Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 1809–1819
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Chemically modified tetracyclines selectively inhibit IL-6 expression in osteoblasts by decreasing mRNA stability

Keith Kirkwood[a,b,c,*], Thomas Martin[a], Stelios T. Andreadis[d], Young Joon Kim[a,e]

[a]*Department of Periodontics and Endodontics, State University of New York at Buffalo, 250 Squire Hall, 3435 Main Street, Buffalo, NY 14214-3008, USA*
[b]*Department of Oral Biology, State University of New York at Buffalo, Buffalo, NY 14214-3008, USA*
[c]*Department Pharmacology and Toxicology, State University of New York at Buffalo, Buffalo, NY 14214-3008, USA*
[d]*Department of Chemical Engineering, State University of New York at Buffalo, Buffalo, NY 14214-3008, USA*
[e]*Department of Periodontics, Chonnam National University, Kwang-Ju, South Korea*

Received 29 November 2002; accepted 12 May 2003

Abstract

In bone biology, interleukin (IL)-6 is an autocrine/paracrine cytokine which can induce osteoclasts formation and activation to help mediate inflammatory bone destruction. Previous studies have shown that tetracycline and its derivatives have potentially beneficial therapeutic effects in the prevention and treatment of metabolic bone diseases by modulating osteoblast and osteoclast activities. Our previous studies indicated that non-antimicrobial chemically modified tetracyclines (CMTs) can dose-dependently inhibit IL-1β-induced IL-6 secretion in osteoblastic cells. In the present study, we explored the molecular mechanisms underlying the ability of doxycycline analogs CMT-8 and its non-chelating pyrazole derivative, CMT-5 to affect IL-6 gene expression in murine osteoblasts. Steady-state IL-6 mRNA was decreased with CMT-8 (ca. 50%) but not by CMT-5 when stimulated by IL-1β. CMT-8 regulation of IL-1β-induced IL-6 gene expression was further explored. CMT-8 did not affect IL-6 promoter activity in reporter gene assays. However, the IL-6 mRNA stability was decreased in the presence of CMT-8. These effects require *de novo* protein synthesis as they were inhibited by cycloheximide. Western blot analysis indicated that CMT-8 did not affect p38 mitogen-activated protein kinase, c-*jun* NH_2-terminal kinases, or extracellular signal-regulated kinases (1 and 2) phosphorylation in response to IL-1β. These data suggest that CMT-8 can modulate inhibit IL-1β-induced IL-6 expression in MC3T3-E1 cells at the post-transcriptional level affecting IL-6 mRNA stability. These observations may offer a novel molecular basis for this treatment of metabolic bone diseases that are mediated by IL-6.

Keywords: IL-6; IL-1β; Osteoblasts; Gene expression; Chemically modified tetracyclines

1. Introduction

Bone is a dynamic tissue that constantly undergoes a remodeling process where bone resorption and bone deposition are balanced. When chronic inflammation occurs in bone, this balance is disrupted favoring net bone loss. Diseases involving an intimate combination of bone loss and inflammation include rheumatoid arthritis (RA) periodontal disease, and other metabolic bone diseases. Inflammatory cytokines, such as IL-1α and IL-1β, tumor necrosis factor (TNF)-α [1,2], and IL-17 [3] have all been shown to promote tissue destruction. In addition to these factors, members of the IL-6 family of cytokines have been shown to perturb bone and cartilage metabolism [4]. For example, leukemia inhibitory factor (LIF) and oncostatin M (OSM) have been shown to promote cartilage degradation *in vitro* and IL-6, LIF [5], OSM [6], IL-11 [7] have all been shown to mediate tissue destruction in various experimental models of bone resorption.

IL-6 is a pleiotropic cytokine that elicits a wide variety of immune/inflammatory responses including B- and T-cell activation, stimulation of fever, and release of acute phase response proteins [8]. Bone resorptive agents, such as parathyroid hormone (PTH), TNF-α, and IL-1β, have all been shown to stimulate IL-6 production and secretion in osteoblasts [9,10]. Proinflammatory cytokines, including

* Corresponding author. Tel.: +1-716-829-2110; fax: +1-716-837-7623.
E-mail address: klkirk@acsu.buffalo.edu (K. Kirkwood).

Abbreviations: IL, interleukin; CMT, chemically modified tetracycline; MAP, mitogen-activated protein; RT–PCR, reverse transcription–polymerase chain reaction; ActD, actinomycin D; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00450-7

those of the IL-6 family, involved in bone formation and remodeling, converge on the expression of receptor-activated NF-κB ligand (RANKL), and its decoy receptor, osteoprotegerin (OPG) [11,12]. RANKL and OPG expression from stromal/osteoblastic cells increases osteoclastogenesis through activation of the RANKL cognate receptor, RANK, located on osteoclast precursor cells [13,14]. Thus, inhibition of IL-6 expression presents an attractive target for the treatment of metabolic bone diseases.

