Increased Levels of Catalase and Cathepsin V/L2 but Decreased TIMP-1 in Keratoconus Corneas: Evidence that Oxidative Stress Plays a Role in This Disorder

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PURPOSE. The mRNA levels of antioxidant enzymes, matrix metalloproteinases, cathepsin V/L2, and tissue inhibitor of matrix metalloproteinases (TIMPs) were determined in keratoconus and normal corneas. Protein levels or enzyme activities were analyzed when RNA levels were different.

METHODS. A total of 25 physiologic (normal) and 32 keratoconus corneas were studied. mRNAs were analyzed by semiquantitative reverse transcription-polymerase chain reaction and Southern blot analysis. Proteins were assessed by immunohistochemistry and/or Western blot analysis. Catalase activity was measured in corneal extracts. Antioxidant enzymes examined were catalase, superoxide dismutase (SOD)-1, SOD3, glutathione reductase, glutathione S-transferase and aldehyde dehydrogenase 3A1. Degradative enzymes examined were cathepsin V/L2 and matrix metalloproteinase (MMP)-1, -2, -7, -9, and -14. Tissue inhibitor of matrix metalloproteinase (TIMP)-1, -2, and -3 were also examined.

RESULTS. Keratoconus corneas exhibited a 2.2-fold increase of catalase mRNA level (P < 0.01) and 1.8-fold of enzyme activity (P < 0.03); a 1.5-fold increase of cathepsin V/L2 mRNA (P < 0.05) and abnormal protein distribution; and a 1.8-fold decrease of TIMP-1 mRNA (P < 0.0001) compared with normal (physiologic) corneas. RNA levels for other antioxidant and degradative enzymes were similar between normal and keratoconus corneas.

CONCLUSIONS. Keratoconus corneas have elevated levels of cathepsins V/L2, B, and -G, which can stimulate hydrogen peroxide production, which, in turn, can upregulate catalase, an antioxidant enzyme. In addition, decreased TIMP-1 and increased cathepsin V/L2 levels may play a role in the matrix degradation that is a hallmark of keratoconus corneas. The findings support the hypothesis that keratoconus corneas undergo oxidative stress and tissue degredation. (Invest Ophthalmol Vis Sci. 2005;46:823–832) DOI:10.1167/iovs.04-0549

Keratoconus (KC) is a significant clinical problem worldwide and a leading indication for corneal transplantation.1–5 KC corneas have significant thinning that leads to keratometric steepening and irregular astigmatism. The onset is usually in the teens to early twenties, after which it can progress to cause moderate to severe vision loss.6 The reported incidence of KC is approximately 1 in 2000 in the general population, but is 300 times higher in patients with Down syndrome.7,8,9 Studies suggest that oxidative stress is involved in KC.5–9 KC corneas have increased levels of inducible nitric oxide synthase (iNOS), nitrotyrosine (a marker for peroxynitrite, ONOO−), malondialdehyde (MDA),10,11 and glutathione S-transferase,12 and decreased activities of extracellular superoxide dismutase (SOD3, EC-SOD)13 and aldehyde dehydrogenase class 3 (ALDH3A1).14 These types of abnormalities are associated with elevated levels of superoxide radicals (O2−), hydrogen peroxide (H2O2) and hydroxyl radicals (OH•), commonly referred to as reactive oxygen species (ROS; Fig. 1). The accumulation of ROS can greatly damage cells by reacting with proteins, DNA, and membrane phospholipids. Normally, the cornea’s natural antioxidant enzymes eliminate the ROS before they damage cells. These include SODs, catalase, glutathione reductase, and glutathione peroxidase (Fig. 1). Although antioxidant activities and protein levels have been examined in normal and keratoconus corneas,5,6,8 the mRNA levels for these enzymes have not been studied in KC corneas.

