Cepharanthin, a Biscoclaurine Alkaloid, Prevents Destruction of Acinar Tissues in Murine Sjögren's Syndrome

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ABSTRACT. ***Objective.*** Our previous study suggested that suppression by cepharanthin of tumor necrosis factor-α (TNF-α)-induced matrix metalloproteinase-9 (MMP-9) could prevent destruction of the acinar structure in the salivary glands of patients with Sjögren's syndrome (SS). In this study, we observed that *in vivo* administration of cepharanthin prevented severe damage to acinar tissues in the murine model of human SS.

Methods. Cepharanthin was intraperitoneally administered to thymectomized female NFS/*sld* mice. Inflammatory lesions in the salivary and lacrimal glands were then examined histologically. Expression of phosphorylated IκB-α, MMP-9, and type IV collagen was analyzed immunohistochemically. The apoptotic cell death of acinar cells was determined.

Results. Although extensive mononuclear cell infiltration and destruction of acinar tissue in salivary and lacrimal glands were observed in control mice, significant improvement of these lesions was evident in mice treated with cepharanthin. Immunohistochemical analysis revealed that p65, phosphorylated IκB-α, and MMP-9 were more strongly stained in the acinar cells of control mice than in cepharanthin-treated mice. Although no staining for type IV collagen was observed in the acinar tissues of control mice, continuity of staining for type IV collagen was observed in acinar tissues of cepharanthin-treated mice. Destruction of acinar tissues was attributed to the induction of apoptosis, suggesting that cepharanthin inhibits apoptosis by suppressing phosphorylation of IκB-α, followed by prevention of MMP-9 activation.

Conclusion. Our findings suggest that cepharanthin may be a promising agent for use in preventing destruction of acinar tissues in murine SS. (J Rheumatol 2006;33:912–20)

Key Indexing Terms:

SJÖGREN'S SYNDROME	CEPHARANTHIN	ACINAR TISSUE
MATRIX METALLOPROTEINASE-9	NUCLEAR FACTOR-κB	TYPE IV COLLAGEN

Sjögren's syndrome (SS), one of the most common rheumatic diseases[1], is characterized by 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 this disease. The expression of mRNA for various cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1ß (IL-1ß), IL-2, and interferon-γ (IFN-γ) has been detected in the salivary glands of humans as well as in those of experimental animals during the development of SS[3,4].

Establishment of the normal acinar structure of salivary glands is fully dependent on the integrity of extracellular matrices, including the basement membrane[5]. 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 (MMP) could lead to the disruption of acinar tissue. Because cytokines, including TNF-α and IL-1ß, have been shown to stimulate the production of MMP[6,7], it is conceivable that cytokines contribute to the destruction of the basement membrane, which in turn leads to disruption of the acinar structure of the salivary and lacrimal glands. Moreover, structural changes in the basement membrane of the salivary glands and increased levels of latent and active MMP-9 in the saliva have recently been described in patients with SS[8,9].

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Together, these observations support a finding[10] that MMP-9 is implicated in the pathogenesis of SS.

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-α, a super-repressor form of IκB-α [an inhibitory protein of transcription nuclear factor-κB (NF-κB)], cDNA-transfected NS-SV-AC clone lost its responsiveness to TNF-α in terms of MMP-9 production[11]. In addition, suppression of TNF-α-induced MMP-9 production restored the normal *in vitro* morphogenesis of NS-SV-AC cells, even when they were cultured on type IV collagen-coated plates in the presence of both TNF-α and plasmin. Therefore, it seems likely that inhibition of TNF-α-induced MMP-9 production in acinar cells may lead to restored integrity of the acinar structure in SS salivary glands.

Based on these considerations, we formulated the working hypothesis that identification of drugs that suppress TNF-α-induced production of MMP-9 would be a promising strategy for therapeutic intervention in SS. We reported that cepharanthin, a bisbenzylisoquinoline (biscoclaurine) alkaloid extracted from *Stephania cephalantha Hayata*, effectively suppressed TNF-α-induced MMP-9 production, which resulted in the restoration of normal *in vitro* morphogenesis of NS-SV-AC cells, even when they were cultured on type IV collagen-coated plates in the presence of TNF-α and plasmin[12].

To investigate the effectiveness of cepharanthin in preventing destruction of acinar tissues in patients with SS, we examined the *in vivo* effect of cepharanthin on development and progression of acinar destruction in murine SS. We found that cepharanthin exerted a therapeutic effect; namely, an improvement of the destructive lesions in both the salivary and lacrimal glands was observed.