Tetracycline and its analogs have been shown to inhibit bone resorption in organ culture when induced by PTH, prostaglandin E_2 (PGE_2), or bacterial lipopolysacchride (LPS) [15–17]. Other studies have shown that tetracyclines inhibit excessive connective tissue degradation in several pathological conditions, including periodontitis and streptozotocin-induced diabetes [15,18]. Tetracyclines are potent inhibitors of extracellular matrix metalloproteinases (MMPs), including the three collagenases MMP-1, MMP-8, and MMP-13, and two gelatinases MMP-2 and MMP-9 [19–23]. More recently, tetracyclines and their analogs have been shown to inhibit inflammatory mediators, such as nitric oxide synthase [24,25], PGE_2 production [26], and possibly other cytokines [27]. Our group has also presented evidence that cytokine-induced IL-6 secretion is regulated by CMTs [28].

Since tetracycline is well known for its ability to bind divalent cations, such as calcium and zinc, and affect intracellular calcium concentrations, we evaluated the abilities of CMTs, which lack antimicrobial activity to affect osteoblast IL-6 secretion from MC3T3-E1 osteoblast cells. Previous data from our laboratory had indicated that regulation of intracellular calcium stores were critical to stimulus-secretion coupling in osteoblasts [29–31]. Herein, we show that, CMT-8 and but not CMT-5, an imidazole derivative, can decrease IL-6 gene expression when induced by IL-1β by a mechanism that requires *de novo* protein synthesis in a manner that decreases IL-6 mRNA stability.

2. Experimental design and methods

2.1. Materials

CMTs were obtained from CollaGenex, Inc., under a material transfer agreement between SUNY at Buffalo and CollaGenex, Inc. Human IL-1β was obtained from Sigma.

2.2. Cell culture of MC3T3-E1 cells

MC3T3-E1 osteoblastic cells were obtained from RIKEN. Cells were cultured in DMEM (Invitrogen Life Technologies), supplemented with 10% fetal bovine serum (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 in air at 37°. Osteoblast phenotypic mRNAs, including bone sialoprotein and osteocalcin, were routinely assayed by Northern blot analysis to verify osteoblastic phenotype expression in these cells.

2.3. Cell culture of primary rat calvarial cells

Osteoblast-enriched cell preparations were obtained from Sprague–Dawley 21- or 28-day fetal rat calvaria by sequential collagenase digestion (type II; Life Technologies) in Bone Cell Buffer, pH 7.4, as described previously [32]. The resultant cells from the third and fourth 15-min collagenase digestions have osteoblastic character, including high alkaline phosphatase activity, and the ability to form collagen and bone *in vitro* [33]. These cells were pooled and cultured in BGJb media (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37° in humidified atmosphere of 5% CO_2/95% air.

2.4. Northern blot analysis of osteoblast gene expression

For detection of mRNA from control and CMT-treated MC3T3-E1 osteoblastic cells, we employed Northern blot using methods as described previously [28,29]. Briefly, total RNA was isolated using Trizol reagent (Life Technologies). Total RNA was visualized for intactness by ethidium bromide staining following gel electrophoresis. RNA was quantitated by spectroscopy (SmartSpec 3000, Bio-Rad). High specific activity cDNA probes were generated from cloned templates using random primer kit (Stratagene). Rat IL-6 cDNA clone was obtained as a gift from Dr. J. Goldie, McMaster University, Hamilton, Ont. In all experiments, 15–20 μg of total RNA was electrophoresed on 1% formaldehyde denaturing gel and transferred to Nytran nylon membranes (Schleicher&Schuell) using the Turboblotter apparatus (Schleicher&Schuell). All blots were normalized for loading with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. After hybridization with the appropriate probes, membranes were washed at high stringency and hybridized probe was quantitated using Bio-Rad's phosphoimager system and Quantity One software (Bio-Rad).

For analysis of mRNA stability in the presence or absence of CMT-8 and -5, we used the DNA polymerase inhibitor, actinomycin D (ActD). In these experiments, cells were pretreated with CMT-8 for 1 hr and then treated with IL-1β for 4 hr. Subsequently, actinomycin D was added to prevent further transcription. Total RNA was harvested and analyzed as outlined above after 0, 1, 2, and 4 hr of treatment with actinomycin D (2 μg/mL).

To determine whether protein synthesis was required for the CMT-mediated effects on IL-6 mRNA levels, MC3T3-E1 cells were incubated with cycloheximide (CHX; 2 μg/mL) prior to treatment with CMT-8 and IL-1β. Northern blot analysis for IL-6 and GAPDH expression was conducted as described above.

2.5. Reverse transcription–polymerase chain reaction (RT–PCR) analysis

Total RNA (5 μg) from MC3T3-E1 or primary rat calvarial cells was used for cDNA synthesis with oligo (dT) 12–18 primers and Superscript II (RNaseH-) (Life Technologies) in reverse transcription (RT) reactions. 2 μL of RT product was used as a template for polymerase chain reaction (PCR) amplification of IL-6 and GAPDH gene products. PCR primers were commercially synthesized (Sigma-Genosys) for IL-6 (GenBank accession # NM-031168). Primer sequences that were employed were 5′-ATGAAGTTCCTCTCTGCAAGAGACTT-3′ for forward sequence and 5′-ATCACGGTTTGCCGAGTAGACTCT-3′ for reverse sequence. Non-regulated GAPDH (GenBank accession # AF106860) sequences 5′-CACCATGGAGA-AGGCCGGGG-3′ for forward primer sequence and 5′-GACGGACACATTGGGGGTAG-3′ for reverse primer sequence. RT–PCR products of 638 base pairs (bp) for IL-6 and 418 bp for GAPDH were separated and analyzed by gel electrophoresis. Resulting images were captured using a Gel-Doc (Bio-Rad) imaging system equipped with UV light and a gel scanner. PCR results were quantitated using Bio-Rad's phosphoimager system and Quantity One software to assess relative differences. RT–PCR products were assessed by subcloning RT–PCR products into pGEM-T (Promega) and DNA sequencing to determine authenticity.

