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TNF-α inhibits aquaporin 5 expression in human salivary gland acinar cells *via* suppression of histone H4 acetylation

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Abstract

Sjögren's syndrome is a systemic autoimmune disease characterized by reductions in salivary and lacrimal secretions. The mechanisms underlying these reductions remain unclear. We have previously shown that TNF-α plays an important role in the destruction of acinar structures. Here we examined TNF-α's function in the expression of aquaporin (AQP) 5 in human salivary gland acinar cells. Immortalized human salivary gland acinar (NS-SV-AC) cells were treated with TNF-α, and then the expression levels of AQP5 mRNA and protein were analysed. In addition, the mechanisms underlying the reduction of AQP5 expression by TNF-α treatment were investigated. TNF-α-treatment of NS-SV-AC cells significantly suppressed the expression levels of AQP5 mRNA and protein, and reduced the net fluid secretion rate. We examined the expression and activation levels of DNA methyltransferases (Dnmts) in NS-SV-AC cells treated with TNF-α. However, no significant changes were observed in the expression or activation levels of Dnmt1, Dnmt3a or Dnmt3b. Although we also investigated the role of NF-κB activity in the TNF-α-induced suppression of AQP5 expression in NS-SV-AC cells, we detected similar TNF-α suppression of AQP5 expression in non-transfected cells and in a super-repressor form of IκBα cDNA-transfected cell clones. However, interestingly, chromatin immunoprecipitation analysis demonstrated a remarkable decrease in levels of acetylated histone H4 associated with the AQP5 gene promoter after treatment with TNF-α in NS-SV-AC cells. Therefore, our results may indicate that TNF-α inhibition of AQP5 expression in human salivary gland acinar cells is due to the epigenetic mechanism by suppression of acetylation of histone H4.

Keywords: Sjgren's syndrome • salivary gland acinar cells • TNF-α • aquaporin 5 • histone

Introduction

Sjögren's syndrome (SS), one of the most common rheumatic diseases [1], is characterized by the eventual total replacement of the acinar structure by marked infiltration of lymphocytes into the salivary and lacrimal glands [2]. The pathogenesis of this selective and progressive destruction of the acinar structure in these glands is not yet fully understood. However, accumulated evidence indicates a close relationship between cytokine expression in salivary and lacrimal gland tissues and the development and progression of this disease. The expression of mRNAs for various cytokines (*e.g.* tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2 and interferon-γ) has been detected in human salivary glands, as well as in those of experimental animals, during the development of SS [3, 4].

AQPs are specific water channels that allow the rapid transcellular movement of water in response to osmotic/hydrostatic pressure gradients [5]. AQP5, cloned from rat submandibular glands, is present in the water-transporting epithelia of the trachea, eyes, lungs, and lacrimal and salivary glands [6]. In human salivary glands, AQP5 has been topographically localized to the apical membranes of acinar cells [7], and it stimulates the outflow of water into the acinar lumen. In fact, a reduction in salivary gland secretion has been observed in mice harboring a mutant AQP5 channel [8]. In the salivary and lacrimal glands of SS patients, AQP5 expression in the plasma membrane was found to be reduced [9], or AQP5 distribution had changed from the apical

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membrane to the basal membrane [10]. The mechanisms underlying AQP5 dysfunction in the salivary and lacrimal glands of SS patients are not yet fully understood. Since suppression of AQP5 gene expression by TNF-α has been detected in mouse lung epithelial cells *in vitro* [11], TNF-α is expected to be a key factor in reduced AQP5 expression in salivary acinar cells.

Recent studies have indicated that the hypermethylation of CpG islands within the promoter and 5′ regions of genes is an important epigenetic mechanism for suppressing gene expression [12–14]. DNA hypermethylation may directly affect the basal transcriptional machinery by altering DNA secondary structure and inducing chromosome remodeling *via* the methyl-group binding proteins and histone deacetylase, thereby leading to transcriptional repression [15]. We have recently demonstrated that an immortalized normal human salivary gland ductal cell (NS-SV-DC) clone, which lacks AQP5 expression, acquires AQP5 gene expression in response to treatment with 5-aza-2′-deoxycytidine (5-Aza-CdR), a DNA demethylating agent [16], indicating that epigenetic modifications by DNA methylation and demethylation affect the expression levels of many genes.

On the other hand, deacetylation of histones results in a net increase in positively charged lysines and arginines at the N-terminal tail of the histones [17], thus inducing a tighter non-covalent linkage between the positively charged histones and the negatively charged DNA [18]. Consequently, transcription factors have difficulty accessing their DNA-binding sites [19], with a reduction or silencing of gene transcription. Thus, it has been reported that trichostatin A (TAS), an inhibitor of histone deacetylase (HDAC), alone induced the re-expression of methylated genes in pancreatic cancer cell lines, suggesting that the state of histone acetylation can influence gene expression [20].

Based on the above findings, in this study we examined AQP5 expression in a human salivary gland acinar cell clone in order to determine whether or not TNF-α suppresses this type of expression, and we investigated the mechanisms involved in the suppression of AQP5 expression by TNF-α in an acinar cell clone.

Materials and methods

Cells and media

The characteristics of the immortalized normal human salivary gland acinar (NS-SV-AC) and ductal (NS-SV-DC) cell clones used here have already been described in detail elsewhere [21, 22]. This cell clone was cultured at 37°C in serum-free keratinocyte medium (Gibco BRL, Grand Island, NY, USA) in an incubator with an atmosphere containing 5% CO_2.

