reverse transcribed to cDNA using GoScript reverse transcriptase (Promega), according to the manufacturer’s protocol. Analysis of mRNA level was performed on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using GoTag qPCR Master Mix (Promega). The primer sequences used for real-time PCR and the expected size of the amplification products were as follows:

- 5’-ACTGCAATGACCTGCAATGTT-3’ (forward) and 5’-TCATGCAACCTGGAAGG-3’ (reverse) for rat Cxcl1 (NM_033845); 124 bp.
- 5’-GGGAAATCTGTCGTGACAT-3’ (forward) and 5’-GGGCCCTGAGCACTCTC-3’ (reverse) for rat Actb (NM_031144); 76 bp.

PCR conditions used were 95°C, 2 minutes; followed by 95°C, 15 seconds; 60°C, 1 minute for 40 cycles. All data were calculated by the ΔΔCt method with the level of Actb mRNA as normalization control.

**ELISA for Cell Culture Supernatant**

TR-MUL5 cells were seeded into a six-well plate at a density of 4 × 10⁵ cells per well and incubated for 24 hours. The cells were serum-starved for 17 hours and stimulated with acrolein (0, 10 µM, 25 µM, 50 µM) for 24 hours. The cell culture supernatant was collected and centrifuged at 1000g for 10 minutes at 4°C. CXCL1 levels in the supernatant were measured using a sandwich ELISA kit (R&D Systems), according to the manufacturer’s protocol.

**Specimens**

Vitreous fluid samples were collected from the eyes of 12 patients with PDR (seven males and five females; mean age, 58.2 ± 4.1 years) who had undergone pars plana vitrectomy for prolonged vitreous hemorrhage and/or tractional retinal detachment resulting from macular lesions. Control vitreous fluid samples were collected from the eyes of eight patients with nondiabetic ocular diseases (four males and four females; mean age, 68.6 ± 3.5 years), that is, idiopathic epiretinal membrane (ERM) and idiopathic macular hole (MH). Undiluted vitreous samples were collected into sterile tubes and were frozen rapidly at ~80°C. Clinical characteristics of the patients are shown in Table 1. Fibrovascular tissues surgically removed...
from the patients with PDR (two males and one female; mean age, 52.7 ± 6.9 years) were used for immunofluorescence microscopy. All experiments were conducted in accordance with the tenets of the Declaration of Helsinki, following an approval from the institutional review committee of Hokkaido University Hospital (institutional review board #015-0124, #014-0293). Written informed consent was obtained from all patients after an explanation of the purpose and procedures of this study.

**ELISA for Vitreous Fluid**

CXCL1 protein levels in the vitreous samples were measured using an ELISA kit for human CXCL1 (R&D Systems). The vitreous samples were used without dilution.

**Immunofluorescence Microscopy**

Paraffin sections of fibrovascular tissues were deparaffinized and hydrated through exposure with xylene and graded alcohols followed by water. After microwave-based antigen retrieval in 10 mM citrate buffer (pH 6.0), sections were incubated in 10% normal goat serum (Thermo Fisher Scientific) for 30 minutes and then incubated with a primary rabbit polyclonal antibody against CXCL1 (1:100, 12335-1-AP; Proteintech, Rosemont, IL, USA) or rabbit polyclonal antibody against CXCR2 (1:100, bs1629R; Bioss, Woburn, MA, USA) and a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:50, NCL-L-GFAP-GA5; Leica Biosystems, Wetzlar, Germany) at 4°C overnight before exposure to Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (1:400; Thermo Fisher Scientific) for 1 hour at room temperature. Serial sections were incubated with normal mouse IgG (2 μg/mL; Agilent) and normal rabbit IgG (1 μg/mL; R&D Systems) as negative control instead of primary antibodies.

**Nuclei** were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Roche Applied Science, Indianapolis, IN, USA), and photomicrographs were taken with a fluorescence microscope (BIOREVO, BZ-9000, Keyence, Japan).

