Involvement of autoimmunity to REG, a regeneration factor, in patients with primary Sjögren's syndrome

Short title: Anti-REG autoantibodies in Sjögren's syndrome

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Summary

The regenerating gene (*Reg*) was originally isolated as a gene specifically overexpressed in regenerating pancreatic islets and constitute a growth factor family. *Reg* gene product (Reg) is important in the pathophysiology of various human inflammatory diseases. Recently, possible involvement of human REG in regeneration of salivary ductal epithelial cells of patients with primary Sjögren's syndrome (SS) was reported. However, the expression of the *REG* family genes in minor salivary glands (MSG) and the occurrence of anti-REG Iα autoantibodies in SS patients were obscured. In this study, we examined the expression of *REG* family genes in the MSG of SS and screened anti-REG Iα autoantibodies in SS. The mRNA levels of *REG* family genes in MSG were quantified using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and REG Iα expression in the MSG was analysed by immunohistochemistry. The mRNA level of *REG Iα* in the MSG of SS patients was significantly higher than that of control. REG Iα protein was highly expressed in SS ductal epithelial cells. Anti-REG Iα autoantibodies in the sera were found in 11% of SS. All the MSG in anti-REG Iα autoantibody positive group showed REG Iα expression, whereas only 40% showed REG Iα expression in anti-REG Iα autoantibody negative group. The anti-REG Iα autoantibody positive group showed significantly lower saliva secretion and higher ratio of grade 4 (by Rubin-Holt) in sialography. These data strongly suggest that autoimmunity to REG Iα may play a role in the degeneration of MSG ductal epithelial cells in primary SS.

Introduction

Primary Sjögren's syndrome (SS) is a chronic autoimmune disease of unknown aetiology, characterized by lymphocytic infiltration of salivary and lacrimal glands, leading to xerostomia and xerophthalmia, and characterized by the presence of a variety autoantibodies directed against organ- and non-organ-specific autoantigens. It is appreciated that the production of autoantibodies is an antigen-driven immune response, as certain autoantibodies are disease specific, contain multiple epitopes and the autoimmune response is perpetuated and augmented via intra- and intermolecular spreading against the same or other autoantigens. It is unknown whether any of the autoantibodies has a direct pathogenic potential or they merely participate in a secondary response to salivary glands that are already damaged by another process.

The regenerating gene, *Reg*, was originally isolated as a growth factor from a cDNA library of rat regenerating pancreatic islets [1-3]. *Reg* gene expression has also been identified outside the pancreas. Subsequently, many *Reg*-related proteins have been identified in human and other animals. The *Reg* family genes constitute a multi-gene family consisting of four subtypes [4]. In human, 5 functional *Reg* genes, i.e., *REG Iα* [1, 2], *REG Iβ* [5], REG III [6], *HIP/PAP* [7, 8], and *REG IV* [9] have been isolated.

Reg family proteins function as acute phase reactants, lectins, anti-apoptotic factors, and growth agents and include growth factors. These proteins are primarily involved in cell proliferation and differentiation, inflammation, diabetes, and carcinogenesis [4, 10]. Type I (and type II) Reg proteins are expressed in regenerating islets [4, 11, 12]. Type III Reg proteins have been suggested to be involved in cellular proliferation in intestinal

cells, hepatic cells, and neuronal cells. Importantly, mouse Reg III was shown to be a Schwann cell mitogen that accompanies the regeneration of motor neurons [13], and Reg protein functions as a neurotrophic factor for motor neurons [14]. It was reported that REG I protein was expressed in ductal epithelial cells in the minor salivary glands (MSG) of patients with SS [15]. However, which *REG* family gene(s) were expressed in MSG in SS patients was obscured.

Autoantibodies against REG (αREG) were found in some diabetic patients [16, 17]. However, the occurrence of αREG in SS patients was obscured. Presence of αREG might compromise regeneration of damaged ductal epithelial cells. Expression of REG could be a key event in autoimmunity. This hypothesis is supported by the fact that the αREG retarded β-cell proliferation *in vitro* [16].

