Role of Matrix Metalloproteinases 2 and 9 in Lacrimal Gland Disease in Animal Models of Sjögren's Syndrome

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PURPOSE. Chronic inflammation of the lacrimal gland results in changes in the composition of the extracellular matrix (ECM), which is believed to compromise tissue repair. We hypothesized that increased production/activity of matrix metalloproteinases (MMPs), especially MMP-2 and -9, in inflamed lacrimal glands modifies the ECM environment, therefore disrupting tissue repair.

METHODS. The lacrimal glands from female MRL/lpr and male NOD mice along with their respective control strains were harvested and divided into three pieces and processed for histology, immunohistochemistry, zymography, Western blotting, and RNA analyses. In another study, MRL/lpr mice were treated for 5 weeks with a selective MMP2/9 inhibitor peptide or a control peptide. At the end of treatment, the lacrimal glands were excised and the tissue was processed as described above.

RESULTS. There was a 2.5- and 2.7-fold increase in MMP2 gene expression levels in MRL/lpr and NOD mice, respectively. Matrix metalloproteinase 2 and 9 enzymatic activities and protein expression levels were significantly upregulated in the lacrimal glands of MRL/lpr and NOD mice compared to controls. Treatment with the MMP2/9 inhibitor resulted in decreased activity of MMP-2 and -9 both in vitro and in vivo. Importantly, MMP2/9 inhibitor treatment of MRL/lpr mice improved aqueous tear production and resulted in reduced number and size of lymphocytic foci in diseased lacrimal glands.

CONCLUSIONS. We conclude that MMP2/9 expression and activity are elevated in lacrimal glands of two murine models of Sjögren's syndrome, suggesting that manipulation of MMP2/9 activity might be a potential therapeutic target in chronically inflamed lacrimal glands.

Keywords: matrix metalloproteinases, Sjögren's syndrome, extracellular matrix, lacrimal gland, MMP2/9

The main function of the lacrimal gland is to produce secretions that lubricate and protect the ocular surface. Inadequate quantity or quality of tear production leads to aqueous deficiency resulting in dry eye syndrome.^{1–3} Dry eye syndrome due to lacrimal gland deficiency is associated with pathological conditions such as autoimmune diseases (Sjögren's syndrome, sarcoidosis, and rheumatoid arthritis),^{4–6} organ transplantation (chronic graft-versus-host disease),⁷ and viral infections (hepatitis, human immunodeficiency virus) or as a result of aging.⁸ Chronic inflammation of the lacrimal gland leads to keratoconjunctivitis sicca or dry eye disease, which is mainly characterized by increased infiltration of immune cells, increased release of proinflammatory cytokines, and loss of tear-producing parenchymal cells.^{1–3,9–11}

Inflammation of the lacrimal gland changes the epithelial phenotype, sensitizes nerve endings, and induces loss and death of apical epithelial cells.^{12,13} Remarkably, murine lacrimal gland has an ability to repair itself upon experimentally induced injury.¹⁴ Lacrimal gland injury/inflammation induces the process called epithelial-mesenchymal transition (EMT). During EMT, epithelial cells lose cell-cell attachment and polarity and epithelial-specific markers, undergo cytoskeletal remodel-

ing, and gain a mesenchymal phenotype.^{14,15} During the repair phase, extracellular matrix (ECM) is remodeled and replaced with new collagen fibers, proteoglycans, and elastin fibers, which restore the structure and function of the lacrimal gland.¹⁵

The ECM, made up of fibrillary proteins and proteoglycans, acts as a substrate for the acinar epithelial cells' attachment within the lacrimal gland.^{16–19} In healthy lacrimal glands, ECM matures, persuading the differentiation of the epithelial cells and restoration of lacrimal gland acinar structure integrity.²⁰ During pathological conditions, ECM triggers signaling mechanisms for selective development and differentiation during tissue injury and repair processes.²¹ Increased degradation of ECM structures of the lacrimal gland has been implicated in the pathogenesis of Sjögren's syndrome.^{22,23} In diseased glands (during chronic lacrimal gland inflammation), the ECM is remodeled and epithelial cell proliferation is initiated. However, maturation of the ECM and epithelial cell differentiation stay incomplete, and lacrimal gland acinar structure remains unrestored.^{15,23} Alterations in the composition of the ECM in the salivary glands of Sjögren's syndrome patients as well as in animal models are well documented.^{24–28} Thus it has been

postulated that during diseased/inflamed conditions, abnormal ECM maturation, expression of cell matrix and adhesion molecules, and/or epithelial-ECM signaling hinder tissue repair in the lacrimal gland.²⁹⁻³²

