The Role of Fractalkine as an Accelerating Factor on the Autoimmune Exocrinopathy in Mice

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PURPOSE. Sjögren’s syndrome (SS) is an organ-specific autoimmune disease caused by the progressive loss of exocrine glands and is associated with several autoimmune phenomena. Various research studies have been performed, and many molecules have been suggested as responsible for the pathogenesis of SS. Here the authors show the increased expression of fractalkine (CX3CL1) in lacrimal glands of SS model mice. Among more than 50 known chemokines, fractalkine is the sole member of the CX3C family and has unique structural and functional attributes. The purpose of this study was to analyze the role of fractalkine in exocrine glands.

METHODS. The expression of fractalkine in the lacrimal glands of thymectomized NFS/sld mice was investigated by immunohistochemistry and RT-PCR. To confirm the effects of fractalkine in exocrine glands, tissue-specific fractalkine transgenic mice were generated using the salivary amylose promoter.

RESULTS. The results demonstrated the upregulated fractalkine expression in thymectomized NFS mice. Furthermore, the lacrimal and salivary gland-specific fractalkine transgenic mice showed the expression of fragmented fractalkine and lymphocytic infiltration in their lacrimal and submandibular glands. Interestingly, the dominant population was B cells in the lacrimal glands, whereas B cells and CD4+ T cells were infiltrated in the submandibular glands. These mice also demonstrated slightly decreased tear and salivary secretion compared with wild-type mice.

CONCLUSIONS. Based on these results, it may be that fractalkine contributes to the development of SS, especially in lymphocyte migration to exocrine glands, and that it accelerates the disease in association with other molecules. (Invest Ophthalmol Vis Sci. 2009;50:4753–4760) DOI:10.1167/iovs.08-2596

Sjögren’s syndrome (SS) in humans is an organ-specific autoimmune disorder characterized by lymphocyte infiltration and destruction of the lacrimal and salivary glands, accompanied by systemic production of autoantibodies to ribonucleoprotein particles SS-A/Ro and SS-B/La.1,2

In several animal models for the investigation of SS in humans, SS is known to occur spontaneously in autoimmune-prone mice such as the NZB/NZW/F1 and MRL/lpr strains3,5 and to occur in nonautoimmune-prone NFS/sld mice thymectomized 3 days after birth (3d-Tx).6 All 3d-Tx NFS/sld mice developed autoimmune lesions in the lacrimal and salivary glands, starting at 3 weeks of age, and the disease was chronic and progressive.6,7 A previous report found a 120-kDa α-fodrin autoantigen in the salivary gland tissues from SS model mice and identified autoantigen-specific T cells.8 Various factors, including the effect of estrogen deficiency,7 the reactivation of Epstein-Barr virus,10 and autoantigens against muscarinic acetylcholine receptors11,12 have been reported; however, the mechanism of the development of SS remains obscure.

Chemokines are small chemoattractant cytokines that induce leukocyte accumulation at inflammatory sites and modulate inflammatory activities through the recruited cells.13 According to NH2-terminal cysteine motifs, chemokines can be grouped into four families: C, CC, CXC, and Cys-X-X-Cys (CX3C). Fractalkine is a member of the CX3C family.14,15 This molecule can exist in two forms, either membrane anchored or as a shed 95-kDa glycoprotein. The soluble fractalkine has potent chemoattractant activity for T cells and monocytes, and the cell-surface-bound protein, which is induced on activated primary endothelial cells, promotes strong adhesion of those leukocytes. Fractalkine is considered especially important for the recruitment of Th1 type cells and is a strong candidate for directing pathologic mononuclear cell infiltration in the muscosa of patients with glomerulonephritis or Crohn’s disease.16,17 Roles of fractalkine in diseases have been reported using fractalkine-deficient mice.18,19 but the relation between fractalkine and SS has not been reported.

