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Authors
Brown, Donald J
Lin, Brian
Chwa, Marilyn
et al.

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Elements of the nitric oxide pathway can degrade TIMP-1 and increase gelatinase activity

Donald J. Brown, Brian Lin, Marilyn Chwa, Shari R. Atilano, Dae W. Kim, M. Cristina Kenney

Department of Ophthalmology, College of Medicine, University of California, Irvine, Orange, CA

Purpose: Keratoconus is a non-inflammatory thinning disorder of the corneal stroma. Recently, we showed that these corneas contain inducible nitric oxide synthase and an accumulation of nitrotyrosine, representing oxidative damage from peroxynitrite. Previously, we suggested that keratoconus corneas and their cell cultures have alterations in a gelatinase system with increased matrix metalloproteinase-2 (MMP-2) activity and decreased tissue inhibitor of metalloproteinase-1 (TIMP-1). This study examines whether a peroxynitrite donor (3-morpholinosydomine N-ethylcarbamide, SIN-1) or nitric oxide donor (S-nitroso-N-acetylpenicillamine, SNAP) could alter TIMP-1 and/or MMP-2 in vitro.
Methods: Normal stromal fibroblasts were cultured in the presence or absence of either SIN-1 or SNAP for varying times. These cultures were analyzed by western and northern blot analyses, gelatin zymography, and a quantitative gelatinase/MMP assay.
Results: In vitro, SIN-1 treatment led to protein nitration, increased RNA levels of TIMP-1 and MMP-2, and loss of TIMP-1 immunostaining, but did not diminish gelatinase activity. SNAP treatment led to activation of MMP-2 and significantly increased gelatinase/MMP activity, without a change in TIMP-1 levels.
Conclusions: Our data show that peroxynitrite or nitric oxide can decrease TIMP-1 and increase gelatinase activity, respectively. This demonstrates a relationship between elements of oxidative stress and tissue degradation in human corneal fibroblasts. This effect may play a significant role in the stromal thinning that occurs in keratoconus.

Keratoconus is a leading indication for corneal transplantation with a reported incidence of 1 in 2,000 individuals [1-4]. Clinically, keratoconus is characterized as a non-inflammatory thinning disorder that occurs in younger adults, leads to irregular corneal astigmatism, and decreased visual acuity [4-6]. It is not uncommon for the keratoconus corneal stroma to become less than one-quarter its normal thickness thereby leading to extensive distortion. The mechanisms of this thinning appear to be related to increased proteinase activities [7-12], decreased proteinase inhibitors [13,14], increased oxidative damage [15], and apoptosis [16].

The nitric oxide pathway involves the formation of nitric oxide from arginine and nitric oxide synthase. Nitric oxide is a mediator in many complex cellular processes in ocular tissues [17]. Increased levels of nitric oxide have cytotoxic effects that are mediated by peroxynitrite [18], which can be localized by the accumulation of a specific marker, nitrotyrosine [19-22]. Recently, we reported that keratoconus corneas have elevated levels of inducible nitric oxide synthase (iNOS) and accumulate nitrotyrosine when compared to normal corneas or corneas affected by other diseases [15]. The appearance of iNOS is usually associated with the generation of high levels of nitric oxide [23], which in turn can react with superoxide molecules to form peroxynitrite. The nitrotyrosine within keratoconus corneas is indicative of peroxynitrite formation and the deposition of nitrated protein(s) [24]. We hypothesize that the reactive nitrogen species, nitric oxide and peroxynitrite, which are present in keratoconus corneas are likely involved in keratoconus pathology. The effects of these reactive nitrogen species can be analyzed by using either the nitric oxide donor, S-nitroso-N-acetylpenicillamine (SNAP), or the peroxynitrite donor, 3-morpholinosydomine N-ethylcarbamide (SIN-1), in tissue culture systems [25,26].

In earlier studies, we reported that the human cornea contains matrix metalloproteinases (predominantly MMP-2) and its inhibitors, tissue inhibitors of matrix metalloproteinase (TIMPs) [13,27-31]. MMP-2 is part of the MMP family of which there are over 20 MMP members, each with different substrate preferences, regulation, and tissue specificity [32]. Presently there are 4 TIMP molecules that inhibit the various MMPs [33]. In keratoconus, MMP-2 was shown to be activated more easily than enzyme from normal corneas [7,8] and, in vitro, keratoconus corneal cells had increased MMP-2/TIMP-1 ratios [13]. However, it has been controversial as to whether MMP-2 and TIMP-1 play a prominent role in keratoconus [7-9,11,28]. Within inflammatory disease processes, it has been demonstrated that nitric oxide and/or peroxynitrite can alter MMP and/or TIMP levels [25,34,35]. Keratoconus corneas have increased degradative enzyme activities but it is also a non-inflammatory disorder that lacks macrophages or other inflammatory cells [36]. We wanted to determine if reactive nitrogen species could change MMP-2 or TIMP-1 production by the corneal fibroblasts themselves.

