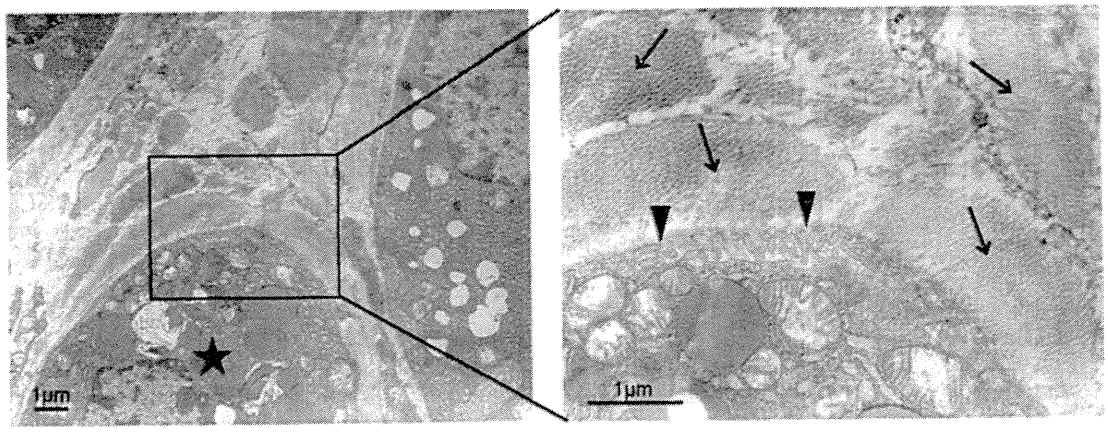


Figure 7. Electron microscopy of lacrimal gland in 50-week-old *Sod1*$^{-/-}$ mice. Note the loss of polarity of the acinar epithelial cell (**star**) with the microvilli (**arrowhead**) and secretary vesicles facing the mesenchymal area, which has abundant collagen fibers (**arrow**). **Right panel** is an enlargement of the boxed region of the **left panel**.

μL/g) (Figure 8B). Not only the aqueous tear but also the total protein secretion measured at 50 weeks was significantly less in the *Sod1*$^{-/-}$ mice (0.920 ± 0.968 μg/mL/g/min) compared with the WT mice (3. 433 ± 2.467 μg/mL/g/min) ($P = 0.024$) (Figure 8C). Cotton thread test measurements revealed mean tear quantity values of 0.083 ± 0.067 mm/g and 0.124 ± 0.065 mm/g in the *Sod1*$^{-/-}$ mice and WT mice, respectively. We also found that 87.5% of the *Sod1*$^{-/-}$ mice and 5.9% of the WT mice at 50 weeks were below the cutoff value. In addition, 5.4% of the SOD1 knockout mice and 2.7% of the WT mice at 10 weeks had dry eye disease (data not shown).

We observed, by transmission electron microscopy examination, that the secretory vesicles in the lacrimal gland acinar cells appeared as gray-black, electron-dense, round-oval bodies. We noted a relative accumulation of secretory vesicles in the acinar epithelia in *Sod1*$^{-/-}$ mice from 10 to 50 weeks (Figure 8D). After quantifying the density of secretory vesicles, we noted a significant accumulation of secretory vesicles in the acinar epithelial cells from 10 weeks (558.14 ± 90.04 vesicles per frame) to 50 weeks (709.80 ± 91.25 vesicles per frame) in the *Sod1*$^{-/-}$ mice ($P = 0.03$). The number of secretory vesicles did not change significantly in the WT mice from 10 weeks (284 ± 90.82 vesicles per frame) to 50 weeks (460.60 ± 125.46 vesicles per frame) as shown in Figure 8E ($P = 0.076$). The differences in the number of secretory vesicles per area between the *Sod1*$^{-/-}$ mice and the WT mice at 10 weeks were statistically significant ($P = 0.006$). The mean number of secretory vesicles was also significantly higher in the *Sod1*$^{-/-}$ mice at 50 weeks than the WT mice at 50 weeks ($P = 0.003$) (Figure 8E).

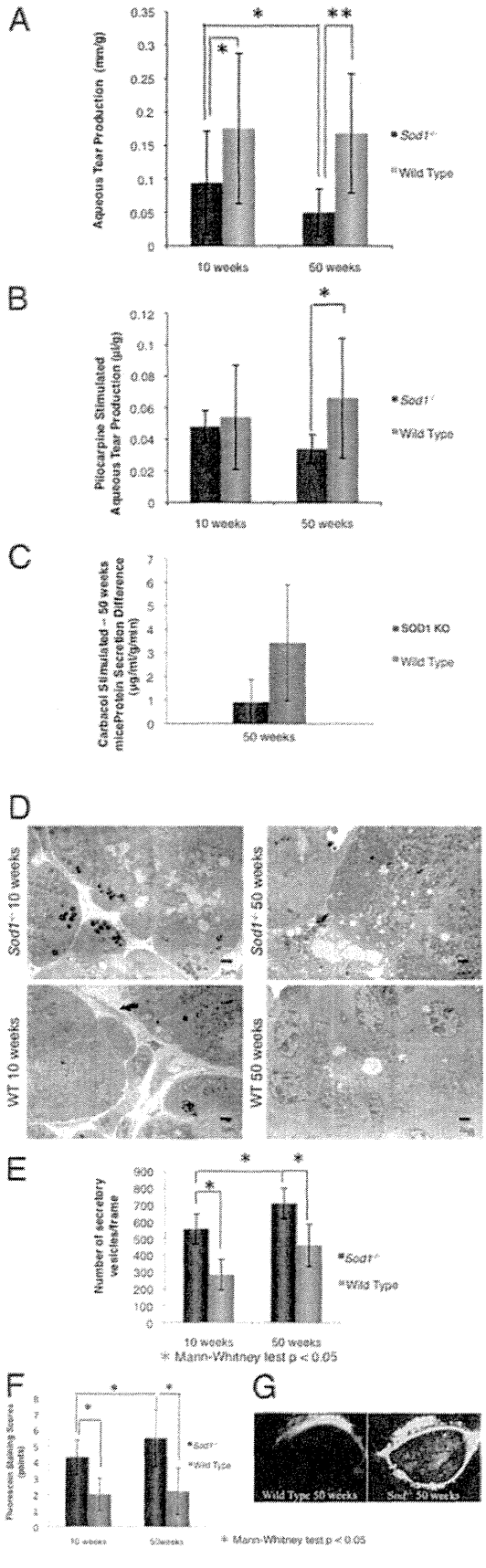
Decreased tear output has been shown to be associated with establishment of a dry eye ocular surface milieu, leading to ocular surface epithelial damage.[7] The mean fluorescein staining score in the *Sod1*$^{-/-}$ mice was significantly higher ($P = 0.001$) than the WT mice at 10 (2 ± 1 points) and 50 weeks (2.21 ± 1.42 points). The mean fluorescein score also showed a significant increase ($P = 0.026$) from 10 (4.3 ± 1.06 points) to 50 weeks (5.5 ± 1.76 points) in the *Sod1*$^{-/-}$ mice (Figure 8F). Representative corneal fluorescein stainings at 50 weeks are shown in Figure 8G. Whereas corneal epithelium from the WT mice displayed no or minimal punctate staining, the corneal epithelium in all *Sod1*$^{-/-}$ mice had marked corneal epithelial damage.

Evidence for Presence of Age-Related Increase of Oxidative Stress and Morphologic Alterations in Human Lacrimal Glands

Human samples were studied under institutional review board permission at Keio University School of Medicine. H&E and Mallory staining revealed acinar unit atrophy; interstitial, periacinar, and periductal fibrosis; and cystic duct dilatation with inflammatory cells in the lacrimal gland specimens from older individuals, which were not observed in the younger individuals (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Staining of the lacrimal gland specimens in the older individuals with anti–4-HNE antibodies showed marked staining of acinar units, indicating increased lipid oxidative damage compared with the scanty staining observed in the younger group of individuals. There was also comparably more and prominent staining for 8-OHdG, indicating extensive DNA damage in the lacrimal gland specimens of the older individuals compared with the younger individuals. Lacrimal gland specimens from older individuals also showed marked infiltration with CD45-positive inflammatory cells compared with the younger individuals.

Discussion

Previous experimental animal studies proposed that *Sod1*$^{-/-}$ caused an elevated oxidative stress status, resulting in various aging phenotypes, such as muscle[36] and skin atrophy,[37] bone weakness,[38] fat liver deposits, hepatic carcinoma,[39] and hemolytic anemia.[40,41] In humans, oxidative stress has been reported to be involved in many systemic diseases, including Parkinson's disease,[42] Alzheimer's disease,[43–45] amyotrophic lateral



sclerosis,[46] cardiovascular diseases,[47,48] cancer,[39,49,50] and ischemic disorders due to oxygen reperfusion injury followed by hypoxia.[51,52] Oxygen free radicals and antioxidant systems have been demonstrated to be potentially important in the pathogenesis of ocular diseases, such as cataract,[53] uveitis,[54] retinopathy of prematurity,[55] AMD,[56] keratitis,[57] keratoconus, and bullous keratopathy.[58] The role and relation of oxidative stress in the pathogenesis of dry eye disease have not been investigated in an aging animal model or in humans before.

