Oxidative Stress-Mediated Induction of MMP-1 and MMP-3 in Human RPE Cells

Claudia S. Alge-Priglinger,1,2 Thomas Kreutzer,1 Katja Obholzer,1 Armin Wolf,1 Martin Mempel,5 Marcus Kernt,1 Anselm Kampik,1 and Siegfried G. Priglinger1,2

PURPOSE. In early exudative age-related macular degeneration (AMD), segmental thinning of Bruch’s membrane is associated with ingrowth of choroidal neovascularization into the subretinal space. To determine whether there is a link between oxidative stress and extracellular matrix (ECM) degradation by the retinal pigment epithelium, the present study focused on the effect of oxidative stress on MMP-1 and MMP-3 expression, two enzymes with substrate specificity for components of Bruch’s membrane.

METHODS. Cultured human RPE cells were exposed to oxidative stress. To investigate the role of signal transduction proteins, cells were pretreated with the specific inhibitors SB202190 or PD98059. Secreted MMP-1 and MMP-3 were detected by ELISA, MMP-2, and MMP-9 by zymography. Expression of mRNA was determined by quantitative real-time RT-PCR. ECM degradation by retinal pigment epithelium was assessed by immunofluorescence microscopy.

RESULTS. Oxidative stress increased MMP-1 and MMP-3 protein release but reduced MMP-2 activity. Real-time RT-PCR disclosed increases of MMP-1 and MMP-3 mRNA after oxidative stress with no modulation of TIMP-1. MMP-2 and MMP-9 mRNA was slightly enhanced. PD98059, an inhibitor of ERK1/2, markedly reduced MMP-1 expression, whereas SB202190, an inhibitor of p38 MAPK, was less effective. MMP-3 expression was attenuated by both inhibitors. Oxidative stress--stimulated type I collagen degradation by RPE cells was reduced by simultaneous treatment with a synthetic MMP-inhibitor or a neutralizing antibody against MMP-1.

CONCLUSIONS. MMP-1 and MMP-3 in the retinal pigment epithelium are inducible by oxidative stress. The directional shift in the MMP-1/-TIMP-1 ratio is associated with increased type I collagen degradation. This may be an important mechanism contributing to the pathogenesis of early exudative AMD. (Invest Ophthalmol Vis Sci. 2009;50:5495–5503) DOI:10.1167/iovs.08-3193

Bruch’s membrane is a pentalaminated extracellular matrix (ECM) interposed between the retinal pigment epithelium and the choriocapillaris. Besides providing a semipermeable diffusion barrier, it acts as a support element and attachment site for the retinal pigment epithelium. Bruch’s membrane can be divided ultrastructurally into five layers: the basement membrane of the retinal pigment epithelium, an inner collagenous zone, an elastic zone, an outer zone of collagen, and an outermost basement membrane elaborated by the endothelial cells of the choriocapillaris. The collagen layers contain collagen fibrils types I, III, and V.1

Alterations in composition and thickness of Bruch’s membrane are frequently observed in age-related macular degeneration (AMD).2,3 Whereas diffuse and focal thickening of Bruch’s membrane is a hallmark of atrophic AMD,4 breaks and segmental thinning of Bruch’s membrane at the sites of new vessel formation are observed in eyes with exudative AMD.5–7 Although the presence of such Bruch’s membrane lesions seems to be a prerequisite for the ingrowth of choroidal neo-vascularizations (CNV) into the subretinal space,6,7 the pathogenic events contributing to breaks in Bruch’s membrane remain elusive.

One of the most widely accepted theories implicates oxidative stress in the pathogenesis of the disease,8 and a number of studies indicate that in AMD, dysregulation of ECM turnover contributes to the alterations found in Bruch’s membrane.9–10 Matrix metalloproteinases (MMPs) are a family of at least 23 enzymes that are secreted by cells and cleave almost all protein components of the extracellular matrix.11,12 Hence, they have been assigned essential roles in modeling and remodeling the ECM in normal and disease states.13,14

