

Miwako Saito · Yasuhiro Ota · Hiroyuki Ohashi  
Yoshiaki Dei · Kumiko Shimoyama · Daisuke Suzuki  
Hideharu Hayashi · Noriyoshi Ogawa

## CD40-CD40 ligand signal induces the intercellular adhesion molecule-1 expression through nuclear factor-kappa B p50 in cultured salivary gland epithelial cells from patients with Sjögren's syndrome

Received: July 26, 2006 / Accepted: October 23, 2006

**Abstract** The purpose of this study is to clarify signal transduction of expression of the intercellular adhesion molecule-1 (ICAM-1) via CD40-CD40 ligand in salivary glands of patients with Sjögren's syndrome (SS). We used cultured salivary gland epithelial cells (SG cells) from 15 SS patients and 8 controls obtained by labial minor salivary gland biopsy. First, ICAM-1 expression was determined with reverse transcriptase–polymerase chain reaction and flow cytometry in the presence or absence of soluble CD40L (sCD40L). Next, SG cells were transfected with plasmids of pGL1.3-Luc inserted with promoter region of ICAM-1, pGL1.3kB<sup>-</sup>-Luc mutated in nuclear factor kappa-B (NF-κB) binding site of pGL1.3-Luc and pNF-κB-Luc by lipofection method. Luciferase activity of the cells was measured in the presence or absence of sCD40L and an NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC). Finally NF-κB family proteins of cell nuclear extracts were determined. ICAM-1 expression was significantly enhanced with sCD40L at the mRNA and protein level. Activity of pNF-κB-Luc and pGL1.3-Luc was significantly elevated by stimulation with sCD40L and suppressed by PDTC. NF-κB p50 protein level was elevated by stimulation with sCD40L and suppressed by PDTC. Our results suggest that sCD40L enhances the ICAM-1 expression by activation of NF-κB p50 in the SS SG cells.

**Key words** CD40 ligand · Intercellular adhesion molecule-1 · Nuclear factor-kappa B · Salivary gland epithelial cells · Sjögren's syndrome

### Introduction

Sjögren's syndrome (SS) is an autoimmune disease of exocrine glands, involving particularly salivary and lacrimal glands. Sjögren's syndrome may occur alone (primary SS), or in association with a variety of other connective tissue diseases (secondary SS).<sup>1</sup> Sjögren's syndrome can be a systemic disease in which patients also have problems with vasculitis, arthritis, gastrointestinal tract, kidneys, lungs and muscles. Sjögren's syndrome is related to rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In SS patients, inflammatory process in the salivary gland may affect peripheral blood and other organs. However, how the autoimmune process develops is not clear. We tried to analyze autoimmune reaction of the SS salivary gland by using an in vitro culture system.

In the SS salivary gland, high expression of CD40 ligand (CD40L) was identified in infiltrating lymphocytes, which are composed of mainly CD4<sup>+</sup> T cells and partly CD8<sup>+</sup> T cells.<sup>2</sup> CD4<sup>+</sup> T cells overexpress Th1 type cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN-γ), and major histocompatibility complex (MHC) class II molecules.<sup>1,3–5</sup> Th1 cytokines induce expression of CD40 on the surface of salivary gland epithelial cells.<sup>2,5</sup> CD40 is usually expressed on monocytes, dendritic cells, endothelial cells, and epithelial cells,<sup>6</sup> and the expression of CD40 in the SS salivary gland is significantly higher than that in controls.<sup>2,6</sup> This suggests the possibility that there is CD40-CD40L interaction in salivary gland of SS patients, which may contribute to the development of inflammation of the SS salivary gland.<sup>5</sup> For example, high expression of intercellular adhesion molecule-1 (ICAM-1)/CD54 in SS salivary gland cells was reported.<sup>2,7</sup> Therefore we tried to elucidate the signal transduction mechanism for the ICAM-1 expression via CD40-CD40L in SS salivary gland cells.

For this purpose we used cultured salivary gland epithelial cells (SG cells) obtained by labial salivary gland biopsy from SS patients. First, we investigated the expression of ICAM-1 of SG cells by reverse transcriptase–polymerase chain reaction (RT-PCR) and flow cytometry. Next, we ex-

M. Saito (✉) · Y. Ota · Y. Dei · K. Shimoyama · D. Suzuki ·  
H. Hayashi · N. Ogawa  
Third Department of Internal Medicine, Hamamatsu University  
School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan  
Tel. +81-53-435-2267; Fax +81-53-434-2910  
e-mail: miwako.t@nifty.com

H. Ohashi  
Omaezaki Municipal Hospital, Shizuoka, Japan

amined the signal transduction mechanism for the expression of ICAM-1 through CD40-CD40L interaction by using transfection. We found that nuclear factor- $\kappa$ B (NF- $\kappa$ B) is involved in the expression of ICAM-1. Finally, NF- $\kappa$ B subunit involved in the ICAM-1 expression was identified.

## Materials and methods

### Patients and controls

Fifteen patients (13 females and 2 males; age 36–70 years; mean  $\pm$  standard deviation  $55.1 \pm 12.8$  years) fulfilling the European classification criteria for SS<sup>8</sup> were recruited from University Hospital, Hamamatsu University School of Medicine (Shizuoka, Japan). Biopsy of minor salivary glands was performed as a routine part of the diagnostic evaluation of SS. The patient profile is shown in Table 1. As controls, minor salivary glands specimens were obtained from 8 healthy individuals (7 females and 1 male; age 30–76 years;  $57.2 \pm 19.5$  years) who had xerostomia, but whose tissues were histologically normal, and showed negative serology. Also as controls, normal parotid glands were obtained from 4 patients (2 females and 2 males; age 16–53 years;  $36.1 \pm 17.3$  years) with squamous cell carcinoma of the oral cavity who had neither radiotherapy nor chemotherapy before surgery.

### Reagents

Defined Keratinocyte-SFM with epidermal growth factor (EGF), bovine pituitary extract (BPE), OPTI-MEM 1 reduced serum medium and penicillin/streptomycin were obtained from Invitrogen (Palo Alto, CA, USA). Hydrocortisone and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma (St. Louis, MO, USA). Recombinant human IFN- $\gamma$  was provided by Shionogi Pharmaceuticals (Osaka, Japan). Recombinant human soluble CD40L was purchased from Alexis (Lausen, Switzerland).

### Primary cultures of salivary gland epithelial cells

SG cells for primary cultures were established from labial salivary glands or parotid glands. Cells were cultured based

on the previously described method.<sup>9</sup> Briefly, tissues were rinsed with cold, sterile phosphate-buffered saline (PBS) containing 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin and were minced into fragments of  $\sim 1$  mm<sup>3</sup>. One fragment of each tissue was placed in a collagen-type 1 coated 24-well tissue culture plate (Iwaki, Tokyo, Japan) and cultured in SG culture medium, at 37°C in 5% CO<sub>2</sub>. The medium was the mixture of Defined Keratinocyte-SFM with EGF, 25  $\mu$ g/ml BPE, 0.4  $\mu$ g/ml hydrocortisone, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. When confluent, the monolayer cells were trypsinized and subcultured into collagen-type 1 coated 6-well tissue culture plates (Iwaki). SG cells at passage 3 were seeded in 6-well culture plates until 60%–70% confluent, and used for the experiments.