We found here that fractalkine was expressed in lacrimal glands of 3d-Tx NFS/sld mice that developed autoimmune lesions in their lacrimal glands. As described, fractalkine has been reported to play an important role in various autoimmune diseases. Therefore, we further studied the role of fractalkine in SS by using tissue-specific fractalkine transgenic (Tg) mice.

METHODS

All experiment procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the animal welfare committees of Tsurumi University, Tokushima University, and Tokyo New Drug Research Laboratories II of Kowa Co. Ltd.

Mice

Female NFS/N-strain mice carrying the mutant gene sld20 were reared in a pathogen-free mouse colony at Tokushima University and given...
food and water ad libitum. Thymectomy was performed on day 3 after birth in NFS/sld mice, which led to the development of lacrimal and salivary gland mononuclear infiltration.6,20

Generation of Transgenic Mice

Mouse fractalkine cDNA was obtained from the spleens of C57BL/6 mice. Fractalkine cDNA was ligated to salivary amylase promoter and Simian virus (SV) 40 polyA signal. C57BL/6 mice were used to obtain fertilized eggs, and fragments containing mouse fractalkine cDNA were microinjected into the pronucleus of fertilized eggs using the standard method. When the mice were 4 weeks of age, DNA was extracted from a piece of the tail of each mouse and was used for PCR analysis to check for the presence of the integrated transgene. Two primers, MFK766S (CCAGAGAAGTCTCTAGGGTC) and SV40pAA (TACCA-CATTGTAGAGGTTTTAC), were used for PCR.

Measurement of Tear Secretion and Salivary Secretion

Mice were anesthetized intraperitoneally with a mixture of 36 mg/kg ketamine and 16 mg/kg xylazine and then were injected intraperitoneally with 5 mg/kg (for tear secretion) or 1 mg/kg (for salivary secretion) pilocarpine-HCl (sanpilo 1%; Santen Pharmaceutical Co. Ltd., Osaka, Japan). Modified Schirmer tests were performed to measure the secreted tears by cotton thread (Zone quick; Showa Pharmaceutical Co. Ltd., Osaka, Japan) during each 1-minute period after an injection of pilocarpine for 15 minutes after the injection. The saliva secreted into the oral cavity was carefully collected using capillaries (ringcaps; Hirschmann Laborgerate GmbH & Co. KG, Eberstadt, Germany) during each 1-minute period after an injection of pilocarpine for 15 minutes after the injection.

Histology

Mice (27 weeks of age) were anesthetized with diethyl ether and killed. Lacrimal and salivary glands were obtained. The sections (4 μm) were stained with hematoxylin and eosin. Histologic grading of the inflammatory lesions was performed according to a previously described method.21

Some glandular epithelial cells were stained with anti–fractalkine antibodies (Zymed Laboratories, South San Francisco, CA) as the second antibody for 1 hour. Sections were treated with a freshly prepared solution of 0.05% 3,3′-diaminobezidine and 0.005% H2O2 and were counterstained with methyl green. For immunofluorescence staining of fractalkine Tg mice, frozen sections were fixed in 4% paraformaldehyde. They were then incubated for 1 hour with the following antibodies: PE-conjugated anti-B220 (RA3-6B2; BD Biosciences, San Jose, CA), FITC-conjugated anti-CD3 (145–2C11; BD Biosciences), PE-conjugated anti-CD4 (GK1.5; BD Biosciences), and FITC-conjugated anti-CD8 (53–6.7; BD Biosciences). The sections were analyzed by fluorescence microscopy (BX50; Olympus, Tokyo, Japan).

