Role of Metallothioneins 1 and 2 in Ocular Neovascularization

Shinsuke Nakamura,1 Masamitsu Shimazawa,1 Yuki Inoue,1 Shinsuke Takata,1 Yasushi Ito,1 Kazuhiro Tsuruma,1 Tsunehiko Ikeda,2 Akiko Honda,3 Masahiko Satoh,3 and Hideaki Hara1

1Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, Gifu, Japan
2Department of Ophthalmology, Osaka Medical College, Osaka, Japan
3Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, Nagoya, Japan

Correspondence: Hideaki Hara, Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan; hidehara@gifu-pu.ac.jp.
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Purpose. The incidence of blindness is increasing, in part, because of abnormal ocular neovascularization. Anti-VEGF therapies have yielded impressive results; however, they are not a cure for blindness. Recently, metallothioneins (MTs) 1 and 2 have been implicated in the process of angiogenesis. Therefore, we investigated whether MT-1 and MT-2 were also involved in ocular neovascularization.

Methods. The concentrations of MT-1 and MT-2 (hereafter MT-1/2) were observed by ELISA. We examined the role of MT-1/2 in ocular neovascularization by using both an oxygen-induced retinopathy (OIR) model and a laser-induced choroidal neovascularization (CNV) model. We investigated the localization of MT-1/2 in retina. Furthermore, we investigated the expression of hypoxia-inducible factor (HIF)-1α and VEGF in OIR. In vitro, we investigated the degradation of HIF-1α.

Results. The MT-1/2 were significantly elevated in proliferative diabetic retinopathy patients. Ocular neovascularization, which was induced in both the OIR model and the CNV model, was decreased in MT-1/2 knockout (KO) mice. We confirmed that although MT-1/2 was expressed throughout the murine retina, its expression levels were highest in the endothelial cells. Further, OIR enhanced MT-1/2 expression in the retina. Interestingly, in the OIR model, both HIF-1α and VEGF levels were significantly decreased in the retina of MT-1/2 KO mice. In addition, we found that knockdown of MT-1/2 accelerated ubiquitination of HIF-1α.

Conclusions. These results indicate that MT-1/2 are involved in retinal and choroidal neovascularization, and that MT-1/2 might be a new therapeutic target in diseases in which ocular angiogenesis is implicated.

Keywords: HIF-1α, metallothionein, neovascularization, VEGF

Pathologic neovascularization in the eye is the major cause of blindness in retinopathy of prematurity (ROP), diabetic retinopathy (DR), and age-related macular degeneration (AMD). The abnormal vessels resulting from neovascularization cause cumulative increase in vision loss, and this debilitating disease is becoming a serious public health problem. At the onset of ocular neovascularization, tissue hypoxia leads to increased production of angiogenic growth factors (particularly vascular endothelial growth factor, VEGF), which trigger the pathologic overgrowth of new vascular networks. Vascular endothelial growth factor is a key angiogenic and vasopermeability factor that is upregulated in ROP, DR, and AMD, where it has been shown to promote blood–retinal barrier (BRB) breakdown and neovascularization. In fact, anti-VEGF therapy is an effective treatment for ocular ischemic diseases, such as ischemic central retinal vein occlusion, 9 DR, 10 and AMD.11 However, relapse following intravitreal administration of anti-VEGF agents is a major problem.12 Thus, additional therapeutic strategies for ocular diseases need to be developed; these will likely emerge from the discovery of new causative factors.

