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Runt-related gene 2 is involved in the inhibition of matrix metalloproteinase-13 expression by roxithromycin in human gingival epithelial cell cultures

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Background and Objective: Matrix metalloproteinase (MMP)-13 has wide substrate specificity compared with other MMPs and appears to be involved in periodontitis. Previously, we reported that roxithromycin (RXM) inhibits vascular endothelial growth factor expression induced by tumour necrosis factor-α in human periodontal ligament cells, but little is known about the effect of RXM on MMP-13 expression in human gingival epithelial cells. We therefore examined the effect of RXM on MMP-13 mRNA expression and production in cultured human gingival epithelial cells.

Material and Methods: Human epithelial cell lines (Ca9-22, TU4, SCCTF and HSC-3) were plated in tissue culture dishes. Then, the culture supernatants and sediments were collected and the production of MMP-13 was analysed using enzyme-linked immunosorbent assay; the expression of MMP-13 mRNA and runt-related gene 2 mRNA was assessed using reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR. We also studied the effect of Runx2 short interfering RNA (siRNA) on the induction of MMP-13.

Results: Roxithromycin downregulated the induction of MMP-13 in Ca9-22 cells. Roxithromycin suppressed the expression of MMP-13 mRNA not only in Ca9-22 cells, but also in other human epithelial cell lines. Roxithromycin strongly inhibited the expression of Runx2 mRNA. Furthermore, Runx2 siRNA inhibited the induction of MMP-13 in Ca9-22 cells.

Conclusion: These results indicate that RXM suppresses MMP-13 via the downregulation of Runx2 in human gingival epithelial cell cultures.

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases collectively capable of degrading several distinct classes of pericellular substrates, such as growth factors, cytokines, chemokines and their receptors and components of the extracellular matrix (ECM). Matrix metalloproteinases play important

roles, not only under physiological conditions, but also under pathological conditions (1). It has been reported that inflammatory cytokines, such as interleukin (IL)-1 and tumour necrosis factor (TNF)-α, induce the expression of MMPs in human gingival fibroblasts, periodontal ligament cells and epithelial cells (2–5). Furthermore, the proteolytic activity of MMPs in the extracellular space is controlled by specific tissue inhibitors of metalloproteinases (TIMPs; 2). It seems that the balance between the level of MMPs and that of TIMPs is important for remodelling in periodontal tissue (6,7). It is now recognized that the degradation of ECM in gingival tissue, such as collagen, during active periodontitis is due in part to MMPs derived from host cells. In addition, it was recently reported that MMP-8 (collagenase-2), -9, -13 (collagenase-3) and -14 are more sensitive to inhibition by doxycycline and chemically modified non-antimicrobial tetracycline derivatives compared with MMP-1 (collagenase-1) and -2 (8,9). These findings suggest that the regulation of MMP-8, -9, -13 and -14 is important for the healing mechanism in periodontitis.

The structure of the upstream regulatory region of the MMP-13 gene and the position of the major transcription factor binding sites has been identified (10). Runt-related gene 2 (Runx2) (also called core binding factor α1, CBFA1) is a transcription factor with a major role in bone formation, which acts via a *runt* domain binding sequence. Runx2 recognizes the Osteoblast-specific element 2 motif in the MMP-13 promoter region, and has been linked to MMP-13 induction in osteoblasts and chondrocytes. Indeed, knockout mice deficient in Runx2 do not express MMP-13 during fetal development, highlighting its importance for MMP-13 induction *in vivo* (11).

Previously, we reported that roxithromycin (RXM), a 14-member macrolide antibiotic, inhibits TNF-α-induced vascular endothelial growth factor (VEGF) expression in human periodontal ligament cells (12). Vascular endothelial growth factor seems to be involved in the pathogenesis of periodontitis, particularly gingival periodontitis accompanied by heavy neovascularization (12,13). However, no information exists about the effect of RXM on MMP-13 mRNA expression. To the best of our knowledge, this is the first study to demonstrate that RXM inhibits the induction of MMP-13 by blocking Runx2, which is essential for MMP-13 expression.