It was reported that the expression of *Reg* family genes was regulated by several factors such as nicotinamide [18, 19], glucocorticoids [18, 20], nutrient factors [21], interleukin (IL)-6 [12, 18], IL-8 [22], IL-11 [23], IL-22 [24], interferon (IFN)γ [25], IFNβ [12], and CINC-2β [26]. We examined the mRNA levels of IL-6 and IL-8, a major proinflammatory cytokine produced in MSG in SS patients [27-30], in MSG specimens. We also examined mRNA levels of IL-6 receptor and gp130 in MSG specimens.

In the present study, we found the over-expression of *REG Iα*, *IL-6*, and *IL-8* mRNAs in MSG of SS patients and possible involvement of autoimmunity to REG in patients with primary Sjögren's syndrome.

Materials and methods

Patients

This study was approved by the Nara Medical University Hospital. After informed consent, a total of 117 patients with primary SS were enrolled for the study at Nara Medical University Hospital during 2001 to 2009. All the patients were fulfilled the diagnostic criteria for definite SS proposed by the Research Committee on SS of the Ministry of Health and Welfare of the Japanese Government (1999) [31], and the diagnosis was also based on the diagnostic criteria proposed by the American–European Consensus Group criteria for SS [32]. The enrollment procedure and study protocol were in compliance with the Declaration of Helsinki. We determined serum levels of amylase, IgG, HbA1c, SS-A/SS-B autoantibodies, antinuclear antibody (ANA) titer, and rheumatoid factor. We also determined the sialographic staging based on the criteria of Rubin and Holt, the histological grade of MSG, presence of keratoconjunctivitis sicca, abnormality of the tear production as determined by the Schirmer's test (<5 mm after 5 min), and abnormality of saliva production as determined by Saxon's test. SS-A/SS-B autoantibodies were measured with an ELISA kit (MBL, Tokyo, Japan). The histological features of labial salivary gland biopsy were evaluated according to Greenspan's histopathologic grading [33], and we classified grade 4 with germinal centers as grade 5. The presence of keratoconjunctivitis was evaluated by fluorescence staining test or Rose Bengal test according to van Bijsterveld score. The relationships among these clinicopatholocal factors and the occurrence of αREG were analysed.

MSG tissue specimens

MSG tissue was obtained from 53 Japanese patients with primary SS (1 male, 52 females; mean age 55 ± 2.0 years, range 15-80 years) in the 117 Japanese SS patients, and also from 25 healthy Japanese controls (13 males, 12 females; mean age 39 ± 3.7 years, range 6-76 years). The tissue specimens were fixed with 10% formalin/phosphate-buffered saline (PBS) and embedded in paraffin.

Patient's sera

Serum samples were collected from 117 Japanese with primary SS patients (two males, 115 females; mean age 56 ± 1.4 years, range 9-83 years), including 53 patients who kindly provided MSG tissue specimens, and 271 healthy Japanese controls (81 males, 190 females; mean age 33 ± 0.63 years, range 20-64 years), and were divided into aliquots and stored at -80 °C until used in screening of αREG.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

MSG tissue was obtained from 23 Japanese patients with primary SS and from 25 healthy Japanese controls for analysing mRNA by RT-PCR. Total RNA was isolated from formalin-fixed, paraffin-embedded MSG tissue specimens using RNeasy FFPE kit (QIAGEN, Hilden, Germany). The isolated RNA was reverse transcribed to the cDNA using High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA) for the template of real-time PCR as described [34, 35]. The cDNA was subjected to PCR with the following primers: *β*-actin (**NM_001101**) sense primer, 5'-GCGAGAAGATGACCCAGA-3' and antisense primer, 5'-CAGAGGCGTACAGGGATA-3'; *REG Iα* (**NM_002909**) sense primer, 5'-AGGAGAGTGGCACTGATGACTT-3' and antisense primer