Primary Culture of Lacrimal Gland Cells

Each mouse was anesthetized with diethyl ether and killed. Lacrimal glands were harvested and minced, washed, and placed in 0.76 μg/mL EDTA, 4.9 μg/mL L-ascorbic acid (Wako Pure Chemical, Osaka, Japan), and 4.9 μg/mL glutathione (Wako). The solution was placed in a shaking water bath at 37°C for 15 minutes, and the fragments were washed and placed in a mixture of collagenase (type 1; 750 U/mL) (Wako) and hyaluronidase (type IV; 500 U/mL) (Sigma) dissolved in DMEM/F12 (Invitrogen, Carlsbad, CA) containing 10% FBS. The first digest suspension was passed through a sterile 100-mm nylon mesh filter, redigested for 2 hours using the same digestion procedure, and passed through a 100-μm nylon mesh filter once again. The growth medium used in this study was serum-free medium supplemented with 5 ng/mL recombinant epidermal growth factor (Invitrogen).

Semiquantitative RT-PCR Analysis

Sequences of the specific sense and antisense oligonucleotide primer pairs were as follows: mouse fractalkine, CCGCGTTCTTCCATTTGTA and GCTTCTCAAACTTGCCACCAT; human fractalkine, TGAAGAATGGCAACATCAG and TCTGTGCTCCGTGGGAAAGA; β-actin, CTCTTTGATGTACGCAGATTTG and GTGGGCCGCTCTAGGCAACAA.

Immunoblotting

Lacrimal glands and salivary glands were obtained from fractalkine Tg and wild-type (WT) mice and were homogenized in lysis buffer (50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.1% SDS, and 1% triton X-100). Trans-
ferred membranes were first blocked and then incubated with the mouse anti-fractalkine (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin (C-11; Santa Cruz Biotechnology) for 1 hour. Membranes were incubated with anti-goat IgG peroxidase conjugate (Biossorb, Carlsbad, CA) for 1 hour. Bound protein was developed with reagent (Western Lighting Chemiluminescence Reagent Plus; PerkinElmer, Boston, MA), and the signals were detected (Fluor-S Multilimage; Bio-Rad, Hercules, CA).

**Statistical Analysis**

Data are expressed as the mean ± SEM. Student’s t-test was used to determine the significance of differences for tear secretion and lymphocytic infiltration in the lacrimal glands.

**RESULTS**

**Expression of Fractalkine in Lacrimal Glands of NFS/sld Mice**

Tx-NFS/sld mice showed massive mononuclear infiltration in exocrine glands and dysfunction of these glands.\(^2\) RT-PCR analyses demonstrated the upregulated fractalkine in Tx-NFS/sld mice lacrimal glands (Fig. 1a). Fractalkine expression was observed in the ductal cells in the lacrimal gland of Tx-NFS/sld mice (Fig. 1d), but this upregulation of fractalkine was not detected in non-Tx NFS/sld mice (Fig. 1c). We also analyzed the expression of fractalkine in cultured lacrimal gland cells;

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**FIGURE 2.** Generation and characterization of tissue-specific fractalkine Tg mice. (a) To direct fractalkine expression to the lacrimal glands and salivary glands that secrete amylase, we linked the mouse fractalkine cDNA to the human salivary amylase promoter. The fragment containing 1.2 kbp fractalkine Nheli-XbaI and the fragment containing 0.3 kbp SV40 polyA Nheli-XbaI were ligated to amylase promoter. (b) Fractalkine expression in the lacrimal glands, salivary glands, pancreas, and liver was detected by Western blot analysis. Numbers represent mass (kDa), as determined using a molecular weight marker. (c–f) Histologic analysis. Sections were stained with hematoxylin and eosin. (c, e) Inflammatory lesions in the lacrimal and salivary glands were characterized by focal mononuclear cell infiltration. In contrast, (d, f) cell infiltration was not observed in littermates. LG, lacrimal gland; SG, submandibular gland. Scale bar, 10 μm. Original magnification, ×200.)
fractalkine expression was increased with TNFα stimulation (10 ng/mL; Fig. 1b). As described, fractalkine differs greatly from other chemokines in that it can exit the cell as a soluble form or as a membrane-bound form. Based on previous reports and our results here, we speculate that fractalkine is upregulated by inflammation in lacrimal glands and may play a role in leukocyte infiltration into inflammatory lesions.

