Effects of Oxidative Stress on the Conjunctiva in Cu, Zn-Superoxide Dismutase-1 (Sod1)–Knockout Mice

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PURPOSE. A healthy conjunctiva secreting mucins is essential for maintaining the integrity of the ocular surface epithelium. We used Cu, Zn-superoxide dismutase 1–deficient mice (Sod1+/− mice) and investigated the effect of oxidative stress on the tear function, conjunctival phenotype, and ocular surface mucin expression.

METHODS. Fifty-week-old C57/B6 wild-type (WT) and Sod1+/− mice were used for evaluations of the tear film breakup time and periodic acid Schiff staining of the conjunctival specimens to detect goblet cell densities in the conjunctiva. Immunohistochemistry stainings with anti-Muc5AC, anti-Muc1, anti−4-hydroxy-2-nonenal, and anti−8-hydroxy-2′-deoxyguanosine antibodies were also performed. The mRNA expression levels of Muc1, Muc5AC, Spdef, involucrin, and transglutaminase 1 were quantified with real-time RT-PCR.

RESULTS. The mean goblet cell density in the aged Sod1+/− mice was significantly lower than the aged WT mice. The mean number of Muc5ac-positive cells was significantly lower in the aged Sod1+/− mice compared with the aged WT mice. The conjunctival epithelium in the aged Sod1+/− mice displayed marked staining with lipid and DNA oxidative stress markers. The mRNA expression of transglutaminase 1 and involucrin in the aged Sod1+/− mice was significantly higher than the aged WT mice. The Spdef mRNA expression in the aged Sod1+/− mice was also significantly lower than the aged WT mice.

CONCLUSIONS. Elevated oxidative stress status appears to affect the conjunctival differentiation and alter the conjunctival epithelial phenotype with aging in the Sod1+/− mice.

Keywords: oxidative stress, conjunctiva, mucin, Sod1

The conjunctival epithelium is composed of stratified non-keratinizing cells that cover the ocular surface. Conjunctiva is essential to attain a healthy ocular surface and to maintain the visual function. In the fornical conjunctiva, goblet cells are densely located and secret mucin including Muc5ac, which stabilizes the tear film. Conjunctival epithelium also expresses membrane-tethered mucins, which alter the conjunctival surface wettability.

The prevalence of dry eye disease varies between 7.8% and 14.6% based on the large epidemiologic studies in the United States.1 Dry eye, which could deteriorate visual function,2 has been reported to be a major public health issue and have an important impact on quality of life.3,4

In the 2007 International Dry Eye Workshop (DEWS) report, the core mechanisms of dry eye are explained by tear hyperosmolarity and tear film instability.5 Tear film instability can lead to irregular astigmatism and increased higher-order aberrations, resulting in decreased visual functions.6,7

Aging has been reported to result from deleterious damage to cells and tissues by free radicals. An imbalance between cell damage by free radicals and radical-scavenging antioxidant systems results in a condition that is called oxidative stress, which is associated with many age-related diseases and is also considered as a major factor in the process of senescence.7 Superoxide dismutase (SOD) is one of the well-known antioxidant systems, and SOD is composed of three isozymes: SOD1, SOD2, and SOD3. Among them, SOD1 is widely distributed in the tissues and represents 90% of the total SOD activity.8,9 We previously showed that Cu, Zn-Sod knockout (Sod1−/−) mice had decreased tear secretion with lacrimal gland and ocular surface damage and concluded that Sod1−/− mice are a good model for studying age-related dry eye disease.10

Conjunctival alterations in the Sod1+/− mice have not been studied in detail thus far. It has been reported that the differentiation into goblet cells in the mouse tracheobronchial and gastrointestinal epithelium is highly regulated by sterile alpha motif (SAM)-pointed domain epithelial-specific transcription factor (Spdef). A recent study showed that Spdef is also required for conjunctival goblet cell differentiation and that Spdef−/− mice have significantly increased corneal surface fluorescein staining.11
In the current study, we investigated the age-related tear film and conjunctival changes including conjunctival differentiation and transmission electron microscopy in Sod1−/− mice.

**METHODS**

**Ethics Statement**

All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by Tokyo Dental College Ichikawa Hospital Ethics Committee for Animal Research.

