Lecithin-Bound Superoxide Dismutase in the Prevention of Neutrophil-Induced Damage of Corneal Tissue

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PURPOSE. To evaluate the effects of a lipophilic analog of superoxide dismutase (SOD) in the prevention of polymorphonuclear leukocyte (PMN)-induced damage to corneal epithelial cells in vitro and in bacterial corneal ulcers in vivo.

METHODS. Immortalized human corneal epithelial cells (T-HCEC) were cocultured with human PMNs activated with N-formyl-methionyl-leucyl-phenylalanine for 18 hours, after which lactate dehydrogenase (LDH) activity of the supernatant was measured as a marker of cellular damage. The inhibitory effects of lecithin-bound SOD (PC-SOD) and unmodified SOD, as well as PMNs pretreated with anti-CD 18 monoclonal antibody, were compared with untreated control. The retention of each drug on the ocular surface of healthy volunteers was measured by flow cytometry using brush cytology samples. The protective effects of a 0.1% solution of PC-SOD on Pseudomonas aeruginosa corneal infection in guinea pigs were assessed by inflammatory grading scores and histology.

RESULTS. Both PC-SOD and SOD effectively suppressed PMN-induced LDH release in T-HCEC in a dose-dependent manner. LDH release was also attenuated when PMNs were pretreated with anti-CD 18 antibodies, suggesting that adhesion molecules were involved in the process. Brush cytology of conjunctival samples showed that PC-SOD was retained longer on the ocular surface compared with unmodified SOD. PC-SOD significantly prevented excessive tissue damage by infiltrating PMNs in P. aeruginosa corneal infection, whereas in control eyes, perforation of the cornea occurred by 6 days.

CONCLUSIONS. PC-SOD was effective in attenuating PMN-related tissue damage to corneal tissue both in vitro and in P. aeruginosa infection in guinea pigs. (Invest Ophthalmol Vis Sci. 1998;39:30–35)

Polymorphonuclear leukocytes (PMNs) play a key role in host defense against bacterial and fungal infections. However, because of the nonspecific nature of the cytotoxic agents released by activated PMNs, they are often responsible for damage to surrounding host tissue. This is also evident from the fact that PMNs have been implicated in the pathogenesis of noninfectious disease processes such as gout,1 rheumatoid arthritis,2 glomerular nephritis,3 and inflammatory bowel disease.4 Although the elimination of invading pathogens is an imminent problem, excessive damage to host tissue in the eye can lead to devastating consequences resulting from the opacification of transparent tissue. The ideal defense system in ocular infections, therefore, is selective targeting of pathogens with minimal attack to host tissue. The development of broad-range antibiotics has enabled the coverage of a variety of organisms, whereas corticosteroids are often used to minimize excessive inflammation. However, the use of steroids is not without certain side effects, and a therapeutic agent with a more specific anti-inflammatory effect would be of value.

Earlier works have linked PMN-induced tissue damage to various proteolytic enzymes,5 whereas recent studies have increasingly focused on reactive oxygen metabolites (ROMs) produced by PMNs as the most potent agents involved.6,7 The identification of ROMs as the toxins involved in PMN-induced tissue injury made possible the development of a new class of therapeutic agents aimed at neutralizing the highly reactive oxygen species. One of the most studied antioxidants as a potential therapeutic agent is superoxide dismutase (SOD), which enzymatically reduces the superoxide radical to hydrogen peroxide. SOD has been shown to be effective in reducing posts ischemic damage in striated muscles8 and hyperoxic tissue damage in the lungs.9 However, only high concentrations of SOD have been found to inhibit PMN-induced luminol-enhanced chemiluminescence of superoxide produced in vitro by the xanthine-xanthine oxidase system.10 This suggests that the relatively short life span of ROMs and poor accessibility of antioxidants to the site of reaction, may pose problems when contemplating the clinical use of SOD. A lipophilic SOD analog bound with lecithin (PC-SOD) was developed to increase cell membrane affinity so that maximal pharmacologic activity may be achieved at the site of oxidative damage.11 The current study was designed to demonstrate the efficacy of PC-SOD as a topical agent in suppressing PMN-related damage in corneal tissue both in vitro and in vivo.