2.6. Transient transfection and reporter gene assays

MC3T3-E1 cells were cultured to 50% (ca.) confluency in 60 mm^2 dishes, washed twice with Opti-MEM MEDIA (Life Technologies) mixture according to manufacturer's instructions. Equal molar amounts of the various pCAT-Basic expression vectors (ca. 2 μg) were transfected along with a fixed amount (1.5 μg) of the pSV-β-galactosidase (pSVβgal) control vector (Promega). Cells were incubated for 12–24 hr with the DNA–lipofectamine mixture, washed twice, and then incubated with α-MEM media with 2% fetal bovine serum. CMT-8 (10 μg/mL each) were added ±IL-1β (1 ng/mL). After 24 hr, the cells were harvested, washed, and soluble extracts prepared by repeated freeze–thawing in 0.15 mL 0.25 M Tris, pH 8.0, and used for determination of β-galactosidase and CAT activities as described previously [31]. Results were analyzed by autoradiography using Bio-Rad Phosphor Imaging and Quantity One software.

2.7. Western blot analysis

MC3T3-E1 cells were exposed to CMT-8 (10 μg/mL) for 30 min, then stimulated with IL-1β (1 ng/mL) for 30 and 60 min time points. Cells were rinsed with ice-cold PBS and whole cell lysates prepared in SDS–PAGE buffer (Bio-Rad). Protein concentrations were measured by Bradford's method (Bio-Rad). Ten micrograms of each sample was electrophoresed on 10% denatured SDS–PAGE gels, transferred to nitrocellulose membranes (Bio-Rad). Antibodies against phosphorylated and non-phosphorylated forms of p38, c-*jun* NH_2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinases (Cell Signaling Technologies) were used as primary antibodies in these studies. Primary antibodies were detected using HRP-conjugated secondary antibodies and LumniGlo (Cell Signaling Technologies) chemiluminescence detection and quantitation by Fuji imaging system.

3. Results

3.1. CMT-8, but not CMT-5, decreases steady-state IL-6 mRNA levels in MC3T3-E1 cells and primary rat osteoblastic cells

Northern blot analysis and semi-quantitative RT–PCR analysis were used to determine CMT-8 and -5 effects on IL-1β-induced IL-6 expression in MC3T3-E1 cells. IL-6 mRNA species of ∼2.4 kb was detected in MC3T3-E1 cells by Northern blot analysis (Fig. 1A). RT–PCR analysis of IL-6 reproducibly identified a 638 bp fragment which was authenticated by sequencing as IL-6 gene product (Fig. 1B). As shown previously in these cells, IL-1β increases steady-state mRNA levels [9,34]. When cells are pretreated with CMT-8, this agent decreased IL-1β-induced IL-6 steady-state mRNA levels in these cells. When osteoblastic cells were pretreated with CMT-5, repression of IL-1β-induced IL-6 gene expression was not observed. When levels of GAPDH were used to normalize the data, an approximate 50% reduction in IL-6 mRNA with CMT-8 treatment is seen compared to IL-1β-treated cells ($N = 4$). In primary rat calvarial osteoblastic cells ($N = 3$), CMT-8 had similar effects on IL-1β-induced IL-6 expression (Fig. 2). CMT-5 was not able to inhibit IL-1β-induced IL-6 gene expression in primary cultures as observed in MC3T3-E1 cells. Relative fold differences are shown above each figure when compared to the unregulated GAPDH control.

3.2. De novo protein synthesis is required for CMT-8 repression of IL-1β-induced IL-6

To explore further the mechanism underlying the ability of CMT-8 to inhibit IL-1β-induced IL-6 expression, we performed experiments in the presence of the protein synthesis inhibitor CHX. As shown in Fig. 3, CHX blocked the ability of CMT-8 to inhibit IL-1β-induced IL-6 mRNA production. In addition, CHX was able to dramatically affect IL-6 mRNA levels in the presence or absence of IL-1β (lanes 6 and 7). When CHX is used as a protein synthesis inhibitor a 'superinduction' phenomenon is often