Some of the proinflammatory mediators increased during dry eye disease include matrix metalloproteinases (MMPs), cytokines, elastases, and other proteolytic enzymes that destroy components of the ECM and damage the growth factors and their receptors, which are essential for tissue repair and cell differentiation.^{22,23,33} Matrix metalloproteinases belong to a family of zinc-requiring neutral endopeptidases that are involved in remodeling of ECM and basement membrane during various physiological and pathological processes.³⁴ Matrix metalloproteinases are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs), and disturbances in the delicate balance between MMPs and TIMPs lead to tissue destruction in various inflammatory and malignant diseases.³⁴⁻³⁷ In Sjögren's syndrome, there is increased production of MMP-3 and MMP-9 by the ocular surface.^{38,39} In dry eye syndrome, epithelial cells secrete MMP-9 upon activation of transforming growth factor β (TGF- β),⁴⁰ resulting in disruption of the apical cornea epithelial cell tight junctions, which causes corneal barrier dysfunction⁴¹⁻⁴³ and accelerates corneal desquamation.⁴⁴ Along with epithelial cells, elevated levels of MMP-2 and MMP-9 are also strongly associated with the inflammatory cells, which invade diseased lacrimal glands.^{11,45,46} Several studies showed that downregulation of MMP-9 activity using chemical inhibitors or using MMP-9 knockout mice improved lacrimal gland function.^{47,48} However, the specific roles of MMPs and their inhibitors (TIMPs) in regulation of ECM during dry eye disease are yet to be clearly defined. Modulation of ECM molecules to treat dry eye condition has not been explored due to inadequate information on the role of ECM during the development of the disease. Therefore, further understanding of the role of ECM remodeling in disease progression could lead to new promising treatment strategies for patients suffering from chronic dry eye disease.

In spite of its critical role in lacrimal gland development and function, and even though changes in ECM might exert pathological effects, until now, very few reports^{22,23} have characterized the changes in the ECM that accompany Sjögren's syndrome in the lacrimal gland. Previous studies in patients with Sjögren's syndrome suggested that increased activity and expression of MMP-2, MMP-9, and MMP-3 trigger the destruction of the lacrimal and salivary glands.⁴⁹⁻⁵¹ Matrix metalloproteinase 3 is an activator of the precursor of MMP-9 (pro-MMP-9). Increases in MMP-3 expression and secretion usually precede MMP-9 expression and activation, which marks the importance of MMP-9 as a key effector molecule for ECM degradation.^{52,53} This led us to hypothesize that, in chronically inflamed lacrimal glands, continuous ECM remodeling caused by the increased expression of MMP-2 and -9 disrupts tissue repair.

In this study, we have investigated the changes in lacrimal gland MMP-2 and MMP-9 expression and activity in two mouse models of Sjögren's syndrome: NOD and MRL/lpr mice. We provide evidence that activity and expression levels of MMP-2 and MMP-9 are increased in NOD and MRL/lpr mice compared to age- and sex-matched controls. Modulating MMP-2 and -9 activity using a specific inhibitor peptide not only decreased their activity *in vivo* but also decreased the size of the lymphocytic foci in diseased lacrimal glands and increased tear production.

MATERIALS AND METHODS

The miRNeasy isolation kit and QuantiNova SYBR Green PCR mix were purchased from Qiagen (Valencia, CA, USA). The

RNA to cDNA conversion kit, SuperScript First-Strand synthesis system or RT² First Strand Kit was obtained from Life Technologies (Carlsbad, CA, USA) or Qiagen. Running buffer, transfer buffer, NuPage 4% to 12% Bis-Tris gels, and Invitrolon polyvinylidene fluoride (PVDF) membranes (0.45 μ m) were procured from Invitrogen (Carlsbad, CA, USA), while the blocking buffer was from Li-Cor Biosciences (Lincoln, NE, USA). All reagents for zymography were purchased from Life Technologies. Phenol red-impregnated cotton threads were obtained from Zone-Quick, Lacrimedics (Eastsound, WA, USA). Antigen retrieval solution and 4',6-diamidino-2-phenylindole (DAPI) were from Vector Laboratories (Burlingame, CA, USA), whereas the donkey serum was purchased from Jackson ImmunoResearch (Westgrove, PA, USA). The following primary antibodies were used: mouse monoclonal antibody against MMP-2 (1:100; Abcam, Inc., Cambridge, MA, USA); rabbit polyclonal antibody against MMP-9 for immunofluorescence (1:100; NeoBioLab, Inc., Woburn, MA, USA); goat polyclonal antibody against MMP-9 for Western blotting (sc-6841; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rat monoclonal antibody against heparan sulfate proteoglycan (clone A7L6, 1:400; Millipore, Billerica, MA, USA); and mouse monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000; Abcam, Inc.). Fluorescein isothiocyanate (FITC)- or tetramethylrhodamine (TRITC)-conjugated secondary antibodies (1:100; Jackson ImmunoResearch) were used for immunodetection.