METHODS

Cell Culture
An immortalized human corneal epithelial cell line (T-HCEC)12 was used in a lactate dehydrogenase (LDH)-release cytotoxicity...
assay. T-HCEC was cultured in supplemented hormone epithelial medium (SHEM) consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 with 10% fetal calf serum, 5 µg/ml insulin, 0.1 µg/ml cholera toxin, 10 ng/ml human epidermal growth factor, and 40 µg/ml gentamicin as reported previously.15 All products were purchased from Life Technologies (Gaithersburg, MD), except for insulin (Sigma Chemical, St. Louis, MO). Cells were cultured in 250-ml flasks (Iwaki Glass, Tokyo, Japan), and passed in 1:4 split ratios (approximately 10^3 cells/cm²) after confluence using 0.05% trypsin, 0.02% ethylenediaminetetraacetic acid in Ca2+, Mg2+-free phosphate-buffered saline, pH 7.4. Cells were then passed to 96-well plates at a seeding density of 10^4 cells/well and incubated for 48 hours before experiments.

**Isolation of Human Polymorphonuclear Leukocytes**

Polymorphonuclear leukocytes were isolated from the venous blood obtained from a healthy human volunteer. After allowing erythrocytes to settle in 15% dextran-saline, the supernatant layer containing leukocytes was centrifuged in Ficoll-Paque (Pharmacia, Uppsala, Sweden) for 25 minutes at 400g at room temperature. The supernatant containing lymphocytes was discarded, and contaminating erythrocytes within the pellet were lysed by hypo-osmosis with distilled water on ice (30 seconds). This procedure was repeated twice, and PMNs were collected by centrifugation and resuspended in SHEM medium at a concentration of 2 × 10^6 PMN/ml. Anti-CD 18 antibodies (7E4; Cosmo Bio, Tokyo, Japan) were added (10 µg/10^6 PMNs) to a separate set of PMNs to determine whether integrins were involved in PMN cytotoxicity. We used mouse immunoglobulin G1 monoclonal antibody immunized with purified gp90-160 complex, which blocks β2 integrin-dependent cell adhesion.14

**Cytotoxicity Assay**

T-HCEC was pretreated with either SOD (1 or 10 µg/ml) or PC-SOD (1 or 10 µg/ml) for 30 minutes before PMN application. SOD (human recombinant SOD) was obtained from Asahi Chemical (Shizuoka, Japan), and PC-SOD was a kind gift from Dr. R. Igarashi (Division of Drug Delivery Systems, Institute of Medical Science, St. Marianna University, Kawasaki, Japan). Each molecule of PC-SOD has four lecithin molecules attached, and the specific activities of the enzymes are 3467 U/mg for unmodified SOD and 2876 U/mg for PC-SOD.11 Cells in the experimental group and the anti-CD 18 group were left untreated.

Polymorphonuclear leukocytes were added to T-HCEC at a final concentration of 2 × 10^6 PMNS/well. One set of T-HCEC not receiving PMN treatment served as a control (spontaneous release), whereas LDH release by PMNs alone was measured by incubating PMNs in empty wells. PMNs were then stimulated with N-formyl-methionyl-leucyl-phenylalanine (100 nM final concentration) and incubated at 37°C, 5% CO₂ for 18 hours. N-Formyl-methionyl-leucyl-phenylalanine applied in vitro mimics PMN response to infectious and inflammatory challenges by binding to specific surface receptors on PMNs.15 Release of cytoplasmic LDH into the culture medium after co-incubation with PMNs was measured as an index of T-HCEC damage. Fifty-microliter aliquots of T-HCEC supernatant were obtained, and LDH activity was measured by spectrophotometry using a commercially available kit (Cyto Tox 96; Promega, Madison, WI) according to the protocol provided by the manufacturer. In brief, the assay quantitatively measures LDH activity released into the medium by measuring the absorbance of a red formazan product enzymatically produced by the conversion of a tetrazolium salt. The amount of color formed is proportional to the amount of lysed cells.

Results were expressed as a percentage of total LDH activity, obtained by subtracting spontaneous LDH released from maximal LDH released by cells treated with the lysis buffer supplied with the kit.

