Editorial Review

Cytokines, Adhesion Molecules, and Immune Deviation in Autoimmune Salivary Gland Disease

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The immunopathology of most autoimmune diseases has been well defined, but the mechanisms responsible for the breakdown of self-tolerance and which lead to the development of autoimmune lesions are still unclear. Primary Sjögren's syndrome in humans is known to be an autoimmune disease characterized by diffuse lymphoid cell infiltration in the exocrine organs, involving in particular the salivary and lacrimal glands. The pathogenesis of this syndrome remains unclear, but the majority of infiltrating cells in the salivary glands were CD4⁺ T cells both in humans and rodents. Since many cytokines and adhesion molecules are involved in the development of T cell-mediated autoimmunity, we have investigated local cytokine, and adhesion molecule gene expression using animal model for primary Sjögren's syndrome in MRL/lpr, and NFS/sld mice. Overexpression of interleukin-1(IL-1) β and tumor necrosis factor(TNF)- α , interferon(IFN)- γ , IL-6, IL-10, IL-12(p40) was detected in the salivary gland, and the up-regulation of ICAM-1, LFA-1, CD44, Mel-14 mRNA was found in accordance with autoimmune sialadenitis. In addition, we have analysed the expression of T cell antigen receptor(TCR)V β transcripts in the salivary gland tissues. Transcript for V β 8 was predominantly detected in the T cell infiltrating sialadenitis from the onset of the disease, suggesting that CD4⁺ T cells bearing TCR V β 8 are play essential roles to recognize unknown autopeptide in the autoimmune salivary gland disease.

Key words: Sjögren's syndrome, cytokine, adhesion molecule, T cell receptor, Animal model

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Introduction

There are possible pathways in which cytokines and cell adhesion molecules might play a crucial part in autoimmune disease processes. Cytokines which directly influence the immune system, such as IL-2, IL-4, IL-6, interferon(IFN)- γ , IL-10, and IL-12(p40), can obviously affect immune processes and thus play an important role in induction of autoimmunity. Cytokines play central roles on the regulation of immune responses, and also regulate their production and function of another(1-5), indicating that up-regulation of cytokine genes may be responsible for the development of various autoimmune diseases(6-10). An imbalance in the normally regulated cytokine system may affect the development and regression of autoimmune pathology. On the other hand, particular interest has focused on the up-regulation and enhanced expression of cell surface adhesion molecules at inflammatory lesions in a variety of tissues, where several adhesion molecules seem to play major roles in the development of autoimmune pathogenesis(11,12). In particular, intercellular adhesion is an essential process, and involves specific T

cell triggering to increase the affinity between intercellular adhesion molecule-1(ICAM-1) and lymphocyte function-associated antigen-1(LFA-1)(13). In addition, it has been suggested that autoreactive T cells bearing CD4 molecule may recognize unknown self antigen and play a crucial role in the pathogenesis of autoimmunity. Thus, clonally expanded T cells using a restricted T cell receptor(TCR) gene segments may be essential in the pathogenesis of various autoimmune diseases(14-16).

Primary Sjögren syndrome in humans is an autoimmune disease characterized by diffuse lymphoid cell infiltrates in the salivary and lacrimal glands, resulting in symptoms of dry mouth and dry eye due to insufficient secretion(17,18). The histopathological changes in the labial gland biopsy are characterized by focal and/or diffuse lymphoid cell infiltrate and parenchymal destruction. The spectrum of presentation of the disease is broad, ranging from the organ-localized dysfunction of exocrine gland to systemic complications such as liver, kidney and lung involvement(19). Moreover, a significant proportion of the patients may develop malignant lymphoproliferative disorders such as B cell lymphoma