Transfection of NS-SV-AC cells with a mutant form of IκBα cDNA

The IκBα double-point mutant (positions 32 and 36) construct (S32/36A) was described by Traenckner and colleagues [23], and an expression vector that contained S32/36A and pRc/CMV was the kind gift of J.F. Peyron, Facult Mdecine Pasteur, France. The empty vector used for the generation of the control cells was the pRc/CMV vector, which was purchased from Invitrogen (San Diego, CA, USA). NS-SV-AC cells (2×10^4) in 35-mm plastic Petri dishes (Falcon Labware, Oxnard, CA, USA) were used for the transfection experiment with a Superfect reagent (Qiagen, Valencia, CA, USA). The transfection procedures were performed as recommended by the manufacturer (Qiagen). In brief, NS-SV-AC cells were incubated with either a Superfect reagent-expression vector complex or a Superfect reagent-empty vector complex. Following three weeks' selection in growth medium containing 800 μg/ml G418 (Gibco BRL, Grand Island, NY, USA), three resistant colonies were isolated and the expression of the mutated form of IκBα was examined as described previously [24]. These cell clones (ACmI-1, -2, and -3) were maintained in culture medium with 800 μg/ml G418 for more than 6 months.

RNA isolation, RT-PCR and quantitative real-time PCR

The total cellular RNA was isolated after the NS-SV-AC cells were treated with TNF-α using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from 5 μg of total RNA using the Advantage cDNA PCR Kit (Clontech, Palo Alto, CA, USA). The sense and anti-sense primers for AQP5, AQP3, Dnmt1, Dnmt3a, Dnmt3b and GAPDH, respectively, were as follows: 5′-CAAGGCCGTGTTCGCAGAGTTCT-3′ and 5′-TCTTCCGCTCTTCCCGCTGCTCC-3′, 5′-CCTTTGGCTTTGCTGTCACTC-3′ and 5′-ACGGGGTTGTTGTAGGGGTCA-3′, 5′-GATCGAATTCATGCCGGCGCGTACCGCCCCAG-3′ and 5′-ATGGTGGTTTGCCTGGTGC-3′, 5′-GGGGACGTCCGCAGCGTCACAC-3′ and 5′-CAGGGTTGGACTCGAGAAATCGC-3′, 5′-CCTGCTGAATTACTCACGCCCC-3′ and 5′-GTCTGTGTAGTGCACAGGAAAGCC-3′, 5′-ACGCATTTGGCTGTATTGGG-3′ and 5′-TGATTTTGGAGGGATCTCGC-3′.

The PCR reactions were conducted in a DNA Thermal Cycler, model TP-3000 (Takara, Otsu, Japan). After 1 min. of denaturation at 94°C, 35 cycles of PCR were performed (94°C for 30 sec. and 68°C for 2 min.), followed by a final 3-min. extension at 72°C.

For the quantitative real-time PCR, equal aliquots (1 μl) of cDNA were amplified according to the manufacturer's TaqMan universal (50 μl) PCR master mix protocol using RT-PCR ABI PRISM 7000 (Applied Biosystems Japan Ltd., Tokyo, Japan). The primer set and TaqMan probe mixture used for PCR were purchased from Applied Biosystems (AQP5: Hs00387048_m1). The data were normalized using RT-PCR GAPDH primers (Applied Biosystems).

Western blot analysis

Crude plasma membranes, examined for AQP5 expression, were prepared from TNF-α-treated or untreated NS-SV-AC cells using a Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The whole cell lysates, examined for Dnmts, were prepared with lysis buffer containing 20 mM HEPES (pH 7.2), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 14,000 × *g* for 15 min. at 4°C, and the insoluble debris was discarded. Crude membrane extracts or cell lysates containing 20 μg of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gel, and then the samples were transferred to a nitrocellulose membrane. The membranes were blocked

with 2% skim milk and incubated with goat anti-human AQP5 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-human Dnmt1 antibody (Santa Cruz Biotechnology), rabbit anti-human Dnmt3a antibody (Santa Cruz Biotechnology), rabbit anti-human Dnmt3b antibody (Santa Cruz Biotechnology) or rabbit anti-human IκBα antibody (Santa Cruz Biotechnology). After intervening rinses with PBS, the antibody was detected using a chemiluminescence Western Blotting Kit (Amersham, Tokyo, Japan) according to the manufacturer's instructions.

Measurement of net fluid secretion rate

The net fluid secretion rates in the control NS-SV-AC cells and in cells treated with TNF-α (10 ng/ml) for 48 hrs were measured using a modified form of the original method [25]. In brief, the NS-SV-AC cells were grown to confluence on 6-well Transwell-Col culture chambers (Coster, Cambridge, MA, USA). The apical fluid was replaced with 400 μl of hyperosmotic medium (400 mOsm, *i.e.* 100 mM sucrose in medium), and the medium from the basolateral chamber was replaced with fresh isosmotic medium (300 mOsm). After 4 hrs of incubation, the liquid on the apical side was collected, and its volume was measured with a calibrated pipette; the net fluid secretion rate was then determined as described previously [26].

DNA methyltransferase activity assay

Cells were seeded on 100-mm plastic Petri dishes (Falcon Labware). Twenty-four hours after the seeding, the cells were treated with or without TNF-α (10 ng/ml) for 1–12 hrs, and the nuclear extracts were then obtained by a previously described method [24]. Ten μg of nuclear extracts from each condition were used for determining DNA methyltransferase activity by using the EpiQuik™ DNA Methyltransferase Activity Assay Kit (Epigentek Group Inc, NY, USA) according to the manufacturer's instructions.

Histone extraction and acetylation assay

Acid extraction of histones was performed according to the manufacturer's protocol for acetylated histones H3 and H4 extraction (Epigentek Group Inc). Acetylation assay was conducted by the method described by the manufacturer (Epigentek Group Inc). The acetylation levels of histones H3 and H4 were calculated by the formula indicated by the manufacturer. Protein samples containing histone were analysed by Western blotting as described above. The primary antibodies recognizing acetyl-histone H4 (P62805), and total histone H4 were obtained from Millipore (Temecula, CA, USA). After intervening rinses with PBS, the antibody was detected using a chemiluminescence Western Blotting Kit (Amersham) according to the manufacturer's instructions.

