cytokines are known to be expressed by a variety of cell types, particularly the myeloid-lineage cells [22,23]. BAFF is synthesized as a membrane-bound or secreted protein, while APRIL exists solely in the secreted form [24]. BAFF binds to three receptors - BAFF receptor (BAFF-R), transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA) - which are expressed by B cells, whereas APRIL binds to TACI and BCMA [25].

BAFF and APRIL are thought to mediate the regulation of B cell maturation, survival, CD40L-independent antibody production, and isotype switching through BAFF-R and TACI [22,23,26,27]. Because overexpression of BAFF is known to induce B cell hyperactivation and autoimmunity in mice [28], BAFF has been considered a promoting factor in the pathogenesis of several autoimmune and allergic diseases. In fact, elevated serum levels of BAFF were observed in patients with rheumatoid arthritis (RA) [29], systemic lupus erythematosus (SLE) [30], primary Sjögren's syndrome (pSS) [31,32], inflammatory myositis (IM) [33], systemic sclerosis (SSc) [34], bronchial asthma [35], and atopic dermatitis [36], and serum BAFF levels were associated with their clinical activity. In contrast, overexpression of APRIL has not been associated with autoimmunity in mice but leads to enhanced IgM production, T cell-independent type 2 humoral responses, and T cell proliferation [37]. On the other hand, a lack of APRIL is associated with an increased percentage of $CD44^{hi}CD62L^{low}$ effector memory T cells and impaired class switching to IgA [38,39]. Although APRIL has been found to be elevated in patients with autoimmune diseases, including SLE [40], pSS [32], and multiple sclerosis [41], it is still under debate whether APRIL has a role in human autoimmunity, and its circulating levels do not parallel those of BAFF.

The aim of this study was to investigate the contribution of BAFF and APRIL in the pathogenesis of IgG4-RD. We assessed serum levels of BAFF and APRIL by ELISA to analyze their association with clinical manifestations, serological parameters, and treatment.

Materials and methods

Patients

All patients were recruited from the Department of Rheumatology and Clinical Immunology, Kyoto University Hospital, Kyoto, Japan. Patients with IgG4-RD (n = 18; 5 females, 13 males; mean age, 68.6 ± 12.3 years; range, 37 to 79 years) were included in the study. Patients were diagnosed on the basis of clinicopathologic findings [3,4], clinical findings (diffuse/focal enlargement or mass formation, nodular/thickened lesions in one or more organs), elevated serum IgG4 (> 135 mg/dL), and histopathologic features including infiltration of lymphocytes and IgG4-positive (+) plasma cells (IgG4+plasma cells/IgG+plasma cells > 40% and/or IgG4+ plasma cells > 10 cells in 5 high-power fields) with typical tissue fibrosis or sclerosis. None of the patients met the criteria for sarcoidosis, Castleman's disease, Wegener granulomatosis, malignant lymphoma, or pSS. Serum samples were obtained before (n = 13) and after (n = 5) glucocorticoid (GC) treatment, and the serum from six patients before GC treatment was drawn repeatedly during GC treatment. Ten healthy individuals and thirteen individuals with pSS were enrolled and served as healthy and disease controls, respectively. All of the patients with pSS had signs and symptoms that satisfied the Japanese Ministry of Health criteria for the diagnosis of pSS. All patients and healthy volunteers provided informed consent in accordance with the Declaration of Helsinki, before providing samples. This study was approved by the Medical Ethics Committee of Kyoto University Graduate School of Medicine.

Measurement of serum levels of BAFF and APRIL

Serum levels of BAFF were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), and serum levels of APRIL were determined using an ELISA kit (BioVendor Laboratory Medicine, Modrice, Czech Republic). All serum samples were stored at -20°C until use.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0a software (GraphPad Software, Inc., San Diego, CA, USA). Nonparametric tests were performed using the Mann-Whitney U test for comparison of the two groups. Correlations were determined by Spearman's correlation. A value of $P < 0.05$ was considered statistically significant. Data are shown as mean ± standard definition (SD).

Results

Clinical, laboratory, and histological features of immunoglobulin G4-related disease (IgG4-RD)

We measured serum levels of BAFF and APRIL in 18 patients with IgG4-RD before GC therapy (cases 1 to 13) and after GC therapy (cases 14 to 18) (Table 1). The duration of GC treatment ranged from five days to thirteen years in five patients (cases 14 to 18). Twelve of eighteen (66.7%) patients with IgG4-RD were elderly men. Serum IgG4 levels were > 135 mg/dL in all patients. Biopsy specimens from affected tissues were obtained from 15 of 18 patients, and abundant IgG4-bearing plasma cell infiltration with lymphoplasmacytic infiltrates and sclerosis was observed in all patients. Three patients (cases 7, 14, and 17), who did not agree to undergo biopsy had their conditions diagnosed as

Table 1 Clinical characteristics of patients with immunoglobulin G4-related disease (IgG4-RD).

Case	Age	Serum IgG4 (mg/dL)	RF	ANA	Clinical manifestation	Biopsy/IgG4/IgG ratio
1	73	2890	< 6	40	Lymph, Mikulicz's disease Prostatitis	Prostate/0.60
2	76	2210	< 6	40	Mikulicz's, RPF	Submandibular gland/0.40
3	79	1460	< 6	160 (SS-A)	IN, IP, Küttner's tumor, Lymph, RPF	Submandibular gland/0.73
4	66	1090	30.3	40	AIP, IN, Renal pseudotumor	Kidney/0.70
5	73	592	< 6	320	IN, IP, Lymph, RPF, Sialadenitis	Submandibular gland/0.43
6	62	736	52.0	< 40	Sialadenitis, Lymph	Parotid gland/0.30*
7	77	738	< 6	< 40	Mikulicz's disease	ND.
8	74	389	< 6	< 40	Retro-orbital tumor	Retro-orbital tumor/0.48
9	76	760	< 6	40	AIP, Periureteritis	Ureter/0.38
10	52	383	< 6	< 40	Küttner's tumor	Submandibular gland/0.57
11	70	724	< 6	< 40	Küttner's tumor, Lymph	Submandibular gland/0.40
12	46	675	26.8	80	Mikulicz's disease	Lachrymal gland/0.41
13	37	533	< 6	< 40	Mikulicz's disease	Lachrymal gland/0.50
14	77	655	< 6	80	RPF	ND
15	76	458	< 6	< 40	AIP, RPF	Retroperitoneal/0.70
16	62	315	< 6	40 (SS-A)	AIP, RPF	Pancreas/0.43
17	79	309	ND	< 40	RPF	ND
18	79	1960	65	40	Orbital tumor, Lymph, Lung nodule	Orbital tumor/0.59

AIP, autoimmune pancreatitis; ANA, antinuclear antibody; IN, interstitial nephritis; IP, interstitial pneumonia; Lymph, lymphadenopathy; ND, not determined; RF, rheumatoid factor; RPF, retroperitoneal fibrosis; SS-A, anti-SS-A antibody; *, IgG4-positive plasmacytes > 100/high-power fields.

IgG4-RD on the basis of hyper-IgG4 γ-globulinemia and typical clinical findings after other diseases were ruled out. Test results of four patients (cases 4, 6, 12, and 18) were positive for rheumatoid factor (RF), those of 10 patients (cases 1 to 5, 9, 12, 14, 16, and 18) were positive for antinuclear antibody (ANA), and those of two patients (cases 3 and 16) were positive for anti-SS-A antibody. Retroperitoneal fibrosis (RPF) was the most frequent clinical manifestation in our cohort.

Increased serum BAFF and APRIL in IgG4-RD

As shown in Figure 1a, serum levels of BAFF in patients with IgG4-RD before GC therapy (n = 13, 1.512 ± 0.393 ng/mL) were significantly higher than those in healthy controls (n = 10, 0.904 ± 0.262 ng/mL) (P < 0.01), and there were no significant differences in the serum levels of BAFF between patients with IgG4-RD before GC therapy and patients with pSS (n = 13, 1.820 ± 0.954 ng/mL) (P = 0.383). Serum levels of BAFF in patients with IgG4-RD after GC therapy (n = 5, 0.749 ± 0.283 ng/mL) were lower than in those before GC therapy (P < 0.01). In contrast, as shown in Figure 1b, serum levels of APRIL in patients with IgG4-RD before GC therapy (n = 13, 3.736 ± 3.271 ng/mL) were significantly higher than those in the healthy controls (n = 10, 1.327 ± 1.259 ng/mL) (P < 0.01), however, unlike BAFF, the levels were significantly lower than those in patients with pSS (n = 13, 11.250 ± 7.418 ng/mL) (P < 0.01).

Inverse correlation between serum APRIL and IgG4 in patients with IgG4-RD

While no significant correlation was found between serum levels of BAFF and IgG4 in patients with IgG4-RD before GC therapy (n = 13, r = -0.159, P = 0.603) (Figure 2a), a significant inverse correlation was found for APRIL before GC therapy (n = 13, r = -0.626, P = 0.022) (Figure 2b).

No correlation between number of affected organs and serum BAFF or APRIL in patients with IgG4-RD

To assess the association between serum BAFF/APRIL and disease severity, we counted the number of affected organs from a list of 10 organs (lacrimal glands, salivary glands, lung, pancreas, kidney, retroperitoneum, lymph node, thyroid glands, prostate, and orbit), which are known to be involved in IgG4-RD. As shown in Figure 3a, a significant correlation between the number of affected organs and serum levels of IgG4 was found in patients with IgG4-RD (n = 18, r = 0.638, P = 0.004). However, as shown in Figure 3b and 3c, no significant correlation was found between the number of affected organs and serum levels of BAFF (n = 13, r = -0.307, P