**Retention of PC-SOD on the Ocular Surface**

To determine whether PC-SOD is maintained on the ocular surface longer than unmodified SOD, conjunctival epithelial cells were collected by brush cytology 5 minutes after instilling 10 µl of 0.1% PC-SOD or SOD in healthy volunteers. Approximately 10^5 conjunctival epithelial cells were obtained and suspended in 1 ml of Dulbecco's modified Eagle's medium/Ham's F-12 medium at 4°C. Cells were then incubated with a mouse anti-CuZn-SOD monoclonal antibody (a kind gift from Ube Kosan Co., Yamaguchi, Japan) for 30 minutes on ice and then incubated with a fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragment of a goat antimouse antibody (Bio Source Int., Camarillo, CA) as previously described.17 Nonspecific mouse immunoglobulin G1 served as a negative control. The flow cytometer was run at a fixed laser output (600 mW at 488 nm) after standardization. Background fluorescence was determined using control mouse immunoglobulin G1, and conjunctival samples were run with the FITC photomultiplier voltage adjusted so that histograms obtained from the forward angle rather than side scatter-gated cells yielded 2% to 7% background autofluorescence intensity in the range reserved for cells positively stained with FITC. Each sample contained 2 to 3 × 10^3 cells, and fluorescence intensity was displayed on a log scale. The handling of human tissue samples complied with the tenets of the Declaration of Helsinki, and appropriate consent and approval were obtained before all experiments were conducted.

**In Vivo study**

Albino Hartley guinea pigs of both sexes, each weighing 400 to 450 g, were used and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The strain of *Pseudomonas aeruginosa* used in this study (serotype I, elastase (+), alkaline protease (+)) was isolated from a patient with a severe corneal ulcer. The strain was stored in 10% skim milk medium at −70°C until use. A 0.1-ml sample of the medium was cultivated in 5 ml of tryptosoy broth (Eiken Chemical, Tokyo, Japan) with reciprocal shaking at 1 Hz and 32°C for 12 hours. *P. aeruginosa* were collected by centrifugation at 3500 rpm for 20 minutes. The organisms were washed twice with sterile 0.9% saline and then resuspended to a concentration of 10^8 to 10^9 CFU/ml using a standard curve relating viable counts to optical density at 600 nm.

After the guinea pigs were anesthetized topically with 0.4% oxybuprocaine hydrochloride and systemically with pentobarbital sodium (25 mg/kg), bacterial cells (1 × 10^9 colony-forming units in 5 µl of sterile 0.9% saline) were injected into the central stroma of the right corneas of 10 guinea pigs by using a 30-gauge needle connected to a 25-µl microsyringe. The animals were then divided into two treatment groups (n = 5 each). The PC-SOD group was treated with topical 0.3%
TABLE 1. Criteria and Scoring of Corneal Lesions After Inoculation of Organisms

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<thead>
<tr>
<th>Macroscopic Finding</th>
<th>Severity</th>
<th>Score</th>
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<tr>
<td>Ulcer</td>
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<tr>
<td></td>
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<tr>
<td>Descemetocele</td>
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<td>Opacity</td>
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<td>1</td>
</tr>
<tr>
<td></td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>Dense</td>
<td>3</td>
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<td>0.5</td>
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<tr>
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</tr>
<tr>
<td></td>
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<td>2.0</td>
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Area (ratio of lesion to whole cornea) Subscore

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<th>Area</th>
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<td>0.8</td>
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ofloxacin (Tarivid; Santen Pharmaceuticals, Osaka, Japan) and 0.1% PC-SOD, and the control group received topical 0.3% ofloxacin and saline. The minimum inhibitory concentration of ofloxacin against this strain was less than 0.5 μg/ml. Eye drops were initiated after characteristic ring abscesses accompanied by corneal ulcers were observed in all infected eyes 24 hours after injection. Eye drops were instilled 6 times a day with a 5-minute interval between the application of ofloxacin and PC-SOD or saline. The time of drug application was between 9 AM and 7 PM at 2-hour intervals for a total of six applications a day. The corneal alterations were observed macroscopically and biomicroscopically for as long as 6 days. After the observation period, eyes were enucleated after the animals were killed with an overdose of intraperitoneal pentobarbital sodium, fixed with 10% formalin, embedded in paraffin, and stained by hematoxylin and eosin.

The grade of corneal damage was quantified according to a corneal damage index (CDI) as follows: CDI = (ulcer score × area) + (opacity score × area) + hypopyon score (Table 1). Statistical analysis was performed using the Mann-Whitney test.