**Table 1. Characteristics of the Patients**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, y</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Case</th>
<th>Age, y</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>HbA1c, %</th>
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<td>Female</td>
<td>MH</td>
<td>5</td>
<td>76</td>
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<td>PDR</td>
<td>7.5</td>
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<td>PDR</td>
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TRD, tractional retinal detachment; VH, vitreous hemorrhage.

**Table 2. Primer Sequences and the Expected Size of the Amplification Products**

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<td>Cxcr2</td>
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<td>Reverse GGCAGGTACAACTGTAATTTG</td>
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<tr>
<td>Glut</td>
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<td></td>
<td>Reverse TCCGAGGTTCTATCTGGGA</td>
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<tr>
<td>S100a16</td>
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<tr>
<td></td>
<td>Reverse GTCCGTACACCTGGTTTCACA</td>
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<tr>
<td>VIM</td>
<td>Forward CAGTCATCTACCTGGAAGT</td>
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<td></td>
<td>Reverse GAGTGCGTCTCAACCAGAGGG</td>
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<tr>
<td>Rip1</td>
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<td>Reverse ACTGTCACCCAGATTCGAC</td>
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<td>Rpe65</td>
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<td>Rho</td>
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<td>Reverse CGACGAGACTCCTCATTCCAGA</td>
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Acrolein Promotes Glial Cell Migration

**ELISA for Vitreous Fluid**

CXCL1 protein levels in the vitreous samples were measured using an ELISA kit for human CXCL1 (R&D Systems). The vitreous samples were used without dilution.

**Immunofluorescence Microscopy**

Paraffin sections of fibrovascular tissues were deparaffinized and hydrated through exposure with xylene and graded alcohols followed by water. After microwave-based antigen retrieval in 10 mM citrate buffer (pH 6.0), sections were incubated in 10% normal goat serum (Thermo Fisher Scientific) for 30 minutes and then incubated with a primary rabbit polyclonal antibody against CXCL1 (1:100, 12335-1-AP; Proteintech, Rosemont, IL, USA) or rabbit polyclonal antibody against CXC2R (1:100, bs1629R; Bioss, Woburn, MA, USA) and a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:50, NCL-LGFAP-GA5; Leica Biosystems, Wetzlar, Germany) at 4°C overnight before exposure to Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (1:400; Thermo Fisher Scientific) for 1 hour at room temperature. Serial sections were incubated with normal mouse IgG (2 μg/mL; Agilent) and normal rabbit IgG (1 μg/mL; R&D Systems) as negative control instead of primary antibodies.

**For immunocytochemistry of TR-MUL5**, cells were seeded into a six-well plate with cover glass and fixed with 4% paraformaldehyde for 15 minutes and permeabilized by 0.1% Triton X-100 for 10 minutes. Cells were incubated in 10% normal goat serum for 30 minutes and then incubated with a primary mouse monoclonal antibody against glutamine synthetase (1:200, MAB302; Merck, Darmstadt, Germany) at 4°C overnight prior to the exposure to Alexa Fluor 546 goat anti-mouse IgG (1:400) for 1 hour at room temperature.

**Nuclei** were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Roche Applied Science, Indianapolis, IN, USA), and photomicrographs were taken with a fluorescence microscope (BIOREVO, BZ-9000, Keyence, Japan).
Reverse-Transcription PCR

Total RNA was extracted from TR-MUL5 cells and reverse transcribed to cDNA using GoScript reverse transcriptase (Promega). RT-PCR was performed using Ex Taq hot start version (Takara Bio Inc.) on Mastercycler nexus (Eppendorf, Hamburg, Germany). The primer sequences and the expected size of the amplification products are listed in Table 2. PCR conditions used were 94°C, 2 minutes; followed by 94°C, 30 seconds; 60°C, 30 seconds, 72°C 30 seconds for 40 cycles. H2O was used instead of cDNA as nontemplate control. The products were electrophoresed in 2% agarose gels, stained with SYBR safe (Thermo Fisher Scientific), and visualized with UV irradiation.