Metallothionein (MT) is a cysteine-rich metal-binding protein that has four major isoforms: MTs 1, 2, 3, and 4. In mammals, MTs 1 and 2 (hereafter MT-1/2) are found in most organs, whereas MT-3 is expressed in the brain, and MT-4 is most abundant in certain stratified squamous epithelial tissues. Metallothionein is involved in various physiological processes such as detoxification of toxic metals and scavenging of free radicals, and is implicated in various diseases such as breast cancer, diabetes, Alzheimer’s disease, transitional cell carcinoma, and Parkinson’s disease. In particular, several reports have suggested that MT-1/2 play a role in protection from ischemia–reperfusion injury to the heart and focal cerebral ischemia. We have already reported that MT-1/2 protected against retinal damage in mice, and other researchers have detected that MT-1/2 have major regulatory functions in the angiogenic process in a mouse model of cortical freeze injury. Additionally, MT-1/2 knockout is associated with impairment of both flow recovery and capillary angiogenesis in a mouse model of acute hindlimb ischemia.

Our objective in the present study was to evaluate the role of MT-1/2 in ocular neovascularization. To this end, we measured MT-1/2 and VEGF in vitreous fluid from patients with proliferative diabetic retinopathy (PDR). Ocular neovascularization was evaluated in MT-1/2 KO mice using both the oxygen-induced retinopathy (OIR) model and the laser-induced choroidal neovascularization (CNV) model. Using the OIR
TABLE. Data From Patients With Proliferative Diabetic Retinopathy and Other Ocular Diseases

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Macular Hole</th>
<th>Proliferative Diabetic Retinopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Age, y, mean ± SEM</td>
<td>67.0 ± 1.8</td>
<td>58.4 ± 2.7</td>
</tr>
<tr>
<td>Number of female patients</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Macular edema</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>+ Proliferative membrane</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>+ Traction membrane</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Traction membrane</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>+ TRD</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>+ MTRD</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>+ EMTRD</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Proliferative membrane</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>+ Tractional RD</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>+ NVD</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>+ NVE</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>+ PVR</td>
<td>1</td>
<td></td>
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<tr>
<td>Alone</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Complications of each patient were shown in clinical findings. TRD, tractional retinal detachment; MTRD, macular tractional retinal detachment; EMTRD, elsewhere macular tractional retinal detachment; RD, retinal detachment; NVD, neovascularization on the disc; NVE, neovascularization elsewhere on the disc; PVR, proliferative vitreoretinopathy.

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model, we also investigated whether MT-1/2 modulated the retinal expression of VEGF and hypoxia-inducible factor (HIF)-1α. Additional in vitro experiments were performed in human retinal microvascular endothelial cells (HRMECs) to determine the role of MT in the angiogenic response to ischemia.

MATERIALS AND METHODS

Preparation of Vitreous Fluid Samples

The collection of vitreous fluid was performed according to the tenets of the Declaration of Helsinki after receiving the approvals from the institutional review committee of Osaka Medical College. Furthermore, we obtained informed consent from 39 patients. Vitreous samples were collected from 39 eyes undergoing pars plana vitrectomy for the treatment of macular hole and DR at Osaka Medical College Hospital. Undiluted vitreous samples were collected from 39 eyes of 39 individuals with either macular hole (MH; 19 eyes) or proliferative diabetic retinopathy (PDR; 20 eyes). None of the patients with an idiopathic MH was affected with diabetes. Details of the patients are shown in the Table.

Vitreous specimens were collected in sterile tubes, placed immediately on ice, centrifuged at 200g for 5 minutes to separate the cell contents, and then homogenized and immediately stored at −80°C until analyzed.

MT-1/2 Measurement in Human Vitreous Fluid

We measured the concentration of MT-1/2 in human vitreous fluid with the metallothionein ELISA kit (Frontier Science Co., Ltd., Hokkaido, Japan). The antibody epitope in the kit recognizes the N-terminals and could react against MT-1 and -2, which have common N-terminals sequence. We added 50 μL standard or assay sample (Frontier Science Co., Ltd.) to each well and then added the antibody solution (50 μL, Frontier Science Co., Ltd.) to the same wells. The plate was covered, incubated at 4°C for 1 hour, and washed. Then, we added 100 μL of the diluted second antibody solution to each plate wells and incubated for 1 hour at room temperature. The optical density of the wells containing the eight standard concentrations of MT was measured using a microplate reader at 450 nm (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

