

MMP-14 and TIMP-2 Overexpression Protects against Hydroquinone-Induced Oxidant Injury in RPE: Implications for Extracellular Matrix Turnover

Oscar Alcazar,¹ Scott W. Cousins,² and Maria E. Marin-Castaño¹

PURPOSE. To investigate whether overexpression of MMP-14 and/or TIMP-2 would overcome the effect of nonlethal oxidant injury with hydroquinone (HQ) on MMP-2 activity.

METHODS. Human MMP-14 and TIMP2 cDNA were cloned into a mammalian expression vector. Transient transfections were performed on human ARPE-19 cells. The cells were incubated 48 hours after transfection with a nonlethal dose of HQ for either 6 or 18 hours and then were collected for protein determination or RNA isolation. MMP-2 protein and activity were determined by Western blot and zymography. The extracellular matrix (ECM) components type I and type IV collagen and laminin were analyzed by Western blot analysis and real-time PCR.

RESULTS. HQ for 6 hours modestly decreased MMP-2. MMP-2 recovered only after co-overexpression of MMP-14 and TIMP-2, but activity further decreased after HQ for 18 hours. MMP-14 or TIMP-2 overexpression alone contributed as much as the co-overexpression to the recovery of MMP-2 activity. MMP-2 protein seemed not to be altered. Type I collagen and laminin transcriptional levels remained unaffected, whereas type IV collagen transcripts decreased with HQ. Transfection with MMP-14 and/or TIMP-2 contributed to the return of type IV collagen levels to normal. On the other hand, type I and IV collagens and laminin protein accumulated after HQ treatment, an effect prevented by transfection.

CONCLUSIONS. MMP-14 and TIMP-2 contribute to the maintenance of adequate levels of MMP-2 activity in ARPE-19 cells after oxidant injury. In addition, changes in ECM components may result as a consequence of MMP-2 activity and may be relevant to the progression of dry AMD. (*Invest Ophthalmol Vis Sci.* 2007;48:5662-5670) DOI:10.1167/iovs.07-0392

Age-related Macular Degeneration (AMD) is a chronic degenerative disorder of the retina that constitutes the leading cause of blindness in the elderly. It is a multifactorial disorder in which age is the predominant risk factor. However, there is a growing body of evidence¹⁻³ showing that environmental factors such as cigarette smoking may contribute to the

early development of this degenerative disease. In this regard, epidemiologic studies⁴⁻⁶ have suggested that cigarette smoking may influence the development and severity of AMD, including an increased risk for AMD in people exposed to passive smoking. Quinones are suggested to be the major source of oxidative injury in cigarette smoke. Hydroquinone (HQ) is an oxidant of special relevance due to its ubiquitous presence not just in cigarette smoke but also in foodstuffs and air pollution.^{7,8}

The initial pathogenic target of AMD is the retinal pigment epithelial (RPE) cells and the adjacent Bruch's membrane (BrM).⁹ The early stage of AMD is characterized by the formation of drusen, as a result of the dysregulation in the turnover of extracellular matrix (ECM) molecules.¹⁰ The normal anatomy and physiology of ECM in most tissues requires continuous turnover of collagen and other matrix components by a tightly regulated balance in the production of matrix molecules like collagen, laminin, MMPs, and TIMPs.^{11,12} Relatively small dysregulation on the ratio of these factors can produce profound changes in the ECM, including thickening and deposit formation.^{13,14} Matrix metalloproteinases (MMPs) are the principal ECM-degrading proteinases, whose activity is mainly regulated by proteins called tissue inhibitors of metalloproteinases (TIMPs). However, the activity of MMP-2 is regulated by another metalloproteinase, MMP-14, in combination with TIMP-2.^{15,16}

Our laboratory has shown that oxidant-induced RPE dysfunction with a nonlethal dose of HQ results in abnormal MMP-2 activity, which may contribute to the development of abnormal ECM turnover.¹⁷ MMP-2 is the key enzyme for ECM turnover in Bruch's membrane (BrM) and is synthesized as an inactive zymogen pro form (pro-MMP-2).¹⁸ The transmembrane metalloproteinase MMP-14 is well known to activate MMP-2 in a specific manner.¹⁵ It is believed that at least two MMP-14 molecules are involved in the activation of the inactive pro-MMP-2 into active MMP-2 by proteolytic cleavage.¹⁹ The first MMP-14 molecule binds TIMP-2 to function together as an adaptor for pro-MMP-2. Once this ternary complex is formed, an additional MMP-14 molecule acts as an activator to cleave the pro form and release the active MMP-2.^{15,19} The stoichiometry of the interaction in the complex has been reported to be pivotal in the activation of MMP-2.¹⁹ In this regard, a molar ratio of MMP-14 to TIMP-2 in the range 3:1 to 3:2 seems to be optimal, whereas an excess of TIMP-2 may inhibit the cleavage of pro-MMP-2 into MMP-2.¹⁹

Recently, our investigations have shown evidence that oxidant injury induced by other oxidant reduced MMP-2 activity, which was rescued, not by MMP-14 overexpression alone, but by addition of TIMP-2 to the culture medium.²⁰ In our study we sought to span these preliminary observations by evaluating both short-term (6 hours) and long-term (18 hours) exposure to a nonlethal oxidant dose of HQ. The purpose of this study was to investigate whether overexpression of MMP-14 and/or TIMP-2 overcomes the effect on MMP-2 activity of nonlethal oxidant injury by HQ. We generated two constructs (MMP-14 and TIMP-2), using the same expression vector to create a real

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Supported by National Eye Institute Grant R01-EY015249-01A1, Minority Supplemental Grant EY015249-01A1S1, and an unrestricted grant from Research to Prevent Blindness.

Submitted for publication April 2, 2007; revised August 24, 2007; accepted October 16, 2007.

