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Roxithromycin inhibits tumor necrosis factor-α-induced matrix metalloproteinase-1 expression through regulating mitogen-activated protein kinase phosphorylation and Ets-1 expression

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Background and Objective: In periodontitis, matrix metalloproteinases (MMPs) are upregulated in response to locally released inflammatory cytokines, resulting in pathologic processes. Roxithromycin is a 14-membered ring macrolide antibiotic with broad-spectrum antibacterial effects against oral pathogens and immunomodulatory effects. Recently, we reported that roxithromycin inhibits tumor necrosis factor (TNF)-α-induced vascular endothelial growth factor expression in human periodontal ligament (HPDL) cell cultures. In the present study, we examined the effect of roxithromycin on TNF-α-induced MMP-1 production by HPDL cells.

Material and Methods: Cultured cells were incubated with 1% fetal bovine serum for 24 h, followed by treatment with 10 ng/ml TNF-α, 10 μM roxithromycin, and mitogen-activated protein kinase inhibitor at various concentrations. Culture supernatants and sediments were collected at different time-points and used for enzyme-linked immunosorbent assays, and northern and western blot analyses.

Results: In HPDL cell cultures, roxithromycin strongly inhibited TNF-α-induced MMP-1 mRNA expression and production. The inhibition of MMP-1 gene expression by roxithromycin was dependent on *de novo* protein synthesis and was regulated at the transcriptional level. Roxithromycin significantly inhibited TNF-α-induced c-Jun N-terminal kinase activation (JNP) and marginally inhibited

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extracellular signal-regulated kinase (ERK) 1/2 activation, but not p38 mitogen-activated protein kinase activation. Furthermore, roxithromycin reduced the induction of Ets-1, one of the critical factors in MMP-1 transcription.

Conclusion: Roxithromycin inhibits TNF-α-mediated MMP-1 induction through the downregulation of ERK1/2 and JNK activation and the subsequent reduction of Ets-1, suggesting that roxithromycin may have therapeutic use in periodontitis and other chronic inflammatory conditions involving MMP-1 induction.

Matrix metalloproteinases (MMPs) are capable of degrading extracellular matrix components (1) and are thought to play an important role in matrix degradation in several inflammatory diseases, including rheumatoid arthritis and periodontitis (2,3). Based on their substrate specificity and structure, MMPs can be classified into the following subgroups: collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), membrane-type MMPs (MMP-14 to -17, -24, and -25), matrilysins (MMP-7 and -26) and other MMPs (MMP-12, -19 to -23, -27, and -28) (4,5). MMP-1 is responsible for the initial breakdown of the fibrillar collagen network and plays an important role in the remodeling of collagenous connective tissue in various physiological and pathological situations (6). The level of MMP-1 is significantly higher in inflamed gingiva (attributable to severe gingivitis or periodontitis) than in healthy gingiva (7,8), demonstrating that MMP-1 may be relevant to collagenolysis in adult periodontitis. Recently, it was reported that a polymorphism in the promoter region of the MMP-1 gene could be a risk factor for severe chronic periodontitis (9). These findings strongly suggest that MMP-1 is a major factor in the tissue destruction associated with periodontitis.

Inhibitors of MMPs may be useful in the therapy of inflammatory diseases that involve the MMP-mediated destruction of connective tissue (10,11) and in corneal allograft acceptance mediated by the Fas/FasL interaction (12). At present, several devices for the local delivery of drugs, such as those of the tetracycline family, have been used to treat periodontitis (13). In addition to their antimicrobial effects, tetracyclines inhibit the induction of several inflammatory mediators, such as MMPs and tumor necrosis factor (TNF)-α, in a wide variety of cells (14). Recently, we reported that roxithromycin, which is one of a family of macrolide antibiotics, inhibits TNF-α-induced vascular endothelial growth factor (VEGF) expression in human periodontal ligament (HPDL) cell cultures (15). It is known that macrolide antibiotics exert both local and systemic anti-inflammatory effects, in addition to their antimicrobial activity, in the management of respiratory tract infections (16,17). However, owing to their pharmacokinetic characteristics, macrolide antibiotics, such as roxithromycin, have prominent penetration into periodontal tissue (18). Furthermore, roxithromycin inhibits biofilm formation by *Pseudomonas aeruginosa* (19) and enhances the invasion of inflammatory cells into the biofilm of *Staphylococcus aureus* (20). As subgingival microbial plaque is a multispecies biofilm (21,22), roxithromycin may be effective against biofilm in the subgingival area and may regulate the quorum-sensing signal in periodontal pathogens.