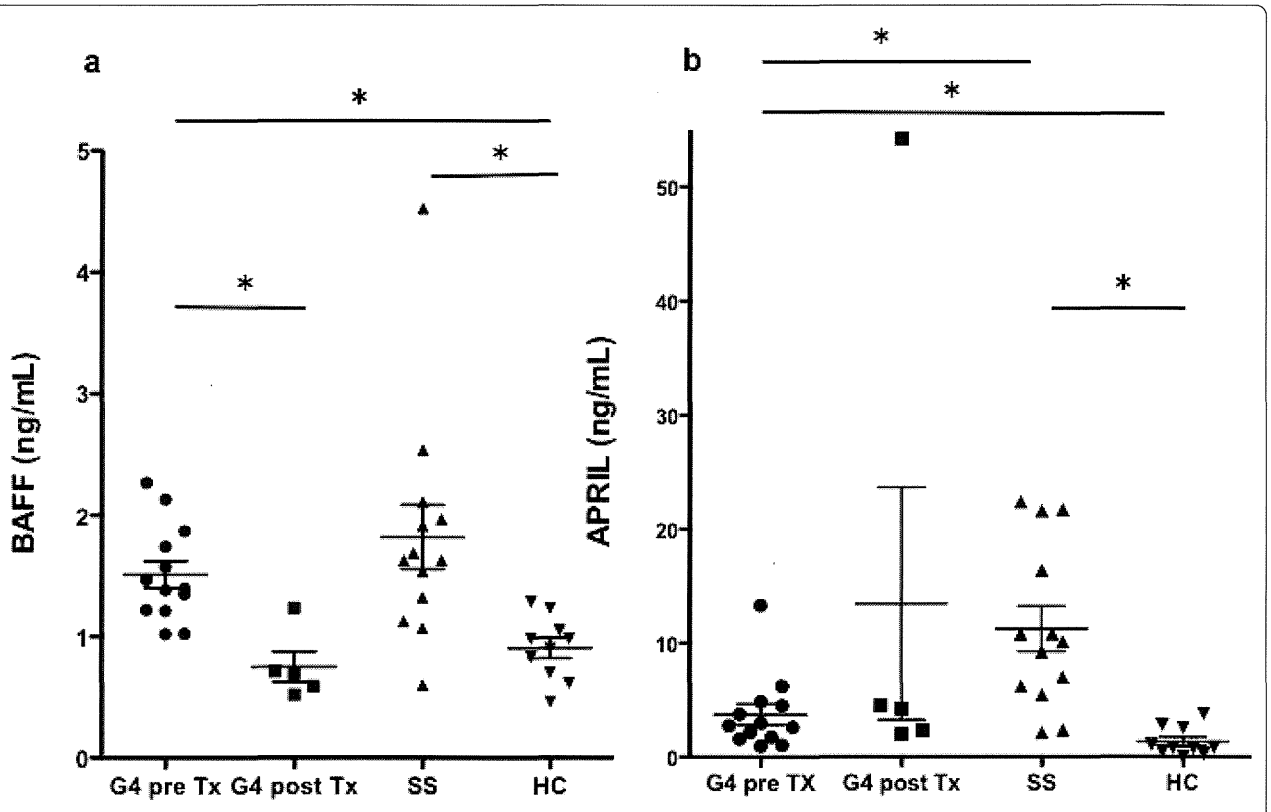

Figure 1 Serum levels of BAFF and APRIL in IgG4-RD. **(a)** Serum levels of BAFF in patients with IgG4-RD before (G4 pre Tx) and after (G4 post Tx) treatment with glucocorticoids (GC). **(b)** Serum levels of APRIL in patients with IgG4-RD before (G4 pre Tx) and after (G4 post Tx) treatment with GC. Mean ± Standard deviation (SD) are shown. *, $P < 0.01$. HC, healthy controls; SS, patients with Sjögren syndrome; Tx, treatment.

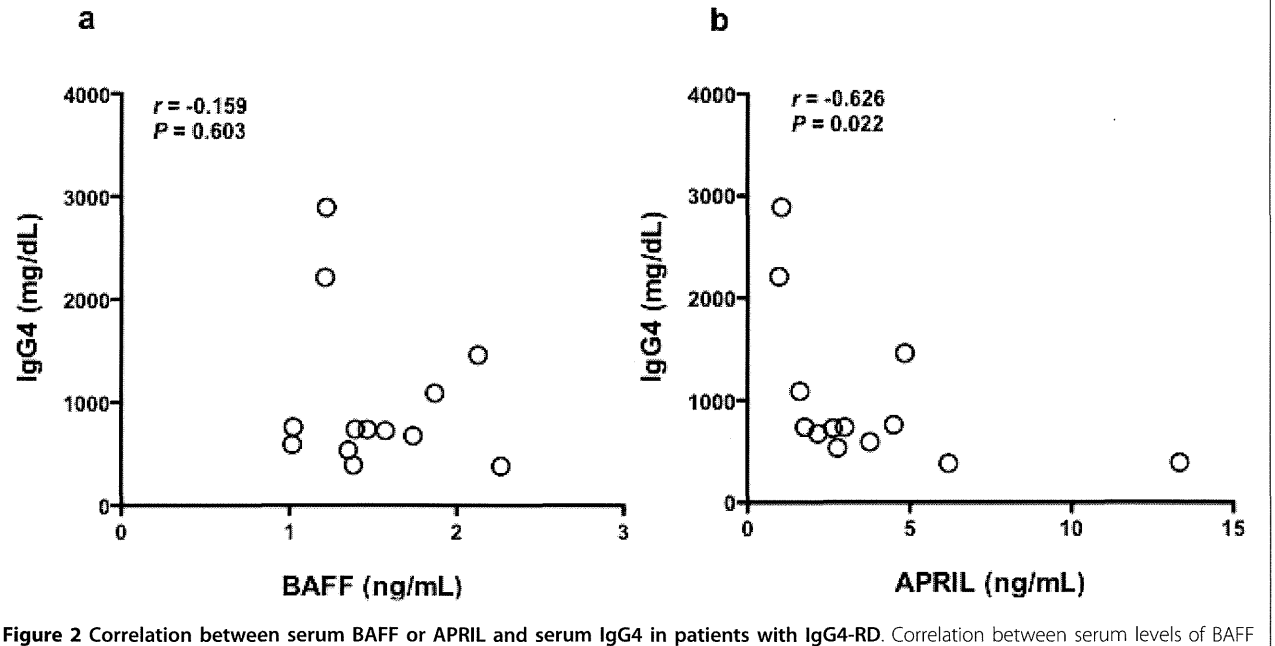

Figure 2 Correlation between serum BAFF or APRIL and serum IgG4 in patients with IgG4-RD. Correlation between serum levels of BAFF **(a)** or APRIL **(b)** and IgG4 in patients with IgG4-RD.

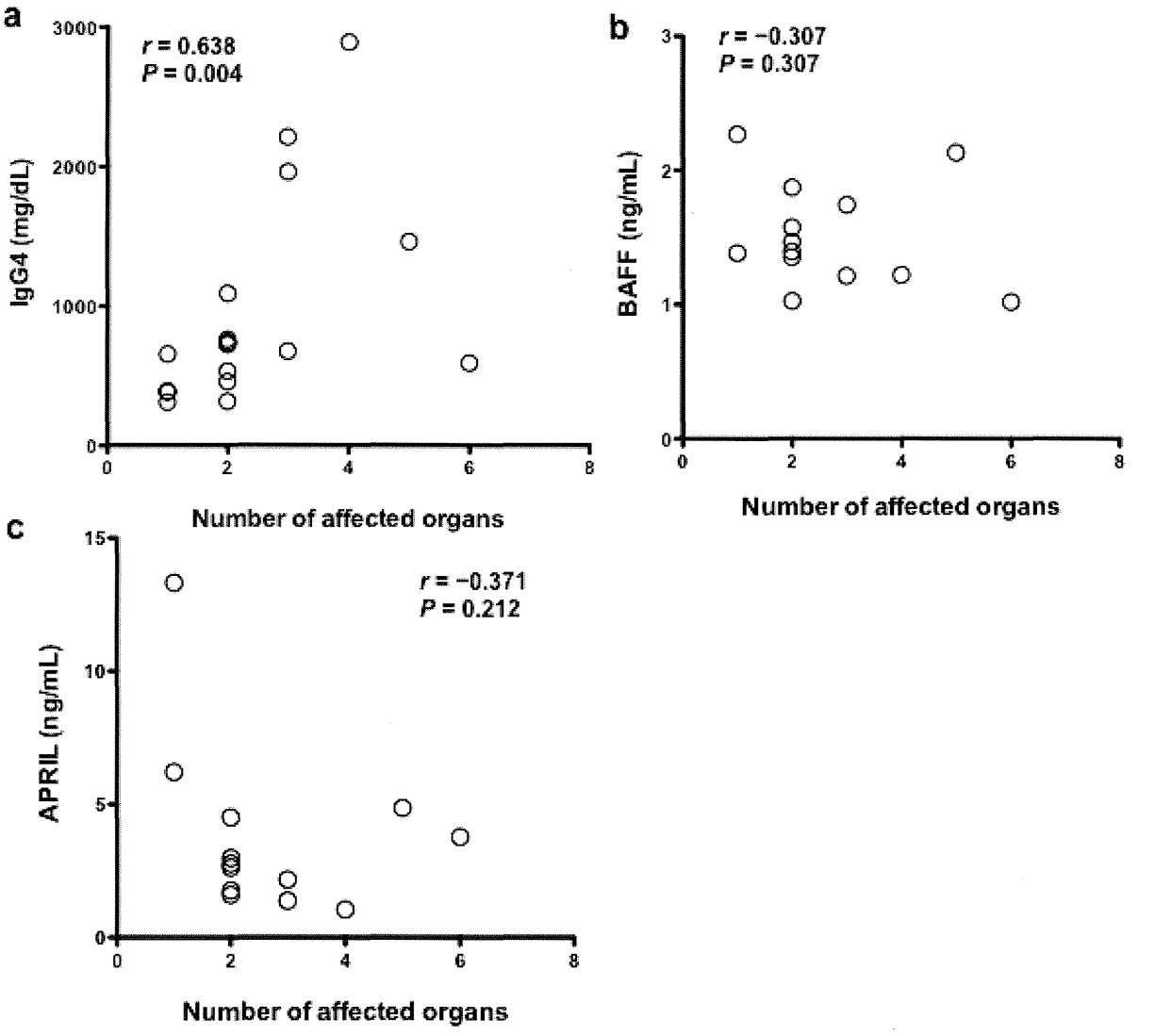

Figure 3 Correlation between organ involvement and serum IgG4, BAFF, or APRIL in patients with IgG4-RD. Correlation between the number of affected organs and IgG4 **(a)**, BAFF **(b)**, and APRIL **(c)** in patients with IgG4-RD.

= 0.307) or APRIL (n = 13, r = -0.371, P = 0.212) in patients with IgG4-RD before GC therapy.

Changes in serum BAFF and APRIL in patients with IgG4-RD during GC therapy

Six patients with IgG4-RD were treated with oral prednisolone (0.6 mg/kg/day) for two weeks, tapering thereafter by 10% of the dose every two weeks, and serum samples were drawn repeatedly to monitor serum BAFF and APRIL during treatment (Figure 4). After treatment, serum levels of BAFF dramatically declined to levels observed in healthy controls in most cases; however, the levels increased again during follow-up (Figure 4a). In contrast, serum levels of APRIL did not decrease during treatment and increased markedly after treatment in most cases (Figure 4b).