In the present study, we examined the relationship between nitric oxide and peroxynitrite, important components

Correspondence to: Donald J. Brown, Ph.D., Department of Ophthalmology, University of California Irvine Medical Center, 101 The City Drive, Orange, CA, 92868; Phone: (714) 456-7368; FAX: (714) 456-5073; email: dbrown@uci.edu

of oxidative stress, and MMP-2 and TIMP-1, molecules which may be involved in corneal degradation. Treatment of cultures with the peroxynitrite donor, SIN-1, caused fragmentation of TIMP-1 protein, upregulation of TIMP-1, and MMP-2 RNA but did not alter gelatinase activity. The treatment of cultures with a nitric oxide donor, SNAP, led to significantly increased gelatinase activity without an apparent change in either TIMP-1 or MMP-2 RNA levels. Our data suggest that a relationship exists between elements of the nitric oxide pathway (nitric oxide, peroxynitrite) and a matrix degradation pathway involving MMP-2/TIMP-1. These observations are consistent with alterations known to occur in keratoconus corneas. Further, nitric oxide and peroxynitrite have distinctly different effects on MMP-2 and TIMP-1. In keratoconus corneas, the action of elements from the nitric oxide pathway [15] may be related to increased activity of degradative enzymes and subsequent stromal thinning.

METHODS

Cell cultures and cell fractionation: Normal corneas (n=14, with an age-range of 20-64 years) were received within 24 h after death from the National Disease Research Interchange (Philadelphia, PA). After removal of the corneal epithelial and endothelial layers, primary stromal cell cultures were established [37]. Duplicate third passage cultures were grown to confluence in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum. Normal stromal cells were plated into 60 mm dishes at $5x10^5$ cells/plate. Plated cells were incubated at 37 °C in 1 ml of serum-free MEM with 1 or 10 mM 3-morpholinosydomine N-ethylcarbamide (SIN-1) or S-nitroso-N-acetylpenicillamine (SNAP, both from Calbiochem, San Diego, CA) for different intervals (15 min, 4 h, 8 h, 18 h, 24 h, or 72 h). At each time point, the media were collected and stored at -70 °C for further use. For western blot or dot blot analyses, the cell layers were rinsed with cold PBS-EDTA buffer, collected in 500 µl of immunoprecipitation (IP) buffer [38], centrifuged at 4 °C for 30 min, and then frozen at -70 °C. For northern blot analysis, cultures were treated with SIN-1 and SNAP as described above, rinsed with cold PBS-EDTA buffer and RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA).

Western blot analyses: The BCA protein assay (Pierce, Rockford, IL) was performed to assess protein concentration in each sample and equal amounts of protein were added to each lane. For western blotting, aliquots from the culture media or where indicated, recombinant TIMP-1 (R&D Systems, Minneapolis, MN), were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The antibodies used in this study were a specific monoclonal antibody to nitrotyrosine (clone 1A6, Upstate Biotechnology, Lake Placid, NY), TIMP-1 monoclonal antibody to the carboxyl terminal region (clone MAB13437, Chemicon International, Temecula, CA), TIMP-1 polyclonal antibody to the TIMP-1 loop 1 (AB8122, Chemicon International), and TIMP-1 polyclonal antibody to the amino terminal half of human TIMP-1 (clone AB8228, Chemicon International). The blots were incubated in Tris-saline containing 0.5% Tween 20 and 5% bovine serum albumin (antibody buffer) overnight at 4 °C to block non-specific binding. Primary antibody at 1 µg/ml in antibody buffer was applied to the membranes and then incubated at room temperature for 1 h. The blots were washed with Tris-Tween-saline (TTBS) and incubated with alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody at 1:5,000 dilution in antibody buffer for 1 h. After extensive washing in TTBS, the blots were developed with Immuno-Star chemiluminescent substrate buffer (Bio-Rad). As controls, some western blots were blocked with the recombinant TIMP-1 protein or nitrotyrosine.

Northern blot analysis of cell cultures: Normal stromal fibroblast cultures (n=6) were either untreated or treated with 1 mM SIN-1 or SNAP for 18 h. RNA was isolated and aliquots were separated on formaldehyde agarose gels (1.2%). The RNA was transferred by capillary action to Hybond N+ membrane (Amersham, Arlington Heights, IL) and crosslinked to the membrane with a Stratalinker (Stratagene, La Jolla, CA). The probes were generated by random priming the cDNA of TIMP-1, MMP-2, or β-actin. Hybridizations were conducted at 68 °C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA). Washings were at room temperature with 2X SSC/0.1%SDS (sodium chloride/sodium citrate buffer/sodium dodecyl sulfate), 0.2X SSC/0.1%SDS, and 2X SSC. The hybridized probes were visualized by phosphorimaging and autoradiography. Band density was analyzed by Imagequant software from the digitized phosphorimage (Amersham Pharmacia, Piscataway, NJ). The samples were normalized to β-actin RNA.

