Treatment with the Biscoclarine Alkaloid Cepharanthine Significantly Increases Salivary Secretion in Primary Sjögren's Syndrome Patients

Tomoko YAMANOI, Keiko AOTA, Yukihiro MOMOTA, Masayuki AZUMA

キーワード：Primary Sjögren's syndrome, NF-κB, MMP-9, Type IV collagen, Acinar structure

Abstract: Objective: Our previous findings suggested that the suppression of tumor necrosis factor-alpha (TNF-α)-induced matrix metalloproteinase (MMP)-9 production by the biscoclarine alkaloid cepharanthine could prevent the destruction of the acinar structure in the salivary glands of murine Sjögren's syndrome. Here, we examined the effect of cepharanthine on the salivary secretion in primary Sjögren's syndrome (pSS) patients.

Methods: In this single-center, open-label pilot study, 29 patients with pSS (28 women, 1 man) received 6 mg/day orally cepharanthine for 12 months. Standard clinical assessments and stimulated salivary flow were examined at baseline and each month for 12 months in all 29 patients. In eight of the patients, inflammatory lesions in the salivary glands were histologically investigated before and after the cepharanthine treatment. We analyzed the expressions of p65, phosphorylated IkB-α, MMP-9, and type IV collagen immunohistochemically.

Results: All patients completed the study without any adverse events. A significant increase in salivary flow was observed after the cepharanthine treatment compared to baseline. The serological analysis revealed that the 14 patients with an anti-Sjögren's-syndrome-related antigen A (anti-SSA/Ro) antibody value that was either negative or <64 U/ml responded significantly well to this treatment, whereas the 15 patients with anti-SSA/Ro antibody values >64 U/ml did not. The immunohistochemical analysis demonstrated that although p65, phosphorylated IkB-α, and MMP-9 were more strongly stained in the acinar cells of the patients at baseline compared to the staining at the completion of cepharanthine treatment, the continuity of type IV collagen was observed following the cepharanthine treatment. These results indicate that cepharanthine could inhibit the phosphorylation of IkB-α, followed by the prevention of MMP-9 activation and the stabilization of type IV collagen.

Conclusions: Our findings suggest that cepharanthine could be a promising agent for improving salivary secretion in pSS patients.

Trial registration: This study was registered at Tokushima University Hospital (Registration number: 2437, November 26, 2012).
Introduction

Primary Sjögren’s syndrome (pSS), one of the most common rheumatic diseases\(^1\), is characterized by the eventual total replacement of the acinar tissue by a marked infiltration of lymphocytes into the salivary and lacrimal glands\(^2\). The pathogenesis of this selective and progressive destruction of the acinar structure in salivary and lacrimal glands is not fully understood. The evidence to date indicates a close relationship between cytokine expression in salivary and lacrimal gland tissues and the development and progression of pSS. The expression of mRNA for various cytokines such as tumor necrosis factor-alpha (TNF-\(\alpha\)), interleukin-1 beta (IL-1\(\beta\)), IL-2, and interferon-gamma (IFN-\(\gamma\)) has been detected in the salivary glands of humans as well as in those of experimental animals during the development of pSS\(^3,4\).

The establishment of the normal acinar structure of salivary glands is fully dependent on the integrity of extracellular matrices, including the basement membrane\(^5\). The basement membrane consists mainly of type IV collagen and laminin, and its synthesis and degradation are tightly regulated by proteolytic enzymes and their inhibitors\(^6,7\). However, the disruption of acinar cell-basement membrane interactions by an excessive production of proteolytic enzymes, such as matrix metalloproteinases (MMPs), could lead to the disruption of the acinar tissue. As cytokines (including TNF-\(\alpha\) and IL-1\(\beta\)) have been shown to stimulate the production of MMPs\(^6,7\), it is conceivable that cytokines contribute to the destruction of the basement membrane, in turn leading to a disruption of the acinar structure of the salivary glands. Moreover, structural changes in the basement membrane of the salivary glands and increased levels of latent and active MMP-9 in the saliva were described in patients with SS\(^6,9\). Together these observations support the concept that MMP-9 is involved in the pathogenesis of SS\(^10\).