Disclosure: O. Alcazar, None; S.W. Cousins, None; M.E. Marin-Castaño, None

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TABLE 1. PCR primers

Target Gene	Forward Primer	Reverse Primer	Size (bp)	Reference
<i>MMP14</i>	ATGTCTCCCGCCCA	TCAGACCTTGTCAGCAGG	1748	
<i>TIMP2</i>	ATGGGCGCGCG	TTATGGTCTCGATGTCGA	662	
Collagen I ($\alpha 1$)	CTCCGGCTCCTGCTCTTA	GCACAGCACTCGCCCTCC	235	22
Collagen IV ($\alpha 1$)	ACTCTTTGTGATGCACCA	AAGCTGTAAGCGTTTGGTA	150	23
Laminin ($\alpha 4$)	CTCCATCTCACTGGATAATGGTACTG	GACACTCATAAAGAGAAGTGTGGACC	362	24

co-overexpression, to gain a better control over the stoichiometry in the cotransfection of MMP-14 and TIMP-2. We analyzed in parallel the potential implications of the overexpression of MMP-14 and/or TIMP-2 on ECM molecules known to be substrates for MMP-2, such as collagens and laminin. Our interest was to investigate HQ due to its environmental ubiquitous presence in Western societies in general and in cigarette smoke in particular. Our data suggest that both MMP-14 and TIMP-2 (alone or in combination) contribute to the maintenance of adequate levels of MMP-2 activity and ECM turnover balance in human ARPE-19 cells when challenged for a long time (18 hours) with the cigarette smoke-related oxidant HQ.

MATERIALS AND METHODS

Cell culture materials and a transfection reagent (LipofectAMINE 2000) were purchased from Invitrogen-Gibco (Carlsbad, CA). All other reagents were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO). The human retinal pigment epithelium ARPE-19 cell line used was purchased from the American Type Culture Collection (ATCC, Manassas, VA).

cDNA Cloning of MMP-14 and TIMP-2 and Construct Generation

Total cellular RNA was isolated (TRI reagent; Sigma-Aldrich) based on the GTC-acidic phenol method. The cDNA corresponding to MMP-14 was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from human kidney as the template. Primers (forward: 5'-ATGTCTCCCGCCCA-3'; reverse: 5'-TCAGACCTTGTCAGCAGG-3') were constructed based on the published coding region sequence of the human mRNA for matrix metalloproteinase 14 (MMP-14; GenBank accession no. NM_004995; <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). For TIMP-2, primers (forward: 5'-ATGGGCGCGCG-3'; reverse: 5'-TTATGGTCTCGATGTCGA-3') were constructed based on the published coding region sequence for human tissue metalloproteinase inhibitor 2 (TIMP-2; GenBank Accession No. NM_003255; <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The single cDNA fragment obtained was TA-cloned into a mammalian expression vector (pTAR-GET; Promega, Madison, WI) using standard molecular biology techniques and subsequently verified by sequencing.

Cell Culture and Transfection

ARPE-19 cells of passages 20 to 22 were plated at subconfluent density on T-75 (75 cm²) flasks and grown to confluence in maintenance medium (Dulbecco's modified Eagle's medium [DMEM]-Ham's F12 [1:1 vol/vol] supplemented with 10% fetal bovine serum [FBS], 100 μ g/mL penicillin/streptomycin, and 0.348% Na₂HCO₃). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. For the experiments, confluent cells were split and plated at subconfluent density (2 \times 10⁵ cells) and grown to confluence.

Transient transfections were performed when the cells were near confluence with a total of 2 μ g of DNA per well in six-well plates (Lipofectamine 2000; Invitrogen-Gibco). The expression of the recombinant protein was allowed for 48 hours, at which time the cells were

either treated with HQ or collected for protein and/or RNA isolation. Total RNA was then isolated (TRI reagent; Sigma-Aldrich), and protein content was determined with a kit (Bio-Rad, Hercules, CA), based on the Bradford method.

HQ Injury

The cells of the same passages were plated at the same subconfluent density (2 \times 10⁵ cells) onto six-well plates. At the time of confluence, the cells were prepared for the experiment by changing the maintenance medium to the assay medium (i.e., maintenance medium without phenol red) for 2 days. This medium was then replaced with assay medium, which was supplemented with 1% FBS instead of 10% for 1 day. Subsequently, the medium was changed to the assay medium supplemented with 0.1% FBS. At this time, 100 μ M HQ was added for either 6 or 18 hours. Culture medium was withdrawn, and the cells were washed two times with phosphate-buffered saline (PBS). After that, fresh assay medium supplemented with 0.1% FBS was added for 24 hours. The cells were harvested for protein (with lysis buffer M-PER; Pierce, Rockford, IL) or RNA (TRI reagent; Sigma-Aldrich) assessment. Supernatants were also collected to measure MMP-2 and TIMP-2 protein expression and activity. Protein was quantified in all samples, as described in the previous section.

Quantitative Real-Time PCR

ARPE-19 cells were cultured and maintained as previously described.²¹ Total RNA was isolated from the cells (TRI reagent; Sigma-Aldrich). Real-time PCR was performed with a thermal cycler system (LightCycler; Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Reverse transcription was performed with 1 μ g RNA in accordance with the manufacturer's protocol (Reverse Transcription System; Promega). The PCR experiments were performed using SYBR green as the interaction agent (Roche Diagnostics). Each 20 μ L PCR contained cDNA template, SYBR Green PCR master mix, and 0.5 μ M of each gene-specific primer. PCR was performed using pairs of specific primers for either type I (chain α -1) or type IV (chain α -1) collagens, or laminin (chain α -4). Primer sequences and size of the PCR product for each targeted gene are described in Table 1. The specificity of PCR amplification products was assessed by dissociation melting-curve analysis and subsequent agarose gel electrophoresis. The ribosomal RNA control reagent kit was used to detect the 18S ribosomal RNA gene, which represented an endogenous control. Each sample was normalized to the 18S transcript content. The standard curves were generated with serially diluted solutions (0.001–100 ng) of mRNA from cultured ARPE cells. PCR assays were conducted in duplicate for each sample. Data are expressed as the mean \pm SEM of results in four independent experiments.

Western Blot Analysis

Ten or 40 μ g protein extracts were denatured in Laemmli sample buffer followed by 5 minutes of boiling and then resolved on a 10% or 8% Tris-glycine gel (Novex, San Diego, CA). After electrophoresis (120 V for 2 hours), the proteins were transferred in 1 \times transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol [\sim pH 8.4]) to a nitrocellulose membrane (Hybond-ECL; GE Healthcare; Piscataway, NJ), with constant current of 100 mA for 2 or 3 hours. The membranes were then blocked in 5% nonfat dry milk TBS solution for 1 hour at

room temperature. The blots were incubated overnight at 4°C with one of the following antibodies: AB19012 (Chemicon International, Temecula, CA), M61403 (Biosensing International, Saco, ME), 1310-01 (Southern Biotechnology, Birmingham, AL), MAB13405 (Chemicon International), MAB3328 (Chemicon International), or AB19078 (Chemicon International). The membranes were washed three times with TBS solution including Tween-20 (TBS-T) incubated with horseradish peroxidase-linked donkey anti-mouse, donkey anti-goat or donkey anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature and then washed four times in TBS-T. Detection of the immunoreactive bands was performed with the chemiluminescent reagent luminol (Santa Cruz Biotechnology). The bands were scanned and quantitated by densitometry (ImageJ 1.17 software; National Institutes of Health [NIH], Bethesda, MD; available by ftp at zippy.nimh.nih.gov/ or at <http://rsb.info.nih.gov/nih-image/>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