MATERIALS AND METHODS

Mice and experimental design. A female NFS/N mouse strain carrying the mutant gene *sld*[13] was reared in our specific pathogen-free mouse colony. Animals were given access to food and water *ad libitum*. Mice were purchased from the Central Institute for Experimental Animals, Kawasaki, Japan. A thymectomy was performed on Day 3 after birth [(3 day)-Tx] in these NFS/*sld* [(3 day)-Tx NFS/*sld*] mice, because this murine model of primary SS spontaneously develops a form of the disease that shares many of the characteristics observed in patients with SS[14]. All the (3 day)-Tx NFS/*sld* mice developed SS-like symptoms in the salivary and lacrimal glands at the age of 4 weeks, and time lapse of the onset of disease was not observed[14]. A total of 25 (3 day)-Tx NFS/*sld* mice were investigated, consisting of control (saline administration, n = 9) and treated (10 μg cepharanthin/mouse, n = 5; 100 μg cepharanthin/mouse, n = 11) mice. Cepharanthin was administered intraperitoneally 5 times a week at a dose of 10 μg/mouse or 100 μg/mouse from 4 to 10 weeks of age. The submandibular, sublingual, and parotid salivary glands, as well as the lacrimal glands, from each mouse were surgically excised under halothane anesthesia (10 weeks of age). Care of the mice was in accord with institutional guidelines on animal welfare.

Histology. The salivary and lacrimal glands were fixed with 4% phosphate-buffered formaldehyde (pH 7.2) and prepared for histologic examination. Sections were stained with hematoxylin and eosin. Histological grading of inflammatory lesions was conducted according to the method proposed by White and Casarett[15] in the following manner: a score of 1 indicated that 1- to 5-foci mononuclear cells were seen (> 20 cells per focus); 2 indicated that > 5 such foci were seen, but without significant parenchymal destruction; 3 indicated that multiple confluent foci were seen with moderate degeneration of the parenchymal tissue; and 4 indicated extensive infiltration of the glands by mononuclear cells and extensive parenchymal destruction. The slides were scored by 2 independent, well trained pathologists in a blinded manner.

Immunohistochemical staining for p65, phosphorylated IκB-α, MMP-9, and type IV collagen in salivary and lacrimal glands. To investigate the effects of cepharanthin on enhanced expression of p65 (a subunit of NF-κB), phosphorylated IκB-α, and MMP-9 in the acinar tissues of the SS model mice, formalin-fixed paraffin-embedded samples of salivary and lacrimal glands were examined. Sections were dewaxed in xylene and rehydrated in graded ethanols according to standard procedures. Antigen retrieval was performed by incubating 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, Santa Cruz, CA, USA), rabbit polyclonal antibody to phosphorylated IκB-α peptide (Santa Cruz), rabbit polyclonal antibody to MMP-9 peptide (Sigma, St. Louis, MO, USA), and rabbit polyclonal antibody to mouse type IV collagen (Chemicon International, Temecula, CA, USA). The second antibody used was an antibiotinylated rabbit IgG (Vectastain kit). Negative controls for each material were processed in the same manner, using a nonimmunized rabbit IgG (Dako, Carpinteria, CA, USA) instead of the primary antibody. All histological analysis was done using blinded samples.

Apoptosis assay. The fixed sections were also subjected to an apoptosis assay. Apoptotic cells were identified using the terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) method (ApopTag *in situ* apoptosis detection kit; Oncor, Gaithersburg, MD, USA). In brief, apoptotic cells were labeled with digoxigenin-conjugated dUTP using terminal deoxynucleotide transferase according to the manufacturer's instructions, and they were then detected with anti-digoxygenin antibody conjugated with peroxidase. Slides were incubated with peroxidase substrate in order to visualize the peroxidase-positive cells, and then the cells were counterstained with hematoxylin. The peroxidase-positive cells on each slide were then counted in 5 high-power fields.

RESULTS

Pathology. Since it has been reported that the lesions first appeared in 4-week-old mice[14], we initiated the administration of cepharanthin in mice that were 4 weeks of age. During and after the experiment, no obvious adverse effects by cepharanthin, such as weight loss or shortening of lifespan, were observed in any of the mice. Most of the pathologic characteristics seen in patients with SS can be attributed to the presence of focal and periductal accumulation of mononuclear cells with or without parenchymal destruction in both the salivary and lacrimal glands[16,17]. As shown in Figure 1, periductal inflammatory lesions resembling those in cases of human primary SS in the submandibular (panels A and B), lacrimal (E and F), parotid (data not shown), and sublingual (data not shown) glands developed exclusively in control (3 day)-Tx NFS/*sld* mice. Extensive inflammatory lesions with parenchymal destruction were observed frequently in these groups. The number of inflammatory lesions in the submandibular, sublingual, parotid, and lacrimal glands was 3.2 ± 1.2, 1.6 ± 1.6, 3.0 ± 1.3, and 2.4 ± 0.7, respectively. However, (3 day)-Tx NFS/*sld* mice receiving cepharanthin showed a dramatic improvement in their submandibular (C and D), lacrimal (G and H), parotid (data not shown), and sublingual (data not