We demonstrated that although NS-SV-AC (a Simian virus 40-immortalized normal human salivary gland cells with an acinar phenotype) cells produced a large amount of MMP-9 in response to TNF-\(\alpha\), a super-repressor form of IkB-\(\alpha\) (an inhibitory protein of NF-\(\kappa\)B) cDNA-transfected NS-SV-AC clone lost its responsiveness to TNF-\(\alpha\) in terms of MMP-9 production\(^11\). In addition, the suppression of TNF-\(\alpha\)-induced MMP-9 production restored the normal in vitro morphology of NS-SV-AC cells, even when they were cultured on type IV collagen-coated plates in the presence of both TNF-\(\alpha\) and plasmin\(^11\). It thus seems likely that the inhibition of TNF-\(\alpha\)-induced MMP-9 production in acinar cells may lead to the restored integrity of the acinar structure in pSS salivary glands.

Based on these considerations, we formulated the working hypothesis that the identification of drugs that suppress the TNF-\(\alpha\)-induced production of MMP-9 would be a promising strategy for therapeutic interventions in pSS. Cepharanthine, a bisoclaraine alkaloid extracted from the plant Stephania cephalantha Hayata, has been used widely for the treatment of patients with leukopenia\(^12\), nasal allergy\(^13\), and venous snake bites\(^14\). Although the exact mechanism has not been elucidated, cepharanthine exerts immunomodulatory effects by enhancing the cytotoxic effect of natural killer cells and macrophages\(^15,16\), suggesting that cepharanthine may play a role in the regulation of signaling pathways of cytokines. Therefore, in this study, we aimed to examine the effect of cepharanthine on the salivary secretion of pSS patients.

We demonstrated that cepharanthine effectively suppressed TNF-\(\alpha\)-induced production of MMP-9, which resulted in the restoration of normal acinar structures in both an in vitro culture system and an in vivo murine Sjögren’s syndrome model\(^17,18\). In the present study, we investigated the efficacy and safety of the use of cepharanthine in pSS patients. We found that the oral administration of cepharanthine significantly increased the production of salivary flow in pSS patients, at least in part through the suppression of the NF-\(\kappa\)B signaling pathway.

Patients and Methods

Study design and patients

This was a single-center open-label study. A total of 29 patients with pSS were enrolled in the study, and all patients fulfilled the Revised Japanese Criteria for SS, including histopathologic criteria\(^19\). The study protocol was approved by the Ethics Committee of Tokushima University Hospital. All patients provided written informed consent to participate in the study. Patients who had another known autoimmune disease or had been treated previously with steroids or cholinergics were excluded. The use of any type of systemic drug for the relief of xerostomia was not permitted during the study. For each patient, oral cepharanthine (6 mg/day, three times a day after every meal) was administered every day for 12 months. At the time point of their enrollment in the study, none of the patients had any systemic manifestations of SS such as fatigue, joint pain, severe parotid gland swelling, lymphadenopathy, or articular, cutaneous, respiratory, or muscular diseases\(^20\).

Laboratory assessment

Baseline evaluations, including a medical history, physical examinations, and measurements of complete blood cell counts with differential counts, biochemical analyses, and assessments of C-reactive protein (CRP), the erythrocyte sedimentation rate (ESR), anti-SSA/Ro antibody, anti-SSB/La antibody, and salivary scintigraphy were performed.
These laboratory examinations were performed at 3–6-month intervals during the study.