### RNA isolation, cDNA synthesis, RT-PCR, and real-time PCR

Reverse transcriptase-PCR was used to measure the messenger ribonucleic acid (mRNA) of ICAM-1, CD40, CD40L, vascular cell adhesion molecule-1 (VCAM-1), CD80, CD86, Fas, Fas ligand (FasL), E-selectin, and  $\beta$ -actin (B.A.). RNA was extracted from harvested SG cells stimulated for 72 h with soluble CD40L (sCD40L) 800 ng/ml or IFN- $\gamma$  500 U/ml using Perfect RNA, Eukaryotic Mini kit (Eppendorf, Germany). Density of recovered total RNA was determined by the  $A_{260/280}$  ratio. RNA was synthesized to complementary deoxyribonucleic acid (cDNA) by using first-strand cDNA Synthesis (Roche Diagnostics Corp., Indianapolis, IN, USA). Reaction volume was 25  $\mu$ l. One  $\mu$ l cDNA, 0.5  $\mu$ l Taq polymerase, 2  $\mu$ l each sense and antisense oligonucleotide primers (Table 2),<sup>10,11</sup> 2.5  $\mu$ l deoxynucleoside triphosphate (dNTP) Mix, 1.5  $\mu$ l MgCl<sub>2</sub> and 2.5  $\mu$ l PCR buffer were used for DNA amplification by RT-PCR. Amplification was carried for 35 cycles, which included 1 min at 94°C, 2 min at 60°C (CD40, CD40L, Fas, B.A.) or 55°C (CD80, CD86, FasL, ICAM-1, VCAM-1, E-selectin, B.A.), and 2 min at 72°C. Amplified DNA was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light and photographed. Next, we quantitatively analyzed the expression of ICAM-1 mRNA by real-time PCR with RNA which was extracted as described above, both before and after stimulating with sCD40L. A relative quantification using the Taq Man method was performed as real-time PCR, with Taq Man

**Table 1.** Subjects

	Primary SS (n = 8)	Secondary SS <sup>a</sup> (n = 7)	Controls (n = 8)
Age (years, mean $\pm$ SD)	50.2 $\pm$ 10.7	60.7 $\pm$ 13.5	57.2 $\pm$ 19.5
Sex (Female/Male)	7/1	6/1	7/1
Biopsy focus score (mean $\pm$ SD)	4.2 $\pm$ 3.8	3.4 $\pm$ 2.7	0
Anti-Ro (SS-A) positive (%)	50.0	85.7	0.0
Anti-La (SS-B) positive (%)	12.5	14.3	0.0
Saxon test (g, mean $\pm$ SD)	1.55 $\pm$ 1.22	1.04 $\pm$ 0.42	2.35 $\pm$ 2.53

SS, Sjögren's syndrome

<sup>a</sup>Patients with secondary SS: 2 patients with systemic lupus erythematosus, 4 patients with rheumatoid arthritis, and 1 patient with mixed connective tissue disease

**Table 2.** Polymerase chain reaction primer sequences

Primer		Sequence
β-actin	Sense	5'-TACATGGCTGGGGTGTGAA-3'
	Antisense	5'-AAGAGAGGCATCCTCACCC-3'
CD40	Sense	5'-CAGAGTTCAGTCAAACGGAATGCC-3'
	Antisense	5'-TGCCTGCCTGTTGCACAACC-3'
VCAM-1	Sense	5'-ATGACATGCTTGAGCCAGG-3'
	Antisense	5'-GTGTCTCCTTCTTGACACT-3'
ICAM-1	Sense	5'-TATGGCAACGACTCCTTCT-3'
	Antisense	5'-CATTTCAGCGTCACCTTGG-3'
E-selectin	Sense	5'-CTCTGACAGAAGAAGCCAAG-3'
	Antisense	5'-ACTTGAGTCCACTGAAGCCA-3'
Fas	Sense	5'-CACTTCGGAGGATTGCTCAACA-3'
	Antisense	5'-TATGTTGGCTCTTCAGCGCTA-3'
Fas ligand	Sense	5'-CTGGGGATGTTTCAGCTCTTC-3'
	Antisense	5'-CCTCCATCATCACCAGATCC-3'
CD80 (B7-1)	Sense	5'-ACTCGCATCTACTGGCAAAGGA-3'
	Antisense	5'-ATGGGAGCAGGTTATCAGGAAAA-3'
CD86 (B7-2)	Sense	5'-TGGTGCTGCTCCTCTGAAGATTC-3'
	Antisense	5'-ATCATTCTGTGGGCTTTTTGTG-3'
CD40L	Sense	5'-ACATAACAACCAAACCTTCTCC-3'
	Antisense	5'-AGATGTTGTTTTACTGCTGGC-3'

VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule

Gene Expression Assay (Assay ID: Hs00164932\_m1) (Applied Biosystems, Foster City, CA, USA) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) as endogenous control gene on the ABI Prism 7700 Sequence Detection System (Applied Biosystems), and analyses were performed with Comparative  $C_T$  method.<sup>12</sup>

