

**Targeting TNF- $\alpha$  suppresses the production of MMP-9  
in human salivary gland cells**

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## **Abstract**

**Objective:** Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a pleiotropic cytokine that plays an essential role in inflammation and apoptosis. Our previous study suggested that TNF- $\alpha$ -induced activation of matrix metalloproteinase-9 (MMP-9) resulted in the destruction of acinar tissue in the salivary glands of patients with Sjögren's syndrome (SS) via disruption of the acinar cell-basement membrane. Recently, a wide array of biological agents has been designed to inhibit TNF, including etanercept and adalimumab.

In this study, we demonstrate the suppressive effect of anti-TNF agents on TNF- $\alpha$ -induced MMP-9 production in NS-AV-AC, an immortalized human salivary gland acinar cell line.

**Materials and Methods:** NS-AV-AC cells were treated with etanercept or adalimumab after TNF- $\alpha$  treatment. MMP-9 production and enzymatic activity were, respectively, visualized by real-time PCR and ELISA assay, and evaluated by gelatin zymography, and apoptosis was evaluated by DNA fragmentation assay.

**Results:** TNF- $\alpha$  induced the production of MMP-9 in NS-SV-AC cells. However, this production was greatly inhibited by treatment with etanercept or adalimumab. In addition, TNF- $\alpha$ -induced DNA fragmentation was prevented by treatment with etanercept or adalimumab.

**Conclusions:** These results may indicate that anti-TNF agents would have therapeutic efficacy for preventing destruction of the acinar structure in the salivary glands of patients with SS.

## 1. Introduction

Sjögren's syndrome (SS), one of the most common rheumatic diseases,<sup>1</sup> is characterized by the eventual total replacement of the acinar structure by marked lymphocytic infiltrates in the salivary and lacrimal glands.<sup>2</sup> The pathogenesis of this selective and progressive destruction of the acinar structure in salivary glands is not yet fully understood. However, accumulated evidence indicates a close relationship between cytokine expression in salivary gland tissue and the development and progression of this disease.<sup>3,4</sup> 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 experimental animals during the development of SS.

Establishment of the normal acinar structure in salivary glands is fully dependent on the integrity of extracellular matrices, including the basement membrane.<sup>5</sup> 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. However, disruption of acinar cell–basement membrane interactions by excessive production of proteolytic enzymes, such as matrix metalloproteinases (MMPs), could lead to the disruption of the acinar tissue. MMP expression is regulated by growth factors, cytokines and hormones, as well as by interactions with extracellular matrix (ECM) proteins.<sup>6</sup> Endogenous inhibitors, such as tissue inhibitors of matrix metalloproteinases (TIMPs), also function to counterbalance MMP activity.<sup>7</sup> Because cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , have been shown to stimulate the production of collagenases,<sup>8,9</sup> it is

conceivable that cytokines contribute to destruction of the basement membrane, which in turn leads to the disruption of the acinar structure of the salivary gland. Moreover, structural changes in the basement membrane of salivary glands and increased levels of latent and active MMP-9 in saliva have recently been demonstrated in SS patients.<sup>10,11</sup> Taken together, these observations support the previous finding that MMP-9 is implicated in the pathogenesis of SS.<sup>12</sup>

Recently, we demonstrated that although NS-SV-AC cells (an SV40-immortalized normal human acinar cell clone) produced a large amount of MMP-9 in response to TNF- $\alpha$ , an NS-SV-AC clone transfected with a super-repressor form of I $\kappa$ B $\alpha$  (srI $\kappa$ B $\alpha$ )-complementary DNA (cDNA) lost its responsiveness to TNF- $\alpha$  in terms of MMP-9 production.<sup>13</sup> In addition, suppression of TNF- $\alpha$ -induced MMP-9 production restored the normal *in vitro* morphogenesis of acinar cells even when they were cultured on type IV collagen-coated plates in the presence of both TNF- $\alpha$  and plasmin. Moreover, an immunohistochemical study using salivary gland tissue from SS patients indicated that acinar cells adjacent to the lymphocytic infiltrate exhibited enhanced expression of both MMP-9 and NF- $\kappa$ B compared with those distant from infiltrated lymphocytes as well as those in normal salivary glands.<sup>13,14</sup> It therefore seems likely that inhibition of TNF- $\alpha$ -induced MMP-9 production in acinar cells leads to the restored integrity of the acinar structure in SS salivary glands.