**Salivary flow measurement**

The primary end point of this study was the evaluation of the increased volume of whole salivary flow. Whole saliva was collected as recommended in the Revised Japanese Criteria (decreased salivary secretion = flow rate <10 ml/10 min in a chewing gum test)\(^2\). Stimulated whole salivary secretions during a 10-min chewing gum test were collected. The stimulated salivary secretions were examined every month for 12 months in all 29 patients, and the highest salivary volume obtained from each patient was assigned to each patient.

**Histology**

Minor salivary glands of each patient’s lower lip were taken by lip biopsy, fixed with 4% phosphate-buffered formaldehyde (pH 7.2) and prepared for histological examination. The histological grading of any inflammatory lesions identified in the salivary gland samples was conducted according to the method proposed by Greenspan et al.\(^2\) in the following manner: a grade of 0 indicated that lymphocytes and plasma cells per 4 mm\(^2\) in salivary gland tissues were absent; grade 1 indicated that a slight infiltration of these cells was recognized in the tissues; grade 2 indicated that a moderate infiltration of these cells or less than one focus (a focus is an aggregate of ≥ 50 lymphocytes, histiocytes, and plasma cells per 4 mm\(^2\)) was observed in the tissues; grade 3 indicated that one focus per 4 mm\(^2\) was seen in the tissues; grade 4 indicated that more than one focus per 4 mm\(^2\) was detected. The slides were scored by two independent, well-trained pathologists in a blind manner.

**Immunohistochemical staining for p65, phosphorylated IxB-α, MMP-9, and type IV collagen in the patients’ salivary glands**

To investigate the effects of cefaphanithine on the expressions of p65 (a subunit of NF-κB), phosphorylated IxB-α, MMP-9, and type IV collagen in the acinar tissues, we examined formalin-fixed paraffin-embedded samples of the patients’ salivary glands. Sections were dewaxed in xylene and rehydrated in graded ethanol according to standard procedures. Antigen retrieval was performed by incubating the sections immersed in 10 mM citric acid in a microwave oven at 100°C.

The primary antibodies used were rabbit polyclonal antibody to human p65 (Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal antibody to phosphorylated IxB-α peptide (abcam, Cambridge, UK), rabbit polyclonal antibody to MMP-9 peptide (abcam), and rabbit polyclonal antibody to mouse type IV collagen (Proteintech, Rosemont, IL).

The secondary antibody used was a biotinylated goat antirabbit IgG (Vectastain kit, Vector Laboratories, Burlingame, CA). Negative controls for each material were processed in the same manner, using a nonimmunized rabbit IgG (Dako, Carpinteria, CA) instead of the primary antibody. All histological analyses were done using blinded samples.

**Statistical analysis**

The statistical analysis was performed using the Wilcoxon matched-pairs test. P-values <0.05 were considered significant.

**Results**

**Patient characteristics**

The baseline clinical characteristics of the individual pSS patients are summarized in Table 1. The mean (SD) age of the 29 patients was 61.7 (13.7) years, and the median disease duration was 31.7 ± 42.7 months. All patients received the full dose of cefaphanthine (6 mg/day for 12 months), and the data of all 29 patients were included in the safety and efficacy analyses. Clinical assessments of disease activity were performed at the study entry and every month thereafter for 12 months, but no significant changes in the patients’ quality of life or activities of daily living, such as increased fatigue or joint pain, or aggravation of severe parotid gland swelling, peripheral neuropathy or interstitial lung disease were observed.

**Assessment of salivary gland function**

As shown in Table 1, all of the patients except Patient 1 demonstrated a stimulated salivary flow <10 ml/10 min at baseline. The stimulated salivary volume from each patient was measured every month for 12 months, and the efficacy of cefaphanzine on the salivary flow was estimated on the basis of an increase in stimulated salivary volume. As a consequence, as can be seen in Figure 1, the daily cefaphanzine treatment resulted in a significant increase of salivary flow in 23 of the 29 patients (5.3 ± 4.5 ml/10 min at 12 months vs. 3.1 ± 2.9 ml/10 min at baseline, P<0.05). The remaining six patients, who did not respond to the cefaphanzine treatment, showed stable salivary secretion during the study (Figure 1).