5'-TAGGAGACCAGGGACCCACTG-3'; *REG Iβ* (**NM_006507**) sense primer, 5'-GCTGATCTCCTCCCTGATGTTC -3' and antisense primer, 5'-TGTCAGTGATCTTGGTTTGAA-3'; *REG III* (**AB161037**) sense primer, 5'-GAATATTCTCCCCAAACTG-3' and antisense primer, 5'-GAGAAAAGCCTGAAATGAAG-3'; *HIP/PAP* (**NM_138937**) sense primer, 5'-AGAGAATATTCGCTTAATTCC-3' and antisense primer, 5'-AATGAAGAGACTGAAATGACA-3'; *REG IV* (**AY007243**) sense primer, 5'-ATCCTGGTCTGGCAAGTC-3' and antisense primer, 5'-CGTTGCTGCTCCAAGTTA-3', *IL-6* (**NM_000600**) sense primer, 5'-GGTACATCCTCGACGGCATC-3' and antisense primer, 5'-GCCTCTTTGCTGCTTTCACAC-3', *IL-8* (**NM_000584**) sense primer, 5'-TAGCAAAATTGAGGCCAAGG-3' and antisense primer, 5'-GGACTTGTGGATCCTGGCTA-3' *IL-22* (**NM_020525**) sense primer, 5'-GCAGGCTTGACAAGTCCAACT-3' and antisense primer, 5'-GCCTCCTTAGCCAGCATGAA-3', *IL-22 receptor* (*IL-22R*) (**NM_021258**) sense primer, 5'-CTACATGTGCCGAGTGAAGA-3' and antisense primer, 5'-ACATATCTGTAGCTCAGGTA-3', *IFNβ* (**NM_002176**) sense primer, 5'-CATTACCTGAAGGCCAAGGA-3' and antisense primer, 5'-CAGCATCTGCTGGTTGAAGA-3', *IFNγ* (**NM_000619**) sense primer, 5'-ATTCGGTAACTGACTTGAATGTCC-3' and antisense primer, 5'-CTCTTCGACCTCGAAACAGC-3', *IL-11* (**NM_000641**) sense primer, 5′-TCTCTCCTGGCGGACACG-3′ and antisense primer, 5′-AATCCAGGTTGTGGTCCCC-3′, *CXCL1* (**NM_001511**) sense primer, 5'-GAAAGCTTGCCTCAATCCTG-3' and antisense primer,

5'-TCCTAAGCGATGCTCAAACA 3', *IL-6 receptor* (*IL-6R*) (**X12830**) sense primer, 5′-TGAGCTCAGATATCGGGCTGAAC-3′ and antisense primer, 5′-CGTCGTGGATGACACAGTGATG-3′, and *gp130* (**NM_002184**) sense primer, 5′-AGGACCAAAGATGCCTCAACT-3′ and antisense primer, 5′-TTGGACAGTGAATGAAGATCG-3′. All the PCR primers were synthesized by NGRL (Sendai, Japan). Real-time PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Boston, MA) and Thermal Cycler Dice Real Time System (Takara, Otsu, Japan) as described [35]. PCR was performed with an initial step of 3 min at 95 °C followed by 40 cycles of 3 s at 95 °C and 20 s at 60 °C for *β-actin*, *REG III*, and *HIP/PAP*, 40 cycles of 3 s at 95 ˚C and 20 s at 64 ˚C for *REG Iα, REG Iβ*, and *REG IV*, 45 cycles of 3 s at 95 ˚C and 20 s at 62 ˚C for *IL-6* and *IL-22,* 45 cycles of 3 s at 95 ˚C and 20 s at 60 ˚C for *IL-8*, *IL-22R*, *IL-6R*, and *gp130*, and 45 cycles of 3 s at 95 ˚C and 20 s at 63 ˚C for *IFNβ*, *IFNγ*, *IL-11* and *CXCL1*. The level of target mRNA was normalized to the mRNA level of *β-actin* as an internal standard.

Immunohistochemical staining for REG Iα protein.

Sections were prepared on glass slides with a 7 μm thickness, processed by deparaffinisation/rehydration, and thereafter fixed in cold acetone at 4 °C for 10 min. The antigen retrieval step was performed using Bond Epitope Retrieval Solution 1 (citrate-based pH 6.0 solution). The tissue slices were incubated with an anti-human REG I protein monoclonal antibody [2, 36] (1:250) as a primary antibody overnight at 4 °C in a humidified environment. After washing, the antibody was detected by the Bond polymer method [Autoimmunostainer Bond MAX (Mitsubishi Chemical Medience Co., Tokyo, Japan)].