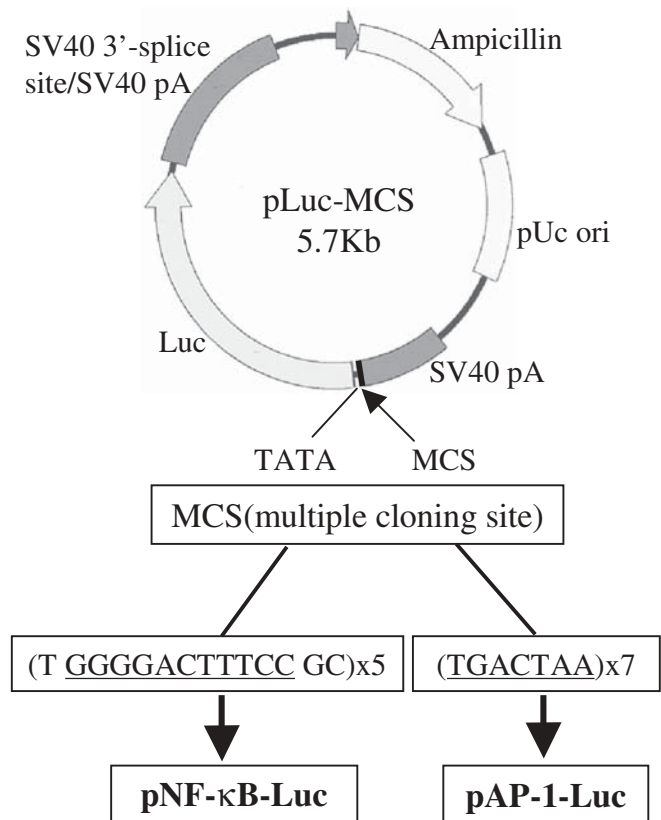
#### Flow cytometry

Harvested SG cells, which stimulated for 72 h with sCD40L 800 ng/ml or IFN- $\gamma$  500 U/ml, were transferred to tubes at the concentration of  $5 \times 10^4$  cells/tube and incubated in each 10  $\mu$ l antibodies at 4°C for 30 min. Antibodies used were as follows: phycoerythrin (PE) mouse IgG<sub>1,κ</sub>, PE mouse IgG<sub>2b,κ</sub>, fluorescein isothiocyanate (FITC) mouse IgG<sub>1,κ</sub>, FITC anti-human CD40, PE anti-human CD62E, PE anti-human CD54 (ICAM-1), PE anti-human CD106 (VCAM-1), PE anti-human CD40L (CD154), FITC anti-human CD80 (B7-1), PE anti-human CD86 (B7-2), and PE anti-human HLA-DR from BD Biosciences Pharmingen (San Diego, CA, USA), PE anti-human FasL, and PE anti-human Fas from Medical & Biological Laboratories (Nagoya, Japan), and PE anti-hamster IgG from Immunotech (Marseille, France). After washing twice with PBS and fluorescence activated cell sorting (FACS) buffer (5% fetal calf serum, PBS, human IgG, and 0.05% NaN<sub>2</sub>) was added, the cells were filtered through nylon mesh (Mesh 380, Tokyo screen, Tokyo, Japan) and then analyzed by flow cytometry (EPICS XL, Beckman Coulter, Fullerton, CA, USA).

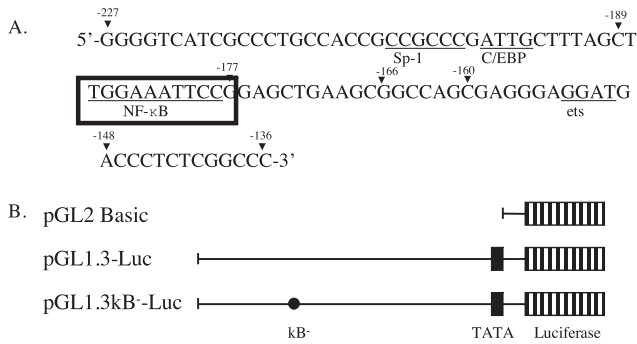
#### Plasmids

Plasmids of pNF- $\kappa$ B-Luc (PathDetect NF- $\kappa$ B cis-Reporting System) and pAP-1-Luc were purchased from Stratagene (La Jolla, CA, USA) (Fig. 1). The luciferase reporter vec-

#### The pLuc-MCS plasmid



**Fig. 1.** Circular map of pLuc-MCS plasmid and sequences of the enhancer elements of plasmids (pNF- $\kappa$ B-Luc, pAP-1-Luc). The pLuc-MCS plasmid is designed for the convenient insertion of enhancer elements upstream of the TATA box and the firefly luciferase gene. This plasmid features a multiple cloning site (MCS) for insertion of double-stranded oligonucleotides. The enhancer element of NF- $\kappa$ B and AP-1 is inserted in the MCS of “pNF- $\kappa$ B-Luc” and “pAP-1-Luc,” respectively



**Fig. 2A,B.** Functional consequences of mutating the intercellular adhesion molecule-1 (ICAM-1) promoter. **A** ICAM-1 sequence from -227 to -136 relative to the start of transcription, comprising the tumor necrosis factor (TNF)- $\alpha$  regulatory region, is shown. Potential transcription factor binding site is identified based on sequence homologies to their published consensus sequence: NF- $\kappa$ B (-176 to -187). **B** pGL1.3-Luc was made by inserted ICAM-1 promoter into the luciferase reporter vector (pGL2 Basic) and pGL1.3kB<sup>-</sup>-Luc was mutated NF- $\kappa$ B binding site from TGGAAATTCC to TctAgATTtag in the pGL1.3-Luc, as detailed in Materials and methods

tor, pGL2 Basic was obtained from Promega (Madison, WI, USA). The plasmids pGL1.3-Luc and pGL1.3kB<sup>-</sup>-Luc were kindly provided from Dr. Thomas P. Parks (Department of Inflammatory Disease, Boehringer Ingelheim Pharmaceuticals, CT, USA) (Fig. 2).<sup>13,14</sup> “pGL1.3-Luc” was made as follows: the *KpnI*-*Sall* fragment of pBHLuc1.3 containing 1344 base pairs of the ICAM-1 upstream region (-1353 to -9 relative to the start of transcription) was cloned into the luciferase reporter vector pGL2 Basic that had been digested with *KpnI* and *XhoI*. “pGL1.3kB<sup>-</sup>-Luc” was made as follows: the NF- $\kappa$ B binding site was mutated from TGGAAATTCC to TctAgATTtag in the context of the full-length reporter construct pGL1.3-Luc. “pSV- $\beta$ -galactosidase control vector” was obtained from Promega.

### Transfection

SG cells were seeded in 6-well culture plates until 60%–70% confluence. Plasmid DNA (pNF- $\kappa$ B-Luc, pAP-1-Luc, pGL2 Basic, pGL1.3-Luc, or pGL1.3kB<sup>-</sup>-Luc) was transfected by lipofection method. Cells were transfected with 2 ml of OPTI-MEM 1 reduced serum medium containing 2  $\mu$ g of DNA, by using 5  $\mu$ l of Lipofectin reagent (Invitrogen) or 6  $\mu$ l of FuGENE 6 transfection reagent (Roche Diagnostics) for 4–6 h at 37°C as previously described,<sup>15</sup> and then medium was changed with 1.5 ml of SG medium. Cells were incubated for 72 h under the stimulation with or without sCD40L 800 ng/ml or IFN- $\gamma$  500 U/ml or sCD40L 800 ng/ml and PDTC 100  $\mu$ M. Transfection efficiency was normalized using cotransfection with plasmid containing pSV- $\beta$ -galactosidase control vector. Transfected cells were subsequently harvested for determination of luciferase and  $\beta$ -galactosidase activities.

### Luciferase and $\beta$ -galactosidase assay

The transfected cells were washed twice with PBS and then 400  $\mu$ l of lysis buffer (supplied in Promega’s luciferase assay kit) was added to each well. After 15 min at room temperature, attached cells were scraped off from the dish. Cells and lysates were transferred to microcentrifuge tubes and performed a single freeze-thaw and centrifuged at 12,000  $\times$ g for 2 min at 4°C, then the supernatant was transferred to a new tube. Luciferase activity was determined by using 20  $\mu$ l of the lysate and 100  $\mu$ l of luciferase assay substrate (Promega) in the luminometer (Lumicounter700, Microtec NITI-ON, Chiba, Japan).  $\beta$ -Galactosidase assay was carried out using 150  $\mu$ l of the lysates as described in the protocol. The luciferase activity of each well was expressed as ratio to  $\beta$ -galactosidase activity in order to standardize transfection efficiency.

### Determination of NF- $\kappa$ B subtypes in nuclear extracts by transcription factor assay

After stimulating for 48 h with sCD40L 800 ng/ml, IFN- $\gamma$  500 U/ml or sCD40L 800 ng/ml and PDTC 100  $\mu$ M, SG cells were used for nuclear extract preparation by Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Then NF- $\kappa$ B activation was measured by using TransAM NF- $\kappa$ B Family (Active Motif) according to the manufacturer’s instructions.