Gelatin zymography of cell cultures: Gelatin zymography and techniques of MMP activation were performed as described previously [7,27]. The culture media from normal stromal cells were fractionated on non-reducing 10% acrylamide Tris-glycine gels with 0.1% gelatin (Invitrogen, Carlsbad, CA). Gels were soaked in 1% Triton X-100 for 30 min, rinsed and incubated overnight at 37 °C in the assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, and 0.02% NaN_3. Gels were stained with Coomassie blue R-250 (EM Science, Gibbstown, NJ) and destained in acetic acid/methanol (10%/10% v/v). Gelatinase bands appeared as clear bands against a blue background. In some cases the media samples were treated with p-aminophenylmercuric acetate (APMA, Sigma Chemical Co., St. Louis, MO) at a final concentration of 2 mM for one h at 37 °C. APMA cleaves the amino terminus to convert the latent MMP to its activated form. These samples were then analyzed by zymography and the gelatinase activity assay kit.

Gelatinase activity assay of cell cultures: After SIN-1 or SNAP treatment, gelatinase/MMP-2 activity in normal stromal fibroblast culture media (n=5) was analyzed using the MMP-Gelatinase Activity Assay Kit (Chemicon International, Temecula, CA). Media samples (50 µl) or the positive control provided by the manufacturer were incubated at 37 °C for 4 h in 96 well plates with 160 µl of 1X biotinylated "Gelatinase Substrate". Then 100 µl of the media/biotinylated "Gelatinase Substrate" mixtures were added to different wells of a rehydrated biotin binding plate and incubated for 30 min at 37 °C.

The plates were washed 5 times with diluted assay buffer. Diluted streptavidin-enzyme conjugate (100 µl) were added and incubated at 37 °C for 30 min. Again, plates were washed 5 times with diluted assay buffer. Substrate solution (100 µl) was added to each well and incubated at 37 °C for 20 min. Optical density was measured at 450 nm on a microplate reader (Perkin-Elmer Lambda Reader). Samples were analyzed in duplicate. Control wells contained all components except the biotinylated "Gelatinase Substrate". Results were statistically analyzed by GraphPad Prism using an ANOVA analysis with Dunn's multiple comparison test.

RESULTS

SIN-1 (a peroxynitrite donor) or SNAP (a nitric oxide donor) were added to normal cell cultures (n=6) for varying periods of time to determine their effects upon TIMP-1 and gelatinase (MMP-2) activity. Figure 1 shows the effect of 1 mM SIN-1 and SNAP on the appearance of nitrated proteins in these cultures. As expected, addition of SIN-1 (peroxynitrite) but not SNAP (nitric oxide) led to the appearance of at least one protein recognized by the monoclonal antibody to nitrotyrosine in as little as 4 h of exposure. This experiment indicated that SIN-1 caused a similar effect seen in keratoconus corneas (accumulation of nitrotyrosine). Therefore, we proceeded to examine this system for any effect that SIN-1 or SNAP might have on MMP-2 and TIMP-1, proteins that have also been implicated in keratoconus.

RNA from cultures treated overnight in the presence of SIN-1 or SNAP was harvested and analyzed by northern analysis with TIMP-1 and MMP-2 hybridization probes. The density of each band was measured and standardized to β-actin (Figure 2). SIN-1 treatment, but not SNAP, led to an increase in the amount of both TIMP-1 and MMP-2 RNA in these cultures ($P<0.01$).

To test whether this increase in TIMP-1 RNA was reflected at the protein level, western blots were performed using monoclonal TIMP-1 antibody (clone MAB13437) and stromal cell lysates (Cell) and culture media (CM) from normal corneal fibroblast cultures (n=6) following treatment with 1 mM SIN-1 or SNAP (Figure 3). Equal amounts of protein, as determined by the BCA protein assay, were loaded onto each lane.

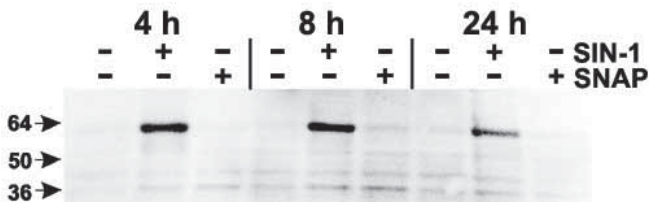

Figure 1. SIN-1 leads to nitrotyrosine accumulation. Cultures of normal stromal cells were cultured with or without either 1 mM SIN-1 or 1 mM SNAP. The cells were harvested at 4 h, 8 h, or 24 h and investigated by western analysis using a monoclonal antibody to nitrotyrosine. The secondary antibody alone shows some background staining (not shown). A prominently stained band in the SIN-1 treated cell cultures that migrated near the 64 kDa marker was noted.