Screening of αREG

Recombinant human REG Iα protein (20 μg) [16] was electrophoresed on a 12.5% SDS-polyacrylamide gel (9 cm × 7 cm × 0.1 cm) with a constant current at 20 mA/gel for 100 min and electrotransferred onto a PVDF membrane using a semidry electro-blotter as described [16, 37]. After blocking with 5% nonfat dry milk, the membrane was incubated with patient or control serum, which had been diluted 1,024-fold with 5% nonfat dry milk, using a screener blotter (Screener Blotter Mini 56, Sanplatec, Osaka, Japan) [16, 37, 38]. The membrane was then rinsed with phosphate-buffered saline containing 0.10% Tween 20 and incubated with goat anti-human IgG labeled with horseradish peroxidase (American Qualex, San Clemente, CA) at 1/1,600 dilution. The signals were visualized by using an ECL detection system (GE healthcare, Buckinghamshire, UK) as described [16, 37, 38]. The band intensities from positive blots were analysed by Image J software (National Institute of Health, Bethesda, MD). The density was standardized using the value of an internal control sample treated as a relative value as described [16, 37].

Statistics

All values are presented as means ± standard error. Differences between two groups were analysed with Mann–Whitney *U*-test. A P value of <0.05 was considered statistically significant. The frequency distribution of positive values (mean + 3SD for control) was compared by the χ^2-test.

Results

REG gene expression in the MSG

We extracted total RNA from formalin fixed paraffin-embedded specimens and analysed mRNA levels of all the *REG* family genes (*REG Iα*, *REG Iβ*, *REG III*, *HIP/PAP*, and *REG IV*) using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 1). No *REG Iβ* mRNA was detected neither in the control nor the SS MSG. The mRNA levels of *REGIII*, *HIP/PAP*, and *REG IV* were not different between the control and SS MSG. In contrast, the mRNA level of *REG Iα* in the MSG of SS patients was significantly higher than that of control (P= 0.036).

Immunohistochemical staining of the MSG with SS for REG Iα protein

We then analysed REG Iα protein expression in MSG of SS patients by immunohistochemistry. REG Iα protein was strongly stained in ductal epithelial cells in 28 of 53 samples (53%), whereas acinar cells were immunostained only in two samples for REG Iα (Fig. 2a, b). In REG Iα positive samples, the intensity of staining was not associated with the degree of inflammation, fibrosis, or acinic atrophy (data not shown).

Cytokine/chemokine(s) gene expression in the MSG

As IL-6 and IL-8 are reported to induce *REG Iα* mRNA *in vitro* [18, 21, 22] and *in vivo* [39], and IL-11 [23], IL-22 [24], IFNγ [25], IFNβ [12] and CINC-2β [26] are also reported to induce *REG Iα* mRNA. IL-6R and gp130 are known as a signal transducer of IL-6. We measured *IL-6*, *IL-8 IL-11*, *IL-22*, *IL-22R*, *IFNγ*, *IFNβ*, *CXCL1* (human homologue of *CINC-2β*), *IL-6R* and *gp130* mRNAs in the MSG by quantitative RT-PCR. As shown in Fig. 3a, the *IL-6* mRNA level in the SS MSG was significantly higher than that in normal MSG. The *IL-8* mRNA level in SS MSG was also higher than

that in normal MSG (Fig. 3b). The mRNA levels of *IL-11*, *IL-22*, *IL-22R*, *IFNγ, CXCL1, IL-6R* and *gp130* were not significantly different between the two groups (Fig. 3c-j). The mRNA of *IFNβ* was not detected in SS MSG (Fig. 3g). We performed correlation analyses of expression of cytokine mRNAs and *REG Iα* mRNA and found that IL-6 mRNA expression was significantly correlated with *REG Iα* mRNA expression (data not shown).