VEGF Measurement in Human Vitreous Fluid

The concentration of VEGF in the human vitreous fluids was measured by using the VEGF enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, Inc.). Sample diluent (50 μL; Thermo Fisher Scientific, Inc.) and 50 μL of either the standard control or a 10-fold-diluted vitreous fluid sample were added to each well, incubated for 2 hours at room temperature, and washed. One hundred microliters of anti-human VEGF biotinylated antibody reagent (Thermo Fisher Scientific, Inc.) was added to each well, incubated for 1 hour at room temperature, and washed. Streptavidin-HRP Reagent for ELISA (Thermo Fisher Scientific, Inc.) was added to each well, incubated for 30 minutes at room temperature, and then washed. One hundred microliters of TMB Substrate solution (Thermo Fisher Scientific, Inc.) was added to each well, and the plate was developed in darkness at room temperature for 30 minutes. Next, 100 μL stop solution (Thermo Fisher Scientific, Inc.) was added, and concentrations were determined at 450 nm using the microplate reader.

Animals

MT-1/2 KO mice (null mutation of MT-1 and MT-2 genes), in which OLA129 and C57BL/6 strains were mixed, were kindly provided by the Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuen University. We produced MT-1/2 hetero KO mice by mating MT-1/2 KO mice with wild-type C57BL/6 mice. Then, we used the littersmates offspring of male and female MT-1/2 hetero KO mice, which were kept under lighting conditions of 12-hour light and 12-hour dark. All investigations were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

OIR Model

The OIR model was produced as previously described. At postnatal day 7 (P7), mice and their mothers were placed in a custom-built chamber and exposed to an atmosphere of 75% ± 1% oxygen for 5 days at a temperature of 24 ± 2°C. Oxygen was continuously monitored with an oxygen controller (PRO-OX 110; Reming Bioinstruments Co., Redfield, SD, USA). On P12, animals were returned to room air (21% O2) until P17.

Retinal Angiography

Mice were deeply anesthetized intraperitoneally with sodium pentobarbital (Nembutal; Dainippon-Sumitomo Pharmaceuti-
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by preincubation with 10% normal goat serum. We used sections were washed in 0.01 M PBS for 10 minutes, followed in accordance with the following protocol. Briefly, tissue histochemistry. Immunohistochemical staining was performed of a cavitation bubble, which are shown as green labels in analyzed images and corresponded well to the pathologic neovascularization area (including tortuous and dilated blood vessels and abnormal vascular structure). To evaluate the capillary-free area, retinal revascularization was measured. The central capillary dropout area around the optic disc was quantified from the digital images in masked fashion using Metamorph in the same way as previously reported. 

**CNV Model**

Mice were anesthetized with pentobarbital (50 mg/kg), and pupils were dilated with 0.5% tropicamide (Santen, Osaka, Japan). Laser photocoagulation (647 nm, 120 mW, 100 ms, 50 μm; MC500; NIDEK, Kyoto, Japan) was performed on the left eye of each animal on day 0. This unit irradiates semiconductor laser. The laser color is red. Six laser spots were applied around the optic nerve with a slit-lamp delivery system and a cover glass as a contact lens. The morphologic point of the laser injury was the appearance of a cavitation bubble, which correlated with the disruption of Bruch’s membrane.