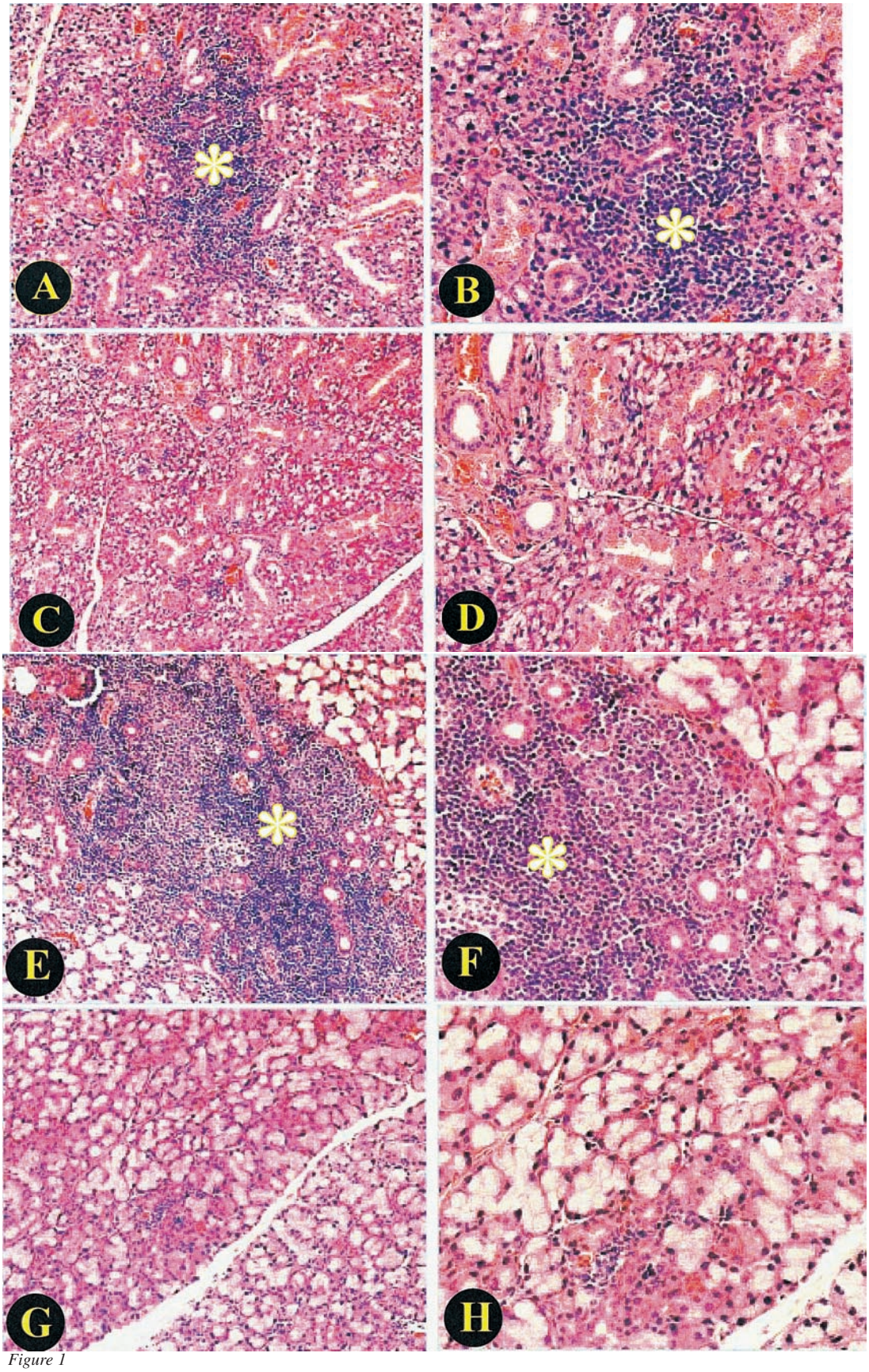


Figure 1

Figure 1 (opposite). Histology of inflammatory lesions in the submandibular (A–D) and lacrimal (E–H) glands of mice treated with (C, D, G, H) or without (A, B, E, F) cepharanthin (100 μg/mouse). Prominent periductal inflammatory infiltration was observed in each gland (A, B, E, F). Asterisks show infiltrated mononuclear cells. Mice receiving cepharanthin 100 μg showed significant reduction in mononuclear cell infiltrates and destruction of acinar tissues in submandibular (C, D) and lacrimal (G, H) glands. Similar histologic findings were observed in sublingual and parotid glands (data not shown). (Original magnifications: A, C, E, G, ×100; B, D, F, H, ×200)

shown) lesions; moreover, periductal infiltration of the mononuclear cells and parenchymal destruction was rarely seen in the (3 day)-Tx NFS/*sld* mice treated with 100 μg cepharanthin (C and D, G and H), and was only slightly more frequent in those treated with 10 μg cepharanthin (data not shown). The number of inflammatory lesions in the submandibular, sublingual, parotid, and lacrimal glands was 0.9 ± 1.6, 0 ± 0, 1.1 ± 1.0, and 0.7 ± 1.0, respectively, in the mice treated with 100 μg cepharanthin.