A wide array of biological agents has been designed to inhibit TNF, such as anti-TNF antibodies, and soluble receptors that bind and neutralize TNF have been developed for the treatment of inflammatory and

autoimmune diseases.<sup>15</sup> Anti-TNF agents include infliximab, etanercept and adalimumab. Infliximab is a chimeric mouse/human anti-TNF- $\alpha$  monoclonal antibody composed of a murine variable region and a human IgG1 constant region. Etanercept is composed of the extracellular portion of the 2 human type II TNF receptors linked to the Fc portion. Adalimumab is a fully humanized anti-TNF- $\alpha$  monoclonal antibody generated by recombinant DNA techniques, and its structure is indistinguishable from the normal human IgG1.<sup>16</sup>

In the present study, we examined the effect of etanercept and adalimumab on the TNF- $\alpha$ -induced MMP-9 production in NS-SV-AC cells. Based on the results, we anticipate that anti-TNF agents will be effective in terms of both inhibiting the release of TNF from lymphocytes and suppressing the TNF- $\alpha$ -induced MMP-9 production.

## **2. Materials and methods**

### **2.1. Cell culture**

The characteristics of NS-SV-AC cells have been described in detail elsewhere.<sup>17</sup> This cell clone was cultured at 37°C in serum-free keratinocyte medium (Gibco®, Life Technologies, Carlsbad, CA, USA) in an incubator with an atmosphere containing 5% CO<sub>2</sub>.

### **2.2. Cell growth assay**

Cells ( $1 \times 10^4$ /well) were grown in 96-well plates (Falcon, Oxnard, CA, USA) in serum-free keratinocyte medium in the presence of etanercept or adalimumab at concentrations of 0, 10, 20, 50 or 100  $\mu$ g/ml for 72 h. After the appropriate incubation periods, an MTT reagent (BioAssay

Systems, Hayward, CA, USA) was added to each well and incubation was continued for 4 h. The cells were dissolved in Solubilization Solution (BioAssay Systems) and read at 570 nm in a microtiter plate reader.

### **2.3. RNA Isolation and real-time quantitative PCR**

Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml= $1.2\times 10^{-9}$  mol/L) and then were treated with etanercept (2.5, 5, 10, 20, or 50  $\mu$ g/ml) (1  $\mu$ g/ml= $6.67\times 10^{-9}$  mol/L) or adalimumab (2.5, 5, 10, 20, or 50  $\mu$ g/ml) (1  $\mu$ g/ml= $6.76\times 10^{-9}$  mol/L) for 48 h. Total cellular RNA was isolated with TRIzol reagent (Invitrogen®, Life Technologies, Carlsbad, CA, USA). The expression levels of mRNAs for MMP-9, TIMP-1 and GAPDH were quantitatively analyzed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan) by using TaqMan® Universal PCR Master Mix (Applied Biosystems) and Assays-on-Demand™ Gene Expression Products (Applied Biosystems) according to the manufacturer's recommendations. Using 96-well plates, 50  $\mu$ l PCR reaction mixtures containing 25  $\mu$ l of the 2 $\times$  TaqMan Universal PCR Master Mix and 2.5  $\mu$ l of the 20 $\times$  TaqMan Assays-on-Demand™ Gene Expression Products were prepared. The thermal cycler protocol consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. An analysis of the relative gene expression data was performed using the  $2^{-\Delta\Delta CT}$  method on Sequence Detection System Software (Applied Biosystems). The fold-change in the studied gene expression, normalized to an endogenous control, was calculated using the formula  $RQ = 2^{-\Delta\Delta CT}$ . The relative expression levels of MMP-9 mRNAs were expressed as a fold-increase in GAPDH mRNA expression.

#### **2.4. Enzyme-linked immunosorbent assays (ELISA)**

Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then were treated with etanercept (2.5, 10, 20, or 50  $\mu$ g/ml) or adalimumab (2.5, 10, 20, or 50  $\mu$ g/ml) for 48 h. The concentrations of MMP-9 were determined with commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Optimal absorbance was read at 450 nm in a microtiter plate reader.