Surprisingly, no increase in either cell associated or secreted TIMP-1 was noted in the SIN-1 treated cultures. In fact, in the 18 h SIN-1 treated cultures, some samples (2/6) showed two additional lower molecular weight bands. These bands likely represent degradation products of the TIMP-1 molecule, since their presence was accompanied by loss of band intensity associated with the 28 kDa TIMP-1 in the media. The presumptive TIMP-1 fragments were found only in the longer incuba-

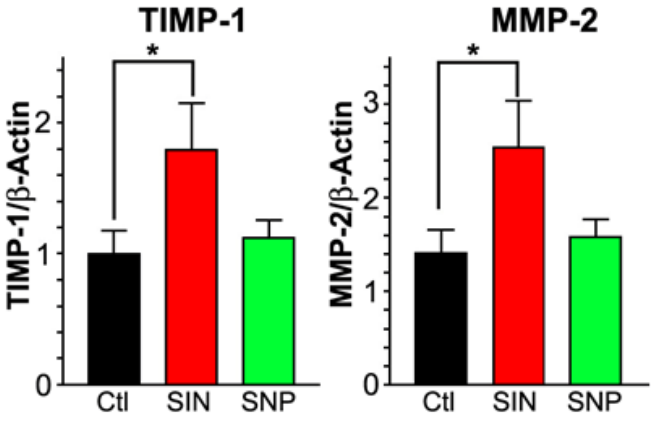

Figure 2. SIN-1 treatment increases mRNA levels of TIMP-1 and MMP-2. Normal stromal fibroblasts were cultured with or without 1 mM SIN-1 or 1 mM SNAP for 18 h. RNA was harvested from each culture and analyzed by northern analysis with probes generated by random priming the cDNA of TIMP-1, MMP-2, or β-actin. Signals for each hybridized probe were obtained with phosphorimaging and the TIMP-1 and MMP-2 normalized by comparison to β-actin. SIN-1 treated cultures showed significant increased of mRNA levels for both TIMP-1 and MMP-2 ($p<0.01$). SNAP treated cultures had mRNA levels similar to untreated.

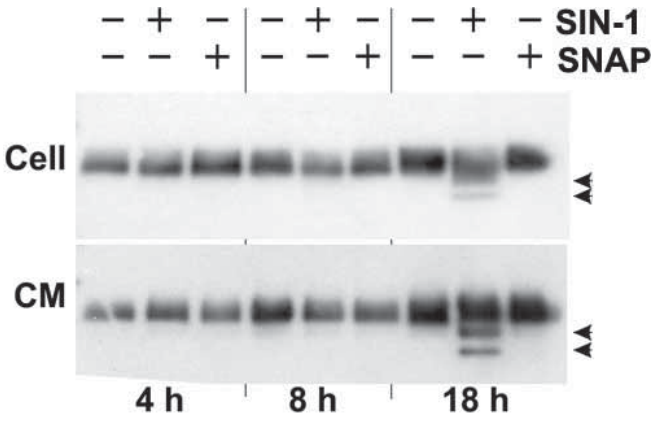

Figure 3. TIMP-1 protein is degraded after SIN-1 treatment of cell cultures. This is a representative western blot to identify TIMP-1 protein in normal cell cultures after incubation with or without 1 mM SIN-1 or 1 mM SNAP. Samples were separated into two fractions, the cell lysate (Cell) and culture media (CM). The TIMP-1 band migrates at 28 kDa. Note that in the 18 h SIN-1 treated samples, there were lower molecular weight bands, representing TIMP-1 fragments (arrowheads).

tion periods and not in the 4 h or 8 h cultures. The band densities of the 8 h cultures were quantitated and normalized to total protein loaded onto the gel (data not shown). The band density of the SIN-1 treated samples and SNAP treated samples, which in this sample appeared somewhat reduced, were not significantly altered from control levels when all samples were considered.

The next series of experiments were designed to address whether the apparent loss of TIMP-1 required the presence of intact, viable cells. Culture media from confluent cultures of corneal fibroblasts were collected and centrifuged to remove cells. Then SIN-1 or SNAP was added to this serum-free conditioned media for varying periods of time. At each time point, an aliquot was analyzed by western analysis with the TIMP-1 antibody to the carboxyl terminal region (clone MAB13437). After 72 h, the TIMP-1 protein staining decreased in the SIN-1 treated samples (Figure 4A), while the serum-free conditioned media incubated without either donor maintained a constant level of TIMP-1. Media treated with SNAP showed results that were indistinguishable from control media (data not shown). This result strongly suggested that SIN-1 may degrade the TIMP-1 molecule in the conditioned media.