Animals and Treatment

MRL/MpJ-Fas $\langle$ lpr $\rangle$ /J (MRL/lpr; female, 12 weeks old) and NOR/LtJ (NOD; male, 13 weeks old) mice and their respective age- and sex-matched MRL/MpJ (MRL/++) and BALB/cJ control mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Tufts Medical Center Animal Care and Use Committee. Animals were euthanized and the exorbital lacrimal glands were harvested and divided into several pieces to be used for histology, immunofluorescence, zymography, or RNA extraction for RT-PCR analysis.

In another set of experiments, MRL/lpr mice (female, 9 weeks old) received daily IP injections (100 μ g/mouse) of either a control peptide (STT peptide: H-Ser-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Ser-OH) or MMP2/9 inhibitor (CTT peptide: H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys-OH)^{54,55} for 5 weeks. STT and CTT peptide were synthesized using solid state synthesis with F-moc as the protecting group. The peptides were cyclized by air oxidation and purified using HPLC. At the end of treatment, the animals were euthanized and the lacrimal glands removed. One piece of the lacrimal gland was fixed and processed for histology or immunohistochemistry. The remainder of the tissue was homogenized, and the resulting tissue lysates were used for zymography.

RNA Extraction and Quality Control

RNA was extracted using the RNeasy kit according to the manufacturer's protocol. Briefly, lacrimal glands were homogenized with lysis reagent and incubated at room temperature for 5 minutes. To the homogenate, chloroform was added and then centrifuged at 12,000g at 4°C. Next, the upper aqueous layer was collected and mixed with 1.5 volumes of 100% ethanol, which was run through an RNeasy MinElute spin column at 8000g for 15 seconds. Then, buffer RWT, buffer RPE, 80% ethanol were added subsequently, one after another, to the RNeasy MinElute spin column and centrifuged at 8000g for

15 seconds. Lastly, 20 μ L RNase-free water was added to the spin column and RNA was collected. The RNA purity and quantity was analyzed using NanoDrop 1000. The samples were then stored at -20°C until use.

Real-Time Quantitative PCR

Total RNA was extracted, as described above, and first-strand cDNA was synthesized from 3 μ g total RNA using random hexamer primers and SuperScript First-Strand synthesis system. SYBR Green real-time PCR for MMP-2 and MMP-9 was performed on an Mx4000 Multiplex Quantitative PCR System in a total volume of 20 μ L using the QuantiNova SYBR Green PCR mix. Each reaction was performed in triplicate using the following thermocycler conditions: 95°C for 10 minutes to denature the DNA and activate the Taq polymerase; amplification for 40 cycles of denaturation (95°C , 10 seconds), annealing (58°C , 5 seconds), and extension (72°C , 26 seconds). A single fluorescence reading was taken at each extension step. The relative quantification of mRNA was performed using the standard curve method. The absence of nonspecific amplification was confirmed by dissociation curve analysis. In all samples, rRNA18s (housekeeping gene) expression was also assessed in duplicates. The results are presented as relative values, ratio of the number of copies in the assessed gene MMP-2 (or MMP-9) and rRNA18s.

Immunohistochemistry

Lacrimal gland pieces were fixed overnight at 4°C in 4% formaldehyde in phosphate buffered saline (PBS, containing in mM: 145 NaCl, 7.3 Na_2HPO_4 , and 2.7 NaH_2PO_4 at pH 7.2) and processed for paraffin embedding. Paraffin sections of the lacrimal gland (6 μ m) were deparaffinized and rehydrated in graded alcohol solutions. For histopathology experiments, slides were processed for hematoxylin and eosin (H&E) staining.⁵⁶ For immunofluorescence experiments, the slides were subjected to microwave pretreatment (15 minutes) with antigen retrieval solution. Following washing with PBS (3 $\times$), nonspecific binding sites were blocked for 30 minutes using 10% normal donkey serum. The slides were then incubated overnight at 4°C with the indicated primary antibody. Sections without primary antibody were included as negative controls. Next, slides were incubated for 60 minutes at room temperature with the appropriate secondary antibody. Finally the slides were covered with mounting medium containing DAPI. Sections were viewed using a microscope equipped for epillumination (Nikon UFXII Epi-Illuminator; Melville, NY, USA) or a Zeiss LSM 780 laser scanning confocal microscope (Thornwood, NY, USA) with the attached Zeiss Observer Z1 microscope. The $\times 63$ oil Plan Apo objective was used.