In this study, we obtained evidence, for the first time, that roxithromycin markedly suppresses TNF-α-induced MMP-1 expression by blocking two mitogen-activated protein kinase (MAPK) cascades – the extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK) cascades – and subsequently downregulates transcription factor Ets-1 in HPDL cell cultures.

Material and methods

Specimens and probes

Recombinant human TNF-α was purchased from Endogen (Cambridge, MA, USA). Roxithromycin was provided by Eisai Ltd (Tokyo, Japan). Cycloheximide and actinomycin D were obtained from Sigma-Aldrich (St Louis, MO, USA). U0126, SB203580, and SP600125 were obtained from Calbiochem (Bad Soden, Germany). Synthetic oligonucleotides complementary to human MMP-1 and Ets-1 mRNA were used as primers for the polymerase chain reaction (PCR). The PCR products were ligated into pGEM-T (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 cells. The inserts of the extracted plasmids were sequenced using a genetic analyser (ABI PRISM™310; Perkin Elmer Co., Emeryville, CA, USA); the sequences exactly matched the fragment sequences in GenBank. A human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA clone was provided by Dr S. Sakiyama (23).

Cell culture

A specimen of normal periodontal ligament tissue was obtained from the extracted tooth of a male patient (21 yr of age) using a protocol approved by the Ethics Committee of Kagoshima University Graduate School of Medical and Dental Sciences, with the informed consent of the donor. HPDL tissues were carefully removed from the middle third of the root surface of the tooth using a scalpel. They were then transferred to 35-mm culture dishes (Nunc, Roskilde, Denmark) and incubated in α-minimal essential medium (α-MEM; Flow Laboratories, Mclean, VA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA), 2 mM L-glutamine, and antibiotics (on primary cultures: 100 U/ml penicillin,

100 μg/ml streptomycin and 10 μg/ml amphotericin B; on subculture: 200 μg/ml kanamycin) (24). Cover glasses were placed over the HPDL tissue to prevent floating. The samples were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When the HPDL cells became confluent, the cells were transferred to 90-mm cell culture dishes in 0.05% trypsin/0.53 mM EDTA solution (Gibco, Life Technologies, Grand Island, NY, USA). The confluent HPDL cells were passaged as the first passage, and HPDL cells at the second passage were stored in liquid nitrogen for use in subsequent experiments. The HPDL cells were used between the 5th and 10th passages.

Cell suspensions (1×10^6 cells) were seeded in 90-mm cell culture dishes in α-MEM containing 10% fetal bovine serum. Confluent cells (3×10^6 cells per dish) were pretreated for 24 h with α-MEM containing 1% fetal bovine serum. The cells were subsequently washed three times with α-MEM, followed by the addition of TNF-α in α-MEM containing 1% fetal bovine serum. To examine the effect of roxithromycin on the induction of MMPs, the cultured cells were simultaneously treated with 10 ng/ml TNF-α, plus various concentrations of roxithromycin, in α-MEM containing 1% fetal bovine serum. After incubation for various periods of time, the culture supernatants and sediments were collected and used for the following experiments.

RNA isolation and northern blot analysis

Total RNA was isolated from the cells by the acid guanidinium–phenol–chloroform method (25). Isolated RNA was fractionated in 1.2% agarose gels containing 0.66 M formaldehyde and transferred to a nylon membrane (Zeta-Probe; Bio-Rad Laboratories, Hercules, CA, USA) by electroblotting (26). The RNA was cross-linked to the membrane with an ultraviolet (UV) linker (Funa-UV-Linker; Funakoshi, Tokyo, Japan). The membrane was prehybridized overnight at 42°C in a solution of 50% formamide, 1% sodium dodecyl sulfate (SDS), 4 × SSPE (1 × SSPE: 180 mM NaCl, 10 mM $Na_2HPO_4{\cdot}7H_2O$, and 1 mM EDTA), 0.5% skim milk, and 0.5 mg/ml salmon-sperm DNA. The cDNA probes were labeled with [α-^{32}P]dCTP using a random-primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany) and were added to the prehybridization solution to give $\approx 10^6$ counts per minute (c.p.m.)/ml. After hybridization for 8 h at 42°C, the membrane was washed and exposed to an imaging plate for analysis using a Bioimaging Analyzer (BAS 1000 Mac; Fuji Photo Film Co., Tokyo, Japan).

Detection of MMP-1 and tissue inhibitor of metalloproteinase (TIMP)-1 by enzyme-linked immunosorbent assay (ELISA), and measurement of MMP-1 activity

The concentrations of MMP-1 and TIMP-1 in the culture supernatants were measured with ELISA kits (Amersham Biosciences UK Ltd, Bucks., UK) according to the manufacturer's instructions. MMP-1 activity in the supernatants was measured using the MMP-1 Biotrak activity assay system (Amersham Biosciences UK Ltd), according to the manufacturer's instructions.