MMP-2 Activity

Conditioned culture medium was collected after treatment and clarified by 30 minutes of centrifugation at 15,000g and 4°C. Protein concentration was determined and MMP-2 activity assessed on 10% zymography gels (Novex, San Diego, CA), as described previously.²⁵ Briefly, 10 µg of protein extracts from each experimental condition was used. The gels were incubated for 18 hours in 50 mM Tris buffer, allowing determination of total proteolytic MMP-2 activity with no interference from their associated tissue inhibitors. Densitometry was performed with the Image J 1.17 densitometry program (NIH).

Reverse Zymography

Ten micrograms of protein was diluted as needed in Laemmli buffer and combined with an equal volume of Tris-glycine SDS (Novex). Samples were electrophoresed at 75 V for 2.5 hours on a standard separating gel composed of 2.25 mg/mL porcine gelatin, 0.25 M Tris-HCl (pH 8.8), 0.125% SDS, 1 µL/mL TEMED, 0.4 mg/mL ammonium persulfate, 15% acrylamide, 0.4% bisacrylamide, and 100 ng/mL proenzyme MMP-2 (EMD Biosciences, Inc., San Diego, CA). After electrophoresis, the gels were incubated in 1× zymogram renaturing buffer (Novex) with gentle agitation for 3 hours at room temperature, with the solution replaced every hour. Then, the renaturing buffer was replaced with 100 mL 1× developing buffer (Novex). The gels were then incubated at 37°C overnight. Each gel was stained with 0.5% Coomassie blue G250 in 30% methanol/10% acetic acid for 4 hours followed by four washes of destaining solution containing 30% methanol/10% acetic acid for 1, 15, 30, and 60 minutes. The gels were further destained in 1% Triton X-100 solution for 1 hour and stored in distilled water until densitometry was performed. They were then analyzed by densitometry with ImageJ 1.17 software (NIH).

MMP-14 Activity Assay

ARPE cells were grown in 24-well plates and transiently transfected with pTARGET-MMP14 construct with transfection reagent (Lipofectamine 2000; Invitrogen-Gibco). Forty-eight hours after transfection, the medium was replaced with 250 µL of extraction buffer from an MMP-14 activity assay system (Biotrak; GE Healthcare) and incubated for 15 minutes at 4°C. The supernatant was then assayed for MMP-14 activity according to the manufacturer's instructions for the ELISA.

Statistical Analysis

All experiments were performed three to four times on cultured cells, with reproducible results. Data are expressed as the mean ± SEM. Statistical comparisons were performed with one-way ANOVA and the Tukey multiple comparison as a post hoc test when differences were significant. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Overexpression of MMP-14 and TIMP-2 in ARPE-19 Cells

The human retinal pigment epithelium cell line ARPE-19 of passages 20 to 22, was assayed to set the ideal transfection conditions with a transfection reagent (Lipofectamine 2000; Invitrogen-Gibco). A mammalian expression vector (pTARGET; Promega) harboring the reporter gene green fluorescence protein (*GFP*) was used for this purpose. In our hands, the transfection efficiency under optimal conditions was ~65%, as determined with a fluorometer (data not shown). Human MMP-14 and TIMP-2 cDNAs were cloned into the vector pTARGET to generate the constructs for the study.

The cell lysates and conditioned mediums (supernatants) were collected 48 hours after transfection of the ARPE-19 cells with the mammalian expression constructs pTARGET-MMP-14 or pTARGET-TIMP-2. Three different samples corresponding to transient transfections with either MMP-14 or TIMP-2 were screened by Western blot analysis for protein expression. Cell lysates were used to analyze MMP-14, whereas TIMP-2 was evaluated in supernatants. Mock transfections were performed in parallel with the empty vector (pTARGET) as the control. We decided to use mock transfection as the ideal control to rule out the possibility of any potential effect due to the vector backbone. Transfection with pTARGET-MMP-14 showed MMP-14 protein levels $2.46\% \pm 0.63\%$ ($n = 3$, $P < 0.05$) higher than that in mock-transfected control cells (Fig. 1A). Similarly, overexpression of TIMP-2 accounted for a $4.11\% \pm 0.24\%$ ($n = 3$, $P < 0.001$) increase in the level of protein compared with that in mock-transfected control cells (Fig. 1B). The level of MMP-14 or TIMP-2 overexpression was normalized against α -actin, a loading control for total protein content.

The moderate level of TIMP-2 overexpression attained is of relevance, as it is well accepted that high levels of TIMP-2 expression induces an inhibitory effect on MMP-2 activation.¹⁹ The ternary molecular complex among MMP-14, TIMP-2, and pro-MMP-2 (inactive form) requires an MMP-14 to TIMP-2 molar ratio that is not too low for efficient cleavage of the MMP-2 proform into its active form.^{15,19} Therefore, the level of overexpression shown for TIMP-2 under our established transfection conditions is adequate to investigate its role regarding MMP-2 activation.

Aside from analyzing whether the constructs used for the transfections were properly translated into protein, we verified that the recombinant protein was functional. For this purpose, MMP-14 activity was assayed by ELISA on cell monolayers as described in the Materials and Methods section. Cells transfected with MMP-14 showed activity levels 2.28 ± 0.15 ($n = 3$, $P < 0.05$) times higher than the control (Fig. 1C). TIMP-2 activity was assessed in supernatants by reverse zymography. Cells transfected with TIMP-2 demonstrated activity levels 2.22 ± 0.09 ($n = 3$, $P < 0.05$) times higher than the control (Fig. 1D).