and macroglobulinemia(20,21). It has been assumed that a combination of immunologic, genetic, and environmental factors may play a key role on development of the autoimmunity in the salivary glands(22), but pathogenetic mechanisms of the autoimmune salivary gland disease are still unclear. Although we have previously proposed murine models inducing autoimmune sialadenitis(23-27), there has not been an established appropriate animal model specific for Sjögren's syndrome in humans. Several animal models for studying autoimmune sialadenitis are known to develop spontaneously in autoimmune-prone mice(28-31). It has been reported that autoimmune sialadenitis most similar to human disease develop spontaneously in MRL/lpr mice in addition to the aggressive lymphadenopathy and generalized autoimmune disorder(30,31). We have investigated spontaneously occurring autoimmune sialadenitis in MRL/lpr mice, and in a newly established animal model for Sjögren's syndrome in NFS/sld mice from various pathogenetic viewpoints(32-35). To elucidate the mechanism of murine autoimmune salivary gland disease, we have analyzed the expression of cytokine and adhesion molecule genes on development and progression of the disease, and repertoire of TCR $V\beta$ genes of tissue infiltrating lymphocytes in the salivary glands.

The aim of this editorials is to review these studies performed in the mouse models for Sjögren's syndrome which have investigated the role of cytokines, adhesion molecules, and T cell receptor(TCR) repertoires in contributing to the pathogenesis of autoimmune salivary gland disease.

Histopathology of autoimmune salivary gland disease in animal model for Sjögren's syndrome

MRL/Mp-lpr/lpr(MRL/lpr) mice are known to develop severe autoimmune diseases in general with lymphadenopathy at a relatively young age (>15 wk), whereas organ-localized autoimmune sialadenitis develop more younger age(>12 wk). Histological sections from the salivary glands of MRL/lpr mice at ages ranging from 1 to 8 months were examined for signs of inflammatory infiltrates. Very early inflammatory infiltrates were present in the salivary glands of few mice aged 2 months, and then typical signs of autoimmune sialadenitis similar to those in Sjögren's syndrome in human(36,37) were frequently observed aged 3 months or more. Severely inflammatory lesions with parenchymal destruction in the salivary glands in MRL/lpr mice were found at aged 5- or 6-month-old. Immunohistochemically, the major proportion of infiltrating cells were CD3⁺, and CD4⁺, whereas lesser number of cells were CD8⁺ during the course of inflammatory lesion in the salivary glands.

We have recently established a new animal model for primary Sjögren's syndrome in NFS/sld mutant mice thymectomized 3 day after birth(3dTX)(35), bearing an autosomal recessive gene with sublingual gland differen-

tiation arrest(38). This mouse model is the first to develop spontaneously occurring autoimmune lesions exclusively localized in the salivary and lacrimal glands. Histopathological findings in the salivary glands after 4-week-old mice having periductal inflammatory infiltration are quite similar to those in human disease. Immunohistochemical analysis to identify cell populations revealed that a major proportion of infiltrating cells were CD3⁺, and CD4⁺ with a small number of CD8⁺, and B220⁺ cells from the onset of disease.

Expression of cytokine genes on the development of autoimmune salivary gland disease

Local cytokine gene expression in vivo has been analyzed by direct analysis of RNA obtained from the salivary gland tissues in both MRL/lpr, and 3dTX NFS/sld mice. The expression of cytokine genes(IL-1 β , TNF- α , IL-2, IFN- γ , IL-4, IL-6, IL-10, IL-12) was assessed using reverse transcriptase polymerase chain reaction (RT-PCR) in addition to the immunohistochemical analysis. Tissues were homogenized in guanidine thiocyanate solution, and total RNA preparation was performed as previously(39). The amplified product was visualized by UV fluorescence after staining with ethidium bromide. PCR products were blotted on a nylon membrane and hybridized to ³²P labeled cDNA cytokine probes. In MRL/lpr mice, we found significant TNF- α , IL-1 β , IL-10, and IL-12 gene expression in the salivary glands before the onset of typical inflammatory infiltrates, whereas other cytokine genes examined such as IL-2 and IL-6 did not detected in the salivary glands at 1 or 2 month of age. Subsequently, remarkable high expression of IL-6 gene observed in the salivary glands at 3 months of age when typical inflammatory lesions histopathologically similar to human sialadenitis began to develop. In addition, expression of these cytokine genes was constantly detected with advancing age until 8-months-old. To examine the particular cells responsible for various kinds of cytokine production, immunohistochemical analysis was performed using biotin-avidin immunoperoxidase method. Anti-TNF-, anti-IL-6-, and IL-10-positive mononuclear cells were detected within the periductal inflammatory infiltrates in the salivary glands at 3 month of age, and more increased number of positive cells were observed with aging(Fig. 1). Other cell components including epithelial cells in the salivary glands were all negative for anti-cytokine antibodies. The other cytokines examined were all negative in any cell components in the salivary glands of MRL/lpr mice. In 3dTX NFS/sld mice, we found the up-regulation of local cytokine genes(IL-1 β , TNF- α , IL-2, IFN- γ , IL-6, IL-10, IL-12) in the salivary glands from the very early inflammatory lesions, while expression of IL-4 gene did not detected during the course of disease. Of importance is that cytokine genes such as IL-1 β , TNF- α , IFN- γ , IL-10, and IL-12 were expressed in the salivary gland tissues before the onset of autoimmune sialadenitis in