**Choroidal Fluorescein Angiography**

Two weeks after laser injury, mice were perfused with 1 mL fluorescein-conjugated dextran (MWt = 2000 kDa, Sigma-Aldrich Corp.) as reported previously.33-34 Eyes were enucleated and fixed in 4% paraformaldehyde for 12 hours. The retinal pigment epithelium–choroid–sclera complex was flat mounted with Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA). In vivo, mice were euthanized using sodium pentobarbital intraperitoneally at 80 mg/kg, and the eyeballs were quickly removed. The retinas were quickly frozen in dry ice. Next, the cornea and lens were removed. Furthermore, we extracted both retinae and intravitreous fluids. For protein extraction, the tissue was homogenized in cell lysis buffer using a homogenizer (Phyiscoyen; Microtec Co., Ltd., Chiba, Japan). The lysate was centrifuged at 12,000g for 20 minutes and the supernatant used for this study. The protein concentration was measured by comparison with a known concentration of bovine serum albumin using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). A mixture of equal parts of an aliquot of protein and sample buffer with 10% 2-mercaptoethanol was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated protein was then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA). For immunoblotting, the following primary antibodies were used: MT-1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), HIF-1α (1:500; Novus Biologicals Ltd., Littleton, CO, USA), VEGF (1:200; Merck Millipore, Billerica, MA, USA), and β-actin mouse monoclonal antibody (1:10,000; Sigma-Aldrich, Tokyo, Japan). The secondary antibody used was either goat anti-rabbit horseradish-peroxidase (HRP) conjugated (1:2000) or goat anti-mouse HRP conjugated (1:2000). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.). The band intensity was measured using a Lumino Imaging Analyzer (FUJifilm, Osaka, Japan). Information on the antibodies used appears in Supplementary Table S1.

**Immunoblotting**

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**Immunohistochemistry**

The enucleated eyes were fixed in 4% paraformaldehyde overnight at 4°C, soaked in 25% sucrose for 24 hours at 4°C, and embedded in optimum cutting temperature (OCT) compound (Sakura Finetechincal Co., Ltd., Tokyo, Japan). These tissues were immediately frozen with liquid nitrogen and stored at −80°C. Serial transverse sections were cut on a cryostat to a thickness of 10 μm and placed on slides (MAS COAT; Matsunami Glass Ind. Ltd., Osaka, Japan) for immunohistochemistry. Immunohistochemical staining was performed in accordance with the following protocol. Briefly, tissue sections were washed in 0.01 M PBS for 10 minutes, followed by preincubation with 10% normal goat serum. We used antibodies CD-31 (1:50; AnaSpec, Inc., San Jose, CA, USA) and MT-1/2 (1:100; Zymed Laboratories, Inc., South San Francisco, CA, USA) within Can Get Signal immunostain solution A (Toyobo Co., Ltd., Osaka, Japan). Anti-CD-31 antibody was applied to the sections overnight at 4°C. After washing the sections with 0.01 M PBS, immunoreactivity was visualized by incubation for 1 hour at room temperature with goat anti-mouse antibody (secondary antibody) conjugated with Alexa 546 (1:2000; Invitrogen Japan K.K., Tokyo, Japan). After washing with 0.01 M PBS in a similar way, anti-MT-1/2 antibody was applied overnight at 4°C, and incubation was performed for 1 hour at room temperature with goat anti-rabbit antibody (secondary antibody) conjugated with Alexa 488 (1:2000; Invitrogen Japan K.K.). At the end of immunostaining, Hoechst 33342 (1:1000) was added to the samples for 30 minutes. The fluorescent images were taken with a confocal microscope (FV10i; Olympus). Information on the antibodies used appears in Supplementary Table S1.

**In Vitro Cobalt Binding Assay**

Primary HRMECs were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan) and cultured in four batches in CS-C medium (DS Pharma Biomedical Co., Ltd.) containing culture boost (growth factors) at 37°C in a humidified atmosphere containing 5% CO2. Cells were treated with 300 μM CoCl2 for 0, 6, 12, and 24 hours and then washed twice with PBS. The nuclear and cytosol fractions were extracted, and Western blot was performed to confirm the expression of HIF-1α and MT-1/2. Each fraction was prepared using Plasma Membrane Protein Extraction Kit (BioVision, Milpitas, CA, USA) according to the manufacturer’s protocol. Following loading of equal amounts of protein, we performed Western blot using anti-c-Jun antibody (Cell Signaling Technology, Beverly, MA, USA) and anti-β-
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Transfection