Immunofluorescence staining for acetylated histone H4

Cells grown on type I collagen (Nippi Inc., Tokyo, Japan)-coated coverglasses were washed with PBS three times, fixed in acetone at 4°C for 10 min. and incubated for 1 hr at 37°C with rabbit polyclonal antibody to acetyl-histone H4 (Millipore) at a dilution of 1:200. After three rinses with PBS, coverglasses were incubated for 1 hr with fluorescein-conjugated goat anti-rabbit IgG (1:200 dilution; Invitrogen). Coverglasses were mounted with Flupr™ Aqueous Mounting Medium (Lab Vision Corporation, Fremont, CA, USA). As a negative control, omission of primary antibody was used.

Chromatin immunoprecipitation (ChIP) assay

NS-SV-AC cells were treated with TNF-α (10 ng/ml) for indicated periods of time. ChIP assays were performed according to the Upstate Biotechnology ChIP kit (Upstate Biotechnology, Lake Placid, NY, USA). Immunoprecipitated DNA samples using an anti-acetyl-histone H4 were resuspended in H_2O and fractions used for PCR. Specific primers for AQP5 was designed to amplify the proximal promoter regions as follows: 5′-GGGAATTTCGGTTTGGGAGA-3′ (forward), 5′-CCCGTCCGAACCACGTAAC-3′ (reverse). Input and immunoprecipitated DNA were PCR amplified and analysed in triplicate.

Statistical analysis

The statistical analysis was performed with the Mann–Whitney *U*-test; *P* values of less than 0.05 were considered to indicate statistical significance.

Results

AQP5 expression was suppressed by TNF-α in human salivary gland acinar cells

RT-PCR was used to examine AQP5 mRNA expression in immortalized NS-SV-AC cells. NS-SV-AC cells were incubated with TNF-α at the concentrations ranging from 0 to 50 ng/ml for 8 hrs. As shown in Figure 1A, untreated cells clearly expressed AQP5 mRNA (739 bp), whereas cells incubated with TNF-α showed a significant dose-dependent decrease in the expression of AQP5 mRNA. Quantitative RT-PCR was employed for further examination of expression levels of AQP5 mRNA associated with TNF-α treatment in NS-SV-AC cells. As shown in Figure 1B, a statistically significant decrease in AQP5 mRNA expression was detected after TNF-α treatment. In addition, treatment of NS-SV-AC cells with TNF-α (10 ng/ml) caused a significant decrease of AQP5 expression in a time-dependent manner (Fig. 1C). Quantitative RT-PCR also demonstrated the inhibition of AQP5 expression in TNF-α-treated NS-SV-AC cells in a time-dependent manner (Fig. 1D). Because the expression of AQP3 is important for the fluid-secreting function of acinar cells [27], we examined the expression of AQP3 mRNA in the same samples that expressed AQP5 mRNA. Expression levels of AQP3 mRNA (372 bp) remained unaffected by TNF-α treatment (Fig. 1C). In the above RT-PCR experiments, equal loading of RNA samples was demonstrated for the housekeeping gene GAPDH