### Statistical analysis

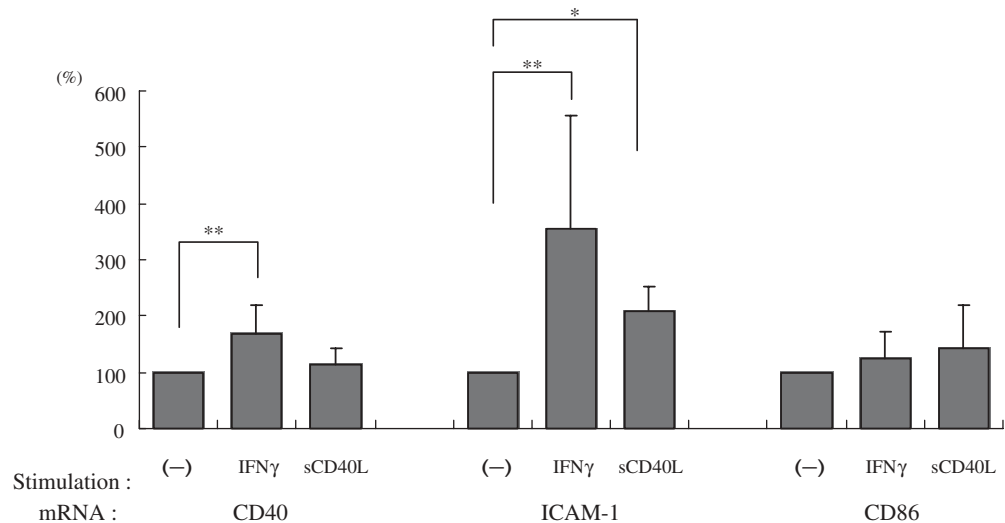
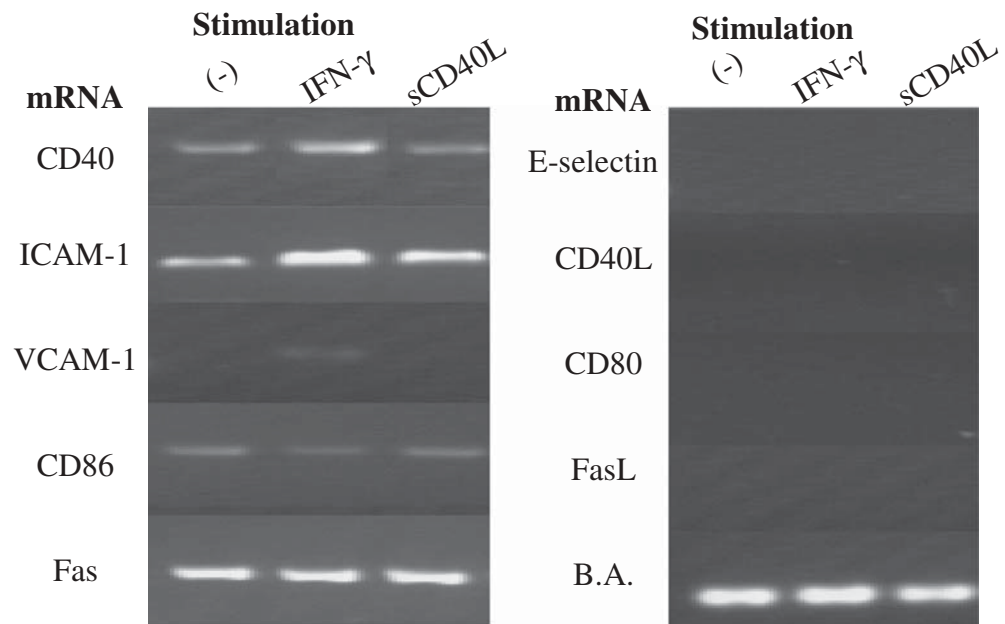
Correlations between SS patients and controls, and before and after stimulation were analyzed by the Wilcoxon *t*-test. Results were expressed as mean  $\pm$  standard deviation (SD) and *P* < 0.05 was considered statistically significant.

## Results

### Expression of ICAM-1 mRNA in SG cells by RT-PCR

First, we analyzed various immunoregulatory molecules which are thought important in the pathogenesis of SS, including co-stimulatory molecules (CD40, CD40L, CD86, and CD80), adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and apoptosis-related proteins (Fas and FasL), in SG cells.<sup>7,16,17</sup> For this purpose, mRNA expression of CD40, ICAM-1, VCAM-1, Fas, CD86, CD40L, E-selectin, CD80, and FasL was studied by RT-PCR in SG cells from 6 SS patients and 9 controls. The focus score of these 6 patients ranged from 1.0 to 7.3 (3.5  $\pm$  2.7). Figure 3 represents the mRNA expression in SG cells from an SS patient (focus score 6.1). SS SG cells expressed mRNA of CD40, ICAM-1, CD86 and Fas, but did not express mRNA of CD40L, E-selectin, CD80 and FasL, and mRNA of VCAM-1 was occasionally expressed. Polymerase chain reaction products were analyzed by densitometry, and gene expression levels

**Fig. 3.** Expression of mRNA with interferon- $\gamma$  (IFN- $\gamma$ ) or sCD40L stimulation in salivary gland epithelial cells from an SS patient. The expression of CD40, ICAM-1, VCAM-1, CD86, Fas, E-selectin, CD40L, CD80, FasL and B.A. mRNA in salivary gland epithelial cells from a Sjögren's syndrome (SS) patient (focus score 6.1), was examined by reverse transcription-polymerase chain reaction (RT-PCR) after the 72-h stimulation with each of IFN- $\gamma$  500 U/ml and soluble CD40L (sCD40L) 800 ng/ml. Enhanced expression of ICAM-1 mRNA was observed after stimulation with IFN- $\gamma$  and sCD40L



**Fig. 4.** Densitometric analysis of PCR products. PCR products of CD40, ICAM-1, and CD86 were electrophoresed on an agarose gel. Each band of the PCR products was analyzed by densitometry. Results were expressed as the ratio of density with stimulations to that without stimulations. CD40 mRNA expression was enhanced with IFN- $\gamma$  stimulation (160%,  $n = 11$ ,  $P = 0.0029$ ), but not enhanced with soluble