The *Sod1*$^{-/-}$ mouse has been shown by us to be a good model for studying retinal oxidative stress changes, which were found to be strongly related to the morphologic and functional retinal alterations similar to human AMD.[4]

In this study, we investigated whether histopathologic alterations existed in the lacrimal glands of the *Sod1*$^{-/-}$ mice and whether these changes translated into functional glandular disturbances, causing dry eye disease. Because the amount and activity of *Sod1* are the highest among the three isozymes in humans, it seemed reasonable to hypothesize that the lack of *Sod1* would accelerate oxidative stress and age-related pathologic changes in the lacrimal glands of the *Sod1*$^{-/-}$ mice.[59]

We observed extensive lipid and DNA oxidative stress damage in the lacrimal gland acinar epithelia, which appeared to increase with aging from 10 to 50 weeks in both the knockout and WT mice. The DNA damage seemed to be more extensive in the old *Sod1*$^{-/-}$ mice compared with the old WT mice. Elevation of serum 8-OHdG provided additional evidence on general cellular DNA damage in the *Sod1*$^{-/-}$ mice. Oxidative stress-related cellular DNA damage has previously been demonstrated in heart, brain, muscles, liver, red blood cells, and other organs in several age-related diseases, including cardiovascular disorders, neurodegenerative diseases, and cancer.[25,39,43,47,48,60,61]

Our observations suggested an accelerated oxidative lipid and DNA damage in the lacrimal glands of *Sod1*$^{-/-}$

Figure 8. Changes in lacrimal gland secretory functions and corneal epithelial damage over time in the *Sod1*$^{-/-}$ and WT mice. **A:** Weight-adjusted aqueous tear production measurements were significantly lower in the *Sod1*$^{-/-}$ mice compared with the WT mice at 10 weeks and 50 weeks. A significant timewise decrease of tear production from 10 to 50 weeks was also observed in the *Sod1*$^{-/-}$ mice ($P = 0.026$). **B:** There is significantly lower tear production with pilocarpine stimulation in the *Sod1*$^{-/-}$ mice at 50 weeks. **C:** At 50 weeks the amount of protein produced, after carbachol stimulation, by the *Sod1*$^{-/-}$ mouse group was lower compared with the WT group. **D:** There is marked timewise accumulation of secretory vesicles in the lacrimal glands of the *Sod1*$^{-/-}$ mice. **E:** There is significant accumulation of secretory vesicles in the acinar epithelial cells from 10 weeks to 50 weeks in the *Sod1*$^{-/-}$ mice ($P = 0.030$). The number of secretory vesicles did not change significantly in the WT mice from 10 weeks to 50 weeks ($P = 0.076$). The differences in the number of secretory vesicles/area between the *Sod1*$^{-/-}$ and WT mice at 10 weeks were statistically significant ($P = 0.006$); the mean number of secretory vesicles was considerably higher in the *Sod1*$^{-/-}$ mice at 50 weeks than the WT mice at 50 weeks ($P = 0.003$). **F:** Changes in corneal epithelial damage scores assessed by fluorescein staining in the *Sod1*$^{-/-}$ and WT mice. A statistically significant timewise increase in the fluorescein staining score was observed in the *Sod1*$^{-/-}$ mice. Note the significantly higher scores in the *Sod1*$^{-/-}$ mice compared with WT mice at 50 weeks. **G:** Representative photomicrographs of fluorescein staining test in the 50-week-old mice. Note the extensive corneal epithelial damage in the *Sod1*$^{-/-}$ mouse compared with the WT mouse at 50 weeks. Error bars indicate SD from at least five independent samples per group of three separate experiments.

mice compared with WT mice, also strengthening our belief that these changes might have very well resulted from accumulation of reactive oxygen species in the lacrimal gland cellular architecture, especially in mitochondria. Accumulation of reactive oxygen species has been shown and linked to mitochondrial alterations in humans and animal models of age-related diseases[44,46,62–65] with striking disturbances in the mitochondrial architecture, including mitochondrial swelling, rupture of membranes, and disruption of cristae changes, which were also observed in our *Sod1*$^{-/-}$ mice.

Such alterations in the mitochondrial cytoskeleton have also been linked to activation of apoptotic signals, initiating cell death.[66–69] We showed an increase in the process of apoptotic cell death over time in the lacrimal glands of the *Sod1*$^{-/-}$ and WT mice by confirming increased TUNEL and caspase-3 staining with a comparatively greater extent of staining in the old *Sod1*$^{-/-}$ compared with old WT mice. Fragmentation of nuclei and vacuolar changes in the lacrimal gland acinar epithelia provided further ultrastructural evidence of the presence of apoptosis as a possible mechanism of cell death in this study. This observation differs from that of Hashizume et al,[70] who found both apoptosis and necrosis in the retina of the *Sod1*$^{-/-}$ mice. We believe this difference in cell death mechanisms is attributable to light exposure protected ocular anatomical location of the lacrimal gland.

We think increased caspase-3 staining overtime in our knockout mice is an important observation because even a small amount of caspase-3 activation has been shown to be sufficient to initiate genomic DNA breakdown, leading to apoptotic cell death.[71] Nonapoptotic functions of caspase-3 include induction of inflammation through lymphocyte proliferation and antigen presentation.[72,73]

We noted an altered "inflammation status" in the lacrimal gland tissue with significant increases in the lacrimal gland inflammatory cell infiltrate densities in both *Sod1*$^{-/-}$ and WT mice overtime, with the difference becoming more significant in the old knockout than the old WT mice. These observations were consistent with previous studies that showed increased focal infiltrates in lacrimal glands with a variety of inflammatory cells with aging.[74–79] In this study, we observed that the inflammatory cells were predominantly CD4$^+$ T cells at 50 weeks in the *Sod1*$^{-/-}$ mice. We previously reported that the conjunctival and lacrimal gland tissues of the *Sod1*$^{-/-}$ mice also became simultaneously infiltrated with CD45 and CD4$^+$ cells from 30 weeks and predominate the lacrimal gland and conjunctival tissues densely at 50 weeks (without evidence of corneal infiltration), with a simultaneous decrease in goblet cell density, Muc5ac mRNA expressions in RT-PCR, and a decrease in tear quantity from approximately 30 weeks becoming significant at 50 weeks (unpublished data). The lacrimal/conjunctival or the corneal tissues of the *Sod1*$^{-/-}$ mice lacked any marked infiltration at 10 weeks, which excludes the possibility of an inflammatory process being the cause of corneal staining observed in the mice at that age. The few inflammatory cells observed were cells tracking or patrolling the lacrimal gland tissues.

Inflammatory cells have been reported to release several cytokines that play an important role by initiating or further adding to the process of apoptotic cell death.[80–82] Likewise, lacrimal gland epithelial cells in an inflamed environment have been shown to express cytokines in dry eye disease and with aging.[83] This study noted significant increases in two cytokines in the old knockout mice compared with the old WT mice: TNF-α and IL-6. TNF-α has indeed been demonstrated in increased amounts in the lacrimal glands of old but not young mice.[11] Increased TNF-α and IL-6 concentrations have also been shown in dry eye syndromes in previous studies.[7,8,11,84–86] The reported roles for TNF-α include induction of inflammation and cell death, and those for IL-6 include induction of inflammation and fibrosis.[83,87–90] The cytokine alterations observed in this study go along well and are consistent with increased inflammatory cell density in our knockout mice model with aging. Again, consistent with the cytokine alterations, we observed an exaggerated fibrosis of the interstitium in the lacrimal glands of the old *Sod1*$^{-/-}$ mice, which was also comparatively more extensive than the lacrimal glands of the old WT mice.

To extensively study the mechanisms involved in the process of fibrosis, we decided to investigate the changes in EMT markers in both WT and *Sod1*$^{-/-}$ mice. EMT plays a crucial role not only in physiologic conditions, such as embryonic development or tissue remodeling, but also in pathologic conditions, such as cancer and organ fibrosis.[91] Pathologic EMT has been reported to be induced by inflammatory cytokines, reactive oxygen species, hypoxia, UV irradiation, and nicotine.[92,93] EMT-inducing inflammatory cytokines include transforming growth factor β, TNF-α, and IL-6,[94,95] the last two of which were significantly increased in tears and serum of the 50-week-old *Sod1*$^{-/-}$ mice. We believe that these inflammatory changes invited EMT with increased collagen lay down, fibrosis, and loss of glandular acinar units in due course. A previous report also showed that the EMT in lacrimal gland was caused by human ocular chronic graft-*versus*-host disease.[96]

Draper et al found increased inflammatory cell infiltration, acinar atrophy, and fibrosis among age-related alterations in lacrimal glands of rats.[76] In the human lacrimal glands, Damato et al and Obata et al described atrophy of secretory acini, periductal fibrosis, and increased inflammatory cell infiltration with aging,[75,78,79] similar to our observations, which included interlobular fibrosis, cystic dilatation of ducts, atrophy of secretory acini, and inflammatory cell infiltration and were more prominent and extensive in the knockout mice in this study. Attempts to quantify these phenotypic alterations in the lacrimal gland acinar units revealed significant decreases in the secretory acinar unit density in the *Sod1*$^{-/-}$ mice, a parameter that we thought would reflect the process of acinar atrophy. Apoptosis of acinar and ductal epithelia of the lacrimal glands along with glandular atrophy have been previously proposed as a possible mechanism for the impairment of glandular secretory function.[33,97] A striking ultrastructural observation was related to the extensive accumulation of secretory vesi-

cles in the lacrimal gland acinar epithelia in the *Sod1*$^{-/-}$ mice overtime compared with the WT mice. These observations suggest that the lacrimal glands may be unable to secrete tears in the presence of marked mitochondrial alterations in the *Sod1*$^{-/-}$ mice because mitochondria are the cellular powerhouses important for normal tear secretions.[27,98] Whereas the mitochondria in the lacrimal glands of the *Sod1*$^{-/-}$ mice showed striking ultrastructural alterations, which might lead to decreased tear production, further evidence from future studies simultaneously looking into lacrimal gland ATP levels, mitochondrial membrane potentials, and Ca^{++} currents across lacrimal gland acinar epithelial membranes may provide essential proof of whether the mitochondrial dysfunction in the presence of phenotypic mitochondrial alterations is linked to tear production decrease or not. Surprisingly, we found a significant decrease in the weight-adjusted aqueous tear production, pilocarpine-stimulated tear output, and total protein secretion capacity of the lacrimal gland in the old *Sod1*$^{-/-}$ mice compared with the old WT mice.

Decreased aqueous tear and protein output by the lacrimal gland and increased corneal epithelial damage are universally well-known features of the dry eye disease in animals and humans.[99–101] The higher corneal epithelial damage observed in the *Sod1*$^{-/-}$ mice reflects both the detrimental effects of decreased tear production on the ocular surface and possible oxidative stress damage on cell membrane lipids. *Sod1* is an abundant Cu- and Zn-containing protein present in cytosol,[67] nucleus, peroxisomes, and mitochondrial intermembrane space.[62,102] Its primary function is to act as an antioxidant enzyme, lowering the steady-state concentrations of superoxide.[103] Our results suggest that oxidative stress is not merely an associated phenomenon but may be an integral and primary cause of age-related dry eye disease in the *Sod1*$^{-/-}$ mice model. Adding further to our surprise were the similar observations in young and old human lacrimal gland specimens showing extensive lipid and DNA oxidation, inflammatory cell infiltration, fibrosis, and cystic duct dilatation in the aged individuals compared with the young individuals. Whereas the lacrimal gland samples from the human young and old individuals showed striking differences in relation to increased staining for oxidative stress markers and increased fibrosis and glandular atrophy, which were more prominent in the old individuals, tear function differences still need to be proven, although previous reports indicated a decrease in tear secretion with aging.[104–106] Future studies looking into differences among reflex tearing, total tear protein secretion, and histopathologic differences of lacrimal gland samples obtained for diagnostic purposes in young and old individuals may increase our understanding of whether the *Sod1*$^{-/-}$ mice are relevant to the human disease or may be useful to identify novel therapies for age-related dry eye disease.