**Laboratory assessments**

Twenty-two of the 29 patients were anti-SSA/Ro antibody-positive, whereas the number of anti-SSB/La-positive patients was only five. In addition, the baseline CRP and ESR values of all of the patients were not abnormally high (Table 1). We next examined the effects of cefaphanzine on the values of anti-SSA/Ro antibody, anti-SSB/La antibody, CRP, and ESR,
Table 1  Characteristics of the 29 pSS at baseline and the volume of salivary secretion at 12 months

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Disease duration (months)</th>
<th>Volume of salivary secretion (ml/12 min)</th>
<th>Scintigraphy findings</th>
<th>Anti-SSA/Ro antibody value</th>
<th>Anti-SSB/La antibody value</th>
<th>CRP</th>
<th>ESR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>73</td>
<td>8</td>
<td>11</td>
<td>not relevant to pSS</td>
<td>4</td>
<td>-</td>
<td>0.2</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>71</td>
<td>16</td>
<td>1.5</td>
<td>relevant to pSS</td>
<td>-</td>
<td>-</td>
<td>&lt;0.05</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>64</td>
<td>12</td>
<td>5</td>
<td>relevant to pSS</td>
<td>64</td>
<td>1</td>
<td>&lt;0.05</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>71</td>
<td>3</td>
<td>1.5</td>
<td>relevant to pSS</td>
<td>64</td>
<td>1</td>
<td>&lt;0.05</td>
<td>n.d</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>59</td>
<td>9</td>
<td>1.5</td>
<td>relevant to pSS</td>
<td>-</td>
<td>-</td>
<td>&lt;0.05</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>24</td>
<td>2</td>
<td>7</td>
<td>relevant to pSS</td>
<td>1</td>
<td>-</td>
<td>&lt;0.05</td>
<td>n.d</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>47</td>
<td>6</td>
<td>2.5</td>
<td>relevant to pSS</td>
<td>16</td>
<td>-</td>
<td>0.67</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>36</td>
<td>120</td>
<td>7</td>
<td>relevant to pSS</td>
<td>64</td>
<td>-</td>
<td>&lt;0.05</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>47</td>
<td>22</td>
<td>4.5</td>
<td>relevant to pSS</td>
<td>-</td>
<td>-</td>
<td>&lt;0.05</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>60</td>
<td>48</td>
<td>1</td>
<td>relevant to pSS</td>
<td>64</td>
<td>-</td>
<td>0.06</td>
<td>n.d</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>60</td>
<td>60</td>
<td>5.2</td>
<td>relevant to pSS</td>
<td>-</td>
<td>-</td>
<td>&lt;0.05</td>
<td>14</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>75</td>
<td>3</td>
<td>3</td>
<td>relevant to pSS</td>
<td>64</td>
<td>-</td>
<td>&lt;0.05</td>
<td>n.d</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>41</td>
<td>48</td>
<td>0.5</td>
<td>relevant to pSS &gt;256</td>
<td>64</td>
<td>-</td>
<td>&lt;0.05</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>54</td>
<td>48</td>
<td>0.1</td>
<td>relevant to pSS</td>
<td>64</td>
<td>-</td>
<td>0.21</td>
<td>54</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>76</td>
<td>4</td>
<td>9</td>
<td>relevant to pSS</td>
<td>4</td>
<td>1</td>
<td>&lt;0.05</td>
<td>32</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>66</td>
<td>12</td>
<td>0</td>
<td>relevant to pSS</td>
<td>64</td>
<td>-</td>
<td>&lt;0.05</td>
<td>46</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>77</td>
<td>36</td>
<td>0.2</td>
<td>relevant to pSS</td>
<td>64</td>
<td>-</td>
<td>0.47</td>
<td>n.d</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>54</td>
<td>36</td>
<td>1</td>
<td>relevant to pSS &gt;256</td>
<td>64</td>
<td>1</td>
<td>2.07</td>
<td>n.d</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>73</td>
<td>4</td>
<td>1.8</td>
<td>relevant to pSS &gt;256</td>
<td>-</td>
<td>1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>67</td>
<td>84</td>
<td>0.4</td>
<td>relevant to pSS</td>
<td>-</td>
<td>-</td>
<td>0.23</td>
<td>15</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>57</td>
<td>9</td>
<td>7.5</td>
<td>not relevant to pSS</td>
<td>-</td>
<td>0.23</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>72</td>
<td>6</td>
<td>5</td>
<td>relevant to pSS</td>
<td>16</td>
<td>0.06</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>65</td>
<td>1</td>
<td>1.4</td>
<td>relevant to pSS</td>
<td>16</td>
<td>0.11</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>74</td>
<td>6</td>
<td>5</td>
<td>not relevant to pSS</td>
<td>1</td>
<td>&lt;0.05</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>78</td>
<td>12</td>
<td>1</td>
<td>relevant to pSS</td>
<td>64</td>
<td>2</td>
<td>0.05</td>
<td>45</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>48</td>
<td>30</td>
<td>0</td>
<td>relevant to pSS</td>
<td>64</td>
<td>-</td>
<td>&lt;0.05</td>
<td>10</td>
</tr>
<tr>
<td>27</td>
<td>F</td>
<td>70</td>
<td>96</td>
<td>3.2</td>
<td>relevant to pSS</td>
<td>-</td>
<td>0.06</td>
<td>n.d</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>72</td>
<td>8</td>
<td>0.5</td>
<td>relevant to pSS</td>
<td>64</td>
<td>0.22</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>66</td>
<td>180</td>
<td>0</td>
<td>relevant to pSS</td>
<td>64</td>
<td>0.11</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