Effect of Overexpression of MMP-14 and TIMP-2 on MMP-2 Activity after 6-Hour HQ Injury

Our laboratory has demonstrated that the induction of oxidant injury to the ARPE-19 cells diminishes the activity of MMP-2.¹⁷ To investigate whether MMP-14 or TIMP-2 is involved in the impairment of MMP-2 activity, we sought to overexpress both molecules, alone and in combination. Of note, the DNA amount used in the cotransfections with MMP-14 and TIMP-2 in ARPE-19 cells was limited by the cytotoxicity shown at high doses of transfection reagent (Lipofectamine2000; Invitrogen-Gibco). Therefore, we used the same total DNA amount for the

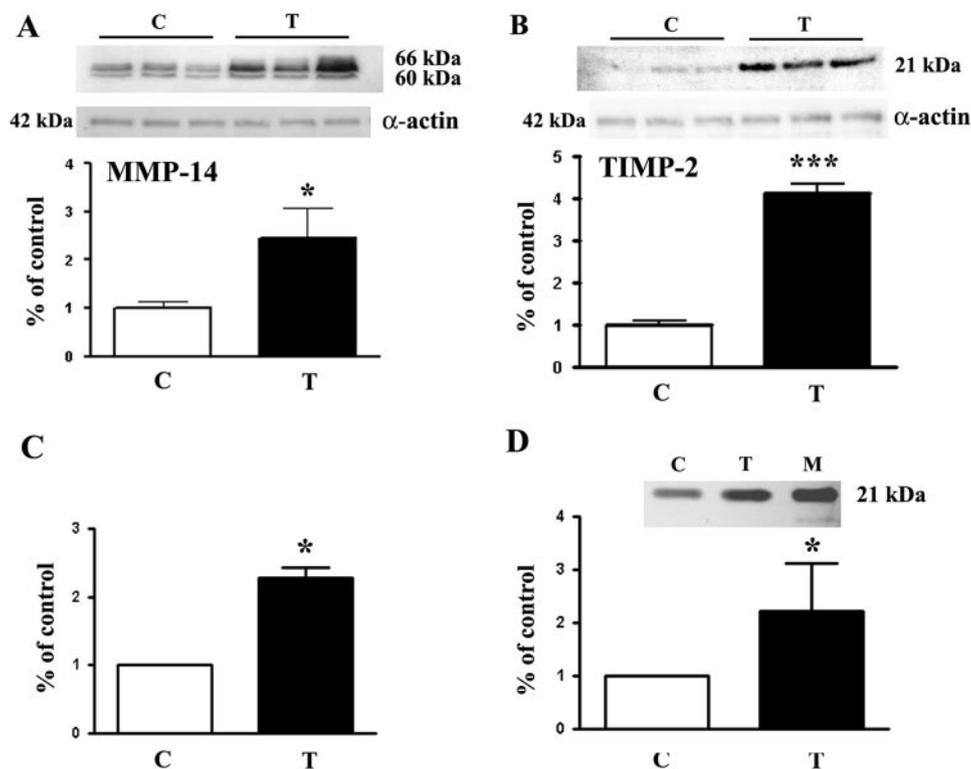


FIGURE 1. MMP-14 and TIMP-2 overexpression in ARPE-19 cells. Transient transfections were performed, and conditioned media and cell monolayers were harvested 48 hours after transfection. Gene expression was quantified by densitometry. (A) MMP-14 protein expression evaluated in cell lysates by Western blot. *Top:* Western blot from a representative experiment. The number on the *right* represents the molecular mass of the protein. *Bottom:* average of three independent transfections. C, mock-transfected control; T, transfected cells. (B) TIMP-2 protein expression evaluated in conditioned media by Western blot. *Top:* Western blot from a representative experiment. The number on the *right* represents the molecular masses of the proteins. *Bottom:* average of three independent transfections. (C) MMP-14 activity assessed by ELISA on cell monolayers. The data represent the average of three independent experiments. (D) TIMP-2 activity evaluated in conditioned media by reverse zymography. *Top:* Reverse zymogram from a representative experiment. M, TIMP-2 marker. *Bottom:* averages of results in three independent experiments. Results are expressed as the mean \pm SEM. * $P < 0.05$, *** $P < 0.001$ compared with control cells.

cotransfection and the two single transfections (i.e., MMP-14 or TIMP-2 alone).

The ARPE-19 cells were subjected to short-term (6 hours) exposure to 100 μ M HQ (a nonlethal dose of this oxidant) 48 hours after transfection. MMP-2 Western blot analysis and zymograms were performed on supernatants. The MMP-2 activity modestly decreased after exposure of mock-transfected ARPE-19 cells to HQ for 6 hours ($36.77\% \pm 5.09\%$ of control, $P < 0.05$, $n = 3$; Fig. 2A). Previous studies by our group have demonstrated a more dramatic effect of HQ on MMP-2 activity.¹⁷ However, those observations were performed on ARPE-19 cells that were not transfected.

A significant change was not induced by overexpression of either MMP-14 or TIMP-2 alone. However, co-overexpression of both molecules led to recovery of the MMP-2 activity to levels comparable to that in cotransfected cells not treated with HQ (Fig. 1A). That the total amount of DNA in the cotransfection equaled that of the single transfections may explain why cotransfecting both molecules did not raise the MMP-2 activity any higher than in control cells.

Analysis of MMP-2 protein expression in supernatants by Western blot showed no significant change after overexpression with MMP-14 or TIMP-2, alone or in combination (Fig. 2B). Protein expression values were normalized against α -actin, a loading control for total protein. This result confirms previous observations made by our group¹⁷ in nontransfected ARPE-19

cells that treatment with HQ does not induce any significant change in the protein level of MMP-2.

Effect of Overexpression of MMP-14 or TIMP-2 on MMP-2 Activity after 18-Hour HQ Treatment

Given that the oxidative injury is an accumulative process with severity increasing with time, we sought to test the effect of overexpression of MMP-14 and/or TIMP-2 in a more prolonged time frame of HQ exposure. The ARPE-19 cells were subjected to 18 hours' incubation with a nonlethal dose of HQ (100 μ M) 48 hours after transfection. MMP-2 Western blot analysis and zymograms were evaluated on supernatants. Treatment with HQ for 18 hours induced a more pronounced impairment in MMP-2 activity ($51.85\% \pm 5.96\%$ of control, $P < 0.05$, $n = 3$; Fig. 3A) when compared with the effect caused after 6 hours' HQ exposure (Fig. 2A). An important finding is that MMP-14 and TIMP-2 overexpression, alone or in combination, contributed to the recovery of MMP-2 activity to mock-transfected control levels. Given that ARPE-19 cells were transfected at nearly confluence and a potential "dilution" effect with cell division is minimal, a longer incubation time with HQ may also represent a longer time for recombinant MMP-14 and TIMP-2 to be expressed. Therefore, higher levels of MMP-14 and TIMP-2 proteins may, at least in part, contribute to the shown effect of