We were unable to disclose when exactly the sequence of mechanistic events resulted in the phenotype observed in the lacrimal glands of the SOD1 knockout mice, which possibly led to dry eye and ocular surface disease. Relevant lacrimal gland pathophysiological and tear function examinations in the *Sod1*$^{-/-}$ mice performed more frequently in future studies will provide useful information.

In conclusion, we demonstrated that the lack of *Sod1* led to increased oxidative lipid and DNA damage, increased $CD4^+$ T-cell inflammation, and EMT in the lacrimal glands of the current mouse model, interfering with glandular secretory functions, which resulted in dry eyes and translated into an ocular surface disease.

Acknowledgments

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Oxidative Stress Induced Inflammation Initiates Functional Decline of Tear Production

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Abstract

Oxidative damage and inflammation are proposed to be involved in an age-related functional decline of exocrine glands. However, the molecular mechanism of how oxidative stress affects the secretory function of exocrine glands is unclear. We developed a novel *mev-1* conditional transgenic mouse model (*Tet-mev-1*) using a modified tetracycline system (Tet-On/Off system). This mouse model demonstrated decreased tear production with morphological changes including leukocytic infiltration and fibrosis. We found that the *mev-1* gene encodes Cyt-1, which is the cytochrome b_{560} large subunit of succinate-ubiquinone oxidoreductase in complex II of mitochondria (homologous to succinate dehydrogenase C subunit (SDHC) in humans). The *mev-1* gene induced excessive oxidative stress associated with ocular surface epithelial damage and a decrease in protein and aqueous secretory function. This new model provides evidence that mitochondrial oxidative damage in the lacrimal gland induces lacrimal dysfunction resulting in dry eye disease. Tear volume in *Tet-mev-1* mice was lower than in wild type mice and histopathological analyses showed the hallmarks of lacrimal gland inflammation by intense mononuclear leukocytic infiltration and fibrosis in the lacrimal gland of *Tet-mev-1* mice. These findings strongly suggest that oxidative stress can be a causative factor for the development of dry eye disease.

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Introduction

Dry eye disease is a deficiency in tear instability, mainly induced by low tear production, and a functional decline of the lacrimal gland induced by age-related chronic inflammation [1–3]. Such age-related chronic inflammation supported the reported prevalence of dry eye disease [4–8]. However, the molecular mechanism of age-related lacrimal gland inflammation is unclear. The main cause of chronic inflammation is postulated to involve oxidative stress, and the main endogenous source of oxidative stress is the electron transport chain in mitochondria [9]. The *mev-1* mutant of the nematode *Caenorhabditis elegans* has a genetic dysfunction in complex II of the mitochondrial electron transport chain [10] and overproduces a superoxide anion (O_2^-) from the mitochondria [11]. The lifespan of this *mev-1* mutant decreases dramatically as oxygen concentrations are increased from 1 to 60% [12]. In addition, *mev-1*-like dominant negative SdhC ($SdhC^{171E}$) increases oxidative stress and reduces the lifespan in *Drosophila* [13].

To determine whether mouse lacrimal gland functional decline is related to oxidative-stress-induced inflammation, a *mev-1* conditional transgenic mouse (*Tet-mev-1*) was established with a modified tetracycline system (Tet-On/Off system) [14], which equilibrates transgene expression to endogenous levels [15]. Excessive oxidative stress induces mitochondrial respiratory chain dysfunction and results in excessive apoptosis leading to low birth weight and growth retardation in *Tet-mev-1* mice [14]. Using this mouse model, we found that the lacrimal gland of *Tet-mev-1* mice produced more O_2^- and oxidative protein than the lacrimal gland of wild type mice. This new model provides evidence that mitochondrial oxidative damage in the lacrimal gland induces lacrimal dysfunction resulting in dry eye disease.

Methods

Animals and Materials

C57BL/6L and *Tet-mev-1* mice were bred and maintained under specially pathogen free (SPF) conditions in the Center of Genetic Engineering for Human Disease (CGHED) (Tokai University School of Medicine, Kanagawa, Japan). Doxycycline was administered in a drinking water mix (dose: 2 mg/ml). All mice used in analyses were 3 month old males.

Histopathology

Under the operating microscope, the lacrimal gland and submandibular salivary gland were surgically excised after death. A portion of each dissected specimen was immediately embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA) and snap frozen in pre-cooled isopentane at −80°C. The remainder of the tissues was analyzed after being fixed in 4% paraformaldehyde or 10% neutral buffered formalin and embedded in paraffin wax.

HE staining and Azan staining. Five micrometer-thick paraffin embedded sections fixed in 4% paraformaldehyde were cut and stained with HE. Additionally, 5 μm-thick paraffin embedded sections fixed in 10% neutral buffered formalin underwent Azan staining to evaluate the severity of fibrosis in the lacrimal gland.

Immunohistochemical analysis of DNA damage due to oxidative stress (8-OHdG). The 5 μm-thick paraffin embedded sections fixed in 4% paraformaldehyde were cut and stained with a mouse anti-8-OHdG monoclonal antibody (Japan Institute for the Control of Aging [JaICA], Shizuoka, Japan) to analyze DNA damage due to oxidative stress [16,17]. After removal of paraffin, the sections were placed in 10 mM citrate buffer solution and autoclaved at 121°C for 10 min. After blocking with 10% normal goat serum (Vector Laboratories, Burlingame, CA), sections were first blocked with Avidin/Biotin blocking reagent (Vector Labs) and then with a mouse on mouse blocking reagent (M.O.M.™). Blocking with the anti-mouse IgG blocking reagent (Vector Laboratories) was completed overnight at 4°C. Sections were exposed to diluted mouse anti-8-OHdG monoclonal antibody (1:10). Antibody binding was detected with a horse anti-mouse IgG ABC kit (Vector Laboratories) according to the manufacturer's protocol. The bound antibodies were visualized by the addition of diaminobenzidine tetrahydroxychloride.

Analysis of the mononuclear cell fraction using histochemical staining (CD4, CD8, CD19 and F4/80). Immunohistochemical analysis was performed according to a standard protocol with a panel of mouse monoclonal antibodies specific for CD4, CD8, CD19 and F4/80, (eBioscience, San Jose, CA) [18,19]. Briefly, 8 μm-thick frozen sections were air dried, fixed in acetone for 20 min at room temperature, and rehydrated in phosphate-buffered saline (PBS). Nonspecific binding was inhibited by incubating the specimens with 5% goat serum in PBS for 30 min at room temperature. The sections were incubated with the optimally diluted primary antibody at room temperature for 2 h, followed by incubation with a peroxidase-conjugated rabbit anti-mouse IgG antibody (Histofine® Simple Stain Rat MAX PO (M)) (Nichirei Biosciences Inc, Tokyo, Japan) for 45 min. The bound antibodies were visualized by the addition of diaminobenzidine tetrahydroxychloride. All steps were followed by three washes with PBS. Nuclei were counterstained with hematoxylin for 1 min [20].

Quantitative real-time RT-PCR

RNA extraction. An acid guanidinium-phenol-chloroform method was used to isolate RNA from tissues and cultured cells. The following protocol describes isolation of RNA from mouse lacrimal gland tissue. Immediately after removal from the animal, the tissue was minced on ice and homogenized (at room temperature) with 0.85 ml of 4 M guanidinium thiocyanate (GTC) in a glass-Teflon homogenizer and subsequently transferred to a 15 ml polypropylene tube with 2 ml of 4 M GTC, 0.15 ml of 10% sarcosyl and 0.72 μl of 2-mercaptoethanol. A total of 0.3 ml of 2 M sodium acetate, pH 4, 3 ml of phenol (water saturated), and 0.6 ml of chloroform-isoamyl alcohol mixture (24:1) were sequentially added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 7000 rpm for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 3 ml of isopropanol, and then placed at −20°C for at least 2 h to precipitate the RNA. Centrifugation at 7000 rpm for 20 min at 4°C was again performed and the resulting RNA pellet was washed in 3 ml of 70% ethanol and centrifuged at 7000 rpm for 20 min at 4°C. After centrifugation, the RNA pellet was air-dried (1 h) at room temperature. After drying, 88 μl 0.1% diethyl pyrocarbonate (DEPC) in distilled water was added to the pellet. The solution was transferred to a 2 ml Eppendorf tube with 2 μl DNase (20 U), 10 μl DNase buffer and 0.5 μl RNase inhibitor (Pharmacia) and was heated for 30 min at 37°C. After cooling on ice, the solution was added to 400 μl of a chloroform-phenol mixture (1:1) and 300 μl of 0.1% DEPC in distilled water. After 20 min on ice, the solution was centrifuged at 12000 rpm for 20 min at 4°C. The aqueous phase was transferred to a fresh tube with 35 μl 3 M sodium acetate and 1 ml 100% ethanol. After mixing, this solution was placed at −20°C for 30 min and centrifuged at 12000 rpm for 20 min at 4°C. The sediment was washed with 400 μl 70% ethanol and centrifuged at 12000 rpm for 5 min at 4°C. The sediment was air-dried for 1 h at room temperature and 100 μl 0.1% DEPC in distilled water was added.

Complementary DNA (cDNA) preparation and quantitative real-time RT-PCR. First strand complementary DNA (cDNA) was synthesized from 4.0 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. RT-PCR primers and an appropriate probe were chosen by the Universal Probe Library (UPL) Assay Design Center web service. Quantitative real-time RT-PCR was performed with pre-designed primers (Nihon Gene Research Laboratories, Sendai, Japan) and a TaqMan® probe (Applied Biosystems, Foster City, CA, USA) for the housekeeping gene GAPDH (NM 008084.2) (forward primer [FP]: AGCTTGTCATCAACGGGAAG, reverse primer [RP]: TTTGATGTTAGTGGGGTCTCG) (UPL probe: #9) as an endogenous control to normalize the expression data for each gene: IL-1β (NM 008361.3) (FP:TGTAATGAAAGACGGCACACC, RP:TCTTCTTTGGGTATTGCTTGG) (UPL probe #78), tumor necrosis factor (TNF-α) (NM 013693.2) (FP:TGCCTATGTCTCAGCCTCTTC, RP:GAGGCCATTTGGGAACTTCT) (UPL probe #49), IL-6 (NM 031168.1) (FP:GCTACCAAACTGGATATAATCAGGA,RP:CCAGGTAGCTATGGTACTCCAGAA) (UPL probe #6), IL-10 (NM 010548.1) (FP:CAGAGCCACATGCTCCTAGA,RP:TGTCCAGCTGGTCCTTTGTT) (UPL probe #41) and interferon-γ (IFN-γ) (NM 008337.3) (FP:ATCTGGAGGAACTGGCAAAA, RP:TTCAAGACTTCAAAGAGTCTGAGGTA) (UPL probe #21). Quantitative real-time RT-PCR was completed using the TaqMan® Gene Expression Assay and the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems).

