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

M. Cristina Kenney,1,2 Marilyn Cbwa,1 Sbari R. Atilano,1 Annie Tran,1 Marilee Carballo,1 Mebrnoosh Saﬁbizadeh,2,5 Vasili Vasiliou,4 Wakako Adachi,5 and Donald J. Brown1

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 < 0.05) and 2.8-fold decrease of protein (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 degeneration. (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.5 The reported incidence of KC is approximately 1 in 2000 in the general population, but is 300 times higher in patients with Down syndrome.1,2,4

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),2,6 and glutathione S-transferase,6 and decreased activities of extracellular superoxide dismutase (SOD3, EC-SOD)6 and aldehyde dehydrogenase class 3 (ALDH3A1).5,6 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,5,6,7 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, 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.
TIMPs have not been studied.

Although KC corneas do not have extensive scarring\textsuperscript{29,30} or inflammatory cell infiltrates,\textsuperscript{29} considerable degradation of the extracellular matrix occurs. Increased gelatinase-matrix metalloproteinase (MMP) activities are found in KC corneas.\textsuperscript{31–35} Recent investigations show relationships between ROS, antioxidant enzymes, and MMPs. For example, IIT-1080 fibrocoma cells that overexpress SOD2 had increased RNA levels of MMP-2, -3, -7, -9, -10, and -11.\textsuperscript{34} In cultured human dermal fibroblasts, ROS increased MMP-1 RNA,\textsuperscript{35} induced MMP-2 RNA, and decreased tissue inhibitor of metalloproteinase (TIMP)-2 RNA levels\textsuperscript{36} leading to increased gelatinase activity of the MMPs.\textsuperscript{37} In cultured human corneal fibroblasts, the addition of exogenous peroxynitrite (ONOO\textsuperscript{-}), known to be elevated in KC corneas,\textsuperscript{38} resulted in upregulation of MMP-2 RNA levels and loss of TIMP-1 immunostaining.\textsuperscript{39} Although KC corneas have evidence of increased ROS production, cytotoxic by-products, and gelatinase activity,\textsuperscript{9,31–35} the RNA levels of the MMPs or TIMPs have not been studied.

In this investigation, the RNA levels, protein distribution, and activity for various antioxidant enzymes, degradative enzymes, and TIMP inhibitors were analyzed. KC corneas had twofold increases of RNA and enzyme activity for catalase, a major antioxidant enzyme that eliminates hydrogen peroxide, and decreased tissue inhibitor of metalloproteinase (TIMP)-2 activity.\textsuperscript{39} Moreover, in addition to the elevated levels of cathepsin B and -G,\textsuperscript{18,19,21,24,25} we demonstrate an upregulation and abnormal protein distribution for cathepsin V/L2. These cathepsins can stimulate hydrogen peroxide, which in turn can upregulate catalase,\textsuperscript{39,42} cause oxidative damage, and induce apoptosis.\textsuperscript{22,23,43,44} Finally, lower levels of the inhibitor TIMP-1 and increased cathepsin V/L2 provide causative mechanisms by which stromal and basement membrane degradation might occur in keratoconus corneas.