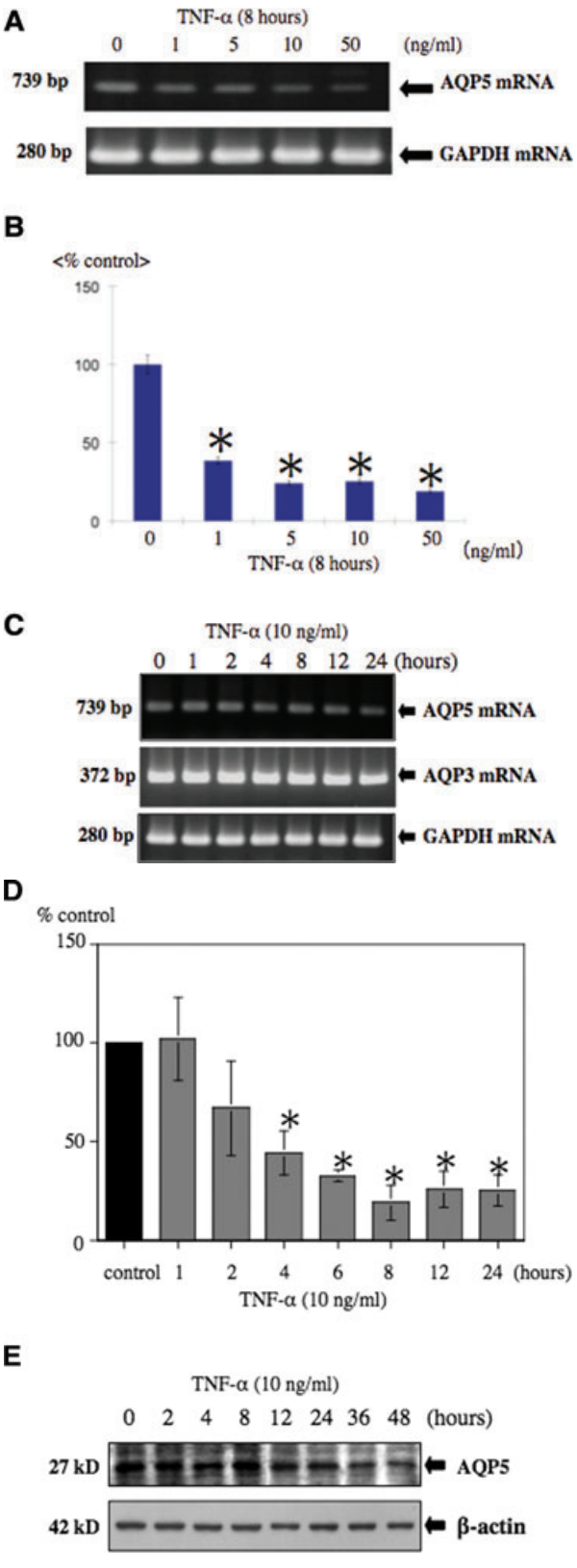

Fig. 1 TNF-α suppression of AQP5 expression in NS-SV-AC cells. (**A**) RT-PCR analysis of the expression of AQP5 mRNA in NS-SV-AC acinar cells. The cells expressed AQP5 mRNA (739 bp) under baseline conditions. After exposing the cells to TNF-α for 8 hrs, AQP5 mRNA expression decreased in a dose-dependent manner. Equal loading of RNA samples was demonstrated for the housekeeping gene GAPDH (280 bp). (**B**) Steady-state levels of AQP5 mRNA measured using quantitative real-time PCR. The primers and probes used for these experiments are described in 'Materials and methods'. The level of AQP5 mRNA expression in NS-SV-AC cells was compared with that (100%) under the basal condition. Each bar represents at least three separate mRNA isolations performed in duplicate. TNF-α-treated NS-SV-AC acinar cells exhibited significantly decreased AQP5 mRNA expression at the doses ranging from 1 to 50 ng/ml. *: statistically significant at $P < 0.05$ (Mann–Whitney *U*-test). (**C**) RT-PCR analysis of the expression of AQP5 mRNA in NS-SV-AC acinar cells. After exposing the cells to 10 ng/ml TNF-α, AQP5 mRNA expression decreased in a time-dependent manner. Equal loading of RNA samples was demonstrated for the housekeeping gene GAPDH (280 bp). Expression levels of AQP3 mRNA (372 bp) remained unaffected by TNF-α treatment. (**D**) Steady-state levels of AQP5 mRNA measured using quantitative real-time PCR. The level of AQP5 mRNA expression in NS-SV-AC cells was compared with that (100%) under the basal condition. Each bar represents at least three separate mRNA isolations performed in duplicate. TNF-α-treated NS-SV-AC acinar cells exhibited significantly decreased AQP5 mRNA expression in a time-dependent manner. *: statistically significant at $P < 0.05$ (Mann–Whitney *U*-test). (**E**) Western blot analysis of the AQP5 protein in crude plasma membranes from TNF-α-treated NS-SV-AC acinar cells. Significantly decreased expression of AQP5 protein (molecular weight: 27 kD) was clearly observed in NS-SV-AC cells after treatment with 10 ng/ml TNF-α (from 36 hrs to 48 hrs of treatment). These results were similar to those observed by RT-PCR analysis.

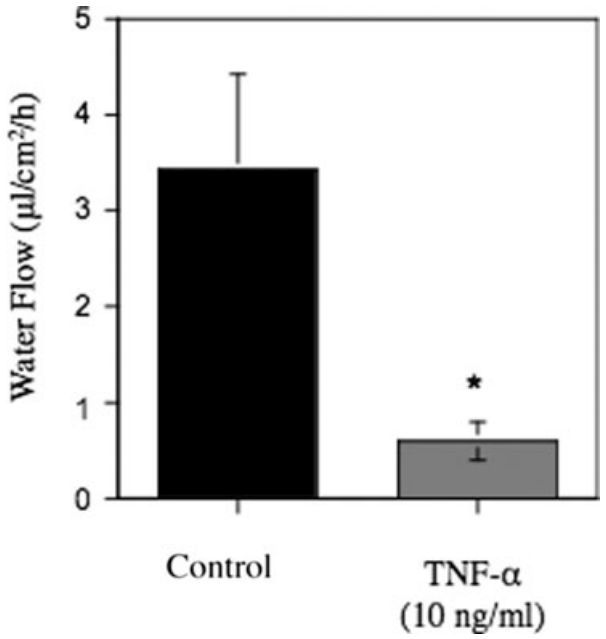

Fig. 2 Net fluid secretion rates across NS-SV-AC monolayers. Net fluid secretion rates of control (untreated NS-SV-AC cells) and TNF-α (10 ng/ml)-treated NS-SV-AC cells were measured using a hyperosmotic medium (400 mOsm) on the apical side and isosmotic medium on the basolateral side. The results are expressed as fluid flow in μl secreted/cm^2/hr, and are the means ± S.D. of three separate experiments performed in triplicate. The results were analysed using the Mann–Whitney *U*-test. $^*P < 0.05$ compared with control cells.

mRNA. To detect the production of AQP5 protein, crude plasma membranes were subjected to Western blot analysis during preparation. As can be seen in Figure 1E, AQP5 protein expression was significantly lower in the TNF-α-treated NS-SV-AC cells than in untreated cells. Thus, AQP5 expression was found to be suppressed, in both time- and dose-dependent manners, by TNF-α in human salivary gland acinar cells.

Fluid secretion rate was reduced by TNF-α in NS-SV-AC cells

To determine whether or not the down-regulation of AQP5 expression in TNF-α-treated NS-SV-AC cells affects water's ability to permeate the cell membrane, we measured the transepithelial net fluid secretion rate in NS-SV-AC cells that were either treated or untreated with TNF-α. The net movement of fluid across NS-SV-AC cells was measured in the presence of a transepithelial (apical > basal) osmotic gradient. After a 4-hr incubation period, the net fluid secretion rate of the control NS-SV-AC cells was approximately 3.8 μl/cm^2 per hour, whereas that of TNF-α-treated NS-SV-AC cells was significantly reduced, to a rate of 0.7 μl/cm^2 per hour (Fig. 2). These results indicate that the suppression of AQP5 expression in NS-SV-AC cells might be related to a reduction in water permeability.

TNF-α did not affect the expression or activity of DNA methyltransferases

We have previously demonstrated that an immortalized NS-SV-DC clone that lacks AQP5 expression acquires AQP5 gene expression in response to treatment with 5-aza-2′-deoxycytidine (5-Aza-CdR) *via* the hypomethylation of the AQP5 promoter region [16]. Because hypermethylation of the promoter region of the target genes is known to inhibit gene expression, we hypothesized that the suppression of AQP5 in the TNF-α-treated cells may have been caused by the induction of DNA methyltransferases. Therefore, we examined the expression levels of three Dnmt members: Dnmt1, Dnmt3a and Dnmt3b, in NS-SV-AC cells by using both RT-PCR and Western blot analyses. As shown in Figure 3A and B, no significant changes were observed in the mRNA and protein expression levels of the three Dnmts in the TNF-α-treated NS-SV-AC cells. Although TNF-α did not induce an up-regulation of Dnmt mRNA or protein expression, it is possible that TNF-α activates Dnmt enzymatic activity. Thus, we investigated Dnmt activity by using a DNA Methyltransferase Activity Kit. As shown in Figure 3C, no changes in enzymatic activity were detected in the TNF-α-treated NS-SV-AC cells. This suggests that DNA methylation is not involved in the TNF-α-induced suppression of AQP5 expression in NS-SV-AC cells.