Isolation of mitochondria

Mitochondria were isolated from mouse lacrimal glands using a standard procedure involving differential centrifugation [21,22]. After washing with ice-cold PBS, the lacrimal glands were minced in a volume of isolation buffer (210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, and 5 mM Tris-HCl, pH 7.4). The minced lacrimal glands were homogenized in isolation buffer at 800 rpm with 30 strokes using a Teflon homogenizer. The homogenate was centrifuged at 2000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and centrifuged at 14000 rpm for 10 min at 4°C. The mitochondria-containing pellet was suspended in TE buffer (50 mM Tris-HCl pH 7.4 and 0.1 mM EDTA).

Measurement of activity of complexes I and II of the electron transport chain

The activity of NADH-coenzyme Q oxidoreductase (complex I) and succinate-coenzyme Q oxidoreductase (complex II) in mitochondria was measured as previously described [22,23]. Tissues were homogenized in isolation buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl). The resulting homogenate was centrifuged at 250× g for 10 min to remove debris. The supernatant was further centrifuged at 31000× g for 20 min. The pellet was suspended in isolation buffer. Complex I activity was assayed by measuring NADH-sensitive NADH-cytochrome *c* reductase activity at 37°C in 200 µl 0.1 M Tris–SO_4 buffer at pH 7.4, containing 0.32 mg cytochrome *c* and 1 mM sodium cyanate. Complex II activity was assayed by measuring malonate-sensitive succinate-cytochrome *c* reductase activity. The reference cuvette contained 20 µl of 20% sodium malonate solution.

Measurement of O_2^-

Production of O_2^- was measured using the chemiluminescent probe 2-methyl-6-p-methoxyphenylethynyl-imidazopyrazinone (MPEC) (ATTO Co., Tokyo, Japan). MPEC has an advantage of low background relative to 3, 7-dihydro-2-methyl-6-(4-methoxyphenol) imidazole [1, 2-a] pyrazin-3-one (MCLA), which is generally used [15,23–25]. A total of 40 µg of intact mitochondrial fraction was added to 1 ml assay buffer (50 mM HEPES-NaOH, pH 7.4 and 2 mM EDTA) containing 0.7 µM of MPEC. The solutions were placed in a photon counter with an AB-2200 type Luminescencer-PSN (ATTO Co.) and measured at 37°C. The rates of O_2^- production were expressed as counts per second.

Measurement of carbonylated protein

Carbonylated protein as an indicator of oxidized protein was detected by an enzyme linked immunosorbent assay (ELISA) [25]. Isolated mitochondrial proteins from the lacrimal gland were treated with 10 mM DNPH. A total of 250 ng of mitochondrial protein in 50 mM $NaHCO_3$ was coated on an enhanced protein-binding ELISA plate (Caster) by incubating at 4°C for 8 h. Nonspecific binding to the plate was minimized by blocking the wells with 100 µl blocking buffer (3% BSA and 0.1% NaN_3 in PBS) at 37°C for 1 h. After the supernatant was removed, 100 µl of anti-DNP antibody diluted with buffer G (0.1% BSA, 0.1% gelatin, 0.1% NaN_3 and 1 mM $MgCl_2$ in PBS) was added to each well and incubated at 37°C for 1 h. After the supernatant was removed, the plate was washed four times with PBS and 100 µl of horseradish peroxidase-conjugated secondary antibody diluted with 0.05% Tween 20 in PBS was added followed by incubation at 37°C for 1 h. The plate was washed four times to remove the unbound secondary antibody. After 100 µl of ELISA coloring solution (0.0156 M $C_6H_8O_7$, 0.1 M $Na_2HPO_4{\cdot}12H_2O$, 0.4 mg/ml o-phenylenediamine dihydrochloride and 0.2 µl/ml 30% H_2O_2) was added to each well, the reaction was terminated by the addition of 100 µl of 1 M H_2SO_4. The absorbance was measured using a computer-controlled spectrophotometric plate reader (Spectra Max 250: Molecular Devices) at a wavelength of 492 nm.

Corneal fluorescein staining

Corneal fluorescein staining was performed as described by Rashid et al. [26]. Sodium fluorescein (1%) was applied to the cornea of mice. Three minutes later, eyes were flushed with PBS to remove excess fluorescein, and corneal staining was evaluated with a hand slit lamp (Kowa, Tokyo, Japan) using cobalt blue light. Punctate staining was recorded using a standardized grading system of 0 to 3 for each of the three areas of the cornea [27–29].

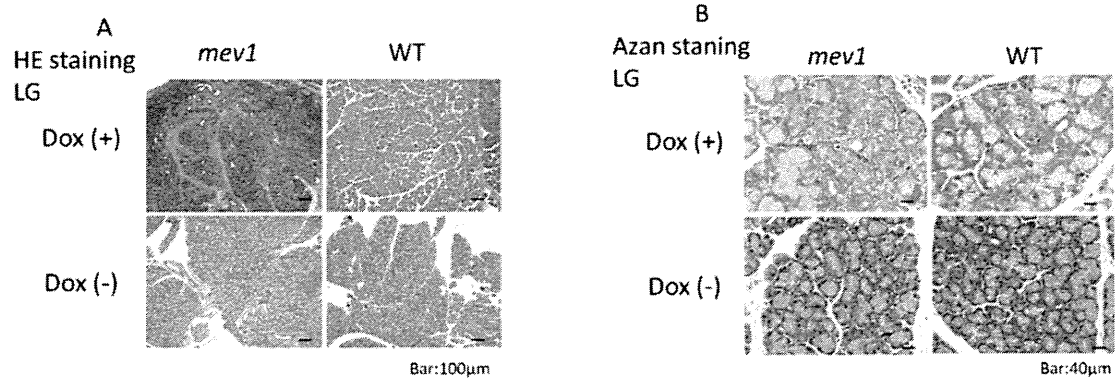
Aqueous tear measurement

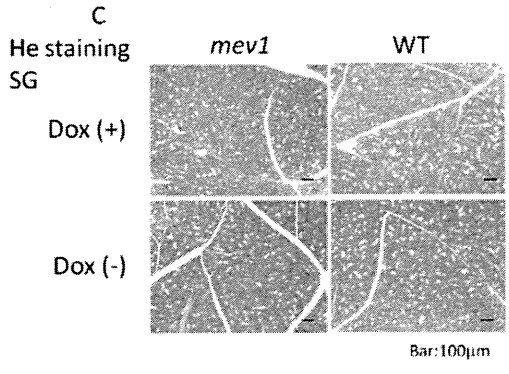
For 3 min, tears (0.5 µl) from each mouse were collected in a microcapillary tube. Tear volume was measured using capillary length (mm). Tear volume was normalized against the body weight of each mouse and the experiments were performed three times to validate the tear measurement.

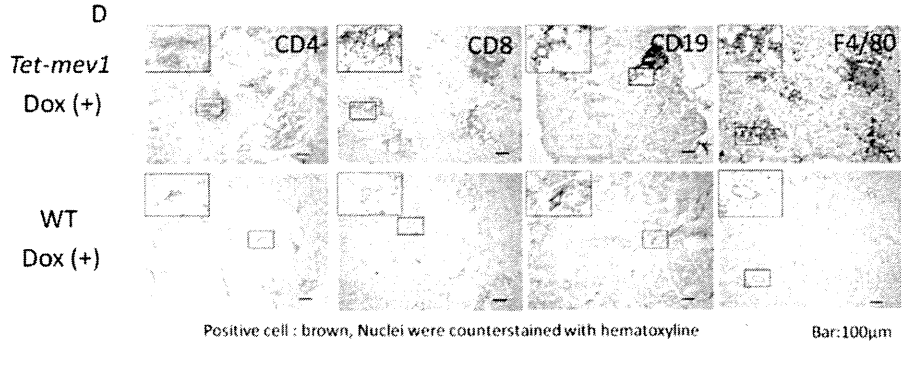
Results

Histopathology of the lacrimal glands revealed no inflammation in *Tet-mev-1* mice without Dox (*Tet-mev-1*/Dox(−)) or in wild type mice (C57BL/6J) with Dox (WT/Dox(+)) or without Dox (WT/Dox(−)) at 3 months old. *Tet-mev-1*/Dox(+) mice typically had multifocal inflammation and fibrosis around acinar cells in the lacrimal gland (Fig. 1a, b). However, histopathology of the salivary glands showed no inflammation in all mice (Fig. 1c). Moreover, although the superoxide anion was overproduced in the whole body of *Tet-mev-1*/Dox(+) mice, other main internal organs examined (i.e., liver, heart, kidney, lung and brain) did not have an inflammatory response (data not shown). To clarify the inflammatory status, we investigated the immunostaining by cell surface antigens (CD4, CD8, CD19, and F4/80). Various immunocytes such as cytotoxic T cell, helper T cells, activated B cells, and pan-macrophages had infiltrated the inflammatory focus (Fig. 1d). This inflammation was not observed in WT/Dox(+) mice, which suggested that doxycycline administration did not cause inflammation in the lacrimal gland. In addition, quantitative real-time RT-PCR analysis of the cytokines in the lacrimal gland showed an increase in inflammatory cytokines including TNFα, IL-6 and INFγ, which may be related to the inflammatory reaction in the lacrimal gland of *Tet-mev-1*/Dox(+) mice. Expression of the anti-inflammatory cytokine IL-10 was increased. (Fig. 1e, f).