Discussion

This is the first study to demonstrate both an increase in BAFF and APRIL levels in patients with IgG4-RD as well as the differential effects of GC treatment on BAFF and APRIL in patients with IgG4-RD. Promotion of B cell activation, plasmacyte differentiation, and germinal center formation by BAFF and APRIL [42], and ectopic germinal center formation in lacrimal and salivary glands from patients with MD [43] suggest that inappropriate BAFF and APRIL may contribute to progressive plasmacyte infiltration and ectopic germinal center

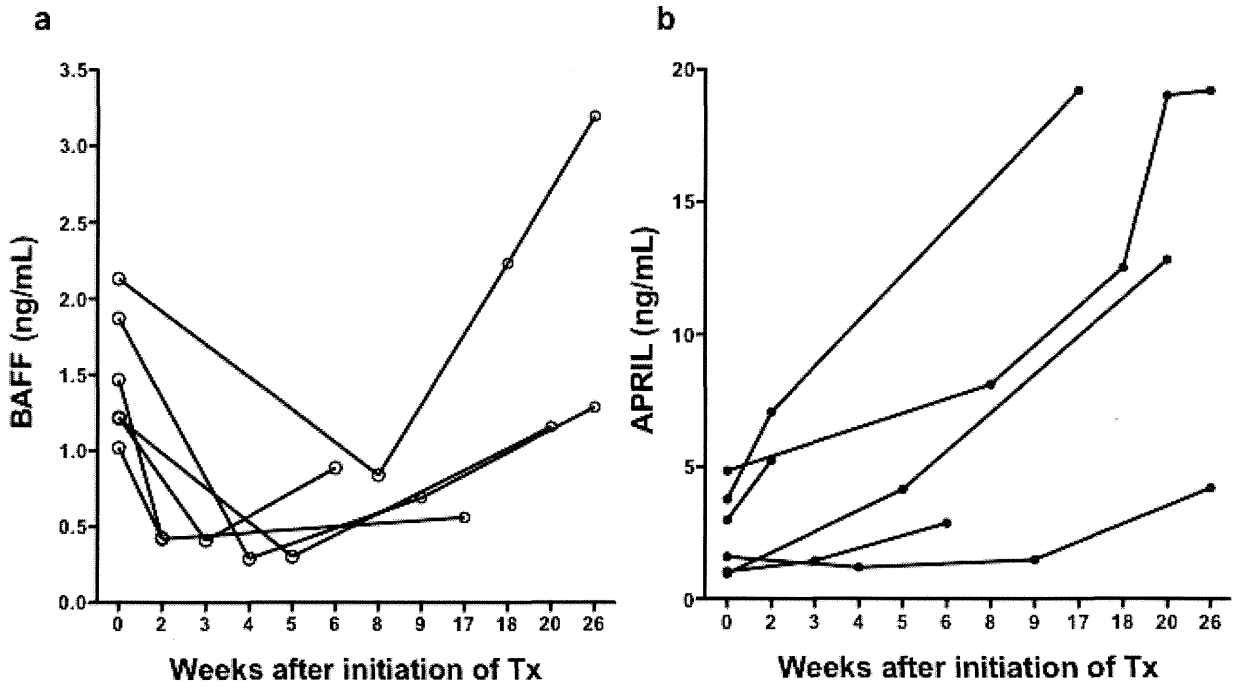

Figure 4 Changes in serum levels of BAFF and APRIL in patients with IgG4-RD during GC therapy. Serum levels of BAFF **(a)** and APRIL **(b)** were monitored during and after GC therapy (n = 6).

formation in the target organs of patients with IgG4-RD. In addition, it has been demonstrated that BAFF and APRIL enhance class switching to produce IgG4 and IgE in the presence of IL-4 [44,45]. Previous studies have shown that production of Th2 cytokines, such as IL-4, IL-5, and IL-13 was augmented in the tissue of patients with AIP [20]. Therefore, increased expression of both cytokines may contribute to the pathogenesis of IgG4-RD in concert with cognate Th2 cells. In particular, three (cases 3, 4 and 12) out of five patients (cases 3, 4, 10, 11 and 12) with high BAFF levels (> 1.5 ng/mL) had positive test results for autoantibodies (for example, RF or anti-SS-A antibody), which is consistent with previous studies showing that serum BAFF levels were correlated with positive results for serum autoantibodies in patients with RA [29], SLE [30], pSS [31], or IM [33]. BAFF might also play a role in the breakdown of B cell tolerance in patients with IgG4-RD. Recently, it has been reported that serum levels of BAFF were higher in patients with AIP than in those with pancreatic cancer or chronic pancreatitis [46]. The same studies also demonstrated that serum BAFF levels were significantly correlated with serum levels of IgG and IgG4 in patients with AIP, which was not observed in our cohort. The relatively small number of patients in our study or the enrollment of different subsets of patients might explain this discrepancy.

Of interest, we found an inverse correlation between serum APRIL and serum IgG4 levels in patients with IgG4-RD. In SLE, a similar inverse correlation between serum APRIL levels and anti-double-stranded DNA antibody titers has been reported [47], and serum APRIL was inversely associated with disease activity. Thus, APRIL might serve as a protective factor against the progression of IgG4-RD. In our cohort, serum IgG4 levels were significantly correlated with the number of affected organs, clearly indicating that serum IgG4 levels reflect disease severity. Although no significant correlation existed between serum BAFF levels and serum immunoglobulins or the number of affected organs, GC treatment dramatically reduced serum levels of BAFF as well as serum IgG4. In patients with AIP, reduced serum levels of BAFF after 12 weeks of GC treatment have been reported [46], a result similar to our finding. Thus, serum BAFF levels might reflect the clinical activity of this disease. During long-term (up to 26 weeks) follow-up, we observed that the elevated levels of serum BAFF reoccurred in most cases; however, no patients suffered clinical relapse. Further follow-up study will be needed to clarify the relationship between the reoccurrence of elevated serum BAFF and clinical relapse in IgG4-RD. In contrast to the rapid reduction of BAFF by GC treatment, serum levels of APRIL increased over the course of treatment. Similar changes in serum levels of BAFF and APRIL have been reported in patients with

GC-treated SLE [47]. Furthermore, no significant correlation was observed between serum BAFF levels and serum APRIL during GC treatment in our patients (data not shown). Thus, universal but distinct mechanisms might exist to control the expression of BAFF and APRIL during GC treatment.

Conclusions

We demonstrate for the first time that serum BAFF and APRIL levels are increased in patients with IgG4-RD and that the levels of both are indirectly related to clinical activity. Our findings add to the body of knowledge on the role of BAFF and APRIL in the pathogenesis of IgG4-RD. Further longitudinal studies with larger numbers of patients are required to determine the role of the BAFF/APRIL system and to determine whether BAFF and APRIL might serve as therapeutic targets in IgG4-RD.

Abbreviations

AIP: autoimmune pancreatitis; ANA: anti-nuclear antibody; APRIL: a proliferation-inducing ligand; BAFF: B cell-activating factor of the tumor necrosis factor family; BCMA: B cell maturation antigen; BLys: B-lymphocyte stimulator; CA II: carbonic anhydrase II; ELISA: enzyme-linked immunosorbent assay; GC: glucocorticoid; Ig: immunoglobulin; IgG4-RD: immunoglobulin G4-related disease; IL: interleukin; IM: inflammatory myopathy; MD: Mikulicz's disease; PSTI: pancreatic secretory trypsin inhibitor; pSS: primary Sjögren's syndrome; RA: rheumatoid arthritis; RF: rheumatoid factor; RPF: retroperitoneal fibrosis; SD: standard deviation; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; TACI: transmembrane activator and calcium-modulating cyclophilin ligand interactor; TALL-1: TNF and apoptosis leukocyte-expressed ligand-1; Th 2: T helper 2; TNF: tumor necrosis factor; TRDL-1: TNF-related death ligand 1.

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Authors' contributions

KK and DK were responsible for the study design, acquisition, analysis and interpretation of data, and manuscript preparation. YH, KK, NY, HY, KO, TF, and TM participated in enrollment of patients and assisted in interpretation of data. All authors have read and approved the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

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The Antiaging Approach for the Treatment of Dry Eye

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Abstract: Dry eye is one of the most common eye disorders affecting millions of people. It causes ocular irritation or discomfort, and decreases functional vision, causing a dramatic deterioration in the quality of life. Although new treatments such as the P2Y2 agonist or cyclosporine eye drops have been developed and a certain level of patient satisfaction can now be obtained, no fundamental treatment has been developed. Currently, there is no therapy available to recover lacrimal function to its normal status. Recent progress in the understanding of aging has laid the foundations for a new way of thinking about intervention of the aging process. Because dry eye is accelerated by aging, a useful approach for the prevention or treatment of dry eye may be to interfere with the aging process. In the scientific community, there is a global consensus that calorie restriction can extend the life span of various kinds of animals, establishing an intervention to aging. Another important hypothesis believed to be involved in aging is the free radical theory. According to these theories, the aging process may be managed by controlling levels of calories or reactive oxygen species. In this review, these 2 important aging theories, calorie restriction and free radical aging, are examined, and we discuss how to apply these theories to the prevention and treatment of dry eye.

Key Words: aging, calorie restriction, dry eye, reactive oxygen species

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The number of patients with dry eye is rapidly increasing around the world. An explanation for this has yet to be elucidated, but several factors, such as the spread of visual display terminal use, longer working hours, shortened sleeping time, increased stress levels, environmental factors, and increased longevity are considered to be at least part of the reason for this increase. Although the incidence of dry eye in the elderly population is high, even high school students can exhibit symptoms of dry eye syndrome.[1–3] Among visual display terminal workers in Japan, the incidence of dry eye is ~22% in women and >10% in men,[1] suggesting there are potentially >24 million patients with dry eye in Japan. Dry eye causes ocular discomfort and irritation, but also decreases functional visual acuity, which potentially interferes with work efficiency.[4–7] Although new treatments such as the P2Y2 agonist or cyclosporine have been developed along with the widely used punctal plug application, which have obtained a certain level of patient satisfaction,[8,9] there is no fundamental treatment for dry eye that recovers lacrimal functions to normal levels. Therefore, the key question for the development of novel treatments is what kind of new research approach should we take?

Of the many causes of dry eye, aging is one of the most important factors in the pathogenesis of dry eye. It has been well established that people lose tear function with age and that dry eye occurs less often in children.[10] We previously reported that 73% of the elderly population exhibit dry eye syndrome, which was far higher than expected.[3] In ophthalmology, there are many age-related diseases such as cataracts, macular degeneration, glaucoma, pterygium, conjunctival chalasis, diabetic retinopathy, retinal vein occlusion, and senile ptosis in addition to dry eye, which account for >80% of all eye diseases. Therefore, aging is an important risk factor for dry eye disease.

Recent advances in the understanding of aging have highlighted a new way of thinking about intervening in the aging process. It had been considered that the aging process was so complex that we could not interfere with it until a complete understanding of aging was realized. However, in the scientific community, there is a global agreement that calorie restriction (CR) can extend the life span of various kinds of animals, establishing an actual intervention to aging.[11,12] One key point about CR is that it also suppresses the incidence of age-related diseases such as cancer, diabetes, and cardiovascular events. Age-related diseases can be either postponed or treated through the lifestyle practice of CR or through CR mimetics according to the CR theory.[11] Interfering with aging through antiaging strategies today is a tremendous advancement in the aging science. In addition to the CR theory, the free radical theory is another important hypothesis thought to be involved in aging.[13] According to this theory,

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the aging process can be controlled through the management of reactive oxygen species (ROS). Hence, this review describes these 2 important aging theories, CR and free radical aging, and explores how to apply these theories for the prevention and treatment of dry eye.