Decreased salivary function was also estimated by salivary scintigraphy according to the method reported by the Revised Japanese Criteria for SS(19).

Pat.: Patient; W: woman; M: man; CRP: mg/ml; ESR: mm/hour. n.d: not determined. —: negative.

and the findings of salivary scintigraphy at the baseline versus after 12 months of treatment; no significant changes were observed in these parameters (data not shown).

However, interestingly, the 14 patients with anti-SSA/Ro antibody values that were either negative or <64 U/ml responded well to the cephaparine treatment: their stimulated salivary flow demonstrated a significant increase after 12 months of cephaparine treatment, whereas the 15 patients with anti-SSA/Ro antibody values >64 U/ml did not show such an improvement (Figure 2). With regard to the values of anti-SSB/La antibody, CRP and ESR, there was no significant relationship between the increase in salivary flow and the values of these parameters.

Histological grading of inflammatory lesions

The minor salivary glands of the lower lip were histologically investigated at the baseline in all 29 patients, and the inflammatory lesions identified in the salivary glands of eight patients, (Patients 1, 2, 4, 5, 7, 10, 12, and 13) were subjected to a histological examination both before and after cephaparine treatment. Most of the histological characteristics seen in pSS patients can be attributed to the presence of focal and periductal accumulations of

Fig. 1 Stimulated whole salivary secretion at baseline and after 12 months of cephaparine treatment. Stimulated salivary secretion was estimated by a 10-min chewing gum test. Cephaparine significantly increased the salivary secretions after 12 months (5.3 ± 4.5 ml/10 min) compared to the baseline values (3.1 ± 2.9 ml/10 min).
Fig. 2 The 14 patients with anti-SSA/Ro antibody values that were either negative or <64 U/ml showed a significant increase in salivary secretion in response to cephapranthine therapy (A), whereas the 15 patients with anti-SSA/Ro antibody values >64 U/ml demonstrated no significant increase in salivary flow (B).