Fig. 1: Immunohistochemical detection of infiltrating mononuclear cells positive for anti-IL-10 monoclonal antibodies in the salivary gland of MRL/lpr mice at 5 months (Immunoperoxidase staining).

both mouse model. IL-1 and TNF are produced by monocytes and macrophages, which are important mediators of immunological events. Local production of IL-1 and TNF has been suggested to be responsible for tissue injury in the affected organs in autoimmune diseases(40,41). The increase in gene expression of IL-1 β and TNF has been demonstrated in the renal lesion in MRL/lpr mice(42). Moreover, it has been shown that IL-1 and TNF could induce IL-6 expression in various organs in autoimmune diseases(43). It is possible that unregulated cytokine gene expression may be responsible for the induction of organ-localized autoimmunity. IL-6 was originally found in the culture supernatants of mitogen- or antigen-stimulated peripheral mononuclear cells and subsequently identified as a T cell-derived factor involved in the final maturation of B cells into antibody-producing cells (44,45). It has been suggested that an IL-6 autocrine mechanism plays a critical role in abnormal polyclonal activation of B cell in autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)(45,46). Moreover, a large amount of IL-6 was detected in synovial fluid of patients with RA(47). IL-6 is also demonstrated to activate T cells to induce IL-2 production, and function as a late-acting killer helper factor in the induction of cytotoxic T cell(48,49). In addition, age-associated increase in IL-6 has been demonstrated in MRL/lpr mice, suggesting that abnormal expression of the IL-6 gene may play an important role in the development of polyclonal B cell activation and glomerulonephritis(50). We found high levels of IL-10 and IL-12 p40 mRNA expression in autoimmune salivary gland diseases before the onset of autoimmunity in both MRL/lpr and NFS/sld mice. IL-10 has a multiple suppressive effect on various effector phases of the immune responses(51-53). It has been shown that IL-10 has a direct inhibitory effect on T cell proliferation by specifically downregulating IL-2 mRNA expression and IL-2 production by T cells and T cell clones belonging to different T helper subsets(54). Our

experiments demonstrated that local expression of IL-10 mRNA was consistently detected throughout the autoimmune salivary gland disease in both MRL/lpr and NFS/sld mice, suggesting that endogenous IL-10 produced by CD4⁺ T cells stimulates antigen-specific immune responses that were associated with up-regulation of MHC class-II mRNA in the salivary glands. Several studies have demonstrated that IL-12 has a striking effect on increasing production of IFN- γ from CD4⁺ T cells(55,56). IL-12 has been demonstrated to support proliferation of activated T cells by an IL-2-independent mechanism(57). It can be considered that IFN- γ production is primarily stimulated by IL-12 production from accessory cells as well as inflammatory cytokines such as IL-1 β and TNF- α . These data suggest that IL-12 may act as a stimulant that can be essential regulatory molecules on developing autoimmunity in the salivary glands in both MRL/lpr and NFS/sld mice. We can conclude that abnormal cytokine regulation may be responsible for the development and acceleration of autoimmunity, leading to tissue damage in the salivary glands. It is strongly suggested that in murine autoimmune sialadenitis local production of these cytokines may be involved in the cascade events that initiate and accelerate organ-localized autoimmunity in the salivary glands of both MRL/lpr and NFS/sld mice.