CR THEORY

The average and maximum life span of many species, from yeast and nematodes to rodents and monkeys, can be increased by up to 50% by reducing calorie intake compared with ad libitum food intake.[11] Microarray analysis demonstrated that CR affects gene expression patterns during aging and provides for a healthier life.[14] Some of the molecular mechanisms that regulate the effects of CR have been described, such as suppression of insulin-like growth factor/insulin signaling, activation of sirtuin molecules, and suppression of mammalian target of rapamycin (Fig. 1).[15–17] CR has been shown to reduce the incidence of age-related diseases such as cancer, cardiovascular disease, and immune disorders.[19–21] Consequently, the physiological changes caused by CR contribute toward improved health and provide increased longevity.[21] The CR lifestyle is difficult to follow as a part of daily life, so aggressive research is under way to seek an alternative to CR by mimicking the molecular effects in the body.[22–24] Possible CR mimetic products include various kinds of polyphenols such as resveratrol, quercetin, and butein.[22] Resveratrol upregulates the activity and transcription of sirtuins, regulatory proteins involved in aging, and thus may provide beneficial effects to mimic CR. There are several ways to stimulate the antiaging sirtuin gene, such as exercise and increased NAD, which has the same effect as CR or resveratrol treatment (Fig. 2). Consequently, the activation of sirtuins interacts with various transcriptional molecules, resulting in the expression and maintenance of the antiaging effect.[25,26] Several researchers and pharmaceutical companies are currently developing

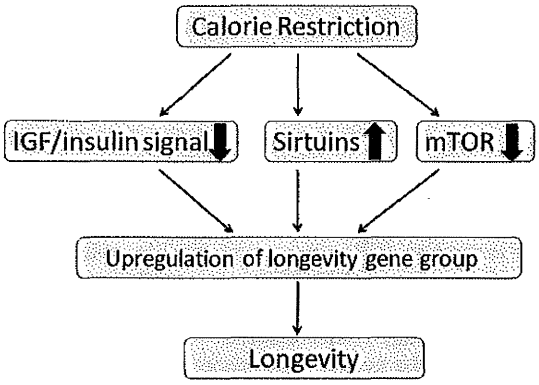

FIGURE 1. CR decreases IGF/insulin signaling, increases sirtuin activity, and suppresses mammalian target of rapamycin (mTOR), resulting in various kinds of gene upregulation associated with longevity. IGF, insulin-like growth factor. Adapted from Tsubota et al[18] with permission from S. Karger AG, Basel.

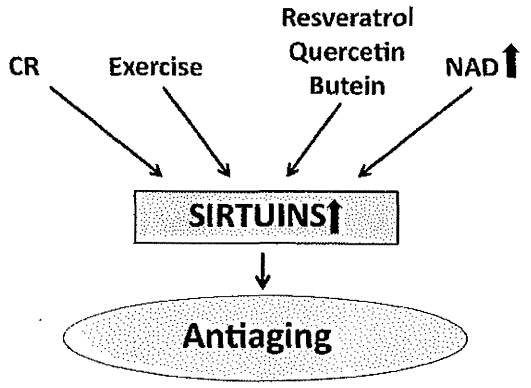

FIGURE 2. Sirtuins can be stimulated in a variety of ways to activate longevity-related gene expression. Reprinted from Tsubota et al[18] with permission from S. Karger AG, Basel.

effective CR mimetic molecules for the prevention and treatment of diseases. In humans, studies have clearly shown that high calorie intake over time is a risk factor for various age-related diseases as observed in metabolic syndrome.[27] Although it has not been elucidated whether CR prevents age-related eye diseases, it is highly possible and could be a significant intervention for the treatment of blinding diseases.[28–30] According to the CR theory, aging and age-related diseases can be controlled by the simple concept of CR.

FREE RADICAL THEORY

The free radical theory was first proposed by Harman[13] in 1956 and is considered a major process in aging (Fig. 3). The free radical theory and ROS, terms used almost interchangeably, describe the attack of key cell molecules such as proteins, lipids, and DNA by free radicals. ROS are generated in the mitochondria, cytoplasm, and extracellular components. The overproduction of ROS in the mitochondria shortens the nematode life span.[31] In contrast, overexpression of

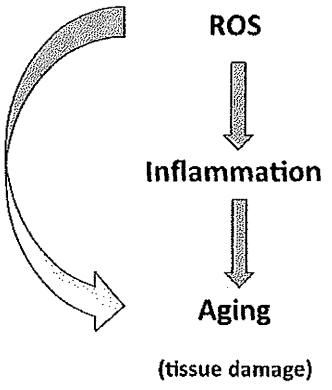

FIGURE 3. ROS induce many changes in lipids, proteins, and DNA, resulting in aging. ROS also mediate inflammation that contributes to the aging process. Reprinted from Tsubota et al[18] with permission from S. Karger AG, Basel.

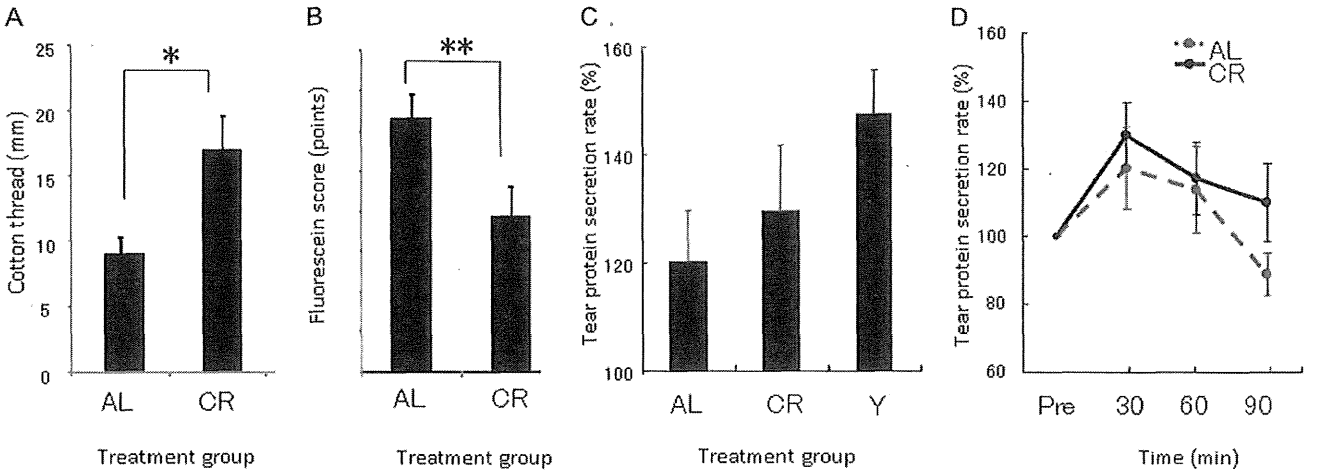

FIGURE 4. Tear function and morphological change by CR effect. A, Cotton thread test. AL, ad libitum group; CR, caloric restriction group. *P = 0.02. B, Fluorescein score, **P = 0.01. C, Tear protein secretion increasing rate (percentage of before stimulation) by Carbachol stimulation. Y, young group. D, Time course of tear protein secretion increasing rate by Carbachol stimulation. Reprinted from Kawashima et al[34] with permission from Elsevier Limited.

superoxide dismutase (SOD) and catalase, which are antioxidant enzymes, can increase the life span of *Drosophila* and mice.[32,33] Of these, ROS generated by mitochondria are considered the primary type, but photo-induced ROS are also important. In dermatology, aging of the skin may be caused by overexposure to light and is called photoaging, where ROS are critical factors. Because exposure to light and ultraviolet light is a risk factor for cataracts and age-related macular degeneration (AMD), photoaging might be an important mechanism in the ocular aging process. According to the free radical theory, the control of ROS can slow the aging process and suppress age-related diseases.

CR AND DRY EYE

The effects of CR on eye disease processes have been analyzed in several studies.[28–30] We believe that the concept of CR for the prevention or treatment of dry eye has potential, although it has not been applied widely. Thus, we undertook preliminary studies using CR for the treatment of dry eye in a rat aging model. The tear volume, measured by a cotton thread, decreased in aging rats. However, in a 6-month study on aging rats fed a 70% reduced calorie diet, the reduction of tear volume was maintained to some extent (Fig. 4).[34] This observation increased our understanding of the recovery of lacrimal gland dysfunction caused by aging, although the molecular

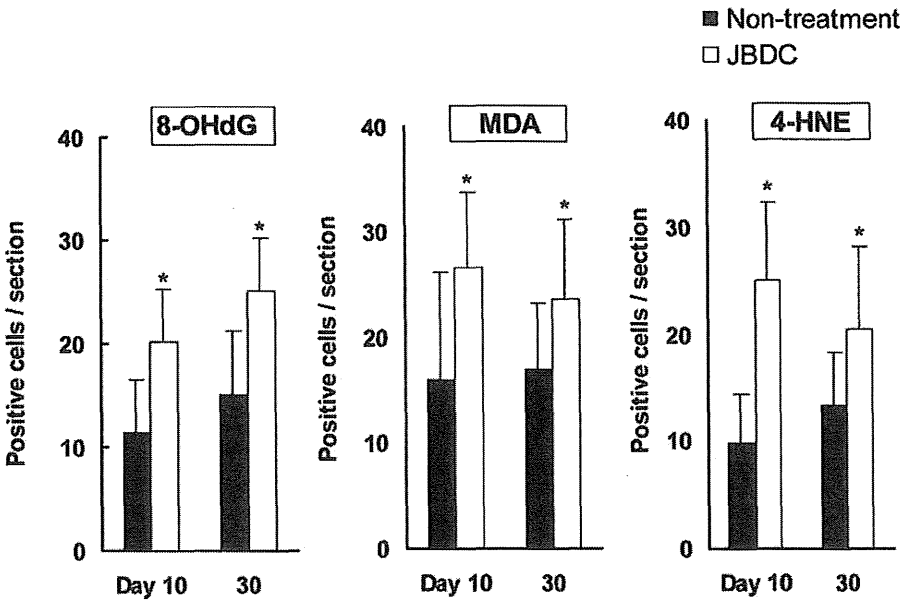

FIGURE 5. Changes in oxidative stress markers in the corneal epithelia in dry eye. Note the increase of 8-OHdG, MDA, and 4-HNE in the cornea with rat swing model of dry eye. Data represent the mean ± standard error of 16 corneas. *P < 0.05 versus the nontreatment group. 4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxy-2-deoxyguanosine; JBDC, swing under dry conditions; MDA, malondialdehyde. Reprinted from Nakamura et al[45] with permission from the Association for Research in Vision and Ophthalmology.