Cells can also be damaged from aldehydes formed during ROS-mediated lipid peroxidation.10–13 Aldehydes are relatively stable compared with free radicals and can diffuse to attack distant target sites. These aldehydes are highly reactive and can covalently interact with proteins and DNA to form adducts that alter signal transduction, gene expression, and proliferation. Glutathione S-transferase and ALDH3A1, which are abundant in the human cornea, detoxify aldehydes by GSH-conjugation and oxidation to their corresponding acids,14–16 respectively, thereby protecting the cornea from lipid peroxidation damage (Fig. 1).

Aldehydes disrupt the membranes of lysosomes and cells releasing lysosomal proteolytic enzymes, including cathepsins.17 Studies have demonstrated that KC corneas have increased levels of cathepsin B and -G, and lysosomal enzymes (acid esterases, acid phosphatases, and acid lipases).18–21 These cathepsins can increase hydrogen peroxide production and oxidative stress and mediate apoptosis.22–26 More recently, the basement membrane–associated cathepsin V/L2 has been detected in normal human corneas (Adachi W, et al. IOVS 1999;40:ARVO Abstract 2063).27,28 Although epithelial basement membrane disruption is a hallmark of keratoconus, the expression and distribution of cathepsin V/L2 have not been determined in KC corneas.
KC corneas, resulted in upregulation of MMP-2 RNA levels and a key element of oxidative stress. Moreover, in addition to major antioxidant enzyme that eliminates hydrogen peroxide, twofold increases of RNA and enzyme activity for catalase, the MMPs. In cultured human corneal fibroblasts, the addition of the inhibitor TIMP-1 and increased cathepsin V/L provide and gelatinase activity, the RNA levels of the MMPs or cornea. Moreover, we analyzed a large number of specimens and used small fraction of tissues used in this study may not represent intact thelum during processing. Therefore, although it is possible that a

**MATERIALS AND METHODS**

**Isolation of RNA and Protein from Normal and Keratoconus Corneas**

Normal human corneas (n = 8) were obtained from the National Disease Research Institute (NDRI) within 24 hours of death. Keratoconus corneas (n = 12) were collected from ophthalmologists within 24 hours after surgery. The study was approved according to the tenets of the Declaration of Helsinki for research involving human subjects. Corneas were snap frozen and was performed according to the tenets of the Declaration of Helsinki and informed consents were obtained from participants and the study was performed according to the tenets of the Declaration of Helsinki for research involving human subjects. Corneas were snap frozen and stored at -70°C until use. Care was taken to handle tissues gently to ensure that epithelium and endothelium would remain intact. Based on histology of frozen tissues, <1 in 20 corneas lose epithelium or endothelium during processing. Therefore, although it is possible that a small fraction of tissues used in this study may not represent intact corneas, we believe most specimens accurately represent the human cornea. Moreover, we analyzed a large number of specimens and used corroborating techniques such as Western blot analysis, immunohistochemistry, and activity assays to verify our RNA results. Finally, similar RNA isolation and RT-PCR methods have been applied successfully in other corneal studies. It should be noted that in studies of human diseases, there is often individual variation that may due to the heterogeneity or severity of the disease. This is a drawback of any human corneal tissue investigation. Therefore, we always examine larger number of corneas, do not pool samples and study multiple parameters (i.e., levels of RNA, protein and/or activities).

The corneas were pulverized individually to a fine powder under liquid nitrogen with the use of a mortar and pestle. One milliliter of extraction reagent (TRIZol; Invitrogen, Carlsbad, CA) was added to the powder, and the sample was warmed to room temperature and homogenized. Chloroform (0.2 mL) was added, and the samples were centrifuged at 11,000 rpm for 15 minutes at 4°C to separate the aqueous and organic phases. Isopropanol (0.5 mL) was added to the aqueous phase for 10 minutes at room temperature to precipitate the RNA. Samples were centrifuged at 11,000 rpm for 15 minutes at 4°C. Pellets were washed with 75% ethanol, dried in a vacuum, and resuspended in RNase-free water. The yield of RNA was estimated by optical density at 260/280 nm.