**Animals**

Seventeen Sod1−/− male mice with a C57BL6 background and 14 C57BL6 strain wild-type (WT) male mice were examined at 10 and 50 weeks in this study. The Sod1−/− mice were from the Tokyo Metropolitan Institute of Gerontology and the WT C57BL/6 mice were purchased from Japan Clea (Osaka, Japan).

**Measurement of Tear Film Breakup Time**

After instillation of 1 μL 0.5% sodium fluorescein, excess fluorescein was wiped away. After spontaneous blinking, the time until the appearance of a dark area representing tear film breakup (BUT) was measured three times, and the averaged value was used for analysis.

**Conjunctival Specimen Collections**

Animals were euthanized using a combination of 6 mg/mL ketamine and 4 mg/mL xylazine at 10 and 50 weeks. The conjunctiva was removed from the eyeball using fine scissors and forceps. Samples were divided and fixed in 4% buffered paraformaldehyde, stored in 2.5% glutaraldehyde in 0.1 M phosphate for electron microscopy, RNAlater (Applied Biosystems, Carlsbad, CA, USA) for RT-PCR, or Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) for immunohistochemistry.

**Histopathologic Assessment of Conjunctival Specimens**

All conjunctival specimens for immunohistochemistry detecting ocular surface mucins were immediately stored in OCT. The frozen OCT blocks were cut at a thickness of 6 μm. To evaluate the goblet cells, conventional periodic acid Schiff (PAS) staining was performed.

**Goblet Cell Density Quantifications**

Five randomly selected nonoverlapping areas in each specimen from the inferior eyelid in 890 × 705-μm frames were digitally photographed (Axioplan2 imaging; Carl Zeiss, Jena, Germany). A total of five images from each Sod1−/− or WT mice were taken where the photographer was masked to the mouse genetic information. The number of goblet cells per section was counted manually, and scores from the samples were determined for mean goblet cell density calculations.

**Fluorescent Immunohistochemistry Staining for the Ocular Surface Mucins**

To evaluate the localization and expression levels of Muc1and Muc5ac in the ocular surface, fluorescent immunohistochemistry was performed as follows: briefly, cryosections (6 μm) from the mouse eyeball were fixed in 4% paraformaldehyde for 20 minutes. After blocking with 1% PBS containing 2% donkey serum, sections were incubated overnight with primary antibodies. After washing with PBS, the sections were incubated for 30 minutes with secondary antibodies and observed using a fluorescence microscope (Carl Zeiss). For negative controls, isotype control IgG was applied instead of primary antibody. The specimens were immunostained with the following primary antibodies: mouse anti-Muc5ac antibody (2 μg/mL, 14-0041, MS-145-P0; Thermo, Cheshire, UK) and rabbit anti-Muc1 (0.2 mg/mL, ab8878; Abcam, Cambridge, MA, USA). The secondary antibody was fluorescein isothiocyanate–conjugated anti-rabbit IgG antibody (0.0075 mg/mL; Jackson Immunoresearch Laboratories, West Grove, PA, USA). DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA) was used for nuclear staining.

**Immunohistochemistry Staining for Oxidative Stress Markers**

Oxidative stress–induced lipid peroxidation was assessed by immunohistochemical detection of 4-hydroxy-2-nonenal (4-HNE); 4-HNE is a well-known major lipid peroxidation product. Oxidative DNA damage was investigated by immunohistochemical staining with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibodies. The avidin–biotin–peroxidase complex method was used in immunostainings. Tissues were fixed overnight in a 4% buffered paraformaldehyde solution and processed for paraffin embedding. Four-micrometer sections were cut from paraffin wax blocks, mounted on precoated glass slides, deparaffinized, and rehydrated. To block nonspecific background staining, conjunctival sections were treated with normal horse serum (Vector Laboratories) for 2 hours at room temperature. An antigen unmasking procedure was not performed. The tissues were then treated with mouse anti–8-OHdG monoclonal antibodies at a concentration of 10 μg/mL diluted with horse blocking serum (Japan Institute for the Control of Aging [JaICA], Shizuoka, Japan) and anti–4-HNE monoclonal antibodies at a concentration of 25 μg/mL diluted with horse blocking serum (JaICA) for 2 hours at room temperature. For the negative controls, the primary antibody was replaced with mouse IgG1 Isotype control (MOPC-21; Sigma-Aldrich Corp., St. Louis, MO, USA). Endogenous peroxidase activity was blocked using 3.0% H2O2 in methanol for 30 minutes. The sections were washed in PBS buffer, incubated for 30 minutes with biotin-labeled horse anti-mouse IgG serum (Vector Laboratories), followed by avidin–biotin–alkaline phosphatase complex treatment (Vector Laboratories) for 30 minutes. The sections were washed in PBS buffer, developed in prepared 3,3′-diaminobenzidine chromogen solution (Vector Laboratories), lightly counterstained with hematoxylin for 4 minutes at room temperature, washed with tap water, dehydrated, and mounted.