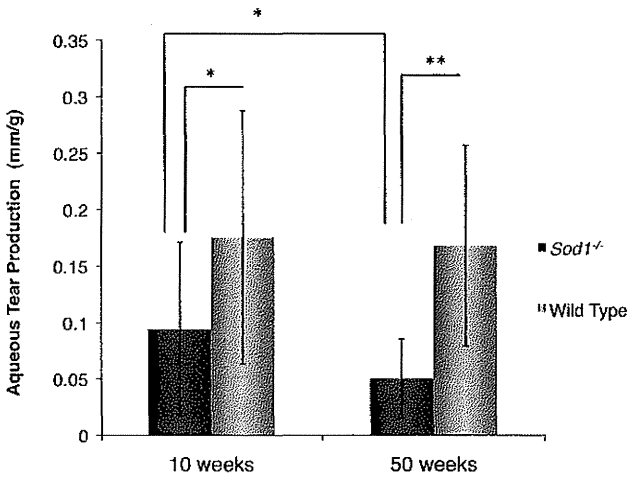

FIGURE 6. Tear decrease in SOD-deficient mice at the age of 10 weeks and 50 weeks compared with the normal controls. Weight-adjusted aqueous tear production measurements were significantly lower in the $Sod1^{-/-}$ mice compared with the wild-type mice at 10 weeks and 50 weeks. $*P < 0.05$, $**P < 0.01$ versus the wild-type group. A significant timewise decrease of tear production from 10 to 50 weeks was also observed in the $Sod1^{-/-}$ mice ($P = 0.026$). Reprinted from Kojima et al[47] with permission from Elsevier Limited.

mechanisms remain unknown. This research is ongoing using various animal models such as the aging mouse model. Thus, the high-fat diet model is being studied to seek molecular changes in the lacrimal gland both in aging and CR-treated conditions.

The strategy of using resveratrol for the treatment of dry eye is also appealing. We performed a preliminary study on the effect of resveratrol in a lipopolysaccharide-induced uveitis model and diabetes model. Resveratrol upregulated sirtuin activity in the retina and suppressed the inflammation in the retina.[35] Adhesion of leukocytes to the blood vessels was also downregulated by resveratrol. We are currently studying the effect of resveratrol in a dry eye animal model to assess its effects on suppression of inflammation of the lacrimal gland and the ocular surface. Because inflammation is considered a major contributing factor to the pathogenesis of dry eye, this may be a new approach for the control of dry eye in addition to other treatments such as cyclosporine A or other anti-inflammatory agents. Our preliminary data suggested that resveratrol treatment could increase the tear volume in various dry eye animal models.

OXIDATIVE STRESS AND DRY EYE

The relationship between oxidative stress and dry eye has been well described in the literature. A large double-blind prospective Age-Related Eye Disease Study (AREDS) showed the efficacy of antioxidant supplementation to suppress the progression of AMD,[36,37] and AMD is now considered to be an oxidative stress and inflammatory disease. Potential risk factors that suggest oxidative stress involvement for the pathogenesis of AMD include smoking, increased body mass index, sun exposure, and accumulation of iron, lead, or cadmium.[38–40] Toxic heavy metals are considered to be key for the excess production of free radicals, and their involvement with other age-related eye diseases has been observed, such as increased lead levels in glaucoma patients.[41] In the AREDS2 study, vitamins A, C, and E; zinc; copper; lutein; zeaxanthin; and eicosapentaenoic acid/docosahexaenoic acid (EPA/DHA) were the major components of the antioxidative supplements.[42] They may also act as anti-inflammatory agents, but research is limited on the mechanism of action of these food factors. We previously reported that these molecules can induce both antioxidant and anti-inflammatory effects.[43,44]

In a rat dry eye model, the accelerated oxidation of protein, fat, and DNA in the ocular surface was observed using the rat swing model (Fig. 5).[45,46] Suppression of the blink function exposes the ocular surface to ambient oxygen and induces direct oxidation of the cellular components. Then, we investigated the role of increased free radicals on inducing dry eye in the SOD1 knockout (KO) mouse. SOD is an important enzyme for the control of O_2^- levels, and an increase in oxidative stress in the retina has been reported in SOD1 KO mice, inducing retinal aging. SOD1 KO mice developed a compromised ocular surface and a decrease in tear volume.[47] Thus, oxidative stress may be involved in the deterioration of ocular surface cells and dysfunction of the lacrimal gland (Fig. 6). Studies are ongoing in the various

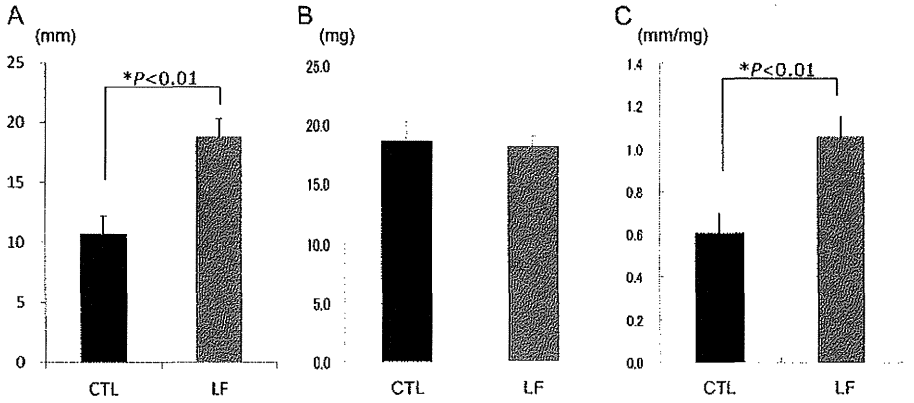

FIGURE 7. Tear secretion volume. A, Tear secretion. B, Lacrimal gland weight. C, Tear secretion corrected by lacrimal gland weight at 6 months. The volume of tear secretion was significantly larger in the lactoferrin (LF) group compared with the control (CTL) group. $*P < 0.01$, n = 10, average ± standard error of mean). Reprinted from Kawashima et al.[54]

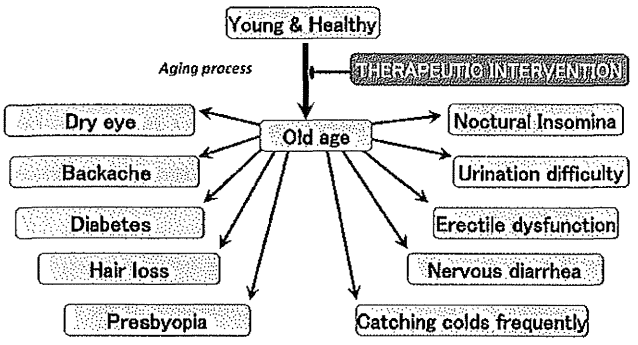

FIGURE 8. Therapeutic approach to aging to combat age-related conditions or diseases. Reprinted from Tsubota et al[18] with permission from S. Karger AG, Basel.

oxidative stress models to confirm the relationship between ROS and dry eye.[48]

Tobacco smoking is believed to be a risk factor for dry eye, and it may be attributable to oxidative stress. Clinical studies have shown that tobacco smoking induces ROS in the ocular surface by initiating inflammation, resulting in dry eye.[49–51] It was reported that the consumption of EPA/DHA is associated with the incidence of dry eye,[52] and administration of EPA is beneficial for the treatment of dry eye. Lactoferrin, a protein found in tears, has antioxidant properties. Preliminary studies demonstrated that oral lactoferrin consumption might increase the tear volume and improve dry eye symptoms (Fig. 7).[53,54] Oxidative stress of the ocular surface may be controlled by other key molecules found in tear fluid such as prealbumin, selenoprotein, and other proteins.[55–58]

FUTURE DIRECTIONS

Discoveries of the aging mechanism have provided evidence-based interventions to our lifestyle, resulting in the possibility of antiaging medicine. Searching for the "fountain of youth" throughout history is no longer an unreachable dream but reality through proven scientific background with a fundamental approach.[12,59] Diseases of aging such as presbyopia, dry eye, diabetes, and hair loss have been approached as separate conditions and treated by respective physicians in those specialties. Yet, they are likely all caused by aging, and thus, intervention of the aging process may be a useful treatment.[60] In this model, intervention in aging was shown to delay or possibly treat the age-related diseases (Fig. 8). Dry eye can now be targeted by this approach and elucidating the mechanism is currently under way. This approach opens up new therapeutic options for dry eye and the future is promising.

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Cell Injury, Repair, Aging, and Apoptosis

Age-Related Dysfunction of the Lacrimal Gland and Oxidative Stress

Evidence from the Cu,Zn-Superoxide Dismutase-1 (Sod1) *Knockout Mice*

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An imbalance between free radical generation and radical scavenging antioxidant systems results in oxidative stress, which has been associated with cell injury observed in many age-related diseases. The superoxide dismutase (SOD) family is a major antioxidant system, and deficiency of Cu,Zn-superoxide dismutase-1 (Sod1) in mice leads to many different phenotypes that resemble accelerated aging. In this study we examined the morphologic features and the secretory functions of the lacrimal glands in *Sod1*$^{-/-}$ mice. Lacrimal glands showed atrophy of acinar units; fibrosis; infiltration with CD4^{+} T cells, monocytes, and neutrophils; increased staining with both 4-hydroxy-2-nonenal and 8-hydroxy-2′-deoxyguanosine; increases in apoptotic cells; and the presence of the epithelial-mesenchymal transition in senescent *Sod1*$^{-/-}$ mice. Electron microscopy findings revealed evidence of epithelial-mesenchymal transition, presence of swollen and degenerated mitochondria, and the presence of apoptotic cell death in the lacrimal glands of senescent *Sod1*$^{-/-}$ mice. These alterations were also associated with the accumulation of secretory vesicles in acinar epithelial cells, decreased production of both stimulated and nonstimulated tears, and a decline in total protein secretion from the lacrimal glands. Our results suggest that *Sod1*$^{-/-}$ mice may be a good model system in which to study the mechanism of reactive oxygen species–mediated lacrimal gland alterations. *(Am J Pathol 2012, 180: 1879–1896; DOI: 10.1016/j.ajpath.2012.01.019)*

Aging is associated with damage to tissues by free radicals. An imbalance between generation of free radicals and radical scavenging antioxidant systems results in oxidative stress, a condition that has been associated with cell injury observed in many age-related diseases and is also considered a major factor in the process of senescence.[1] One of the well-known antioxidant defense systems is superoxide dismutase (SOD), an enzyme system that 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.[2,3]

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Previously, Imamura et al[4] reported that *Sod1* gene knockout in a mouse model was associated with signs of oxidative stress–related retinal damage and features of age-related macular degeneration (AMD), an aging disease of the human retina.

The prevalence of AMD in the US population 40 years or older is estimated to be 1.47%.[5] Even more prevalent than AMD is another age-related ophthalmic disorder, the dry eye disease, the prevalence of which varies from 3.98% to 9.80% in the United States.[6] Dry eye, which is a visually disabling disease,[6] has been reported to be a major public health issue in many societies with a significant effect on quality of life and especially on the visual quality of patients with this disorder.[6]

Evidence from mouse models and human studies of dry eye disease showed decreased tear production, corneal epithelial damage, and lacrimal gland inflammation as important alterations in the pathogenesis of dry eye disease.[7–13] With the aim to investigate the eligibility of the *Sod1* knockout (*Sod1*$^{-/-}$) mice as a model for age-related dry eye disease, we studied the functional and histopathologic alterations of the lacrimal gland in the *Sod1*$^{-/-}$ mice, comparing the results with wild-type (WT) mice. We also investigated the histopathologic changes in human lacrimal gland samples obtained soon after death from young and elderly individuals.