Although there is some overlap in substrate specificity between the subgroups, the most specific are the collagenases (MMP-1, -8, and -13), which have the unique ability to denature native interstitial collagen types I, II, and III. Of these enzymes collagenase-1/MMP-1 is the most ubiquitously expressed subtype. MMP-1 has been implicated in cancer metastasis,13,15 melanoma angiogenesis,16 and premature aging of the skin after infrared and ultraviolet radiation.17,18 MMP-3, which belongs to the subgroup of stromelysins, can activate latent MMP-1 and degrade collagen types II, III, IV, and V as well as noncollagen matrix proteins such as laminin, elastin, fibronectin, and proteoglycans, most of which are found in the ECM surrounding the retinal pigment epithelium and in Bruch’s membrane.19,20 Increased MMP-3 levels are found in destructive inflammatory disease such as juvenile rheumatoid arthritis.21,22 In the eye, MMP-3 overexpression has been implicated in corneal invasion of pterygium head fibroblasts23 and in the pathogenesis of proliferative vitreoretinal disorders.24 Cultured RPE and choroidal endothelial cells secrete MMP-1, -2, -3, and -9, and all four subtypes have been immunohistochemically localized in Bruch’s membrane.10

Like other MMPs, MMP-1, -2, -3, and -9 are regulated at multiple levels, including transcription, proenzyme activation, and enzyme inhibition.25 The latter is a result of natural antagonists known as tissue inhibitors of metalloproteinase (TIMPs), which bind to the latent or active enzyme in a 1:1 complex. For example, an imbalance between MMP and TIMP activity is a likely cause of progressive pannus invasion and irreversible tissue damage in inflammatory disease such as rheumatoid arthritis26 and scleral matrix dissolution in scleritis.27 In the retinal pigment epithelium, expression and activity of MMPs

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has been shown to be modulated by various cytokines and growth factors, including transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Additionally, in nonocular cell types, the induction of MMP-1 and MMP-3 after ultraviolet light exposure and oxidative stress has been documented. However, it must be noted that the effects of various stimuli, including cytokines, growth factors, and oxidative stress on MMP expression, are dependent on the cell types under study.

In the past most studies on the role of MMPs in AMD have focused on MMP-2 and MMP-9. The localization of MMP-2 and MMP-9 to the areas of CNV and to the enveloping Bruch’s-like membrane led to the assumption that MMP-2 and MMP-9 may be cooperatively involved in the progressive growth of CNV in AMD. In contrast, increased MMP-2 activity was proposed to contribute to the thickening of Bruch’s membrane, as seen in atrophic AMD. Little, however, is known about the regulation of MMP-1 and MMP-3 in RPE cells after oxidative injury, though the primary substrates of the enzymes are major components of Bruch’s membrane. To define a possible role of MMP-1 and MMP-3 in the pathogenesis of AMD, this investigation focused on the effect of oxidative stress on expression of MMP-1 and MMP-3, its influence on ECM degradation, and the potential mechanism of induction.

**Materials and Methods**

**Isolation of Human RPE Cells and Human RPE Cell Culture**

Eyes from six human donors were obtained from the Munich University Hospital Eye Bank and processed within 4 to 16 hours of death. None of the donors had a known history of eye disease. Methods for securing human tissue were humane, included proper consent and approval, complied with the Declaration of Helsinki, and were approved by the local ethics committee. Human RPE cells were harvested and cultured according to the procedure described previously. ARPE-19 cells were obtained from ATCC (Rockville, MD), routinely passed once a week, and maintained in DMEM/Ham’s F12 (Biochrom Ltd., Cambridge, UK) supplemented with 10% FCS at 37°C and 5% CO2. For experiments cells were maintained for 4 days after having reached confluence. Thereafter, the regular medium was replaced with serum-free medium for 16 hours. Hydrogen peroxide (H2O2; Sigma, St. Louis, MO) at doses as indicated in the figure legends was then added to the cells for 1 hour in serum-free medium. The medium then was replaced by 1 mL fresh medium without serum for the indicated times.

**Enzyme-Linked Immunosorbent Assay**

MMP1 and MMP3 concentrations in conditioned media were determined with colorimetric immunoassays performed according to the instructions of the manufacturers. ELISA systems were purchased from GE Healthcare (Buckinghamshire, UK; MMP-1 Biotrak Assay, sensitivity 1.7 ng/mL; MMP-3 Biotrak activity assay, sensitivity 0.5 ng/mL). Optical density of each 96-well plate was read at 405 nm on a microplate reader (Versamax; Molecular Devices, Sunnyvale CA). Mean ± SD from several experiments was calculated, and the data were analyzed for significance by the paired Student’s t-test. P < 0.05 was considered to be statistically significant.

