Augmented Expression of CD44 Splice Variants in Lymphoproliferative Disorder of the Lacrimal Gland in Sjögren's Syndrome

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Abstract: We explored the involvement of CD44 isoforms in lymphoproliferative disorder (LPD) of the lacrimal gland of a Sjögren's syndrome patient with unilateral LPD. The CD44 variant with the v6 exon was selectively detected from infiltrating lymphocytes in the gland with LPD, but not from infiltrating lymphocytes in the normal lacrimal gland, suggesting that the CD44 v6 variant exon may be closely associated with the development of LPD in Sjögren's syndrome. **Jpn J Ophthalmol 1997;41:312–318** © 1997 Japanese Ophthalmological Society

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Introduction

Sjögren's syndrome is a chronic autoimmune disease characterized by lymphoid infiltration into the lacrimal and salivary glands with destruction of acini and ductal cells. Exocrine gland destruction and/or dysfunction causes dry eyes and dry mouth, the main symptoms of this disorder. Although most patients suffer from exocrine gland dysfunction, only some develop systemic autoimmune lesions.

Lymphoproliferative disorders (LPD), including malignant lymphoma, develop in Sjögren's syndrome patients with greater frequency than in age-matched normal controls. Lymphomas are generally non-Hodgkin's lymphoma of B-lymphocyte origin.[1-3]

Cell adhesion molecules may be involved in the dissemination of cancer and lymphoma; CD44, a cell surface glycoprotein found in several isoforms, influ-

ences cellular adhesion, lymphocyte homing,[4,5] and lymphocyte activation.[6] The standard CD44 isoform (80-90 kD), which is the smallest CD44 protein, is expressed chiefly on hematopoietic cells[5,7] without any insertion and has a high affinity for hyaluronate.[8-10] Splice variant forms have additional domains in the membrane-proximal extracellular region of the CD44 standard form.[11-13] The epithelial form (130–150 kD), which has insertions of exons v3 to v10, is weakly expressed on normal epithelial cell subsets, but is highly expressed in carcinomas.[14] The splice variant containing only the v6 exon is overexpressed in highly metastatic pancreatic and mammary carcinoma in rat cell lines.[15,16] This variant form not only confers metastatic capability in carcinoma,[17] but is also overexpressed in aggressive non-Hodgkin's lymphomas.[18]

We recently encountered a rare form of Sjögren's syndrome in a patient suffering from unilateral enlargement of the lacrimal gland. Both lacrimal glands were biopsied and examined for the expression of CD44 isoforms using reverse transcriptase-polymerase chain reaction (RT-PCR) and immuno-

histochemical analysis. We found that expression of the CD44 variant containing the v6 exon was enhanced in LPD, but not in the sample without LPD, suggesting association of this specific isoform with the development of LPD in Sjögren's syndrome.

Subject and Methods

Patient and Samples

A 64-year-old woman who had been suffering from dryness of the skin and mouth first noticed bilateral parotid gland enlargement in 1988. The chewing gum test (9 mL/10 minutes), salivary gland scan (grade II),[19] and lip biopsy (grade II)[20] were done; results of a Schirmer test with anesthesia were 10 mm/5 minutes in each eye. Mild keratoconjunctivitis sicca was also found. The diagnosis was primary Sjögren's syndrome, according to the Japanese criteria.[21] A palpable mass corresponding to the left lacrimal gland appeared on the left upper eyelid in 1991. In 1992, with the patient's written informed consent, the mass was resected; a right lacrimal gland biopsy was also done (Figure 1). The patient's clinical course is described elsewhere.[22] Unfixed tissue blocks were placed in OCT compound (Miles, Naperville, IL, USA), snap-frozen in liquid nitrogen, and stored at −80°C. Cryostat serial sections (4 μm) from each block were transferred to glass slides for immunohistochemical analysis. Consecutive sections were subsequently placed in sterile Eppendorf (Madison, WI, USA) tubes for RNA extraction. Cut sections taken before and after RNA extraction were also stained with hematoxylin and eosin, using the standard method.
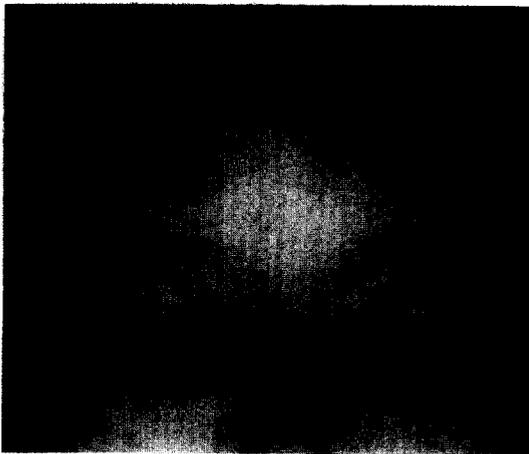
Figure 1. Sjögren's syndrome patient with palpable mass on left upper eyelid.