Histological grading of inflammatory lesions. Figure 2 shows a summary of the median grade of lesions with inflammatory infiltration of the mononuclear cells that developed in the submandibular, sublingual, parotid, and lacrimal glands of (3 day)-Tx NFS/*sld* mutant mice (n = 25). Administration of cepharanthin 100 μg/mouse significantly improved the grade of the inflammatory lesions ($p < 0.05$) in each gland; moreover, 10 μg cepharanthin per mouse also showed a propensity to decrease the grade of the inflammatory lesions in salivary glands, although this finding was without statistical significance. In the lacrimal glands, this dosage of cepharanthin had a significant effect on prevention of inflammatory infiltration of mononuclear cells ($p < 0.05$).

Immunohistochemical detection of p65, phosphorylated IκB-α, MMP-9, and type IV collagen. As shown in Figure 3, there was enhancement of p65 (panels A and B), phosphorylated IκB-α (E and F), and MMP-9 (I and J) expression in SS acinar cells located near infiltrated mononuclear cells, where destruction of the acinar structure seems to occur. In addition, the continuity of staining for type IV collagen, one of the major components of the basement membrane, was disrupted in SS acinar tissues adjacent to the infiltrated mononuclear cells (panel M). However, administration of 100 μg cepharanthin/mouse dramatically diminished the intensity of staining for p65 (panels C and D), phosphorylated IκB-α (G and H),

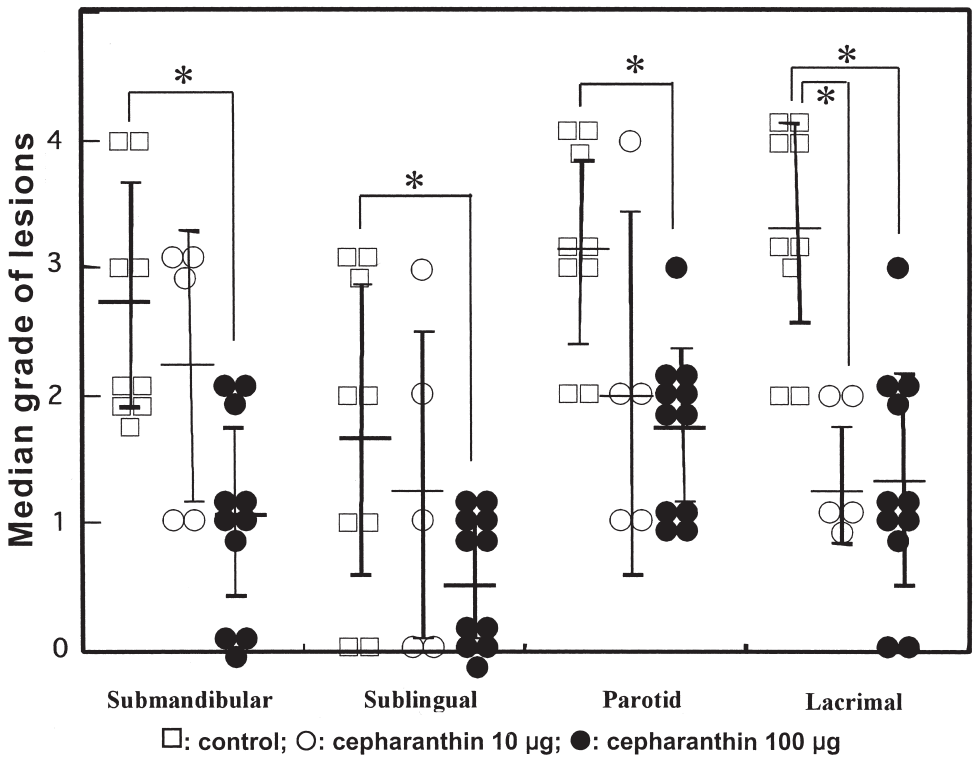
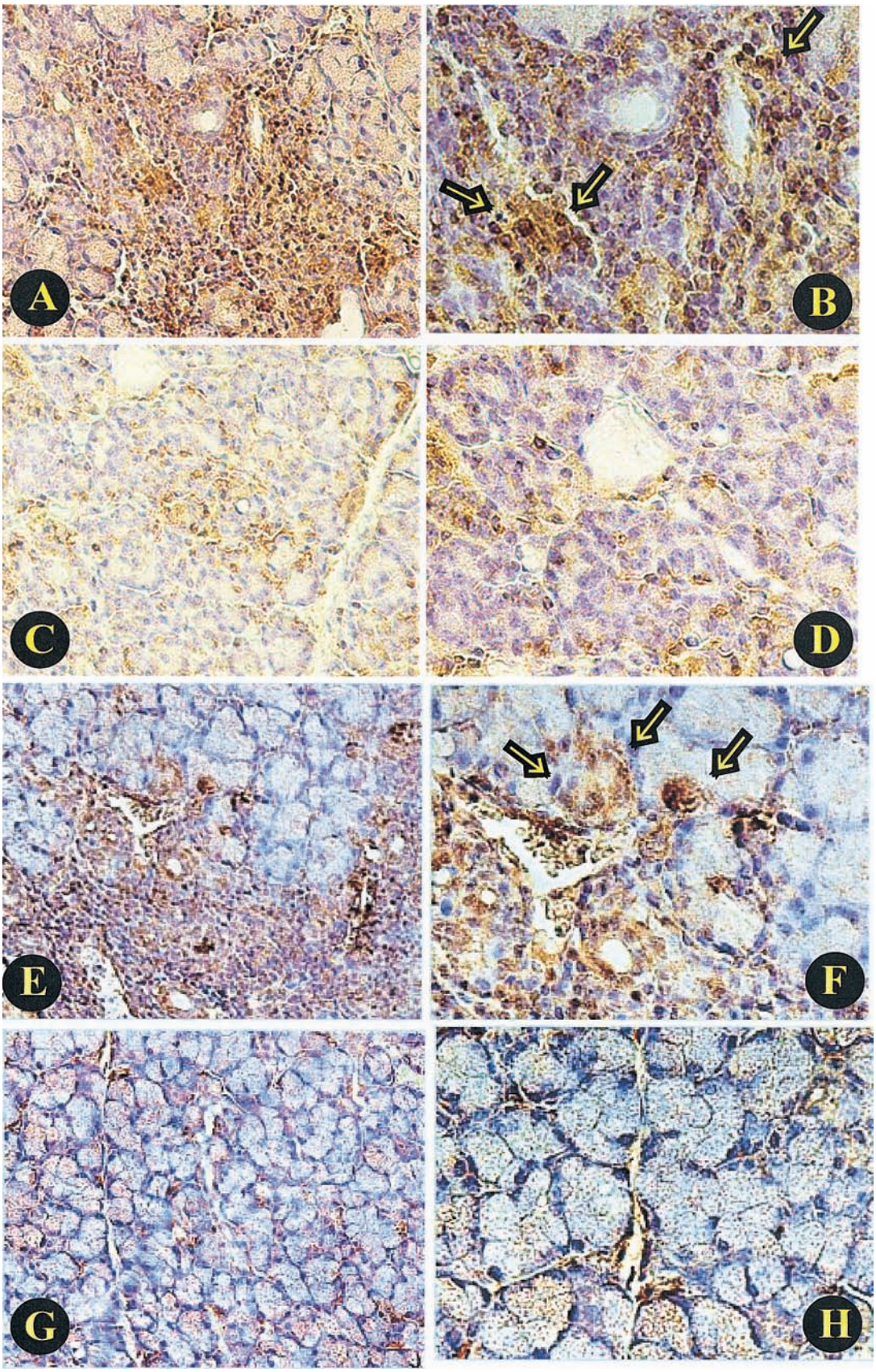