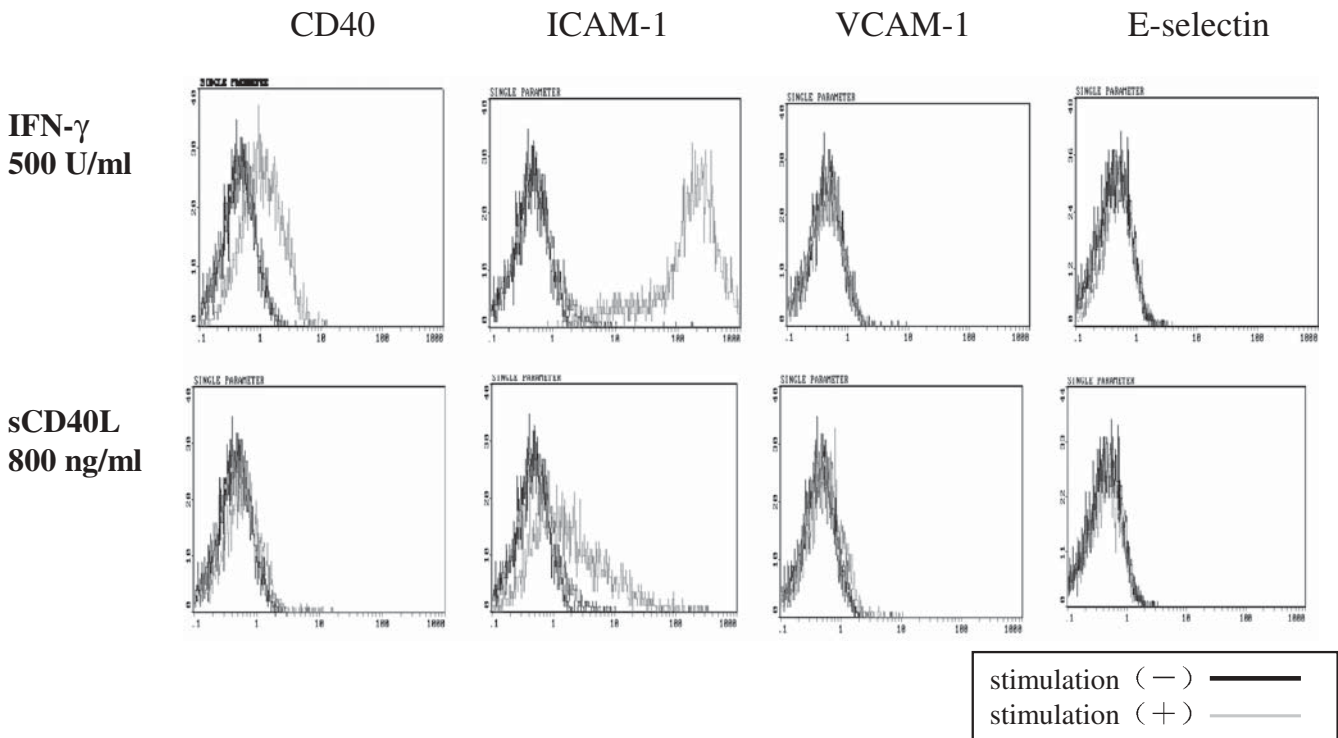
CD40L (sCD40L) stimulation ( $n = 6$ ). ICAM-1 mRNA expression was enhanced with IFN- $\gamma$  stimulation (360%,  $n = 12$ ,  $P = 0.0011$ ) and sCD40L stimulation (200%,  $n = 6$ ,  $P = 0.015$ ). CD86 mRNA expression did not change with any stimulations ( $n = 5$ ). Mean  $\pm$  SD is indicated. \* $P < 0.05$ , \*\* $P < 0.01$

were standardized against nonstimulation control and compared. As shown in Fig. 4, ICAM-1 expression was significantly elevated with stimulation of IFN- $\gamma$  ( $n = 12$ ,  $P = 0.0011$ ) or sCD40L ( $n = 6$ ,  $P = 0.0145$ ). There was no significant difference in each gene expression between SS patients and controls (data not shown).

To confirm above results, we analyzed the expression of ICAM-1 mRNA by real-time PCR. ICAM-1 mRNA expression was significantly enhanced with sCD40L stimulation compared to non-stimulation control (sCD40L stimulation  $6.60 \pm 4.59$ , nonstimulation 1.0,  $n = 7$ ,  $P = 0.008$ ).

#### Flow cytometric analysis of ICAM-1 expression on SG cells

SG cells from 5 SS patients and 7 controls were studied for ICAM-1 expression by flow cytometry. The focus score of these 5 patients ranged from 1.0 to 6.1 ( $2.2 \pm 2.2$ ). CD40 expression was slightly enhanced with IFN- $\gamma$  stimulation, but was not with sCD40L stimulation. ICAM-1 expression was enhanced with sCD40L stimulation from  $0.747 \pm 0.376$  to  $1.98 \pm 1.10$  (mean fluorescence intensity: MFI,  $P = 0.0015$ ) and with IFN- $\gamma$  stimulation from  $0.747 \pm 0.376$  to  $194 \pm 171$  (MFI,  $P = 0.0011$ ) (Fig. 5). There was no significant differ-



**Fig. 5.** Effects of IFN- $\gamma$  or sCD40L on the surface expression of CD40, ICAM-1, VCAM-1, and E-selectin on cultured salivary gland epithelial cells (SG cells). Flow cytometric analysis of CD40, ICAM-1, VCAM-1, and E-selectin expression in the presence or absence of stimulation

with IFN- $\gamma$  500 U/ml or sCD40L 800 ng/ml on SG cells was performed. CD40 expression was slightly enhanced with IFN- $\gamma$  stimulation, but was not with sCD40L stimulation. ICAM-1 expression was enhanced with IFN- $\gamma$  and sCD40L stimulation

ence in the enhancement of ICAM-1 expression between SS and controls by stimulations such as IFN- $\gamma$  or sCD40L (data not shown).

Analysis of NF- $\kappa$ B involvement in ICAM-1 expression by CD40L with using transfection

Next, in order to analyze the involvement of NF- $\kappa$ B in signal transduction of the expression of ICAM-1, we used transfection with plasmid containing NF- $\kappa$ B binding site, and NF- $\kappa$ B inhibitor. Transfection was performed with SG cells from 9 SS patients and 4 controls. The focus score of these 9 patients ranged from 1.0 to 12 ( $4.0 \pm 3.9$ ). CD40L stimulation enhanced pNF- $\kappa$ B-Luc activity in concentration-dependent manner (Fig. 6). Moreover, an NF- $\kappa$ B inhibitor, PDTC,<sup>18,19</sup> suppressed pNF- $\kappa$ B-Luc activity. Activity of pAP-1-Luc was not changed with CD40L stimulation (data not shown).

When SS SG cells were transfected with pGL1.3-Luc, luciferase activity was enhanced with IFN- $\gamma$  stimulation from  $0.99 \pm 0.22$  to  $15.8 \pm 26.5$  ( $n = 13$ ,  $P = 9.4 \times 10^{-7}$ ) (data not shown) and with sCD40L stimulation from  $1.00 \pm 0.21$  to  $1.49 \pm 0.24$  ( $n = 11$ ,  $P = 5.25 \times 10^{-5}$ ) (Fig. 7). On the other hand, the activity was suppressed by PDTC to 70% of the activity before stimulation ( $0.98 \pm 0.40$ ,  $n = 5$ ,  $P = 0.015$ ). When SS SG cells were transfected with pGL1.3-KB<sup>-</sup>-Luc, which was mutated in NF- $\kappa$ B binding site, luciferase activity was suppressed from  $1.00 \pm 0.21$  (pGL1.3-Luc activity with

no stimulation) to  $0.71 \pm 0.29$  ( $n = 11$ ,  $P = 0.0009$ ) and was not enhanced with sCD40L stimulation. There was no significant difference in the enhancement of ICAM-1 expression in the presence or absence of any stimulation between SS and controls (data not shown). These results suggest that NF- $\kappa$ B would be involved in signal transduction of ICAM-1 expression.