Cell Cultures

T-cell lines were cultured in RPMI-1640 and A-431 in DMEM (Dulbecco's Modified Eagle medium) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Control cell lines examined were: Jurkat (T-cell line, negative for CD44);[18] CEM (T-cell line, positive for CD44 variant containing exon v6);[18] and A-431 (human skin epidermal cell line, positive for CD44 variant containing exons v3–v10).[23]

RNA Extraction and RT-PCR

Frozen sections from each specimen block, and the cultured cell lines, were placed in sterile Ependorf tubes for RNA extraction. The samples were homogenized with Trizol Reagent (Gibco) and chloroform was added. After thorough mixing, they were centrifuged at 12 000g for 15 minutes at 4°C. The aqueous phase was removed and mixed with isopropanol at room temperature for 10 minutes. After centrifugation again (12 000g for 10 minutes at 4°C), the pellet was washed with 75% ethanol and centrifuged (7,500g for 5 minutes at 4°C). The resulting RNA was stored in diethyl pyrocarbonate water at −20°C for further processing.

For cDNA synthesis, 100 ng of total RNA was incubated at 65°C for 5 minutes, then immediately chilled on ice. A reaction mixture of 20 units of ribonuclease inhibitor (Takara Shuzo, Kyoto); 10× PCR buffer (500 mmol/L KCl, 200 mmol/L Tris-HCl buffer (pH 8.4), 25 mmol/L $MgCl_2$, 1 mg/mL of bovine serum albumin); 1.25 mmol/L dNTPs (deoxyribonucleoside triphosphates; dATP, dCTP, dGTP, dTTP; Pharmacia LKB Biotechnology, Uppsala, Sweden); 10× hexanucleotide mixture (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA); 0.1% dithiothreitol (Aldrich Chemical, Milwaukee, WI, USA); and 3 units reverse transcriptase-derived Rous-associated virus 2 (Takara Shuzo) were added to the RNA solution and incubated at 42°C for 60 minutes. After incubation, the solution was heated at 94°C for 5 minutes and chilled on ice. Synthesized cDNAs were diluted with autoclaved distilled water and stored at −20°C. For each PCR reaction, 50 μL of diluted cDNA was used for clinical samples and 10 μL was used for cell lines. This permitted 10 amplifications from each sample. For the PCR assay, the cDNA mixture was combined with 10 pmol/L of 5′ and 3′ primers, 1.25 mmol/L of dNTPs, and 1 unit of ther-

Table 1. Sequence of Primers and Probes Used for PCR

Primer	Probe Sequence
H1	5' GACACATATTGCTTCAATGCTTCAGC 3'
H2	5' GATGCCAAGATGATCAGCCATTCTGGAAT 3'
H3	5' CCTGAAGAAGATTGTACATCAGT 3'
V1	5' AGGCAACTCCTAGTAGTACAACG 3'
V3	5' CCAGAGAAGACTCCCATTCG 3'
GAPDH 5' primer	5' CCATGGAGAAGGCTGGGG 3'
GAPDH 3' primer	5' CCAAAGTTGTCATGGATGACC 3'
GAPDH probe	5' TCAAGATCATCAGCAATGCC 3'

mostable Taq polymerase (Boehringer Mannheim). Light mineral oil was added to prevent evaporation.