**Transmission Electron Microscopic Examination**

Conjunctival specimens were immediately fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4), immersed for 4 hours at 4°C, and washed three times with a 0.1 M PBS solution. The samples were then postfixed in 2% osmium tetroxide, dehydrated in a series of ethanol and propylene oxide, and embedded in epoxy resin. One-micrometer sections were stained with methylene blue, and the conjunctival tissues were thin sectioned on an ultratome (LKB, Gaithersburg, MD, USA) with a diamond knife. Sections were collected on 150-mesh grids, stained with uranyl acetate and lead citrate, examined, and photographed using an electron microscope (model 1200 EXII; JEOL, Tokyo, Japan).
Quantitative RT-PCR for Ocular Surface Mucin and Spdef

Mouse conjunctival tissues were preserved overnight in RNA later (Applied Biosystems) after prompt excision. Tissues were then transferred into ISOGEN (Nippon Gene, Tokyo, Japan) and homogenized well. Total RNA was extracted, cleaned up, and treated with DNase using the RNeasy mini kit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). SYBR Green–based quantitative real-time PCR was performed using OneStepPlus system (Applied Biosystems). Mouse glyceraldehyde-3-phosphate (GAPDH) (sense 5'-TGA CGT GCC GCC TGG AGA AA-3', antisense, 3'-AGT GTA GCC CAA GAT GCC CTT CAG-5'), Muc1 (sense 5'-CTT TCA GAA GAC TCC GCC AG-3', antisense 3'-GGC CAA GAC TGA TTC AGA GC-5'), Muc5ac (sense 5'-AAA GAC ACC AGT AGT CAC TCA GCA A-3', antisense 3'-CTG GGA AGT CAG TGT CAA ACC A-5'), transglutaminase 1 (sense 5'-AAA GAC ACC AGT AGT CAC TCA GCA A-3', antisense 3'-AAC TCA TCC GTG TGG TGC TC-5'), and Spdef (sense 5'-TTG GAT GAG CAC TCG TCA G-3', antisense 3'-AAA AGC ACC TTC TGC ACG TT-5') primers were used. Data were normalized to GAPDH.

Statistical Analyses

The Mann-Whitney U test was performed to compare the tear film break-up time (TBUT), staining area, goblet cell density, and mRNA expression level between different mouse groups. P < 0.05 was considered statistically significant. SPSS ver.19 (IBM Corp., New York, NY, USA) software was used for all statistical analyses.

RESULTS

Lack of SOD1 Accelerates Ocular Surface Damage and Deteriorates Tear Functions

To evaluate tear functions, the cotton thread test and BUT measurements were performed. Although there was no significant difference in weight-adjusted tear volume between the 10-week-old WT and 10-week-old Sod1−/− mice, the tear volume in the 50-week-old Sod1−/− mice was significantly lower than the 50-week-old WT mice (Supplementary Table S1). The mean BUT values in the 10-week-old WT and Sod1−/− mice were 3.2 ± 1.6 and 3.0 ± 1.7 seconds, respectively. There was no significant difference in the BUT values between the 10-week-old WT and the 10-week-old Sod1−/− mice (P = 0.7865). The mean BUT values in the 50-week-old wild and Sod1−/− mice were 3.2 ± 1.2 and 2.1 ± 1.1 seconds, respectively. The mean BUT values in the 50-week-old Sod1−/− mice were significantly shorter than the 50-week-old WT mice (P = 0.0149) (Fig. 1A). Fluorescein staining was performed to evaluate the ocular surface damage. There was no significant difference in the mean fluorescein staining scores between the 10-week-old WT and 10-week-old Sod1−/− mice (P = 0.3552). The mean fluorescein staining scores in the 50-week-old Sod1−/− mice was significantly higher than the 50-week-old WT mice (P = 0.0015; Supplementary Table S1).