Material and methods

Cell culture

Four human epithelial cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) with 5% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 2 mM L-glutamine, 10 000 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 and 95% air atmosphere. The cell lines were Ca9-22 cells derived from a primary lower gingival carcinoma (14), TU4 cells from an upper gingival carcinoma (15), SCCTF cells from tongue carcinoma (16) and HSC-3 cells from lymph node metastases of a primary carcinoma of the tongue (obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The proliferation of Ca9-22 cells in the presence of RXM (Sigma, St Louis, MO, USA) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. The Ca9-22 cells (2.5×10^3 per well) were seeded onto 96-well culture plates (Nunc, Roskilde, Denmark) in DMEM with 1% FBS for 24 h, and then treated with various concentrations of RXM for different times. After treatment, the cells were incubated with MTT for 4 h. Then, the cells were lysed and solubilized using dimethyl sulphoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader (model 450; Bio-Rad Laboratories, Richmond, CA, USA).

Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

The Ca9-22 cells (7×10^5 per well) were seeded onto 100 mm tissue culture dishes (Nunc) in DMEM supplemented with 1% FBS for 24 h. The supernatants were collected for enzyme-linked immunosorbent assay (ELISA). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (5 μg) was used to synthesize single-stranded cDNA via superscript III RNase H^--reverse transcriptase (Invitrogen). The reagents were incubated at 50°C for 60 min and the reaction was halted by heating at 70°C for 15 min. The cDNA mixture was then amplified with Go Taq Green Master Mix (Promega, Madison, WI, USA) for 25 [MMP-13 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH)] or 30 thermal cycles (Runx2). The PCR working conditions consisted of an initial denaturation at 94°C for 2 min, followed by cycles of 94°C for 30 s, primer annealing at 55°C (MMP-13 and GAPDH) or 51°C (Runx2) for 30 s and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR products were analysed using 2% ethidium bromide-stained agarose gel electrophoresis. To control for variation in gel loading, GAPDH mRNA expression was analysed over the same number of cycles and under the same PCR running conditions. The results were expressed as the relative mRNA accumulation, with GAPDH mRNA as an internal standard, and analysed. The primers used were as follows: MMP-13, sense 5′-CTA TGG TCC AGG AGA TGA AG-3′, antisense 5′-AGA GTC TTG CCT GTA TCC TC-3′; Runx2, sense 5′-ACC ATG GTG GAG ATC ATC GC-3′, antisense 5′-CAT CAA GCT TCT GTC TGT GC-3′; GAPDH, sense 5′-ACA CAT GCC ATC ACT GCC GCC-3′, antisense 5′-GCC TGC TTC ACC ACC TTC TTG-3′.

Real-time RT-PCR

Real-time RT-PCR was used to quantify mRNA expression with SYBR

Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan). The optimized thermocycling parameters used were 95°C for 1 min, 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then 95°C for 2 min and 65°C for 1 min. The real-time RT-PCR was performed on a Line Gene (BioFlux, Tokyo, Japan) fluorescence quantitative detection system, and the MMP-13, Runx2 and GAPDH gene expression was quantified from standard curves. The quantity of MMP-13 and Runx2 mRNA in each sample was subsequently normalized to the quantity of GAPDH mRNA and expressed as the fold change over the unstimulated control.

ELISA

After stimulation with RXM or minocycline (MINO; Sigma), the concentration of MMP-13 protein in the culture supernatants was measured using an ELISA system (Amersham Biosciences, Piscataway, NJ, USA). Preliminary experiments showed that a 1:2 dilution of the test sample was required for the standard curve. Therefore, supernatants from human epithelial cell cultures were examined at this dilution. Other experimental procedures were conducted according to the manufacturer's instructions.

Gene silencing with short interfering RNA (siRNA)

The Ca9-22 cells were plated the day before transfection and grown to between 50 and 60% confluence in 60 mm plates. Transfection was carried out with Runx2 siRNA or scramble siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Transfected cells were cultured for 48 h. The MMP-13 mRNA expression was examined using real-time RT-PCR, and MMP-13 protein production was measured using ELISA as outlined above.

Statistical analysis

Each assay was carried out in triplicate. The significance of differences between the results of each test and the respective control values was determined using Student's unpaired *t*-test.