Detection of αREG in SS patient sera

The sera from 117 patients with primary SS and 271 controls were screened for αREG. A percentile graph of the relative αREG values is shown in Fig. 4. The relative αREG values for all controls, barring six individuals, were within the mean+3SD value. Henceforth, we treated this mean+3SD value (3.9) of the controls as the cut-off value for all related data analyses. Eleven % (13 of 117) of patients with primary SS subjects tested positive for αREG, whereas only 2.2% (6 of 271) were positive in controls (P = 0.00019 in χ^2-test).

The relationships among clinicopathological factors

The αREG positive group showed significantly lower saliva secretion (0.64±0.29 g/2 min) than the negative group (1.54±0.17 g/2 min) in Saxon's test (P = 0.0073) (Table 1). The ratio of a destructive stage (stage 4 based on the criteria of Rubin and Holt) in sialography in the αREG positive group was significantly higher than that in the αREG negative group (Table 2). In the patients with primary SS, no correlation was found in age, sex ratio, serum levels of SS-A/SS-B autoantibody, antinuclear antibody titer, rheumatoid factor, amylase, IgG, HbA1c between the αREG positive group and the

αREG negative group (Table 1). Kerato-conjunctivitis sicca, Schirmer's test also showed no significantly difference between two groups. In the histological features of labial salivary gland biopsy according to Greenspan's grade, there was no significant difference in the αREG positive group and the αREG negative group (Table 2). We also analysed about extra glandular disease, systemic or severe disease (skin rash, raynaud phenomenon, arthralgia, thyroid gland disease, interstitial pneumonia, primary biliary cirrhosis, renal tubular acidosis, peripheral neuropathy and lymphoma), there was no significant difference in two groups (data not shown).

Relationship between REG Iα protein expression and αREG

Twenty-one % of patients (11 of 53) who were examined REG Iα protein expression in MSG by immunohistochemistry tested positive for αREG. All the 11 samples showed REG Iα expression in the αREG positive group, whereas only 40% (17 of 42) showed REG Iα expression in MSG in the αREG negative group (P=0.00043) (Table 3).

Discussion

In the present study, we found that REG Iα protein was expressed in the ductal cells of MSGs from patients with primary SS and that saliva secretion was reduced in primary SS patients with the αREG. Although the aetiology of SS is still unclear, it is thought to be an autoimmune disease characterized by marked ductal cell destruction with inflammatory cell infiltration in MSG [40]. It was reported previously that REG Iα is expressed not only in various human inflammatory diseases such as gastritis [41], pancreatitis [42] and colitis, but also in various experimental models of inflammation in animal tissues [39, 43]. Thus, it is most likely that inflammation, regardless of whether or not it is autoimmune-associated, is a key event triggering REG Iα expression in many tissues. Therefore, it is an interesting question as to whether REG Iα overexpression is associated directly with the immune disorder in patients with primary SS. We performed RT-PCR analyses of all the *REG* family genes in MSG and found that *REG Iα* mRNA was specifically overexpressed in MSG specimens from SS patients (Fig. 1). These results support the idea that *REG Iα* mRNA overexpression is associated with inflammation triggered by autoimmune disorder such as SS.

It was reported that *Reg* gene expression was regulated by several factors such as nicotinamide [18, 19], glucocorticoids [18, 20], nutrient factors [21], IL-6 [12, 18], IL-8 [22], IL-11 [23], IL-22 [24], IFNγ [25], IFNβ [12], and CINC-2β [26]. Among the major inflammatory mediators involved in the induction of the inflammation of the salivary glands with primary SS, IL-6 is an important proinflammatory cytokine in relation to the infiltration of lymphocyte [27, 28]. In addition, the presence of IL-8 also was reported in the salivary glands of SS [29, 30].

We therefore examined the mRNA levels of *IL-6*, *IL-8* and other

cytokine/chemokine(s), which were reported to induce *REG Iα* mRNA in MSG specimens. The mRNA levels of *IL-6* and *IL-8* in the MSG of SS patients were significantly higher than those of the control. The mRNA levels of *IL-11*, *IL-22*, *IL-22R*, *IFNγ,* and *CXCL1* were not significantly different between the two groups. The mRNA of *IFNβ* was not detected in SS MSG. These results suggest that the up-regulation of cytokines, especially IL-6 and IL-8, induces the over-expression of *REG Iα* gene in SS MSG. IL-6R and gp130 are known as a signal transducer of IL-6. We examined the mRNA levels of *IL-6R* and *gp130* in SS and normal MSG. The mRNA levels of *IL-6R* and *gp130* were not significantly different between the two groups (Fig. 3i, j), suggesting that the increase of IL-6 in the salivary gland of SS patients (Fig. 3a) can function as a switch of the IL-6/gp130 signaling system to induce *REG Iα* gene expression.