Western Blotting

Lacrimal gland pieces were homogenized in 0.4 mL ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors). The supernatant containing proteins was collected by centrifugation at 24,000g for 30 minutes.

Proteins were loaded and separated using NuPage 4% to 12% Bis-Tris gels followed by transfer onto Invitrolon PVDF membranes. The membranes were then incubated at room temperature for 60 minutes in a blocking solution consisting of 5% wt/vol nonfat dry milk prepared in TBST (TBS, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Next, the membranes were incubated with diluted primary antibody in either 5% wt/vol BSA or nonfat dry milk, 1 $\times$ TBST, 0.1% Tween

20 overnight at 4°C with gentle agitation. After washing with TBST, the blots were incubated for 1 hour at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at a dilution of 1:5000 in blocking buffer. The HRP-labeled protein bands were detected using the chemiluminescent detection system. As a gel loading control, the membrane was also probed with an antibody against GAPDH.

Zymography

The activity of the gelatinases, MMP-2 and MMP-9, in lacrimal gland tissue extracts was determined by SDS-PAGE gelatin zymography. Briefly, 30 μ g proteins from homogenized tissue samples were mixed with sample buffer (1:1, vol:vol) and electrophoresed through 10% zymogram (gelatin) gel. Gels were incubated in renaturing buffer and developing buffer at room temperature for 30 minutes each and subsequently at 37°C overnight in developing buffer. Following 24-hour incubation, the gels were placed in freshly made developing buffer at 37°C . After 48-hour incubation, gels were stained with SimplyBlue SafeStain, and proteolytic activity was detected as a clear band against a blue background. The activity of MMP-2 and MMP-9 was determined by densitometric scanning of the bands using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Metalloproteinase 2 and 9 activity was expressed in arbitrary units.

An in vitro experiment was performed on MRL/lpr mouse lacrimal gland lysates to test the MMP2/9 inhibitors using zymography. MRL/lpr lacrimal gland lysates (60 μ g) were mixed with sample buffer (1:1) and electrophoresed through 10% zymogram (gelatin) gel. Gels were incubated in renaturing buffer for 30 minutes. Following, the zymogram was divided into three parts. One part was incubated with developing buffer (negative control); another part was incubated with the STT control peptide (100 μ M made in developing buffer); and the remaining part was incubated with the CTT inhibitor peptide (100 μ M made in developing buffer). Following incubation for 36 hours, the gels were placed in freshly made developing or inhibitor or control peptide solutions, respectively, and this step was repeated again after 36 hours. After incubation for 4 days, the gels were stained with SimplyBlue SafeStain and the proteolytic activity was detected as a clear band against a blue background.

Measurement of Aqueous Tear Production

Mice were anesthetized with isoflurane and tear production was measured using phenol red-impregnated cotton threads as previously described.¹⁴ The threads were applied in the lateral canthus of the ocular surface for 10 seconds. Contact with tears changes the color of the thread from yellow to red. The length of wetting was measured in millimeters under a dissecting microscope.

Measurement of Lymphocytic Foci Number and Size

Lymphocytic foci number and size were evaluated with a Nikon UFXII microscope coupled to a SPOT digital camera. The number of lymphocytic foci was counted in five to eight nonconsecutive sections per gland. Lymphocytic focus was defined as focus harboring 50 or more lymphocytes. To determine the size of lymphocytic foci, total area of tissue sections was first measured; then individual lymphocytic focus sizes were determined. The percentage of the area occupied by lymphocytes was calculated by summing the areas of individual

FIGURE 1. Gene expression of MMP-2 in MRL/lpr and NOD mice compared to their respective control strains. RNA isolated from lacrimal glands was used for reverse transcription and PCR quantification using primers specific to MMP-2 and 18s (control gene). *Left:* Matrix metalloproteinase 2 mRNA levels in MRL/lpr mice compared to their control strain MRL/+. *Right:* Quantitative data of MMP-2 mRNA levels in NOD mice and their control strain BALB/c. Data are presented as mean $\pm$ SEM ($n = 5$).

foci in a given section, divided by the total section area and multiplied by 100. The slides (5–8 nonconsecutive sections/gland) were masked, and two individuals performed the histologic analysis as a blindfolded experiment.