Results

Effect of RXM on MMP-13 induction in Ca9-22 cells

To examine the effect of RXM on MMP-13 induction in gingival epithelial cells, we first performed RT-PCR (Fig. 1, upper panel). The MMP-13 mRNA expression was downregulated by treatment with 6.25 μM RXM. Then, we carried out real-time RT-PCR to evaluate MMP-13 mRNA transcription (Fig. 1, lower panel). Treatment with 6.25 or 25 μM RXM for 48 h led to an about 25 or 50% decrease in MMP-13 mRNA expression in Ca9-22 cells, respectively. Roxithromycin treatment did not change the level of MMP-13 mRNA expression until 24 h (data not shown). These results suggest that RXM inhibits MMP-13 at the transcription step.

We performed ELISA to examine the effect of RXM on MMP-13 production (Fig. 2). We confirmed that 6.25–50 μM RXM for 48 h strongly inhibited MMP-13 production, whilst 1.56 μM RXM did not inhibit MMP-13 induction significantly at the transcription or protein levels (data not shown). These results suggest that RXM inhibits MMP-13 induction in human gingival epithelial cells.

Effect of RXM on Ca9-22 cell viability

To determine whether RXM affects the growth of Ca9-22 cells, we evaluated cell viability using the MTT assay. Roxithromycin at concentrations up to 50 μM did not inhibit the growth of Ca9-22 cells for 48 h (Fig. 3), suggesting that the inhibitory effect of RXM on MMP-13 induction in Ca9-22 cells was not due to cytotoxicity.

Effect of RXM on MMP-13 mRNA expression in various human epithelial cells

To determine whether the inhibition of MMP-13 induction by RXM was specific to Ca9-22 cells, we performed real-time RT-PCR (Fig. 4). We confirmed

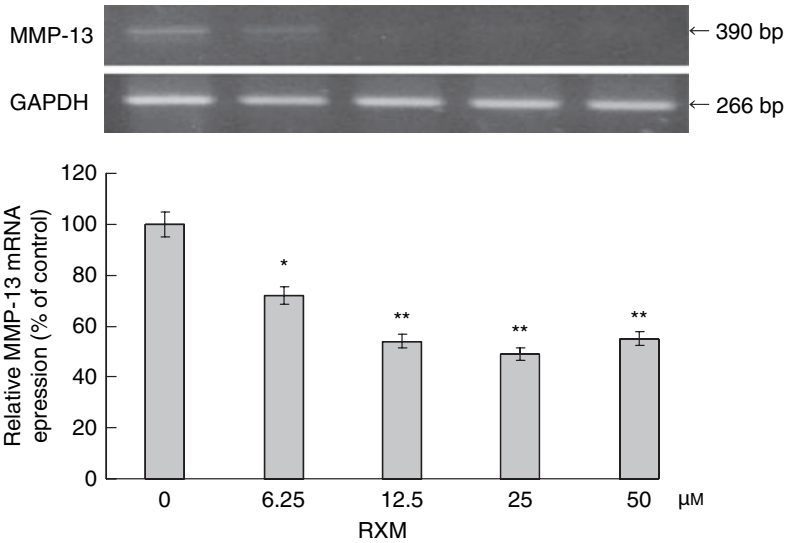

Fig. 1. Roxithromycin inhibited MMP-13 mRNA expression in Ca9-22 cells. The Ca9-22 cells were treated with the indicated concentration of RXM for 48 h. Upper panel, total RNA was extracted and analysed using RT-PCR. Each amplified product was electrophoresed on a 2% agarose gel, and then stained with ethidium bromide and photographed under ultraviolet light. Lower panel, the same RNA sample was analysed using real-time RT-PCR. Each assay was carried out in triplicate. *$p < 0.05$ and **$p < 0.01$ vs. the control culture (0 μM). Two additional experiments gave results similar to those shown here.

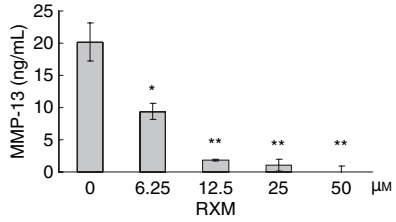

Fig. 2. Roxithromycin inhibited MMP-13 production in Ca9-22 cells. The Ca9-22 cells were treated with the indicated concentration of RXM for 48 h. Culture medium was collected, and the concentration of MMP-13 protein was determined using ELISA. Each assay was carried out in triplicate. $*p < 0.05$ and $**p < 0.01$ vs. the control culture (0 μM). Two additional experiments gave results similar to those shown here.