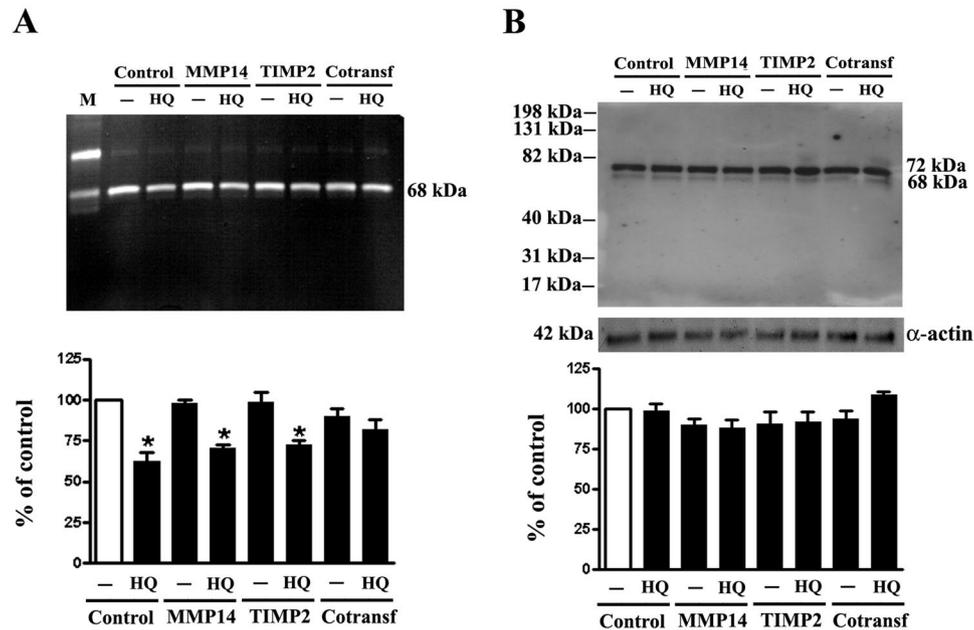


FIGURE 2. MMP-2 activity and protein expression in transfected ARPE-19 cells exposed to HQ for 6 hours. MMP-2 activity and protein expression in transfected ARPE-19 cells exposed to HQ. Cells were transfected with empty vector (control), MMP-14, or TIMP-2 or cotransfected with MMP-14+TIMP-2 (Cotransf). Forty-eight hours after transfection, the cells were incubated in the absence or presence of 100 μ M HQ for 6 hours. Thereafter, conditioned media (supernatants) were collected, and the protein content quantified. **(A)** MMP-2 activity evaluated in supernatants by zymography. *Top*: gelatin zymogram from a representative experiment. M, a commercial preparation of MMP-2 enzyme used as a marker. *Bottom*: average results of three independent experiments. **(B)** MMP-2 expression evaluated in supernatants by Western blot. *Top*: representative Western blot for evaluation of MMP-2 protein present in the supernatants. Numbers on the *left* represent the molecular masses of the marker. α -Actin was the loading control. *Bottom*: averages of three independent experiments normalized against the loading control. Results are expressed as the mean \pm SEM. * $P < 0.05$ compared with control cells.

the single transfections, preventing the impairment of MMP-2 activity in the presence of HQ.

Protein expression analysis of MMP-2 in supernatants by Western blot showed no change in the levels of protein (Fig. 3B). Similar to incubations of ARPE-19 cells with HQ for a 6-hour period, there was no apparent variation in the inactive pro-MMP-2 form (72 kDa) or the active MMP-2 form (68 kDa). Protein-expressed values were normalized against α -actin, a loading control for total protein.

Alteration of Type IV Collagen Transcripts and Protein Expression by HQ

Type IV collagen is an important component of the extracellular matrix in BrM. This type of collagen is recognized to be a substrate and is cleaved by MMP-2. Therefore, we decided to investigate whether HQ has any effect on type IV collagen transcripts and/or protein expression and any potential influence of overexpression MMP-14 or TIMP-2.

ARPE-19 cells were transiently transfected with expression constructs for MMP-14 and TIMP-2, as described in the Materials and Methods section. Expression was allowed for 48 hours, and the cells were then exposed to 100 μ M HQ for a period of 18 hours. Cell lysates were used to examine collagen type IV. Quantitative real-time-PCR revealed a significant decrease of 32.8% (70.43 ± 7.6 ng vs. 104.85 ± 6.3 ng) in the level of type IV collagen α -1 chain transcripts (Table 2). Overexpression of either MMP-14 or TIMP-2, or a combination of both, showed no change in the level of transcripts compared with the control. Data were normalized against 18S rRNA transcript content. Table 2 depicts a pattern that correlates with MMP-2 activity after the various transfections, showing

evidence of a potential link between MMP-2 activity and the transcriptional regulation of type IV collagen. One might speculate that reduced MMP-2 activity, regardless of measured protein level, is associated with increased collagen IV protein and that reduced collagen IV mRNA may be a compensatory effect.

Protein analysis of type IV collagen in cell lysates by Western blot showed two bands in the upper molecular range (~198 kDa), probably corresponding to different combinations of α -1 (IV) and α -2 (IV) chains (Fig. 4). The immunoblots revealed a clear accumulation of type IV collagen after 18 hours of treatment with 100 μ M HQ. Cell transfections with MMP-14, TIMP-2, or a combination of both molecules did not modify the normal levels of type IV collagen expression in the control cells. Transfected cells exposed to HQ did not show any increase in type IV collagen protein whatsoever. Protein expressed values were normalized against α -actin, a loading control for total protein.

HQ-Induced Accumulation of Type I Collagen

Type I collagen is another ECM component present in BrM that is susceptible to digestion by MMP-2. Similar to type IV collagen, we sought to determine any effect that HQ and overexpression of MMP-14 or TIMP-2 might have on this structural component of the ECM.

Forty-eight hours after transfection, ARPE-19 cells were incubated with 100 μ M HQ for 18 hours before cell lysis and analysis. Quantitative real-time-PCR revealed no significant regulation of the α -1 chain of type I collagen by HQ in control or transfected cells (Table 3). Data were normalized against 18S rRNA transcript content.