**Generation of Tissue-Specific Fractalkine Transgenic Mice**

To address the role of fractalkine in lacrimal and salivary glands, we constructed tissue-specific fractalkine Tg mice in which the expression of mouse fractalkine was regulated by the human salivary amylase promoter (Fig. 2a). The approximately 25-kDa band was specifically found in Tg mice salivary glands, and bands of the same size were detectable in the lacrimal glands of WT and Tg mice. A specific band of lacrimal bands was found around 50 kDa in Tg mice (Fig. 2b). This 50-kDa form was not detected in the salivary glands of WT or Tg mice. We also detected 25-kDa bands in the pancreas and liver and a 50-kDa band in the pancreas; however, a clear difference was not found between WT and Tg mice. Given that full-length fractalkine weighs approximately 95-kDa, these bands may be a cleaved form, as reported in a former study. These data indicated that WT mice expressed the 25-kDa form of endogenous fractalkine but did not express the 50-kDa form in lacrimal glands; therefore, we clearly detected the 25-kDa form only in salivary glands and the 50-kDa form in lacrimal glands. Figure 2b also showed exogenous fractalkine was specifically expressed in lacrimal and salivary glands in this Tg mouse.

**Histologic Analysis**

Representative histologic findings were observed in lacrimal and submandibular glands with mononuclear infiltration in fractalkine Tg mice (Figs. 2c, e), whereas infiltrated cells were not seen in littermate lacrimal or submandibular glands (Figs. 2d, f). These infiltrations demonstrated a role of fractalkine in the migration of leukocytes in lacrimal and submandibular glands. We also analyzed sublingual glands and parotid glands for mononuclear infiltration but did not detect it (data not shown). There was no difference between male and female with respect to inflammation. Older mice tended to show greater frequency of mononuclear infiltration than younger mice in lacrimal and submandibular glands. The mice used in Figures 2c to 2f were 27 weeks of age. In previous reports of aging and SS, SS is shown to be a female-dominant disease with a late age of onset. Furthermore, severe autoimmune lesions through Fas-mediated apoptosis with aging was demonstrated in a murine SS model.

**Subset of Infiltrated Lymphocytes**

To investigate the phenotypic characterization and the role of the cells that infiltrate the lacrimal and submandibular glands, the population of infiltrated cells in both glands was analyzed in fractalkine Tg mice (27 weeks of age). Frozen sections were subjected to immunofluorescence staining. This revealed the dominant presence of B cells characterized by a positive reaction for B220 in lacrimal glands (Fig. 3a), and CD4+ or CD8+ T cells were minimally observed (Fig. 3b). In contrast, T cells and B cells characterized by a positive reaction for CD3 and B220 were observed in submandibular glands (Fig. 3c). T cells and B cells seemed to be distributed in clusters in the focal infiltrates. Furthermore, most T cells were CD4+, and few CD8+ T cells were detected (Fig. 3d).

**Tear and Salivary Secretion in Fractalkine Tg Mice**

Tear and salivary secretion was measured at four age points in each mouse (16, 20, 28, and 33 weeks for tear secretion and 15, 20, 29, and 33 weeks for salivary secretion).
When 5 mg/kg pilocarpine was injected intraperitoneally into anesthetized WT mice, the cumulative amount of tears secreted in 15 minutes was 2.1 ± 0.2 mm/g, 2.5 ± 0.2 mm/g, 3.0 ± 0.5 mm/g, and 3.4 ± 0.5 mm/g at 16, 20, 28, and 33 weeks of age, respectively. On the other hand, in fractalkine Tg mice, induced tear secretion in 15 minutes was 2.2 ± 0.2 mm/g, 2.1 ± 0.2 mm/g, 2.0 ± 0.2 mm/g, and 2.4 ± 0.6 mm/g at 16, 20, 28, and 33 weeks of age, respectively, and secretion was slightly decreased in older mice. Statistical significance determined using Student’s \( t \)-test comparing WT and fractalkine Tg mice was \( P = 0.91, P = 0.09, P = 0.10, \) and \( P = 0.22 \) for each age point, and no statistically significant difference was detected. Figure 4a shows the cumulative amounts of tears secreted in 15 minutes, and Figures 4b to 4e show the tear output for each 1-minute period after pilocarpine injection.