The protein was recovered from the organic phase by the addition of 0.5 mL 100% ethanol for 3 minutes at room temperature and then centrifugation at 2000 rpm for 5 minutes at 4°C. Isopropanol (1.5 mL) was added to the supernatant for 10 minutes at room temperature followed by centrifugation at 11,000 rpm for 10 minutes at 4°C. The protein pellets were washed three times in 0.3 M guanidine hydrochloride in 95% ethanol. Pellets were dried in a vacuum and stored at -70°C until further use.

**Reverse Transcription–Polymerase Chain Reaction Analysis**

For these experiments, RNA samples from individual corneas were isolated, and the samples were not pooled together (8 normal and 12 KC). Two micrograms of RNA were reverse transcribed in an 80-μL reaction volume containing 500 μM dNTPs, 2.5 μM random hexamer primers, 20 U RNase inhibitor, and 200 U reverse transcriptase (SuperScript II; Invitrogen). Reactions were performed for 10 minutes at 25°C, 45 minutes at 42°C, and 5 minutes at 95°C, followed by cooling to 4°C. cDNA samples were subjected to PCR using specific primers (Table 1). Primers were designed by computer (Primer 3 Internet software program; Whitehead Institute, Cambridge, MA), and their specificities were confirmed by a BLAST Internet software-assisted search of nonredundant nucleotide sequence database (National Library of Medicine, Bethesda, MD).

Polymerase chain reactions (PCR) were performed with 5 to 25 ng of reverse-transcribed RNA. Taq polymerase buffer (200 μM deoxyribonucleoside triphosphates, 1.25 U Taq polymerase (Promega Biotech, Madison, WI), and 250 nM forward and reverse primers in a total volume of 50 μL. The conditions for the PCR reactions are described in Table 1. For semiquantitative PCR, samples were amplified in the linear range, which was established by using serial cDNA dilutions and varying the number of cycles. Amplified products were separated by electrophoresis in 2% agarose gels and visualized under ultraviolet light after staining with ethidium bromide. All samples were normalized to β2-microglobulin (β2MG) amplification. Routine PCR control analyses without reverse transcriptase (water control) or with normal human genomic DNA as a template were negative. Blots were scanned by densitometry with phosphorimaging (Typhoon; Molecular Dynamics, Sunnyvale, CA) and bands were standardized to β2MG levels. Statistical analysis results were analyzed with the nonparametric Mann-Whitney test (InStat software program; GraphPad Software, San Diego, CA).

Attempts to perform RT-PCR for glutathione peroxidase were not successful, but immunohistochemistry with specific antibody was performed.
Table 1. RTPCR Primers, Annealing Temperatures and Hybridization Oligonucleotides Used for Antioxidant Enzymes, Degradative Enzymes and Inhibitors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temp. (°C)</th>
<th>Cycles (n)</th>
<th>Size (bp)</th>
<th>Internal Oligonucleotide</th>
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<tr>
<td>ALDH3A1</td>
<td>GAGCGAGCTTCACTTAAA</td>
<td>GATTCGTTGGCTGGAGAA</td>
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<td>38</td>
<td>251</td>
<td>ND</td>
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<td>CATase</td>
<td>GAGCTTCTCCTCCTGCTCA</td>
<td>GGGAAGATCGTCAGGTCAA</td>
<td>56</td>
<td>33</td>
<td>279</td>
<td>ND</td>
</tr>
<tr>
<td>SOD1</td>
<td>GAGCTTCTCCTCCTGCTCA</td>
<td>GGGAAGATCGTCAGGTCAA</td>
<td>56</td>
<td>33</td>
<td>279</td>
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<tr>
<td>SOD3</td>
<td>GAGCTTCTCCTCCTGCTCA</td>
<td>GGGAAGATCGTCAGGTCAA</td>
<td>56</td>
<td>33</td>
<td>279</td>
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<td>41</td>
<td>337</td>
<td>ND</td>
</tr>
<tr>
<td>GST-pi</td>
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<td>GCAGCAAGTCCAGCAGGTTTAGTC</td>
<td>55</td>
<td>40</td>
<td>ND</td>
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<td>56</td>
<td>36</td>
<td>515</td>
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<tr>
<td>MMP-1</td>
<td>GGTTCTCAAAGTTGTAGGTGGG</td>
<td>ND</td>
<td>56</td>
<td>36</td>
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<tr>
<td>MMP-1</td>
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<td>ND</td>
<td>56</td>
<td>36</td>
<td>515</td>
<td>ND</td>
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<tr>
<td>CV/L2</td>
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<td>58</td>
<td>40</td>
<td>926</td>
<td>ND</td>
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<tr>
<td>TIMP-1</td>
<td>GTATTTGTTCAAGGATGGGAAGTC</td>
<td>GCAGGATGTCATAGGTCACGTAGT</td>
<td>58</td>
<td>40</td>
<td>926</td>
<td>ND</td>
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<td>TIMP-1</td>
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<td>40</td>
<td>926</td>
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<tr>
<td>TIMP-2</td>
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<td>40</td>
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<tr>
<td>TIMP-3</td>
<td>GTATTTGTTCAAGGATGGGAAGTC</td>
<td>GCAGGATGTCATAGGTCACGTAGT</td>
<td>58</td>
<td>40</td>
<td>926</td>
<td>ND</td>
</tr>
</tbody>
</table>