Western blot analysis

Equal aliquots of conditioned media were analyzed for MMP-1 by western blotting. Protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). For SDS-polyacrylamide gel electrophoresis, samples were boiled for 5 min in SDS sample buffer (2% SDS, 25 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, and 10% glycerol) and loaded onto 10% polyacrylamide gels. The separated proteins were electroblotted onto a Hybond-P polyvinylidene fluoride membrane (Amersham Biosciences UK Ltd). The membrane was incubated with polyclonal rabbit antihuman MMP-1 (1 : 2000) (Chemicon International, Temecura, CA, USA) and subsequently with horseradish peroxidase-conjugated goat antirabbit immunoglobulin G (New England Biolabs, Beverly, MA, USA). The immunoreactive bands were detected using an electrochemiluminescence kit (ECL Plus; Amersham Biosciences UK Ltd) according to the manufacturer's instructions.

For the detection of Ets-1 expression, HPDL cells were lysed in lysis buffer (40 mM Hepes, pH 7.4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol), and protein samples were prepared, electrophoresed, and transferred to a membrane as for the media samples; polyclonal rabbit antihuman Ets-1 (1 : 2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody. The blots were stripped and reprobed with rabbit antihuman γ-tubulin to confirm equal protein loading.

For the analysis of MAPK activation, HPDL cells were lysed in SDS sample buffer containing 50 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM Na_2VO_3, and protease inhibitor cocktail (Roche, Mannheim, Germany), and protein samples were prepared, electrophoresed, and transferred to a membrane as for the media samples; rabbit antihuman phosphorylated ERK1/2, p38 MAPK, or JNK antibody (1 : 1000) (New England Biolabs) were used as the primary antibody. As a control, the amount of total MAPKs was determined in the same samples using all three rabbit antibodies, namely those against human ERK1/2, p38 MAPK, and JNK (New England Biolabs).

Results

Roxithromycin inhibited MMP-1 induction in HPDL cells in response to TNF-α

We first examined the effect of TNF-α on the expression of MMP-1 mRNA in HPDL cells. As shown in Fig. 1A, the MMP-1 mRNA level increased in a dose-dependent manner within a range of 0.01–10 ng/ml TNF-α at 8 h of culture. The time course of TNF-α-induced MMP-1 mRNA expression is shown in Fig. 1B. The MMP-1 mRNA level began to increase after 1 h of

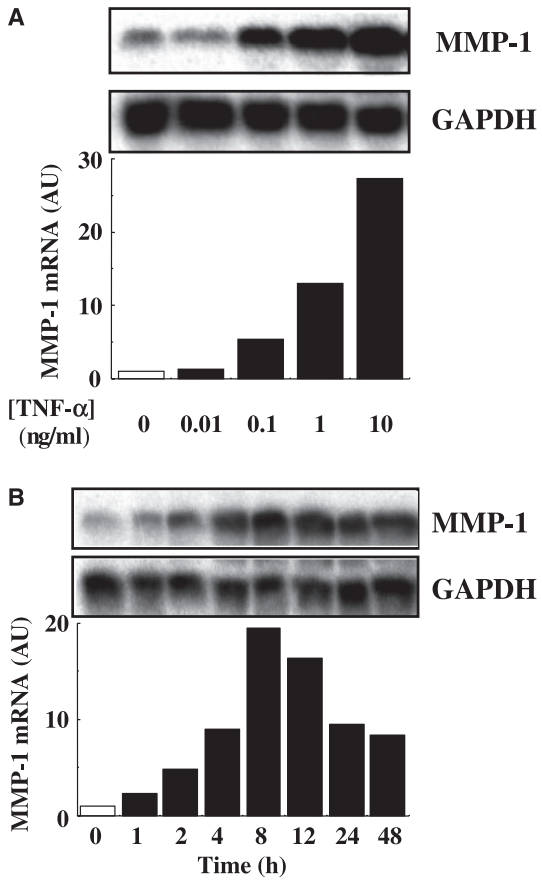

Fig. 1. Matrix metalloproteinase-1 (MMP-1) expression in human periodontal ligament (HPDL) cells in response to tumor necrosis factor-α (TNF-α). (A) Dose–response. Confluent HPDL cells were stimulated with 0–10 ng/ml TNF-α for 8 h. Total RNA was analysed by northern blotting with a radiolabeled MMP-1 probe. The results are expressed as the relative mRNA accumulation compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Two additional experiments gave results similar to those shown. (B) Time course experiment. Confluent HPDL cells were stimulated with 10 ng/ml TNF-α for 0–48 h. Total RNA was analyzed by northern blotting with a radiolabeled MMP-1 probe. The results are expressed as the relative mRNA accumulation compared with the internal standard, GAPDH mRNA. Two additional experiments gave results similar to those shown.