Tear secretion and cumulative amount of tear secretion were slightly decreased in fractalkine Tg mice for each 1-minute period at each age point, but no statistically significant difference was observed.

The cumulative amounts of salivary secretion in 15 minutes were 10.0 ± 1.1 μL/g, 9.5 ± 0.7 μL/g, 7.5 ± 0.8 μL/g, and 9.1 ± 0.4 μL/g at 15, 20, 29, and 33 weeks of age, respectively. In fractalkine Tg mice, induced amounts of salivary secretion in 15 minutes were 8.5 ± 0.5 μL/g, 8.8 ± 0.6 μL/g, 7.8 ± 0.5 μL/g, and 6.7 ± 1.6 μL/g at 16, 20, 28, and 33 weeks of age, respectively. Statistical significance determined using Student’s \( t \)-test comparing WT and fractalkine Tg mice was \( P = 0.24, P = 0.47, P = 0.76, \) and \( P = 0.15, \) at each age point, respectively. Figure 5a shows the cumulative amounts of saliva secreted in 15 minutes, and Figures 5b to 5e show the saliva output for each 1-minute period after pilocarpine injection. Statistically significant differences between WT and fractalkine Tg mice were not detected even in older mice in cumulative amounts of saliva.

**DISCUSSION**

The roles of fractalkine in lacrimal and salivary glands and in the SS model mice were investigated in this study. Fractalkine triggers the adhesion of mononuclear cells and is a potent mononuclear cell-directed chemoattractant. A previous study reports the expression and distribution of fractalkine in human renal inflammation and showed that expression correlated with leukocyte infiltration subsets. An antagonist of fractalkine inhibited the chemotaxis of activated leukocytes isolated from nephritic glomeruli, significantly reduced leukocyte infiltration in the glomeruli, and markedly attenuated proteinuria. Immunoneutralization of the fractalkine receptor CX3CR1 also prevented crescentic glomerulonephritis. Considering these previous reports, we focused on the role of fractalkine in the inflammation of exocrine glands in this study. Immunohistochemistry showed the upregulation of fractalkine in lacrimal glands of SS model mice, Tx-NFS/sld mice.

**FIGURE 4.** Tear secretion in WT and fractalkine Tg mice. (a) Cumulative amount of tear secretion in 15 minutes in response to 5 mg/kg pilocarpine administered intraperitoneally in WT mice (n = 5) and fractalkine Tg (FKTG) mice (n = 5) at 16, 20, 28, and 33 weeks of age. (b–e) Tear output for each 1-minute period after injection at each age point is represented by the symbols and lines.
RT-PCR using primary cultured cells of lacrimal glands also demonstrated the induction of fractalkine expression by TNFα. Although the cells used in RT-PCR were a mixture of various kinds of cells in lacrimal glands, fractalkine seemed to be expressed in epithelial cells around the duct in the histologic analysis. The organ-specific fractalkine fragment in salivary glands of nonobese diabetic (NOD) mice that are spontaneous experimental SS model mice has been reported recently.24 These data indicate the involvement of fractalkine with mononuclear infiltration and inflammation.