As shown in Figure 3, extensive periductal inflammatory lesions and parenchymal destruction were apparent in the minor salivary glands of Patients 1, 2, 4, and 13 (Figure 3A, C, E, and F). Interestingly, these four patients receiving cephapranthine treatment showed an improvement in the salivary gland lesions: periductal infiltration of mononuclear cells and parenchymal destruction were rarely observed in these lesions (Figure 3B, D, F, and H).

In addition, cephapranthine therapy inhibited the progression of the lesions in Patients 5, 10, and 12 (data not shown). Unfortunately, progression of the lesions was seen in one patient (Patient 7). Table 2 summarizes the histological grading of the lesions in eight patients.

Immunoehistochemical detection of p65, phosphorylated IkB-α, MMP-9, and type IV collagen

As shown in Figure 4, there was enhanced expression of p65 (Figure 4A), phosphorylated IkB-α (Figure 4C), and MMP-9 (Figure 4E) in pSS acinar cells near infiltrated mononuclear cells, where destruction of the acinar structure seemed to have occurred. In addition, the continuity of staining for type IV collagen, one of the major components of the basement membrane, was disrupted in pSS acinar tissues adjacent to the infiltrated mononuclear cells (Figure
Table 2  Histological grading of inflammatory lesions in 8 patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Baseline</th>
<th>After 12 months</th>
<th>Histological changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Grade 1</td>
<td>Grade 0</td>
<td>Improvement of lymphocyte infiltrations and destruction of acinar tissues</td>
</tr>
<tr>
<td>2</td>
<td>Grade 4</td>
<td>Grade 2</td>
<td>Improvement of lymphocyte infiltrations and destruction of acinar tissues</td>
</tr>
<tr>
<td>4</td>
<td>Grade 4</td>
<td>Grade 3</td>
<td>Improvement of lymphocyte infiltrations</td>
</tr>
<tr>
<td>5</td>
<td>Grade 1</td>
<td>Grade 1</td>
<td>No progression of the lesions</td>
</tr>
<tr>
<td>7</td>
<td>Grade 2</td>
<td>Grade 3</td>
<td>Progression of the lesions</td>
</tr>
<tr>
<td>10</td>
<td>Grade 4</td>
<td>Grade 4</td>
<td>No progression of the lesions</td>
</tr>
<tr>
<td>12</td>
<td>Grade 3</td>
<td>Grade 3</td>
<td>No progression of the lesions</td>
</tr>
<tr>
<td>13</td>
<td>Grade 3</td>
<td>Grade 1</td>
<td>Improvement of lymphocyte infiltrations</td>
</tr>
</tbody>
</table>

Histological grading was conducted according to the method proposed by Greenspan et al. The slides were scored by two independent, well-trained pathologists in a blind manner.

![Histological images](image.png)

4G, arrows). However, the cepharanthine treatment markedly diminished the intensity of staining for p65 (Figure 4B), phosphorylated IxB-α (Figure 4D), and MMP-9 (Figure 4F).

The cepharanthine treatment also restored the continuity of the type IV collagen (Figure 4H, arrows), indicating that cepharanthine strengthens the integrity of the acinar structure.

Fig. 4 Immunohistochemical detection of p65 (A, B), phosphorylated IxB-α (C, D), MMP-9 (E, F), and type IV collagen (G, H) in glands from Patient 1 at baseline (A, C, E, G) and after 12 months of cepharanthine therapy (B, D, F, H). Acinar cells adjacent to the infiltrated mononuclear cells showed highly intense staining for p65 (A), phosphorylated IxB-α (C), and MMP-9 (E), compared to acinar cells in the glands treated with cepharanthine (B, D, F). A lack of continuity of type IV collagen was observed in acinar tissues located near infiltrated mononuclear cells (G, arrows), whereas cepharanthine treatment restored the continuity of type IV collagen in acinar tissues (H, arrows). Similar immunohistochemical findings were observed in Patients 2, 4, and 13 (data not shown). (Original magnifications: A–H, ×400). When a nonimmunized rabbit IgG was used instead of the primary antibody, no significant staining was observed (data not shown).
by preventing destruction of the basement membrane.