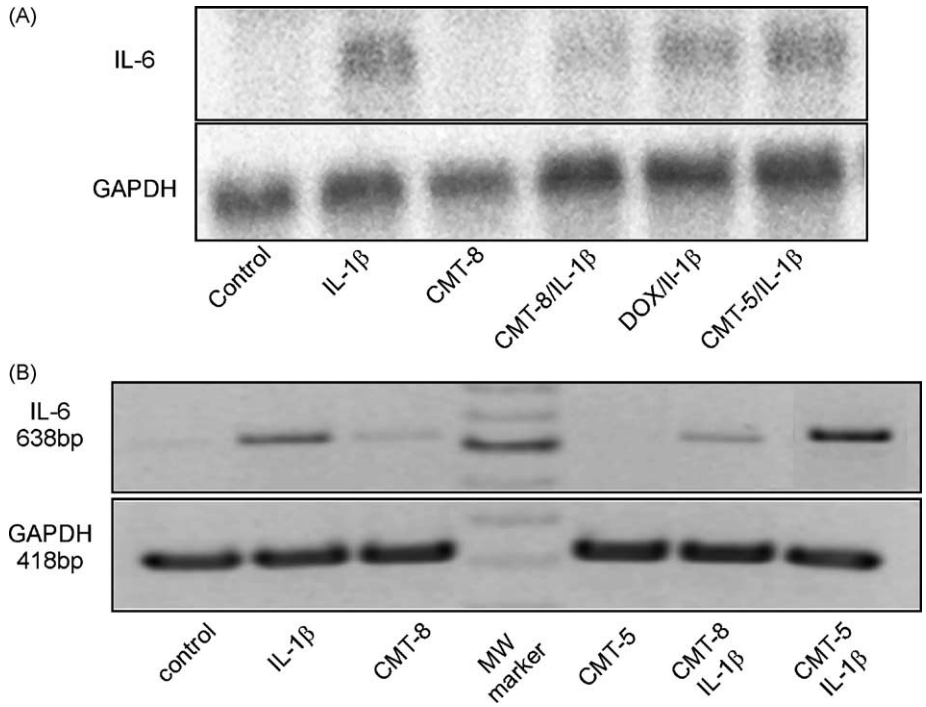

Fig. 1. Effect of CMTs and doxycycline MC3T3-E1 cell gene expression. (A) MC3T3-E1 cells were cultured in the presence or absence of CMT-8 or CMT-5 (10 μg/mL) and then exposed to IL-1β (1.0 ng/mL) for 18 hr. Total RNA was purified, separated on agarose gels, blotted onto nylon membranes, and sequentially hybridized with ^{32}P-labeled cDNA probes specific for IL-6 and GAPDH. (B) MC3T3-E1 cells were cultured in the presence or absence of CMT-8 or CMT-5 (10 μg/mL) and then exposed to IL-1β (1.0 ng/mL) for 18 hr. Semi-quantitative RT–PCR was performed on total RNA from treated cells. Agarose gels were used to analyze RT–PCR products and quantitated using Quantity One software of digitized images.

observed. With a variety of cytokine genes, including IL-6, the explanation most often used to explain this is treatment of cells with CHX causes the ribosomes to 'freeze' on the mRNA, potentially shielding it from degradation by cytoplasmic RNases [35].

3.3. CMT-8 does not affect expression of IL-6 promoter in MC3T3-E1 cells

To determine if CMT-8 can affect IL-6 gene promoter activity, transient transfection with rat IL-6 promoter constructs in MC3T3-E1 cells were performed. Following transient transfection of MC3T3-E1 cells with the −225 IL-6 promoter construct containing the reporter CAT gene or the −138 construct, cells were incubated in the CMT-8 in the presence or absence of IL-1β for 24 hr and then assayed for CAT activity. Figure 4 shows that cells transfected with either the −225 or the −138 constructs expressed relatively low constitutive CAT activity. Both IL-6 promoter constructs were stimulated in the presence of IL-1β (1 ng/mL) by 3-fold (ca.), for the −225 and −138 construct, respectively (N = 3). However, when CMT-8 was added to cells, this agent was not able to neither increase basal CAT activity nor inhibit IL-1β-stimulated CAT activity in a significant manner ($P = 0.87$, paired Student's *t*-test). Preliminary experiments indicated that empty vector pCAT-Basic (control) had no detectable CAT activity under non-treated conditions (data not shown).

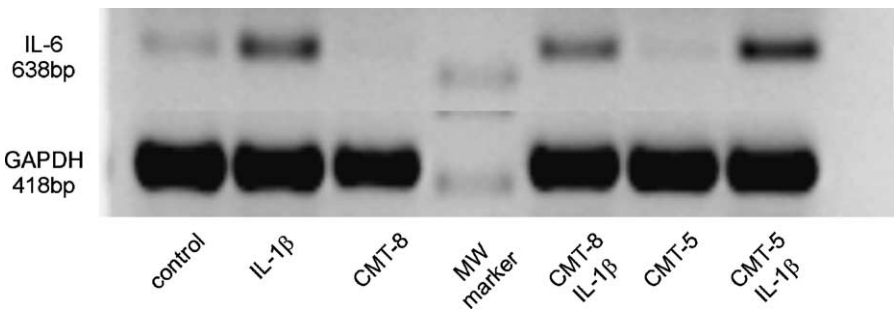

Fig. 2. Effect of CMTs on IL-6 steady-state mRNA levels in primary rat calvarial osteoblastic cells. Primary rat calvarial osteoblastic cells were cultured in the presence or absence of CMT-8 or CMT-5 (10 μg/mL) and then exposed to IL-1β (1.0 ng/mL) for 18 hr. Semi-quantitative RT–PCR was performed on total RNA from treated cells. Agarose gels were used to analyze RT–PCR products and quantitated using Quantity One software of digitized images.