Data Presentation and Statistical Analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). The data consisting of two groups were analyzed by two-tailed Student's *t*-test. All analyses were performed using GraphPad Prism Version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Quantitative-PCR of MMP-2 and -9 in MRL/lpr and NOD Mice

Changes in gene expression levels of MMP-2 and -9 in diseased lacrimal glands compared to controls were determined by quantitative (q)RT-PCR. The MMP-2 mRNA level was significantly elevated in MRL/lpr lacrimal gland samples, 2.7-fold ($P < 0.05$), compared to mRNA level in control tissue samples (MRL/+, Fig. 1). Similarly, lacrimal gland tissue samples from NOD mice also had a 2.5-fold higher MMP-2 mRNA expression level compared to BALB/c mice (Fig. 1). Several primers for MMP-9 were tested to study gene expression; however, we were unable to quantify the changes in MMP-9 between diseased and control groups.

Enzymatic Activity of MMP-2 and -9 in MRL/lpr and NOD Mice

To verify the gene expression data, we performed zymography studies to detect changes in MMP-2 and MMP-9 protein activity. Enzymatic activity of MMP-2 and -9 in MRL/lpr and NOD mice in lacrimal gland homogenates was measured. During zymography, MMPs were denatured with SDS, exposing their active site, which allows both the latent (pro-MMP) and active forms of MMPs to exhibit gelatinolytic activity. As shown in Figure 2, active MMP-2 (62 kDa) was significantly increased 3.2-fold in MRL/lpr mice lacrimal glands compared to control MRL/++ mice ($P < 0.05$). However, there was no significant change in MMP-9 (82 kDa) activity levels between the diseased (MRL/lpr) and control mice (MRL/++). Similarly, a 2.3-fold increase ($P < 0.05$) in active MMP-2 levels was observed in NOD mice compared to BALB/c mice, whereas the activity of MMP-9 (active form) was significantly increased, by 2.1-fold, in NOD mice compared to their control strain (Fig. 2). In contrast, the

activity of pro-MMP-9 was significantly downregulated in NOD mice (4.69 ± 0.49) compared to BALB/c mice (16.88 ± 1.53 , $P < 0.0001$). These results indicate that the activity of MMP-2 is significantly upregulated in chronically inflamed lacrimal glands of MRL/lpr and NOD mice, whereas MMP-9 activity could be measured only in NOD samples.

Immunohistochemical Staining of MMP-2 and -9 in the Lacrimal Gland of MRL/lpr and NOD Mice

We assessed the expression of MMP-2 and MMP-9 proteins in the MRL/lpr, NOD, and control (MRL/+ and BALB/c) mouse lacrimal glands using immunohistochemistry techniques (Figs. 3A–S). To better distinguish epithelial acinar and ductal structures in the lacrimal gland we also used antibody to heparan sulfate (Figs. 3D, 3H, 3L, 3P). Heparan sulfate is an extensively sulfated glycosaminoglycan that is highly abundant in the lacrimal gland basal membrane. We found low levels of MMP-2 and -9 expression in the lacrimal gland epithelial cells of control MRL/+ (Figs. 3A–D) and BALB/c (Figs. 3I–L) mice, whereas in the diseased glands of MRL/lpr (Figs. 3E–H) and NOD mice (Figs. 3M–P), MMP-2 and -9 levels were strongly upregulated. A strong upregulation of MMP-9 expression was found in the lacrimal gland epithelial and the inflammatory cells within the foci (Figs. 3E, 3G, 3H; 3M, 3O, 3P, white asterisk) in both MRL/lpr and NOD mouse models of Sjögren's syndrome. Metalloproteinase 2 expression was mostly restricted to the epithelial cells within the damaged acini, surrounding the inflammatory foci (Figs. 3F–H, 3N–P). Very little or no expression of MMP-2 was found within the infiltrating cells (Figs. 3E, 3N, white stars). In addition, double-labeling experiments revealed the presence of single labeled cells—MMP-2⁺ cells, MMP-9⁺ cells—and double positive cells, MMP-2⁺/MMP-9⁺ cells (see example in Figs. 3Q–S). We also found that some cells in the lacrimal gland stroma showed strong surface MMP-2⁺/MMP-9⁺ staining, most likely reflecting release of MMPs into the stroma (Fig. 3S, inset). These data suggest that both MMP-2 and -9 protein expression levels were increased in chronically inflamed lacrimal glands of MRL/lpr and NOD mice.