NF-κB is not involved in the suppression of AQP5 by TNF-α

Nuclear factor-kappa B (NF-κB) is a transcriptional factor that up-regulates numerous genes. It is activated by many stimulators, including TNF-α. Therefore, we examined the possibility that the stimulation of NF-κB activity in turn suppresses AQP5 expression in NS-SV-AC cells in response to TNF-α. To examine the effects of NF-κB activation on AQP5 expression, we established mutant IκBα-transfected cell lines (designated as ACmI). As shown in Figure 4A, the ACmI-1 cell clone expressed both wild-type (wt-IκBα; 36 kD) and mutant-type (sr-IκBα; 38 kD) IκBα proteins. Although the expression of wild-type IκBα protein decreased in response to TNF-α treatment, mutant-IκBα protein was stably detected even after treatment with TNF-α. These results are similar to those described in a previous report by our group [24]. Thus, the results indicated that mutant IκBα cDNA was indeed transfected into NS-SV-AC cells. After NS-SV-AC cells and the ACmI-1 cell clone were incubated with TNF-α, the AQP5 mRNA expression level was confirmed by using real-time RT-PCR. The expression levels of AQP5 mRNA in the ACmI-1 cell clone were significantly down-regulated by TNF-α treatment (Fig. 4B) compared to those in NS-SV-AC cells. Western blot analysis was carried out to detect AQP5 protein expression in these two types of cell clone. As can be seen in Figure 4C,

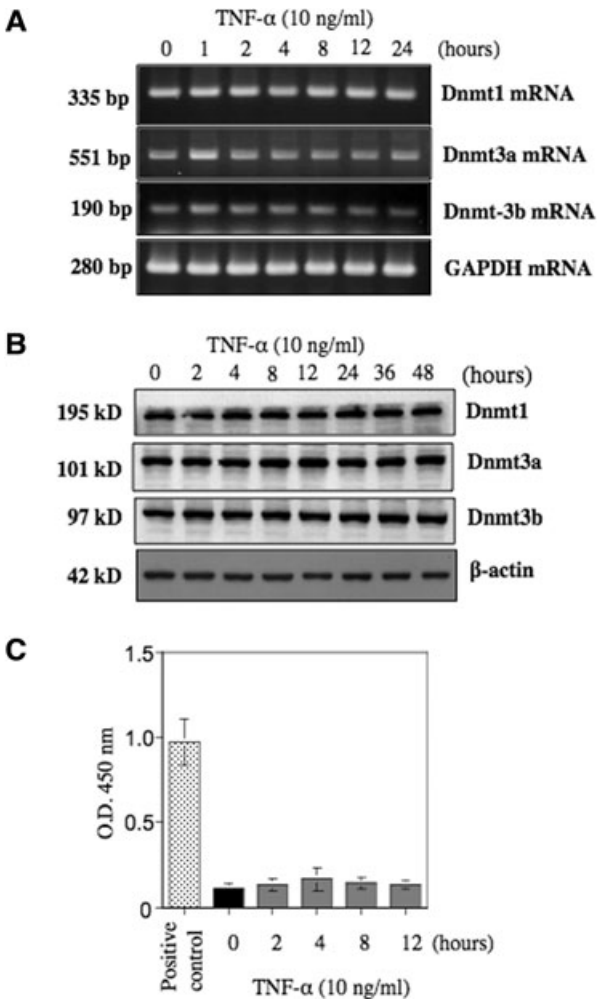

Fig. 3 Expression and activation levels of DNA methyltransferases in TNF-α-treated NS-SV-AC acinar cells. (**A**) RT-PCR analysis of the expression of Dnmt1, Dnmt3a and Dnmt3b mRNAs in TNF-α-treated NS-SV-AC acinar cells. NS-SV-AC acinar cells expressed Dnmt1, Dnmt3a and Dnmt3b mRNAs under the basal condition. After exposure to 10 ng/ml TNF-α, the expression levels of the three Dnmt mRNAs showed no significant changes. (**B**) Western blot analysis of Dnmt1, Dnmt3a and Dnmt3b proteins in TNF-α-treated NS-SV-AC acinar cells. There were no marked increases in the expression levels of these three Dnmts with TNF-α treatment. These results were similar to those observed by RT-PCR analysis. (**C**) Methylation activity assay of TNF-α-treated NS-SV-AC acinar cells. The methylation activity of TNF-α-treated NS-SV-AC cells was measured by using the EpiQuik^{TM} DNA Methyltransferase Activity Assay Kit. The results are expressed as optical density at a wavelength of 450 nm, and are the means ± S.D. of three separate experiments. The results were analysed using the Mann–Whitney *U*-test. $^{*}P < 0.05$ compared with control cells.