#### **2.5. Gelatin zymography**

Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then were treated with etanercept (2.5, 10, 20, or 50  $\mu$ g/ml) or adalimumab (2.5, 10, 20, or 50  $\mu$ g/ml) for 48 h. Before the assay, conditioned medium was collected. MMP-9 activity was determined with a commercially available gelatin-zymography kit (Cosmo Bio Co., Tokyo, Japan) according to the manufacturer's instructions. Briefly, 5  $\mu$ l of the conditioned medium samples was diluted in sample buffer (4% SDS, 125 mM Tris-HCl; pH 6.8, 20% glycerol, and 0.001% bromophenol blue) and subjected to electrophoresis on a 7% SDS-PAGE gel copolymerized with gelatin (0.15%) as the substrate. After electrophoresis, the gels were washed with 2% Triton X-100 solution for 1 h and incubated for 30 h on Tris CaCl<sub>2</sub> pH 7.4 buffer at 37°C. Then, the gels were stained with 0.05% Coomassie Brilliant Blue G-250 and destained with 25% methanol and 7% acetic acid solution. Gelatinolytic activities were detected as unstained bands against the background of the Coomassie blue-stained gelatin. Images were captured using a UV transilluminator (FASIII; Toyobo Co., Ltd., Osaka, Japan).

#### **2.6. DNA Fragmentation assay**

Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then treated with etanercept (10, 50  $\mu$ g/ml) or adalimumab (10, 50  $\mu$ g/ml) for 48 h. DNA Fragmentation was determined with a commercially available Apoptosis Ladder Detection Kit (Wako, Osaka, Japan).  $1 \times 10^6$  cells were collected and then enzyme activator solution, RNase solution and protein digestion enzyme solution were added. After incubating the cells at 50°C for 30 min, DNA extraction solution and isopropanol were added. The solution was then left at room temperature for 15 min. After centrifugation at 10,000 $\times$ g at room temperature for 10 min, the resulting DNA was dissolved in TE Buffer. Finally, the purified DNA was electrophoretically separated on 1.5% agarose gel and visualized by ethidium bromide staining.

## **2.7. Fluorescence-activated cell sorting analysis of apoptosis**

Apoptosis was analyzed using a fluorescence-activated cell sorting (FACS) Muse™ Cell Analyzer (Merck, Ltd., Tokyo, Japan) and an Annexin V & Dead Cell Kit (Merck, Ltd.).

## **2.8. Statistical analysis**

The statistical analysis was performed by Mann-Whitney *U*-test; Values of  $p < 0.05$  were considered to be statistically significant.

# **3. Results**

## **3.1. Effects of etanercept or adalimumab on cell growth**

The growth kinetics of NS-SV-AC cells treated with various concentrations of etanercept or adalimumab were investigated by MTT assay for up to 3 days. As shown in Figure 1, no remarkable cytotoxicity was observed when NS-SV-AC cells were treated with 10~100  $\mu$ g/ml of



etanercept or adalimumab.

### **3.2. Effects of etanercept or adalimumab on the production of TNF- $\alpha$ by NS-SV-AC cells**

Since TNF- $\alpha$  stimulates the expression of MMP-9, we performed an ELISA to determine the effect of etanercept or adalimumab on the production of TNF- $\alpha$  by NS-SV-AC cells. We found that etanercept and adalimumab had no effect on the production of TNF- $\alpha$  by NS-SV-AC cells (data not shown).

### **3.3. Relative levels of TNF- $\alpha$ -induced MMP-9 and TIMP-1 mRNA expression in NS-SV-AC cells.**

Figure 2 shows the expression of MMP-9 mRNA in NS-SV-AC cells pretreated for 24 h with TNF- $\alpha$  (20 ng/ml), followed by treatment for 48 h with TNF- $\alpha$  alone or a combination of etanercept (2.5, 5, 10, 20, or 50  $\mu$ g/ml) or adalimumab (2.5, 5, 10, 20, or 50  $\mu$ g/ml) and TNF- $\alpha$ . The NS-SV-AC cells demonstrated a significant increase in the expression of MMP-9 mRNA in response to TNF- $\alpha$ . Both etanercept and adalimumab suppressed the expression of MMP-9 mRNA when administered simultaneously with TNF- $\alpha$ . The suppressive effects of etanercept and adalimumab on the MMP-9 mRNA level were not altered by the use of different concentrations of either agent.

On the other hand, as shown in Figure 3, TIMP-1 mRNA expression was slightly augmented by TNF- $\alpha$  treatment ( $P=0.049$ ), and both etanercept and adalimumab suppressed the expression of TIMP-1 mRNA. The value of the MMP-9/TIMP-1 mRNA ratio was increased when NS-SV-AC cells were treated with TNF- $\alpha$ , and was decreased by treatment with etanercept

or adalimumab (Table 1).

