Superoxide Dismutase Isoenzymes in the Normal and Diseased Human Cornea

Anders Behndig,1 Kurt Karlsson,2 Bengt O. Jobansson,3 Thomas Brännström,4 and Stefan L. Marklund2

PURPOSE. The human cornea, a tissue much exposed to oxidative stress, is rich in extracellular superoxide dismutase (SOD). In this study, the contents and distributions of the SOD isoenzymes in the normal human cornea were compared with those in corneas affected by keratoconus and bullous keratopathy.

METHODS. The central and peripheral parts of normal human corneas were analyzed separately. Central corneal buttons were obtained from patients with keratoconus and bullous keratopathy who were undergoing primary keratoplasty or retransplantation. SOD enzymatic activities were determined by a direct spectrophotometric method, and extracellular SOD and the cytosolic Cu- and Zn-containing SOD (CuZn-SOD) proteins were determined with ELISA and studied with immunohistochemistry.

RESULTS. The total SOD content, and particularly the extracellular SOD content, was lower in the central than in the peripheral normal cornea. CuZn-SOD and extracellular SOD were demonstrated in all three corneal layers. CuZn-SOD was found in cells, whereas extracellular SOD appeared to be localized on cell surfaces, in basal membranes, and in the stroma. In keratoconus, corneal levels of extracellular SOD were half those in the control corneas, whereas CuZn-SOD and the mitochondrial Mn-containing SOD levels were normal. In bullous keratopathy, apart from edematous dilution, SOD isoenzyme levels were essentially normal. In a remarkable finding, the same pattern in SOD isoenzyme levels as in the original disease was also found at retransplantation.

CONCLUSIONS. Extracellular SOD and CuZn-SOD show markedly different distribution patterns within the human cornea. Extracellular SOD activity in the central cornea is halved in keratoconus, compared with that in normal control corneas. The finding of a similar reduction at retransplantation in keratoconus suggests reduced corneal extracellular SOD synthesis in cells of the host as a cause of the low enzyme levels. (Invest Ophthalmol Vis Sci. 2001;42:2293–2296)

The eye, with its intense exposure to light, its mostly slow tissue turnover rates, and its optical demands requiring exact tissue organization, is potentially vulnerable to oxidant stress. This is particularly true of the cornea, which is avascular, absorbs the major part of the UV light entering the eye’s optical system,3 and has considerable variation in oxygenation over a 24-hour period.4 Such a tissue is likely to need a strong defense against oxygen free radicals. We have shown7 that superoxide dismutase (SOD) is relatively abundant in the human cornea, with a 50:50 ratio between the cytosolic Cu- and Zn-containing SOD (CuZn-SOD)4 and the extracellular SOD (EC-SOD).5 Considering the low cellularity of the cornea, the third SOD isoenzyme, Mn-containing SOD (Mn-SOD),6 located in the mitochondrial matrix, is also relatively abundant. Because the substrate of the SOD isoenzymes, the superoxide anion, poorly penetrates membranes, all three SOD isoenzymes exert their actions in their respective compartments. Therefore, to understand the protection of the tissue against superoxide radicals, specific analysis of all three SOD isoenzymes is necessary.

Keratoconus (KC) is traditionally regarded as a noninflammatory, ectatic corneal degeneration.7 Still, the disease shares many features with inflammatory corneal disorders, including degradation of the extracellular matrix of the superficial stroma by elevated degradative enzymes,8 wound-healing and stress-related proteins,9 and altered proteinase inhibitors.10–12 Recent investigations have indicated a role for oxygen free radicals in the pathogenesis of the disease.13

Bullous keratopathy (BKP) is characterized by corneal edema, caused by insufficient corneal endothelial pump function.14 Loss of corneal endothelial cells, probably through apoptosis15 is a causative factor behind the disease. Oxygen free radicals have been shown to induce corneal endothelial cell apoptosis,16 which could indicate that oxygen free radicals also have a role in the pathogenesis of BKP.

We compared normal corneal SOD levels to those in KC and BKP and in corneal transplants from earlier transplantations in patients with an original diagnosis of KC or BKP who were undergoing retransplantation (KC-RT and BKP-RT). We also determined the distribution of the SOD isoenzymes within the normal human cornea with special attention to EC-SOD.

MATERIALS AND METHODS

Ocular Tissues

The tenets of the Declaration of Helsinki for the collection of human material were followed in this investigation. The investigation was approved by the research ethics committee of Umeå University, Umeå, Sweden. Normal human corneas were obtained from the Department of Pathology or Forensic Medicine (four corneas) and from the Cornea Bank, Århus Kommunehospital, Denmark (seven corneas), within 24 hours after death, from donors without any known eye disease. The Århus corneas were stored for up to an additional 24 hours at 8°C before dissection. The corneas were excised under a dissecting microscope, excluding the limbal vessels. The overall sample diameter was 11 mm. To obtain a central portion comparable to that from corneal transplantations, the central part of each specimen was punched out with a 7-mm trephine, and the central part and the peripheral ring were analyzed separately.