Expression of adhesion molecule genes in autoimmune salivary gland diseases

Using RT-PCR and Southern blot analysis, significant ICAM-1 gene expression was detected in the salivary glands before the onset of inflammatory infiltration in the autoimmune salivary gland disease in both MRL/lpr and NFS/sld mice(Fig. 2). Its expression was contiguously observed until late stage of the diseases. Expressions of LFA-1, CD44, and Mel-14 mRNAs were also detected in the salivary glands during the course of autoimmune diseases(Fig. 3) Immunohistochemically, ICAM-1-positive cells were observed mainly in vascular endothelial cells in the early stage of the autoimmune salivary gland disease. With advancing stage of the disease, a considerable number of ICAM-1-positive cells were seen in the inflammatory lesions in addition to the endothelial cells. LFA-1, CD44-, and Mel-14-positive cells were constantly detected within the inflammatory lesions during the course of diseases in both MRL/lpr and NFS/sld mice. It is known that intercellular adhesion is an essential process and involves specific T cell triggering to increase the affinity between ICAM-1 and LFA-1(13). Increase in gene expressions of adhesion molecules including ICAM-1, LFA-1, CD44, and Mel-14 was detected throughout the inflammatory lesions in the salivary glands. ICAM-1-positive cells were first observed in vascular endothelial cells before the onset of inflammatory lesions, indicating that the ICAM-1/LFA-1 pathway may play an important role for the initiation of

Fig. 2: Expression of IL-10, IL-12(p40), IFN- γ , and β -actin mRNA in the salivary glands of MRL/lpr mice of various ages demonstrated by Southern blot analysis of RT-PCR products.

Fig. 3: Expression of ICAM-1, LFA-1, Mel-14, and CD44, and β -actin mRNA in the salivary glands of 3dTX NFS/sld mice of various ages demonstrated by Southern blot analysis of RT-PCR products.

autoimmune salivary gland diseases.

We have analysed the splenic CD4⁺ T cells bearing activation markers in order to clarify whether self-reactive cells are activated or not in the periphery. FACS analysis showed that many activation markers (CD44, CD45RB^{low}, LFA-1, Mel-14^{low}) were up-regulated in the spleens of newly established animal model for Sjögren's syndrome. Previous studies demonstrated that mature T cell populations include cells that recognize and respond self-antigens, but these cells are normally harmful because they are not activated (58). It has been reported that only very small fractions of the large numbers of potential disease-causing T cells are spontaneously activated in T cell receptor transgenic model of murine models for autoimmune diseases (59,60).

Analysis of T cell receptor V β gene segment in autoimmune salivary gland disease

A recent study demonstrated that a predominant genes of TCR V β repertoires is present in the labial salivary glands in the patients with Sjögren's syndrome (61). We examined the TCR V β repertoire of infiltrating T cells in the salivary glands of MRL/lpr and NFS/sld mice using flow cytometry (FACS), immunohistochemistry, and RT-PCR analysis. Infiltrating lymphocytes isolated from the salivary gland tissues were analyzed by two color immunofluorescence studies using FACSCAN (Becton Dickinson). We can obtain a considerable amount of tissue infiltrating lymphocytes ranged from 7X10⁵/ml to 1.2X10⁶/ml for each salivary gland tissue by Ficoll-isopaque density (1090) gradient centrifugation. The salivary gland lymphocytes were serially harvested, counted, and stained with FITC- or PE-conjugated mAbs. At the same time, spleen cells and thymocytes were also analyzed. FACS analysis of TCR V β using the isolated infiltrating lymphocytes, when gated on CD4, revealed exclusively predominant of V β 8, and a small proportion of V β 6. Predominance of the V β 8⁺ cells was specific in the salivary glands because markedly lesser percentage of the gene was expressed on spleen cells in the same subject, respectively. FACS analysis using other mAbs to TCRs did not reveal any predominant pattern in any organs. Immunohistochemical staining with mAbs was performed on freshly frozen sections. A considerable proportion of infiltrating lymphocytes in the salivary glands were V β 8-positive throughout the inflammatory lesions, whereas a small proportion of V β 6-positive cells was detected. A particular accumulation of cells bearing V β 8 was not detected immunohistochemically in other organs such as spleen, thymus, and lymph nodes in the same subject.