### **3.4. Inhibition of MMP-9 protein expression by etanercept or adalimumab**

With respect to MMP-9 expression at the protein level, ELISA analysis confirmed that etanercept (2.5, 10, 20, or 50  $\mu\text{g/ml}$ ) or adalimumab (2.5, 10, 20, or 50  $\mu\text{g/ml}$ ) inhibited the TNF- $\alpha$ -induced production of MMP-9 protein in NS-SV-AC cells (Figure 4). The NS-SV-AC cells demonstrated a significant increase in the expression of MMP-9 protein in response to TNF- $\alpha$ . Etanercept seemed to be more effective than adalimumab at inhibiting the TNF- $\alpha$ -induced production of MMP-9 protein.

### **3.5. Suppression of MMP-9 activity by etanercept or adalimumab**

As shown in Figure 5, clearance of the gelatin substrate was greatly enhanced in TNF- $\alpha$ -treated NS-SV-AC cells. Consistent with the results of gelatin zymography, both etanercept and adalimumab prevented the ability of TNF- $\alpha$  to stimulate the production of MMP-9.

### **3.6. Suppression of TNF- $\alpha$ -induced apoptosis by etanercept or adalimumab**

Figure 6A shows the DNA fragmentation in NS-AV-AC cells treated with TNF- $\alpha$ . No apoptotic effect was observed in cells treated with etanercept or adalimumab alone. In NS-AV-AC cells, TNF- $\alpha$ -induced DNA fragmentation was prevented by treatment with etanercept or adalimumab. FACS analysis was found to be a sensitive indicator of TNF- $\alpha$ -induced apoptosis and of the efficacy of etanercept or adalimumab in blocking apoptosis. It revealed that 30.3% of the total cells were apoptotic after incubation with TNF- $\alpha$  compared with approximately 14.8% of the cells treated with TNF- $\alpha$  plus

etanercept and approximately 15.4% of the cells treated with TNF- $\alpha$  plus adalimumab (Figure 6B).

#### 4. Discussion

The etiology of SS has not yet been elucidated. Lymphocytic infiltrates are a characteristic histopathologic finding in SS. The presence of cytokines during the formation and proliferation of these lymphocytic infiltrates has been investigated,<sup>4,18</sup> and evidence suggests that proinflammatory cytokines, particularly TNF- $\alpha$ , may play an important role in the pathogenesis of the disease.<sup>19-21</sup> In addition, we examined the proteolytic activity in NS-SV-AC cells, and found that the proteolytic activity estimated by the MMP-2/TIMP-2 ratio was elevated when NS-SV-SC cells were treated with either TNF- $\alpha$  alone or a combination of TNF- $\alpha$  and IL-1 $\beta$ .<sup>14</sup>

We have previously shown that the expression of MMP-9 is up-regulated in SS acinar cells located near infiltrated lymphocytes, where destruction of the acinar structure seems to occur, compared with both the expression in cells distant from the infiltrated lymphocytes and the expression in cells from normal salivary glands.<sup>13</sup> We also found evidence that although NS-SV-AC cells entered apoptosis when cultured on type IV collagen-coated dishes in the presence of TNF- $\alpha$  and plasmin, suppression of TNF- $\alpha$ -induced MMP-9 production by the introduction of srIkB $\alpha$  cDNA corrected the aberrant *in vitro* morphogenesis of these cells.<sup>13</sup> 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.<sup>22,23</sup> Our previous results therefore indicate that MMP-9 would be one of the causal molecules in the destruction of the acinar structure in the salivary glands of SS patients and that suppression of MMP-9 activity in acinar cells may provide a therapeutic strategy for clinical improvement in SS salivary glands.