**Gelatin Zymography**

To detect MMP-2 and MMP-9 activity, culture-conditioned, serum-free media were collected as described and analyzed by zymography. Samples were electrophoresed on SDS-10% polyacrylamide minigels containing gelatin (1 mg/mL). Standards were run in parallel. Gels were washed three times in 2.5% Triton X-100 solution for 10 minutes, followed by overnight incubation in 50 mM Tris-HCl (pH 7.8), 5 mM CaCl2, 0.02 Na2S, and 0.02% Brij 35 at 37°C. Gels were stained with 2.5% Coomassie brilliant blue in 50% methanol and 10% acetic acid for 2 hours and then destained in 40% methanol and 10% acetic acid to reveal zones of lysis within the gelatin matrix. Gels were analyzed by densitometry with a software package (AIDA, Raytest, Pforzheim, Germany) to determine relative gelatinolytic activity. At least three independent experiments were performed in duplicate to reproduce results.

**RNA Isolation and Real-Time Quantitative RT-PCR**

Total RNA was isolated from 35-mm Petri dishes by the guanidinium thiocyanate-phenol-chloroform extraction method (Stratagene, Heidelberg, Germany). cDNA was synthesized from total cellular RNA using a reaction mix (First Strand Synthesis Superscript III; Invitrogen-Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. In brief, 1 µg total RNA was reverse transcribed in a 20-µL volume containing 10 µL 2X RT reaction mix and 2 µL enzyme mix for 1 hour at 42°C. cDNA was then incubated at 85°C for 5 minutes, followed by the addition of 1 µL RNase inhibitor (all from Invitrogen-Life Technologies) for another 20 minutes at 37°C. Quantitative real-time PCR was performed on a sequence-detection system (LightCycler 1.5; Roche, Basel, Switzerland) using heat-activated Taq DNA polymerase (LightCycler TaqMan Master; Roche), according to the manufacturer’s protocol. Primers and respective probes were designed with the probe finder software on the Roche Applied Biosciences Web site (https://qpcr.probefinder.com/roche2.html). The primer pairs used are listed in Table 1. The cycling conditions were as follows: after an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. The quantity of mRNA expression was analyzed by standard curve quantification for

**Table 1. Primer Pairs Used for Quantitative Real-Time RT-PCR**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Left</td>
<td>1079–1100</td>
<td>5’-CAGAGATGAGTCCGCGGTTTTC-3’</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1135–1154</td>
<td>5’-GGGTTGCTCGTTAGACACAT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’-ATAACGCAGCGTTATATTA-3’</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Left</td>
<td>2148–2166</td>
<td>5’-AGGCACCTTTGAAAGAGTG-3’</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>2190–2210</td>
<td>5’-ACAAGATATTTCCTGGAGG-3’</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Left</td>
<td>1194–1220</td>
<td>5’-TTCCAGCTATTGGTGGGAAA-3’</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1264–1284</td>
<td>5’-GAACTATCTTGCGCAGCG-3’</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Left</td>
<td>129–145</td>
<td>5’-GGCAGGCCCAGTGGTTCACA-3’</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>173–192</td>
<td>5’-GGGTTGACGGAGGCGCTC-3’</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Left</td>
<td>603–622</td>
<td>5’-TACCAGGGGGATGATAAACG-3’</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>654–673</td>
<td>5’-TTCTGGGCTGGGAGGCTAC-3’</td>
</tr>
<tr>
<td>β2-Microglobulin</td>
<td>Left</td>
<td>58–76</td>
<td>5’-TCCAGGAATTGGACTTTCCATTC-3’</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>121–143</td>
<td></td>
</tr>
</tbody>
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the target gene and the β2-microglobulin mRNA in the same sample using a software package (LightCycler Relative Quantification, version 1.0; Roche). All measurements were performed in duplicate. Controls consisting of bidistilled H2O were negative in all runs. Experiments were repeated at least five times.