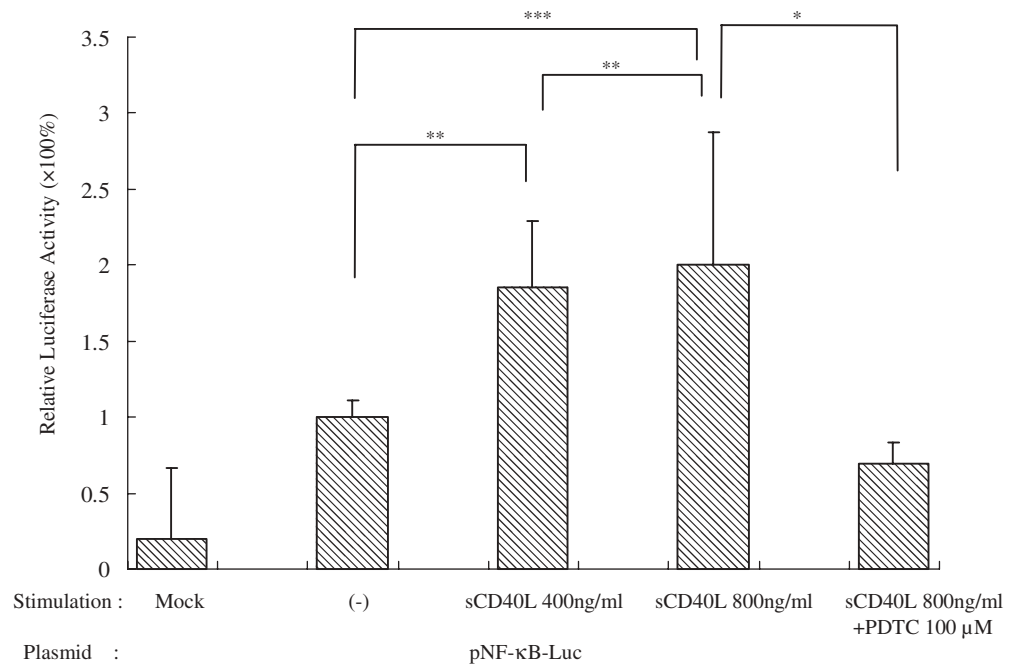
Analysis of NF- $\kappa$ B subunits involved in ICAM-1 expression by NF- $\kappa$ B transcription factor assay

Because NF- $\kappa$ B consists of several subunits, we tried to determine which subunit is involved in ICAM-1 expression in SG cells. For this purpose, NF- $\kappa$ B family activities in nuclear extracts were analyzed by using SG cells from 2 SS patients and 2 controls. The focus scores of the 2 patients were 12 and 1.0. Activity of NF- $\kappa$ B p50 was increased with sCD40L stimulation ( $P = 0.02$ ) and was suppressed with PDTC ( $P = 0.02$ ). The activity of other NF- $\kappa$ B family iso-type was not changed with any stimulations (Fig. 8).

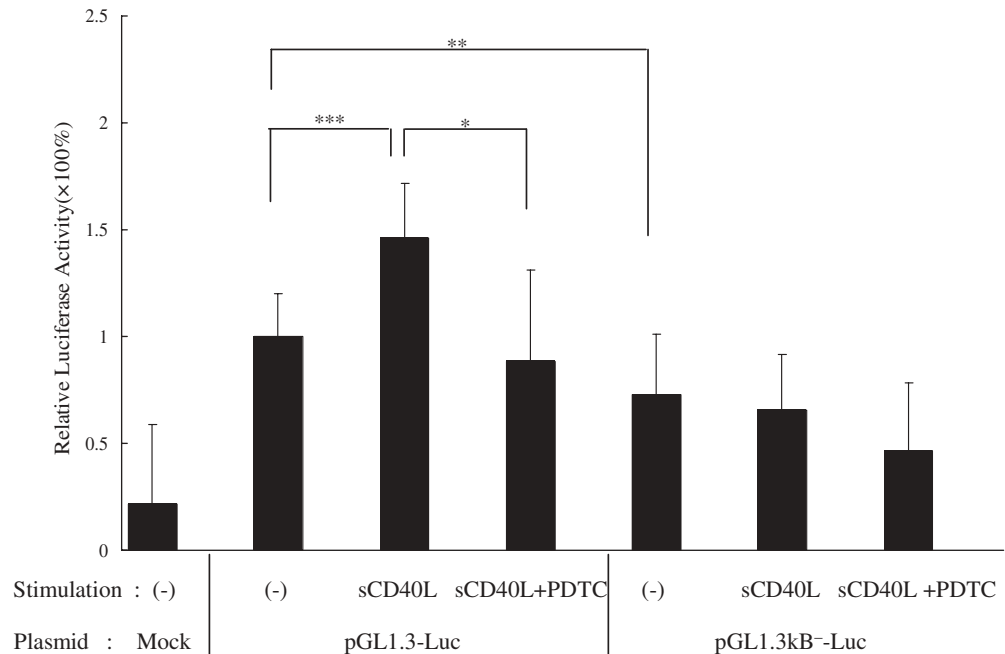
## Discussion

Sjögren's syndrome is a chronic organ-specific autoimmune disease in exocrine glands such as lacrimal and salivary glands. Recently it has been reported that in primary SS the expression of co-stimulatory molecules, such as CD40-

**Fig. 6.** Luciferase activity of pNF- $\kappa$ B-Luc in the presence or absence of sCD40L stimulation. SG cells were transfected with pNF- $\kappa$ B-Luc plasmid in the presence or absence of sCD40L or sCD40L and pyrrolidine dithiocarbamate (PDTC), an NF- $\kappa$ B inhibitor, as detailed in Materials and methods. Soluble CD40L stimulation enhanced pNF- $\kappa$ B-Luc activity in concentration-dependent manner from  $100.0 \pm 11.5$  to  $185.1 \pm 43.6$  (sCD40L 400ng/ml,  $P = 0.0059$ ) or to  $200.7 \pm 87.5$  (sCD40L 800ng/ml,  $P = 0.0087$ ) ( $n = 7$ ). Moreover, PDTC suppressed pNF- $\kappa$ B-Luc activity from  $200.7 \pm 87.5$  to  $69.2 \pm 13.6$  (56.6%,  $P = 0.015$ ). Bars represent luciferase activity, which was normalized to  $\beta$ -galactosidase activity and standardized against nonstimulation control. Mean  $\pm$  SD is indicated. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$