Materials and Methods

Animals

Seventeen *Sod1*$^{-/-}$ male mice with C57BL/background and 14 C57BL6 strain WT male mice were examined at 10 and 50 weeks in this study. The *Sod1*$^{-/-}$ mice were received from the Tokyo Metropolitan Institute of Gerontology and the WT C57BL/6 mice were purchased from Japan Clea (Osaka, Japan). *Sod1*$^{-/-}$ mice were backcrossed to *Sod2*$^{flox/flox}$[14] for two generations to obtain the *Sod1*$^{-/-}$, *Sod2*$^{flox/flox}$. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Aqueous Tear Production Measurements

Aqueous tear production was measured with phenol red–impregnated cotton threads (Zone-Quick, Showa Yakuhin Kako Co., Ltd., Tokyo, Japan) without anesthesia. The validity of this test in mice has been previously described.[15] The threads were held with a jeweler forceps and then immersed into the tear meniscus in the lateral canthus for 60 seconds. The length of wetting of the thread was measured in millimeters. Aqueous tear production was weight adjusted by dividing the amount of total aqueous tear produced in 60 seconds by weight. In a separate experiment to determine the incidence of dry eye disease, 40 *Sod1*$^{-/-}$ male mice and 118 WT male mice aged 50 weeks underwent weight and aqueous tear production measurements. To be able to define the incidence of dry eye disease in mice, we defined dry eye disease as a cotton thread test value of <0.1 mm/g and a corneal fluorescein staining score exceeding 3 points. We then calculated the percentages of mice with dry eye disease in each group.

Ocular Surface Epithelial Damage Assessment

Corneal fluorescein staining was evaluated with slit lamp biomicroscopy using cobalt blue light after instillation of 2 μL of 0.5% sodium fluorescein. Excess of fluorescein was wiped from the lateral tear meniscus. The cornea was examined with a handheld slit lamp 2 minutes after fluorescein instillation. Punctuate staining was recorded using a grading system of 0 to 3 points for superior, central, and inferior corneal areas. The fluorescein staining scores ranged from a minimum of 0 to a maximum of 9 points.

Pilocarpine-Stimulated Aqueous Tear Production Measurements

Tear secretion was stimulated 3 minutes after anesthesia (6 mg/mL of ketamine and 4 mg/mL of xyladine) by intraperitoneal injection of 0.06% pilocarpine solution (3 mg/kg; Santen Pharmaceutical Co., Ltd., Osaka, Japan), a nonselective muscarinic receptor agonist of the parasympathetic nervous system. Tears were collected from the lateral canthus for 15 minutes using 5 μL of graded capillary microglass tubes (Hirschmann Laborgerate and GmbH & Co., Eberstadt, Germany), and graticule readings were recorded at the end of each measurement.

Lacrimal Gland Carbachol–Stimulated Total Protein Secretion Measurements

The nerves in the lacrimal gland provide the major stimuli for secretion of proteins, electrolytes, and water.[16–18] To test the total protein secretion from the lacrimal glands, we exposed lacrimal gland fragments to cholinergic agonists and measured the amount of protein secreted in response to carbachol, a drug that binds and activates the acetylcholine receptor. Mice lacrimal glands were weighed before being cut into small fragments of 1 to 2 mm with a scalpel blade. The fragments were washed in 5 mL of saline solution containing 116 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.81 mmol/L $MgCl_2$, 1.01 mmol/L NaH_2PO_4, 26.2 mmol/L $NaHCO_3$, and 5.6 mmol/L dextrose (pH 7.4), maintained at 37°C and vigorously bubbled with 95% O_2 and 5% CO_2 in a beaker for 10 minutes. The solution was changed three times and discarded. The gland fragments were then incubated in 1 mL of saline for 10 minutes at 37°C, and the saline was removed and replaced with fresh medium. This cycle was repeated three times, and the saline was collected after each 10-minute incubation. The protein level measured in these samples represented the basal total protein secretion from the glands. After another 10 minutes of incubation, the medium was removed and saved, and the protein levels in these solutions represented the stimulated total protein secretion in response to carbachol. One gland was used in each experiment. The lacrimal gland samples were analyzed for total protein with a Coomassie protein assay kit (Pierce, Rockford, IL). Bovine serum

albumin was used as the standard protein, and standards were run with each assay. Protein concentrations were determined from the standard curves measured with each assay. The assays were performed in a microplate reader (model EL 808; Bio-Tek Instruments, Winooski, VT) at 595 nm. Both samples and standards were read in duplicate in 96-well flat-bottomed microplates (Costar; Corning Inc., Corning, NY). Total protein concentration was determined with the software provided by the manufacturer (KC4 version 2.7; Bio-Tek Instruments). Stimulated protein secretion was calculated by subtracting the basal protein secretion levels from the post–carbachol-stimulated protein secretion levels. The readings from the plate reader were then converted to micrograms per milliliter per minute.

Tear Fluid Collections

A total of 10 μL of 0.1 mol/L PBS was introduced onto the ocular surface by a micropipette and then collected with a 10-μL glass capillary tube (Hirschmann Laborgerate and GmbH & Co) from the lateral canthus. This procedure was performed at 10 and 50 weeks in both *Sod1*$^{-/-}$ and WT mice. Collected tears were stored at −80°C until tear cytokine concentration assessments.

Cytometric Bead Array for Assessment of Inflammatory Cytokines in Tears

The Becton Dickinson Cytometric Bead Array system was used to investigate the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analyses in particle-based immunoassay. Each bead provides a capture surface for a specific protein and is analogous to an individual coated well in an enzyme-linked immunosorbent assay (ELISA) plate. The testing allows the detection of multiple analyses in a small-volume sample. We quantitatively measured IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), interferon (IFN)-γ, tumor necrosis factor (TNF), and IL-12p70 protein levels in tears and serum samples using the mouse inflammation kit (BD Bioscience, Franklin Lakes, NJ).

Blood was collected from the intracardiac space and centrifuged at 9100 × *g* for 5 minutes at 4°C. Serum was separated from the clotted blood and stored at −80°C. After reconstituting the mouse inflammation standards, the cytokine standard mixture (20 μL) and the tear and serum (20 μL) samples were diluted with 30 μL of the assay diluent. The standards and samples were added to a mixture of 50 μL of capture antibody-bead reagent and detector antibody-phosphatidylethanolamine phycoerythrin reagent. The mixture (150 μL) was subsequently incubated for 2 hours at room temperature and washed with 1 mL of wash buffer (from the kit) to remove unbound detector antibody-phycoerythrin reagent. After washing, the samples and standards were centrifuged at 200 × *g* for 5 minutes, and then the supernatants were carefully removed. The bead pellets were resuspended with 300 μL of wash buffer before data acquisition using flow cytometry.

Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data were acquired and analyzed using the Becton Dickinson Cytometric Bead Array software version 1.4 (BD Bioscience).[19]

Lacrimal Gland Specimen Collections

Animals were sacrificed at 10 and 50 weeks. The preauricular lacrimal glands were rapidly removed by trimming the glands from the surrounding tissues. Samples were divided and fixed in 4% buffered paraformaldehyde for stainings or stored in 2.5% glutaraldehyde in 0.1M phosphate for electron microscopy or were prepared for protein secretion analysis.

Histopathologic Assessment of Lacrimal Gland Specimens

All lacrimal gland specimens were immediately fixed in 4% buffered paraformaldehyde, embedded in paraffin wax, cut into 4-μm–thick paraffin sections, and processed according to conventional histological techniques, including H&E and Mallory stainings.[20,21]

Lacrimal Gland Acinar Unit and Secretory Vesicle Density Quantifications

Five randomly selected nonoverlapping areas in each specimen in 890 × 705-μm frames were digitally photographed (Axioplan2imaging; Carl Zeiss, Jena, Germany). A total of five images from each *Sod1*$^{-/-}$ or WT mouse were taken with the photographer masked to the mouse genetic information. The acinar units and secretory vesicles were counted manually, and scores from the samples were averaged as the lacrimal acinar unit density for that lacrimal gland.

Lacrimal Gland Inflammatory Cell Density Assessment

Using an image capturing software (Adobe Photoshop Creative Suite version 8.0.1, San Jose, CA), a subset of color that indicated the stained areas (brown color) was selected from the raw pictures and analyzed using ImageJ version 1.410 (NIH, Bethesda, MD). The density of inflammatory cells in each picture was measured and expressed in pixels.[2]

IHC Staining for Oxidative Stress Markers and CD45 Panleukocyte Antigen

Lipid peroxidation was assessed by immunohistochemical (IHC) detection of 4-hydroxy-2-nonenal (4-HNE). Oxidative DNA damage was investigated by IHC staining with anti–8-hydroxy-2′-deoxyguanosine (8-OHdG) antibodies. The avidin-biotin-peroxidase complex (ABC) method was used in immunostainings. Tissues were fixed overnight in a 4% buffered paraformaldehyde solution

and processed for paraffin embedding. Sections 4 μm thick were cut from paraffin wax blocks, mounted on precoated glass slides, deparaffinized, and rehydrated. To block nonspecific background staining, lacrimal gland sections were treated with normal horse serum (Vector Laboratories, Burlingame, CA) for 2 hours at room temperature. The tissues were then treated with mouse anti–8-OHdG monoclonal antibody at a concentration of 10 μg/mL diluted with horse-blocking serum (Japan Institute for the Control of Aging, Shizuoka, Japan) and anti–4-HNE monoclonal antibody at a concentration of 25 μg/mL diluted with horse-blocking serum (Japan Institute for the Control of Aging) for 2 hours at room temperature. For the negative controls, the primary antibody was replaced with mouse IgG1 Isotype control (MOPC-21; Sigma, St. Louis, MO). Endogenous peroxidase activity was blocked using 3.0% H_2O_2 in methanol for 3 minutes. The sections were 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 3,−3′-diaminobenzidine (DAB) chromogen solution (Vector Laboratories), lightly counterstained with hematoxylin for 4 minutes at room temperature, washed with tap water, dehydrated, and mounted. For CD45 IHC stainings, we used the purified anti-mouse CD45 antibody solution diluted with rabbit blocking serum at a concentration of 10 μg/mL (BioLegend, San Diego, CA) and the peroxidase system Vectastain ABC kit (rat IgG; Vector Laboratories). To block nonspecific background staining, lacrimal gland sections were treated with normal rabbit serum (Vector Laboratories) for 2 hours at room temperature. The tissues were then treated with 10 μg/mL of anti-mouse CD45 for 2 hours at room temperature. For the negative controls, the primary antibody was replaced with rat IgG2B isotype control at the same concentration of the primary antibody (R&D Systems, Minneapolis, MN). Endogenous peroxidase activity was blocked using 3.0% H_2O_2 in methanol for 3 minutes. The sections were incubated for 30 minutes with biotin-labeled rabbit anti-rat IgG serum (Vector Laboratories), followed by avidin-biotin-alkaline phosphatase complex treatment (Vector Laboratories) for 30 minutes. The sections were washed in 0.1M PBS, developed in prepared DAB chromogen solution (Vector Laboratories), lightly counterstained with hematoxylin for 4 minutes at room temperature, washed with tap water, dehydrated, and mounted.