**Cell Lysis, Gel Electrophoresis, and Immunoblotting**

ARPE-19 cells were solubilized for 20 minutes at 4°C in RIPA lysis buffer containing 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL phenylmethylsulfonyl fluoride, 50 kIU aprotinin, 100 mM sodium orthovanadate, and 10 µL/mL protease inhibitor cocktail (Sigma). Cell lysates were then sedimented in a microfuge for 15 minutes at 15,000 g. Soluble supernatant was collected and used for SDS-PAGE. After cell lysis, the supernatant was titrated in reducing SDS-PAGE loading buffer (Invitrogen), treated at 70°C for 10 minutes, separated in a 10% Bis-Tris gel (Invitrogen) with MOPS or MES buffer, according to the manufacturer’s instructions, and transferred to a polyvinylidene fluoride membrane (PVDF; Immobilon P, Millipore, Billerica, MA) for 60 minutes using transfer buffer (Invitrogen). Membranes were blocked (blocking buffer: 20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween20, 0.5% BSA) for 30 minutes at room temperature and incubated at 4°C overnight with the following primary antibodies: anti–ERK1/2 (Cell Signaling, MA), anti–phospho-ERK1/2 (Cell Signaling), anti–JNK (Cell Signaling), anti–p38 and anti–phospho-p38 (Cell Signaling), anti–β-actin (Sigma; 0.25 μg/mL; diluted 1:200 in Trisbuffered saline containing 0.5% BSA and 0.05% Tween20). Blots were washed repeatedly in washing buffer (15 mM NaCl, 50 mM Tris-HCl, 0.05% Tween20 [pH 7.6]) and incubated for 1 hour at room temperature with 0.1 μg/mL peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK) in blocking buffer. Peroxidase activity was detected using chemiluminescence substrate (Pierce, Rockford, IL) and recorded with a chemiluminescence detector (Vilber Lourmat, Eberhardzell, Germany). In dose-finding experiments, the inhibitors SB202190 (Calbiochem, Darmstadt, Germany) and PD98059 (Calbiochem) were used to determine optimal inhibition of p38 and ERK1/2 phosphorylation, respectively.

**Results**

**Effect of H2O2 on MMP-1 and MMP-3 Production by Cultured Primary Human RPE Cells**

MMP-1 was constitutively produced at relatively low levels (5.75 ± 0.12 ng/mL), but oxidative stress led to an enhancement in MMP-1 levels in the cell culture supernatant up to a mean of 2.5-fold (P < 0.01) when exposed to 300 µM H2O2 (Fig. 1A). A dose-dependent enhancement was seen up to a dose of 300 µM H2O2 with no further increase at higher doses.

MMP-3 also was constitutively produced at low levels (0.084 ± 0.025 ng/mL) but was significantly increased to 0.838 ± 0.08 ng/mL (9.8 ± 2.5-fold; P < 0.01) when cultured RPE cells had been exposed to 300 µM H2O2 (Fig. 2A). An

![Figure 1. Oxidative stress enhances MMP-1 production by cultured human RPE cells.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933244/ on 04/04/2018)
increasing MMP-3 release was seen until a dose of 300 µM H2O2. The higher doses studied yielded no further increment.

Next, time-course experiments were performed in which cultured RPE cells were exposed to 300 µM H2O2, and the supernatants were harvested at 12- to 24-hour intervals over 3 days. Oxidative stress stimulated a low level of MMP-1 protein at 12 hours, with a significant increase at 24 and 48 hours (Fig. 1B). The greatest increase was evident at 24 hours, with a subsequent decline to baseline until 72 hours after oxidative injury.

Time-course experiments for MMP-3 protein secretion disclosed kinetics comparable to those of MMP-1 with a small increment at 12 hours, a significant increase at 24 and 48 hours, and a subsequent decline until 72 hours after treatment (Fig. 2B).

**Effect of Oxidative Stress on MMP-1, MMP-3, and TIMP-1 mRNA Expression**

MMP-1 RNA was found to be constitutively expressed in untreated control cells. As depicted in Figure 1C, a dose-dependent increase of MMP-1 mRNA up to 10.57-fold (± 2.1; \( P < 0.01 \)) at 800 µM H2O2 was evident. At all doses of oxidative stress tested, MMP-1 mRNA induction was evident as early as 12 hours, showed the greatest increase at 48 hours, and subsequently declined in the 72-hour observation period (Fig. 1D).

Comparable findings were made for MMP-3 mRNA expression. Twenty-four hours after oxidative stress, quantitative real-time RT-PCR disclosed a significant and dose-dependent up-regulation of up to 26-fold (± 5.1; \( P < 0.01 \)) when compared with untreated controls (Fig. 2C). A marked induction of MMP-3 mRNA was evident as early as 12 hours, reached a peak at 24 hours, and then declined (Fig. 2D). This was in clear contrast to findings made for TIMP-1, the main inhibitor of MMP-1 and MMP-3. No modulation of TIMP-1 mRNA was noted after the same treatment (Figs. 2E, F). Thus, physiologically relevant sources of oxidative stress led to an up-regulation of MMP-1 and MMP-3 but not TIMP-1 in retinal pigment epithelium. For both enzymes, however, the increase in protein level was smaller than that of mRNA.