RESULTS

Cytotoxicity Assay

T-HCEC subjected to PMN-induced damage released LDH into the surrounding medium, which was significantly inhibited by both SOD and PC-SOD at concentrations of 10 μg/ml (Fig. 1). Both agents showed a trend in suppression at 1 μg/ml. Results show that PC-SOD was as effective as unmodified SOD in vitro. Pretreatment of PMNs with an anti-CD 18 antibody also attenuated LDH release, suggesting that adhesion molecules such as LFA-1 and MAC-1, which share the CD-18 antigen, are involved in PMN-induced cytotoxicity.

Flow Cytometry

Flow cytometry of conjunctival brush cytology samples taken at 5 minutes revealed that SOD-associated fluorescence was significantly greater in the PC-SOD group than in the unmodified SOD group, indicating that PC-SOD remained on the ocular surface longer in vivo (Fig. 2). These results are compatible with previous reports demonstrating the greater affinity of PC-SOD to the cellular membrane because of the four phosphatidyl choline (lecithin) moieties attached to the molecule. Taken together, PC-SOD was chosen as the agent of choice in the following in vivo animal study designed to demonstrate the efficacy of SOD derivatives as an adjuvant therapy in microbial corneal ulcers.

In Vivo Study

Characteristic ring abscesses accompanied by hypopyon was observed in all eyes 24 hours after *P. aeruginosa* injection (Figs. 3A, 3B). Staining with sodium fluorescein dye revealed corneal ulcers overlying the abscess, whereas ground-glass-like opacity of the stroma was observed surrounding the ring abscess. In the control group, abscesses characteristically increased in intensity and showed dense liquefactive necrosis. Hypopyon was observed throughout the course of the study, and neovascularization gradually extended from the limbus to the central cornea. Three of five corneas became perforated by day 6, and descemetocele developed in one cornea (Fig. 3D). On the other hand, corneas in the PC-SOD group showed a reduction in severity of the corneal lesions and in hypopyon (Fig. 3C). None of the five corneas became perforated, but descemetocele developed in one cornea.

Statistical analysis revealed a significant difference in CDI between the two groups on day 3 and at the end of the study.
Figure 2. Flow cytometry of conjunctival brush cytology samples (10^5 conjunctival epithelial cells) taken 5 minutes after the application of 10 μl of 0.1% superoxide dismutase or 0.1% lecithin-bound superoxide dismutase. Greater fluorescence is observed in the lecithin-bound superoxide dismutase group because of significantly higher levels of fluorescein isothiocyanate-labeled anti-superoxide dismutase antibody bound to superoxide dismutase molecules remaining on the cell membrane. x-axis, log scale of fluorescence intensity.

(Fig. 4). Histopathologic examination of the control group revealed extensive ulceration (epithelial breakdown) and liquefactive necrosis. Massive inflammatory cell infiltration was observed in all layers of the corneal stroma. High magnification revealed the infiltrating cells to be primarily PMNs (data not shown). Abscesses observed by biomicroscope corresponded

Figure 3. Macroscopic appearances of representative guinea pig corneas treated topically with a combination of 0.3% ofloxacin and saline (control group) or 0.3% ofloxacin and 0.1% lecithin-bound superoxide dismutase. Characteristic ulcers appeared in each cornea 24 hours after injection with a clinical isolate of Pseudomonas aeruginosa (day 1), at which point treatment with both regiments was started (A, B) lecithin-bound superoxide dismutase eye and control eye, respectively, prior to treatment. Note that the cornea treated with lecithin-bound superoxide dismutase on day 6 (C) had underwent less tissue damage compared than control corneum (D), which became perforated by day 6.
with PMN accumulation histopathologically. No normal stromal architecture was found in and around the massive PMN infiltration site. Sections of the cornea that became perforated were covered by fibrinous tissue and innumerable PMNs on the anterior chamber side (Fig. 5A). On the other hand, eyes in the PC-SOD group showed much less corneal damage, although extensive PMN infiltration was observed in all layers of the stroma (Fig. 5B). In fact, CDI score in the PC-SOD group significantly improved by day 6 ($p < 0.05$, Wilcoxon signed rank test), whereas control corneas deteriorated.