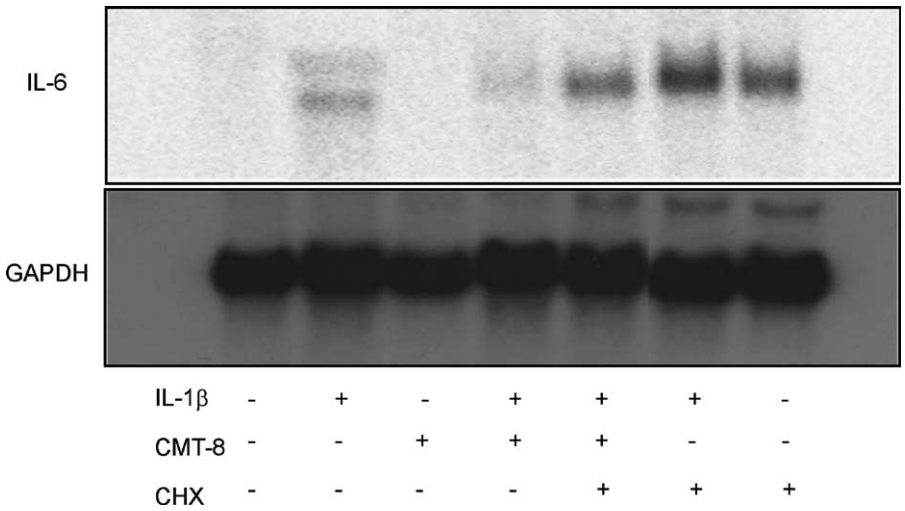

Fig. 3. Effect of CHX on CMT regulation of IL-6 in MC3T3-E1 Cells. MC3T3-E1 cells were treated with IL-1β (1.0 ng/mL) and CMT-8 (10 μg/mL) and in the presence or absence of CHX (2 μg/mL). Total RNA (15 μg) was analyzed by Northern blot using ^{32}P-labeled cDNA probes specific for rat IL-6 and rat GAPDH. See text for details on CHX effects.

CMT-5 was shown not to affect IL-6 gene promoter activity in parallel studies (data not shown).

3.4. CMT-8 accelerates IL-6 mRNA rate of decay in MC3T3-E1 cells

To determine whether the down-regulatory effect of CMT-8 on IL-1β-induced steady-state IL-6 mRNA level was due to decreased stability of IL-6 mRNA, MC3T3-E1 cells were pretreated with IL-1β (1.0 ng/mL), then treated with CMT-8 (10 μg/mL) for 6 hr and, during the last period of incubation, actinomycin D (2 μg/mL) was added for 1, 2, or 4 hr. IL-6 mRNA levels were then analyzed by Northern blot. Actinomycin D blocks further transcription and allows the rates of mRNA decay to be determined. The results indicate that IL-1β increases the half-life of IL-6 (Fig. 5), with calculated half-lives of 90 min, consistent with previous reports where IL-1β prolonged the half-life of IL-6 mRNA [36]. However, in the presence of CMT-8, the half-life of IL-6 was decreased to ∼30 min following normalization to GAPDH which has a relatively long half-life (∼8 hr) [37] (Fig. 5). These results indicate that the rate of IL-6 mRNA decay in MC3T3-E1 cells is accelerated by CMT-8.

3.5. CMT-8 does not affect IL-1β-induced phosphorylation of MAP kinases

To further explore the molecular nature of CMT-8 on IL-6 mRNA stability, we directly measured phosphorylation of p38, JNK, and ERK MAP kinases in MC3T3-E1 cells. MAP kinase activation appears to play a significant role in cytokine mRNA stabilization, including IL-6 [38–40]. As shown in Fig. 6, MC3T3-E1 cells were pretreated with CMT-8 for 30 min and the stimulated by IL-1β. Protein from treated cells was evaluated by Western blot analysis for phosphorylated and non-phosphorylated forms of p38, JNK, and ERK MAP kinases. Results from two independent studies indicated that IL-1β activates both p38 and JNK MAP kinase, but not ERK MAP kinase in the cells. Neither p38 nor JNK phosphorylation was inhibited by CMT-8 (N = 2).

4. Discussion

Tetracyclines have been used for decades as antimicrobial agents. More recently, a new effect of this class of agents has been discovered: inhibition of MMPs, which degrade connective tissue and other host-modulating activities [16,41]. The effects of tetracyclines were found to be independent of their antimicrobial activity in germ-free and conventional animal models, as well as in sterile *in vitro* systems [20]. Additionally, some CMTs, which lack antimicrobial activity, inhibit collagenase, inhibit bone resorption, and stimulate collagen deposition [41]. Other effects include inhibition of expression of iNOS [25], PLA_2 [42], and reduction of cytokine expression [27,43], e.g. TNF-α, IL-8, and IL-12 [25,41]. It should be noted here that CMT-5, a tetracycline analog lacking the important metal-binding domain, is ineffective on many of these pathways.

MC3T3-E1 osteoblastic cells are of the osteoblastic lineage that can undergo cellular differentiation in tissue culture into mature osteoblasts capable of secreting bone matrix proteins, including osteocalcin, consistent with a mature osteoblast phenotype [44]. In the present study, we used MC3T3-E1 cells at the stage of confluency in all experiments, approximately 3–5 days after plating. Thus, results found in these experiments are limited to osteoblasts that are still in the pre-osteoblastic stage and not the mature osteoblastic stage [45].