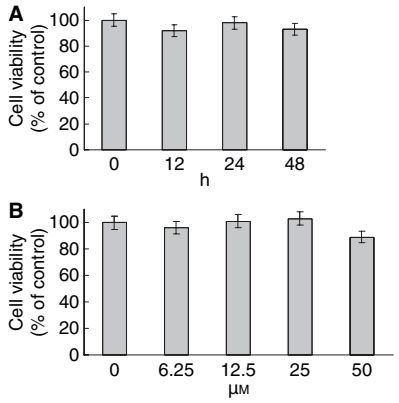

Fig. 3. Roxithromycin did not affect Ca9-22 cell viability. Cell viability was assessed using the MTT assay. The Ca9-22 cells were treated with 50 μM RXM for different periods (A), or with various concentrations of RXM for 48 h (B). Relative viability is shown as the percentage absorbance of the sample with respect to that at the zero point. Each assay was carried out in triplicate. Three additional experiments gave results similar to those shown here.

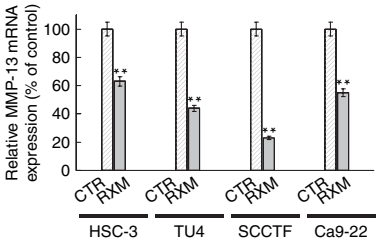

Fig. 4. Roxithromycin inhibited MMP-13 mRNA expression in various human gingival epithelial cells. Each cell line was treated with 50 μM RXM for 48 h. Expression of MMP-13 mRNA was quantified using real-time RT-PCR. Each assay was carried out in triplicate. $**p < 0.01$ vs. the control culture. Similar results were obtained in two additional experiments.

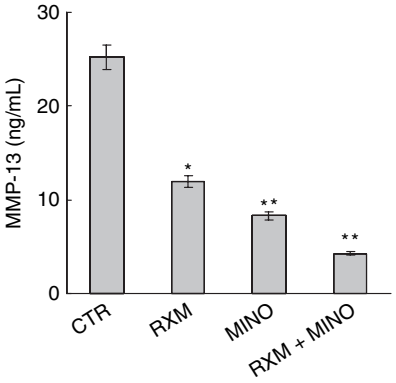

Fig. 5. Roxithromycin and MINO both inhibited MMP-13 protein production in Ca9-22 cells. The Ca9-22 cells were treated for 48 h with or without 25 μM RXM and 100 μM MINO. The culture medium was collected, and the MMP-13 concentration was determined using ELISA. Each assay was carried out in triplicate. $*p < 0.05$ and $**p < 0.01$ vs. the control culture. Two additional experiments gave results similar to those shown here.

that treatment with 50 μM RXM for 48 h inhibited MMP-13 mRNA expression in HSC-3, TU4 and SCCTF cells, which suggests that RXM inhibits MMP-13 gene expression in human epithelial cells.

Effect of RXM and MINO on MMP-13 production in Ca9-22 cells

To compare the effect of RXM in the induction of MMP-13 to that of MINO, we examined the production of MMP-13 protein using ELISA (Fig. 5). Roxithromycin inhibited MMP-13 production to a similar extent to MINO. Interestingly, RXM and MINO showed an additive effect on the inhibition of MMP-13.

Possible involvement of Runx2 in MMP-13 induction in Ca9-22 cells

To clarify the pathway of the inhibitory effect of RXM on MMP-13 signalling, we examined the effect of RXM on the induction of Runx2, which is a crucial transcription factor, using real-time RT-PCR. We confirmed that RXM downregulated the expression of Runx2 mRNA (Fig. 6). Furthermore, Runx2 siRNA also led to an approximately 40% decrease in MMP-13 mRNA expression and protein production compared with scramble siRNA (Figs 7 and 8). In our assay, the maximal inhibition of MMP-13 mRNA expression occurred with 12.5 μM Runx2 siRNA (data not shown).