Lipid and DNA Oxidative Damage Accumulated With Aging in the Mouse Conjunctival Tissue

To evaluate the influence of oxidative stress on nuclei, we performed immunohistochemistry stainings with a DNA oxidative stress marker: anti-8-OHdG (Fig. 1B). Specimens from the 50-week-old Sod1−/− mice exclusively showed dense staining in the nuclei of the conjunctival epithelium compared with the specimens from the WT mice at 10 and 50 weeks and Sod1−/− mice at 10 weeks (Fig. 1B). The mean (μm²) of the positively stained areas with 8-OHdG antibodies was 2080 ± 1578 for WT mice at 10 weeks, 9754 ± 2403 for Sod1−/− mice at 10 weeks, 9047 ± 2795 for WT mice at 50 weeks, and 38,766 ± 4578 for Sod1−/− mice at 50 weeks. The extent of conjunctival staining with 8-OHdG antibodies showed a significant increase (P < 0.0001) in both Sod1−/− and WT mice from 10 to 50 weeks as shown in Figure 1C. Moreover, the extent of staining with 8-OHdG antibodies in the Sod1−/− mice at 50 weeks was significantly higher (P < 0.0001) than the WT mice at 50 weeks (Fig. 1C). To evaluate the lipid oxidative stress in conjunctival tissues, we performed immunohistochemistry stainings with an anti-4-HNE antibody. Specimens from the 50-week-old Sod1−/− mice extensively showed dense staining in the conjunctival epithelium compared with the specimens from the WT mice at 10 and 50 weeks and Sod1−/− mice at 10 weeks (Fig. 1D). The mean (μm²) of the positively stained areas with 4-HNE antibodies in the conjunctival epithelium was 4676 ± 1678 for WT mice at 10 weeks, 15,478 ± 2506 for Sod1−/− mice at 10 weeks, 24,689 ± 2899 for WT mice at 50 weeks, and 60,453 ± 8823 for Sod1−/− mice at 50 weeks. The extent of conjunctival staining with 4-HNE antibodies showed a significant increase (P < 0.0001) in both Sod1−/− and WT mice from 10 to 50 weeks as shown in Figure 1E. Moreover, the extent of staining with 4-HNE antibodies in the Sod1−/− mice at 50 weeks was significantly higher (P < 0.0001) than the WT mice at 50 weeks (Fig. 1E).

Aging Was Associated With a Decrease of Goblet Cells in the Conjunctival Epithelium in Sod1−/− Mice

Conjunctival goblet cells were evaluated with PAS staining. In the 50-week-old Sod1−/− mice, a marked decrease of goblet cells and thickening of conjunctival epithelium were observed (Fig. 2A). The mean number of goblet cells was 56.3 ± 28.5 for WT mice at 10 weeks, 48.8 ± 24.2 for Sod1−/− mice at 10 weeks, 48.3 ± 15.6 for WT mice at 50 weeks, and 16.9 ± 10.2 for Sod1−/− mice at 50 weeks. The mean goblet cell density in the conjunctiva showed a significant decrease (P < 0.0001) in the 50-week-old WT mice from 10 to 50 weeks compared with the samples from 10-week-old WT mice (P = 0.0002). Moreover, the goblet cell density in the 50-week-old WT mice was significantly lower (P < 0.0001) than the WT mice at 50 weeks (Fig. 2B).