The sequences of the primers and probes were specific, and confirmed by a computer search of updated versions of GenBank (Table 1). The reaction was started by denaturing the RNA-cDNA hybrid by heating at 94°C for 30 seconds, annealing the primers at 55°C for 30 seconds, and extending the primers at 72°C for 1 minute. The cycle was repeated 35 times with a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA). After the final cycle, the temperature was maintained at 72°C for 10 minutes to allow reannealing of the amplified products, and the mixture was then chilled. Ten microliters of the reaction mixture was electrophoresed in 1.7% agarose in 1× Tris-acetate/EDTA electrophoresis buffer, containing ethidium bromide. The DNA molecular weight marker VI (pBR328 DNA *Bgl*I-digested + pBR328 DNA *Hinf*I-digested) (Boehringer Mannheim) was run parallel as a molecular weight marker. The visualized DNA was photographed with type 667 film (Polaroid, Cambridge, MA, USA). The

specificity of the amplified bands was validated by their predicted size. The PCR products were transferred onto nylon membranes, hybridized with digoxigenin-labeled probes, and analyzed with an alkaline phosphatase-labeled anti-digoxigen antibody and a chemiluminescent substrate, AMPPD ([3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxetane]; DIG Luminescent Detection Kit; Boehringer Mannheim), according to the manufacturer's instructions. These membranes were then autoradiographed with intensifying screens.

RT-PCR Analysis

The following experiments were designed to precisely determine the mRNA expression of CD44 in the lacrimal glands from our Sjögren's syndrome patient. The needed RNA was extracted from thin frozen sections and cultured cells. cDNAs synthesized from these RNA samples were amplified by PCR using primers and were hybridized with probes. (Glyceraldehyde-3-phosphate dehydrogenase amplification
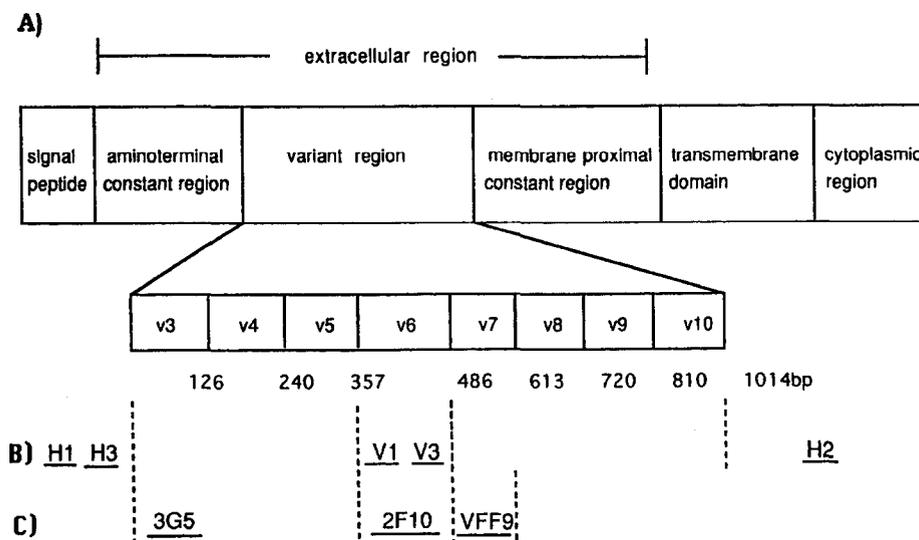
Figure 2. (A) Schematic representation of CD44 molecule. Putative CD44 variants carry epitopes encoded by variant exons v3–v10.[12] (B) Location of epitopes coded with designed primers and probes. (C) Location of epitopes recognized by monoclonal antibodies 3G5, 2F10, and VFF9.

was used to demonstrate that samples contained similar amounts of cDNA. With specific primers (H1 and H2 in Figure 2) and a specific probe (H3 in Figure 2), each cDNA was amplified by PCR and hybridized to detect the standard form of CD44 (Figure 3A). The expression of the standard form was similar in both lacrimal glands. In addition, to detect the variant forms including exon v6 and downstream, the 5' primer was set in part of exon v6 (V1 in figure 2) and H2 oligonucleotide (in Figure 2, Table 1) was used for the 3' primer. The probe (V3 in Figure 2, Table 1) was located in exon v6, which was not cross-linked to the V1 primer above. With these pairs of primers, the PCR products containing only exon v6 are expected to appear on 284 base pairs, and those including exons v6 to v10 on 812 base pairs.