exposure, reached a maximum at 8 h, and decreased gradually until 48 h. To examine the effect of roxithromycin on TNF-α-induced MMP-1 mRNA expression, we treated HPDL cells with TNF-α in the presence of roxithromycin. The MMP-1 mRNA expression was downregulated in a dose-dependent manner at roxithromycin concentrations of 10–100 μM (Fig. 2A). Roxithromycin also marginally inhibited TNF-α-induced MMP-2 mRNA expression (data not shown). In this study, we primarily used a concentration of 10 μM roxithromycin because the usual dose of roxithromycin in humans, 300 mg per adult, results in a maximum concentration of ≈9.6 μM in serum. In the second HPDL cell line, we confirmed the inhibitory effect of roxithromycin on MMP-1 mRNA expression in response to TNF-α (data not shown). In accordance with the gene expression, the TNF-α-induced MMP-1 production was similarly reduced by 10 μM roxithromycin (Fig. 2B,C). To evaluate the inhibitory effect of roxithromycin in greater detail, TNF-α-treated HPDL cells were processed for MMP-1 activity using the MMP-1 Biotrak activity assay system. Roxithromycin, at a concentrraion of 10 μM, inhibited TNF-α-induced MMP-1 activity (Fig. 2D). A cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide showed that the inhibition was not attributable to cytotoxicity (data not shown).

Roxithromycin did not affect the production of TIMP-1

As TIMPs are specific inhibitors of MMPs that participate in controlling the local activities of MMPs, we examined the effect of roxithromycin on TIMP-1 production in HPDL cells. Roxithromycin did not significantly affect the level of TIMP-1 production in the cultures (Fig. 3A); HPDL cells showed spontaneous production of TIMP-1.

Dependence of TNF-α-induced MMP-1 mRNA expression on *de novo* protein synthesis

To determine whether TNF-α-induced MMP-1 expression was dependent on *de novo* protein synthesis, we added cycloheximide to the HPDL cell cultures. Cycloheximide markedly inhibited TNF-α-induced MMP-1 mRNA expression (Fig. 3B). Next, we examined whether MMP-1 expression, in response to TNF-α, was the result of an alteration in the stability of primary transcripts and/or mature cytoplasmic RNA. Actinomycin D completely blocked MMP-1 mRNA expression upon stimulation with TNF-α (Fig. 3B). These findings suggest that MMP-1 expression in response to TNF-α was attributable to increased *de novo* protein synthesis and was regulated, at least in part, at the transcriptional level.

Roxithromycin inhibited the induction of Ets-1

To examine the effect of roxithromycin on the transcriptional regulation of MMP-1 in response to TNF-α, we examined the induction of Ets-1, which is a crucial transcription factor for MMP-1 gene expression. Roxithromycin markedly inhibited Ets-1 production (Fig. 3C) and *ets-1* mRNA expression (Fig. 3D).