Tet-mev-1 mice contain the mutation site of SDHC V69E, which is located within the functional ubiquinone (CoQ)-binding region of complex II [15,30,31]. *Tet-mev-1* mice are conditional transgenic mice and were designed to have decreased affinity of CoQ for complex II in mitochondria, which would induce electron leakage and lead to an increase in production of superoxide anion from complex II in the presence of doxycycline. The activity of complexes I and II in mitochondria of the lacrimal gland was compared between WT/Dox(+) and *Tet-mev-1*/Dox(+) mice. In the mitochondria of the *Tet-mev-1* mouse, only the activity of complex II was decreased, and, thus, reactive oxygen species (ROS) was overproduced from complex II with doxycycline. According to the intended design of the model, complex I activity of the lacrimal gland was not significantly different between WT/Dox(+) and *Tet-mev-1*/Dox(+) mice, and complex II activity in *Tet-mev-1*/Dox(+) mice was significantly lower than in WT/Dox(+) mice ($p = 0.008$, Fig. 2a). The activity of complex II-induced O_2^- production in the lacrimal gland significantly increased in *Tet-mev-1*/Dox(+) mice compared with that in the other types of mice ($p = 0.014$, Fig. 2b). We then measured carbonylated protein as a marker of oxidized proteins, which accumulate in the mitochondrial fractions of wild type mice during aging [25]. Our results showed that carbonylated protein amounts in the lacrimal gland of wild type mice were not significantly different between Dox(+) and Dox(−) mice. Therefore, doxycycline did not affect the quantity of carbonylated protein. Carbonylated protein content was determined by ELISA and the ratio of WT/Dox(+) and *Tet-mev-1*/Dox(+) was three times higher than the ratio of WT/Dox(−) and *Tet-mev-1*/Dox(−) ($p<0.01$, Figure 2c). The compound 8-OHdG







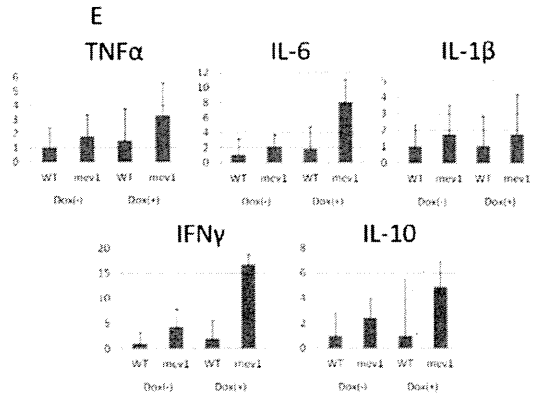


Figure1F : Average of Δ Ct

	WT(-)	mev1(-)	WT(+)	mev1(+)
TNFα	10.65±0.52	9.78±0.62	10.07±1.20	8.93±1.19
IL-6	17.06±1.13	15.96±0.63	16.2±1.56	14.04±1.59
IL-1β	11.3±0.45	10.5±0.82	11.25±0.85	10.52±1.30
IFN-γ	18.51±1.15	16.36±1.80	17.55±1.85	14.45±1.02
IL-10	14.41±0.90	13.13±0.66	14.46±2.19	12.11±1.00

Figure 1. Inflammation of the lacrimal gland in *Tet-mev-1 mice* with Dox. A, HE staining shows that *Tet-mev-1 mice* with Dox (*Tet-mev-1*/Dox(+)) typically have multifocal inflammation. The other types of mice (*Tet-mev-1*/Dox(−), WT/Dox(+) and WT/Dox(−)) have no inflammation in the lacrimal gland. Scale bar, approximately 100 μm. B, Azan staining was used to evaluate the severity of fibrosis in the lacrimal gland. *Tet-mev-1*/Dox(+) only shows fibrosis around acinar cells in the lacrimal gland. Scale bar, approximately 40 μm. C, Histopathology of the salivary glands shows no inflammation in all types of mice. Scale bar, approximately 100 μm. D, In lacrimal glands of *Tet-mev-1*/Dox (+) mice, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ cells (B cells) and $F4/80^+$ cells (pan-macrophage) were observed. Scale bar, approximately 100 μm. E, Proinflammatory cytokines were evaluated by real-time RT-PCR (ratio to WT/Dox(−)). Proinflammatory cytokines (TNF-α, IL-6, IL-1β, and IFN-γ) were increased in *Tet-mev-1*/Dox(+), especially IL-6 and IFN-γ, and IL-10 was also increased. F, Row data about Proinflammatory cytokines evaluated by Real-time RT-PCR is shown.
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accumulates with aging [32], and accordingly, 8-OHdG was used as a marker of oxidative damage in DNA in our study. Immunohistological labeling intensity for 8-OHdG was higher in the lacrimal gland of *Tet-mev-1*/Dox(+) mice compared with that in the other types of mice (Fig. 2d).

The aqueous tear quantity values were 2.26±0.48 mm/g (n = 14), 2.23±0.46 mm/g (n = 6), 2.47±0.60 mm/g (n = 6), and 1.35±0.48 mm/g (n = 8) for WT/Dox(−) mice, *Tet-mev-1*/Dox(−) mice, WT/Dox(+) mice, and *Tet-mev-1*/Dox(+) mice, respectively. The aqueous tear quantity values for *Tet-mev-1*/Dox(+) mice were significantly lower than in the other types of mice (n≥6, ANOVA Tukey's test, p = 0.0024) (Fig. 3a). Corneal fluorescein staining was higher in *Tet-mev-1*/Dox(+) mice compared with that in the other three types of mice (Fig. 3b). The corneal fluorescein staining scores were 0.75±0.89, 1.14±0.90, 0.71±1.25, and 4.50±1.60 for WT/Dox(−) mice, *Tet-mev-1*/Dox(−) mice, WT/Dox(+) mice, and *Tet-mev-1*/Dox(+) mice (all n = 8), respectively. The score ranged from 0 to 9 with a score of 0 indicating normal and a score of 9 indicating a severe corneal punctuate defect. The corneal fluorescein staining score for *Tet-mev-1*/Dox(+) mice was significantly worse than for the other three types of mice (n = 8, ANOVA Tukey's test, p<0.00001) (Fig. 3c).

Discussion

It is well known that lacrimal and salivary gland functions decline with age in humans [33,34]. We first hypothesized that both lacrimal and salivary gland functions decline in *Tet-mev-1*/Dox(+) mice. However, the severe inflammation and fibrosis associated with functional decline occurred in the lacrimal gland, but not in the salivary gland. We hypothesized that the inherent tissue responses to oxidative stress in the lacrimal and salivary glands are different. Pharmacological cholinergic blockade (subcutaneous injection of scopolamine hydrobromide) inhibits lacrimal gland function. It also stimulates inflammatory cytokine production and lymphocytic infiltration in the lacrimal gland. This systemic cholinergic blockade does not induce a nonspecific inflammation at three sites (conjunctival goblet cells, submandibular glands and small intestine) that receive cholinergic innerva-

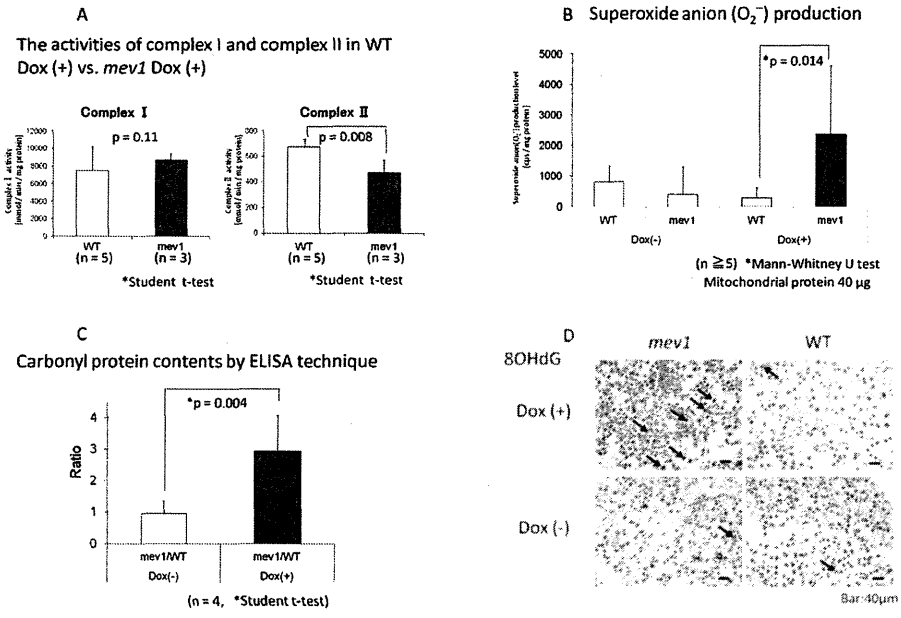

Figure 2. Lacrimal gland in *Tet-mev-1 mice* with Dox has functional depression of mitochondria and excessive O_2^- production. A, The activity of complexes I and II in WT/Dox(+) vs. *Tet-mev-1* mice/Dox(+). NADH-cytochrome c oxidoreductase was applied as an enzymatic indicator of complex I activity, and succinate-coenzyme Q oxidoreductase as an enzymatic indicator of complex II activity. Although there were no differences in the activity of complex I between these mice, complex II was significantly decreased in *Tet-mev-1 mice* with Dox. (WT: n = 5, *Tet-mev-1*: n = 3, NS, not significant; *P<0.01 [Student's t-test]). The vertical bars indicate the standard deviation of the separate experiments. B, Production of O_2^- in the lacrimal gland was significantly increased in *Tet-mev-1*/Dox(+) compared with that in the other types of mice. (n≥5, *P = 0.0014 [Kruskal-Wallis test]). The vertical bars indicate the standard deviation of the separate experiments. C, Carbonyl protein content of the lacrimal gland by ELISA. Each value shows the ratio of *Tet-mev-1* and WT for the relative amount of carbonyl protein in *Tet-mev-1 mice* with or without Dox (n = 4, *P = 0.004 [Student's t-test]). D, Immunohistochemical staining of 8-OHdG: *Tet-mev-1*/Dox(+) shows more positive nuclei (brown, indicated by the arrow) than the other types of mice.
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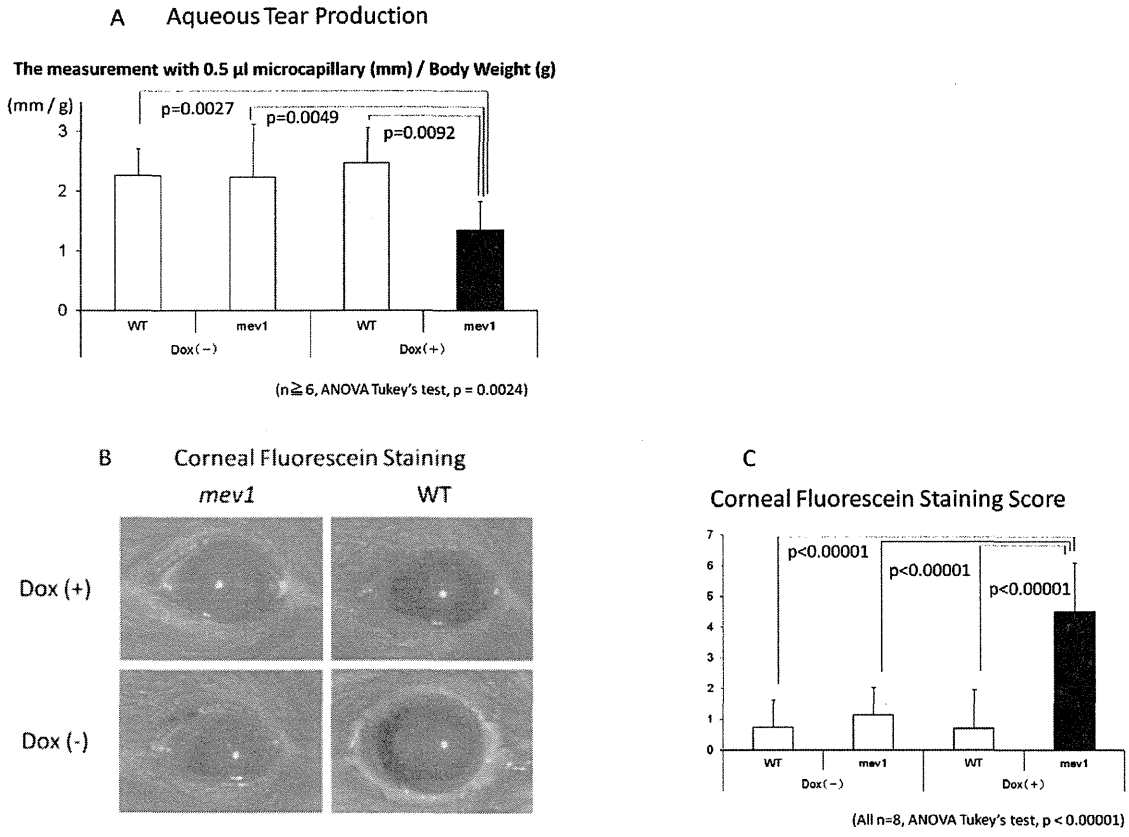