All primers are from 5'-3' ALDH, aldehyde dehydrogenase; CATase, catalase; SOD, superoxide dismutase; GTR, glutathione reductase; GST-pi, glutathione S-transferase; MMP, matrix metalloproteinase; CV/L2, cathepsin V/L2; TIMP, tissue inhibitor of metalloproteinase; B2-MG, B2-microglobulin.

Southern Blot Analysis

PCR products were transferred from 2% agarose gels to positively charged nylon membranes (Hybond N+; Amersham, Arlington Heights, IL) using an alkaline blot procedure. Membranes were hybridized (Table 1) with oligonucleotide probes 5'-end labeled with [32P] adenosine triphosphate (222 Tbq/mmol; NEN, Boston, MA), washed at high stringency, and exposed to x-ray film (X-Omat AR; Eastman Kodak, Rochester, NY). Blots were scanned by densitometry with phosphorimaging (Typhoon; Molecular Dynamics), and bands were standardized to B2-MG levels.

Immunohistochemistry

Normal corneas (n = 10) were obtained from NDRI within 24 hours of death. KC corneas (n = 10) were obtained within 24 hours after a corneal transplantation. On arrival in the laboratory, tissues were rinsed thoroughly in cold phosphate-buffered saline (pH 7.2) and embedded in OCT compound (Tissue-Tek; Sakura Finetec, Torrance, CA) before freezing in liquid nitrogen. Five-micrometer tissue sections were cut with a cryostat (Leica, Deerfield, IL), mounted onto microscope slides, and stored at −80°C until further use.

Immunohistochemistry was performed with the polyclonal antibodies to SOD1 (1:10 dilution; 20 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), catalase (1:500 dilution; Chemicon International, Temecula, CA), glutathione peroxidase (GPX; U.S. Biological, Swampscott, MA), and cathepsin V/L2 (a gift from Drs. Adachi, Ljuubimov and Kinoshita). A monoclonal antibody to ALDH3A1 (1:20 dilution) was also used for immunohistochemical staining. 47 Rhodamine-conjugated secondary antibodies were from Chemicon International.

Tissue sections were thawed and incubated in phosphate-buffered saline (PBS) for 15 minutes. Primary antibody was applied to the tissue and incubated in a humidified chamber for 1 hour. Tissues were washed with PBS and incubated in 50 μl of the appropriate secondary antibody for 1 hour in a humidified, dark chamber. Sections were washed again with PBS and mounted with glycerol/PBS (1:1). The slides were examined and photographed using a fluorescence microscope (Leica) with an attached digital camera. Controls included the use of secondary antibodies only, with available blocking peptides (SOD1) added to the primary antibodies.