Western Blotting

TR-MUL5 cells were lysed in 1× Laemmli buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.01% Bromophenol blue, 5% 2-Mercaptoethanol). The cell lysates were sonicated three times for 5 seconds each on ice and centrifuged at 15,000g at 4°C for 10 minutes. Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific) and adjusted to 2 mg/mL. The samples were boiled at 95°C for 3 minutes, separated by SDS-PAGE, and electroblotted to polyvinylidene fluoride membranes (Merck). Membranes were incubated with 5% skim milk for blocking and then reacted with a rabbit polyclonal antibody against CXCR2 (1/1000, bs-1629R; Bioss) at 4°C overnight and then incubated with goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (1:4000; Promega) at room temperature for 1 hour. Signals were visualized using a SuperSignal west pico chemiluminescent substrate (Thermo Fisher Scientific).

Reactive Oxygen Species (ROS) Detection Assay

Cellular oxidative stress status was measured by using a DCFDA-Cellular ROS Detection Assay Kit (Abcam, Cambridge, MA, USA), according to the manufacturer’s protocol. Briefly, TR-MUL5 cells were seeded into a Black 96-well plate at a density of 1.5 × 10⁴ cells per well and incubated for 24 hours. The cells were serum-starved for 17 hours, stained with 40 μM DCFDA for 45 minutes, and then exposed to 50 μM acrolein. Fluorescence was measured by Infinite 200 PRO (Tecan) after 6 and 24 hours from acrolein stimulation.

Glutathione (GSH) Assay

TR-MUL5 cells were seeded into a 24-well plate at a density of 1 × 10⁵ cells per well and incubated for 24 hours. The cells were serum-starved for 17 hours and stimulated with 10 to 50 μM acrolein for 3 hours. Total GSH levels were measured using a total GSH assay kit (Nikken Seiko Co., Ltd., Shizuoka, Japan), according to the manufacturer’s protocol.
Statistical Analysis

All results are presented as the mean ± SEM. The Student’s t-test was used for pairwise statistical comparisons between groups, and 1-way ANOVA, followed by the post hoc Tukey-Kramer test, if appropriate, was used for multiple comparisons. Differences in the means were considered statistically significant if \( P < 0.05 \).

RESULTS

Acrolein Induces Retinal Glial Cell Migration

To investigate the impact of acrolein on retinal Müller glial cells, cell viability and migration were assessed in TR-MUL5 cells stimulated with acrolein. High-concentration acrolein (100–200 \( \mu \)M) reduced cell viability in comparison with that of glial cells without acrolein stimulation (\( n = 4 \) each; \( P < 0.01 \); Figs. 1A, 1B). However, at lower concentrations (25–50 \( \mu \)M), acrolein induced a 1.3-fold increase of cell viability (\( n = 4 \) each; \( P < 0.01 \); Fig. 1B) and led to an approximate 2.4-fold increase of cell migration (\( n = 16 \) each; \( P < 0.01 \); Figs. 2A, 2B) in TR-MUL5, indicating that sublethal concentration of acrolein increases cell mobility without causing cell death.

To validate the characteristics of TR-MUL5 cells, Müller glial cell marker expression in TR-MUL5 was confirmed by RT-PCR and immunostaining. TR-MUL5 expresses typical Müller cell markers, glutamine synthetase (\( Glul \)), S-100 (\( S100a16 \)), vimentin (\( Vim \)), and CRALBP (\( Rlbp1 \)) (Supplementary Figs. S1A, S1B), but not cell markers for other types of retinal cells (Supplementary Fig. S1C).

Acrolein Causes Inflammatory Chemokine CXCL1 Production in Retinal Glial Cells

To examine global transcriptional profile in retinal glial cells stimulated by a sublethal concentration of acrolein (25 \( \mu \)M), DNA microarray analysis was performed. After normalization and background correction, upregulation of 437 genes (≥2-fold) and downregulation of 822 genes (≤0.5-fold) were revealed in acrolein-stimulated TR-MUL5 cells compared with expression levels of these genes in control conditions. The top five genes upregulated by acrolein are shown in Table 3. Of these, \( Cxcl1 \) was the most highly upregulated gene in TR-MUL5 cells stimulated with acrolein.