Recently, novel anti-TNF agents have been reported to be effective for the treatment of rheumatoid arthritis (RA). This fact strongly suggests that TNF is involved in the pathogenesis of RA.<sup>24</sup> The anti-TNF agents include infliximab, etanercept and adalimumab. Infliximab is a chimeric mouse/human anti-TNF monoclonal antibody composed of a murine variable region and a human IgG1 constant region. Etanercept is composed of the extracellular portion of the 2 human type II TNF receptors linked to the Fc portion. Adalimumab is a fully humanized anti-TNF- $\alpha$  monoclonal antibody generated by recombinant DNA techniques, and its structure is indistinguishable from that of the normal human IgG1. All 3 of these agents are able to bind to a soluble form of TNF and exert potent clinical effects on RA.<sup>25-27</sup> In contrast, infliximab and etanercept did not show such an effect on primary SS.<sup>28,29</sup>

In this study, we used cultures of an SV40-immortalized normal human acinar cell clone for our analysis. We hypothesized that anti-TNF agents may play an important role in the regulation of MMP-9 following TNF- $\alpha$  treatment in NS-SV-AC cells. In our previous study, MMP-9 expression was significantly increased by TNF- $\alpha$  in NS-SV-AC cells at the mRNA and protein levels. In the present study, it was of particular interest that the TIMP-1 expression was slightly increased by TNF- $\alpha$  at the mRNA

level. However, because the degree of increase in TIMP-1 was slight compared with the level of MMP-9, the MMP-9/TIMP-1 ratio was much higher in the presence of TNF- $\alpha$ . Recent studies have demonstrated increased MMP-9/TIMP-1 ratios in whole mixed saliva from patients with primary SS.<sup>30,31</sup> In the present work, we showed that the TNF- $\alpha$ -induced MMP-9 expression was inhibited by anti-TNF agents, and MMP-9/TIMP-1 ratios were reduced approximately 10-fold.

We also examined MMP-9 activity by gelatin zymography. The clearance of the gelatin substrate was greatly enhanced in TNF- $\alpha$ -treated NS-SV-AC cells. Both etanercept and adalimumab were able to prevent TNF- $\alpha$  from stimulating the production of pro-MMP-9. MMP-9 is secreted as a proenzyme and is subsequently activated by multiple enzymes, including cathepsin G, trypsin, stromelysin 1, and plasmin.<sup>32-35</sup> The activation of pro-MMP-9 is an inevitable step in acquiring the properties necessary for degradation of the basement membrane components. We have recently found that the latent form of pro-MMP-9 (92 kD) secreted from TNF- $\alpha$ -induced NS-SV-AC cells was actually converted to the active form (82 kD) in the presence of plasmin.<sup>11</sup>

These findings indicated that anti-TNF agents may prove useful to prevent destruction of the acinar structure. Our results also suggest that etanercept and adalimumab would be promising agents for use in the treatment of salivary gland involvement in patients with SS. However, in previous studies infliximab and etanercept did not show such an effect on primary SS.<sup>29,30</sup> It is worth noting that these prior investigations were performed using local administration of the anti-TNF agents into salivary

glands from the Stensen's duct and Wharton's duct in order to allow sialography. Considering that SS is characterized by the eventual total replacement of the acinar structure by marked lymphocytic infiltrates in salivary and lacrimal glands, it may be more efficient to administer the anti-TNF agents directly under the salivary and lacrimal glands.

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### **References**

1. Alspaugh MA, Whaley WW, Wohl MJ, Bunin JJ, Wohl MJ, Bunin JJ. Sjögren's syndrome. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, editors. Textbook of rheumatology. Philadelphia: WB Saunders: 971–999, 1981.
2. Daniel TE. Labial salivary gland biopsy in Sjögren's syndrome: assessment as a diagnostic criterion in 362 suspected cases. *Arthritis Rheum* 1984; 27(1): 147–156.
3. Hamano H, Saito I, Haneji N, Mitsuhashi Y, Miyasaka N, Hayashi Y. Expression of cytokine genes during development of autoimmune sialadenitis in MRL/lpr mice. *Eur J Immunol* 1993; 23(10): 2387–2391.
4. Fox RI, Kang HI, Ando D, Abrams J, Pisa E. Cytokine mRNA