Western Blot Analysis

From normal (n = 8) and KC (n = 12) corneal extractions, equal amounts of protein, as determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL), were electrophoresed on precast 4% to 20% Tris-glycine sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (Invitrogen). The samples were electrophoresed under nonreduced and reduced (3% dithiothreitol) conditions. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) in a humidified chamber for 1 hour. The membranes were then blocked with a blocking solution (3%) and incubated in a humidified, dark chamber. Sections were incubated with a primary antibody for 1 hour in a humidified, dark chamber. Sections were washed with PBS and incubated in 50 μl of the appropriate secondary antibody for 1 hour in a humidified, dark chamber. The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody, washed, and exposed to x-ray film (X-Omat AR; Eastman Kodak, Rochester, NY). Blots were scanned by densitometry with phosphorimaging (Typhoon; Molecular Dynamics), and bands were standardized to β2-MG levels.

Catalase Activity Assay

Normal (n = 7) and keratoconus (n = 10) frozen corneas were pulverized to a fine powder in liquid nitrogen and extraction buffer (0.1 M potassium phosphate [pH 7.7] and 0.2% Triton X-100) was added. Samples were centrifuged at 12,000 rpm at 4°C for 15 minutes. Protein concentrations were measured by a BCA protein assay kit.

All primers are from 5'-3' ALDH, aldehyde dehydrogenase; CATase, catalase; SOD, superoxide dismutase; GTR, glutathione reductase; GST-pi, glutathione S-transferase; MMP, matrix metalloproteinase; CV/L2, cathepsin V/L2; TIMP, tissue inhibitor of metalloproteinase; B2-MG, B2-microglobulin.

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Catalase activity was measured according to the manufacturer’s protocol (an Amplex Red Catalase kit; Molecular Probes, Eugene, OR). Briefly, catalase standards and 2 μg of each sample (total protein) were incubated in 40 μM H₂O₂ solution, followed by incubation with 100 μM of nonfluorescent reagent (Amplex Red; Molecular Probes) containing 0.4 U/mL horseradish peroxidase (HRP) at 37°C for 30 minutes. At the end of incubation, fluorescence intensity was measured with a laser-based scanning system (FMBIOIII; Hitachi, Yokohama, Japan) with excitation at 535 nm and emission at 590 nm.

Statistical analysis was performed with the unpaired Student’s t-test (one-tailed).

RESULTS

Semi-quantitative RT-PCR was performed with specific primers for six antioxidant enzymes, six degradative enzymes, and three TIMPs (Table 1). All samples were amplified in a linear range established with serial cDNA dilution and varied number of cycles. The gels were scanned, normalized to β₂M, and three genes—catalase, cathepsin V/L2, and TIMP-1—appeared to have altered expression. In those cases, further analyses by Southern blot analysis, Western blot analysis, immunohistochemistry, and/or enzyme activity assays were pursued. These results are presented in Figures 2, 3, and 4.

Catalase

Figure 2 shows the catalase mRNA levels, activity, and protein distribution in KC corneas. Semiquantitative RT-PCR showed that after being standardized to β₂M, catalase RNA levels appeared to be increased in KC corneas (n = 8) compared with normal corneas (n = 8; Fig. 2A). The blots were scanned, and statistical analysis of the results showed that the KC corneas (0.167 ± 0.02) had a 2.2-fold increase of catalase mRNA compared with age-matched normal corneas (0.076 ± 0.01; P < 0.01). Southern blot analysis with a labeled internal oligonucleotide specific for catalase (Table 1) was performed to confirm specificity of the product (Fig. 2B). Whereas these results indicate alterations in the level of mRNA for catalase, it was possible that the level of catalase activity would not be similarly changed. Therefore, to assess the level of enzyme activity, a fluorescent substrate assay was used and the mean ± SEM was calculated. Extracts of normal (n = 7) and KC (n = 10) corneas showed an approximate 1.8-fold increase of catalase activity in KC compared to age-matched normal corneas (P < 0.03). (D) Immunohistochemical analysis of KC corneas stained with rhodamine-conjugated antibodies to catalase. Antibody to catalase stained the epithelial cell cytoplasm with slight staining of the keratocytes and endothelium. Normal corneas had staining patterns similar to those of the KC corneas. No staining was observed when only secondary antibody was used on the tissue sections. E, epithelium; S, stroma; Endo, endothelium. Scale bar, 100 μm.
Catalase Activity in Keratoconus