Western Blot Analysis of MMP-2 and -9 Protein Expression in the Lacrimal Gland of MRL/lpr and NOD Mice

To further confirm that MMP-2 and -9 expression and activity levels are upregulated in diseased lacrimal glands, we assessed the MMP-2 and MMP-9 protein expression using Western blotting. Similar to what was seen with immunostaining, there was a significant increase in the expression of the active forms of MMP-2 and MMP-9 in MRL/lpr and NOD mice compared to MRL/++ and BALB/c mice, respectively (Fig. 4).

FIGURE 2. Enzymatic activity of MMP-2 and MMP-9 in MRL/lpr and NOD mice lacrimal glands compared to their respective control strains. Lacrimal gland lysates from MRL/lpr, MRL/++, NOD, and BALB/c mice were analyzed for enzymatic activity using SDS-PAGE gelatin zymography. *Left:* Matrix metalloproteinase 2 and MMP-9 activities in MRL/lpr and MRL/++ lacrimal glands. The plot shows quantitative analysis of MMP-2 and MMP-9 activities using densitometry. *Right:* Matrix metalloproteinase 2 and MMP-9 activities in NOD and BALB/c mice. The plot shows quantitative analysis of MMP-2 and MMP-9 activities using densitometry. Data are presented as mean $\pm$ SEM ($n = 5$).

Taken together, our data imply that there was a substantial increase in protein expression and activity of MMP-2 and -9 in inflamed lacrimal glands of MRL/lpr and NOD mice, suggesting the involvement of these MMPs in the lacrimal gland tissue injury processes.

Tear Production in MRL/lpr Mice Treated With an MMP2/9 Inhibitor Peptide

Increased activity and expression levels of MMP-2 and -9 in diseased mice prompted us to test the hypothesis that

inhibiting the activity of MMP-2 and -9 might restore lacrimal gland repair and adequate tear production. To test this hypothesis, MRL/lpr mice received daily IP injections (100 μ g/mouse) of either a control peptide (STT peptide) or an MMP2/9 inhibitor peptide (CTT peptide) for 5 weeks. The MMP2/9 inhibitor is a hydrophobic cyclic peptide that acts as a potent, nontoxic, and specific inhibitor of MMP-2 and MMP-9 with a reported IC₅₀ of 10 μ M.^{54,55} Before treatment of the mice with the inhibitor or control peptide, an *in vitro* experiment was performed to test the peptide's inhibitory effect using lacrimal gland cell lysates isolated from diseased

FIGURE 3. Expression of MMP-2 and MMP-9 in lacrimal glands of MRL/++ and MRL/lpr mice. Lacrimal glands were removed and processed for immunohistochemistry. Tissue sections were stained with a mouse polyclonal antibody against MMP-2, a rabbit polyclonal antibody against MMP-9, and a rat monoclonal antibody against heparan sulfate proteoglycan. Cell nuclei were counterstained with DAPI. (A–H) Matrix metalloproteinase 2 and -9 expression in lacrimal gland sections of MRL/++ and MRL/lpr mice; (I–P) Matrix metalloproteinase 2 and -9 expression in BALB/c and NOD mice. (Q–S) Matrix metalloproteinase 2⁺/MMP-9⁺ staining of cells in the lacrimal gland stroma of MRL/lpr mice. Scale bars: 20 μm (A–P); 50 μm (Q–S). *Inset* in (S) is a digital magnification of the area denoted by an *asterisk*.

FIGURE 4. Western blot of MMP-2 and MMP-9 expression in MRL/lpr and NOD mouse lacrimal glands compared to their respective control strains. Lacrimal gland lysates from MRL/lpr, MRL/+, NOD, and BALB/c mice were analyzed for protein expression using SDS-PAGE and Western blotting. Blot shows MMP-2 and MMP-9 active and pro forms in MRL/lpr, MRL/+, NOD, and BALB/c mouse lacrimal gland homogenates.

mice. As shown in Figure 5, the CTT peptide decreased both MMP-2 and MMP-9 activities, whereas zymograms incubated with the control STT peptide or with developing buffer (negative control) showed no effect on MMP-2 and -9 activities.

Following 5 weeks of treatment with the MMP2/9 inhibitor, tear production was significantly increased (8.3 ± 1.6 mm/10 s, $P < 0.05$) in MRL/lpr mice compared to the baseline level (day 1 treatment) (3.6 ± 0.8 mm/10 s). In contrast, there was no statistically significant difference in tear production in MRL/lpr mice treated with the control peptide (5.5 ± 0.98 mm/10 s) relative to the baseline level (3.8 ± 0.8 mm/10 s) (Fig. 6).