**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 by the institutional review boards of University of California Irvine and Cedars-Sinai Medical Center. Informed consents were obtained from patients 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.\textsuperscript{44,45} It should be noted that in studies of human diseases, there is often individual variation that may be 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 RNA-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 0.3 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 (β2-MG) 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 β2-MG 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. RT-PCR Primers, Annealing Temperatures and Hybridization Oligonucleotides Used for Antioxidant Enzymes, Degradative Enzymes and Inhibitors**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Cycles</th>
<th>Annealing Temp. (°C)</th>
<th>Reverse Primer</th>
<th>Internal Oligonucleotide</th>
<th>Gene Forward Primer</th>
<th>Internal Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH3A1</td>
<td>407</td>
<td>56</td>
<td>35</td>
<td>CTTGTCGTTGCTGGAGAA</td>
<td>GCAGGTAGGGACAGTTCACAGG</td>
<td>TTCATGAACAGTGGCCAG</td>
<td></td>
</tr>
<tr>
<td>CATase</td>
<td>279</td>
<td>56</td>
<td>35</td>
<td>GGGAAGCATTAAGGACTGACTG</td>
<td>GGGAAGATCGTCAGGTCAAA</td>
<td>GATCCTGTCAGCCCTGGGTTCTAAGA</td>
<td></td>
</tr>
<tr>
<td>SOD1</td>
<td>300</td>
<td>56</td>
<td>35</td>
<td>TCTCTTGGAGGAGCTGGA</td>
<td>GATCCTGTCAGCCCTGGGTTCTAAGA</td>
<td>GTCCTACGATGATATGACCCTTGTCATC</td>
<td></td>
</tr>
<tr>
<td>SOD3</td>
<td>337</td>
<td>54</td>
<td>41</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GST-pi</td>
<td>786</td>
<td>60</td>
<td>40</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>446</td>
<td>60</td>
<td>36</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>378</td>
<td>60</td>
<td>33</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>507</td>
<td>58</td>
<td>32</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MMP-14</td>
<td>334</td>
<td>60</td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>252</td>
<td>60</td>
<td>33</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>334</td>
<td>60</td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TIMP-3</td>
<td>334</td>
<td>60</td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

**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 [γ-<sup>32</sup>P] adenosine triphosphate (222 Tbp/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 β<sub>2</sub>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, Ljubimov and Kinoshita). A monoclonal antibody to ALDH3A1 (1:20 dilution) was also used for immunohistochemical staining. 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) and probed with a monoclonal TIMP-1 antibody (Chemicon International). A monoclonal antibody to ALDH3A1 (1:20 dilution) was also used for immunohistochemical staining. 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.

**Catalase Activity in Keratoconus**

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.
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 μL 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
Semiquantitative 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 β2-MG 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.* KC corneas had increased catalase mRNA levels and activity but normal protein distribution compared with normal corneas. (A) RT-PCR analysis of gene expression for catalase in normal and KC corneas. Lanes 1 to 8: normal corneas; lanes 9 to 16: KC corneas. H2O lane, water only. Hybridization signal intensities were normalized to values obtained for β2-MG. KC corneas had an ~2.2-fold increase of catalase mRNA compared to age-matched normal corneas (P < 0.01). (B) Southern blot analysis of catalase in normal and KC corneas. Primers for catalase were designed and used to amplify the RNA from individual corneas. After amplification, the products were run on agarose gels and then analyzed by Southern blot analysis using 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) Catalase activities from normal (n = 7) and KC (n = 10) corneas were measured. Note that the KC corneas had ~1.8-fold increase of catalase activity compared with 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 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 $\beta$-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,
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 corneas 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 proteins were loaded in each lane. Each lane represents extracts of individual corneas. Representative Western blot analysis showed that the TIMP-1 band migrating as a 28-kDa protein was decreased in KC corneas compared with normal corneas.
Previous studies reported alterations in various activities of SOD1, ALDH3A1, and SOD3 in normal and KC corneas. (B) Immunohistochemical analysis of KC corneas stained with rhodamine conjugated antibodies to SOD1, ALDH3A1, and GPX. The SOD1 antibody stained the epithelial cell cytoplasm and endothelium. Anti-ALDH3A1 antibody heavily stained the stromal keratocytes and epithelial cells but did not stain the endothelial cells. GPX antibody staining was diffuse in epithelial cells, keratocytes, and endothelial cells. The KC corneas and normal corneas had similar staining patterns for SOD1, ALDH3A1, and GPX (normal cornea sections not shown). No staining was observed when only secondary antibody (IgG only) was used on the tissue sections. E, epithelium; S, stroma; Endo, endothelium; sites of Bowman’s layer disruption where the stroma is in direct contact with the epithelium. Scale bar, 100 μm.