Figure 3. ***Tet-mev-1*/Dox(+) have dry eye disease.** A, Aqueous tear production: Aqueous tear quantity values of *Tet-mev-1*/Dox(+) were significantly lower than those in the other types of mice (n≥6, ANOVA Tukey's test, p = 0.0024). B, *Tet-mev-1*/Dox(+) mice had more corneal fluorescein staining than in the other mice. C, The corneal fluorescein staining score of *Tet-mev-1*/Dox(+) was significantly worse than that in the other types of mice (all n = 8, ANOVA Tukey's test, p<0.00001).
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tions [35]. These results suggest that the lacrimal gland is subject to inflammation by various stimuli in contrast with the salivary gland.

Mitochondria generate ATP through aerobic respiration, whereby glucose, pyruvate, and NADH are oxidized, thus generating ROS as a byproduct. In normal circumstances, the deleterious effects caused by the highly reactive nature of ROS are balanced by the presence of antioxidants. However, high levels of ROS are observed in chronic human diseases such as neurodegeneration [36], digestive organ inflammation [37], and cancer [38]. Recent work exploring the mechanisms linking ROS and inflammation suggest that ROS derived from mitochondria (mtROS) act as signal transducing molecules to trigger pro-inflammatory cytokine production [39]. Cells from patients with TNFR1-associated periodic syndrome (TRAPS) demonstrate that increased mtROS levels influence the transcription of pro-inflammatory cytokines such as IL-6 and TNF. TRAPS manifests as episodes of fever and severe localized inflammation with mutations in TNFR1. Inhibition of mtROS production inhibited MAPK activation and production of IL-6 and TNF in cells from TRAPS patients [40]. The mtROS in *Tet-mev-1*/Dox(+) mice may also directly induce increasing production of TNF-α and IL-6 and continuously induce inflammation in the lacrimal gland.

Protein oxidation is a biomarker of oxidative stress and many different types of protein oxidative modification can be induced directly by ROS or indirectly by reactions of secondary by-products of oxidative stress [41]. Lacrimal gland function has been reported to decrease gradually with aging, leading to reduced tear secretion and dry eye disease in the elderly [3,7]. Aging occurs, in part, as a result of the accumulation of oxidative stress caused by ROS that are generated continuously during the course of metabolic processes. Levels of 8-OHdG as a DNA oxidative stress marker and 4-HNE as a by-product of lipid peroxidation are higher and tear volume is decreased in middle-aged rats. Caloric restriction prevents a decline in lacrimal gland function and morphological changes and might be associated with a reduction in oxidative stress [42].

We confirmed that 8-OHdG immunohistological labeling intensity was higher in the lacrimal gland of *Tet-mev-1*/Dox(+) mice than in other mice types and the ratio of carbonylated protein content in mice with Dox was three times the ratio of mice without Dox. Collectively, mtROS production may damage DNA and induce the accumulation of carbonylated protein in the lacrimal gland.

These biochemical and histochemical data suggest that over-produced superoxide anion from the mitochondria affect directly and/or indirectly oxidative damage and inflammation in the lacrimal gland. It is believed that chronic inflammation of the lacrimal gland is a major contributor to insufficient tear secretion. Chronic inflammation of the lacrimal gland occurs in several

pathologic conditions such as autoimmune diseases (Sjögren syndrome, sarcoidosis, and diabetes) or simply as a result of aging [43]. The relationship between inflammation of the lacrimal gland and tear secretion deficiency has been described [44,45]. IL-1β induces a severe inflammatory response in the lacrimal gland and inhibits lacrimal gland secretion and subsequent dry eye disease [44]. A single injection of interleukin-1 into the lacrimal glands induces reversible inflammation and leads to destruction of lacrimal gland acinar epithelial cells, which results in decreased tear production. However, these inflammatory responses subside and lacrimal gland secretion and tear production return to normal levels [45].

For the dry eye model, we first reported the accelerated oxidation of protein, lipid, and DNA of the ocular surface in the rat swing model [46,47]. Accumulated oxidative damage caused the functional decline of the lacrimal gland and dry eye disease in *Tet-mev-1*/Dox(+) mice. In the lacrimal gland, age-related chronic inflammation, and age-related functional alterations including decreased acetylcholine release and protein secretion, might be related to dry eye diseases [48,49]. Our study clearly demonstrated that oxidative stress from mitochondria induced dry eye disease with morphological changes in the lacrimal gland of mice. In conclusion, reducing oxidative stress might be one of the possible treatments for age-related/ROS-induced dry eye disease.

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Author Contributions

Conceived and designed the experiments: YU MM TI NI. Performed the experiments: YU MM TI. Analyzed the data: YU TK SS KT. Contributed reagents/materials/analysis tools: HO KY YO. Wrote the paper: YU TK.

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Skewed Production of IL-6 and TGFβ by Cultured Salivary Gland Epithelial Cells from Patients with Sjögren's Syndrome

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Abstract

Objective: To determine the cytokine production profile of cultured salivary gland epithelial (SGE) cells obtained from patients with Sjögren's syndrome (SS).

Methods: SGE cells obtained from 9 SS patients and 6 normal controls were cultured in the presence of exogenous IFNγ. Cell proliferation and apoptosis in response to IFNγ were determined by WST1 assay and by FACS analysis. The concentrations of IL-6 and TGFβ secreted into culture supernatants were analyzed by ELISA.

Results: IFNγ did not significantly affect the proliferation or apoptosis of SGE cells. However, IL-6 concentrations were higher, and TGFβ concentrations were lower, in culture supernatants of SGE cells from SS patients than from normal controls.

Conclusion: Cytokine production by SGE cells from SS patients showed a skewed balance compared with normal controls, with increased IL-6 and decreased TGFβ secretion. This imbalance may be critical in the regulation of Treg/Th17 cells and may foster a pathogenic milieu that may be causative and predictive in SS.

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Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by lymphocytic infiltration into the salivary and lacrimal glands [1,2]. This chronic inflammation leads to destruction of the salivary glands and may ultimately result in salivary hypofunction. Although the mechanisms underlying this salivary gland destruction are not clearly understood, a better understanding of the precise molecular mechanisms may lead to the development of specific therapies for SS, similar to cytokine-targeted therapies in patients with rheumatoid arthritis (RA) [3,4].

Cytokines are key molecules that mediate chronic autoimmune inflammatory reactions in the salivary glands of SS patients [5]. Proinflammatory cytokines, such as interferon (IFN) γ, interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF) α, are produced by infiltrating lymphocytes and are involved in the maintenance of chronic inflammation [6–9]. In SS patients, these cytokines can induce the expression of HLA-DR, BAFF, costimulatory molecules such as CD80 and CD86, and/or chemokines in salivary gland epithelial (SGE) cells [10,11]. In addition, we have reported that the production of IFNs can further perpetuate the homing and activation of lymphocytes and the apoptosis of glandular cells [12,13]. In contrast, the absence of transforming growth factor (TGF) β has been reported to lead to systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and SS in TGFβknockout mice [14,15]. TGFβ promotes the differentiation of regulatory-T cells (Treg) [16] and, together with IL-6, plays a crucial role in the induction of Th17 cells [17,18]. Taken together, these findings suggest that cytokine balance plays an important role in chronic inflammation of the salivary glands in SS patients [5]. Moreover, long-term exposure to pro-inflammatory cytokines such as IFNγ and TNFα can result in salivary epithelium dysfunction, leading to hyposalivation. We therefore evaluated cytokine expression profiles in salivary gland epithelial (SGE) cells from SS patients stimulated with IFNγ.

Materials and Methods

Patients and controls

We evaluated 15 patients at Kanazawa Medical University Hospital (Ishikawa, Japan) who were enrolled in the Sjögren's International Collaborative Clinical Alliance (SICCA) Registry; the complete details of this registry have been described [19]. In brief, SICCA is an ongoing longitudinal multisite observational study of a large and growing cohort of uniformly evaluated individuals from ethnically diverse populations, designed to develop standardized classification/diagnostic criteria for SS [20,21]. Each participant in the SICCA cohort is assessed, systemically and extensively, for symptoms and signs related to SS. Of the 15 patients, nine (all women; mean age, 48±14 years) met both the 2002 American-European consensus group (AECG) and the SICCA criteria for SS [21,22], whereas six (all women; mean age, 57±8 years) did not meet either set of criteria and had no objective findings indicative of SS (Table 1). All experimental protocols were approved by the independent ethics committee of Kanazawa Medical University, and all participants provided written informed consent.

Labial minor salivary gland (MSG) biopsies were taken from each patient for diagnostic evaluation of SS, with SG tissue samples processed for further culture of primary epithelial cells. None of these participants had taken any immune suppressants or steroids.