To validate microarray analysis data, mRNA and protein expression levels of CXCL1 were quantified by real-time PCR and ELISA, respectively. \( Cxcl1 \) mRNA expression was significantly increased by acrolein in a dose-dependent manner (\( n = 3 \) each, \( P < 0.05 \), \( P < 0.01 \), Fig. 3A), and CXCL1 protein production was also induced by acrolein stimulation in TR-MUL5 cells (\( n = 3 \) each, \( P < 0.05 \), \( P < 0.01 \), Fig. 3B). In addition, RT-PCR and Western blotting demonstrated the presence of CXCR2, a G-protein–coupled receptor family.
member protein known to be the main receptor for CXCL1, in TR-MUL5 cells (Figs. 4A, 4B).

Accumulation of CXCL1 in Eyes Affected by DR

To determine whether the vitreous level of CXCL1 increased in eyes affected by DR, CXCL1 concentration was measured in vitreous fluid samples obtained from PDR and nondiabetic control eyes with idiopathic ERM and MH. CXCL1 was detectable in all vitreous samples, and it was significantly elevated in vitreous fluids of PDR patients (39.4 ± 4.9 pg/mL, n = 12) compared with that in nondiabetic patients (19.5 ± 3.7 pg/mL, n = 8, P < 0.01, Fig. 5A). CXCL1 and CXCR2 were present in GFAP-positive glial cells in fibrovascular tissues obtained from PDR patients (Figs. 5B, 5C; Supplementary Figs. S2A–D).

CXCL1 Promotes Retinal Glial Cell Migration Through Binding to CXCR2

To investigate the impact of CXCL1 on glial cell migration, cell migration rate was evaluated after stimulation with CXCL1. The cell migration rate increased in the TR-MUL5 cell after CXCL1 exposure in a dose-dependent manner (n = 12 for each concentration, P < 0.01, Figs. 6A, 6B). Furthermore, cell migration induced by acrolein was abrogated by either a CXCL1 neutralizing antibody (n = 4, P < 0.01, Figs. 7A, 7B) or the CXCR2 chemical inhibitor SB225002 (n = 4, P < 0.05, P < 0.01, Figs. 7C, 7D).

Oxidative Stress Promotes CXCL1 Production Induced by Acrolein

Because acrolein is known to induce oxidative stress, and CXCL1 gene transcription is enhanced by oxidative stress, we next analyzed the relationship between oxidative stress and acrolein-induced CXCL1 induction in retinal glial cells. Total GSH levels were reduced (n = 4, P < 0.01, Fig. 8A) and ROS levels were increased (n = 4 each, P < 0.01, Fig. 8B) in TR-MUL5 cells after acrolein stimulation. Furthermore, CXCL1 expression induced by acrolein was abolished by the oxidative stress inhibitor N-acetylcysteine (NAC) in a dose-dependent manner (n = 3, P < 0.01, Figs. 8C, 8D).

Discussion

In the present study, we demonstrated that (1) acrolein exerted potent cellular toxicity at a high concentration; however, sublethal concentration of acrolein slightly induces viability of retinal glial cells; (2) sublethal concentration of acrolein also promotes migration of retinal glial cells; (3) acrolein remarkably induced CXCL1 production in retinal glial cells and blockade of either CXCL1 or CXCR2 reduced the migration of glial cells caused by sublethal acrolein; and (4) acrolein increased oxidative stress, which stimulates CXCL1 production, presumably due to GSH reduction (Fig. 9). The current
Figure 5. CXCL1 accumulation and CXCR2 localization in patients with DR. (A) Levels of CXCL1 in the vitreous fluid samples of idiopathic ERM and idiopathic MH (n = 8) and PDR patients (n = 12, **P < 0.01). (B) Representative fluorescent micrographs of immunofluorescence in fibrovascular tissues. (a) Green, GFAP (Alexa Fluor 488). (b) Red, CXCL1 (Alexa Fluor 546). (c) Blue, counterstaining for the nuclei with DAPI. (d) Merged image. Arrows indicate the colocalization of CXCL1 with GFAP in glial cells. (e) Negative control (mouse and rabbit normal IgG) in sequential sections. Scale bar: 25 µm. (C) Representative fluorescent micrographs of immunofluorescence in fibrovascular tissues. (a) Green, GFAP (Alexa Fluor 488). (b) Red, CXCR2 (Alexa Fluor 546). (c) Blue, counterstaining for the nuclei with DAPI. (d) Merged image. Arrows indicate the colocalization of CXCR2 with GFAP in glial cells. (e) Negative control (mouse and rabbit normal IgG) in sequential sections. Scale bar: 25 µm.
data provide new insights into the effect of acrolein on glial cell migration, a crucial pathological event in the development of DR.14,15