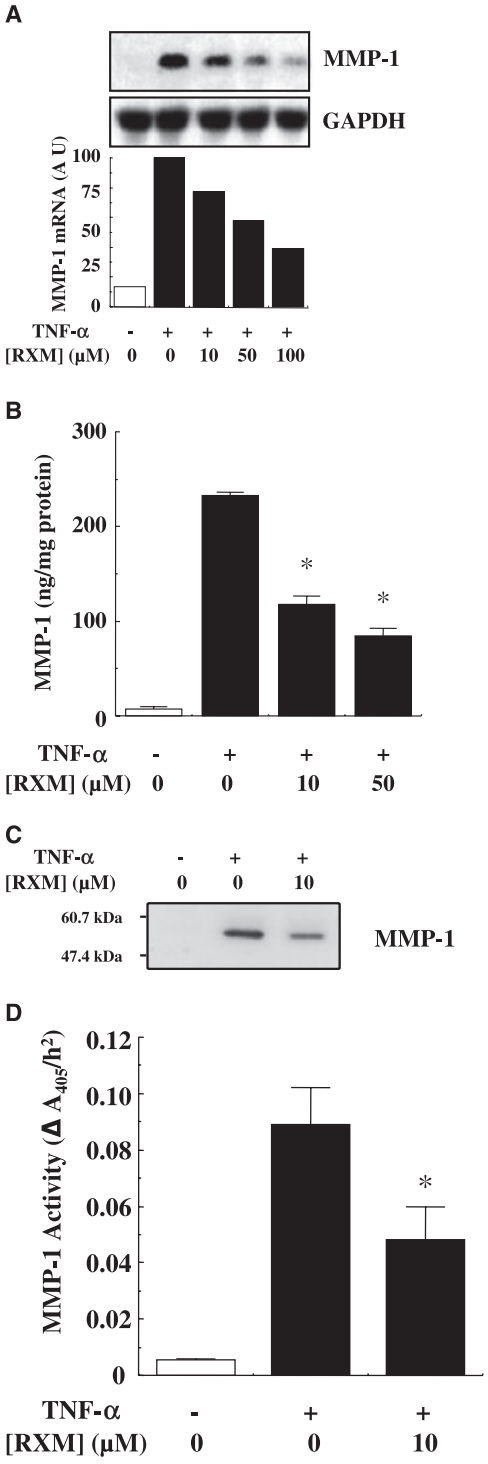

Fig. 2. Inhibitory effects of roxithromycin (RXM) on tumor necrosis factor-α (TNF-α)-stimulated matrix metalloproteinase-1 (MMP-1) induction in human periodontal ligament (HPDL) cells. (A) Roxithromycin inhibits TNF-α-induced MMP-1 mRNA expression. Confluent HPDL cells were stimulated with 10 ng/ml TNF-α in the presence of various concentrations of roxithromycin for 8 h. Total RNA was analyzed by northern blotting with a radiolabeled MMP-1 probe. The results are expressed as the mRNA accumulation relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Two additional experiments gave results similar to those shown. (B) Roxithromycin inhibits TNF-α-induced MMP-1 production [enzyme-linked immunosorbent assay (ELISA)]. Confluent HPDL cells were stimulated with 10 ng/ml TNF-α and various concentrations of roxithromycin for 72 h. Culture supernatants were then collected, and the concentration of MMP-1 was determined by ELISA. The data are expressed as means ± standard deviation for triplicate assays and are representative of three experiments that gave essentially identical results. Differences between the control cultures (TNF-α alone), and the cultures with roxithromycin, are significant at $*p < 0.01$ (Student's *t*-test). (C) Roxithromycin inhibits TNF-α-induced MMP-1 production (western blot). Confluent HPDL cells were stimulated with 5 ng/ml TNF-α and 10 μM roxithromycin for 72 h. The amount of MMP-1 released from the cells into the medium was blotted with antibody to MMP-1. Two additional experiments gave results similar to those shown. (D) Roxithromycin inhibits TNF-α-induced-MMP-1 activation. Confluent HPDL cells were stimulated with 5 ng/ml TNF-α in the presence or absence of 10 μM roxithromycin for 72 h. The amount of active MMP-1 released from cells into the medium was measured by using the MMP-1 Biotrak activity assay system ($*p < 0.01$ vs. TNF-α alone). Two additional experiments gave results similar to those shown.

The downregulation of MMP-1 induction by roxithromycin was mediated by the inhibition of JNK and ERK1/2

To clarify the effect of roxithromycin on the TNF-α signaling pathway, we examined the effect of MAPK inhibitors on TNF-α-induced MMP-1 mRNA expression. The inhibitors SP600125 (a JNK inhibitor), SB203580 (a p38 MAPK inhibitor), and U0126 (an ERK inhibitor), respectively, downregulated TNF-α-induced MMP-1 mRNA expression (Fig. 4A) and MMP-1 protein production (Fig. 4B). Roxithromycin significantly downregulated TNF-α-induced JNK activation and marginally inhibited ERK1/2 activation, dose-dependently, in part, whereas roxithromycin had no effect on p38 MAPK activation stimulated by TNF-α (Fig. 4C).