The HRMECs were seeded at $4 \times 10^4$ cells/well in 12-well plates and incubated for 24 hours at 37°C in 5% CO$_2$ and then transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The HRMECs were transfected with 50 nM small interfering RNA (siRNA) targeting MT-1X (Ambion, Austin, TX, USA) and/or MT-2A (Ambion) for 24 hours. Human retinal microvascular endothelial cells transfected with a nontargeting siRNA (50 nM; Invitrogen) served as a negative control. Samples were then evaluated using an MCE-202 MultiNA (Microchip Electrophoresis System for DNA/RNA Analysis; Shimadzu Corporation, Kyoto, Japan) or Thermal Cycler Dice Real-Time System (Takara, Tokyo, Japan). After total RNA was extracted, real-time RT-PCR was performed using the Prime Script RT reagent kit (Takara) and SYBR Premix EX Tq II (Takara). The mRNA levels of target genes were normalized against GAPDH levels. Primers specific for MT-1 (forward, 5'-TTCTCCTTTGCCTCGAAATGGGAC-3'; reverse, 5'-ACATGGGACAGGCCAAG-3'), MT-2 (forward, 5'-ACTCTTCAGCCCTCTTCCGACG-3'; reverse, 5'-GGTCATTTGCACTCTTTGAC-3'), and GAPDH (forward, 5'-TGTGTCGTCGTGATCTG-3'; reverse, 5'-TTGCTGGAGAAGTCGAC-3') were used in the analysis.

Detection of Ubiquitination

After transfected with each siRNA for 48 hours, HRMECs were treated with 100 μM cycloheximide (Alomone Labs Ltd., Jerusalem, Israel) and incubated for 1 hour to inhibit intracellular protein synthesis. Hypoxia-inducible factor 1α in HRMECs was concentrated by immunoprecipitation using the Pierce Classic IP Kit (Thermo Fisher Scientific, Inc.) with anti-HIF-1α antibody (Novus Biologicals LTD) as the designated protocol. After the immunoprecipitation, immunoblotting was performed with anti-ubiquitin antibody (Cell Signaling Technology, Danvers, MA, USA).

Statistical Analysis

Statistical analyses of in vitro and in vivo experiments were performed with the aid of Statistical Package for the Social Sciences 15.0J for Windows (SPSS Japan, Inc., Tokyo, Japan).

RESULTS

Vitreous Levels of MT-1/2 and VEGF in PDR Patients

We measured the concentrations of MT-1/2 and VEGF in vitreous fluids obtained from patients with MH and PDR. The concentrations of MT-1/2 were significantly higher in PDR patients (mean ± SEM, 9305.0 ± 510.6 pg/mL, n = 20) than in MH patients (7729.9 ± 512.7 pg/mL, n = 19) (Fig. 1A). The concentration of VEGF was also higher in PDR patients than in MH patients (mean ± SEM, 779.4 ± 339.9 pg/mL, n = 20, versus MH; 2.2 ± 1.8 pg/mL, n = 19 [Fig. 1B]).