Although we showed the immunostaining of p65, phosphorylated IkB-α, MMP-9, and type IV collagen in only one patient (Patient 1; Figure 4), the staining patterns in the remaining three patients (Patients 2, 4, and 13) were the same (data not shown). The intensity of staining for p65, phosphorylated IkB-α, and MMP-9 was also suppressed by cephaparine treatment in Patients 5, 10, and 12, but the suppression was less pronounced than that in Patients 1, 2, 4, and 13. The effect of cephaparine on the staining for type IV collagen was similar to the effect on the staining for p65, phosphorylated IkB-α and MMP-9. We used phosphorylated IkB-α as a marker of activation of NF-κB by cytokines such as TNF-α because the phosphorylation of IkB-α by IkB kinase is the essential event in the canonical pathway of TNF-α-mediated NF-κB activation\(^2\). When a nonimmunized rabbit IgG was used instead of the primary antibody, no significant staining was observed (data not shown).

**Discussion**

The main objective of this study was to determine the stimulatory effect of cephaparine on the salivary flow in pSS patients. We therefore focused our attention on the patients’ salivary gland function. Our hypothesis regarding the mechanism involved in the destruction of acinar tissues in pSS salivary glands is that TNF-α-mediated MMP-9 production through the activation of NF-κB signaling disrupts acinar cell-basement membrane interactions. The importance of interactions between the cell and the basement membrane to the survival of cells has also been reported in normal endothelial and prostate cancer cells\(^24,25\). In an earlier investigation of our hypothesis, we observed that the in vitro treatment of normal human acinar (NS-SV-AC) cells with cephaparine enabled these cells to survive on a type IV collagen substrate—even after treatment with TNF-α and cephaparine—via the suppression of a TNF-α-induced production of MMP-9\(^22\).

Our earlier study also demonstrated that the in vivo treatment of a murine model of human SS with cephaparine also diminished the expression of both phosphorylated IkB-α and MMP-9 and maintained the integrity of the type IV collagen, leading to the prevention of the destruction of the acinar structure in salivary glands\(^26\). Thus, to determine the therapeutic effectiveness of cephaparine in pSS patients, we investigated the ameliorating effects of cephaparine in a single-center open-label study. Our analyses revealed that (1) cephaparine exerted a significant increase in the stimulated salivary flow in pSS patients, (2) the patients with an anti-SSA/Ro antibody value that was either negative or <64 U/ml responded well to the cephaparine treatment, and (3) the increase in salivary flow was, at least in part, attributable to the reversion of the destruction of acinar tissues by blocking the NF-κB pathway.

As noted in the Introduction, cephaparine has been widely used for the treatment of patients with leukopenia\(^12\), nasal allergy\(^13\), and venomous snake bites\(^14\). Although the exact mechanism has not been elucidated, cephaparine exerts immunomodulatory effects by enhancing the cytotoxic effects of natural killer cells and macrophages\(^15,16\), suggesting that it may play a role in the regulation of the signaling pathway of cytokines. Indeed, we observed that cephaparine suppresses TNF-α-induced MMP-9 production through the inhibition of NF-κB activity in acinar cells\(^17\). Although we have not yet identified in detail the mechanism involved in the cephaparine-inhibited inhibition of NF-κB activity, except to discern that the blocking of degradation of the IkB-α protein is involved\(^17\), several possibilities can be suggested. Specifically, a decrease in the activity of IkB kinase or the ubiquitination or proteasome-mediated degradation of IkB-α, IkB-β, or IkB-ε could account for the cephaparine-inhibited inhibition of TNF-α-induced NF-κB activation\(^18\).