To confirm the contribution of fractalkine in SS, we generated lacrimal and salivary gland-specific fractalkine Tg mice by using a salivary amylase promoter. These Tg mice showed specific expression of the 50-kDa form of fractalkine in lacrimal glands and the 25-kDa form in salivary glands, respectively (Fig. 2b). These detected signals were considered a fragmented form of fractalkine, as formerly reported by Wildenberg et al.,24 who showed that altered fractalkine cleavage resulted in organ-specific, approximately 17-kDa and 19-kDa fractalkine fragments in salivary glands of NOD mice. The approximately 19-kDa fractalkine fragment was also found in the C57BL/6 strain, and another fractalkine fragment (31-kDa) was demonstrated in the report. We detected the 25-kDa fractalkine fragment in our study using the same antibody, and another report also demonstrated the 25-kDa fractalkine fragment. This difference of band size is considered to be attributed to the kind of molecular marker and condition of electrophoresis. The 25-kDa form of fractalkine was detected in the pancreas in our study, and it may be equivalent to the 31-kDa form in the study by Wildenberg et al.24 Given that Wildenberg et al.24 indicated that approximately 17-kDa and 19-kDa fractalkine fragments cause autoimmunity, it is possible to consider the 25-kDa fractalkine fragment induced mononuclear infiltration in salivary glands, as seen in Figure 2c. The 50-kDa fractalkine fragment was detected in lacrimal glands, and this fragment may explain the mononuclear infiltration in the lacrimal gland as shown in Figure 2c. The organ-specific cleavage of fractalkine may be also indicated in lacrimal glands. Fragment size and organ specificity of fractalkine cleavage should be studied in the future, along with the effect of proteases as analyzed in their study.24 From the results in these Tg mice and Tx-NFS/sld mice, it is speculated that fractalkine may be one of the key molecules to induce mononuclear cell infiltration and inflammation in SS.

Interestingly, the respective proportions of T- and B-cell subsets in lacrimal and submandibular glands were different: B cells were predominant in the lacrimal glands (Figs. 3a, b), and T cells and B cells were observed in the submandibular glands (Figs. 3c, d). It has been demonstrated that increased B-cell subsets in the focal infiltration were observed in lacrimal glands but not in submandibular glands in aged NOD mice.31 It is also reported that most cells in the lymphocyte infiltrate were B cells in lacrimal gland biopsies from patients with SS,25 whereas T-helper cells were prevalent in SS salivary gland...
biopsies. These reports are consistent with our results regarding the distribution of the subset of infiltrated cells. However, another report demonstrated infiltration of CD4+ T cells in SS lacrimal gland biopsies and in NFS/sld mice, and it has been shown that most cells in infiltrated salivary glands were CD4+ T cells. Therefore, it is still unclear whether this observation of prevailing B cells in the lacrimal gland is a general phenomenon, and the difference between the SS model mice strains should be investigated.

These differences of infiltrated cells in lacrimal glands and submandibular glands of fractalkine Tg mice may be explained with the different form of fractalkine detected in both glands. The 25-kDa fractalkine fragment, but not the 50-kDa fractalkine fragment, may have the potential to migrate T cells that mainly express CX3CGR in inflammatory lesions though the role of each fractalkine fragment was not solved. The role of each fragment and organ-specific environment are issues to be analyzed in a future study.

Some interesting issues might provide important insight into the relation between fractalkine and SS. Based on the results of histologic analyses, including the subset of infiltrated cells, it was expected that tear and salivary secretion would be downregulated. Although both tear and salivary secretion decreased slightly compared with WT mice, statistical difference was not detected. The degree of lymphocyte infiltration should determine whether it destroys the lacrimal and salivary glands and contributes to the dysfunction of these glands; fractalkine fragments may not be able to migrate enough lymphocytes to reduce secretion. However, the mono-nuclear infiltration at 27 weeks of age may contribute to the small change in secretion at 33 weeks of age. The main population was B cells and CD4+ T cells in lacrimal glands and salivary glands, respectively, and a few CD8+ cytotoxic cells were observed. Therefore, both glands might not be destroyed by cytotoxic T cells.

From the results in our study, it is likely that fractalkine, including fragmented fractalkine, plays a role in lymphocyte migration in SS and participates in the development of this disease as one of the accelerating factors. In this study, we have demonstrated a new molecule that may assist in the understanding of the pathogenesis of SS.

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