Figure 2C shows the increased catalase activity (P < 0.03) in KC corneas (216.0 ± 37.1 mU catalase activity/μg protein) when compared with normal corneas (120.8 ± 14.6 mU catalase activity/μg protein; Fig. 2C). Within the KC group, the catalase activity levels showed large variations with the range from 94 to 448 mU catalase activity/μg protein. This higher activity level in KC corneas correlated well with the apparent increase in the level of catalase mRNA. Immunohistochemistry with an antibody specific to catalase was performed to determine whether the distribution of the protein was changed in KC corneas. Figure 2D showed that the catalase localized mainly to epithelial cells with slight staining of the keratocytes and endothelium. The catalase staining patterns for normal and KC corneas were similar to each other.

Cathepsin V/L2

Figure 3 shows the increased mRNA levels and altered protein distribution for cathepsin V/L2 in KC corneas. Semiquantitative RT-PCR (Fig. 3A) was performed followed by Southern blot analysis (Fig. 3B) using specific primers and oligonucleotides for cathepsin V/L2 (Table 1). After normalization to the intensity values of β2-MG, the cathepsin V/L2 RNA levels in KC corneas (1.48 ± 0.26) were increased ~1.5-fold compared with age-matched normal corneas (0.97 ± 0.33; P < 0.03). To determine the protein distribution, immunohistochemistry was performed with an antibody specific for cathepsin V/L2 (Fig. 3C). In normal corneas, cathepsin V/L2 was found primarily in association with the epithelial basement membrane (Adachi W,

![Cathepsin V/L2 Diagram]

**Figure 3.** KC corneas had increased cathepsin V/L2 mRNA levels and abnormal protein distribution compared with normal corneas. (A) RT-PCR analysis of gene expression for cathepsin V/L2 in normal and KC corneas. Lanes 1 to 8: normal corneas; lanes 9 to 20: KC corneas. H2O lane, water only. Hybridization signal intensities were normalized to values obtained for β2-MG. The cathepsin V/L2 RNA levels in KC corneas were increased ~1.5-fold compared with age-matched normal corneas (P < 0.03). (B) Southern blot analysis of cathepsin V/L2 in normal and KC corneas. Primers for cathepsin V/L2 were designed and used to amplify the RNA from individual corneas. After amplification, the products were run on agarose gels and then assayed by Southern blot analysis with specific oligonucleotide probes to confirm the specificity of the product. Lanes are as in (A)—RT, negative control without RT; STD, dilution to show linear range. (C) Protein distribution was determined by immunohistochemistry with a cathepsin V/L2 antibody. In normal corneas, cathepsin V/L2 was associated primarily with the epithelial basement membrane. In KC corneas, increased staining with cathepsin V/L2 antibody was observed and the pattern of staining was variable. Some KC corneas had increased staining of the epithelial basement membranes, whereas other corneas showed cathepsin V/L2-positive epithelial cells and stromal cells. No staining was observed when only secondary antibody was used on the tissue sections. E, epithelium; S, stroma; Endo, endothelium. Scale bar, 100 μm.
et al. IOVS 1999;40:ARVO Abstract 2063). In KC corneas, increased staining with cathepsin V/L2 antibody was observed and the pattern of staining was variable. Some KC corneas had cathepsin V/L2-positive epithelial cells, other corneas showed staining of stromal cells, and still others had increased staining of the epithelial basement membrane. These data suggest that there is an increase and redistribution of cathepsin V/L2 expression in KC corneas.