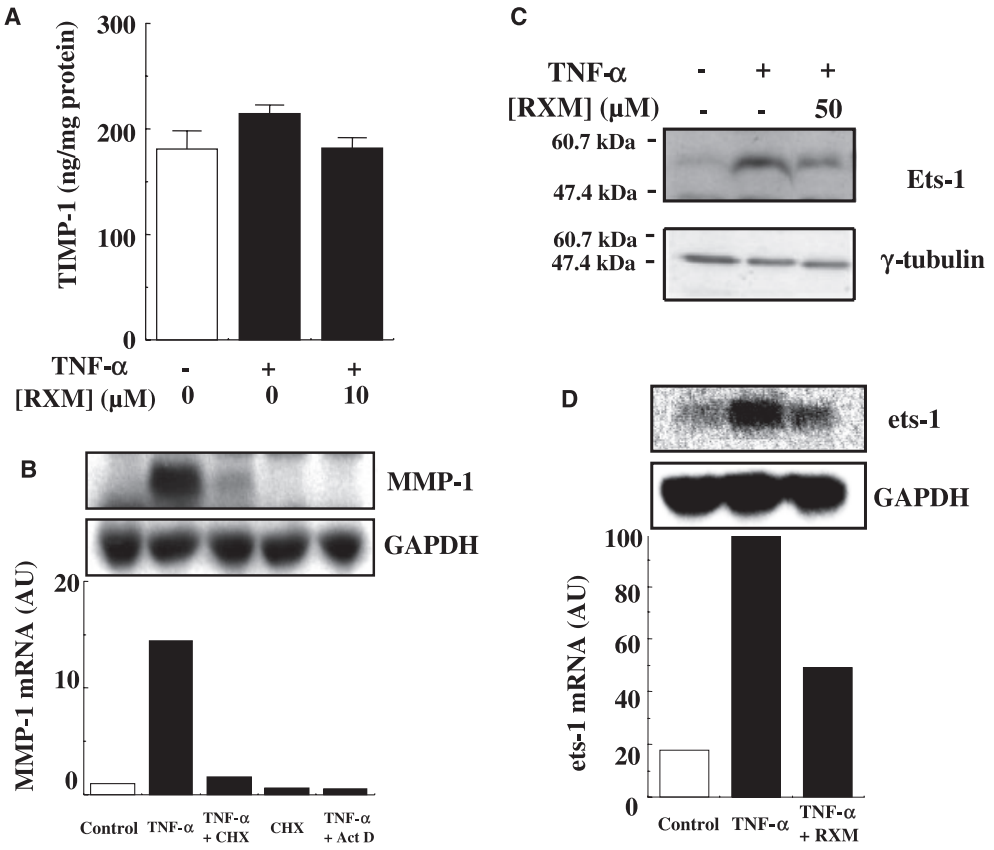

Fig. 3. Inhibitory effect of roxithromycin on the induction of Ets-1. (A) Roxithromycin (RXM) does not inhibit tissue inhibitor of metalloproteinase (TIMP-1) production in human periodontal ligament (HPDL) cells. Confluent HPDL cells were stimulated with 10 ng/ml tumor necrosis factor-α (TNF-α) and 10 μM roxithromycin for 72 h. The amount of TIMP-1 released from cells into the medium was measured by enzyme-linked immunosorbent assay (ELISA). Two additional experiments gave results similar to those shown. (B) Cycloheximide (CHX) and actinomycin D (Act D) inhibit TNF-α-induced matrix metalloproteinase-1 (MMP-1) mRNA expression. Confluent HPDL cells were stimulated for 8 h with 10 ng/ml TNF-α in the presence or absence of 10 μg/ml cycloheximide or 10 μg/ml actinomycin D. Total RNA was analyzed by northern blotting with a radiolabeled MMP-1 probe. The results are expressed as the mRNA accumulation relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Two additional experiments gave results similar to those shown. (C) Roxithromycin inhibits TNF-α-induced Ets-1 production. Confluent HPDL cells were stimulated with 5 ng/ml TNF-α, in the presence or absence of 50 μM roxithromycin, for 24 h. Lysates were analyzed by western blotting with an antibody against Ets-1 (top row) or with an antibody against γ-tubulin (bottom row). The results shown are representative of three independent experiments. (D) Roxithromycin inhibits TNF-α-induced *ets-1* mRNA expression. Confluent HPDL cells were stimulated with 10 ng/ml TNF-α, in the presence or absence of 10 μM roxithromycin, for 4 h. Total RNA was analyzed by northern blotting with a radiolabeled MMP-1 probe. The results are expressed as the mRNA accumulation relative to GAPDH mRNA as an internal standard. Two additional experiments gave results similar to those shown.

Discussion

Periodontitis is an infectious bacterial disease characterized by the destruction of connective tissue, including alveolar bone, and it may eventually lead to tooth loss. In inflamed periodontal tissue, pro-inflammatory cytokines, such as TNF-α and interleukin-1, activate fibroblasts and epithelial cells, which subsequently produce numerous inflammatory mediators such as MMPs (13,14). TNF-α levels were shown to be elevated in the gingival crevicular fluid of adults with periodontitis (27,28). Furthermore, it was reported that MMP-1, -2, -3, -8 and -9 levels were higher in the gingival crevicular fluid and periodontal tissues from clinically diseased sites than in those from healthy sites (7,8,29). Thus, MMPs appear to be associated with the etiology of periodontitis. MMP-1 is a potent protease that initiates the cleavage of triple-helical collagen and makes the collagen fragments susceptible to further degradation by other MMPs (30). Therefore, MMP-1 may be critical to the initiation of collagen degradation in periodontitis.