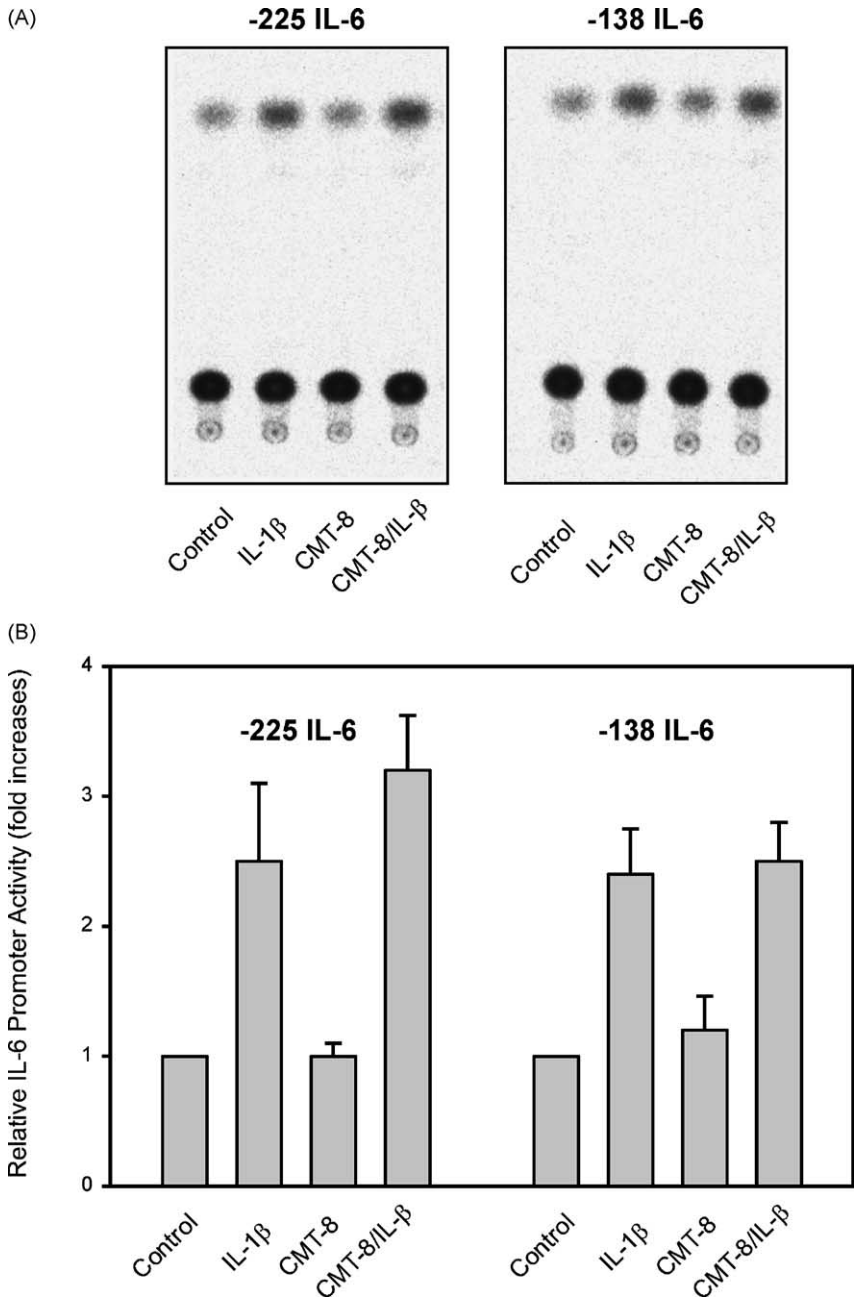

Fig. 4. Effect of CMT-8 on IL-6 promoter region in transient transfection experiments. (A) pCAT-Basic vector containing either the −225 bp through +6 bp fragment or the −138 bp to +6 bp fragment inserted in the forward orientation in front of the bacterial CAT gene was transfected onto MC3T3-E1 cells as described. Cells were then treated with CMT-8 ± IL-1β for 24 hr and assayed for soluble CAT activity. (B) Graphical representation of mean relative CAT activity from transiently transfected MC3T3-E1 cells following normalization to pSVβgal activity (N = 3).

Most of the studies that address gene regulation by CMTs have found that CMT effects occur *via* a post-transcriptional or translation mechanism. For example, iNOS has been shown to be regulated by CMT-3 by a mechanism similar to CMT-8 and -5 regulation of IL-6 where the mRNA of iNOS was decreased in the presence of CMT-3 and doxycycline [25]. PLA_2, an inflammatory mediator, has been shown to be regulated by a post-translational mechanism; however, the exact mechanism has not be elucidated [42]. The results of the present study suggest CMT-8, but not CMT-5, can inhibit IL-6 gene expression by a mechanism consistent with decreasing mRNA stability in osteoblasts. The results in this paper are consistent with the effects on IL-6 gene expression at the post-transcriptional level [39,46,47]. Similar findings were observed with CMT regulation of iNOS in macrophages [25].