Since our immunohistochemical results, in part, demonstrated that cephaparine suppressed the phosphorylation of IkB-α in acinar cells of the salivary glands, these above explanations may have important physiological relevance in pSS patients. Cytokines such as TNF-α and IL-1β released from infiltrated mononuclear cells phosphorylate the IkB-α of acinar cells, followed by the NF-κB-dependent activation of the MMP-9 gene\(^27\). Since secreted pro-MMP-9 is easily activated by the ubiquitously present activators plasmin and trypsin\(^28\), activated MMP-9 preferentially degrades basement membrane components, including type IV collagen. It has been reported that when cells lose contact with parts of the extracellular matrix, such as the basement membrane, the cells enter anoikis, a type of programmed cell death\(^29\). Therefore, blocking the cytokine-mediated signaling pathway could be a promising strategy for bringing about a clinical improvement of the salivary glands of patients with pSS.

Based on the above considerations, a pilot study was conducted using a TNF-α blocker, infliximab, and the results suggested that this drug would be clinically useful for enhancing unstimulated salivary flow\(^30\). However, two multicenter, randomized, double-blind and placebo-controlled trials showed no evidence of efficacy of infliximab on the increase of salivary flow in pSS patients\(^31,32\). Mavragani et al. indicated that an anti-TNF agent, etanercept, augmented the IFN-α pathway in SS patients, resulting in the activation of B cell-activating factor (BAFF)\(^33\). In addition, BAFF, a key molecule in promoting B-lymphocyte activation and survival, was found to be induced by the stimulation of IFN-α in
salivary gland epithelial cells of pSS patients\(^3\).

These findings may thus indicate a reason for the lack of efficacy of anti-TNF agents in pSS patients\(^5\). Moreover, cultured SS salivary gland ductal cells were shown to produce high levels of an IFN-\(\gamma\)-inducible 10-kD protein (CXCL10) and a monokine induced by IFN-\(\gamma\) (CXCL9)—both of which are T cell-attracting chemokines—by the stimulation of IFN-\(\gamma\), a major cytokine present in the SS salivary glands\(^7\). A mechanism involved in the periductal infiltration of T cells may therefore be that T cells expressing CXCR3, the receptor for CXCL10 and CXCL9, accumulate around the ductal structure\(^7\), which results in the periductal infiltration of T cells. It has been reported that these CXCR3 ligand chemokine genes contain p65 NF-\(\kappa\)B-binding sites at the proximal regions\(^8\), and that IFN-\(\gamma\) potentiates TNF-\(\alpha\)-induced CXCL-10 production in human monocytes by increasing the activation of STAT1 and NF-\(\kappa\)B through JAK1 and JAK2\(^9\), it seems likely that cephaparine could inhibit an IFN-\(\gamma\)-induced activation of CXCR3 ligands by preventing the degradation of the 1xB-\(\alpha\) protein, suggesting that cephaparine functions as a proteasome inhibitor. We recently observed that cephaparine could inhibit the production of CXCL10 in IFN-\(\gamma\)-stimulated human salivary gland ductal (NS-SV-DC) cells (unpublished data). Accordingly, these findings might indicate the decrease of T-cell infiltration in the salivary gland tissues of cephaparine-treated pSS patients.

Our present findings suggest that cephaparine would be a promising agent for use in the treatment of salivary gland involvement in patients with pSS. Cephaparine may also have applications in various other diseases in which NF-\(\kappa\)B activation has been shown to mediate pathogenesis, including arthritis and oral lichen planus. These possibilities warrant further investigation.

**Acknowledgements**

We thank all of the patients who participated in this study.

**References**

27) Sato H and Seiki M: Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene 8, 395-405 (1993)
37) Ogawa N, Ping L, Zhenjin L, Takada Y and Sugai
S: Involvement of the interferon-γ-induced T cell-attracting chemokines, interferon-γ-inducible 10-kd protein (CXCL10) and monokine induced by interferon-γ (CXCL9), in the salivary gland lesions of patients with Sjögren’s syndrome. Arthritis Rheum 46, 2730-2741 (2002)