Discussion

Periodontal disease, the major cause of tooth loss in the adult population, is characterized by the destruction of collagen fibres and other matrix constituents of the gingiva, periodontal ligaments and alveolar bone. Connective tissue breakdown appears to be mediated in part by excessive levels of activated MMPs produced by host cells (e.g. neutrophils, macrophages, fibroblasts, osteoblasts and osteoclasts; 17). Matrix metalloproteinase-13 has been shown to be expressed by periodontitis-affected human gingival sulcular epithelium (18), and gingival fibroblasts (19,20) express MMP-13. Whilst many MMPs are synthesized in the sulcular epithelium, gingival tissue specimens and gingival crevicular fluid from adult and localized juvenile periodontitis patients (18,20,21), a few MMPs, such as MMP-13, are more sensitive to inhibition by doxycycline, a drug used to treat periodontitis (8). Previously, we reported the effects of RXM on TNF-α-induced VEGF in human periodontal ligament cells (12), so in this study, we focused on the regulation of MMP-13 induction by RXM in human gingival epithelial cells.

Some antibiotics, including tetracycline and macrolides, inhibit pro-inflammatory cytokines (9,22), and tetracyclines, such as tetracycline hydrochloride and doxycycline, have been evaluated in periodontal clinical studies (23). Although tetracyclines have antibiotic effects against a broad spectrum of bacteria, very high concentrations of doxycycline are needed

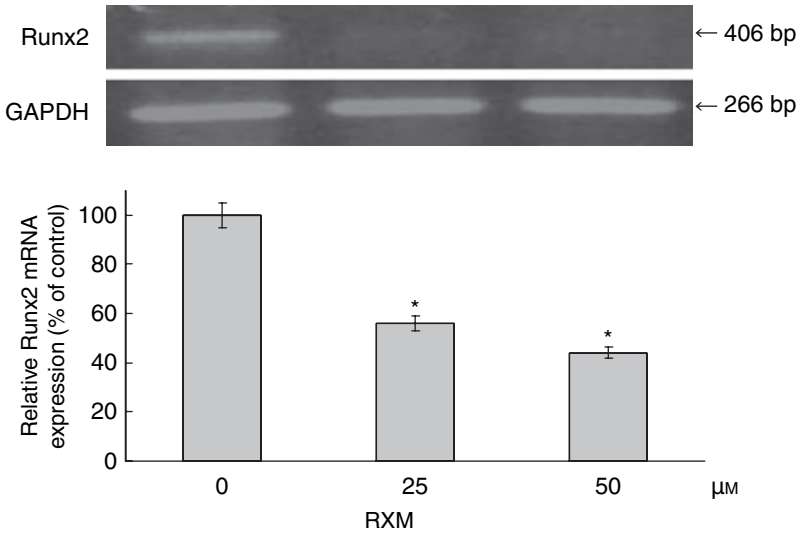

Fig. 6. Roxithromycin inhibited Runx2 mRNA expression in Ca9-22 cells. The Ca9-22 cells were treated with the indicated concentration of RXM for 48 h. The Runx2 mRNA expression was analysed using RT-PCR (upper panel) and real-time RT-PCR (lower panel). $*p < 0.05$ vs. the control culture. Other procedures are described in the legend to Fig. 1. Two additional experiments gave results similar to those shown here.