Immunohistochemical Methods

For immunohistochemical study, acetone-fixed (−20°C acetone for 10 minutes) serial frozen sections, placed on gelatin-coated slides, were incubated with murine monoclonal antibodies to CD2, CD21 (Coulter Immunology, Hialeah, FL, USA), human Leu-CD44(CD44) (Becton-Dickinson Immunocytometry System, San Jose, CA, USA), and the variant forms of CD44 (3G5; anti-v3 (IgG 2b), 2F10; anti-v6 (IgG1) [R&D Systems Europe, Abingdon, UK] and VFF 9; anti-v7 (IgG1) [Bender Med Systems, Vienna, Austria]). Immunoglobulins of the same isotype, with irrelevant antibody activity, were used as negative controls. After rinsing, the sections were reacted with biotinylated goat anti-mouse (IgG + IgM) antibody (Tago, Burlingame, CA, USA), followed by a mixture of avidin and biotin-conjugated peroxidase (Vector Laboratories, Burlingame, CA, USA) and then the substrate, 3', 3'-diaminobenzidine.

Results

In the sample from the right lacrimal gland without LPD, no variant form was detected with the specific pairs of primers or with the probe. The v6 exon isoform was, however, found in the sample of the left lacrimal gland (with LPD); only the v6 exon of all the possible variants based on product size (Figure 3B) was found, clearly showing the difference from the right lacrimal gland.

Among the cell lines used to verify our RT-PCR process in the Jurkat line, which lacks CD44, neither the standard nor variant from of CD44 was detected; in the CEM cell line, which is known to express the variant with v6 exon, the products appeared at approximately 284 bp. The A-431 epidermal cell line,

which expresses the variant form that includes exons v3–v10, demonstrated its products at 812 bp.