- expression in salivary gland biopsies of Sjögren's syndrome. *J Immunol* 1994; 152(11): 5532–5529.
5. Bernfield M, Banerjee SD, Cohn RH. Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide protein (proteoglycan) at the epithelial surface. *J Cell Biol* 1972; 52(13): 674–689.
  6. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001; 17: 463–516.
  7. Woessner JF Jr. MMPs and TIMPs: an historical perspective. *Mol Biotechnol* 2002; 22(1): 33–49.
  8. Dayer JM, Beutler B, Cerami A. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by synovial cells and dermal fibroblasts. *J Exp Med* 1985; 162(6): 2163–2168.
  9. Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, et al. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. *J Clin Invest* 1994; 94(6): 2307–2316.
  10. Konttinen YT, Kangaspunta P, Lindy O, Takagi M, Sorsa T, Segerberg M, et al. Collagenase in Sjögren's syndrome. *Ann Rheum Dis* 1994; 53(12): 836–839.
  11. Hanemaaijer R, Visser H, Konttinen YT, Koolwijk P, Verheijen JH. A novel and simple immunocapture assay for determination of gelatinase-B (MMP-9) activities in biological fluids: saliva from patients with Sjögren's syndrome contains increased latent and active gelatinase-B levels. *Matrix Biol* 1998; 17(8-9): 657–665.
  12. Konttinen YT, Halinen S, Hanemaaijer R, Sorsa T, Hietanen J, Ceponis

- A, et al. Matrix metalloproteinase (MMP)-9 type IV collagenase/gelatinase implicated in the pathogenesis of Sjögren's syndrome. *Matrix Biol* 1998; 17(5): 335–347.
13. Azuma M, Aota K, Tamatani T, Motegi K, Yamashita T, Harada, et al. Suppression of tumor necrosis factor  $\alpha$ -induced matrix metalloproteinase 9 production by the introduction of a superrepressor form of inhibitor of nuclear factor  $\kappa$ B $\alpha$  complementary DNA into immortalized human salivary gland acinar cells: prevention of the destruction of the acinar structure in Sjögren's syndrome salivary glands. *Arthritis Rheum* 2000; 43(8): 1756–1767.
14. Azuma M, Motegi K, Aota K, Hayashi Y, Sato M. Role of cytokines in the destruction of acinar structure in Sjögren's syndrome salivary glands. *Lab Invest* 1997; 77(3): 269–280.
15. Sandborn WJ. Strategies for targeting tumour necrosis factor in IBD. *Best Pract Res Clin Gastroenterol* 2003; 17(1): 105–117.
16. Mitoma H, Horiuchi T, Tsukamoto H, Tamimoto Y, Kimoto Y, Uchino A, et al. Mechanisms for cytotoxic effects of anti-tumor necrosis factor agents on transmembrane tumor necrosis factor  $\alpha$ -expressing cells comparison among infliximab, etanercept, and adalimumab. *Arthritis Rheum* 2008; 58(5): 1248–1257.
17. Azuma M, Tamatani T, Kasai Y, Sato M. Immortalization of normal human salivary gland cells with duct-, myoepithelial-, acinar-, or squamous phenotype by transfection of SV40 orimutant DNA. *Lab Invest* 1993; 69(1): 24–42.
18. Fox PC, Speight PM. Current concepts of autoimmune exocrinopathy:



- immunologic mechanisms in the salivary pathology of Sjögren's syndrome. *Crit Rev Oral Biol Med* 1996; 7(2): 144–158.
19. Fox RI, Tornwall J, Michelson P. Current issues in the diagnosis and treatment of Sjögren's syndrome. *Curr Opin Rheumatol* 1999; 11(5): 364–371.
20. Hunger RE, Muller S, Laissue JA, Hess MW, Carnaud C, Garcia I, et al. Inhibition of submandibular and lacrimal gland infiltration in nonobese diabetic mice by transgenic expression of soluble TNF-receptor p55. *J Clin Invest* 1996; 98(4): 954–961.
21. Koski H, Janin A, Humphreys-Beher MG, Sorsa T, Malmstrom M, Konttinen YT. Tumor necrosis factor- $\alpha$  and receptors for it in labial salivary glands in Sjögren's syndrome. *Clin Exp Rheumatol* 2001; 19(2): 131–137.
22. Aoudjit F and Vuori K. Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-Flip and implication for anoikis. *J Cell Biol* 2001; 152(3): 633–643.
23. Jiang C, Wang Z, Ganther H, Lu J. Caspases as key executors of methyl selenium-induced apoptosis (anoikis) of DU-145 prostate cancer cells. *Cancer Res* 2001; 61(7): 3062–3070.
24. Feldmann M and Maini RN. Anti-TNF- $\alpha$  therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* 2001; 19: 163–196.
25. Bathon JM, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, et al. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. *N Engl J Med* 2000; 343(22): 1586–1593.

26. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. *N Engl J Med* 2000; 343(22): 1594–1602.
27. Weinblatt ME, Keystone EC, Furst DE, Moreland LW, Weisman MH, Birbara CA, et al. Adalimumab, a fully human anti-tumor necrosis factor  $\alpha$  monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMADA trial. *Arthritis Rheum* 2003; 48(1): 35–45.
28. Mariette X, Ravaud P, Steinfeld S, Baron G, Goetz J, Hachulla E, et al. Inefficacy of infliximab in primary Sjögren's syndrome: results of the randomized, controlled Trial of Remicade in primary Sjögren's syndrome (TRIPSS). *Arthritis Rheum* 2004; 50(4): 1270-1276.
29. Sankar V, Brennan MT, Kok MR, Leakan RA, Smith JA, Manny J, et al. Etanercept in Sjögren's syndrome: a twelve-week randomized, double-blind, placebo-controlled pilot clinical trial. *Arthritis Rheum* 2004; 50(7): 2240-2245.
30. Asatsuma M, Ito S, Watanabe M, Takeishi H, Nomura S, Wada Y, et al. Increase in the ratio of matrix metalloproteinase-9 to tissue inhibitor of metalloproteinase-1 in saliva from patients with primary Sjögren's syndrome. *Clin Chim Acta* 2004; 345(1-2): 99–104.
31. Perez P, Kwon YJ, Alliende C, Leyton L, Aguilera S, Molina C, et al. Increased acinar damage of salivary glands of patients with Sjögren's syndrome is paralleled by simultaneous imbalance of matrix metalloproteinase 3/tissue inhibitor of metalloproteinases 1 and matrix metalloproteinase 9/tissue inhibitor of metalloproteinases 1 ratios. *Arthritis*

Rheum 2005; 52(9): 2751-2760.

32. Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, et al. Matrix metalloproteinase-9 (92-kDa gelatinase/type IV collagenase) from HT1080 human fibrosarcoma cells: purification and activation of the precursor, and enzymic properties. *J Biol Chem* 1992; 267(30): 21712–21719.
33. Shapiro SD, Fliszar CJ, Broekelmann TJ, Mecham RP, Senior RM, Welgus HG. Activation of the 92-kDa gelatinase by stromelysin and 4-aminophenylmercuric acetate. *J Biol Chem* 1995; 270(11): 6351–6356.
34. Ogata Y, Enghild JJ, Nagase H. Matrix metalloproteinase-3 (stromelysin) activates the precursor for the human matrix metalloproteinase-9. *J Biol Chem* 1992; 267(6): 3581–3584.
35. Mazzieri R, Masiero L, Zanetta L, Monea S, Onisto M, Garbisa S, et al. Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cellbound reactants. *EMBO J* 1997; 16(9): 2319–2332.

## **Figure Legends**

### **Figure 1**

**The growth properties of NS-SV-AC cells treated with various concentrations of etanercept or adalimumab.**

Cells ( $1 \times 10^4$ /well) were grown in 96-well plates in medium supplemented with etanercept or adalimumab (10~100  $\mu\text{g/ml}$ ) for 72 h. Viable cells were estimated by MTT assay. A significant suppression of cell growth was not detected in NS-SV-AC cells.

## **Figure 2**

### **Effects of anti-TNF agents on the production of TNF- $\alpha$ -induced MMP-9 mRNA in NS-SV-AC cells.**

Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then were treated with etanercept (2.5, 5, 10, 20, or 50  $\mu$ g/ml) or adalimumab (2.5, 5, 10, 20, or 50  $\mu$ g/ml) for 48 h. Expression levels of mRNA for MMP-9 were determined by real-time quantitative PCR. The NS-SV-AC cells demonstrated a significant increase in the expression of MMP-9 mRNA in response to TNF- $\alpha$ . Both etanercept (A) and adalimumab (B) suppressed the expression of MMP-9 mRNA after pretreatment with TNF- $\alpha$ . The statistical analysis was performed with Mann-Whitney *U*-test. \* $P$ <0.05.

## **Figure 3**

### **Effects of anti-TNF agents on the production of TNF- $\alpha$ -induced TIMP-1 mRNA in NS-SV-AC cells.**

Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then were treated with etanercept (2.5, 10, or 50  $\mu$ g/ml) or adalimumab (2.5, 10, or 50  $\mu$ g/ml) for 48 h. Expression levels of mRNA for MMP-9 were determined by real-time quantitative PCR. The NS-SV-AC cells demonstrated a slightly but significant increase in the expression of TIMP-1 mRNA in response to TNF- $\alpha$ . Both etanercept (A) and adalimumab (B) suppressed the expression of TIMP-1 mRNA after pretreatment with TNF- $\alpha$ . The statistical analysis was performed with Mann-Whitney *U*-test. \* $P$ <0.05.