Anti-REG autoantibodies that inhibited pancreatic β-cell replication were detected in Japanese diabetes patients [16]. In the present study, for the first time, we detected autoantibody against REG Iα in Japanese primary SS patients. We evaluated the correlation between αREG and clinicopathological factors of primary SS patients.

The αREG positive group showed significantly lower salivary secretion and higher ratio of a destructive stage in sialography. REG Iα protein was expressed in MSG ductal epithelial cells from nearly half of SS patients, although a few in MSG acinar cells. Interestingly, all the patients in the αREG positive group showed REG Iα expression in MSG ductal cells, whereas only 42% in the αREG negative group showed REG Iα expression in MSG (Table 3). These results suggest that autoimmunity to REG is associated with the regeneration of the ductal epithelial cells of MSG in primary SS patients.

When salivary glands are damaged by inflammation, REG Iα protein may be induced in progenitor cells for MSG ductal/acinar cells such as ductal cells for recovering damaged cell mass by regeneration. Accumulating evidence concerning development of the salivary gland suggests the stem cell population of salivary glands is present in the intercalated duct [44, 45]. Additionally, the proliferation of pancreatic β cell was reported to be attenuated by the diabetic patient sera containing αREG *in vitro* [16]. It is quite possible that αREG attenuates not only growth-promoting affects of REG to fully differentiated acinar/ductal cells but also regeneration of the stem cell population of salivary glands. As a result, salivary functions including saliva secretion could become worse in SS patients with αREG.

Xerostomia is an important clinical concern in oral health and is known to induce various problems including dental caries, periodontitis, denture problems, mastication and swallowing problems, burning sensations, and dysgeusia [46]. Muscarinic agonist medications such as pilocarpine and cevimeline induced salivary secretion from the residual functional tissue [47]. However, these medications provided temporary relief of symptoms and had a limited effect on the recovery of damaged tissues. Accordingly, the development of a novel treatment to restore or regenerate damaged salivary gland tissues is eagerly awaited. It is unclear whether or not a specific signal is required for the regeneration of salivary gland. REG Iα may be a candidate growth factor for regeneration of the salivary gland cells as hepatocyte growth factor is a well-known protein that promotes the regeneration of liver and even protects tissue from damages [48, 49]. Therefore, it is expected that the regenerative growth of ductal epithelial cells serves as a practical therapeutic approach for SS.

In the aspects of SS diagnosis, detection of αREG is not so powerful. However, as

the correlation between the salivary functions and existence of serum αREG (Tables 1 and 2), the αREG detection can be a useful diagnostic marker for the prognosis of the salivary functions such as saliva secretion. In addition, as described in materials and methods, it is required very small volumes of serum (less than 1 μl) to detect αREG.

In conclusion, we showed that *REG Iα* mRNA expressed in the SS patient salivary glands and that REG Iα protein was expressed in the ductal epithelial cells of MSGs from patients with primary SS. Saxon's test clearly revealed that saliva secretion was reduced in primary SS patients with the αREG. Furthermore, there was a correlation between the presence of αREG and REG Iα protein expression on ductal cells of MSG. These data strongly suggest that autoimmunity to REG Iα may play a role in the regeneration of MSG ductal epithelial cells in primary SS.

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Disclosure

None.

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Table 1. General characteristics of patients with primary SS.