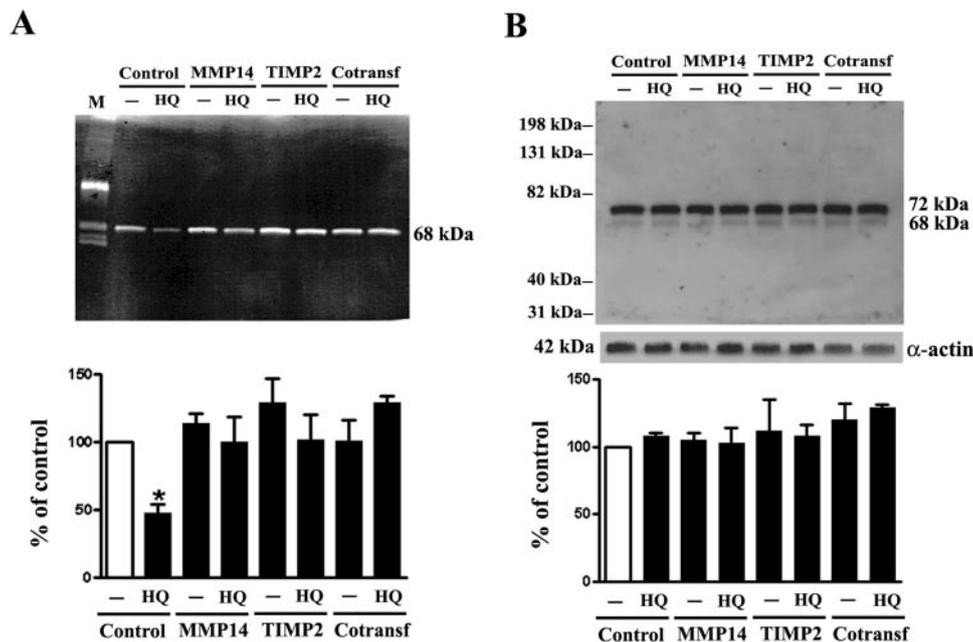


FIGURE 3. MMP-2 activity and protein expression in transfected ARPE-19 cells exposed to HQ for 18 hours. Cells were transfected with empty vector (control), MMP-14, or TIMP-2 or cotransfected with MMP-14+TIMP-2 (Cotransf). Forty-eight hours after transfection, the cells were incubated in the absence or presence of 100 μ M HQ for 18 hours. Thereafter, conditioned media (supernatants) were collected and the protein content quantified. (A) MMP-2 activity evaluated in supernatants by zymography. *Top*: gelatin zymogram from a representative experiment. M refers to a commercial preparation of MMP-2 enzyme used as a marker. *Bottom*: average results of three independent experiments. (B) MMP-2 expression evaluated in supernatants by Western blot. *Top*: representative Western blot for evaluation of MMP-2 protein present in the supernatants. Numbers on the *left* represent the molecular masses of the marker. α -Actin was the loading control. *Bottom*: averages of three independent experiments normalized against the loading control. Results are expressed as the mean \pm SEM. * $P < 0.05$ compared with control cells.

Protein expression analysis of type I collagen performed on cell lysates showed a single band in the upper-molecular-mass range (\sim 198 kDa; Fig. 5). Treatment of control cells with HQ doubled the amount of type I collagen protein ($206\% \pm 18\%$, $n = 3$, $P < 0.001$). This effect is probably due to accumulation of the synthesized protein, given that the transcriptional rate of type I collagen remains unaffected by HQ (Table 3). Cells transfected with MMP-14 and/or TIMP-2 did not show any significant change in type I collagen protein even after exposure to HQ. Protein expression values were normalized against α -actin, a loading control for total protein.

HQ's Effect on Laminin Expression

To complete the study of the effects raised by the oxidant HQ and overexpression of MMP-14 and TIMP-2 on ECM compo-

TABLE 2. Regulation of Collagen IV (α 1-Chain) mRNA by Hydroquinone in Transfected ARPE-19 Cells

Treatment	Collagen IV (α 1) mRNA	
	Amount (ng)	% Change
Mock	104.85 \pm 6.3	
Mock+HQ	70.43 \pm 7.6	-32.8*
MMP14	108.29 \pm 6.2	+3.28
MMP14+HQ	101.67 \pm 5.7	-3.03
TIMP2	109.20 \pm 5.6	+4.15
TIMP2+HQ	102.34 \pm 4.2	-2.40
Cotransf.	98.25 \pm 6.1	-6.29
Cotransf.+HQ	96.02 \pm 4.1	-8.42

Cotransf., cotransfected.

* $P < 0.05$.

ments, we decided to analyze laminin, a noncollagen molecule that is very abundant in BrM. Similar to the type I and IV collagens, laminin is also a potential substrate for MMP-2.

ARPE-19 cells were transfected and treated as described for analysis of the collagens. Protein expression and transcription were allowed for 48 hours after transfection, and the cells were then challenged with 100 μ M HQ for a period of 18 hours. Transcriptional levels of laminin chain α -4, assessed by quantitative real-time PCR, remained unaffected after HQ treatment in the various transfections performed (Table 4). Data were normalized against 18S rRNA transcript content. On the other hand, Western blot analysis of laminin protein expression on cell lysates showed a significant increase ($150\% \pm 11\%$, $n = 3$, $P < 0.05$) when cells were exposed to HQ for 18 hours (Fig. 6). The accumulation of laminin followed the pattern shown by collagens type I and IV, which also accumulated in the presence of the oxidant. Transfections with MMP-14 and/or TIMP-2 exhibited no remarkable differences both in the presence or absence of HQ (Fig. 6). Protein expression was normalized against α -actin, a loading control for total protein.

DISCUSSION

A fairly extensive body of evidence has shown that oxidative stress constitutes a major risk factor in the early phase of the pathogenesis of AMD.^{1,26,27} In the present study we focused on HQ as oxidant stimulus due to its widespread presence in Western societies. We postulate that overexpression of MMP-14 and TIMP-2 in RPE cells may prevent, at least in part, the decrease in MMP-2 activity after incubation with HQ. Consequently, the purpose of this study was to investigate the impact of overexpressing MMP-14 and/or TIMP-2 on HQ-in-