The Role of MT-1/2 in Ocular Neovascularization

To elucidate the role of MT-1/2 in retinal vascular injury associated with the OIR model, we evaluated pathologic neovascularization and the retinal capillary-free area in wild-type (WT) (MT-1/2/+), heterozygous (MT-1/2+/-), and knockout (KO, MT-1/2-/-) mice. As shown in Figure 2A, representative angiographic images of retinal fluorescein showed reduction of retinal pathologic neovascularization in MT-1/2 KO mice (Fig. 2A). Both the number and area of nodes were lower in MT-1/2 KO mice (Figs. 2B, 2C) compared to WT mice. There was no significant difference between WT and heterozygous mice, although a decreasing trend was observed (Figs. 2B, 2C). To evaluate physiological angiogenesis (physiological revascularization after oxygen-induced obliteration), the capillary-free area was measured. The capillary-free area in MT-1/2 KO mice was significantly greater than that in WT mice (Figs. 2D, 2E). Furthermore, we determined the influence of MT-1/2 on CNV by using the laser-induced CNV model. Previously, MT-1/2 was shown to be involved in pancreas function and high glucose levels to promote angiogenesis; however, the blood glucose level in MT-1/2 KO mice was not significantly changed compared with that in WT mice (WT mice: 170.7 ± 3.8 μg/mL [n = 3]; MT-1/2 KO mice: 175.8 ± 5.8 μg/mL [n = 4]). The mean size of the
Figure 2. The role of metallothioneins (MTs) 1 and 2 in retinal and choroidal neovascularization (A). Representative images of retinal fluorescein angiography and analyzed images in wild-type (WT) (MT-1/2+/+) and MT-1/2 knockout (KO) (MT-1/2−/−) mice. Scale bars: 1 mm (upper); 200 μm (lower). Quantitative analysis of pathologic neovascularization was performed in whole-mounted retinas as described in Methods. Both the number of nodes (B) and node area (C) were significantly decreased in MT-1/2 KO mice. The capillary-free area of WT and MT-1/2 KO mice (D) was significantly increased in MT-1/2 KO mice (E). Scale bar: 500 μm. Data are shown as mean ± SEM (n = 4 or 5). *P < 0.05; **P < 0.01 versus WT (Dunnett’s multiple comparison test). WT (MT-1/2+/+), MT-1/2 hetero (MT-1/2+/−), and MT-1/2 KO (MT-1/2−/−) mice were treated to induce laser-induced photocoagulation. (F) Laser-induced choroidal neovascularization was visualized in WT and MT-1/2 KO mice. Scale bar: 50 μm. (G) The size of the choroidal neovascularization area was significantly decreased in MT-1/2 KO mice. Data are shown as mean ± SEM (n = 3 or 4). *P < 0.05 versus WT (Dunnett’s multiple comparison test).
CNV lesions in the eyes of MT-1/2 KO mice was significantly smaller than that observed in WT mice (Figs. 2F, 2G).

Localization and Expression Level of MT-1/2 in the OIR Model

We further examined the cellular distribution of MT-1/2 by immunohistochemistry. Metallothioneins 1 and 2 were found ubiquitously in the retina and colocalized with retinal endothelial cells (Fig. 3A) in both normal and OIR mice. Double labeling for MT-1/2 and CD31 revealed that the MTs in endothelial cells were present in association with neovascularization. We subsequently investigated whether expression of MT-1/2 in the retina was altered in mice with OIR and observed that it was 5.58 times the expression in normal mice (Fig. 3B).

Retinal Expression of HIF-1α and VEGF in MT-1/2 KO Mice

The expression of selected proteins such as HIF-1α and VEGF, which are known to play roles in neovascularization, was measured in the retinas of normal and OIR mice. The levels of both HIF-1α and VEGF were significantly higher in the OIR model than in normal mice (Figs. 4A, 4B). Hypoxia-inducible factor 1α was significantly decreased in MT-1/2 KO mice as well as in the normal and OIR mice (Fig. 4A). The VEGF level was significantly decreased in MT-1/2 KO mice only in the OIR model (Fig. 4B).

Effect of MT-1/2 Against Expression of HIF-1α

To assess induction of HIF-1α protein following treatment with CoCl₂ in HRMECs, we investigated time-dependent HIF-1α expression in the nuclear and cytosolic fractions of HRMEC lysates. Figure 5A shows the purity of each fraction as confirmed by detecting the expression of c-Jun (nucleus) or β3-tubulin (cytosol). Although HIF-1α expression showed no changes in the cytosolic fraction, the levels increased dramatically in the nuclear fraction (Fig. 5A). On the other hand, CoCl₂ increased the expression of MT-1/2 in the cytosol but not in the nucleus (Fig. 5A).