**MMP-2 and MMP-9 Expression in Oxidative Stress-Stimulated RPE Cells**

Because the two remaining MMPs expressed by RPE cells, MMP-2 and MMP-9, have been assigned a role in the maintenance and formation of CNV in AMD, we also sought to determine the expression of MMP-2 and MMP-9 in RPE cells after oxidative injury. Quantitative real-time RT-PCR of the same mRNA as used for assessment for MMP-1 and MMP-3 expression disclosed only minimal modifications in MMP-2 (greatest increase, 1.72 ± 0.21-fold at 800 µM H2O2; \( P = 0.02 \)) and MMP-9 (no significant change at any dose of H2O2).

**Figure 2.** Oxidative stress induces MMP-3 but not TIMP-1 in cultured human RPE cells. (A) Dose dependence of MMP-3 production by hydrogen peroxide–stimulated RPE cells. Monolayers of human RPE cells were exposed to 0 to 800 µM H2O2, and the cell culture supernatant was collected after 24 hours and analyzed for MMP-3 by ELISA. (B) Kinetics of MMP-3 production by H2O2-stimulated RPE cells. Cultured human RPE cells were exposed to 0 or 300 µM H2O2, and the cell culture supernatant was harvested after 12, 24, 48, and 72 hours and analyzed for MMP-3 protein by ELISA. The data are expressed as proportions of MMP-3 concentrations in relationship to the untreated control culture and represent the mean ± SD of duplicate samples from at least three experiments (\( P < 0.05 \)). (C) Dose dependence and (D) time course of MMP-3 mRNA induction by hydrogen peroxide–stimulated RPE cells. (E) Dose dependence and (F) time course of TIMP-1 mRNA expression in RPE cells after oxidative stress. (C–F) Cells were treated as described, and total cellular RNA was analyzed for MMP-3 (C, D) and TIMP-1 (E, F) expression by quantitative real-time RT-PCR. MMP-3 and TIMP-1 mRNA expression was normalized to β2-microglobulin transcript content. Data are presented as relative mRNA expression levels compared with untreated control and represent the mean ± SD of at least five experiments. * \( P < 0.05 \), analyzed by the paired Student’s t-test.
and MMP-9 (greatest increase \(1.64 \pm 0.33\) fold at 800 \(\mu M\) \(H_2O_2\); \(P = 0.02\)) mRNA expression levels 24 hours after oxidative stress (Figs. 3A, B). Because the enzymatic activity of MMP-2 and MMP-9 is characterized by their capability to degrade gelatin, supernatants of RPE cells were, in the next step, analyzed for gelatinolytic activity. Zymography showed one prominent, faint band at approximately 74 kDa (Fig. 3C), corresponding to MMP-2. At 24 hours after treatment with 600 and 800 \(\mu M\) \(H_2O_2\), MMP-2 activity released into the supernatant had diminished to approximately 35\% (\(P < 0.05\)) and 47\% (\(P = 0.05\)), respectively, compared with untreated control. At 48 hours after oxidative stress, MMP-2 activity had returned to control levels. However, we did not detect bands corresponding to RPE cell-associated MMP-9 activity under the cell culture conditions tested. A representative gelatin zymogram is depicted in Figure 3C. Thus, in cultured RPE cells, different MMP subtypes are differentially regulated by oxidative stress.

**Involvement of Extracellular Signal-Regulated Kinase (ERK 1/2) and Stress Activated/p38 MAPK in Oxidative Stress-Stimulated MMP-1 and MMP-3 Expression**