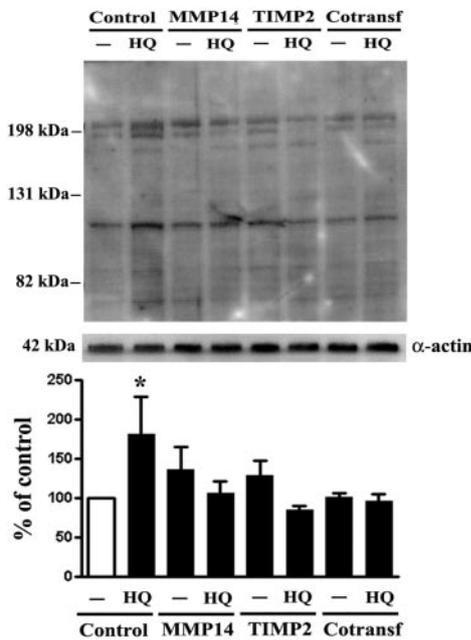


FIGURE 4. Collagen IV expression in transfected ARPE-19 cells exposed to HQ. Cells were transfected with empty vector (control), MMP-14, or TIMP-2 or cotransfected with MMP-14+TIMP-2 (Cotransf). Forty-eight hours after transfection, the cells were incubated in the absence or presence of 100 μ M HQ for 18 hours. Thereafter, the cells were lysed and the protein analyzed by Western blot. *Top*: representative Western blot for evaluation of collagen IV expression in cell lysates. α -Actin was the loading control. *Bottom*: average results of three independent experiments normalized against the loading control. (□) Mock-transfected control. Results are expressed as the mean \pm SEM. * P < 0.05 compared with control cells.

duced MMP-2 impaired activity, and their consequences on extracellular molecules involved in ECM turnover in vitro. Our results showed that prolonged incubation with HQ not only impaired MMP-2 activity in ARPE-19 cells, but also induced an accumulation of collagens I and IV, as well as laminin, all important ECM molecules. All these effects induced by HQ were overcome in vitro by the overexpression of MMP-14 and/or TIMP-2.

The RPE is a central element in the pathogenesis of AMD. Dysfunctional RPE cells may alter the normal turnover of ECM components triggering the thickening of BrM and potential formation of sub-RPE deposits, which are believed to be the origin of drusen in the early phase of dry AMD. Even though the cellular mechanism(s) for dysregulation of ECM turnover in AMD remains unknown, evidence suggests that MMPs and

TABLE 3. Regulation of Collagen I (α 1-Chain) mRNA by Hydroquinone in Transfected ARPE-19 Cells

Treatment	Collagen I (α 1) mRNA	
	Amount (ng)	% Change
Mock	98.32 \pm 6.0	
Mock+HQ	98.70 \pm 2.9	+0.38
MMP14	105.55 \pm 1.9	+7.35
MMP14+HQ	99.44 \pm 6.4	+1.13
TIMP2	101.72 \pm 4.3	+3.45
TIMP2+HQ	99.09 \pm 9.6	+0.78
Cotransf.	96.28 \pm 3.2	-2.08
Cotransf.+HQ	94.99 \pm 3.5	-3.39

Cotransf., cotransfected.

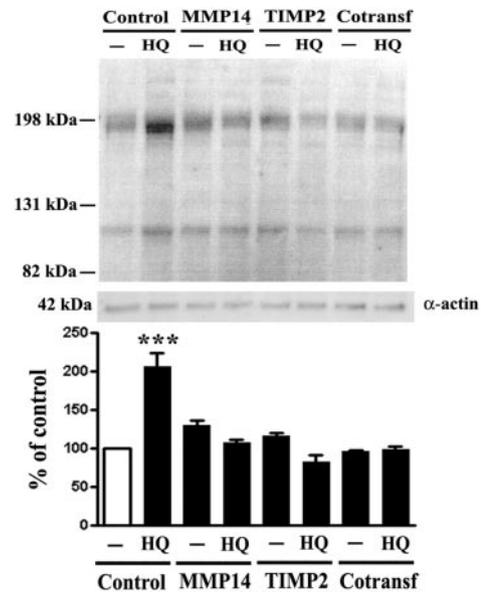


FIGURE 5. Collagen I expression in transfected ARPE-19 cells exposed to HQ. Cells were transfected with empty vector (control), MMP-14, or TIMP-2 or cotransfected with MMP-14+TIMP-2 (Cotransf). Forty-eight hours after transfection, the cells were incubated in the absence or presence of 100 μ M HQ for 18 hours. Thereafter, cells were lysed and protein analyzed by Western blot. *Top*: a representative Western blot for evaluation of collagen I expression in cell lysates. α -Actin was the loading control. *Bottom*: average results of three independent experiments normalized against the loading control. (□) Mock-transfected control. The results are expressed as the mean \pm SEM. *** P < 0.001 compared with control cells.

their tissue inhibitors (TIMPs) play an important role.^{12-14,28} In this regard, we and others have shown that MMP-2 is crucial for the degradation and turnover of ECM in BrM.^{17,29,30} To investigate the effect of oxidant injury with HQ on ECM turnover, we selected the human retinal pigment epithelium cell line ARPE-19 because it is well characterized³¹ and is easily transfected with plasmid vectors. Given the heterogeneous nature of this cell line, we used the same culture conditions and similar passage to make it useful for our experiments.

The activation of MMP-2 (cleavage of the pro form) involves the formation of a trimer composed of TIMP-2 that binds to MMP-14 before association with pro-MMP-2. TIMP-2 concentration is critical for MMP-2 activation, such that insufficient TIMP-2 results in failure to localize pro-MMP-2 to the cell surface, whereas excess TIMP-2 reduces the free MMP-14 needed to activate pro-MMP-2.¹⁹ Based on this model and

TABLE 4. Regulation of Laminin (α 4-Chain) mRNA by Hydroquinone in Transfected ARPE-19 Cells

Treatment	Laminin (α 4) mRNA	
	Amount (ng)	% Change
Mock	98.16 \pm 2.7	
Mock+HQ	101.67 \pm 1.6	+3.57
MMP14	94.83 \pm 1.4	-3.39
MMP14+HQ	95.11 \pm 2.7	-3.11
TIMP2	94.58 \pm 0.7	-3.65
TIMP2+HQ	91.68 \pm 3.9	-6.61
Cotransf.	97.02 \pm 1.8	-1.17
Cotransf.+HQ	93.55 \pm 1.6	-4.70

Cotransf., cotransfected.