We wanted to determine if the loss of TIMP-1 staining might be a direct action of SIN-1, or if this occurred indirectly (i.e., the activation of a protease in the conditioned media that then degrades other proteins). Triplicate aliquots of recombinant TIMP-1 (rTIMP-1, 50 ng) suspended in fresh serum free medium were combined with SIN-1 for varying time periods (Figure 4B,C) and then analyzed by western blotting with 2 different TIMP-1 antibodies. The first antibody (clone MAB13437) was directed to the carboxyl terminal of the TIMP-1 molecule (Figure 4B) and second antibody (AB8122)

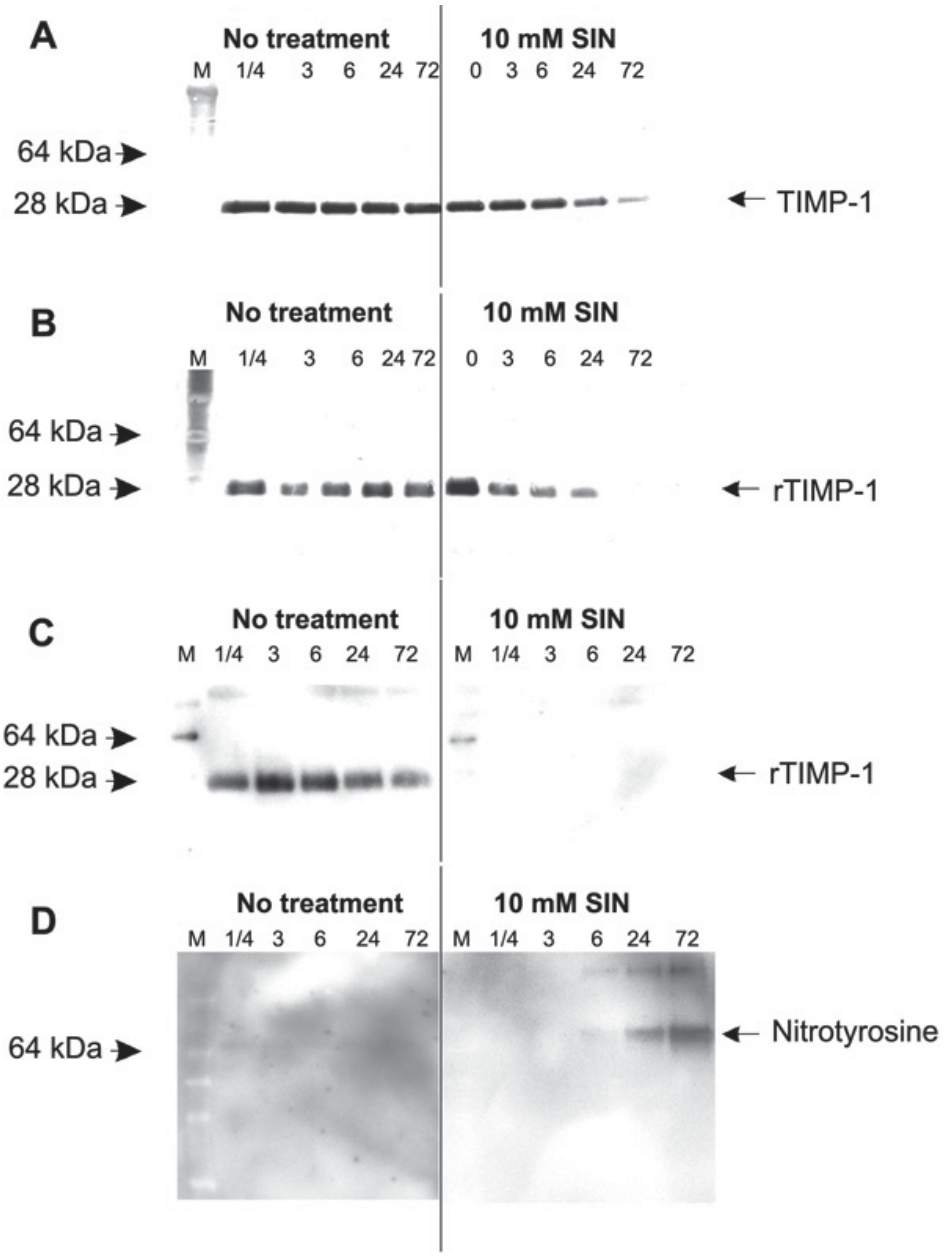

Figure 4. SIN-1 degrades both TIMP-1 and recombinant TIMP-1 (rTIMP-1). **A**: Aliquots of culture media from normal cell cultures were collected, SIN-1 was added for varying time periods, and then western blot analysis with monoclonal TIMP-1 antibody to the carboxyl terminal region was performed. Note that the untreated samples showed stable TIMP-1 levels. In contrast, the SIN-1 treated serum-free media showed less staining of the TIMP-1 protein over time. **B**: Aliquots of recombinant TIMP-1 (rTIMP-1, 50 ng) were exposed to SIN-1 for varying time periods and then analyzed by western blotting with theTIMP-1 antibody to the carboxyl terminal region. Note that the rTIMP-1 protein in the untreated samples remained stable over time. The rTIMP-1 in the SIN-1 treated samples showed decreased staining by 24 h and was not seen by the 72 h time point. **C**: Aliquots of recombinant TIMP-1 (rTIMP-1, 50 ng) were exposed to SIN-1 for varying time periods and then analyzed by western blotting with theTIMP-1 antibody to the loop 1 site of the TIMP-1 molecule. The untreated rTIMP-1 samples were stable over time. The SIN-1 treated rTIMP-1 samples did not stain at all even at the 15 min time period. **D**: The blot from **C** was stripped and reprobed with an antibody to nitrotyrosine. No bands were seen in the untreated samples. After 24 and 72 h treatment with SIN-1, an approximately 68 kDa band was seen.