Furthermore, we used siRNA to determine the roles of MT-1 and/or MT-2 on the ubiquitination of HIF-1α in HRMECs. As shown in Figures 5B and 5C, MT-1 or MT-2 siRNA effectively reduced expression of the target genes. Blocking protein synthesis with cycloheximide showed that HIF-1α ubiquitina-
tion increased following knockdown of MT-1 and/or MT-2 (Fig. 5C). In addition, MT-1/2 double knockdown accelerated the ubiquitination of HIF-1α compared to MT-1 or MT-2 single knockdown (Fig. 5C). To investigate the role of MT-1/2 in synthesis of HIF-1α, we evaluated the activation of eukaryotic initiation factor 4E (eIF4E) involved in the HIF-1α generation pathway.37 However, MT-1/2 double knockdown did not affect synthesis of HIF-1α (Supplementary Fig. S1).

**DISCUSSION**

Here we report that the concentration of MT-1/2 was higher in the vitreous fluid of patients with PDR, known as a retinal angiogenic disease, and that ocular neovascularization in MT-1/2 KO mice was decreased via downregulation of a key factor, HIF-1α. Furthermore, in our in vitro experiments provide evidence that MT-1 and/or MT-2 promote the ubiquitination of HIF-1α in HRMECs. Our findings highlight the importance of MT-1/2 in ocular angiogenesis and provide new insights into pathologic ocular neovascularization.

To investigate the correlation between MT-1/2 and retinal neovascularization, we measured both MT-1/2 and VEGF in the vitreous fluids of patients with MH (as a control group) and PDR. Metallothionein 1 and 2 and VEGF were significantly higher in PDR patients than in control patients (Fig. 1). From the clinical data, we hypothesized that MT-1/2 might play an important role in ocular neovascularization. Reports show that after a cortical injury, the number of capillaries decreases and blood vessel walls become less preserved and discontinuous in MT-1/2 KO mice.28 The results of a previous study suggest that altered angiogenesis might be involved in the impaired response of MT-1/2 KO at the site of freeze lesions. In addition, significantly fewer capillaries were found in MT-1/2 KO mice than in WT animals in a model of hindlimb ischemia.29 Further, the number of cells that stained positive for CD-31, a generally accepted endothelial marker, was significantly lower in Matrigel plugs implanted in MT-1/2 KO mice than in the plugs in WT mice.29 Indeed, retinal neovascularization and CNV in the two ocular neovascularization models (OIR and CNV) were both significantly decreased in MT-1/2 KO mice (Fig. 2). Metallothionein has antioxidative effects, and we have also previously reported that MT-3-deficient mice had exacerbated light-induced retinal damage compared with WT mice.38 However, according to our findings and previous reports, not only MT-1/2– but also MT-3-deficient mice had exacerbated ocular angiogenesis.39 These findings suggest that MT contributes to decrease of HIF-1α and VEGF expression, and this effect was stronger than the protective effects of RPE through antioxidative stress. Therefore, MT might promote angiogenesis. Because the present results agree with the previous reports, we believe that neovascularization in the eye was decreased in MT-1/2 KO mice.

To our knowledge, few reports describing a role for MT-1/2 in ocular vascular biology have been published. In this study, we performed a series of experiments to evaluate the biological roles of MT-1/2 in retinal neovascularization. We confirmed that MT-1/2 expression was localized to retinal endothelial cells and that MT-1/2 expression in the retina increased in the OIR model (Fig. 3). These data correlate with the significant increase of MT-1/2 in the vitreous fluid in PDR patients and suggest that MT-1/2 may have pivotal roles in retinal neovascularization.