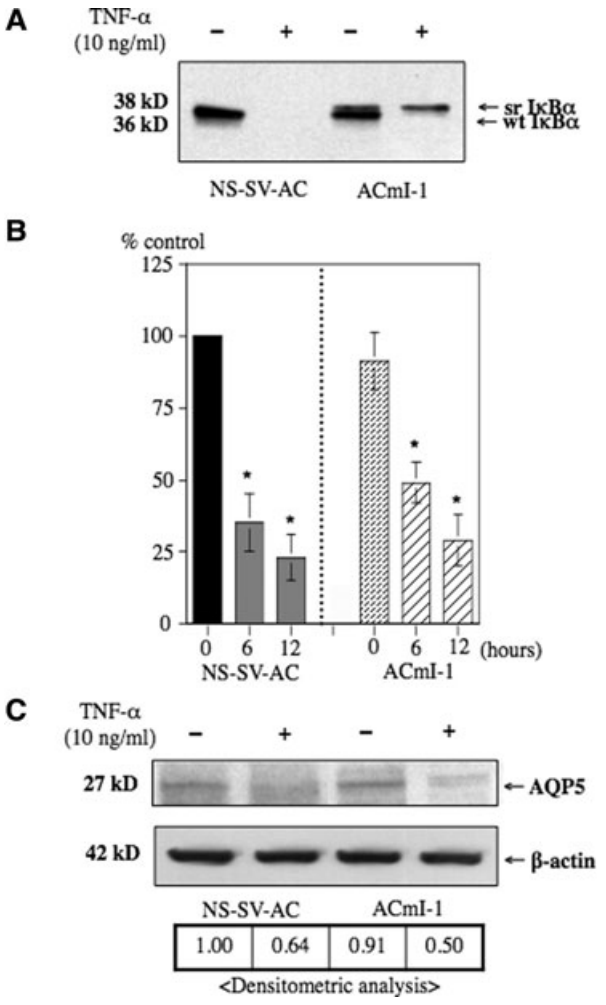

Fig. 4 The relationship between the suppression of AQP5 and NF-κB. (**A**) Western blot analysis of the expression patterns of IκBα in NS-SV-AC cells and ACmI-1 clone. The cells were treated with or without TNF-α (10 ng/ml, 30 min.) and the cytosolic fractions were collected. srIκBα was detected in the ACmI-1 cell clone. (**B**) Steady-state levels of AQP5 mRNA measured using quantitative real-time PCR. The expression levels of AQP5 mRNA in TNF-α-treated NS-SV-AC cells and ACmI-1 clone were compared with those (100%) under the basal condition. Each bar represents at least three separate mRNA isolations performed in duplicate. Both TNF-α-treated NS-SV-AC cells and ACmI-1 clone showed significantly decreased AQP5 mRNA expression. *: statistically significant at $P < 0.05$ (Mann–Whitney *U*-test). (**C**) Western blot analysis of AQP5 protein in TNF-α-treated NS-SV-AC cells and ACmI-1 clone. AQP5 protein expression was significantly decreased in both NS-SV-AC cells and ACmI-1 clone. Densitometric analysis (AQP5/β-actin ratio) revealed the expression levels of AQP5 protein in TNF-α treated NS-SV-AC cells and ACmI-1 clone relative to those of untreated NS-SV-AC cells. These results were similar to those observed with real-time RT-PCR analysis.

NS-SV-AC and ACmI-1 showed almost the same expression patterns of AQP5 protein. Densitometric analysis demonstrated almost identical expression levels of AQP5/β-actin in TNF-α-treated NS-SV-AC and ACmI-1 relative to those in untreated NS-SV-AC cells. These results agreed with those of the quantitative real-time RT-PCR analysis. Thus, it is evident that NF-κB was not involved in the suppression of AQP5 in TNF-α-treated NS-SV-AC cells.

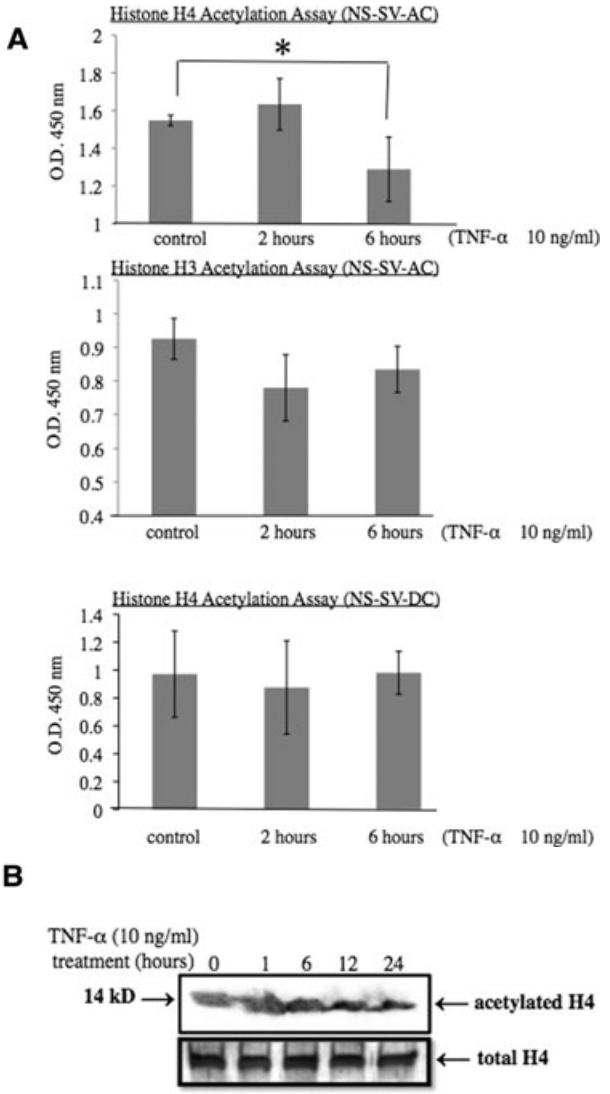

Fig. 5 Deacetylation of histone H4 in TNF-α-treated NS-SV-AC cells. Acetylation levels of histones H3 and H4 were analysed by using EpiQuik™ Global Histone Acetylation Assay Kits. (**A**) Histone H4 acetylation level was significantly suppressed by TNF-α treatment in NS-SV-AC cells. Histone H4 acetylation level was not affected by TNF-α treatment in NS-SV-DC cells. *: statistically significant at $P < 0.05$ (Mann–Whitney *U*-test). (**B**) Western blot analysis of both acetylated and total histone H4 in histone preparations of NS-SV-AC cells following treatment with TNF-α for the indicated times.