to the loop1 portion of TIMP-1 (Figure 4C). After 24 h, the rTIMP-1 protein staining was reduced in the SIN-1 treated samples and by 72 h the rTIMP-1 was undetectable as seen with TIMP-1 antibody to the carboxyl region (Figure 4B). The untreated samples and SNAP treated samples (data not shown) displayed no loss of TIMP-1 signal over this time period. It was possible that the reduced staining could be due to the modification of the protein in a manner in which the antibody no longer bound to the carboxyl terminus site. Therefore, we repeated the experiment with a second antibody that was directed to the loop1 portion of the TIMP-1 molecule (Figure 4C). In this case, untreated rTIMP-1 was detected as a 28 kDa band as expected. The SIN-1 treated rTIMP-1 did not stain at any time period examined. It is possible that SIN-1 treatment acted to modify the protein in a manner that destroys antibody recognition. Attempts to verify our findings with a third antibody to the amino terminus were not successful as the antibody failed to recognize rTIMP-1 by western blot analysis. However, when these samples were examined for the presence of nitrotyrosine (Figure 4D), a 68 kDa band was detected that increased in intensity over the course of the experiment. Presumably this represents nitration of the albumin carrier protein present in the rTIMP-1 preparation. However, no nitrotyrosine staining was associated with the expected size of the rTIMP-1 (28 kDa). This suggested that SIN-1 did not lead to the accumulation of nitrotyrosine in rTIMP-1 or that once modified by nitration, the peptide is rapidly degraded.