The expression of MT-1/2 is ubiquitous and is markedly induced by various stresses, including endoplasmic reticulum stress and oxidative stress.40–44 Moreover, MT has an important role in brain microvascular pericytes, because MTs have protective effects in pericytes.45 In a previous study of angiogenesis, the expression levels of angiogenic factors including VEGF and matrix metalloproteinase-9 (MMP-9) were found to be decreased in MT-1/2 KO smooth muscle cells (SMC).29 Because SMC have a role in remodeling the vasculature and in the maturation of collateral vessels, changes in the expression of angiogenic factors in the SMC have a major effect on angiogenesis. Furthermore, both VEGF and MMP-9 are induced not only in the SMC but also in endothelial cells, which clearly play a central role in vascular biology. Expression of angiogenic factors such as VEGF and MMP-9 were induced in response to hypoxia in an HIF-1α-dependent manner.46 Recent evidence suggests that HIF-1α can be regarded as a “master switch” that regulates oxygen-dependent responses in ocular diseases.47 In fact, HIF-1α downregulation has a pivotal role in inhibition of both retinal neovascularization...
Consistent with this finding, our results showed alterations in HIF-1α and VEGF expression levels in the retina in the OIR model. Specifically, expression of both HIF-1α and VEGF was substantially higher compared to that in normal mice. Interestingly, these increases in the OIR model were significantly inhibited in MT-1/2 KO mice (Fig. 4). The results of the present study clearly indicate the importance of MT-1/2 in regulating angiogenic factors in retinal endothelial cells.

**FIGURE 5.** The role of metallothioneins (MTs) 1 and 2 in the ubiquitination of hypoxia-inducible factor (HIF)-1α. (A) The nucleus and cytosol extract from human retinal microvascular endothelial cells (HRMECs) were analyzed by immunoblotting for HIF-1α and MT-1/2 after treatment with CoCl₂ (300 μM). The same blot was reprobed with an anti-c-Jun antibody for confirmation of nucleus extraction and with an anti-β3-tubulin antibody for confirmation of cytosol extraction. MT-1 (B) and MT-2 (C) mRNA levels in HRMECs treated with MT-1 or MT-2 siRNA. Data are shown as mean ± SEM (n = 7 or 8). **P < 0.01 versus negative siRNA (Student’s t-test). (D) Representative images of HIF-1α ubiquitination immunoblots in HRMECs treated with MT-1 and/or MT-2 siRNA. After blocking of the synthesis of HIF-1α with cycloheximide, it was evident that MT-1 and/or MT-2 knockdown accelerated the ubiquitination of HIF-1α.
To begin characterizing the mechanisms associated with MT-1/2 function in retinal endothelial cells, we evaluated the expression of HIF-1α and VEGF in the nuclear and cytosolic fractions of HRMECs. Using the hypoxia mimic CoCl₂, we found that the stability of HIF-1α was increased in the nuclear fraction and that the expression of MT-1/2 was increased in the cytosol fraction (Fig. 5). Previously, eIF4E activation was shown to be a marker of HIF-1α protein synthesis. These in vitro data are consistent with both clinical and in vivo data suggesting that MT-1/2 are involved in regulation of HIF-1α expression. Under normoxic conditions, HIF-1α is degraded by proteasomes following its polyubiquitination. Other factors including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) are involved in this process. If these factors were upregulated, HIF-1α synthesis was facilitated. To reveal the role of MT-1/2 in the degradation of HIF-1α, we then investigated whether HIF-1α polyubiquitination was affected by knockdown of MT-1 and/or MT-2 under normoxia. Hypoxia-inducible factor 1α (HIF-1α) polypeptidyltransferase cleavage was increased upon knockdown of MT-1 or MT-2, and the knockdown of both proteins further enhanced this process (Fig. 5); activation of eIF4E was not altered by silencing of MT-1 or MT-2. Taken together, these results suggest that knockdown of MT-1 and/or MT-2 increases HIF-1α turnover. We believe that this is the first study to show that MT-1/2 may act as inhibitors of HIF-1α degradation.

In conclusion, we have demonstrated that MT-1/2 contribute to ocular neovascularization through stabilization of HIF-1α in endothelial cells. Thus, MT-1/2 appear to have an aggravating effect on ocular neovascularization. Accordingly, MT-1/2 may be new molecular targets for ocular angiogenic diseases including ROP, DR, and AMD.

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