Corneal buttons were obtained from patients with KC or BKP who were undergoing routine penetrating keratoplasty. All these patients...
had advanced forms of their respective diseases, with typical clinical features that left no doubt about the respective diagnoses. None of the patients with KC had any underlying connective tissue disease or Down syndrome. In the BKP group, three eyes were aphakic with Fuchs dystrophy as the primary cause of the edema, two were aphakic, and five were pseudophakic.

Corneal transplants were obtained from patients with the original diagnosis of KC (n = 7) or BKP (n = 10), who were undergoing retransplantation. In the KC-RT group, the causes for retransplantation were irregular astigmatism (n = 3), haze after photorefractive kerectomy (n = 3), and cornea guttata without edema after rejection (n = 1). In the BKP-RT group, the causes were recurrence of corneal edema due to endothelial failure (n = 6) or after rejection (n = 4). None of the eyes in these two groups showed any signs of an ongoing inflammatory reaction at retransplantation. One of the BKP specimens and two of the BKP-RT specimens showed mild vascular ingrowth; the remainder did not. All samples were weighed, frozen at −80°C, pulverized and homogenized as detailed earlier, and kept at −80°C until analysis.

SOD Analysis

SOD enzymatic activity was determined by using the modified direct spectrophotometric method with K2O2 as described earlier. EC-SOD and CuZn-SOD protein contents were also determined with ELISA.

Specific Enzymatic Activity of CuZn-SOD and EC-SOD

For analysis of CuZn-SOD- and EC-SOD-specific enzymatic activity, diluted corneal extracts were incubated with shaking at 4°C overnight with rabbit antibodies against human EC-SOD or CuZn-SOD immobilized on Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) or with Sepharose 4B alone (sham incubation). After centrifugation, both the SOD activity and the EC-SOD or CuZn-SOD protein content were analyzed. The differences in results for these incubations were used for the calculation of EC-SOD- or CuZn-SOD-specific enzymatic activity for each group of specimens.

Distinction between CuZn-SOD, Mn-SOD, and EC-SOD

Cyanide (5 mM) was used to distinguish between the cyanide-sensitive CuZn-SOD and EC-SOD and the resistant Mn-SOD. To determine the EC-SOD content (expressed as units per gram wet weight), EC-SOD protein determined with ELISA was divided by the specific enzymatic activity for each group of specimens. Finally, the CuZn-SOD activity of the extract was calculated as total SOD activity minus Mn-SOD and EC-SOD activities.

Protein and DNA Analysis

For protein analysis, Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Inc., Hercules, CA) was used, standardized with human serum albumin. DNA concentration was determined with fluorometry as a complex with bisbenzimidazole (33258; Hoechst Marion Roussel, now Aventis SA, Strasbourg, Germany) using calf thymus DNA as a standard.

Immunohistochemistry

Three normal human corneas, three KC corneal buttons, and one BKP corneal button were fixed in buffered formaldehyde solution. The samples were processed for standard immunohistochemical staining according to the peroxidase-anti-peroxidase (PAP) technique, and 4-μm-thick serial sections were photographed in a photomicroscope (Carl Zeiss, Oberkochen, Germany) with a digital microscope camera (DKC-5000; Sony Corp., Tokyo, Japan). Polyclonal rabbit antibodies were used. The EC-SOD antibody was raised against recombinant human EC-SOD and purified by adsorption-desorption to immobilized EC-SOD, and the CuZn-SOD antibody was raised against a synthetic complex with bisbenzimidazole (33258; Hoechst Marion Roussel, now Aventis SA, Strasbourg, Germany). Diluted corneal extracts were incubated with shaking at 4°C overnight and processed for standard immunohistochemical staining.

Table 1. Levels of SOD Isoenzymes in the Normal and Diseased Human Cornea

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mn-SOD</th>
<th>CuZn-SOD</th>
<th>EC-SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human cornea</td>
<td>0.6 ± 0.1</td>
<td>34 ± 6.7</td>
<td>7.8 ± 1.4</td>
</tr>
<tr>
<td>Keratoconus (n = 9)</td>
<td>3.4 ± 0.3</td>
<td>1.3 ± 0.7</td>
<td>0.96 ± 0.2</td>
</tr>
<tr>
<td>Bullous keratopathy§ (n = 10)</td>
<td>3.4 ± 0.1</td>
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</tr>
</tbody>
</table>

All probabilities calculated with Student’s t test.