	αREG		*P* value
	(+) *n*=13	(-) *n*=104	
Age (years)	62 ±4.0	55 ±1.5	NS
Sex ratio (M/F)	0/13	2/102	NS
SS-A (+/-)	11/2	84/16 (n=100)	NS
SS-B (+/-)	7/6	44/56 (n=100)	NS
ANA titer ≥1:320 (+/-)	6/7 (n=13)	60/33 (n=93)	NS
Rheumatoid factor (+/-)	7/3 (n=10)	34/32 (n=66)	NS
Amylase (IU/L)	70 ± 9.3 (n=12)	88 ± 4.1 (n=97)	NS
IgG (mg/dl)	2041 ± 125	1790 ± 57 (n=99)	NS
HbA1c (%)	5.3 ± 0.24 (n=5)	5.2 ± 0.1 (n=16)	NS
Kerato-conjunctivitis sicca (KCS) (+/-)	9/2 (n=11)	62/19 (n=81)	NS
Schirmer's test (≤5 mm/5 min / >5 mm/5 min)	8/4 (n=12)	54/43 (n=97)	NS
Saxon's test (g/2min)	0.64±0.29 (n=12)	1.54±0.17 (n=84)	0.0073

NS: $P \geq 0.05$

Table 2. Relationship among sialography (Rubin-Holt's stage), labial salivary gland biopsy (Greenspan's grade) and, and presence of αREG.

		αREG		*P* value
		(+)	(-)	
Sialography (Rubin-Holt stage)				
0	normal	20 % (2/10)	22 % (16/72)	NS
1	punctate	10 % (1/10)	21 % (15/72)	NS
2	globular	0.0 % (0/10)	18 % (13/72)	NS
3	cavitary	20 % (2/10)	32 % (23/72)	NS
4	destructive	50 % (5/10)	6.9 % (5/72)	0.000097
Labial salivary gland biopsy (Greenspan's grade)				
0		0.0 % (0/12)	1.7 % (1/59)	NS
1		0.0 % (0/12)	3.4 % (2/59)	NS
2		8.3 % (1/12)	6.8 % (4/59)	NS
3		8.3 % (1/12)	14 % (8/59)	NS
4		42 % (5/12)	41 % (24/59)	NS
5 (+germinal centers)*		42 % (5/12)	34 % (20/59)	NS

NS: $P \geq 0.05$. *In the grade 4 group, we classified "grade 5" in which germinal centers were found in the histopathology.

Table 3. Relation between REG Iα expression in the ductal epithelial cells and presence of αREG.

	REG Iα expression in the ductal epithelial cells	
	(+)	(-)
αREG positive (*n*=11)	11 (100%)	0 (0%)
αREG negative (*n*=42)	17 (40%)	25 (60%)

P=0.00043

Figure legends

Fig. 1. Expression of *REG* gene family in MSG. The mRNA level of *REG Iα* in the MSG of SS patients was significantly higher than that of control (*P*=0.036). No *REG Iβ* mRNA was detected neither in the control nor the SS MSG. The mRNA levels of *REG III*, *HIP/PAP*, and *REG IV* were not different between the control and SS MSG. Data are expressed as mean ± SE for each group (n=11-24).

Fig. 2. Immunostaining of REG Iα in MSG tissues. REG Iα expression were seen in regenerated ductal epithelia, together with a few inflammatory cells of heavily inflamed area from SS patients (**a, b**).

Fig. 3. The mRNA levels of *IL-6* (**a**), *IL-8* (**b**), *IL-11* (**c**), *IL-22* (**d**), *IL-22R* (**e**), *IFNγ* (**f**), *IFNβ* (**g**), *CXCL1* (*CINC-2β*) (**h**), *IL-6R* (**i**), and *gp130* (**j**) in MSG. The mRNA level of *IL-6* in the MSG of SS patients was significantly higher than that of the control (*P*=0.0176). The mRNA level of *IL-8* in the MSG of SS patients was also higher than that of the control (*P*=0.0353). The mRNA levels of *IL-11*, *IL-22*, *IL-22R*, *IFNγ*, and *CINC-2β* were not significantly different between the two groups. Data are expressed as mean ± SE for each group (n=21-23). The mRNA of *IFNβ* was not detected in SS MSG.

Fig. 4. Distribution of αREG in primary SS patients. The relative values from Western blot analyses of αREG in 117 patients with primary SS patients and 271 control subjects are shown in percentile graph. Broken line indicates mean+3SD (3.9) of the control samples and values above the mean+3SD were regarded as autoantibody-positive.