**Fig. 7.** Luciferase activity of pGL1.3-Luc with stimulation of sCD40L or sCD40L and PDTC in SG cells. "pGL1.3-luc," luciferase activity was enhanced with sCD40L 800ng/ml stimulation ( $\times 1.49$ ,  $n = 11$ ,  $P = 5.25 \times 10^{-5}$ ), by adding PDTC 100  $\mu$ M, inhibitor of NF- $\kappa$ B, the activity was suppressed to 70% of the activity before stimulation ( $n = 5$ ,  $P = 0.015$ ). "pGL1.3KB-Luc," which was mutated in NF- $\kappa$ B binding site, luciferase activity was suppressed to 70% of the luciferase activity of pGL1.3-Luc with no stimulation ( $n = 11$ ,  $P = 0.0009$ ) and didn't enhance with sCD40L stimulation. Bars represent luciferase activity, which was normalized to  $\beta$ -galactosidase activity and standardized against non stimulation control. Mean  $\pm$  SD is indicated. \* $P < 0.005$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0005$

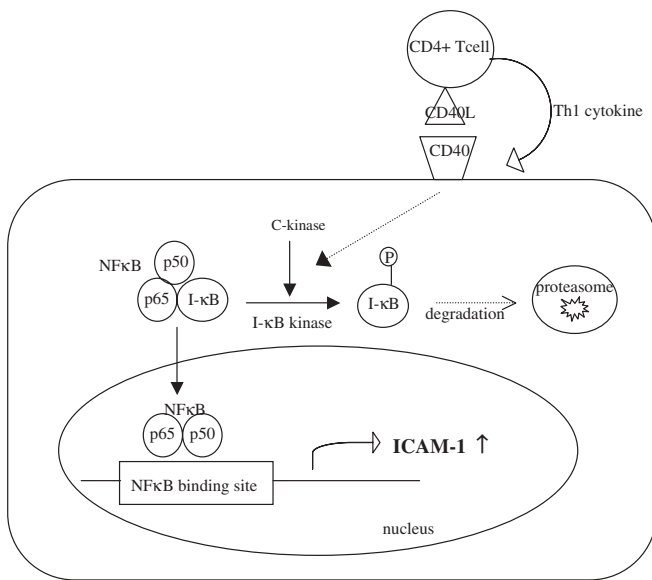
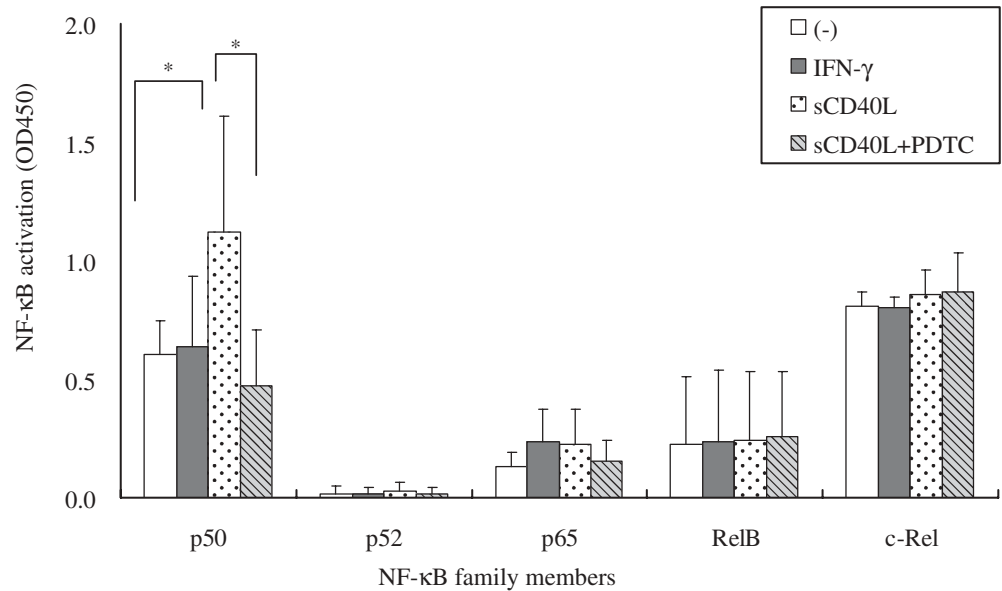


CD40L and adhesion molecules including ICAM-1, is associated with the pathology of SS.<sup>27</sup> In our present study, we used cultured SG cells obtained by labial minor salivary gland biopsy from SS patients and controls. In these SG cells the expression of both CD40 and ICAM-1 was identified with flow cytometry and RT-PCR. It was observed that the expression of ICAM-1 was enhanced by stimulation with sCD40L. Furthermore, by using two types of plasmids, one has NF- $\kappa$ B binding site and the other is mutated at reaction site of NF- $\kappa$ B, it was found that ICAM-1 expression is

associated with NF- $\kappa$ B activation. Finally, NF- $\kappa$ B p50 was significantly increased by sCD40L stimulation and was suppressed by an NF- $\kappa$ B inhibitor PDTC.

These results suggest that CD40-CD40L signal may up-regulate ICAM-1 expression as follows (Fig. 9). First, CD4+ T cells infiltrated in the SS salivary gland overexpress Th1 cytokine.<sup>3-5,20</sup> CD40 expression on SG cells is promoted by these Th1 cytokines.<sup>2,5,6</sup> Next, CD40 on SG cells is stimulated either with CD40L expressed on CD4+ T cells<sup>21</sup> or sCD40L in serum,<sup>22</sup> and the inhibitor kappa B (I- $\kappa$ B) is

**Fig. 8.** NF- $\kappa$ B activity in SG cells by NF- $\kappa$ B transcription factor assay. Activity of NF- $\kappa$ B p50 was enhanced with sCD40L stimulation from  $0.60 \pm 0.14$  to  $1.12 \pm 0.49$  ( $P = 0.021$ ) and suppressed by adding PDTC to  $0.47 \pm 0.24$  ( $P = 0.021$ ) ( $n = 4$ ). Mean  $\pm$  SD is indicated. \* $P < 0.05$



**Fig. 9.** Pathway of NF- $\kappa$ B activation. *I* $\kappa$ B, inhibitor kappa B; *PKC*, protein kinase C; *P*, phosphorus

phosphorylated. Then, NF- $\kappa$ B moves from cytoplasm into nucleus and transcription is activated, and ICAM-1 is expressed. Besides the pathway we studied, it is reported that ICAM-1 expression is induced by the stimulation with IFN- $\gamma$ .<sup>2,23,24</sup> Our results showed that IFN- $\gamma$  is more potent in the induction of ICAM-1 compared to sCD40L. However, these results were obtained under in vitro culture conditions. It would be difficult to say which, IFN- $\gamma$  or sCD40L, is more important in the maintenance of inflammation only from this study. In our study, any activity of NF- $\kappa$ B subtypes was not increased by IFN- $\gamma$  stimulation. Therefore it is considered that the pathway of ICAM-1 expression induced by CD40L mediated NF- $\kappa$ B described above, differs from that by IFN- $\gamma$  stimulation. It is reported that NF- $\kappa$ B is not involved in the pathway of ICAM-1 expression induced

by IFN- $\gamma$  in human alveolar epithelial cells.<sup>24</sup> We think further study would be required to clarify how the ICAM-1 expression is regulated in vivo by various types of stimulations. In any case, ICAM-1 is highly expressed in SG cells of SS patients compared to controls, and ICAM-1 is associated with the development of inflammation in SS salivary glands. Therefore it would be possible to attenuate the chronic inflammation of the SS salivary glands by blocking the ICAM-1 inducing pathway described above.