* P < 0.05,
† P < 0.01,
‡ P < 0.001.
Results

The SOD isoenzymes that were analyzed were localized to different compartments in a tissue (cornea) with low cellularity. For analysis of differences between the groups, the activity of the extracellularly located EC-SOD should be compared on a wet-weight basis but not when related to the tissue DNA content. The intracellularly located CuZn-SOD and Mn-SOD, on the other hand, should be compared relative to the tissue wet weight or total protein content. The EC-SOD activity was lower in the central cornea than in the peripheral part (Table 1), whereas there were no differences in the activities of the intracellular isoenzymes. In KC, the EC-SOD activities were roughly half those found in control corneas, whereas there were no differences in the activities of CuZn-SOD and Mn-SOD. In BKP, EC-SOD was reduced when compared on a wet-weight basis but not when related to the protein content of the cornea. This is explained by the reduced protein content in BKP, which is probably caused by dilution with the edema seen in this disease. There was a slight reduction of CuZn-SOD in BKP and a marked upregulation of Mn-SOD.

In KC-RT the SOD isoenzyme activities were remarkably similar to those seen in KC, with a marked reduction in EC-SOD activity. In BK-RT the EC-SOD and CuZn-SOD activities were similar to those found in BK, but increased Mn-SOD activity was not seen.

Immunohistochemistry showed staining for both EC-SOD and CuZn-SOD in the corneal epithelium, with EC-SOD apparent on the cell surfaces (Fig. 1A) and CuZn-SOD in the cytoplasm of the epithelial cells (Fig. 1B). The stromal EC-SOD staining was slightly weaker in the superficial stroma and denser farther posteriorly (Fig. 1D). Whereas the CuZn-SOD antibody labeled the keratocytes (Fig. 1E), the endothelial layer stained for both EC-SOD (Fig. 1G) and CuZn-SOD (Fig. 1H). The immunohistochemical distribution patterns for the two isoenzymes in KC and BKP were similar to those seen in the normal cornea (not shown).

Discussion

In the present study, EC-SOD was unevenly distributed within the human cornea, with lower levels in the central portion. The levels of EC-SOD in the central cornea in KC were still lower—approximately half the levels found in normal control corneas. A markedly different distribution of the two SOD isoenzymes, EC-SOD and CuZn-SOD, was demonstrated within the human cornea.

EC-SOD has high affinity with sulfated glycosaminoglycans and mainly exists anchored to proteoglycans in the connective tissue matrix, on basal membranes, and on cell surfaces in other tissues. Because the EC-SOD binding varies markedly between different glycosaminoglycans, reduced binding of EC-SOD to the anterior stromal glycosaminoglycans may explain the lower EC-SOD staining of the anterior stroma (Fig. 1D), because the composition of these is different from that in the posterior part of the stroma.

The finding of EC-SOD within the corneal epithelium, stroma, and endothelium indicates a local synthesis of this isoenzyme in all three corneal layers. A plausible explanation for the lowered EC-SOD levels in KC is a reduced synthesis within the corneal epithelium or stroma in this disease. The reduced EC-SOD levels also seen in KC-RT may be explained by reduced synthesis by cells from the host, because in these corneas, the epithelial cells are gradually regenerated from the limbal stem cells of the host, and the keratocytes eventually are also replaced by host cells. Superficial stromal keratocyte apoptosis, which can be triggered by various types of stress to the superficial cornea, may initiate the degradation of the extracellular matrix in the superficial stroma.

Recently, Kenney et al. suggested a hypothesis for KC pathogenesis, involving the superoxide radical nitric oxide (NO) and, in particular, the reaction product of these two, the highly toxic peroxynitrite ONOO⁻. NO is synthesized by keratocytes under stress conditions and in other tissues, destructive inflammation from ONOO⁻ formation is thought to be suppressed by EC-SOD. An interesting note in connection with this is that more ONOO⁻ is formed in the basal epithelium of the KC-affected cornea than in normal control corneas. It could be speculated that the lowered EC-SOD content in KC leads to increased superoxide radical levels in the extracellular space of the basal cornea with enhanced ONOO⁻ formation, which may contribute to the series of events leading to KC.

Furthermore, the changes in KC occur in the central cornea, where EC-SOD levels are lower than in the periphery, and...
the histologic changes in KC are localized mainly to the anterior stroma, the region where EC-SOD staining is immunohistochemically weaker. Despite reduced EC-SOD levels in KC-RT, recurrence of the disease in the transplant is exceedingly rare. This may be explained by the fact that the transplant generally has a higher biological age than the host and that the collagen structure of the transplanted stroma is more rigid and resistant to these degradative mechanisms, analogous to the stabilization of the disease often seen after the age of 30 in patients with KC.7

In BKP, the reduced SOD isoenzyme levels are largely explainable by the edematous dilution of the tissue, perhaps combined with a relative increase in cellularity (seen as an increase in milligrams DNA per milligram protein) due to the minor inflammatory activity. A slight inflammation could also explain the increased Mn-SOD levels in BKP, because Mn-SOD is induced by inflammatory cytokines.37 The essentially normal SOD isoenzyme levels in BKP and BKP-RT also indicate that the lowered EC-SOD levels found in KC is not an unspecific finding seen in any diseased cornea, but rather a specific radical scavenger deficiency that may contribute to KC pathogenesis.

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References