## **Figure 4**

### **Effects of anti-TNF agents on the production of TNF- $\alpha$ -induced MMP-9 protein in NS-SV-AC cells.**

Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then were treated with etanercept (2.5, 10, 20, or 50  $\mu$ g/ml) or adalimumab (2.5, 10, 20, or 50  $\mu$ g/ml) for 48 h. Concentrations of MMP-9 were determined with enzyme-linked immunosorbent assays (ELISA). Both etanercept (A) and adalimumab (B) suppressed the expression of MMP-9 protein after pretreatment with TNF- $\alpha$ . Etanercept appeared to be more effective than adalimumab at inhibiting the TNF- $\alpha$ -induced production of MMP-9 protein. The statistical analysis was performed with Mann-Whitney *U*-test. \**P*<0.05.

### **Figure 5**

#### **Effects of anti-TNF agents on the production of TNF $\alpha$ -induced MMP-9 activity in NS-SV-AC cells.**

Cells were pretreated for 24 h with TNF $\alpha$  (20 ng/ml) and then were treated with etanercept (2.5, 10, 20, or 50  $\mu$ g/ml) or adalimumab (2.5, 10, 20, or 50  $\mu$ g/ml) for 48 h. The MMP-9 activity was determined by using gelatin zymography. The clearance of the gelatin substrate was greatly enhanced in response to TNF $\alpha$ . Both etanercept and adalimumab blocked the stimulation of MMP-9 activity by TNF- $\alpha$ .

### **Figure 6**

#### **Effects of anti-TNF agents on TNF- $\alpha$ -induced apoptosis in NS-SV-AC cells.**

(A) Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then were treated with etanercept (10, 50  $\mu$ g/ml) or adalimumab (10, 50  $\mu$ g/ml) for 48 h. No apoptotic effect was observed in cells treated with etanercept or adalimumab alone. In NS-AV-AC cells, TNF- $\alpha$ -induced DNA fragmentation was prevented by treatment with etanercept or adalimumab.

(B) Cells were pretreated for 24 h with TNF- $\alpha$  (20 ng/ml) and then were treated with etanercept (50  $\mu$ g/ml) or adalimumab (50  $\mu$ g/ml) for 48 h. FACS analysis revealed that 30.3% of the total cells were apoptotic after incubation with TNF- $\alpha$  compared with approximately 14.8% of the cells treated with TNF- $\alpha$  plus etanercept and approximately 15.4% of the cells treated with TNF- $\alpha$  plus adalimumab.

### **Table 1**

#### **Quantitative determination of the MMP-9 and TIMP-1 mRNA expression ratio.**

The value of the MMP-9/TIMP-1 ratio was increased when NS-SV-AC cells were treated with TNF- $\alpha$ , and was decreased by treatment with etanercept or adalimumab.

Table 1. Quantitative determination of MMP-9 and TIMP-1 mRNA expression ratio

A. Etanercept

	MMP-9/ GAPDH	TIMP-1/ GAPDH	MMP-9/TIMP-1
Control	1	1	1
Etanercept	0.73	0.99	0.74
TNF $\alpha$	746.16	1.77	421.56
TNF $\alpha$ + Etanercept (2.5 $\mu$ g/ml)	65.48	1.57	41.71
TNF $\alpha$ + Etanercept (10 $\mu$ g/ml)	47.28	1.43	33.06
TNF $\alpha$ + Etanercept (50 $\mu$ g/ml)	34.19	1.23	27.80

B. Adalimumab

	MMP-9/ GAPDH	TIMP-1/ GAPDH	MMP-9/TIMP-1
Control	1	1	1
Adalimumab	1.01	0.90	1.12
TNF $\alpha$	517.84	1.71	302.83
TNF $\alpha$ + Adalimumab (2.5 $\mu$ g/ml)	64.96	2.04	31.84
TNF $\alpha$ + Adalimumab (10 $\mu$ g/ml)	56.87	1.33	42.76
TNF $\alpha$ + Adalimumab (50 $\mu$ g/ml)	54.49	1.30	41.92