Cell lines and primary cultures of SGE cells from MSGs

Human airway epithelial cells (HBTEC) and human umbilical vein endothelial cells (HUVEC) were obtained from Kurabo Co. Ltd., Osaka Japan. Epithelial cells obtained from the MSGs were cultured immediately after biopsy, as described [13]. In brief, each tissue sample was rinsed with cold sterile phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin and minced into small pieces of approximately 1–2 mm^3. One tissue sample from each subject was placed in a well of a collagen type I-coated 12-well plate (Iwaki, Tokyo, Japan) and cultured in keratinocyte serum-free medium (SFM; Invitrogen Corp., Carlsbad, CA) containing 0.4 μg/ml hydrocortisone and 25 μg/ml bovine pituitary extract (Sigma Chemical Co., St. Louis, MO). The epithelial cell outgrowth of each explant was assessed after 1–2 weeks. Upon attaining confluence, the monolayer cells were subcultured. Cells were rinsed twice with PBS and detached from the substrate by incubation with 0.05% trypsin (Invitrogen) for no longer than 10 min, with cell detachment monitored by light microscopy. Trypsin was inactivated by adding an equal volume of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS). The detached cells were centrifuged at 1500 rpm for 5 min, washed once with PBS, resuspended in culture medium, and reseeded, at a concentration of 8×10^4 cells per well, in a fresh collagen type I-coated 6-well

Table 1. Profile of patients included in the study.

	Sex	Age	Diagnosis	Focus Score (/4 mm^2)	ANA*	Anti SS-A	Anti SS-B
SS.1	F	64	SS	2.3	40	+	–
SS.2	F	67	SS	3.9	1280	–	–
SS.3	F	56	SS	1.8	160	+	+
SS.4	F	44	SS	2.8	320	+	+
SS.5	F	28	SS	3.2	160	+	–
SS.6	F	32	SS	2.4	80	+	+
SS.7	F	58	SS	2.7	160	+	–
SS.8	F	36	SS	2.9	160	+	+
SS.9	F	52	SS	1.2	–	+	–
No.1	F	67	non-SS	0	320	–	–
No.2	F	67	non-SS	0	–	–	–
No.3	F	51	non-SS	0	–	–	–
No.4	F	57	non-SS	0.33	–	–	–
No.5	F	46	non-SS	0	–	–	–
No.6	F	58	non-SS	0	640	–	–

Nine patients (all women; mean age, 48±14 years) met both the 2002 American-European consensus group (AECG) criteria and the SICCA criteria for Sjögren's syndrome (SS), whereas the other six (all women; mean age, 57±8 years) did not (No).
*Titers of anti-nuclear antibody (ANA).
doi:10.1371/journal.pone.0045689.t001

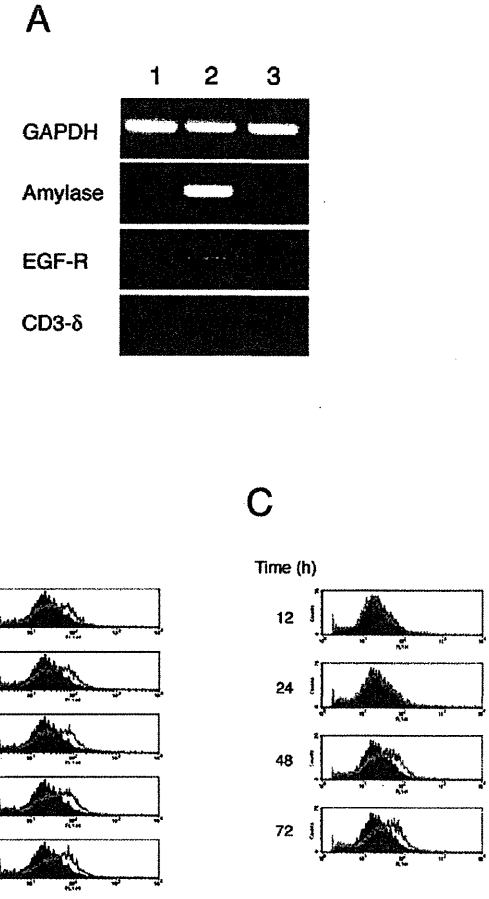

Figure 1. (A) Expression of mRNA in salivary gland epithelial cells. Salivary gland epithelial cells were isolated from SS patients and cultured. Total RNA was isolated from these cells, and EGF-R, α amylase-1, and CD3δ mRNAs were assayed by RT-PCR, as described in the Materials and Methods section. Lane 1: salivary gland epithelial cells, Lane 2: labial salivary gland of the same patient, Lane 3: normal lymph node as a control for CD3δ. **(B and C) Effects of IFNγ on human SGE cells.** SGE cells were incubated with various concentration of IFNγ for 48 hours (B) or with 1000 U/ml of IFNγ for the indicated times (C), and the surface expression of CD40 was examined by FACS analysis.
doi:10.1371/journal.pone.0045689.g001

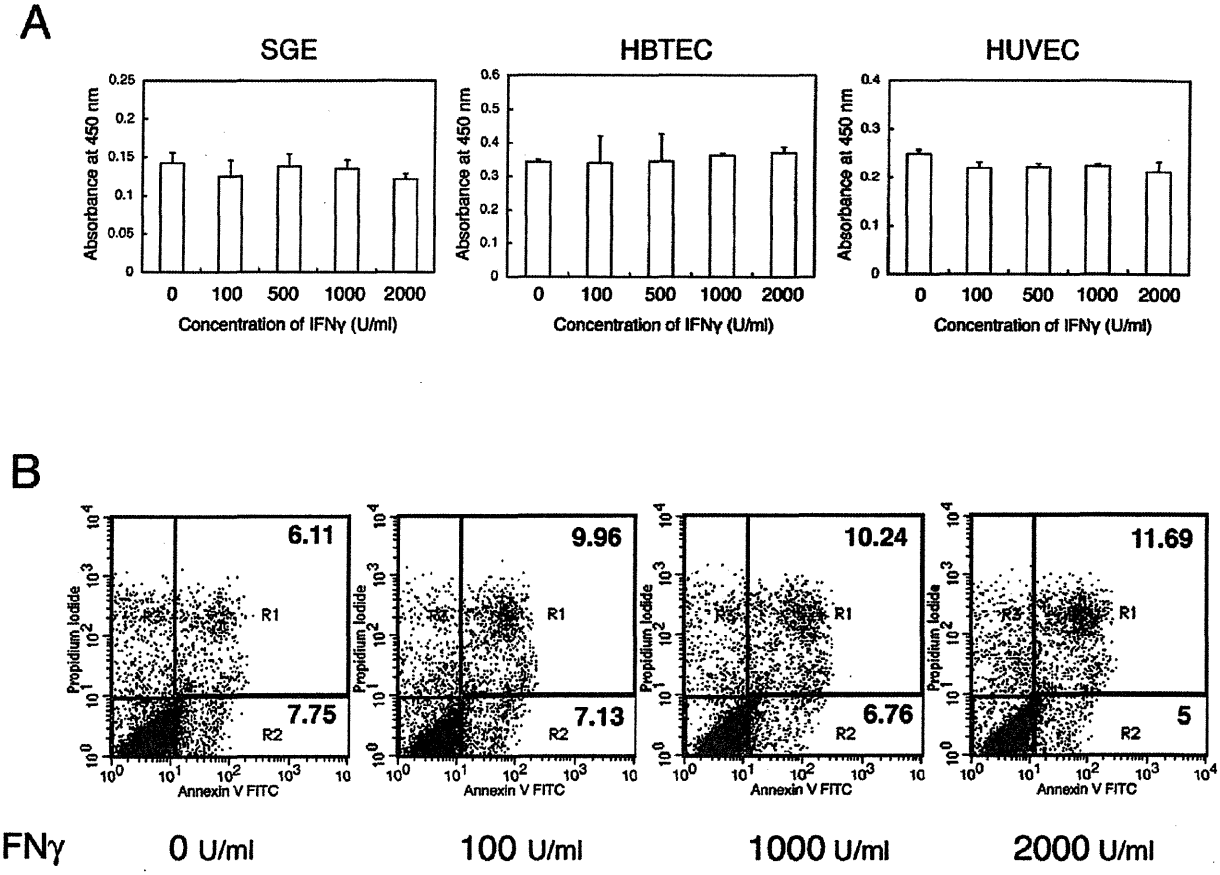

Figure 2. Effects of IFNγ on the proliferation and apoptosis of SGE cells. (A) SGE cells, human airway epithelial cells (HBTEC) and human umbilical vein endothelial cells (HUVEC)were incubated with the indicated concentration of IFNγ, and proliferative responses were assessed at 48 h. Each bar shows mean + SD. IFNγ did not significantly affect the proliferation of any of these cells. The results shown are representative of three independent experiments. (B) SGE cells were incubated with the indicated concentration of IFNγ, and apoptosis was determined at 12 h by flow cytometry. Numbers in R1 and R2 indicate early and late apoptosis, respectively.
doi:10.1371/journal.pone.0045689.g002

plate. Fibroblasts were routinely removed from the cultures by treating the cells with 0.02% ethylenediaminetetraacetic acid (EDTA; Invitrogen).

Immunocytochemistry

The SGE cells were harvested, washed once with PBS, and allowed to adhere to a glass slide in a monolayer. The cells were air dried for 30 min, fixed in 4% paraformaldehyde (PFA) acetone at 4°C for 30 sec, washed 3 times with purified water, incubated in Tris-buffered saline (TBS) for 5 min, and blocked with bovine serum albumin at room temperature for 5 min. After washing, the cells were incubated overnight at 4°C in a moist chamber with primary antibodies against epithelial membrane antigen (EMA, clone E29), cytokeratin 8 (35βH11), and cytokeratin 18 (DC10; Dako, Kyoto, Japan), each at a concentration of 1 μg/ml. Antigen-antibody complexes were detected using a labeled polymer conjugated with alkaline phosphatase (Envision/AP kit, Dako), according to the manufacturer's instructions, visualized by treatment for 10 min with the New Fuchsin chromogen-substrate solution and counterstained with Mayer's hematoxylin. Control slides were incubated with isotype-matched antibodies in TBS in place of the primary antibody; these invariably yielded negative results (data not shown).