RT-PCR analysis demonstrated significant V β 8 gene expression in the salivary gland tissues from the very early stage of inflammatory infiltrates (Fig. 4), and thereafter extensive age-related diversity of TCR V β

Fig. 4: RT-PCR analysis of T cell receptor V β gene expression. Expression of V β 8 mRNA demonstrated in the salivary gland of the very early stage of autoimmune lesion by RT-PCR.

gene usage was found until the late stage of autoimmune lesions. It is suggested that there is a limited heterogeneity of the repertoire of TCR V β genes in the cell population from the salivary gland tissues developing autoimmune sialadenitis in both MRL/lpr and NFS/sld mice. Recent reports have demonstrated evidences that immunological tolerance of T cells to self antigens is induced and maintained by various mechanisms including clonal elimination, clonal anergy, and suppressor activity, can be considered that one of the major mechanisms of self tolerance has been shown to involve clonal elimination of self reactive T cells within the thymus(62-67). It has been known that the cellular basis of T cell tolerance analyzed from the observation that certain TCR V β domains correlated with reactivity to particular antigens and a direct link has been observed between the reactivity to the products of minor lymphocyte stimulatory (Mls) Mls^a allele and TCR V β 6/V β 8.1 expressed on T cells. Mice expressing Mls^a or Mls^c are found to eliminate V β 6/V β 8.1 - or V β 3-bearing T cells from their maturation. Recent studies demonstrated that clonal deletion of potentially self-reactive cells does occur in lpr mice(68). It has been shown that T cells in AKR-lpr/lpr mice have undergone a clonal elimination of V β 8⁺ cells(69). Of importance is that these data appear to show direct evidence that V β 8⁺ cells with failure to delete self-reactive cells are present in developing autoimmune salivary gland diseases. In an experimental autoimmune encephalomyelitis (EAE), it has been known that the repertoire of the autoreactive T cells is restricted. The V β 8.2 and V β 4 segments were dominantly utilized by myelin basic protein-specific T cells in H-2u mice, while V β 17a was frequently observed H-2^s mice(70-72). Recent studies of human autoimmune diseases have provided evidence for oligoclonality by the restriction fragment length polymorphism method in the patients. It was demonstrated that T cells of synovial fluid in the patients with rheumatoid arthritis expressed TCR V β 7 transcripts(73) and brain biopsy specimens from multiple sclerosis preferentially expressed TCR V β 10

gene(74). These data strongly suggest the limited heterogeneity of TCR V β transcripts may be responsible for the development of autoimmune diseases also in humans and rodents. We confirmed evidence that in autoimmune sialadenitis of both MRL/lpr and NFS/sld mice there is a limited usage of TCR V β gene elements on the very early stage of autoimmune lesions, and that V β 8 cells may play an essential role for pathogenesis of autoimmunity in the autoimmune salivary gland diseases.