**SOD1, ALDH3A1, and SOD3**

Previous studies reported alterations in various activities of different antioxidant enzymes in KC corneas. But RNA levels and/or protein distributions were not examined. Figures 5A and 5B showed RNA levels and protein distributions for SOD1 (CuZn-SOD) and ALDH3A1 were similar between KC corneas and normal corneas. Anti-SOD1 antibody stained the cytoplasm of epithelial cells, endothelial cells, and a few of the keratocytes (Fig. 5B). A monoclonal antibody to ALDH3A1 showed immunoreactivity in epithelial cells and keratocytes but not endothelial cells. Although EC-SOD (SOD3) enzyme activities were reportedly lower in KC corneas, the RNA levels were similar as determined by RT-PCR (Fig. 5A). Immunohistochemistry was not performed for EC-SOD (SOD3) since no antibody was commercially available.

KC corneas have elevated levels of catalase, an antioxidant enzyme that eliminates hydrogen peroxide. This prompted us to examine a second antioxidant enzyme known to act on hydrogen peroxide, GPX. Attempts to perform RT-PCR for GPX were not successful but immunohistochemistry with a GPX-specific antibody (Fig. 5B) showed similar positive staining of the epithelial cells, keratocytes, and endothelial cells in both KC and normal corneas.

By semiquantitative RT-PCR, the RNA levels for glutathione reductase (GTR), glutathione S-transferase (GST-pi), MMP-2, MMP-14, TIMP-2, and TIMP-3 were similar between normal and KC corneas (data not shown). The RT-PCR reactions for MMP-7 and MMP-1 resulted in detectable but very low levels of reaction products that could not be quantified. Unfortunately, the primers for MMP-9 yielded only nonspecific bands from the RT-PCR reaction.

**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. Our data, along with others, show that KC corneas have increased catalase, cathepsins, lipid peroxidation related-aldehydes, nitric oxide pathway abnormalities, degradative enzymes, and decreased enzyme inhibitor levels, all of which are important elements for oxidative damage and matrix degradation within tissues.

**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 Chinese hamster ovary 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. IOVS 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, and we believe that their presence supports our hypothesis that KC corneas are oxidatively stressed. Purified cathepsin B can increase H$_2$O$_2$ production in isolated mitochondria, causing a release of cytochrome c and initiating apoptosis. Cathepsins L and B cleave Bid, a proapoptotic BCL-2-family member, leading to cytochrome c release and apoptosis. Cathepsins may play a role in the increased apoptosis in KC corneas. Because evidence shows that cathepsins are involved in H$_2$O$_2$ formation and apoptosis, it is reasonable to speculate that in KC corneas, elevated levels of cathepsins B, G, and -V/L2 lead to increased H$_2$O$_2$ production, which in turn upregulates catalase. These events could be part of a destructive cycle in which excess H$_2$O$_2$ enters the lipid peroxidation pathway and damages lysosomal membranes, leading to additional cathepsin release and subsequently to higher H$_2$O$_2$ and catalase levels. Therefore, the alterations of cathepsins, H$_2$O$_2$, and catalase may be related to each other and may play a significant role in KC.

**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 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.

**References**


**Acknowledgments**

The authors thank Alexander Ljubimov (Ophthalmology Research Laboratories, Cedars-Sinai Medical Center, University of California Los Angeles, Los Angeles, CA), Wakako Adachi, and Shigeru Kinoshita (Kyoto Prefecture University of Medicine, Kyoto, Japan) for the antibody to cathepsin V/L2, Joseph McCord for a critical reading of the manuscript; especially, Ezra Maguen, Anthony Nesburn, Frank Price, Yaron Rabinowitz, Theodore Perl for providing diseased corneas and the National Disease Research Interchange for supplying normal human corneas.