Aged Sod1−/− Mice Showed Alterations in the Ocular Surface Mucin Expression

To evaluate the membrane tethered mucins, Muc1 immunohistochemistry using an anti-Muc1 antibody was performed. The conjunctival tissue in the 10-week-old WT and Sod1−/− mice showed a continuous positive staining pattern in the superficial conjunctival epithelium. Fifty-week-old WT mice also showed a similar immunohistochemical expression. On the other hand, 50-week-old Sod1−/− mice showed marked less staining in the conjunctival epithelium (Fig. 3A). Real-time RT-PCR of the conjunctival tissues showed that Muc1 mRNA expression in the 50-week-old Sod1−/− mice was significantly lower than the 50-week-old WT mice (Fig. 3B; P = 0.0003). To evaluate the secretory mucin, Muc5ac, immunohistochemistry using an anti-Muc5ac antibody was performed. The conjunctival tissues in the 10- and 50-week-old WT and 10-
FIGURE 1. Tear film stability, oxidative DNA, and lipid changes in the conjunctival tissues. (A) Tear film BUT was measured to evaluate the tear film stability. The mean tear film BUT in 50-week-old Sod1−/− mice was significantly shorter than the 50-week-old WT mice. (B) Conjunctival epithelial cell nuclei showed scanty staining with 8-OHdG antibodies in the Sod1−/− and WT mice at 10 weeks. There was a marked increase in nuclear staining from 10 to 50 weeks, exclusively in all Sod1−/− mice. Relatively more conjunctival epithelial cell nuclei stained with anti–8-OHdG antibodies in the Sod1−/− mice at 50 weeks compared with conjunctival specimens from WT mice at 50 weeks. (C) Semiquantitative analysis of the extent of conjunctival epithelial cell staining for 8-OHdG showed a statistically significant increase in the 50-week-old mice group compared with
the 10-week-old group and a significant elevation in staining for the Sod1<sup>-/-</sup> group compared with the WT mice at 50 weeks. Error bars indicate SD from at least five independent samples. (D) Late-phase lipid peroxidation marker 4-HNE stained conjunctival epithelial cells positively showing a dense staining in the 50-week-old Sod1<sup>-/-</sup> mice. The WT mice in the 50-week-old specimens were also stained but to a lesser extent observed in Sod1<sup>-/-</sup> mice. Hematoxylin staining was performed for counterstaining. (E) Semiquantitative analysis of the extent of conjunctival epithelial cell staining for 4-HNE showed a statistically significant increase in the 50-week-old mice group compared to the 10-week group and a significant elevation in staining for the Sod1<sup>-/-</sup> group compared with the WT mice at 50 weeks. Error bars indicate SD from at least five independent samples. *<i>P</i> < 0.05.

week-old Sod1<sup>-/-</sup> mice showed abundant positive staining in the conjunctival epithelium. However, 50-week-old Sod1<sup>-/-</sup> mice showed scanty positive staining in the conjunctival epithelium (Fig. 3C). Real-time RT-PCR of the conjunctival tissues showed that Muc5ac mRNA expression in the 10-week-old Sod1<sup>-/-</sup> mice was significantly higher than the 10-week-old WT mice (<i>P</i> = 0.0192). On the other hand, Muc5ac mRNA expression in the 50-week-old Sod1<sup>-/-</sup> mice was lower than the 50-week-old WT mice (<i>P</i> = 0.0076). In the 50-week-old Sod1<sup>-/-</sup> mice, Muc5ac mRNA expression was significantly lower than 10-week-old Sod1<sup>-/-</sup> mice (<i>P</i> < 0.0001; Fig. 3D).

Electron Microscopy Revealed Marked Ultrastructural Alterations in Aged Sod1<sup>-/-</sup> Mice Conjunctiva

Conjunctival specimens from the 50-week-old WT and Sod1<sup>-/-</sup> mice were observed under transmission electron microscopy. The specimens from the WT mice showed a normal architecture of the conjunctival epithelium with abundant goblet cells (Figs. 4A, 4C). In the Sod1<sup>-/-</sup> mice conjunctival epithelium, marked thickening of the conjunctival epithelium and goblet cell loss were observed (Fig. 4B). Figure 4C shows normal microvilli and goblet cell secretory mucin packages. Flattening of the superficial conjunctival epithelium, blunting of microvilli, and decreased intercellular cohesion between the conjunctival epithelial cells were prominent in the 50-week-old Sod1<sup>-/-</sup> mice (Fig. 4D). Figure 4E shows normal basement membrane architecture in the 50-week-old WT mice. Distortion and thickening of the basement membrane in the 50-week-old Sod1<sup>-/-</sup> mice was also observed beneath the conjunctival basal epithelium (Fig. 4F).

Aging Was Associated With Alterations of mRNA Expression in Keratinization or Goblet Cell Differentiation Markers in Sod1<sup>-/-</sup> Mice