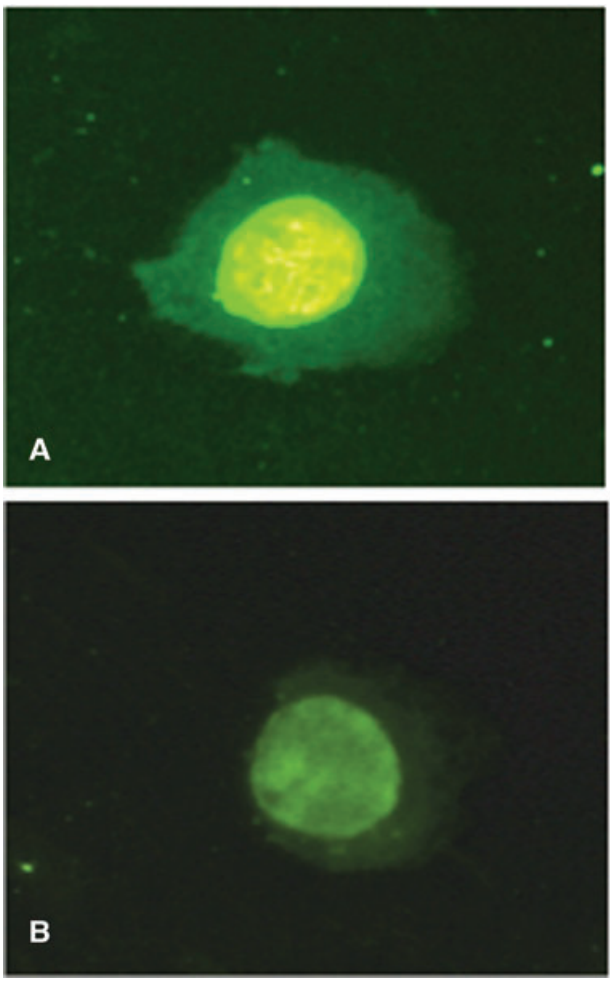

Fig. 6 Indirect immunofluorescence microscopy of acetylated histone H4 in NS-SV-AC cells treated with or without TNF-α. The expression of acetylated histone H4 was specifically observed in the nuclei of NS-SV-AC cells (**A**), however, reduced expression of acetylated histone H4 was evident in NS-SV-AC cells treated with TNF-α for 12 hrs (**B**).

TNF-α reduced the acetylation level of histone H4

Global levels of histone acetylations upon treatment with TNF-α were examined by Acetylation Assay Kits and Western blotting with antibodies specific for acetylated histone H4 (lysines 5, 8, 12, 16) and total histone H4. As shown in Figure 5A, TNF-α treatment of NS-SV-AC cells significantly suppressed the acetylation levels of histone H4, but not of histone H3. When ductal (NS-SV-DC) cells were used as a control, histone H4 acetylation level was not affected by TNF-α treatment. In addition, we determined levels of acetylated histone H4 protein by Western blot analysis. As shown in Figure 5B, treatment with TNF-α decreased levels of acetylated histone H4. The steady-state levels of histone H4 did not change significantly under a variety of conditions. In addition, fluorescence-microscopic examination revealed that nuclear staining of acetylated histone H4 was evident in control cells, however, reduced expression of acetylated histone H4 was detected in TNF-α-treated cells (Fig. 6). When primary antibody was omitted, no positive staining was observed (data not shown).

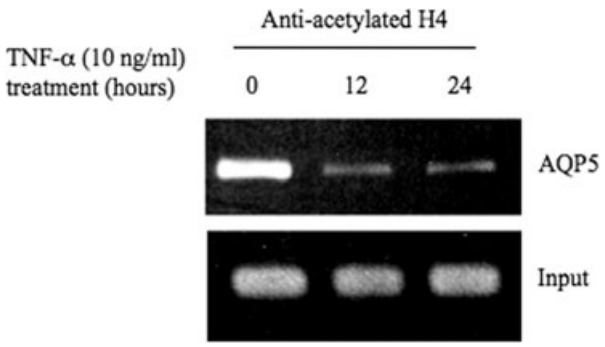

Fig. 7 TNF-α inhibition of acetylated histone H4 in chromatin associated with the AQP5 gene promoter. Cells were treated with TNF-α (10 ng/ml) for indicated periods of time, processed for ChIP assay and analysed by PCR with AQP5 promoter primers. Input controls were obtained by conducting PCR on sonicated genomic DNA before precipitation with antibody to acetylated histone H4. TNF-α reduced the acetylated histone H4 in chromatin associated with the AQP5 gene promoter.

TNF-α inhibits AQP5 expression by suppressing acetylated histone H4 to the AQP5 promoter

The effect of TNF-α on the acetylation of histone H4, which is associated with the AQP5 gene promoter, was examined using ChIP. Cells were harvested 12 and 24 hrs after TNF-α treatment and chromatin was precipitated with antibody against acetyl-histone H4. Subsequent PCR amplification of the AQP5 promoter in cells treated with TNF-α showed that reduced acetylated histone H4 was associated with the suppression of AQP5 gene promoter after treatment with TNF-α (Fig. 7). Therefore, these results suggest that transcription of AQP5 gene is suppressed upon deacetylation of histone H4.