Previous studies indicate that enhancement of MMP-1 and MMP-3 expression by various stimuli involves the coordinated activation of mitogen-activated protein kinase (MAPK) pathways. In a first step, we determined the effects of oxidative stress on activation of the MAPK cascade in ARPE-19 cells. Immunoblot analysis disclosed a dose-dependent activation of the MEK/ERK1/2 pathway (Fig. 4A), which became evident at 300 \(\mu M\) \(H_2O_2\). In control cells and at low concentrations of \(H_2O_2\), phosphorylated p38 MAPK was barely detectable. At the highest dose tested, a marked upregulation of the phosphorylated isoform became evident (Fig. 4A) without affecting the levels of total MEK/ERK1/2 and p38 (data not shown). Expression levels of phosphorylated JNK remained unchanged. Addition of SB202190, a selective inhibitor of the p38 MAPK, or PD98059, an inhibitor of MEK/ERK1/2, specifically suppressed the levels of phosphorylated p38 or MEK/ERK 1/2, respectively. We found that SB202190 in the concentration range tested (1.0–15 \(\mu M\)) specifically suppressed the phosphorylation of p38 MAPK, whereas PD98059, at concentrations greater than 10 \(\mu M\), concomitantly reduced p38 phosphorylation, suggesting less specificity of this inhibitor at a higher dose (data not shown). To determine the contribution of MEK/ERK1/2 and p38 MAPK activation on oxidative stress–mediated MMP-1 and MMP-3 induction, RPE cells in a next step were treated with the respective inhibitors, and MMP-1 and MMP-3 mRNA expression was analyzed by real-time RT-PCR. Oxidative stress–induced upregulation of MMP-1 was completely reversed to control levels after preincubation of RPE cells with concentrations greater than 5 \(\mu M\) of the ERK pathway inhibitor PD98059 (Fig. 4C). The p38 kinase inhibitor SB202190 also attenuated MMP-1 gene activation, but this effect faded with increasing doses of the inhibitor. When compared with cells exposed to oxidative stress without inhibitor in the medium 1.0 \(\mu M\) SB202190 attenuated oxidative stress-induced MMP-1 expression by 75\%, whereas pretreatment with 10 \(\mu M\) SB202190 reduced MMP-1 mRNA by only approximately 30\% (Fig. 4B). Both SB202190 and PD98059 significantly lowered the effect of oxidative stress on MMP-3 mRNA expression in a dose-dependent manner (Figs. 4D, E).

**Effect of Oxidative Stress on MMP-3 Activity**

Active MMP-3 can cleave and, therefore, enzymatically activate pro-MMP-1. To test whether active MMP-3 was present in the conditioned media after oxidative stress, RPE cells were treated as described, and the supernatants were analyzed for active MMP-3 using an ELISA kit that exclusively determines the active form of MMP-3. Although overall relatively low levels of active MMP-3 were detectable in the oxidative-stress conditioned media, at 24 hours oxidative stress yielded a significant increase of active MMP-3 (2.56 \(\pm 0.61\)-fold; \(P < 0.05\)) compared with untreated controls.

**FIGURE 3.** Regulation of MMP-2 (A) and MMP-9 (B) mRNA expression by oxidative stress in cultured human RPE cells. Cultured human RPE cells were treated with 0, 600, or 800 \(\mu M\) \(H_2O_2\) for 1 hour, and total cellular mRNA was analyzed for MMP-2 and MMP-9 mRNA levels by quantitative real-time RT-PCR. MMP-2 and MMP-9 mRNA expression was normalized to \(\beta\)-2-microglobulin transcript content. Data represent the mean \(\pm\) SD of at least five experiments. (C) Oxidative stress reduces MMP-2 activity released by human RPE cells. Cells were treated as described, and after 24 and 48 hours the supernatant was analyzed for MMP-2 protein activity by gelatin zymography. Top: gelatin zymogram from a representative experiment. Lane M: molecular weight standard. Bottom: averages of results of three independent experiments run in duplicate on cultured RPE cells. *\(P < 0.05\), compared with control; analyzed by the paired Student’s \(t\)-test.
controls (Fig. 5). At 48 hours, the active form of the enzyme was still found marginally increased (1.61 ± 0.34fold, P = 0.02) at doses above 600 µM H2O2, whereas at lower doses active MMP-3 had declined to control levels (data not shown). Expression levels are depicted in Figure 5.

Collagen Type I Degradation

To test the ability of oxidative stress-stimulated RPE cells to degrade type I collagen, we assessed the degradation of fluorescein-conjugated type I collagen after oxidative stress. RPE cells were grown on coverslips coated with fluorescein-conjugated type I collagen and exposed to oxidative stress. Forty-eight hours after treatment, application of H2O2 had caused marked degradation of type I collagen, as detected by changes in fluorescence intensity (Figs. 6A, B). To examine whether inhibition of the MAPK pathways could be effective in suppressing the observed effect, cells were exposed to oxidative stress in the presence of MAPK inhibitors. From preliminary dose-finding experiments, we chose 1 µM SB202190 and 5 µM PD98059 as optimal MAPK inhibitor concentrations. H2O2-induced degradation of type I collagen was in part prevented by addition of the p38 and MEK/ERK1/2 MAPK inhibitors SB202190 and PD98059, respectively (Figs. 6C-F). The addition of FN-439, a strong inhibitor of MMP-1, or of a function-blocking anti–MMP-1 antibody to the medium led to a diminished amount of oxidative stress–induced type I collagen degradation (Figs. 6G-J). These results suggest that in vitro oxidative stress via activation of ERK and p38 MAPK induces the release of functionally active MMP-1 by RPE cells, capable of degrading collagen type I.