Histological and Immunocytochemical Analysis

The sample from the right lacrimal gland revealed lymphoid cell infiltration around the acini and ducts; these infiltrating cells were primarily CD2-positive T lymphocytes (Figure 4A). The sample from the left lacrimal gland had almost no acini or ducts, but did show invasive infiltration of CD21-positive B lymphocytes with scattered CD2-positive T lymphocytes (Figure 4A, 4B). The standard CD44 form was expressed on infiltrating cells of both samples, even on
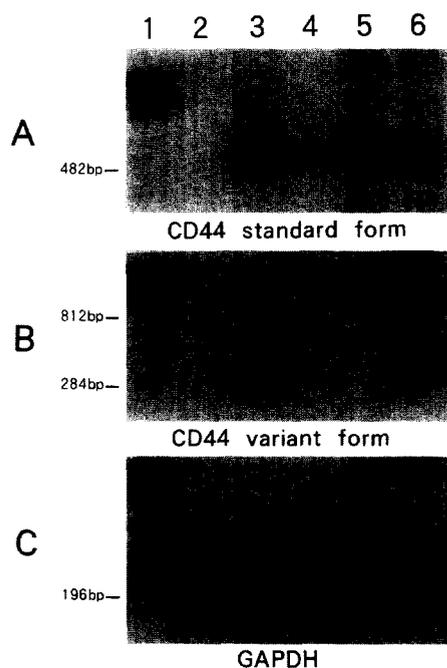
Figure 3. RT-PCR examinations of CD44 isoform mRNAs in lacrimal gland from Sjögren's syndrome patient. cDNA was synthesized from 100 ng of total RNA. Half of the cDNA from lacrimal gland and one-tenth of the cDNA from cell lines were used in PCR with specific primers. Southern blot analysis was then performed on amplified products with oligonucleotide probes. Column 1: DNA molecular weight marker VI. Column 2: Jurkat (CD44-negative T-cell line). Column 3: CEM (CD44 variant form containing v6 exon-positive T-cell line). Column 4: A-431 (CD44 variant form containing v3 – v10 exon-positive human epidermal cell line). Column 5: Right lacrimal gland without LPD. Column 6: Left lacrimal gland with LPD. (A) CD44 standard form was detected with specific primers (H1, H2) and probe (H3). (B) To detect CD44 variant form containing v6 exon, 5' primer (V1) was designed to locate within exon v6; for 3' primer, H2 primer was used. Amplified products were hybridized with v6-specific probe (V3). (C) Amplification and hybridization with GAPDH primers and probe were carried out on each sample.
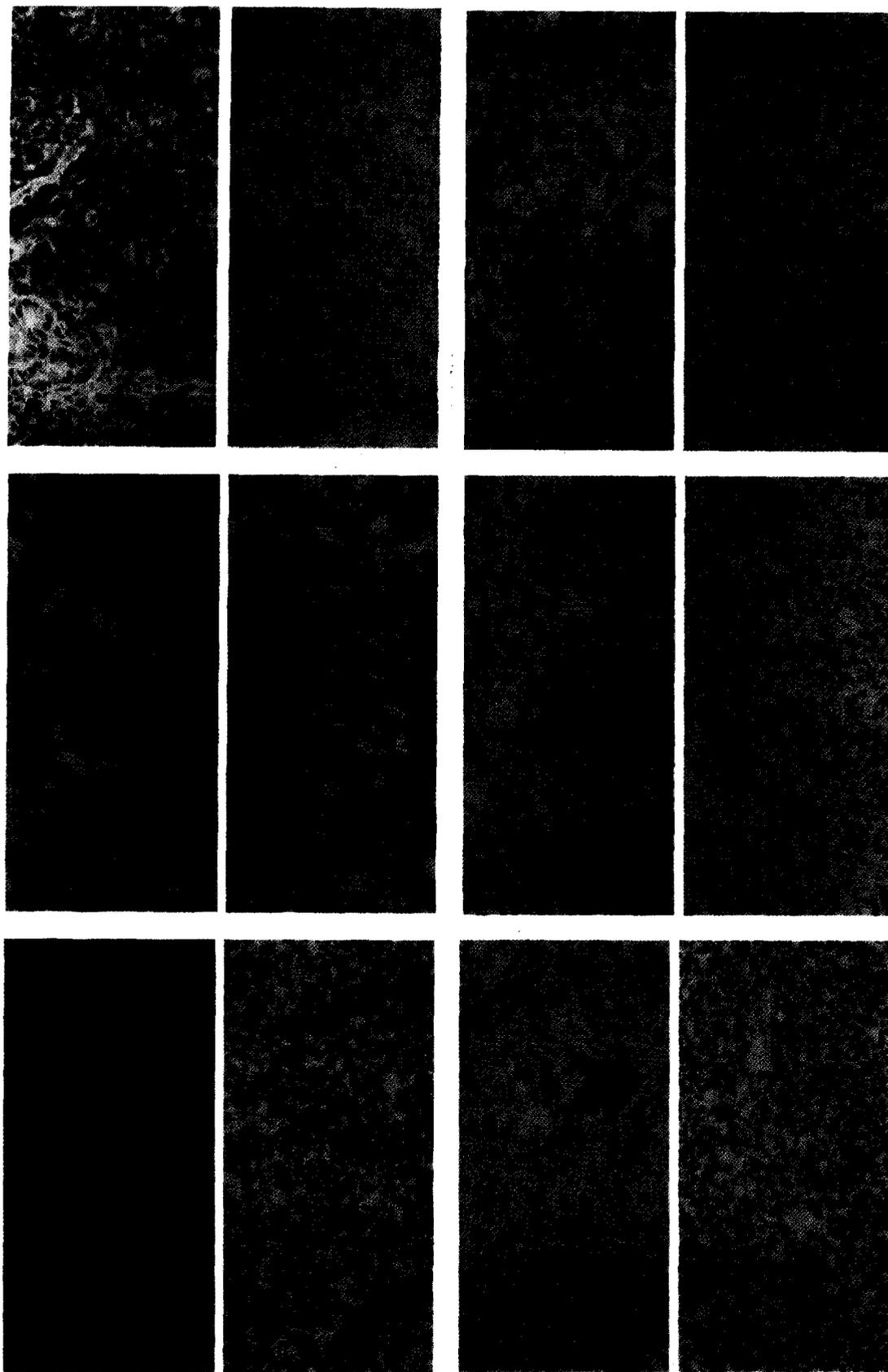
Figure 4. Hostological and immunohistochemical analysis of right (R) and left (L) lacrimal gland sections of Sjögren's syndrome patient. In right lacrimal gland, CD2+ T cells infiltrated and aggregated near acini and ducts (**A**). In left lacrimal gland, structure of acini and ducts was destroyed, and infiltrating cells were mainly CD21+ B cells (**B**). CD44 standard form was expressed on infiltrating cells in both samples (**C**). Variant forms coding exons v3, v6, and v7 were negative on epithelial cells of acini and ducts and on infiltrating cells in right lacrimal gland (**D, E, F**). In left lacrimal gland with LPD, infiltrating cells were positive for exon v6 (**E**), but not for exon v3 or v7 (**D, F**).

the acinar cells and ductal epithelium (Figure 4C). In the CD44 splice variants, infiltrating cells were negative for exons v3, v6, and v7 in the right lacrimal gland (Figures 4D, 4E, 4F), but in the left lacrimal gland (with LPD), exon v6 was diffusely expressed on infiltrating cells with distribution corresponding to the B lymphocytes (Figure 4E). Exons v3 and v7 were not observed (Figures 4D, 4F).