Enzymatic Activity of MMP-2 and -9 in MRL/lpr Treated With an MMP2/9 Inhibitor Peptide

We next evaluated the effect of the peptide inhibitor treatment on protein activity of MMP-2 and MMP-9 in the lacrimal glands of MRL/lpr mice. The enzymatic activities of MMP-2 and -9 in lacrimal gland homogenates prepared from mice treated with the MMP2/9 CTT inhibitor or the control STT peptide were measured using zymography. As shown in Figure 7, active MMP-2 (62 kDa) band was significantly decreased by 35% ($P < 0.05$) in mice treated with the MMP2/9 inhibitor compared to those treated with the control peptide. Similarly, active MMP-9 (82 kDa) band was substantially decreased by 52% ($P < 0.05$) in MMP2/9 inhibitor-treated mice (Fig. 7). These data suggest that treatment with the MMP2/9 inhibitor effectively reduced the activity of MMP-2 and -9 in diseased mice with chronic lacrimal gland inflammation that presumably leads to increased tear production.

FIGURE 5. Evaluating the inhibitor/CTT peptide or control/STT peptide inhibitory effect in vitro on MRL/lpr lacrimal gland lysates. MRL/lpr mouse lacrimal gland lysates were electrophoresed through SDS-PAGE gelatin zymogram and the zymogram divided into three parts; each part was incubated with either developing buffer or STT control peptide or CTT inhibitor peptide. After incubation, the gels were stained with SimplyBlue SafeStain and the proteolytic activity was detected as a clear band against a blue background.

Lymphocytic Foci Number and Size in MRL/lpr Mice Treated With an MMP2/9 Inhibitor Peptide

To examine whether MMP2/9 inhibitor treatment might ameliorate lymphocyte accumulation in lacrimal glands of MRL/lpr mice, lacrimal gland sections were stained with H&E and lymphocytic foci (number and size) were analyzed as described in Materials and Methods. As shown in Figure 8, following 5 weeks of treatment, the average number of lymphocytic foci was significantly lower (1.0 ± 0.25) in MMP2/9 inhibitor-treated MRL/lpr mice compared to those treated with the control peptide (2.32 ± 0.40). In addition, there was a 2.5-fold decrease (3.21 ± 0.85) in the area occupied by the lymphocytic infiltrates in animals treated with the MMP2/9 inhibitor when compared to those treated with the control peptide (6.16 ± 1.04) (Fig. 8). These data show that the number and size of lymphocytic foci were significantly reduced in the lacrimal glands of MRL/lpr mice following treatment with the MMP2/9 inhibitor, which may explain the improvement in lacrimal gland function.

DISCUSSION

In this study, we report that MMP-2 and -9, protein and gene expression levels are upregulated in chronically inflamed lacrimal glands from MRL/lpr and NOD mice, suggesting their possible role in continuous degradation of lacrimal gland ECM and thus impeding initiation of MET and lacrimal gland regeneration. Decrease of MMP-2 and -9 activities with a specific inhibitor peptide improved histologic appearance of the lacrimal gland in MRL/lpr mice along with enhanced aqueous tear secretion. Our data imply that inhibition of MMP2/9 in diseased lacrimal gland results in deposition of an ECM that promotes tissue repair and ultimately increased aqueous tear production.

Metalloproteinases 2 and 9, also known as gelatinases, are involved in the breakdown of ECM proteins during embryonic development and tissue remodeling and regeneration, as well as in disease processes.³⁴ Extracellular matrix is an essential component of stem cell niche and is a crucial regulator of epithelial cell polarity. Matrix metalloproteinase 2 and/or 9 proteolytically digest several ECM proteins including collagen, decorin, elastin, fibrillin, and laminin (expressed in the lacrimal gland) and can also activate several cytokines, chemokines, and growth factors such as pro-TGF- β and pro-TNF α .³⁴ Studies in patients with Sjögren's syndrome suggest that increased activity and expression of MMP-2, -3, and -9 triggers the destruction of the lacrimal and salivary glands.⁴⁹⁻⁵¹ The ratio between MMPs and TIMPs in Sjögren's syndrome patients correlates with degree of inflammatory infiltration and acinar