To quantify the mRNA expression related to goblet cell differentiation and keratinization, real-time RT-PCR was performed. The mRNA expression of Spdef, a transcription factor related to goblet cell differentiation, in the 10-week-old Sod1<sup>-/-</sup> mice was significantly higher than the 10-week-old WT mice (<i>P</i> < 0.0001). The Spdef expression in the 50-week-old Sod1<sup>-/-</sup> mice was significantly lower than the 50-week-old WT mice (<i>P</i> = 0.0016). The Spdef expression in the 50-week-old Sod1<sup>-/-</sup> mice was significantly lower than the 10-week-old Sod1<sup>-/-</sup> mice (<i>P</i> < 0.0001; Fig. 5A). The mRNA expression of involucrin, a keratinization-related protein, in the 50-week-old Sod1<sup>-/-</sup> mice was significantly higher than the 10-week-old Sod1<sup>-/-</sup> mice (<i>P</i> < 0.0001). The involucrin expression in the Sod1<sup>-/-</sup> mice showed a significant reduction from 10 to 50 weeks (<i>P</i> < 0.0001). The mRNA expression of transglutaminase 1, a keratinization-related protein, in the 50-week-old Sod1<sup>-/-</sup> mice was significantly higher than the 50-week-old WT mice (<i>P</i> < 0.0001). The transglutaminase 1 expression showed a significant increase from 10 to 50 weeks in the Sod1<sup>-/-</sup> mice (<i>P</i> < 0.0001).

DISCUSSION

Oxidative stress is defined as the imbalance between production of reactive oxygen species and restoration by antioxidant systems. Previous experimental animal studies proposed that Cu, Zn-SOD knockout (Sod1<sup>-/-</sup>) caused an elevated oxidative stress status, resulting in various aging phenotypes such as muscle atrophy, macular degeneration, fat liver deposits, hepatic carcinoma and hemolytic anemia, skin atrophy, bone loss, exacerbation of Alzheimer’s disease, and rotator cuff degeneration. In humans, oxidative stress has been reported to be involved in many systemic diseases including Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, cardiovascular diseases, cancer, ischemic disorders, oxygen free radical and antioxidant systems have also been demonstrated to be potentially important in the pathogenesis of ocular diseases such as cataract, age-related macular degeneration, glaucoma, and dry eye disease. We previously demonstrated that excessive accumulation of oxidative stress led to histopathologic alterations in the lacrimal glands and deteriorated lacrimal gland functions in the Sod1<sup>-/-</sup> mice.

Healthy conjunctival tissues can contribute to tear film stability. The membrane mucins can alter the surface of conjunctival epithelium to a hydrophilic status, and secreted mucins spread into the aqueous layer and stabilize the tear film. Both membrane and secreted mucins also contribute to the barrier functions against foreign pathogens.

A recent study using in vivo confocal microscopy revealed that there was a negative correlation between goblet cell...
density in the conjunctiva and aging. Previous literature showed that goblet cell densities decreased with various types of dry eyes including Sjögren syndrome, Stevens Johnson syndrome, and graft-versus-host disease. Several reports showed that the incidence of dry eye disease increases with age. The prevalence of dry eye disease (DES) in US women younger than 50 years old was 5.7% and was 9.8% among women older than 75 years old. In the DEWS report, aging is defined as one of the environmental factors to cause dry eye disease.

In the current study, we confirmed a decrease in tear volume and an increase in fluorescein staining score in the 50-week-old Sod1−/− mice. These data were consistent with previous reports from our laboratory. In addition to the tear volume and ocular surface abnormality, the current study revealed that tear film BUT was deteriorated in the 50-week-old Sod1−/− mice. Generally, an unstable tear film is considered to be caused by aqueous tear deficiency, excessive tear evaporation, or alteration of ocular surface mucins. In the current study, we found a significant decrease in the number of goblet cells in aged Sod1−/− mice. Moreover, a decreased intensity of immunohistochemistry stainings with Muc1 and Muc5ac in the aged Sod1−/− mice concomitant with a decrease in mRNA expression levels of Muc1 and Muc5ac was observed. These changes coexisted with accumulation of oxidative stress in the conjunctiva, which might have affected conjunctival mucins and deteriorated the tear film stability.

The conjunctival PAS staining in the Sod1−/− mice showed thickening of the conjunctival epithelium and a decrease of goblet cell density with aging. Transmission electron micro-
FIGURE 4. Transmission electron microscopy evaluations of the conjunctival tissues in the 50-week-old WT and Sod1−/− mice. The WT mice showed normal architecture of the conjunctival epithelium with abundant goblet cells ([A], arrows; [C], asterisks), microvilli ([C], arrows), and a smooth and thin layer of the basement membrane ([E], arrows). On the other hand, marked thickening of conjunctival epithelium and goblet cell loss were observed in the Sod1−/− mice ([B]). Flattening of the superficial conjunctival epithelium, blunting of microvilli ([D], arrows), and decreased cohesion of the conjunctival epithelium ([D], arrowheads) were prominent in the Sod1−/− mice ([D]). Distortion and thickening of the basement membrane in Sod1−/− mice was also observed beneath the conjunctival basal epithelium ([F], arrows).
copy also confirmed the decrease of goblet cells and thickening of the conjunctival epithelium. Transmission electron microscopy observations further revealed weakening of intercellular cohesion in the uppermost layer of conjunctival epithelium. The basement membrane of the conjunctival epithelium in the 50-week-old Sod1/C0/C0 mice was distorted and became thicker compared with the same aged WT mice.