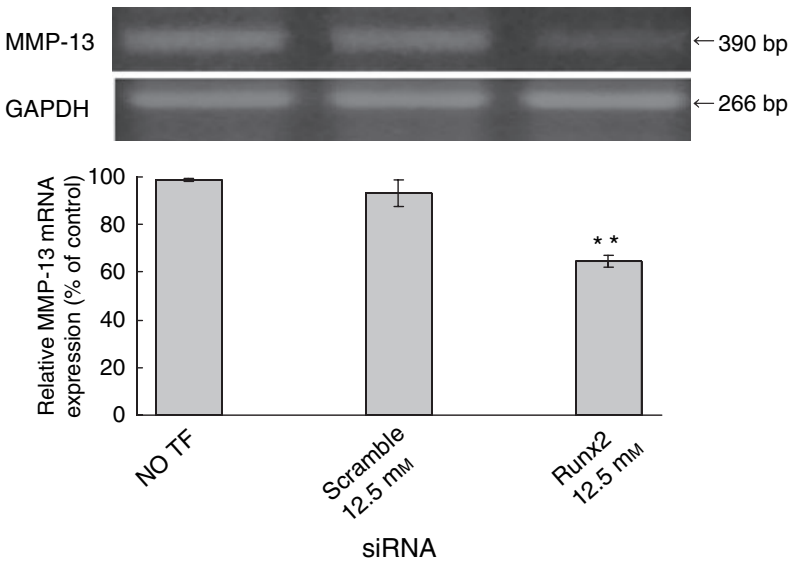

Fig. 7. Short interfering RNA for Runx2 suppressed MMP-13 mRNA expression in Ca9-22 cells. The Ca9-22 cells were transfected with 12.5 μM Runx2 siRNA or scramble siRNA, and then cultured for 48 h. The MMP-13 mRNA expression was analysed using RT-PCR (upper panel) and real-time RT-PCR (lower panel). $**p < 0.01$ vs. the no transfection (NO TF) culture. Similar results were obtained in two additional experiments.

to eliminate *Streptococcus sanguis* in biofilms (24); this organism is one of the primary colonizers of tooth surfaces *in vivo*, forming the basis of dental plaque (25). Bacterial biofilms develop marked resistance to chemical antimicrobial agents and antibiotics, and subgingival microbial plaque, which consists of a multispecies biofilm, causes periodontal disease (26). Macrolide antibiotics can inhibit biofilm formation by *Pseudomonas aeruginosa* (27), and the enhanced invasion of inflammatory cells into biofilms of

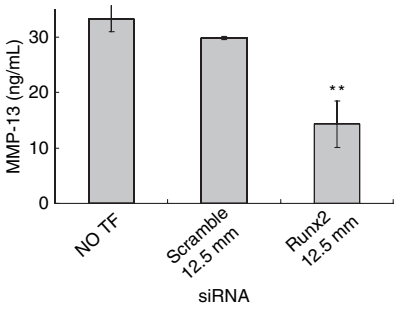

Fig. 8. Short interfering RNA for Runx2 inhibited MMP-13 production in Ca9-22 cells. The Ca9-22 cells were transfected with 12.5 μM Runx2 siRNA or scramble siRNA, and then cultured for 48 h. The MMP-13 concentration in culture medium was quantified by ELISA. No significant difference was observed between no transfection (NO TF) and scramble siRNA. Each assay was carried out in triplicate. $**p < 0.01$, control group vs. treated groups. Two additional experiments gave results similar to those shown here.

Staphylococcus aureus (28). These observations suggest that macrolide antibiotics, including RXM, are also effective against biofilm formation in the subgingival area. Furthermore, systemic antibiotic therapy with RXM should lead to the downregulation of MMP-13 because the usual dose in humans, 300 mg per day, results in a maximal concentration of about 9.6 μM in serum. Based on this finding, we are currently examining the effect of RXM *in vivo*.

It has been reported that IL-1β induces MMP-13 mRNA expression via p38 mitogen-activated protein kinase (MAPK)–Runx2 activation in human chondrosarcoma cells (29), but it has also been reported that transforming growth factor-β induces MMP-13 mRNA expression through p38 MAPK–activator protein 1 activation in human gingival fibroblasts (30). These variable findings regarding MMP-13 expression might be dependent on the target cells and/or stimulants. In this study, we clarified that RXM inhibited MMP-13 expression in part by controlling Runx2 (Figs 6–8). In our assay, however, siRNA for Runx2 inhibited the expression of Runx2 by 40%, as shown by real-time RT-PCR (data not shown).

In conclusion, we demonstrated that RXM inhibits MMP-13 and Runx2 expression in human gingival epithelial cells, and that Runx2 may be involved in MMP-13 expression. Roxithromycin may serve as a new therapeutic agent for periodontitis and other diseases, such as rheumatoid arthritis and cancer. We are undertaking further investigations to elucidate the precise molecular mechanisms of RXM involvement in modulating MMP-13 expression, and further functional analyses with animal models.

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