Recent studies have demonstrated that acrolein, a toxic unsaturated aldehyde, affects the pathogenesis of systemic disorders, such as neurodegenerative diseases,16 cardiovascular diseases,17 and diabetes.18 With respect to the involvement of acrolein in diabetes, acrolein was reportedly increased in the serum of patients with diabetes.19 It was also shown that the urinary level of acrolein was higher in type 2 diabetes patients than in individuals without diabetes.18 By contrast, little is known about the role of acrolein in ocular diseases, including DR. It has been previously reported that FDP-Lys, an acrolein-conjugated protein, was localized predominantly in glial cells of fibrovascular tissues in patients with PDR.11 Furthermore, FDP-Lys immunoreactivity was shown to be markedly enhanced in Müller glial cells of diabetic rats.20–22 Therefore, both human sample observation and experimental evidence have indicated that acrolein is generated and/or accumulates in retinal glial cells under diabetic conditions. Extending the previous findings, the present study demonstrates that acrolein in sublethal concentrations stimulated cell viability and migration of Müller glial cells, whereas the exposure to a high concentration of 100μM exerted a toxic effect on cellular viability. Our current results are consistent with our previous data in cultured retinal endothelial cells.11 Glial cell activation is the initial response during the early stage of DR,14,15 and retinal glial cells proliferate and migrate into the vitreoretinal interface, leading to fibrovascular tissue formation during PDR progression.23,24 Therefore, the current data indicate that acrolein is potentially one of the molecules triggering the retinal glial cell activation in diabetic eyes.

Next, we sought to explore the mechanism by which acrolein promoted glial cell migration. Based on DNA microarray analysis that revealed multiple candidate genes upregulated by acrolein stimulation, we focused on one of the most highly upregulated mRNAs, Cxcl1, and showed that acrolein stimulation also significantly induced CXCL1 protein production in cultured Müller glial cells. CXCL1 is a member of the C-X-C family of chemokines that promotes neutrophil and tumor cell migration through binding to CXCR2.25 It has been previously shown that the vitreous level of CXCL1 increased in eyes affected by PDR26 and in the retinal tissue of diabetic mice.27 In accordance with the previous data, in the current study we also demonstrated that CXCL1 concentration was higher in the vitreous fluid of the patients with DR than in individuals without diabetes. Furthermore, immunofluorescence microscopy and in vitro experiments revealed that CXCL1 was localized in GFAP-positive cells of fibrovascular tissues and CXCL1 promoted glial cell migration, respectively. As we have previously reported,11 FDP-Lys largely accumulates in glial cells of fibrovascular tissues. Taken together, several lines of evidence suggest that CXCL1 stimulates retinal glial cells in an autocrine fashion through its receptor, CXCR2, in response to acrolein under diabetic conditions.