Other studies have shown that tetracyclines can regulate steady-state levels of specific mRNAs. In cultured keratinocytes, gelatinase (MMP-2) gene expression was regulated

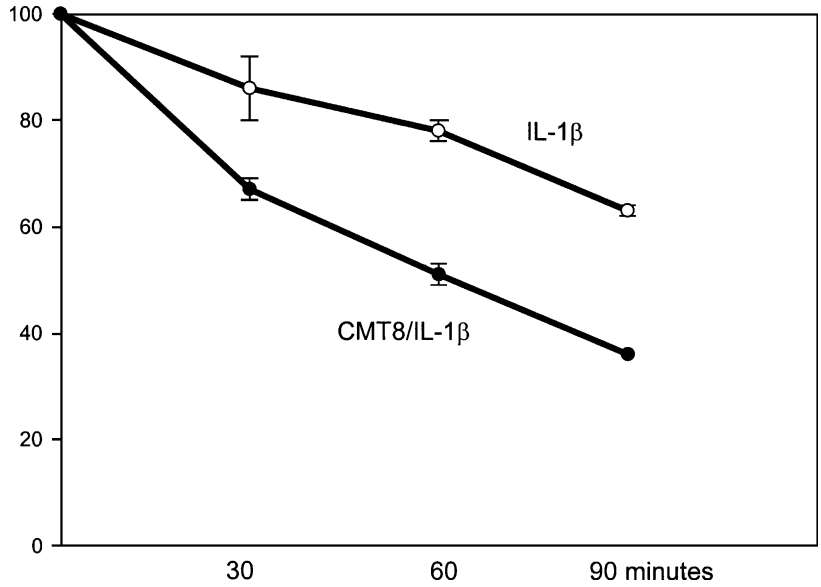

Fig. 5. Effect of CMT-8 on IL-6 mRNA stability in MC3T3-E1. MC3T3-E1 cells were pretreated with CMT-8 (10 μg/mL), then treated with IL-1β (1 ng/mL) for 6 hr. During the last 90, 60, and 30 min of incubation, actinomycin D (2.0 μg/mL) was added. Total RNA (N = 3) was purified, separated on agarose gels, blotted onto nylon membranes, and sequentially hybridized with ^{32}P-labeled cDNA probes specific for IL-6 and GAPDH. The % mRNA remaining from IL-1β ± CMT-8-treated cells following normalization to GAPDH. mRNA half-lives were experimentally determined to be ∼90 min in IL-1β-treated cells, and ∼30 min in CMT-8/IL-1β-treated cells.

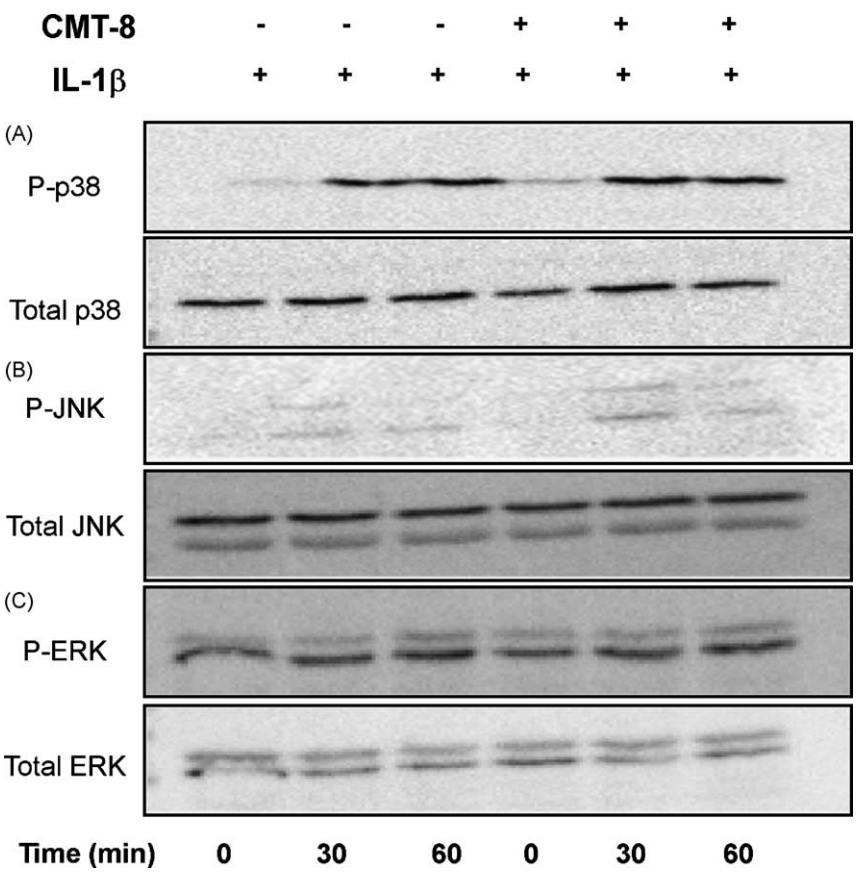

Fig. 6. Effect of CMT-8 on MAP kinase phosphorylation in MC3T3-E1. (A) Activation of p38 (A), JNK (B), or ERK (C) was evaluated in MC3T3-E1 cells in response to IL-1β stimulation was detected by phosphospecific antibodies to phosphorlyated p38 (A), JNK (B), or ERK (C). Effects of CMT-8 were evaluated by pretreatment of cells with CMT-8 followed by IL-1β stimulation. Blots were reprobed with anti-p38 (A), JNK (B), or ERK (C) to verify equal loading.