Figure 2. Median grade ± SD of lesions of submandibular, sublingual, and parotid salivary glands and lacrimal glands in mice treated with 10 μg or 100 μg cepharanthin or without cepharanthin, as follows: Submandibular glands in control mice (n = 9), 2.7 ± 0.9; treated mice (10 μg; n = 5), 2.2 ± 0.9; and treated mice (100 μg; n = 11), 1.1 ± 0.8. Sublingual glands in control mice, 1.6 ± 1.2; treated mice (10 μg), 1.2 ± 1.1; and treated mice (100 μg), 0.5 ± 0.4. Parotid glands in control mice, 3.1 ± 0.6; treated mice (10 μg), 2.0 ± 1.2; and treated mice (100 μg), 1.7 ± 0.3. Lacrimal glands in control mice, 3.3 ± 0.8; treated mice (10 μg), 1.2 ± 0.4; and treated mice (100 μg), 1.2 ± 0.9. Results analyzed by one-way ANOVA. *$p < 0.05$ compared with control mice. Grading of lesions according to the method of White and Cassarett[15].



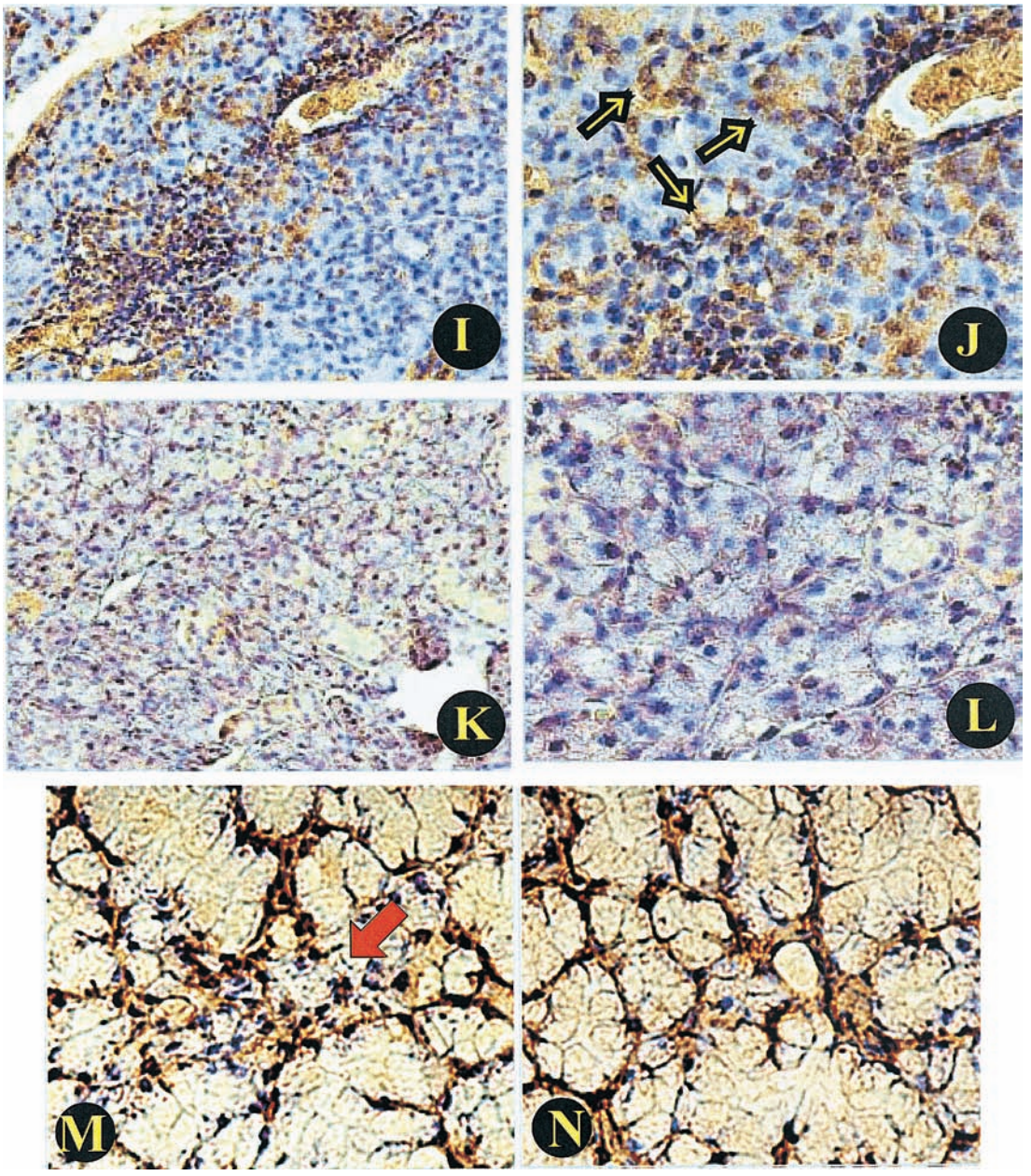


Figure 3. Immunohistochemical detection of p65 (A–D), phosphorylated IκB-α (E–H), MMP-9 (I–L), and type IV collagen (M and N) in lacrimal (A–H and M and N) and submandibular (I–L) glands from mice treated with (C, D, G, H, K, L, and N) or without (A, B, E, F, I, J, and M) cepharanthin (100 μg/mouse). Acinar cells adjacent to the infiltrated mononuclear cells showed highly intense staining (brown color, arrows) for p65 (A and B), phosphorylated IκB-α (E and F), and MMP-9 (I and J), compared with acinar cells in lacrimal (C, D, G, and H) and submandibular (K and L) glands treated with cepharanthin. Lack of continuity of type IV collagen was observed in acinar tissues located near infiltrated mononuclear cells (M, arrow), whereas treatment with cepharanthin restored the continuity of type IV collagen in acinar tissues (N). Brown and blue colors indicate type IV collagen and infiltrated mononuclear cells, respectively. Similar immunohistochemical findings were observed in the sublingual and parotid glands (data not shown). (Original magnifications: A, C, E, G, I, K, ×100; B, D, F, H, J, L, ×200; M and N, ×400)

and MMP-9 (K and L). Further, treatment of (3 day)-Tx NFS/*sld* mice with cepharanthin 100 μg restored the continuity of the type IV collagen, indicating that cepharanthin strengthens the integrity of the acinar structure by preventing destruction of the basement membrane (panels M and N). The intensity of staining for both phosphorylated IκB-α and MMP-9 was also suppressed by cepharanthin 10 μg, but the suppression was less pronounced than that by cepharanthin 100 μg. The effect of cepharanthin 10 μg on the staining for type IV collagen was similar to the effect of this dose on the staining for phosphorylated IκB-α and MMP-9. In Figure 3, although we show the immunostaining of phosphorylated

IκB-α, MMP-9, and type IV collagen in only the lacrimal and submandibular glands, the staining patterns in the other salivary glands were the same (data not shown). We employed phosphorylated IκB-α as a marker of activation of NF-κB by cytokines such as TNF-α because phosphorylation of IκB-α by IκB kinase is the essential event in the canonical pathway of TNF-α-mediated NF-κB activation[18,19].