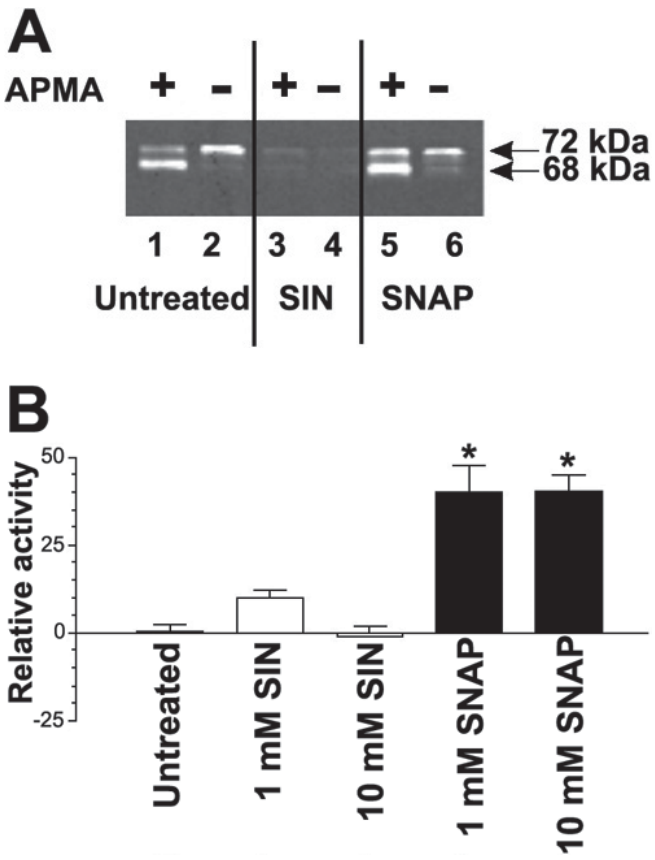

Figure 5. Increased gelatinase activity in SNAP treated cultures. Cells were cultured for 24 h in serum free media with or without SIN-1 (1 mM or 10 mM) or SNAP (1 mM or 10 mM). The media were harvested, treated with 2 mM of p-aminophenylmercuric acetate (APMA) for one h at 37 °C to activate the MMP-2 from the latent form (72 kDa) to the activated form (68 kDa). **A**: This is a representative gelatinase zymogram. Lane 1 shows that after activation with APMA (+), the 68 kDa activated MMP-2 is found. Lane 2 shows that in the control, APMA (-) cultures, the major band of gelatinase activity is at 72 kDa that corresponds to the latent form of MMP-2. Lanes 3 and 4 show that in the 10 mM SIN-1 treated cultures, little gelatinase activity is found in either the APMA (+) or APMA (-) samples. Lane 5 shows that the major band in the 10 mM SNAP treated APMA (+) culture is the 68 kDa activated MMP-2. Lane 6 shows that in the 10 mM SNAP treated, APMA (-) cultures, the major gelatinase band is the latent MMP-2 (72 kDa). **B**: Gelatinase activities found in the APMA (+) activated samples were quantitated by the gelatinase/MMP-2 assay kit. Untreated APMA (+) cultures were normalized to zero. Gelatinase/MMP activities in the 10 mM SIN-1 treated APMA (+) samples were similar to the control APMA (+) samples. Gelatinase/MMP activity in the 1 mM and 10 mM SNAP treated APMA (+) samples were significantly higher compared to control APMA (+) samples (asterisk, $p<0.001$). Assays were performed in duplicate on different normal cultures (n=5).

As TIMP-1 was altered with SIN-1 incubation, we predicted that this might lead to a change in TIMP-1 function and reveal an elevation in gelatinase activity associated with MMP-2 in these cultures. To test this, normal corneal fibroblast cultures (n=5) were treated with SIN-1 (1 mM or 10 mM) or SNAP (1 mM or 10 mM). Culture media were collected for zymography and quantitative measurement of gelatinase activity (Figure 5).

Zymography shows representative aliquots of normal culture media with and without 10 mM of SIN-1 or SNAP (Figure 5A). The latent form of MMP-2, a 72 kDa gelatinase band, is the major band. After APMA activation there is an additional band at 68 kDa representing the activated form of MMP-2. The samples treated with 10 mM SIN-1 showed little gelatinase activity in either the 72 kDa or 68 kDa bands. Interestingly, the SNAP treated media had relatively more of the 68 kDa form of the molecule, even prior to APMA treatment. MMP-9 (92 kDa) was not apparent under any of the conditions examined.

The gelatinase activities in the APMA activated samples were quantitated with a gelatinase/MMP assay kit (Figure 5B). The untreated samples were normalized to zero and compared to those that had been treated. The 10 mM SIN-1 treated samples had very low gelatinase activity, which is consistent with the SIN-1 treated zymogram (Figure 5A). In contrast, the 10 mM SNAP treated samples had significantly increased gelatinase activity compared to untreated or SIN-1 treated cultures ($p<0.001$). Interestingly, even the 1 mM SNAP treated cultures showed significantly increased gelatinase activities ($p<0.001$) compared to untreated samples.

DISCUSSION

Oxidative stress is important in the pathological processes associated with cardiovascular disease, arthritis, and kidney disease [19-22]. Major pathways involved in oxidative damage include lipid peroxidation and nitric oxide metabolites. In

the cornea, nitric oxide has been shown to be involved in inflammation, angiogenesis, and the maintenance of corneal thickness [17,39,40]. We hypothesize that nitric oxide and oxidative stress also play a role in the non-inflammatory corneal disorder, keratoconus. Our previous immunohistochemistry data demonstrated an accumulation of nitrotyrosine (a marker for peroxynitrite, a cytotoxic product of the nitric oxide pathway) within the keratoconus cornea compared to normal corneas or other corneal diseases [15]. Here, we demonstrate that the addition of peroxynitrite to stromal cell cultures leads to the accumulation of nitrotyrosine, mimicking the in vivo observation.

Using this in vitro corneal fibroblast tissue culture system, we demonstrate a relationship between elements of the nitric oxide pathway (nitric oxide and peroxynitrite) and a corneal degradative pathway (TIMP-1 and MMP-2/gelatinase). SIN-1 generates nitric oxide and the superoxide anion, both of which are required for the formation of peroxynitrite [18,20]. At higher SIN-1 concentrations and longer incubation times, we show that TIMP-1 within the conditioned media becomes altered. This modification of TIMP-1 is most likely a direct action, since rTIMP-1 rapidly loses antibody recognition to a loop-specific epitope after SIN-1 exposure. This suggests that the disulfide bond forming the loop is rapidly disrupted (within 15 min) following SIN-1 treatment. The use of an alternate antibody to the carboxyl terminus showed a slow progressive loss of staining, which suggests, but does not prove, a decline in the amount of rTIMP-1. Our observation is consistent with Frears and coworkers [41] who demonstrated that high concentrations of peroxynitrite could reduce by 50% the inhibition of MMP-2 by TIMP-1 and lead to TIMP-1 protein fragmentation.