In the current study, we found notable findings in relation to the mRNA expression in the young and older aged Sod1/C0/C0 mice. Muc5ac and Spdef mRNA expression in the 10-week-old Sod1/C0/C0 mice was significantly higher than the 10-week-old WT mice. On the other hand, Muc5ac and Spdef mRNA expression in the 50-week-old Sod1/C0/C0 mice was significantly lower than the 50-week-old WT mice. These findings suggest a decline in epithelial differentiation with aging in the Sod1/C0/C0 mice. It might be that, with aging, the conjunctival epithelium may gradually undergo an alteration of phenotype due to accumulation of oxidative stress.

To further investigate the mechanism of goblet cell loss in aged Sod1/C0/C0 mice, Spdef mRNA expression was measured. Aged Sod1/C0/C0 mice showed a decrease of Spdef mRNA expression in the conjunctival tissue compared with aged WT mice. Accumulation of oxidative stress in the conjunctival epithelium could alter the Spdef expression and induce phenotypic alterations toward a squamous metaplastic epithelium. Indeed, increased mRNA expression of keratinization-related proteins (transglutaminase 1 and involucrin) in the aged Sod1/C0/C0 mice could be caused by desiccation stress due to

**FIGURE 5.** Real-time RT-PCR evaluations of goblet cell differentiation and keratinization in the conjunctival tissues. Spdef expression in the 50-week-old Sod1/C0/C0 mice was significantly less than the 50-week-old WT mice or the 10-week-old Sod1/C0/C0 mice. Fifty-week-old WT mice showed a higher Spdef mRNA expression than the 10-week-old WT mice (A). Involucrin mRNA expression in the 50-week-old Sod1/C0/C0 mice was higher than the 50-week-old WT mice or the 10-week-old Sod1/C0/C0 mice (B). The mRNA expression of transglutaminase 1 in the 50-week-old Sod1/C0/C0 mice was significantly higher than the 50-week-old WT mice or the 10-week-old WT mice (C). *P < 0.05.
the low wettability of the ocular surface. In epidermal tissue, a cornified cell envelope was formed underneath the plasma membrane and consisted of cornified precursor proteins such as involucrin and loricrin. These precursor proteins are cross-linked by transglutaminase 1. Although the existence of a cornified cell envelope was still unknown in the ocular surface in severe ocular surface diseases, such as Stevens Johnson syndrome and ocular cicatricial pemphigoid, elevation of transglutaminase 1 mRNA expression and increased cornified precursor protein involucrin were reported. A detailed pathogenesis of keratinization-related mRNA and protein up-regulation in severe ocular surface disease is still unknown. Because dry eye disease is one of the major diseases causing squamous metaplasia, dryness of the ocular surface may be involved in the pathogenesis of the keratinization process in severe ocular surface disease. Another study showed that mice with an experimental dry eye model showed increased mRNA expression of cornified envelope precursor proteins, cross-linking transglutaminase 1 enzyme, and decreased mRNA expression of Muc5ac. Aged Sod1−/− mice showed the phenotype of dry eye disease including decreased tear volume, decreased tear stability, and ocular surface damage. It is possible that dry eye in the aged Sod1−/− mice caused a phenotypic alteration in the conjunctival tissues into squamous metaplasia.

A few potential limitations were apparent in our study. Only male mice were analyzed to avoid the effect of menstrual cycle in the study. Because dry eye is more prevalent in women, we need to investigate female mice in the future studies.

In conclusion, accumulation of oxidative stress in the aged Sod1−/− mice induced a decrease of corneal wettability and altered the conjunctival epithelium to a squamous metaplastic phenotype. Further mechanisms of how oxidative stress affects Spdef expression with aging should be investigated in future studies.

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