Discussion

In the present study, RT-PCR analysis demonstrated that the CD44 variant containing the v6 exon was selectively expressed in the left lacrimal gland with LPD, but not in the right lacrimal gland without LPD in a patient with primary glandular Sjögren's syndrome. Immunohistochemical analysis confirmed that CD21-positive B cells reacted with anti-CD44 v6 antibody. CD2-positive infiltrating lymphocytes surrounding the B cell foci, however, did not express the CD44 exon v6 variant. The standard CD44 form was universally expressed on infiltrating lymphocytes both in LPD and non-LPD lesions, irrespective of their cell lineage, as well as on ductal epithelia and acinar cells.

Sjögren's syndrome patients frequently develop bilateral, asymmetrical, painless enlargements of the parotid and/or lacrimal glands.[2] Biopsy specimens show dense infiltration of CD4+CD45RO+T lymphocytes around interlobular ducts with acinar atrophy.[24] B lymphocytes subsequently appear in the lesion, forming follicles. This lymphocyte proliferation may progress to form the characteristic epimyoepithelial islets. Infiltrating B cells are initially polyclonally activated, but oligoclonal or monoclonal populations eventually expand in the lesion. In this present case, the biopsy findings in the two lacrimal glands differed histologically: In the right, there were major T lymphocyte infiltrations around the ducts, and the acinar atrophy was remarkable; In the left lacrimal gland, there was dense B lymphocyte infiltration, with ductal and acinar structures completely destroyed, giving the characteristic appearance of LPD. Immunoglobulin gene rearrangement was not evaluated in this study; however, immunostaining by anti-light chain antibodies showed both isotypes on B cells in the left lacrimal gland, confirming polyclonal expansion or proliferation of B cells.[22]

CD44, a glycoprotein mediating cell-cell and cell-matrix adhesion, has several isoforms that contain additional exons inserted in the extracellular domain.[11-13] The functions of these variant isoforms have not been well defined, but it appears that some are related to tumor progression[18,25,26] and metastasis.[17] The CD44 isoform carrying the v6 exon is particularly linked to tumor dissemination, and is strongly expressed in aggressive non-Hodgkin's lymphomas.[18] In in vitro study, both T and B lymphocytes transiently express this variant form with mitogen or antigen stimulation.[18] In this study, the standard CD44 form was detected in infiltrating T lymphocytes of both lacrimal glands, and in the B lymphocytes of the left (LPD) lacrimal gland. It was also expressed in the ductal epithelia and acinar cells. These findings are consistent with previous reports.[23] In contrast, the variant form, with the v6 exon, was found solely in infiltrated B lymphocytes in the LPD. None of the infiltrating T cells or ductal cells expressed this form. It has been reported that splice variants containing exon v6 are expressed on several normal human epithelia and on carcinoma lines from lung, breast, and colon.[4,23] However, we could not detect CD44 splice variants on Sjögren's syndrome ductal epithelia. Our findings presumably reflect polyclonal B cell activation in the LPD lesion, indicated by the immunohistochemical analysis, although there is also the possibility that enhanced expression of the v6 variant is involved in recruiting activated B cells to the LPD lesion or in the development of LPD.

This is the first study showing enhanced expression of the CD44 v6 exon isoform in infiltrated B cells of LPD in Sjögren's syndrome. It is interesting to speculate that the expression of this isoform in these cells might signal development of LPD and a prelymphomatous lesion. If this is the case, monitoring the expression of the CD44 variant form in the biopsy specimens may be useful in predicting the emergence of LPD in Sjögren's syndrome. However, since this is a study of a single patient; more cases should be examined before any definitive conclusion is drawn. The clonality of infiltrated cells expressing the specific v6 exon variant of CD44 should also be determined precisely by both immunohistochemistry and Southern blot analysis, to identify the light chain restriction.

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