**Tissue Inhibitor of Matrix Metalloproteinase-1**

Figure 4 shows decreased TIMP-1 mRNA and protein levels in KC corneas. Semiquantitative RT-PCR (Fig. 4A) and Southern blot (Fig. 4B) analyses were performed with TIMP-1 primers and oligonucleotides (Table 1). The TIMP-1 mRNA levels (Fig. 4A) were decreased 1.8-fold in KC corneas (0.85 ± 0.42) compared with normal corneas (1.49 ± 0.40; \(P < 0.05\)). Western blot analyses of normal (\(n = 8\)) and KC (\(n = 12\)) corneal extracts were performed to determine whether the altered RNA expression for TIMP-1 was reflected at the protein level (Figs. 4C, 4D). The SDS-PAGE gel stained with Coomassie blue showed that equivalent amounts of protein were loaded in each lane. Each lane represents extracts of individual corneas. (D) Western blot analysis of KC corneal extracts using a monoclonal antibody to TIMP-1. The single 28-kDa band is the correct molecular weight for TIMP-1 (arrowhead). Note the decreased staining for TIMP-1 in the KC corneal extracts compared with normal corneas. The TIMP-1 densities in KC samples (\(n = 10\)) and normal samples (\(n = 8\)) were scanned and standardized to total protein. KC corneas had 2.8-fold a decrease in TIMP-1 protein (0.249 ± 0.088) compared with normal corneas (0.694 ± 0.212). \(P < 0.0001\).
The major antioxidant enzyme upregulated was catalase. Similar findings have been reported in experimental corneal animal models, but this is the first report of catalase activity in human corneas. In this study, expression of catalase mRNA levels along with its enzyme activity was increased significantly in KC corneas. In a lens epithelial cell line conditioned to survive H₂O₂ exposure, the major antioxidant enzyme upregulated was catalase. Similarly, increased levels of catalase were found in H₂O₂-stressed Chinese hamster fibroblasts, A549 human lung adenocarcinoma cells, and human keratocytes. 

**DISCUSSION**

The findings in this study support our hypothesis that oxidative stress and tissue degradation are ongoing processes in KC corneas. Hydrogen peroxide (H₂O₂) is a key element in oxidative damage associated with a variety of diseases. High levels of H₂O₂ are a signal for upregulation of catalase. Cathepsins can increase H₂O₂ production, cleave Bid, cause release of cytochrome c, and initiate apoptosis. In addition, cathepsins can degrade extracellular matrix and destabilize cell membranes. 

**Catalase**

Catalase is an important enzyme in the elimination of H₂O₂ from tissues (Fig. 1). Catalase activity has been reported in experimental corneal animal models, but this is the first report of catalase activity in human corneas. In this study, expression of catalase mRNA levels along with its enzyme activity was increased significantly in KC corneas. In a lens epithelial cell line conditioned to survive H₂O₂ exposure, the major antioxidant enzyme upregulated was catalase. Similarly, increased levels of catalase were found in H₂O₂-stressed Chinese hamster fibroblasts, A549 human lung adenocarcinoma cells, and human keratocytes.
noma cells and U87MG glioblastoma cells. It is suggested that catalase and not GPX is the major antioxidant enzyme responsible for H$_2$O$_2$ degradation. Because H$_2$O$_2$ induces catalase expression, it is likely that KC corneas have elevated levels of this ROS, which could account for some of the oxidative damage associated with KC corneas. Furthermore, under experimental conditions, H$_2$O$_2$ can inhibit SOD, which may play a role in the lower SOD3 activities found in KC corneas. Moreover, the cytotoxic by-products of oxidative stress, that is malondialdehyde (MDA) and peroxynitrite, are present in KC corneas. MDA and peroxynitrite are formed from hydrogen peroxide and superoxide, respectively, both highly reactive oxygen species. Taken together with our findings of increased catalase levels, we believe these data support our hypothesis that ROS production and oxidative stress are important elements in KC.

**Cathepsin V/L2**

Cathepsin V is a recently described addition to the cathepsin family. It is a cysteine proteinase that is found in thymus, testis, and cornea. Cathepsin V is homologous to cathepsin L, a lysosomal enzyme, with substrates that include fibronectin, laminin, collagens, and proteoglycans. Although most corneal proteases are found either intracellularly or associated with the stromal matrix, cathepsin V/L2 is associated with normal corneal epithelial basement membranes (Adachi W, et al. *JOVS* 1999;40:ARVO Abstract S392). Furthermore, although cathepsin V/L2 has weaker collagenolytic activity than cathepsin L, its presence may still correlate with the digested basement membranes and Bowman’s layer seen early in KC corneas. Increased cathepsin V/L2 expression and its redistribution may be related to the increased fragmentation of epithelial basement membranes in the KC corneas.