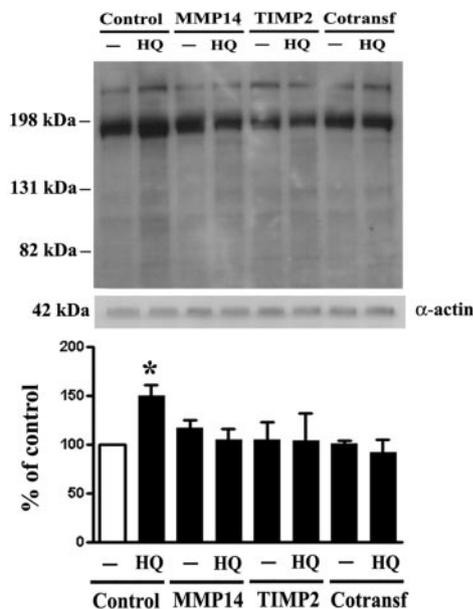


FIGURE 6. Laminin expression in transfected ARPE-19 cells exposed to HQ. Cells were transfected with empty vector (control), MMP-14, or TIMP-2 or cotransfected with MMP-14+TIMP-2 (Cotransf). Forty-eight hours after transfection, the cells were incubated in the absence or presence of 100 μ M HQ for 18 hours. Thereafter, the cells were lysed and the protein analyzed by Western blot. *Top*: representative Western blot for evaluation of laminin expression in cell lysates. α -Actin was the loading control. *Bottom*: average results of three independent experiments normalized against the loading control. (□) Mock-transfected control. The results are expressed as the mean \pm SEM. * P < 0.05 compared with control cells.

previous observations,^{15,19} we decided to use a molar ratio of 3 to 1 (MMP-14:TIMP-2) for the cotransfections. In this way, we attained comparable activity levels of MMP-14 and TIMP-2 in transfected cells and prevented an excess of TIMP-2, which may inhibit the MMP-2 activation.

In agreement with previous observations²⁰ obtained with a different oxidant (myeloperoxidase/H₂O₂), overexpression of MMP-14 alone did not rescue the impaired MMP-2 activity in the short term (i.e., 6 hours' incubation). This finding is interesting to note, as it is believed that different oxidants may use different mechanisms of action. For instance, myeloperoxidase/H₂O₂, unlike HQ, generates reactive chlorinating species that target proteins, nucleic acids, and lipids, oxidizing all these biomolecules. HQ is a redox molecule whose metabolism in the mitochondria interferes with the electron transport,³² damaging the mitochondrial membrane and consequently inducing oxidation of proteins and peroxidation of lipid components in the plasma membrane.^{33,34}

However, longer exposition of transfected ARPE-19 cells to HQ revealed that overexpression of MMP-14 and TIMP-2, alone or in combination, may somehow prevent the decay in MMP-2 activity under oxidative stress. Eighteen versus 6 hours of incubation with HQ implies a longer time for the recombinant MMP-14 and TIMP-2 proteins to be expressed. One might speculate that the rate of the inactive pro-MMP-2 form catalytic cleavage into the active MMP-2 form should increase. If so, it may explain the different observations obtained at 6 and 18 hours' incubation time with HQ. This matter deserves further investigation.

It is intriguing that, when cells are treated with HQ, MMP-2 protein does not change, whereas its activity is recovered by MMP-14 or TIMP-2 overexpression. It is plausible that the antibody used (the best of a few different antibodies tested by

our laboratory) lacks the sensitivity to show clearly significant differences in the protein content of the active form. In this regard, we have found in the literature some reports questioning the quantitative nature of Western blot analysis in certain settings.³⁵⁻³⁷

Behind the RPE lies BrM, which is composed of a central elastic layer surrounded by two collagenous layers. Laminin and collagens type I and IV are among the major components of BrM.^{38,39} During AMD, BrM gets progressively thickened due to deposit formation. Sub-RPE deposits in macular specimens contain basement membrane components, such as collagen type IV. Based on these observations, we sought to investigate relevant ECM molecules that might accumulate and contribute, at least in part, to this deposit formation and ultimately to AMD. Our results clearly demonstrated a significant increase in all three ECM molecules studied—laminin and collagens type I and IV—after exposure to the oxidant stressor HQ. Of note, HQ-induced accumulation of type IV collagen protein shown by Western blot confirms previous data by our group using ELISA and immunohistochemistry on cell lysates and cells, respectively.¹⁷

We demonstrated for the first time that HQ-induced impairment of MMP-2 expression not only correlates with accumulation of type IV collagen, but also that of laminin and type I collagen. These three molecules are not just components of BrM but also are substrates for MMP-2, which is the major RPE enzyme for degrading type I and IV collagens and laminin.^{29,30} The accumulation of these relevant ECM structural proteins when the activity of MMP-2 is impaired by HQ supports the hypothesis that MMP-2 is an enzyme of pivotal importance for maintenance of the ECM balance in BrM. Therefore, nonlethal oxidant injury to RPE by HQ may have dramatic consequences for the ECM. Accumulation of laminin and collagens may contribute, at least in part, to sub-RPE deposit formation and gradual thickening of BrM. On the other hand, transfection of ARPE-19 cells with MMP-14 and/or TIMP-2 prevented the accumulation of all three ECM molecules analyzed (i.e., type I collagen, type IV collagen, and laminin). This observation correlates well with the lack of perturbation in the MMP-2 activity when transfected ARPE-19 cells are incubated in the presence of HQ. These data on ECM molecules provide evidence that ECM turnover decreases in RPE cells after 18 hours of exposure to nonlethal oxidant injury and may help explain the thickening of BrM and later sub-RPE deposit formation in AMD. Our results also highlight the importance of MMP-14 and TIMP-2 molecules in the maintenance of adequate levels of MMP-2 activity, an enzyme that was shown to be sufficient to generate an imbalance in relevant ECM molecules when impaired by an oxidant stressor.

Transcriptional analysis of ECM components was performed by quantitative real-time PCR. From all three ECM components analyzed, only type IV collagen showed evidence of a potential transcriptional regulation. Incubation with HQ diminished the transcriptional rate of type IV collagen, probably as a negative feedback mechanism in response to the accumulation of this molecule. One might speculate that this observation reflects a "protective" attempt by the ARPE-19 cells to prevent a rapid accumulation of type IV collagen in the extracellular space. Whether this decrease in type IV collagen results from a negative feedback by high levels of this accumulated molecule deserves further investigation.

This study expands our previous work on the HQ-induced decrease of MMP-2 activity in the RPE and its consequences. Taken together, our data suggest that MMP-14 and TIMP-2 are molecules that are important in sustaining adequate levels of MMP-2 activity under oxidant stress circumstances. In addition, we demonstrate that defects in the activity of MMP-2 are sufficient to cause an imbalance in the ECM in vitro. At least

three components of the ECM accumulate after oxidative stress induced by HQ, which may be of relevance to the progression of dry AMD. Of importance, overexpression of MMP-14 and TIMP-2 molecules may prevent this defect in ECM turnover. Our findings pave the way for future interventions in patients during the early stages of AMD.

Acknowledgments

The authors thank Sanjoy Bhattacharya for critical reading of the manuscript and Marianne Pons for technical support.

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