Fluorescent IHC Staining for EMT Markers and Inflammatory Cell Markers

Epithelial mesenchymal transition (EMT) has been reported to play a crucial role in fibrosis of tissues.[22,23] To evaluate whether EMT is involved in the pathogenesis of fibrosis in aged *SOD1*$^{-/-}$ mice, mice lacrimal gland specimens were immunostained with epithelial cell marker (E-cadherin) and mesenchymal cell marker [α-smooth muscle actin (SMA)]. To evaluate the continuity of basement membrane in acinar units, type I collagen was immunostained. IHC for EMT markers was performed as described previously.[24]

To differentiate the type of inflammatory cells in the lacrimal gland, specimens were immunostained with CD4, CD11b, and Gr-1 antibodies. Fluorescent IHC was performed as follows. Briefly, cryosections (6 μm) from mouse lacrimal gland were fixed in 4% paraformaldehyde for 20 minutes. After blocking with 1% bovine serum albumin 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, Oberkochen, Germany). For negative control, isotype control IgG was applied instead of primary antibody. The specimens were immunostained with the following primary antibodies: rabbit anti–α-SMA antibody (0.01 mg/mL, ab5694; Abcam, Boston, MA), rat anti–E-cadherin antibody (0.01 mg/mL, ab11512; Abcam), rabbit antitype I collagen antibody (0.01 mg/mL, ab292; Abcam), rat anti-CD4 antibody (0.01 mg/mL, 14–0041; eBioscience, San Diego, CA), rat anti-CD11b (0.026 mg/mL, ab8878; Abcam), and rat anti–Gr-1 (0.01 mg/mL, ab25377; Abcam). The secondary antibodies were fluorescein isothiocyanate–conjugated anti-rabbit IgG antibody (0.0075 mg/mL; Jackson Immunoresearch Laboratories, West Grove, PA) and fluorescein isothiocyanate–conjugated anti-rat IgG antibody (0.0075 mg/mL; Jackson Immunoresearch Laboratories). DAPI (Vector Laboratories) was used for nuclear staining.

Immunofluorescence Staining for Apoptosis Markers

Terminal deoxyribonucleotidyl transferase–mediated deoxyuridine triphosphate digoxigenin nick end labeling (TUNEL) staining was performed using the *in situ* Cell Death Detection Kit, TMR Red (Roche Applied Science, Mannheim, Germany). Initially, 10 μg/mL of proteinase K (Roche Applied Science) in 10 mmol/L Tris/HCl (pH 7.4) was applied to the lacrimal gland specimens and left for 15 minutes at room temperature. After washing the samples with 0.1M PBS twice, TUNEL reaction mixture (Roche Applied Science) was added to the samples and the label solution on the negative control sample, which were then incubated at 37°C for 60 minutes in a dark room. The specimens were rinsed in 1M PBS for 5 minutes three times, and then 100 μL of 0.5 μg/mL of DAPI diluted in Tris-buffered saline and Tween-20 was added on samples for 5 minutes at room temperature. Finally, the specimens were washed with 1M PBS and mounted with aqueous mounting medium Permafluor (Beckman Coulter, Marseille, France). Caspase-3 immunofluorescent staining was performed using the Alexa Fluor 488 Goat Anti-Rabbit SFX Kit (Molecular Probes, Eugene, OR). The samples were initially blocked with four drops of Image-iT FX signal enhancer (Molecular Probes) for 30 minutes and then with 10% normal goat serum (Dako, Tokyo, Japan) diluted in Block-Ace solution (dilution factor 1:25 in 0.1M PBS) for 2 hours. Primary cleaved caspase-3 antibody (dilution factor 1:200; Cell Signaling,

Danvers, MA) was added on the specimens, which were kept refrigerated overnight at 4°C. Subsequently, after wash with 0.1M PBS, the secondary antibody anti-IgG Alexa 488 (dilution 1:200; Molecular Probes) was applied for 45 minutes in a dark incubation chamber. After wash with 0.1M PBS, specimens were coverslipped with fluorescent mounting medium with DAPI (Vectashield; Vector Laboratories). Sections were examined and photographed with an epifluorescence microscope (Axioplan2imaging; Carl Zeiss).

Transmission Electron Microscopy Examination

Lacrimal gland specimens were immediately fixed with 2.5% glutaraldehyde in 0.1M PBS (pH 7.4) immersed for 4 hours at 4°C, and then washed three times with 0.1M 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 lacrimal gland tissues were thin sectioned on an Ultratome (LKB, Gaithersburg, MD) 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).

Serum 8-OHdG Concentrations Assessed by ELISA

A commercially available 8-OHdG ELISA kit (Japan Institute for the Control of Aging) was used to determine the serum 8-OHdG concentration, as reported previously.[25] A total of 200 μL of serum was used for 8-OHDG measurements.

Lacrimal Gland IHC Staining for Oxidative Stress and Inflammatory Cell Markers in Humans

Postmortem human lacrimal gland tissues from 6 individuals 17 to 48 years old (the young group) and 6 individuals 76 to 87 years old (the old group) were donated by Dr. Hiroto Obata. The samples were studied under institutional review board permission at Keio University School of Medicine. All lacrimal gland specimens were immediately fixed in 4% buffered paraformaldehyde, embedded in paraffin wax, cut into 4-μm–thick paraffin sections, and processed according to conventional histologic techniques, including H&E and Mallory staining fibrosis. Oxidative stress–induced lipid peroxidation was assessed by IHC detection of 4-HNE protein adducts. Oxidative DNA damage was investigated by IHC staining of 8-OHdG. The presence of inflammatory cells was investigated by IHC using anti-CD45 antibodies (concentration: 0.01 mg/mL; Dako, Glostrup, Denmark). The ABC method was used for immunostaining. Antigen retrieval was achieved by microwaving in 10 mmol/L sodium citrate buffer for 5 minutes then cooling for 20 minutes. The tissues were then treated with mouse anti–8-OHdG monoclonal antibody at a concentration of 10 μg/mL diluted with horse-blocking serum (Japan Institute for the Control of Aging) and anti–4-HNE monoclonal antibody at a concentration of 25 μg/mL diluted with horse-blocking serum (Japan Institute for the Control of Aging) for 2 hours at room temperature. For the negative controls, the primary antibody was replaced with mouse IgG1 isotype control (MOPC-21; Sigma). Endogenous peroxidase activity was blocked using 3.0% H_2O_2 in methanol for 3 minutes. The sections were 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 DAB chromogen solution (Vector Laboratories), lightly counterstained with hematoxylin for 4 minutes at room temperature (4-HNE stainings), washed with tap water, dehydrated, and mounted.

Quantitative RT-PCR for EMT Markers

Mouse lacrimal glands were preserved overnight in RNA later (Applied Biosystems, Carlsbad, CA) 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 RNeasy mini kit (Qiagen, Valencia, CA). cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). SYBR Green–based quantitative real-time PCR was performed using StepOnePlus system (Applied Biosystems). Mouse glyceraldehyde-3-phosphate (GAPDH) (sense 5′-TGACGTGCCGCCTGGAGAAA-3′, antisense, -3′AGTGTAGCCCAAGATGCCCTTCAG5′-), Snail (sense 5′-TGGAAAGGCCTTCTCTAGGC-3′, antisense, -3′CTTCACATCCGAGTGGGTTT5′-), E-cadherin (sense 5′-GGCTTCAGTTCCGAGGTCTA-3′, antisense, -3′CGAAAA-GAAGGCTGTCCTTG5′-), α-SMA (sense 5′-CTGACAGAG-GCACCACTGAA-3′, antisense, -3′AGAGGCATAGAGGG-ACAGCA5′-) primers were used as templates for GAPDH, Snail, E-cadherin, and α-SMA amplification. Data were normalized to GAPDH.

Results

Lack of SOD1 Accelerates Oxidative Lipid and DNA Damage in the Lacrimal Glands and Causes Elevation of Serum 8-OHdG Levels

Aldehyde molecules generated endogenously during the process of lipid peroxidation have been reported to be associated with oxidative stress in cells and tissues.[26] 4-HNE is one of the best recognized and most studied cytotoxic product of lipid peroxidation.[26]

To evaluate the influence of the *Sod1* knockout on the lipid peroxidation process, we initially performed lacrimal gland IHC stainings with anti–4-HNE antibodies (Figure 1A). Specimens from 50-week-old *Sod1*$^{-/-}$ mice exclusively showed dense staining compared with the specimens from the WT mice at 10 and 50 weeks and *Sod1*$^{-/-}$ mice at 10 weeks (Figure 1A). The mean areas (pixels^2) of positively stained cells were 11.98 ± 4.26 for WT mice