We found that the expression of ICAM-1 in cultured SG cells was enhanced by CD40L stimulation. It was reported that the elevation of sCD40L was observed in serum of SS patients,<sup>22</sup> and that the expression of CD40L on the infiltrating lymphocytes in SS patients was observed.<sup>21</sup> In view of the results and studies, the stimulation with CD40L in vivo is probably the cause of the high expression of ICAM-1 in SS patients. By adding the stimulation with sCD40L to controls, our study showed that the expression of ICAM-1 was enhanced as well. This indicates that the continuous stimulation of SG cells with costimulatory molecules such as CD40L contributes to the SS pathogenesis.

Though it is widely accepted that the transfection is an excellent method to analyze the transcriptional regulation of gene expression, there have been few reports in which the transfection method was applied to SG cells, because it is usually difficult to get stable results when applying the transfection to primary cultured cells. However, we succeeded in the analysis using the transfection method described here. By using this method, transcriptional regulation of any particular gene expression could be examined in primary cultured SG cells.

In the present study, we clarified the pathway contributing to the inflammation of the SS salivary gland, and that CD40L induces ICAM-1 expression in the SS salivary gland epithelial cells via NF- $\kappa$ B p50 activation (Fig. 9). As upregulation of ICAM-1 is associated with SS pathological development, it is hopefully expected to improve pathological conditions of SS patients by suppression of the pathway.



## References

1. Fox RI, Robinson CA, Curd JG, Kozin F, Howell FV. Sjögren's syndrome. Proposed criteria for classification. *Arthritis Rheum* 1986;29:577-85.
2. Dimitriou ID, Kapsogeorgou EK, Moutsopoulos HM, Manoussakis MN. CD40 on salivary gland epithelial cells: high constitutive expression by cultured cells from Sjögren's syndrome patients indicating their intrinsic activation. *Clin Exp Immunol* 2002;127:386-92.
3. Ogawa N, Dang H, Lazaridis K, McGuff HS, Aufdemorte TB, Talal N. Analysis of transforming growth factor beta and other cytokines in autoimmune exocrinopathy (Sjögren's syndrome). *J Interferon Cytokine Res* 1995;15:759-67.
4. Rowe D, Griffiths M, Stewart J, Novick D, Beverley PC, Isenberg DA. HLA class I and II, interferon, interleukin 2, and the interleukin 2 receptor expression on labial biopsy specimens from patients with Sjögren's syndrome. *Ann Rheum Dis* 1987;46:580-6.
5. Kamanaka M, Yu P, Yasui T, Yoshida K, Kawabe T, Horii T, et al. Protective role of CD40 in *Leishmania major* infection at two distinct phases of cell-mediated immunity. *Immunity* 1996;4:275-81.
6. van Kooten C, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol* 2000;67:2-17.
7. Kapsogeorgou EK, Dimitriou ID, Abu-Helu RF, Moutsopoulos HM, Manoussakis MN. Activation of epithelial and myoepithelial cells in the salivary glands of patients with Sjögren's syndrome: high expression of intercellular adhesion molecule-1 (ICAM-1) in biopsy specimens and cultured cells. *Clin Exp Immunol* 2001;124:126-33.
8. Vitali C, Bombardieri S, Moutsopoulos HM, Balestrieri G, Benicivelli W, Bernstein RM, et al. Preliminary criteria for the classification of Sjögren's syndrome. Results of a prospective concerted action supported by the European Community. *Arthritis Rheum* 1993;36:340-7.
9. Ping L, Ogawa N, Sugai S. Novel role of CD40 in Fas-dependent apoptosis of cultured salivary epithelial cells from patients with Sjögren's syndrome. *Arthritis Rheum* 2005;52:573-81.
10. Umehara F, Itoh K, Michizono K, Abe M, Izumo S, Osame M. Involvement of Fas/Fas ligand system in the spinal cords of HTLV-I-associated myelopathy. *Acta Neuropathol (Berl)* 2002;103:384-90.
11. Koga T, Claycombe K, Meydani M. Homocysteine increases monocyte and T-cell adhesion to human aortic endothelial cells. *Atherosclerosis* 2002;161:365-74.
12. Mitchell TJ, Whittaker SJ, John S. Dysregulated expression of COOH-terminally truncated Stat5 and loss of IL2-inducible Stat5-dependent gene expression in Sezary syndrome. *Cancer Res* 2003;63:9048-54.
13. Ledebur HC, Parks TP. Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells. Essential roles of a variant NF-kappa B site and p65 homodimers. *J Biol Chem* 1995;270:933-43.
14. Voraberger G, Schafer R, Stratowa C. Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5'-regulatory region. Induction by cytokines and phorbol ester. *J Immunol* 1991;147:2777-86.
15. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 1987;84:7413-7.
16. Manoussakis MN, Dimitriou ID, Kapsogeorgou EK, Xanthou G, Paikos S, Polihronis M, et al. Expression of B7 costimulatory molecules by salivary gland epithelial cells in patients with Sjögren's syndrome. *Arthritis Rheum* 1999;42:229-39.
17. Abu-Helu RF, Dimitriou ID, Kapsogeorgou EK, Moutsopoulos HM, Manoussakis MN. Induction of salivary gland epithelial cell injury in Sjögren's syndrome: in vitro assessment of T cell-derived cytokines and Fas protein expression. *J Autoimmun* 2001;17:141-53.
18. Moellering D, McAndrew J, Jo H, Darley-Usmar VM. Effects of pyrrolidine dithiocarbamate on endothelial cells: protection against oxidative stress. *Free Radic Biol Med* 1999;26:1138-45.
19. Howard EF, Chen Q, Cheng C, Carroll JE, Hess D. NF-kappa B is activated and ICAM-1 gene expression is upregulated during reoxygenation of human brain endothelial cells. *Neurosci Lett* 1998;248:199-203.
20. Adamson TC, 3rd, Fox RI, Frisman DM, Howell FV. Immunohistologic analysis of lymphoid infiltrates in primary Sjögren's syndrome using monoclonal antibodies. *J Immunol* 1983;130:203-8.
21. van Kooten C, Banchereau J. Immune regulation by CD40-CD40-L interactions. *Front Biosci* 1997;2:d1-11.
22. Goules A, Tzioufas AG, Manousakis MN, Kirou KA, Crow MK, Routsias JG. Elevated levels of soluble CD40 ligand (sCD40L) in serum of patients with systemic autoimmune diseases. *J Autoimmun* 2006;26:165-71.
23. Tsunawaki S, Nakamura S, Ohyama Y, Sasaki M, Ikebe-Hiroki A, Hiraki A, et al. Possible function of salivary gland epithelial cells as nonprofessional antigen-presenting cells in the development of Sjögren's syndrome. *J Rheumatol* 2002;29:1884-96.
24. Chang YJ, Holtzman MJ, Chen CC. Interferon-gamma-induced epithelial ICAM-1 expression and monocyte adhesion. Involvement of protein kinase C-dependent c-Src tyrosine kinase activation pathway. *J Biol Chem* 2002;277:7118-26.