In general, decreased TIMP-1 levels or an alteration of its structure could have multiple ramifications within any diseased tissue. TIMP-1 has diverse roles within cells [42,43]. Notably, TIMP-1 can suppress apoptosis [44-46], and it was shown that keratocytes in the anterior stroma of keratoconus corneas are apoptotic [16].

One of the major functions of TIMP-1 is the inhibition of gelatinase/MMP activity. Since TIMP-1 was fragmented in the presence of peroxynitrite/SIN-1 treated fibroblast cultures, it was reasonable to expect that gelatinase activity would increase in these cultures. It was surprising to find that the gelatinase/MMP activity in the SIN-1 treated samples were similar to untreated cultures. It may be that the exposure to peroxynitrite caused a loss of function of the MMP/gelatinase enzyme through protein oxidation or nitration. Perhaps, as it has been shown that peroxynitrite causes fragmentation of bovine serum albumin[47], degradation and fragmentation of proteins may be a common outcome of peroxynitrite exposure and this leads to altered function. In other systems, it has been shown that peroxynitrite can lead to altered biologic functions via protein modifications including inhibition of tyrosine phosphorylation [18,48-54].

Our findings concerning MMP-2 activity after SIN-1 treatment differ from those presented for tumor cells where peroxynitrite was involved in the activation of MMPs [34,35,55]. In in vitro systems, peroxynitrite can activate proMMP to MMP [35,55], but in our tissue culture system we have no evidence that this activation from the latent form of MMP-2 is occurring and the gelatinase/MMP-2 activity is not quantitatively increased after SIN-1 treatment. We do not know the mechanism of action since we have no evidence that MMP-2 is becoming nitrated or fragmented after SIN-1 treatment. However, we can speculate that as the MMP-2 becomes functionally inactivated there is feedback at the cellular level because SIN-1 treated cells demonstrate upregulation of both MMP-2 and TIMP-1 RNA levels. This may reflect the cellular response to oxidative stress induced by peroxynitrite and suggests a relationship between oxidative stress elements and degradative enzyme activities in corneal fibroblasts.

In the presence of SNAP, a nitric oxide donor, gelatinase activity was significantly higher than in untreated cultures or SIN-1 treated cultures. The mechanism for the elevated gelatinase activity in the SNAP treated cultures is most likely due to activation of the 72 kDa latent form into the 68 kDa active form as seen in the zymogram (Figure 5A). In the SNAP treated cultures, TIMP-1 protein was not fragmented and RNA levels of TIMP-1 and MMP-2 were normal. This finding is not unexpected as other investigators [56,57] have shown that latent MMPs can be activated without autocatalysis by treatment with a number of organomercurials, sulfhydryl alkylating agents, and (more recently) nitric oxide. This activation is accomplished by disrupting the interaction of a critical cysteine residue in the amino terminus normally coordinated with the catalytic zinc moiety in the active site of the molecule. This disruption has often been referred to as the cysteine switch mechanism of activation and can occur without autocatalysis. Our findings are in agreement with another group that showed SNAP could induce the expression of MMP-2 without changing TIMP [25].

In terms of understanding keratoconus, our finding are important because most of the literature regarding oxidative stress and tissue degradation involves inflammatory disease processes such as arthritis, systemic lupus erythromatosis, and cardiovascular disease. In those processes, macrophages, polymorphonuclear cells, and inflammatory cells are involved and these are known to have activated degradative enzyme systems. Keratoconus, lacking macrophages and inflammatory cell infiltrates [36], is not an inflammatory process and yet oxidative stress and tissue degradation is occurring. Our data demonstrate that in response to nitric oxide elements (peroxynitrite and nitric oxide), cultured human corneal fibroblasts are capable of modulating MMP-2 and TIMP-1 levels. Furthermore, for the first time, an in vitro culture system of normal stromal cells has been shown to mimic aspects of the keratoconus cornea (nitrotyrosine accumulation, increased gelatinase activity, and decreased TIMP-1) by the direct addition of nitric oxide and peroxynitrite donors.

In summary, our previous studies showed that keratoconus corneas have both increased nitric oxide (evidenced by elevated levels of inducible nitric oxide synthase) and peroxynitrite (reflected by the nitrotyrosine staining [15]). If the in vitro relationship between nitric oxide elements and

TIMP-1 and MMP-2/gelatinase activity is maintained in the intact corneas, then the presence of nitric oxide and/or peroxynitrite may explain the observed increase in gelatinase activities [58-60] and decreased TIMPs [29] reported to occur in keratoconus corneas. This relationship may play a significant role in the stromal thinning that occurs in keratoconus.

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