Detection of apoptosis. To determine the mechanism involved in the destruction of acinar tissues in murine SS, a TUNEL assay was carried out. As shown in Figure 4, apoptotic cells with nuclear staining (brown color) were abundant in SS acinar cells (panel A), whereas administration of cepharanthin 100 μg/mouse dramatically diminished the apoptotic cell death of acinar cells (B). As quantified in Figure 5, doses of both 10 μg and 100 μg cepharanthin per mouse significantly prevented the apoptotic cell death of acinar cells ($p < 0.05$), indicating that destruction of acinar tissues was, at least in part, due to apoptosis of the acinar cells.

DISCUSSION

Our hypothesis regarding the mechanism involved in the destruction of acinar tissues in SS salivary glands is that TNF-α-mediated MMP-9 production through the activation of NF-κB signaling disrupts acinar cell-basement membrane interactions, which leads to apoptotic death of acinar cells. 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[20,21]. Based on our hypothesis, we recently reported that *in vitro* treatment of human acinar (NS-SV-AC) cells with cepharanthin enabled these cells to survive on a type IV collagen substrate, even after treatment with TNF-α and cepharanthin, via suppression of TNF-α-induced production of MMP-9[12]. Thus, to determine the therapeutic effectiveness of cepharanthin, we investigated the ameliorating effects of cepharanthin following destruction of acinar tissues in a murine model of human SS. We found that cepharanthin significantly reversed the destruction of acinar tissues by preventing the apoptotic cell death of acinar cells.

Consistent with our hypothesis on the molecular mechanism involved in the development of salivary and lacrimal gland lesions in SS[11,12], immunohistochemical staining for phosphorylated IκB-α, MMP-9, and type IV collagen and TUNEL analysis for apoptotic cell death clearly demonstrated the sequential processes occurring in the acinar tissues located near the infiltrated mononuclear cells: cytokines such as TNF-α and IL-1ß released from infiltrated mononuclear cells phosphorylate IκB-α of acinar cells, followed by the NF-κB-dependent activation of the MMP-9 gene[22,23]. Since secreted pro-MMP-9 is easily activated by the ubiquitously present activators plasmin and trypsin-2[24,25], 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[26-28]. Therefore, blocking the cytokine-mediated signaling pathway could be a promising strategy for bringing about clinical improvement of the salivary and lacrimal glands of patients with SS.

Cepharanthin has been widely used for the treatment of patients with leukopenia[29], nasal allergy[30], and venomous snake bites[31]. Although the exact mechanism has not been elucidated, cepharanthin exerts immunomodulatory effects by enhancing the cytotoxic effects of natural killer cells and macrophages[32,33], suggesting that it may play a role in regulation of the signaling pathways of cytokines. Indeed, we have reported that cepharanthin suppresses TNF-α-induced MMP-9 production through the inhibition of NF-κB activity in acinar cells[12]. Although we have not yet identified in detail the mechanism involved in the cepharanthin-induced inhibition of

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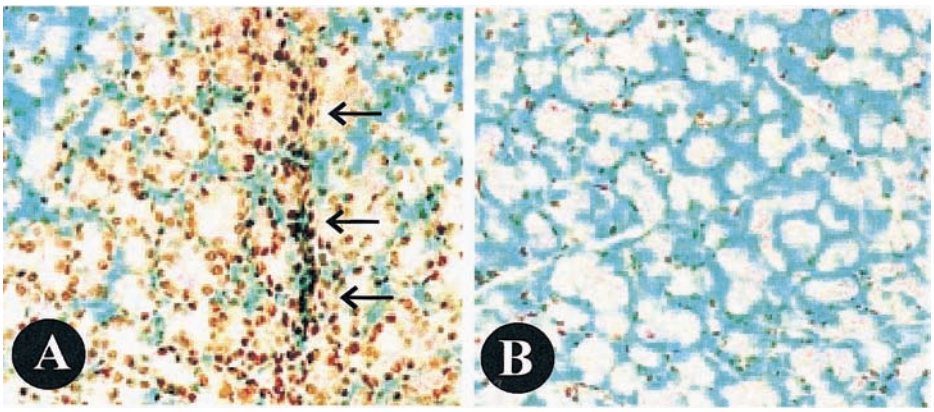

Figure 4. Apoptotic cell death of acinar cells in (3 day)-Tx NFL/*sld* mice. Findings are representative of those observed in 5 high-power fields. Numerous apoptotic cells (brown color) were detected in acinar cells from lacrimal glands (A); after treatment with cepharanthin 100 μg, the number of apoptotic cell deaths decreased dramatically (B). Arrows indicate infiltration of inflammatory cells. In acinar cells of salivary gland tissues, cepharanthin exerted an anti-apoptotic effect similar to that observed in lacrimal glands (data not shown). (Original magnification: A and B ×200)