Discussion

SS is a chronic autoimmune disease characterized by lymphocytic infiltration and the destruction of salivary and lacrimal glands [1, 2]. The biological mechanisms that lead to SS symptoms are poorly understood. Because various degrees of acinar destruction have been observed in the salivary and lacrimal glands of patients with SS [1, 2], it is likely that destruction of the acinar structure plays a key role in the pathogenesis of SS. However, recent reports have suggested that sicca symptoms are due to a functional inhibition of lacrimal and salivary glands rather than to the infiltration and destruction of the glands by T-lymphocytes [28, 29]. Regarding the deficiency of fluid secretion in patients with SS, dysfunction or abnormal distribution of AQP5 in acinar cells has been observed [9, 10]. On the other hand, it is possible that TNF-α plays an important role in the immunopathogenesis of SS; TNF-α might contribute to the decrease in salivary flow in patients with SS, and it may inhibit salivary secretion due to its neurotoxic effect on sympathetic nerves [30]. In some clinical cases, the administration of infliximab, a chimeric anti-TNF-α antibody, to patients with SS has been shown to dramatically improve salivary flow [31]. In this study, we found that AQP5 expression was suppressed in a human salivary gland acinar cell line, and that TNF-α treatment dramatically decreased water flow rate in cultured acinar cells. These results indicated that TNF-α directly inhibits salivary flow from acinar cells, which may be evidence of infliximab's effectiveness, and may also indicate that TNF-α plays an important role in the pathogenesis of primary SS. However, a conflict clinical result showing that the randomized, double-blind, placebo-controlled study of an anti-TNF agent employing a total of 103 patients with SS did not show any evidence of efficacy of infliximab also has been demonstrated [32]. Regarding the inhibitory effect of TNF-α on AQP5 expression, this phenomenon could be considered to be specific in acinar cells, because AQP5 expression in human salivary glands is detected in acinar cells, but not in duct cells [7, 16].

In the present study, we have shown that reduced expression of AQP5 by TNF-α treatment significantly suppressed the transepithelial net fluid secretion rate in NS-SV-AC cells. In general, it is well recognized that fluid, including water, is moved either across the plasma membranes of the cells that comprise the epithelial layer (transcellular transport) or between these cells, through the tight junction complex that forms a barrier (paracellular transport). Although in this study we have not examined the transport pathway whereby TNF-α-mediated suppression of AQP5 inhibits the transepithelial net fluid secretion from NS-SV-AC cells, Kawedia *et al.* have recently shown that lack of AQP5 in the salivary gland resulted in decreased water transport not only through the plasma membrane (transcellular transport) but also through tight junction complex (paracellular transport), and that the transcellular and paracellular pathways act in conjunction rather than in a compensatory manner [33].

Although a strong correlation between promoter methylation and gene silencing has been extensively demonstrated [34–36], the molecular mechanism involved in this methylation-modulated gene inactivation remains unclear. Recent studies have demonstrated that both global DNA hypomethylation and regional hypermethylation occur in tumorigenesis, and certain types of tumours have been shown to exhibit regional hypermethylation in CpG islands associated with the promoter regions of tumour suppressor genes [37, 38]. As regards mammalian DNA hypermethylation, DNA methyltransferase has been considered to play a central role in gene silencing [39]. We have recently demonstrated that an immortalized NS-SV-DC (salivary ductal) cell line lacking in AQP5 expression acquires AQP5 gene expression in response to treatment with a DNA demethylating agent [16]. These findings demonstrate that TNF-α contributes to the hypermethylation of the 5′-region of the AQP5 promoter and that it may inhibit the transcriptional regulation of the AQP5 gene. Although we examined the expression levels of Dnmt1, 3a and 3b, as well as the enzymatic activities of Dnmts, we did not find any marked differences in the TNF-α-treated NS-SV-AC cells. Thus, these results indicate that TNF-α might not exert any effects on the hypermethylation of the promoter region of the AQP5 gene.

In the TNF-α signaling cascade, NF-κB plays an important role in the expression of numerous genes. We examined the contribution of NF-κB in the TNF-α-induced suppression of AQP5 in NS-SV-AC cells by using a super-repressor form of IκBα (srIκBα) cDNA. However, we have found that srIκBα inhibited the nuclear translocation of NF-κB by TNF-α treatment [24], and that in the present study TNF-α suppressed AQP5 expression in the ACmI-1 cell clone. These results indicated that NF-κB exerts no influence on TNF-α-induced AQP5 suppression in salivary gland acinar cells. Although Towne and colleagues demonstrated that TNF-α inhibited AQP5 expression in mouse lung epithelial cell lines by activating NF-κB [11], our results were not consistent with this. This discrepancy may be attributable to differences between the cell types used in the respective studies.

The acetylation and deacetylation status of histones are thought to act as general regulators of chromatin structure that correlates in general with transcriptional activation and repression of the genes, respectively [40]. Actually, in the present study, our investigations provided evidence for general alterations in histone modification by TNF-α, such as the decreased histone H4 acetylation occurring during the suppression of AQP5 expression in NS-SV-AC cells. In addition, ChIP analysis revealed down-regulation of AQP5 gene promoter in association with suppression of acetylated histone H4, therefore suggesting the mechanism that deacetylation of histone H4 proteins acquires the positive charge on lysine residues and allows folding of the AQP5 gene promoter, subsequent inhibition of access by transcription factors, and suppression in AQP5 gene expression. Although the mechanism involved in the suppression of acetylation levels of histone H4 by TNF-α remains unknown, some investigators demonstrated that mitogen-activated protein kinase (MAPK) signaling pathways regulate the expression of transcriptional corepressors, including silencing mediator for retinoid and thyroid receptors (SMRT) and nuclear receptor corepressor (NCoR), both of which possess histone deacetylase activity, such as histone deacetylase 3 (HDAC3) [41–43]. Since it is well known that signaling *via* the TNF receptor leads to alterations in gene expression through the activation of multiple signal transduction pathways that include the activation of the MAPK family, extracellular signal-regulated kinase (ERK) 1/2 and p38 [44], it is conceivable that TNF-α may affect the expression levels of SMRT and NCoR corepressors in NS-SV-AC cells. Indeed, we demonstrated that TNF-α augmented the SMRT gene expression (3.5-fold as compared to control) in NS-SV-AC cells by the analysis of cDNA microarray (unpublished data). Therefore, to elucidate the more precise mechanism whereby TNF-α suppresses the expression of AQP5 in salivary acinar cells, search for signal transduction cascade leading to TNF-α-regulated SMRT expression would be inevitable.

In conclusion, our results showed that TNF-α actually suppresses the AQP5 expression in and the fluid secretion from salivary acinar cells, and that a decrease of histone H4 acetylation level is one of the mechanisms involved in the reduction of AQP5 expression by TNF-α. Based on the above findings, further analysis regarding the regulatory mechanism underlying the SMRT expression by MAPK is now under investigation in our laboratory.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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