In addition, we found that acrolein-induced CXCL1 production was suppressed by NAC, a thiol-containing antioxidant and precursor of GSH, in cultured Müller glial cells. Acrolein and its adduct FDP-Lyso are known to covalently bind to thiols such as GSH. Hence, it is conceivable that acrolein stimulation increased the intracellular oxidative stress, which is a known pathological change in diabetes, through the dysfunction of antioxidant systems in Müller glial cells. An increasing amount of evidence shows that oxidative stress caused by diabetes-induced metabolic abnormalities plays a pivotal role in the pathogenesis of DR.28 Oxidative stress is known to increase histone acetylation and transcription of inflammatory chemokines, including CXCL1.13 Therefore, multiple lines of evidence indicated that acrolein-induced oxidative stress mediated CXCL1 production in Müller glial cells. However, it is also known that the damage-associated molecular pattern molecules (DAMPS) also induce CXCL1 expression in necro-

![Figure 6.](https://example.com/figure6) Impact of CXCL1 protein on retinal glial cell migration. (A) Representative micrographs of TR-MUL5 cell migration stimulated with recombinant rat CXCL1 (0–10 ng/mL). White dotted line indicates the base line, and red solid line indicates the edge of the migrating cells. Scale bar: 250 μm. (B) Cell migration analysis in TR-MUL5 cells stimulated with CXCL1 (0–10 ng/mL, n = 12 each, **P < 0.01).
Figure 7. Impact of blockade for CXCL1-CXCR2 axis on retinal glial cell migration induced by acrolein. (A) Representative micrographs of TR-MUL5 cell migration control stimulated with acrolein with or without CXCL1 neutralization antibody (1–10 μg/mL). White dotted line indicates the base line, and red solid line indicates the edge of the migrating cells. Scale bar: 250 μm. (B) Cell migration analysis in TR-MUL5 cells stimulated with acrolein with or without CXCL1 antibody (1–10 μg/mL, n = 4 each, **P < 0.01). (C) Representative micrographs of TR-MUL5 cell migration stimulated with acrolein with or without CXCR2 inhibitor SB225002 (200 nM) or 0.001% ethanol. White dotted line indicates the base line, and red solid line indicates the edge of the migrating cells. Scale bar: 250 μm. (D) Cell migration analysis in TR-MUL5 cells stimulated with acrolein with or without CXCR2 inhibitor SB225002 (20–200 nM) or 0.001% ethanol as a solvent control (n = 4 each, *P < 0.05, **P < 0.01).
In the current study, sublethal concentration of acrolein slightly increases cell viability; therefore, it is likely that CXCL1 production shown in this study was not derived from DAMPs released by necrotic cells. Alternatively, it was also reported that high mobility group box-1 (HMGB1), a representative DAMPs, is secreted from not only necrotic cells, but also viable cells under pathological conditions, such as brain injury, Parkinson’s disease, and ischemic retina. Therefore, it is possible that DAMPs (e.g., HMGB1) are involved in CXCL1 production by viable glial cells stimulated with acrolein. Further analysis is necessary to clarify the detailed molecular mechanism of CXCL1 induction by acrolein in retinal glial cells.

In summary, the current data provide a novel insight into the role of unsaturated aldehyde acrolein on the migration of Müller glial cells, which eventually contribute to fibrovascular tissue formation, suggesting that acrolein is an attractive molecular target for the prevention of PDR.

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**FIGURE 8.** Role of oxidative stress in retinal glial cell migration induced by acrolein. (A) Total GSH levels in TR-MUL5 cells stimulated with acrolein for 3 hours (10–50 μM, n = 4 each, **P < 0.01). (B) Relative ROS levels in TR-MUL5 cell stimulated with or without acrolein (50 μM) stimulation for 6 and 24 hours (n = 4 each, **P < 0.01). (C) Cxcl1 mRNA expression in TR-MUL5 cells stimulated with acrolein (25 μM) with or without NAC (5 to 500 μM) for 6 hours, analyzed by real-time PCR (n = 3 each, **P < 0.01). (D) Cxcl1 protein in supernatants of TR-MUL5 cells stimulated with acrolein (25 μM) with or without NAC (5–500 μM) for 24 hours, analyzed by ELISA (n = 3 each, *P < 0.05, **P < 0.01).

**FIGURE 9.** A schematic figure depicting proposed mechanism of acrolein-driven Müller glial cell migration.

1. Acrolein
2. Müller cell
3. GSH↓
4. Oxidative stress↑
5. Migration↑
6. CXCL1
7. CXCR2
References