FIGURE 6. Effect of MMP2/9 inhibitor peptide treatment on aqueous tear production in MRL/lpr mice. Female MRL/lpr mice received daily IP injections (100 μ g/mouse) of either a control peptide (STT peptide: H-Ser-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Ser-OH) or MMP2/9 inhibitor (CTT peptide: H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys-OH) for 5 weeks. Aqueous tear production from both eyes was measured using phenol-impregnated cotton threads for 10 seconds. *Left:* Aqueous tear production following 5-week treatment compared to baseline (day 0) in MRL/lpr mice treated with CTT inhibitor peptide. *Right:* Aqueous tear production following 5-week treatment compared to baseline (day 0) in MRL/lpr mice treated with STT control peptide. Data are presented as mean $\pm$ SEM ($n = 3-4$).

structure integrity.⁵⁷ Furthermore, along with the ECM turnover described in NOD mouse lacrimal glands, MMP-2 and -9 protein expression levels as well as activities were upregulated, suggesting involvement in ECM remodeling.^{22,23} Moreover, involvement of MMP-9 activity in EMT has been recently demonstrated in studies reporting that decreased expression of MMP-9 downregulates activation of EMT.⁵⁸⁻⁶⁰

There is currently no universal animal model that mirrors precisely the complexity and chronicity of human Sjögren's syndrome. However, the MRL/lpr and the NOD mouse models are excellent models that mimic different pathologies of this frequent and debilitating autoimmune-driven lacrimal gland deficiency. Both strains develop spontaneous age- and sex-dependent infiltration of autoreactive lymphocytes, which leads to loss of parenchymal tissue resulting in aqueous deficient type of dry eye, hallmarks of human Sjögren's syndrome.⁶¹⁻⁶³ In this report, our data from immunohisto-

chemistry, protein and gene expression, and zymography studies confirm that MMP-2 and -9 activity and expression levels are significantly increased in both mouse models of Sjögren's syndrome. Matrix metalloproteinase 2 and 9 expression levels were upregulated not only in areas of lymphocytic infiltration in NOD and MRL/lpr lacrimal glands, but also in areas that did not have immune cells, which supports earlier findings in the NOD mouse model.²³ This finding suggests that increase in MMP levels throughout the gland induces a general process of ECM degradation and predisposes diseased lacrimal glands to lymphocytic cell invasion/accumulation.

Studies of other tissues in various inflammation models have demonstrated that inhibition of gelatinase activity effectively attenuates lymphocytic infiltration.⁶⁴⁻⁶⁷ Thus specific inhibitors of MMPs were shown to reduce invasiveness and migration of tumor cells.⁶⁸ A specific inhibitor of MMP2/9, the CTT peptide, was shown to selectively decrease endothe-

FIGURE 7. Enzymatic activity of MMP-2 and MMP-9 in MRL/lpr mice treated with STT and CTT peptides. Following 5-week treatment with inhibitor or control peptide, lacrimal glands from MRL/lpr mice were harvested, and the tissue lysates were analyzed for enzymatic activity using SDS-PAGE gelatin zymography. *Top:* Zymogram showing MMP-2 and MMP-9 activities in MRL/lpr mice treated with inhibitor or control peptide for 5 weeks. *Bottom:* Quantitative analyses of MMP-2 and MMP-9 activity using densitometry. Data are presented as mean $\pm$ SEM ($n = 3-4$).

FIGURE 8. Influence of CTT and STT peptide treatment on lymphocyte infiltration in MRL/lpr mice. Following 5-week treatment with the inhibitor peptide or control peptide, lacrimal glands from MRL/lpr mice were harvested and processed for histopathology. The number of lymphocytic foci and their size were determined in five to eight nonconsecutive sections per gland as described in Materials and Methods. *Left:* Changes in number of lymphocytic infiltrate focus following 5 weeks of treatment with control or inhibitor peptide. *Right:* Changes in focus size (% of total) following 5 weeks of treatment with control or inhibitor peptide. Data are presented as mean $\pm$ SEM ($n = 3-4$).

lial and tumor cell migration in vitro as well as tumor progression in vivo.⁵⁴ Similarly, the MMP2/9-specific inhibitor reduced neutrophil and T cell recruitment and improved subsequent liver damage following ischemia-reperfusion.⁶⁹ The data from the present study show that inhibition of MMP2/9 activity significantly improved the structure and secretory function of the lacrimal gland in diseased MRL/lpr mice following 5 weeks of treatment. Together, these data support the hypothesis that increased expression of MMPs by lymphocytic infiltrates and epithelial cells impedes tissue repair and secretion of aqueous tears.

In summary, our results show that modulation of MMP2/9 activity improves tear production and decreases lymphocytic infiltration, suggesting that modulation of MMP2/9 activity could be used as a potential therapeutic target for patients suffering with Sjögren's syndrome dry eye.

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