The cathepsin V/L2 immunostaining patterns varied markedly in the individual KC corneas. In addition to staining of the epithelial basement membrane, some KC corneas had increased cathepsin V/L2 staining in stromal cells and epithelial cells. KC corneas have increased RNA and/or protein levels of cathepsin V/L2, -B, and -G. Increased cathepsin V/L2 expression and its redistribution may be related to the increased fragmentation of epithelial basement membranes in the KC corneas.

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**Tissue Inhibitor of Matrix Metalloproteinase-1**

Both the RNA and protein levels of TIMP-1 were decreased significantly in KC corneas compared with normal corneas, in agreement with previous in vitro experiments showing that KC corneas have decreased TIMP protein levels. Decreased levels of TIMP-1 may be a mechanism by which increased gelatinase activities and increased apoptosis are found in KC corneas. In normal corneal fibroblast cultures, treatment with a peroxynitrite donor (3-morpholinosydnonine-N-ethylcarbamate or SIN-1) caused fragmentation of TIMP-1 protein but did not alter gelatinase activity. Rather, the presence of SIN-1 in normal corneal fibroblast cultures yielded a picture that was similar to features found in KC corneas.

Whether MMPs plays a role in KC is controversial. KC corneal MMP-2 is activated more easily than MMP-2 from normal corneas, and KC corneal cells in vitro have higher levels of MMP-2/TIMP-1 than do normal corneas. In the present study, RNA levels for MMP-2 were normal. If MMP-2 is involved in KC, it may be indirectly through the nitric oxide pathway, since nitric oxide can activate MMP-2 activity of corneal fibroblasts in vivo. In one study, MMP-3 RNA levels are decreased in KC corneas compared with normal or diabetic corneas, but in another report, expression levels of MMP-3 and MMP-10 are normal. KC corneas have increased expression of MMP-14 (MT1-MMP), a membrane-bound MMP that activates MMP-2, but in our study, cathepsin V/L2 was the only degradative enzyme examined that had increased RNA levels.

The enzyme activities for SOD1 and -3 have been described in the human normal and KC corneas. SOD3 binds to heparan sulfate proteoglycans and is localized to the cell membrane and basement membrane in human corneas. Despite the significant decrease in SOD3 activity reported in KC corneas, we found the level of SOD3 mRNA to be unaltered (Fig. 5A). KC corneas also have higher GST enzyme activity and lower ALDH3A1 enzyme activity than do normal corneas, but our data showed that GST and ALDH3A1 RNA levels in KC corneas were similar to those in normal corneas. The apparent lack of correlation between RNA levels and enzyme activities of these genes may be a result of post-translational processes, enzyme turnover rates or genetic polymorphisms. For example, the SWR/J mice have essentially normal mRNA levels but only trace levels of ALDH3A1 activity most likely due to nucleotide and amino acid alterations. Further analysis of the corneal antioxidant enzyme RNAs may demonstrate variations in isoforms or polymorphisms that could account for abnormal enzyme activities while maintaining normal RNA levels.

In summary, our findings of increased catalase expression and activity provide evidence that H$_2$O$_2$-related oxidative stress is involved in KC corneas. Decreased levels of TIMP-1 may play a role in the increased gelatinase activities and apoptosis found in KC corneas. Increased cathepsin V/L2 expression and its redistribution may be related to the increased fragmentation of epithelial basement membranes. Furthermore, cathepsin V/L2 is the third member of this family, along with cathepsins B and -G, which are upregulated and could contribute to the H$_2$O$_2$ burden and oxidative stress. Future studies will further test the hypothesis that oxidative stress may be an important factor in KC pathogenesis and that there is a role for antioxidant therapy in these patients.

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**References**