Discussion

Recent studies using ocular tissue and cell culture systems suggest that MMPs and their inhibitors play a key role in the homeostasis of extracellular matrices in the eye.24–27 The extracellular form of AMD, which is associated with CNV, is characterized by a disruption of structural properties of Bruch’s membrane, thus facilitating neovascular ingrowth into the subretinal space. Triggering signals for CNV are poorly defined and may constitute, for example, modified ECM components,38 oxidative stress,8,39 or cytokines that induce inflammatory reactions, which participate in altered MMP secretion by RPE cells.24,28

We present here a novel pathway by which oxidative stress causes an imbalance in ECM turnover in the retinal pigment epithelium. We demonstrate for the first time that oxidative stress drives RPE cells to strongly express MMP-1 and MMP-3, two MMPs with substrate specificity for components of the extracellular matrix.
Bruch’s membrane. The mechanism by which oxidative stress promoted MMP-1 and MMP-3 induction involved MAPK-dependent signal transduction. Our results further demonstrate that this leads to an imbalance in the MMP-1/TIMP-1 and MMP-3/TIMP-1 ratios because of the lack of TIMP-1 induction by H$_2$O$_2$. Functionally, we observed increased collagen degradation by RPE cells after oxidative stress. Consistent with our in vitro studies, an imbalance between MMPs and inhibitors of MMPs has been described for several nonocular and ocular cell types after different stimuli, such as oxidative damage, UVB irradiation, or cytokine treatment. In vivo, such discrepancies in MMP-1, MMP-3, and TIMP-1 expression have been attributed to various disease states, such as the breakdown of the blood retinal barrier in neurodegenerative disease, the invasiveness of pterygial fibroblasts, or matrix destruction in proliferative vitreoretinopathy. Assuming that in the RPE oxidative stress leads to a directional shift in the MMP-1/TIMP-1 and MMP-3/TIMP-1 balance, this may allude to oxidative stress-mediated proteolysis of certain ECM components surrounding the retinal pigment epithelium.

In the present study, the strong induction of MMP-1 and MMP-3 by oxidative stress stands in clear contrast to the findings made for MMP-2 and MMP-9. In agreement with previous reports by others, we found only minimal induction of MMP-2 and MMP-9 mRNA, together with reduced MMP-2 activity, in RPE cells after oxidative stress. Such differential regulation of subtypes of MMPs by a stressful stimulus was also observed in other cell types. Similarly, in a recent study, Park et al. found an upregulation of MMP-1 and MMP-3, but not of MMP-2, mRNA in dermal fibroblasts after heat shock. However, differential regulation of MMP expression reportedly is cell type-dependent and tissue-specific. For example, in brain vascular endothelial cells or hepatic stellate cells, both MMP-2 and MMP-9 were found to be inducible by oxidative stress. The strong induction of MMP-1 and MMP-3, together with only modest regulation of MMP-2 and MMP-9 expression by oxidative stress, may therefore be a feature specific of the retinal pigment epithelium. Interestingly, the extents to which MMP-1 and MMP-3 mRNA levels increased in response to oxidative stress were much greater than the increase of the proteins in the medium. Furthermore mRNA expression of both MMPs showed clear dose dependence, whereas protein expression exhibited no further significant increment once a certain expression level was reached. Previous genomic and proteomic analyses found that for a substantial number of genes, the absolute amount of protein in a cell is not strongly correlated to the amount of mRNA. Whereas the levels of mRNA are controlled mainly by transcription activity and mRNA stability, the levels of proteins reflect mRNA level, translation efficiency, and posttranslational modification, activation, and degradation. Secretion efficiency may also control the MMP levels in the medium. Further studies are necessary to fully understand the mechanism by which oxidative stress affects the expression and secretion of MMPs in RPE cells.
necessary to elucidate the underlying molecular mechanisms responsible for these findings.