**DISCUSSION**

The ability of PMNs to cause acute inflammation has been attributed to the release of numerous toxic agents, including proteolytic enzymes and reactive oxygen species (ROM). 18 Recently, however, ROM produced by PMNs has attracted much attention because their role represents the most destructive agents involved. 5,7 Unfortunately these reactive substances have no specificity as to their target, and substantial damage can occur in the host tissue and lead to irreversible consequences in the transparent cornea. The mechanisms involved in ROM production are the enzymes NADPH oxidase located on the plasma membrane and myeloperoxidase located within PMN granules, which is released into the extracellular space on activation. Although the final ROM molecule responsible for direct tissue damage is still not clear, both the superoxide radical ($O_2^-$) and hydrogen peroxide ($H_2O_2$) have been well characterized as the primary species generated. 18 Superoxide dismutase and its derivatives act in the dismutation of $O_2^-$ to $H_2O_2$, and thereby attenuating subsequent oxidant formation. In the study, both SOD and PC-SOD significantly suppressed PMN-induced LDH release from cultured corneal epithelial cells (Fig. 1), indicating that $O_2^-$ is involved in cell disruption. Pretreatment of PMNs with an anti-CD18 monoclonal antibody also attenuated PMN-induced LDH release, indicating that adhesion molecules sharing the CD18 molecule (LFA-1, MAC-1) may play a role in PMN injury to T-HCEC. This is a common phenomenon observed in PMN-induced oxidative damage to other tissue, such as the vascular endothelium. 19 The ligand for LFA-1 and MAC-1 is intercellular cell adhesion molecule-1 (ICAM-1), which has been reported to be expressed on corneal epithelial cells and endothelial cells. 20,21 The upregulation of ICAM-1 in the cornea has in fact been suggested as a crucial component in the host defense mechanism against *P. aeruginosa* corneal infection. 22

We further investigated whether the in vivo use of SOD derivatives was feasible for pathologic conditions involving PMN infiltration. *P. aeruginosa* corneal ulcer was chosen as the animal model to be used in the study because activated PMN responses has been reported to play a role in the inflammatory response leading to the tissue destruction observed during ocular *P. aeruginosa* infection. 23 Because ROM may be involved in the bactericidal activities of PMNs, concurrent application of ofloxacin was applied with PC-SOD. As shown in Figure 4, the results were dramatic where control eyes resulted in liquefactive necrosis and perforation of the cornea, whereas eyes receiving PC-SOD suffered significantly less inflammation and retained greater transparency of the cornea. None of the corneas became perforated, leading to a significant improvement in CDI scores (Fig. 4).

**FIGURE 4.** Corneal damage index scores in the lecithin-bound superoxide dismutase group and the control group (mean ± 1 SE, $n = 5$ each). Day 1 indicates the day on which treatment with both regimens were started 24 hours after *Pseudomonas aeruginosa* was injected into the right eye of each animal. The average score was significantly less in corneas treated with 0.1% lecithin-bound superoxide dismutase on days 3 and 6.

**FIGURE 5.** Sections of guinea pig corneas corresponding to those in Figure 3. The cornea in the control group (A) showed perforation of the cornea and massive cellular infiltration in the remaining corneal stroma. The perforated site was covered by fibrinous tissue and innumerable polymorphonuclear leukocytes. On the other hand, the cornea in the lecithin-bound superoxide dismutase group (B) showed much less corneal damage, although extensive polymorphonuclear leukocytes infiltration was observed in all layers of the stroma. Bar = 1 mm.
although descemetocele developed in one eye. Previous reports on the clinical use of SOD have shown some benefit, though SOD used in other studies was not as effective. 24 The variation in effectiveness probably was caused by differences in drug delivery that would greatly affect the local concentration of SOD at the site of inflammation. Studies in which SOD application was effective either directly applied the drug intratracheally to treat lung damage 9 or intravenously applied it with the use of agents such as heparin that enhance SOD concentration. 8 From this standpoint, eye drops are effective in applying high concentrations of SOD to the cornea, but lecithinization offers the additional benefit of decreased clearance and enhanced local concentration.

As with any combination of therapeutic agents, a regimen of medication with different modes of action may have an additive effect in the desired outcome. The greatest advantage of PC-SOD is that the mechanisms involved are different from those of the bacteriocidal effects of antibiotics and the nonspecific immunosuppressive activities of corticosteroids. Because the ultimate goal in the treatment of bacterial ulcers in not only the elimination of invading organisms but also the preservation of transparent medium in the interest of useful vision, the concomitant use of PC-SOD may be of great benefit. The clinical evaluation of PC-SOD in PMN-induced damage of the ocular surface merits additional investigation.

References