Proliferation assay and detection of cell apoptosis

The proliferative responses of SGE cells to IFNγ (R&D Systems) were determined using a WST-1 cell counting kit, and cell apoptosis was determined by flow cytometry as described [23]. Briefly, SGE cells were cultured in collagen type I-coated plates in the presence of IFNγ at 37°C for the indicated times. Ethanol-fixed cell suspensions were centrifuged, followed by the addition of 50 μl RNase solution and 450 μl propidium iodide (final concentration 50 mg/ml). The cells were washed twice and subsequently analyzed by flow cytometry (BD Biosciences, Palo Alto, CA, USA).

Quantification of cytokines in the culture supernatants by ELISA

The concentrations of IL-6 and TGFβ1 secreted into culture supernatants were quantified using ELISA test kits for IL-6 (Immunotech, Marseille, France) and TGFβ1 (R&D Systems, Minneapolis, MN), according to the manufacturers' instructions.

Statistical analysis

Median cytokine concentrations, calculated to minimize the effects of extreme outliers, were compared using non-parametric

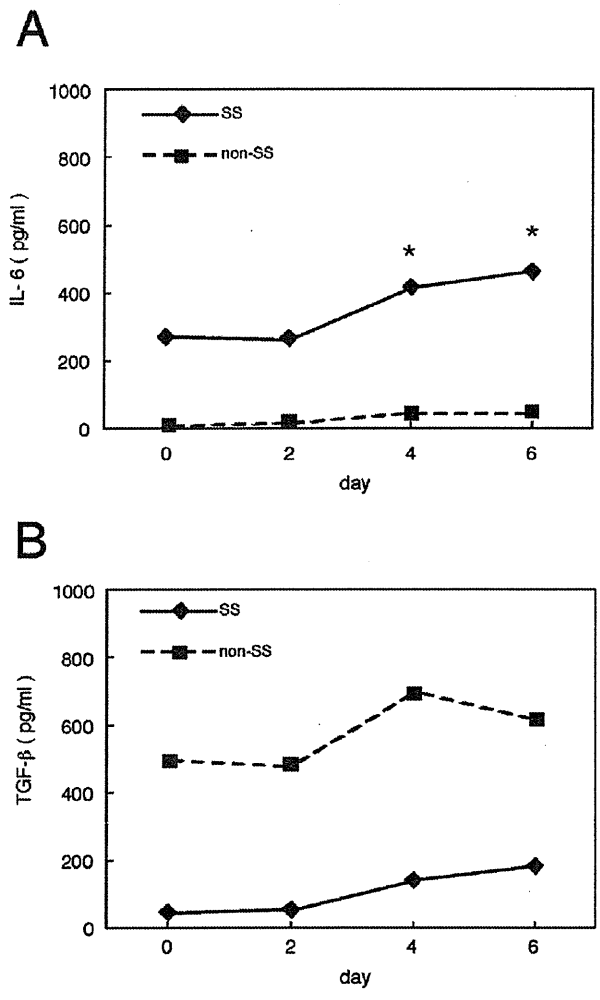

Figure 3. Quantification of cytokines secreted into the culture supernatants of SGE cells. Confluent SGE cells obtained from 9 SS patients and 6 normal controls (non-SS) were incubated in the presence of IFNγ (1000 U/ml), and the culture supernatants were collected on days 0, 2, 4, and 6. Median concentrations (pg/ml) of IL-6 (A) and TGFβ (B) in the supernatants were determined by ELISA and compared by non-parametric Mann-Whitney tests (*, $p<0.05$).
doi:10.1371/journal.pone.0045689.g003

Mann-Whitney tests. A p value<0.05 was considered statistically significant.

Results

Primary cultures of human SGE cells from MSGs

To establish primary cultures of human SGE cells, MSG biopsy samples were washed with PBS, cut into pieces of approximately 1–2 mm^3, and explanted onto collagen type I-coated plastic plates. Within a few weeks, the epithelial cells gradually grew out from the explants. Most of these cells were cuboidal, round, or spindle shaped. RT-PCR showed that these cells expressed EGF-R, but not α-amylase 1 or CD3δ, mRNA (Figure 1A). Immunohistochemical analysis showed that these cells were positive for EMA and cytokeratins-8 and -18 (data not shown), indicating that these cells were primarily ductal epithelial cells, with small amounts of acinar or lymphocytic components. These SGE cells could be maintained in culture medium for at least a few months.

Effects of IFNγ on activation of SGE cells

Previous studies have shown that the concentrations of cytokines such as IFNγ, IL-1β, IL-6, IL-10 and TNFα are increased in SS patients, and that long-term exposure to pro-inflammatory cytokines such as IFNγ and TNFα can lead to dysfunction of the salivary epithelium. To assess the effects of IFNγ on SGE cell activation, SGE cells were incubated with various concentration of IFNγ for 48 hours (Figure 1B) or with 1000 U/ml of IFNγ for the indicated times (Figure 1C), and the expression of CD40 was examined by FACS analysis. We found that CD40 expression on SGE cells was increased in a dose- and time-dependent manner (Figure 1B and C).

Effects of IFNγ on the proliferation and apoptosis of SGE cells

To test the effects of IFNγ on the proliferation of SGE cells, these cells, as well as human airway epithelial cells (HBTEC) and human umbilical vein endothelial cells (HUVEC), were incubated in the presence of the indicated concentrations of IFNγ for 48 h. We found that IFNγ had no effects on the proliferation these three cell types (Figure 2A). To assess the effects of IFNγ on SGE cell apoptosis, these cells were incubated with the indicated concentrations of IFNγ for 12 h. We observed that IFNγ did not significantly affect the early (R1) or late (R2) apoptosis of SGE cells (Figure 2B).

Production of IL-6 and TGFβ by IFNγ stimulated SGE cells

TGFβ induces Foxp3 in naïve T cells, resulting in their differentiation to regulatory T cells (Treg). In contrast, IL-6 switches T cell differentiation from a Treg to a Th17 pathway. We therefore incubated confluent SGE cells with IFNγ (1000 U/ml), and assayed the concentrations of IL-6 and TGFβ secreted into culture supernatants on days 0, 2, 4 and 6. We found that IL-6 concentrations on days 4 and 6 were significantly higher (Figure 3A), and TGFβ concentrations on days 2 and 4 were significantly lower (Figure 3B), in the supernatants of cells from SS patients than from controls.

Discussion

Although the production of cytokines in the salivary glands of SS patients has been evaluated [6–9,12,13], the most important cytokines involved in the pathogenesis of SS have not yet been identified. Since it is difficult to distinguish between cause and effect relationships of cytokine production, due to their complex interactions and the presence of various cells in tissues, we attempted to minimize this problem by using cultured SGE cells. Although previous studies have been hampered by the lack of a suitable in vitro culture system for SGE cells, our culture system, using a non-serum-containing medium, enabled us to examine cellular functions including cytokine production and to maintain human SGE cells as ductal epithelial cells for at least a few months.

Because the salivary glands of SS patients contain T cells that express IFNγ and Stat1 mRNA [24], with these T cells being predominantly Th1 cells [25], the proinflammatory cytokine IFNγ has been considered a principle mediator of inflammation in SS patients, similar to TNFα in patients with RA [3]. Local IFNγ production in the salivary glands may perpetuate inflammation by inducing SGE cells to express HLA-DR, co-stimulatory molecules, cytokines and chemokines, leading to secretory gland dysfunction [2]. We therefore comprehensively examined the effects of IFNγ

on the functions of SGE cells obtained from SS patients. We found that IFNγ activated SGE cells, leading to the increased expression of CD40, but did not significantly affect the proliferation or apoptosis of SGE cells.

Next we examined cytokine production by IFNγ-stimulated SGE cells obtained from SS patients. Although the differences were not significant, IFNγ induced a skewed expression of mRNAs encoding several cytokines, including IL-6, TNFα, TGFα and TGFβ, in SGE cells from both SS patients and normal controls (data not shown). We hypothesized that TGFβand IL-6 may play key roles in the pathogenesis of SS by affecting the balance between Treg and Th17 cells. TGFβ can have pro- or anti-inflammatory effects, depending on the context; i.e., TGFβ promotes the differentiation of naive T cells to Treg cells in the presence of IL-2, while inducing Th17 cells in the presence of IL-6 [16,26,27].

IL-6 is another pleiotropic cytokine that regulates immune responses, hematopoiesis and bone metabolism [28]. IL-6 overproduction has been found to be involved in the pathogenesis of several human autoimmune diseases, including RA and Castleman's disease [29]. Recently, IL-6 was shown to play an important role in T helper differentiation [26]. Stimulation of cultured CD4 T cells with IL-6 and TGFβ potently induced Th17 differentiation. IL-17 has been reported to be involved in the chronic inflammatory processes that occur in many autoimmune diseases, including SS [27,30]. Whereas TGFβ was found to induce naive CD4 T cells to differentiate into Foxp3+ Treg cells, this Treg induction was potently inhibited by IL-6. Rather, IL-6 was found to promote their differentiation into inflammatory Th17 cells, providing further evidence that IL-6 is critical in the regulation of the Treg/Th17 balance [26].

To identify the cytokines that play major roles in the development of salivary gland lesions in SS, we assayed the concentrations of IL-6 and TGFβ secreted into the culture supernatants of IFNγ-stimulated SGE cells. We found that IL-6 concentrations were higher in the supernatants of cells from SS patients than from normal controls, suggesting that IL-6 may be important in maintaining chronic inflammation in SS. These findings are consistent with results showing that IL-6 is highly expressed in SGE, with high focal scores [31]. Moreover, increased IL-6 production may contribute to the presence of abundant IL-17-bearing cells in the salivary glands of SS patients [30].

In contrast, we found that the secretion of TGFβ was lower in supernatants of SGE cells from SS patients than from controls. Since TGFβ has been linked to the generation of Treg cells, it is likely that Th1 cell differentiation and IFNγ production are not suppressed in the salivary glands of SS patients during the initial stages of inflammation.

Our findings suggested that the skewed production of IL-6 and TGFβ in the salivary glands of SS patients may shift the milieu to one more favorable for the propagation of Th17 cells, with correspondingly fewer Foxp3+ Treg cells, and may foster a pathogenic milieu that may be causative and predictive of infiltrative injury in SS patients.

Although the mechanism underlying this cytokine imbalance in SGE cells of SS patients, including reduced TGFβ and increased IL-6 production is currently unknown, modulation of this imbalance may help control the chronic inflammation of the salivary glands occurring in SS patients.

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Author Contributions

Conceived and designed the experiments: TK YH TO HU. Performed the experiments: TK T. Sawaki T. Sakai T. Sato MM HI AN. Analyzed the data: TK TN T. Sawaki T. Sakai T. Sato. Contributed reagents/materials/analysis tools: TK YF M. Tanaka M. Taniguchi YM TF NS. Wrote the paper: TK HU.

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