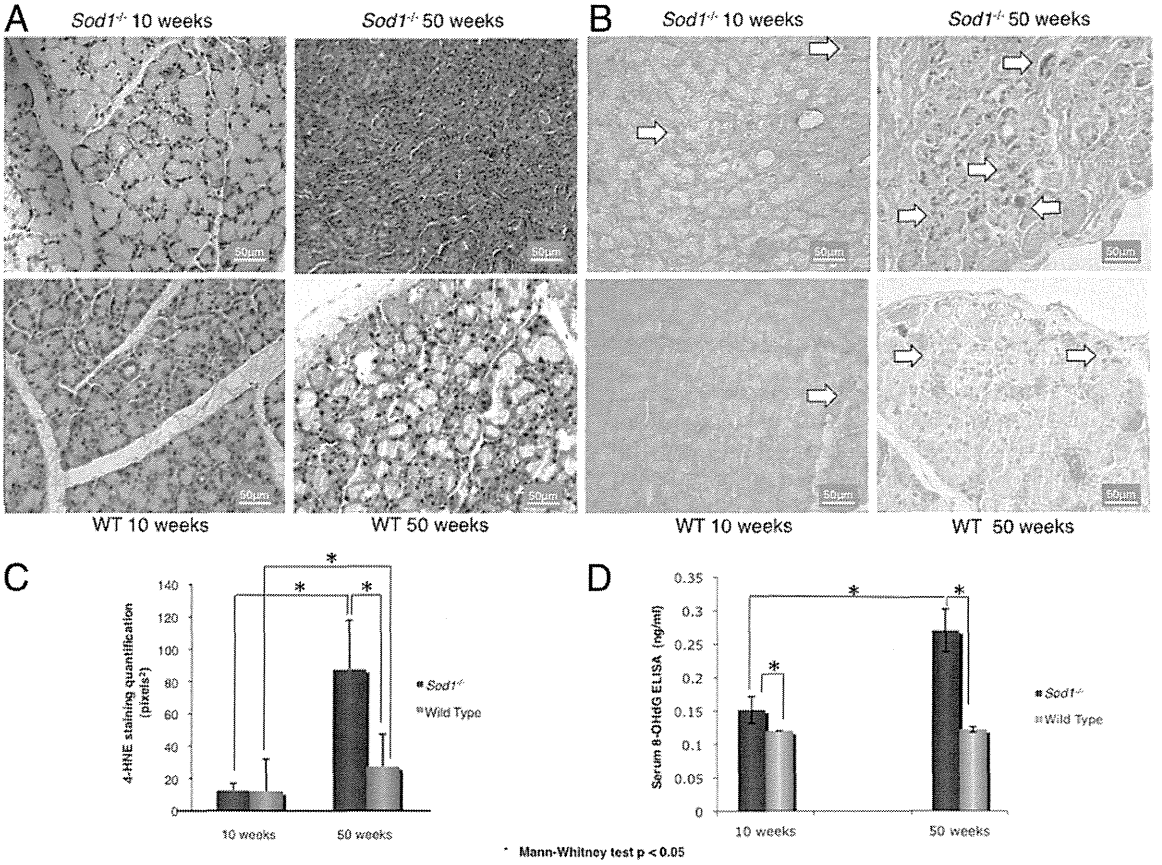

Figure 1. Oxidative lipid and DNA changes in the lacrimal glands and alterations in serum 8-OHdG levels. **A:** Late-phase lipid peroxidation marker 4-HNE stained cells positively, showing a dense staining in the 50-week-old *Sod1*$^{-/-}$ mouse. WT mice specimens were also stained but not to the extent observed in *Sod1*$^{-/-}$ mice. **B:** Acinar 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 acinar cellular nuclei stained with anti–8-OHdG antibodies in the *Sod1*$^{-/-}$ mice at 50 weeks compared with lacrimal gland specimens from WT mice at 50 weeks. **C:** Semiquantitative analysis of the extent of cellular staining for 4-HNE revealed a statistically significance increase in the 50-week-old mice group compared with the 10-week-old group and a significant elevation in staining for the *Sod1*$^{-/-}$ mice group compared with the WT mice at 50 weeks ($P < 0.0001$). Error bars indicate SD from at least five independent samples. **D:** The mean 8-OHdG serum concentrations were significantly higher in the *Sod1*$^{-/-}$ than the WT mice at 10 ($P < 0.05$) and 50 weeks ($P = 0.008$). Note the significant elevation of serum 8-OHdG concentration from 10 to 50 weeks in the *Sod1*$^{-/-}$ mice. Error bars indicate SD from at least five independent samples per group of two separate experiments.

at 10 weeks, 12.29 ± 4.64 for *Sod1*$^{-/-}$ mice at 10 weeks, 27.23 ± 12.37 for WT mice at 50 weeks, and 87.43 ± 30.37 for *Sod1*$^{-/-}$ mice at 50 weeks. The extent of lacrimal gland staining with 4-HNE antibodies showed a significant increase ($P < 0.0001$) in both *Sod1*$^{-/-}$ and WT mice from 10 weeks to 50 weeks as shown in Figure 1C. 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 (Figure 1C).

8-OHdG is a well-known marker for oxidative stress–induced DNA damage.[26] To assess the cellular DNA damage, we next performed IHC with anti–8-OHdG antibodies. Acinar 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 in all *Sod1*$^{-/-}$ mice. Relatively more acinar cellular nuclei stained with anti–8-OHdG antibodies in the *Sod1*$^{-/-}$ mice at 50 weeks compared with lacrimal gland specimens from WT mice at 50 weeks (Figure 1B). Serum 8-OHdG assessment has been shown to be a reliable marker for elevated systemic oxidative stress status.[26] To investigate this possibility, we performed ELISA to determine serum 8-OHdG concentrations in the *Sod1*$^{-/-}$ and WT mice at both 10 and 50 weeks. The mean 8-OHdG concentrations were significantly higher in the *Sod1*$^{-/-}$ than the WT mice at 10 ($P < 0.05$) and 50 weeks ($P = 0.008$).

A significant timewise increase was seen in the mean serum 8-OHdG concentration in the *Sod1*$^{-/-}$ mice from 10 to 50 weeks. The mean values for serum 8-OHdG concentrations were 0.12 ± 0.01 ng/mL for WT mice at 10 weeks, 0.15 ± 0.02 ng/mL for *Sod1*$^{-/-}$ mice at 10 weeks, 0.12 ± 0.01 ng/mL for WT mice at 50 weeks, and 0.27 ± 0.03 ng/mL for *Sod1*$^{-/-}$ mice at 50 weeks (Figure 1D).

Oxidative Lipid and DNA Damage Is Associated with Ultrastructural Mitochondrial Alterations

At organelle level, we could observe, by transmission electron microscopy examination, certain ultrastructural changes in the mitochondria, which are the power houses of the living cells.[27] Whereas the mitochondria did not show any phenotypic alterations from 10 to 50

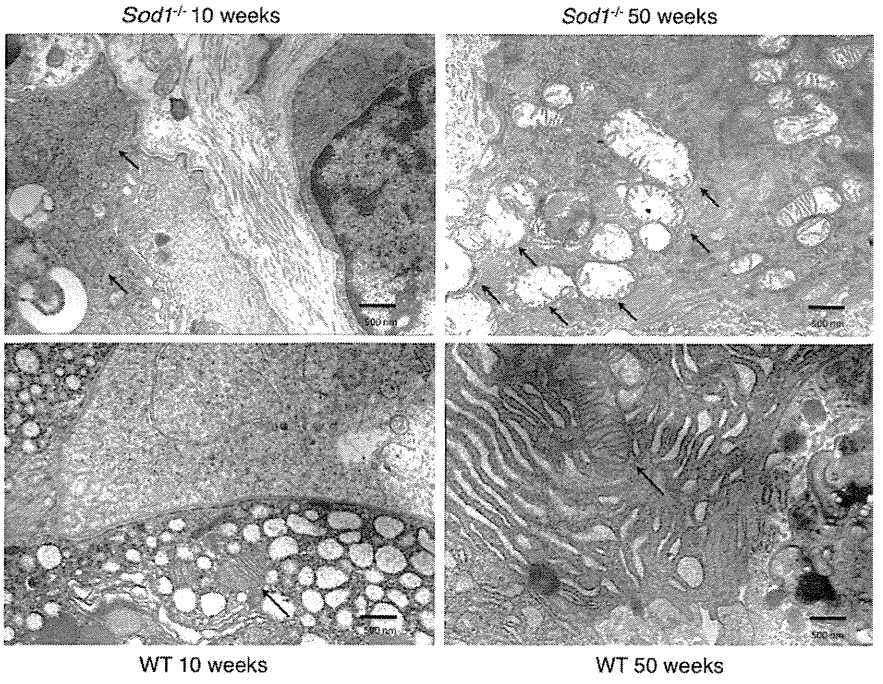

Figure 2. Ultrastructural mitochondrial alterations. Transmission electron microscopic examination of WT and *Sod1*$^{-/-}$ mice at 10 and 50 weeks revealed marked ultrastructural changes in the mitochondria. Whereas the mitochondria did not show any phenotypic alterations from 10 to 50 weeks in the WT mice, mitochondrial swelling, disorientation, shortening, and disorganization of cristae were noted at 50 weeks in the *Sod1*$^{-/-}$ mice.

weeks in the WT or the *Sod1*$^{-/-}$ mice at 10 weeks, mitochondrial swelling, disorientation, shortening, and disorganization of cristae were noted prominently at 50 weeks in 88.2% of the *Sod1*$^{-/-}$ mice (Figure 2).

Sod1 *Knockout Is Also Associated with Increased Inflammatory Response in the Lacrimal Gland and Elevation of Inflammatory Cytokines in Tears and Serum*

Oxidative damage has been reported to be associated with induction of inflammation.[28–30] To check whether the aforementioned oxidative stress changes were associated with increased lacrimal gland inflammation, we performed IHC stainings with CD45 antibodies. CD45 is a panleukocyte marker and has been shown to be a good marker for staining of T lymphocytes, B lymphocytes, granulocytes, monocytes, and macrophages.[31] Whereas specimens from the 10-week-old *Sod1*$^{-/-}$ and WT mice showed scanty staining of inflammatory cells with anti-CD45 antibodies, there was intense staining in all specimens from the *Sod1*$^{-/-}$ mice at 50 weeks, where inflammatory cell infiltrates around glandular ducts and several foci of inflammation could be observed. Relatively more inflammatory infiltrates were observed in specimens from the 50-week-old *Sod1*$^{-/-}$ mice compared with the WT mice (Figure 3A). To differentiate the type of inflammatory cells in the 50-week-old *Sod1*$^{-/-}$ mice, we performed further IHC stainings with CD4, CD11b, and Gr-1 antibodies. CD4 is a marker of helper T cells, whereas CD11b and Gr-1 are markers for neutrophils and monocytes, respectively.

Using the ImageJ and Adobe Photoshop computer software, we quantified the total inflammatory cell counts in each specimen. The mean CD45^{+} inflammatory cell density showed a significant timewise increase from 10 to 50 weeks in both the *Sod1*$^{-/-}$ and WT mice ($P < 0.0001$ and $P = 0.0031$, respectively). The mean inflammatory cell density was significantly higher in the *Sod1*$^{-/-}$ mice at 50 weeks compared with the WT mice ($P < 0.05$) (Figure 3B). The mean CD4-, CD11b-, and Gr-1–positive inflammatory cell densities were significantly higher in the *Sod1*$^{-/-}$ mice compared with the WT mice at 50 weeks. The mean CD4-positive cell density was significantly higher than the density of other inflammatory cells in the *Sod1*$^{-/-}$ mice at 50 weeks (Figure 3B).

To investigate the inflammatory cytokine alterations in the serum and tears, we performed Cytometric Bead Array evaluating the changes in six cytokines, including IL-6, IL-10, IFN-γ, TNF-α, MCP-1, and IL-12p70. Among them, the mean serum IL-6 concentration in the *Sod1*$^{-/-}$ mice showed a significant ($P = 0.009$) timewise increase from 10 to 50 weeks (9.96 ± 12.95 pg/mL to 33.62 ± 14.98 pg/mL). The mean serum TNF-α levels were also significantly higher ($P = 0.009$) in the *Sod1*$^{-/-}$ mice (10.89 ± 3.23 pg/mL) compared with the WT mice at 50 weeks (5.6 ± 3.73 pg/mL). There was a significant ($P = 0.016$) timewise increase in the mean TNF-α serum concentration from 10 (5.13 ± 6.39 pg/mL) to 50 (10.89 ± 3.23 pg/mL) weeks in the *Sod1*$^{-/-}$ mice (Figure 3C).

The mean tear IL-6 concentration also showed a significant increase in the *Sod1*$^{-/-}$ mice from 10 to 50 weeks ($P = 0.002$). The IL-6 concentration was significantly higher ($P = 0.028$) in the *Sod1*$^{-/-}$ at 50 weeks (36.88 ± 29.23 pg/mL) compared with the WT mice at 50 weeks (14.08 ± 11.65 pg/mL). The mean tear TNF-α concentration increased significantly from 10 to 50 weeks in both the *Sod1*$^{-/-}$ (9.96 ± 12.95 pg/mL and 33.62 ± 14.98 pg/mL, respectively) and the WT mice (6.82 ± 10.38 pg/mL and 46.11 ± 17.86 pg/mL, respectively). The mean tear TNF-α concentration was significantly higher in