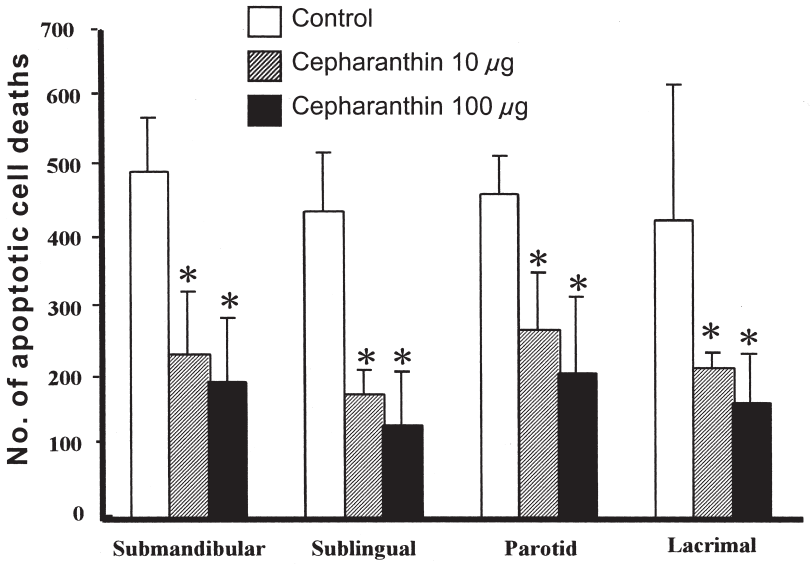

Figure 5. Effect of cepharanthin on apoptotic cell death of acinar cells in salivary and lacrimal glands. Data represent the mean apoptotic cell death ± SD as follows: Submandibular glands in control mice (n = 9), 498 ± 71; treated mice (10 μg; n = 5), 234 ± 80; and treated mice (100 μg; n = 11), 194 ± 83. Sublingual glands in control mice, 438 ± 77; treated mice (10 μg), 176 ± 32; and treated mice (100 μg), 132 ± 70. Parotid glands in control mice, 464 ± 51; treated mice (10 μg), 269 ± 71; and treated mice (100 μg), 208 ± 100. Lacrimal glands in control mice, 426 ± 174; treated mice (10 μg), 214 ± 18; and treated mice (100 μg), 166 ± 61. Results were analyzed by one-way ANOVA. *$p < 0.05$ compared with control mice.

NF-κB activity, except to discern that blocking of degradation of the IκB-α protein is involved, several possibilities can be suggested. Specifically, a decrease in the activity of IκB kinase or the ubiquitination or proteasome-mediated degradation of IκB-α, IκB-ß, or IκB-ε could account for the cepharanthin-mediated inhibition of TNF-α-induced NF-κB activation[34]. Since our results showed that cepharanthin suppressed the phosphorylation of IκB-α in acinar cells of the salivary and lacrimal glands, these explanations may have important physiological relevance *in vivo*. A TUNEL assay revealed that, as a result of inhibition of MMP-9 activation, cepharanthin enabled acinar cells in the murine salivary and lacrimal glands to survive in the extracellular matrix.

Another effective agent for treatment of SS could be a monoclonal antibody to TNF-α, infliximab. The mechanism by which infliximab improves salivary flow is the direct inhibition of TNF-α molecules. This suggests infliximab would be clinically useful for enhancing unstimulated salivary flow[35]. However, although infliximab may be able to inhibit the TNF-α-mediated activation of NF-κB, it would not be able to interrupt the signaling pathway mediated by IL-1, which also stimulates the expression of NF-κB-mediated genes. Consistent with this notion, a recent randomized, double-blind, placebo-controlled study of an anti-TNF-α agent found no evidence of efficacy of infliximab in primary SS[36]. Unlike infliximab, cepharanthin inhibits TNF-α-stimulated NF-κB activation by preventing degradation of the IκB-α protein in immortalized human salivary gland acinar (NS-SV-AC) cells[12], suggesting that cepharanthin functions as a proteasome inhibitor. Accordingly, cepharanthin might be able to suppress the expression of genes mediated through NF-κB activation by TNF-α and IL-1.

Our results show that acinar cells in the salivary and lacrimal glands of (3 day)-Tx NFS/*sld* mice exhibited both marked enhancement of phosphorylated IκB-α and MMP-9 expression, and disruption of the type IV collagen, resulting in their apoptotic cell death. Treatment of (3 day)-Tx NFS/*sld* mice with cepharanthin also diminished the expression of both phosphorylated IκB-α and MMP-9 and maintained the integrity of the type IV collagen, leading to prevention of destruction of the acinar structures in salivary and lacrimal glands. Thus cepharanthin treatment might provide an efficient and useful therapeutic approach for countering the destruction of acinar tissue in salivary and lacrimal glands of patients with SS.

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