Adoptive transfer of autoimmune salivary gland disease into SCID mice

SCID mouse strain exhibits defective recombination of antigen receptor genes leading to an early arrest in T and B lymphocyte development(75). Construction of a functional human immune system in the SCID mouse has been pursued by introducing human lymphopoiesis by grafting fetal human thymus, lymph nodes and transferring immunocompetent lymphocytes from patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (76-78). We attempt to reconstruct organ-localized autoimmune lesions into SCID mice using the isolated mononuclear cells directly obtained from the inflamed submandibular salivary gland tissues of MRL/lpr mice. In order to determine whether MRL/lpr autoimmune sialadenitis can be transferred into SCID mice, the 1×10^6 isolated mononuclear cells directly obtained from the submandibular salivary gland tissues of MRL/lpr mice were intraperitoneally injected with no treatment. Sjögren's syndrome-like lesions in the salivary and lacrimal glands developed 8 weeks after the injection in SCID mice. This pathology resembles human Sjögren's syndrome, features of which develop in the submandibular(92%) parotid(92%) salivary glands, and in the lacrimal glands(75%) of SCID mice. Mild periductal inflammatory infiltrates were observed in the sublingual glands(33%). No other organs in each animal were affected in general histologically. Mice that received spleen cells from C3H/He(H-2^b) and BALB/c(H-2^d) donors did not develop any lesion

in general. These findings indicate that the inflammatory lesions in both the salivary and the lacrimal glands did not induced by a correspondence to H-2 haplotype. It was of great interest that transfer of the isolated mononuclear cells from inflamed submandibular salivary glands of MRL/lpr mice led to the development of Sjögren's syndrome-like lesions also in the salivary and lacrimal glands in recipient SCID mice. Immunohistochemical studies revealed that major proportion of infiltrating cells in transferred lesions was positive for CD4 and V β 8 and lesser number was positive for V β 6. These results demonstrate that CD4-and V β 8-bearing T cells may play crucial role on triggering the autoimmunity in the salivary and lacrimal glands. To confirm the TCR V β repertoire in Sjögren's syndrome-like lesions transferred into SCID mice, we have used RT-PCR to study the expression of T-cell antigen receptor β RNA containing particular variable region (V) elements, and found that transcripts for V β 8 was detected in all transferred SCID mice.

We conducted additional experiments to examine the preventive effect against development of these lesions by the isolated cells treated with each monoclonal antibody. It was clearly demonstrated that the treatment with anti-CD4 antibody was effective for prevention of development of inflammatory lesions in the parotid(95%), submandibular(94%), sublingual(100%), and lacrimal(100%) glands. Moreover, the treatment with anti-V β 8 antibody prevented the disease developing the parotid(93%), submandibular(92%), sublingual(94%), and lacrimal(93%) glands. This may indicate that CD4⁺V β 8⁺ T cells play essential roles on developing the diseases to recognize autoantigen in the salivary and lacrimal gland. It has been reported that human organ-localized autoimmune lesion in patients with multiple sclerosis could be transferred into SCID mice(79), but the murine transfer system in these experiments is the first to develop organ-localized autoimmune lesions resembling Sjögren's syndrome into SCID mice intraperitoneally(80). It can be considered that this transfer system is an useful tool for studying the pathogenetic mechanisms of human Sjögren's syndrome. These results imply that murine exocrinopathy resembling Sjögren's syndrome is essentially T-cell mediated, and inducible by activated T cells bearing mainly CD4 and V β 8 molecule, which are able to recognize shared autoantigen in both salivary and lacrimal glands. It is possible that therapies designed with anti-CD4 and TCR V β 8 may be useful in treating autoimmune diseases of the salivary and lacrimal glands. Moreover, we reported recently that antibodies to ICAM-1 in combination with anti-LFA-1 prevented adoptive transfer of Sjögren's syndrome in MRL/lpr mice into SCID mice(81), suggesting that therapies designed with anti-adhesion molecules may also be useful in treating autoimmune salivary gland disease.

Perspectives

Further experimental informations about the pathogenetic mechanisms in autoimmune salivary gland disease, the role of abnormal regulation in other cytokine network and various costimulatory signals, the molecular characteristics of TCR V β gene segment, nature of the etiologic autopeptide in immune recognition, and the function of autoreactive T cells in tissue damage will supply the postulates in new approaches. These postulates will provide a understanding of the basic mechanisms in human Sjögren's syndrome in detail, and also allow the appropriate treatment responsible for autoimmune salivary gland disease.

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