The periodontal ligament is a complex, vascular, and highly cellular soft connective tissue that attaches the tooth roots to the inner wall of the alveolar bone; it is a functionally important tissue in tooth support (31). HPDL cells play an important role in forming new attachments between these tissues as well as in cementogenesis and osteogenesis. The breakdown of the periodontal ligament, together

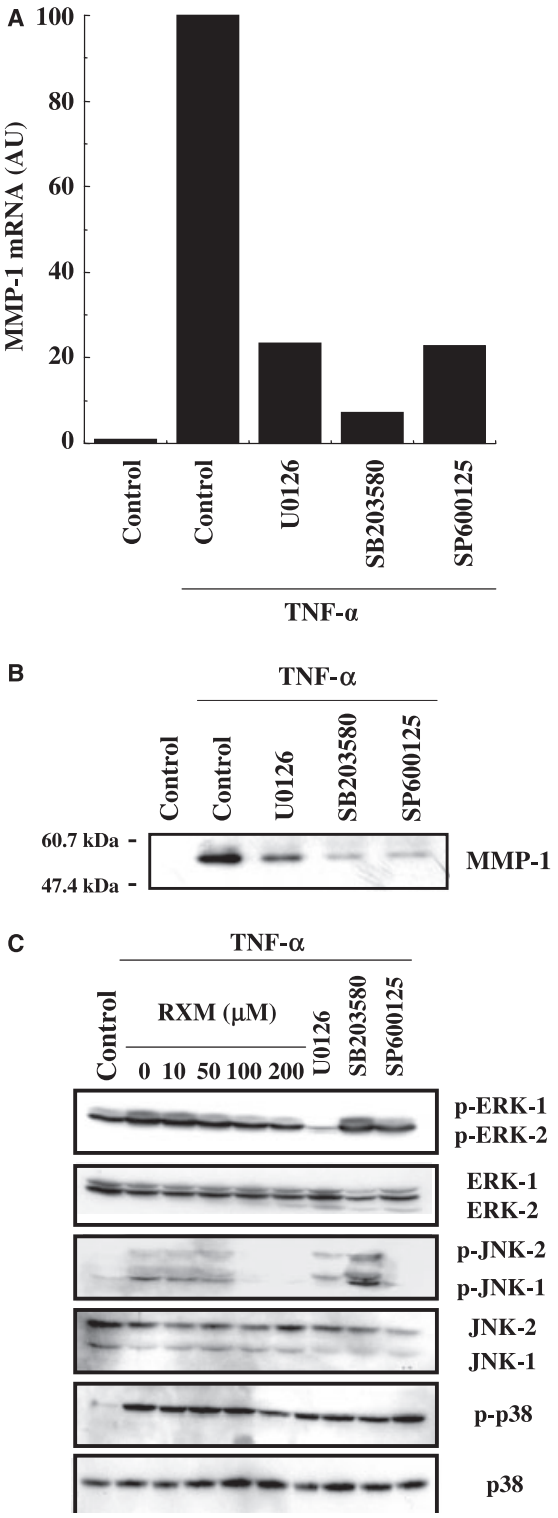

Fig. 4. Inhibitory effect of roxithromycin on matrix metalloproteinase-1 (MMP-1) induction through the regulation of mitogen-activated protein kinase (MAPK) activation. (A) MAPK mediates tumor necrosis factor-α (TNF-α)-induced MMP-1 mRNA expression. Confluent human periodontal ligament (HPDL) cells were pretreated with 20 μM SP600125, SB203580 or U0126 for 30 min and stimulated with 5 ng/ml TNF-α for 8 h. Total RNA was analyzed by northern blotting with a radiolabeled MMP-1 probe. The results are expressed as the mRNA accumulation relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, as an internal standard. Two additional experiments gave results similar to those shown. (B) MAPKs mediate TNF-α-induced MMP-1 production. Confluent HPDL cells were pretreated with 20 μM SP600125, SB203580 or U0126 for 1 h and stimulated with 5 ng/ml TNF-α for 24 h. The MMP-1 released from cells into the medium was blotted with antibody to MMP-1. Two additional experiments gave results similar to those shown here. (C) Roxithromycin (RXM) inhibits MAPK phosphorylation. Confluent HPDL cells were pretreated with 30 μM SP600125, SB203580 or U0126 for 1 h, and stimulated with 5 ng/ml TNF-α for 15 min. Lysates were immunoblotted. In all cases, the upper panels show the western blot analyses using antibodies recognizing the phosphorylated forms of extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK)1/2, or p38 MAPK. The lower panels show western blot analyses using antibodies recognizing total ERK1/2, JNK1/2, or p38 MAPK for control of equal loading. These data are representative of three independent experiments, with similar results obtained on each occasion.

with alveolar bone resorption by MMPs, is likely to contribute to tooth loss. In the present study, we confirmed that TNF-α can induce MMP-1 mRNA expression in HPDL cells (Fig. 1A,B), suggesting that TNF-α derived from inflammatory cells may be involved in the progress of periodontitis via the production of MMP-1 in periodontal ligament tissue.