Currently, three different MAPK pathways have been characterized in detail: ERK 1/2, JNK, and stress-activated p38 MAPK. All three pathways can be activated by oxidative stress to varying degrees, with ERK 1/2 and p38 MAPK playing the most important role in the regulation of MMP expression. This rationale was used to identify the intracellular pathway involved in oxidative stress-stimulated MMP-1 and MMP-3 induction in cultured RPE cells. Several studies have shown activation of MAPK by oxidative stress in the retinal pigment epithelium. Nevertheless, results from these studies are conflicting. For example, Glotin et al. showed that oxidative stress activates the MEK/ERK1/2 but not the p38 and JUN MAPK pathways, whereas others found an activation of the p38 and JUN pathways. Qin et al. report an activation of all three main MAPK pathways in RPE cells. In the present study, levels of phosphorylated JUN remained unchanged, whereas an upregulation of phosphorylated ERK1/2 and p38 MAPK was evident. Different cell culture conditions such as state of confluence, serum starvation, or type, dose, and duration of oxidative stimulus may account for these discrepancies and make an interstudy comparison difficult. Inhibition of ERK 1/2 and p38 phosphorylation was effective in suppressing the levels of MMP-1 and MMP-3 gene activation. PD98059 (an inhibitor of ERK1/2) and SB202190 (an inhibitor of p38) clearly attenuated MMP-3 mRNA expression in a dose-dependent manner. At the lower concentrations studied, MMP-3 transcription was more sufficiently reduced by inhibition of p38 MAPK, but at concentrations greater than 5 μM, both inhibitors were equally effective, suggesting that MMP-3 was transcriptionally enhanced by oxidative stress via both ERK and p38 MAPK pathways. In agreement with this, several previous studies have shown that MMP-1 and MMP-3 induction in response to various stimuli is dependent on both MAPK pathways. The regulation of MMP-1 expression by oxidative stress appeared to involve more complex intracellular pathways. Both inhibitors were effective in attenuating MMP-1 mRNA levels enhanced by oxidative stress: A reduction beyond control levels was disclosed at concentrations greater 5 μM of the ERK1/2 inhibitor PD98059, and a 75% reduction of MMP-1 mRNA was achieved at 1 μM of the p38 MAP kinase inhibitor SB202190. However, it is noteworthy that the inhibitory effect of SB202190 was attenuated with increasing doses. On the one hand, these data clearly support the notion that both pathways may be necessary for optimal MMP-1 enhancement by oxidative stress but with a clear predominance of the ERK1/2 pathway. On the other hand, these findings add to the complexity of the intracellular pathways because previous studies that examined the effects of the MAPK inhibitors on downstream signaling demonstrated variable responses in different species and models. In the present study, the inhibitory effect of SB202190 on MMP-3 expression levels was clearly concentration dependent. In contrast, the suppressive effect of SB202190 on MMP-1 expression diminished with increasing concentrations. Based on this limited survey, these findings suggest that the p38 MAPK pathway may be additionally involved in a feedback inhibition of the stimulatory effect of oxidative stress on MMP-1 expression but does not directly mediate this effect. Clearly, further studies will be necessary to elucidate the exact mechanisms underlying the induction of MMP-1 and MMP-3 in the retinal pigment epithelium.

To summarize, we have reported that in vitro, MMP-1 and MMP-3 are highly inducible by oxidative stress and that the stress-mediated upregulation in the retinal pigment epithelium is essentially mediated by MAPK-dependent signal transduction. The directional shift in the MMP-1/TIMP-1 and MMP-3/TIMP-1 ratio translates into increased type I collagen degradation. This is underscored by the observation that FN-439, an inhibitor of MMPs with specificity for MMP-1, and a function-blocking monoclonal anti-MMP-1 antibody reduced MMP activity and subsequent type I collagen degradation. Collectively, these data support a possible link between oxidative stress and a dysregulated ECM turnover by the retinal pigment epithelium. In vivo the retinal pigment epithelium is exposed to high oxygen fluxes, all factors that downstream result in the exogenous or endogenous generation of radical oxygen species, including H₂O₂. Hence, in the pathogenesis of CNV, oxidative stress as a permanently present stimulus may lead to a sustained release of MMP-1 and MMP-3 by the retinal pigment epithelium, which in turn may contribute to focal proteolysis of Bruch’s membrane. This is corroborated by the immunohistochemical localization of both MMP-1 and MMP-3 in Bruch’s membrane. Based on these assumptions, it is conceivable that these may be an important mechanism contributing to the pathogenesis of early exudative AMD.

References
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