Macrolide antibiotics are effective as therapeutic agents against chronic inflammatory diseases such as diffuse panbronchiolitis and chronic sinusitis (32,33). Roxithromycin, a 14-membered ring macrolide antibiotic, has anti-inflammatory activity (34–36) in addition to antimicrobial activity (37). Roxithromycin inhibits the production of interleukin-6 and -8 in human bronchiolitis (35) and inhibits TNF-α and interleukin-1 production in human monocytes (38). This suggests that roxithromycin modulates local recruitment and activation of inflammatory cells, which may be relevant to its efficacy in inflammatory disorders. In the present study, roxithromycin showed an inhibitory effect on TNF-α-induced

MMP-1 mRNA expression (Fig. 2A), MMP-1 production (Fig. 2B,C), and MMP-1 activation (Fig. 2D). Furthermore, actinomycin D suppressed TNF-α-induced MMP-1 mRNA expression (Fig. 3B). These findings indicate that roxithromycin downregulated the activation of MMP-1, at least in part, at the transcriptional level.

Several potential binding sites for transcription factors, such as AP-1 and Ets-1, are located in the MMP-1 promoter, and it is thought that these factors are associated with MMP-1 expression (39). In the present study, we showed that roxithromycin inhibited the induction of Ets-1. Roxithromycin has also been reported to suppress the activation of AP-1 upon stimulation with TNF-α in HPDL cells (15) and in human nasal polyp fibroblasts (40). These findings suggest that roxithromycin downregulates the transcription of the MMP-1 gene by interfering with Ets-1 and AP-1.

TIMPs are specific cellular inhibitors of MMPs and help to regulate the local activities of MMPs in tissues (41). As TIMP-1 is inducible, and because the transcription of TIMP-1 is AP-1 dependent (42), we focused on the effect of roxithromycin on TIMP-1 induction. Interestingly, roxithromycin did not affect the production of TIMP-1 protein that was induced by TNF-α (Fig. 3A). The overexpression of Jun B, a member of the AP-1 transcription factor family, was reported to cause the induction of two other AP-1 family members – c-Jun and Fra1 – and the enhancement of MMP-1 promoter activity in a rat keratinocyte cell line (43). However, the overexpression of c-Jun or Fra1 did not increase TIMP-1 promoter activity in hepatic stellate cells (44). These observations indicate that differences between the regulation of MMP-1 and TIMP-1 expression may contribute to the combinations of AP-1 members affected by roxithromycin.

Extracellular matrix destruction via the activation of MAPK and MMP-1 is a central event in the pathogenesis of rheumatoid arthritis (45) and periodontitis (46). The expression of MMP-1 is up-regulated by mitogenic growth factors and pro-inflammatory cytokines, which activate different parts of the MAPK pathways (39). It was reported that the activation of ERK1/2 and p38 MAPK resulted in potent induction of MMP-1 in response to TNF-α in human fibroblasts (47) and in transformed human keratinocytes (48). However, little is known about the participation of the JNK cascade upstream of MMP-1 gene expression. In this study, we demonstrated that not only U0126 and SB203580, but also SP600125, clearly blocked the expression of MMP-1 mRNA and protein in response to TNF-α (Fig. 4A,B). Therefore, TNF-α might induce MMP-1 gene expression through the activation of ERK1/2, p38 MAPK, and JNK in HPDL cells. We also showed that roxithromycin suppressed the phosphorylation of ERK1/2 and JNK, but not of p38 MAPK. The phosphorylation of threonine-38 by ERK1/2 strongly increased the transcriptional activity of Ets-1 (49). Furthermore, ERK1/2 and JNK activate c-Jun (39). Thus, it seems that roxithromycin may suppress the induction of MMP-1 in response to TNF-α via the downregulation of ERK1/2 and JNK and the subsequent inhibition of Ets-1 and AP-1 in HPDL cells.

In conclusion, we demonstrated that roxithromycin inhibited TNF-α-induced MMP-1 production in HPDL cell cultures by blocking two MAPK cascades – the ERK1/2 and JNK cascades – and subsequently by downregulating the transcription factor Ets-1. We have shown previously that roxithromycin inhibits TNF-α-induced VEGF expression (15). MMPs directly cause the breakdown of periodontal tissue, and VEGF is involved in the initiation and progression of human periodontitis (50). Given that roxithromycin inhibits MMP-1 and VEGF induction in HPDL cell cultures, it may be possible to use roxithromycin as a new therapeutic reagent for acute and chronic periodontitis.

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