by doxycycline and CMT-1, however, the mechanism of this regulation was not elucidated [23]. It was proposed to involve a direct regulation of mRNA transcription rate or an increase in the rate of decay post-transcriptionally. Another mechanism of indirect regulation of MMP-2 expression by doxycycline and CMT-1 was proposed to involve chelation of divalent cations required for cell adhesions molecules, with subsequent changes in gene expression mediated through signal transduction pathways. Another study has shown that CMT-1 can prevent streptozotocin-induced depression of skin type I procollagen α_1 (I) gene steady-state mRNA levels in a diabetic rat model [48]. Although the mechanism was not elucidated the authors suggested that the type I (α_1) procollagen gene mRNA half-life was prolonged in the presence of CMT-1. *In vivo* data have shown that minocycline can prevent trabecular bone loss induced by ovariectomy (OVX) consistent with effects on bone remodeling in aged rats [49]. Additional *in vivo* data showed that CMT-1 with or without flurbiprofen was able to prevent bone loss associated with loss of gonadal function [50]. Other animal studies have subsequently shown CMT-8 along with bisphosphonates can diminish LPS-induced alveolar bone destruction [49]. In this paper, CMT-8 but not CMT-5 inhibited the potentially catabolic effects of IL-1β, suggesting a novel molecular mechanism of these drugs with the treatment of metabolic bone diseases, such as osteoporosis and periodontal diseases.

Induction of IL-6 in these studies with IL-1β also translated into secretion of IL-6 protein. Our previous results indicated that IL-6 secretion could be inhibited by up to ~50% by CMT-8 and to a lesser extent by doxycycline (~30%) [28]. In these studies, CMT-8 inhibition of IL-1β-induced IL-6 secretion occurred in a dose-dependent manner with an experimentally IC_{50} of 4.4 μg/mL. In subsequent studies, we used 10 μg/mL CMTs as these concentrations were determined not to have any affects on cell viability (preliminary data not shown). The results of the present study were consistent with the regulation of the IL-1β-induced IL-6 steady-state mRNA regulation in osteoblastic cultures (Figs. 1 and 2), suggesting that IL-6 protein levels are decreased due to a decrease in the amount of IL-6 gene expression rather than a decrease in newly translated protein. Studies with MC3T3-E1 cells have shown that IL-1β increases steady-state IL-6 mRNA levels as early as 1 hr and later studies showed that the transcription factors NF-κB and C/EBP-β are needed to mediate the induction of IL-6 gene expression [4]. IL-1β has been shown to increase the mRNA half-life of IL-6 from 30 min to 2 hr [36]. We also observed a similar increase in IL-6 half-life with IL-1β treatment (Fig. 5). Results using actinomycin D to arrest transcription suggest that CMT-8 accelerates IL-6 degradation at the post-transcriptional level rather than transcription at the promoter level. Additional studies addressing the rate of transcription were inconclusive (data not shown), but based upon promoter activity in these cells, we do not feel the rate of mRNA synthesis is dramatically affected. Additional experiments are needed to confirm this interpretation.

Regulation of cytokine mRNA turnover, such as IL-6, is recognized to be one of the centrally important mechanisms of controlling the level of cytoplasmic mRNA, and consequently, gene expression. Rapid mRNA turnover is mediated by *cis*-acting elements that are distributed throughout the mRNA molecule [51]. For example, a common destabilizing element found in the 3′ untranslated region (UTR) of short-lived mRNAs is the AU-rich element (ARE), consisting of multiple copies of the pentanucleotide, AUUUA, and a high content of U residues [52,53]. ARE-directed mRNA decay may be positively or negatively regulated by extracellular stimuli [54,55].

MAP kinases are key enzymes in the signal transduction cascade from the extracellular environment to the nucleus of essentially every eukaryotic cell type [56]. Three groups of MAP kinases have been identified in mammalian cells [57,58]. These are the ERKs, the JNKs, and the p38 MAP kinases. In general, the ERKs are activated by growth factors and hormones whereas both JNKs and p38 MAP kinases are activated by environmental stress and inflammatory cytokines [58,59]. p38 MAP kinase was originally identified as the target of pyridinylimidazole compounds that inhibit the production of inflammatory cytokines from monocytes [60]. Subsequently, p38 MAP kinase has been shown to play a role in a variety of other cellular processes, including prolongation of cytokine mRNA half-life [61,62].

In these studies, we observed that IL-6 mRNA half-life was reduced in the presence of CMT-8. To explore the possibility that CMT-8 could directly affect MAP kinases which are activated in response to IL-1β stimuli, we performed experiments to measure CMT-8 effects on p38, JNK, and ERK MAP kinase phosphorylation when activated by IL-1β. Results shown in Fig. 6 indicate that CMT-8 does not directly affect MAP kinase activation in MC3T3-E1 cells. In these cells, both p38 and JNK are activated in response to IL-1β (Fig. 6A and B), while no effect is observed with ERK in response to IL-1β. These results are similar to that of other studies with osteoblasts or bone marrow stromal cells [63,64]. Thus, data from these studies suggest that MAP kinases are not the direct target of CMT-8 in osteoblasts.

It is assumed that regulation of mRNA stability is mediated through *trans*-acting RNA-binding factors which interact with *cis*-elements, including AREs. ARE-binding regulators of mRNA stability include AUF1, HuR, and tristeraprolin (TTP) [65]. All of these RNA-binding proteins are expressed in MC3T3-E1 cells.[1] Based upon the data obtained in this present study, we propose a mechanism whereby CMT-8 regulation of IL-1β-induced IL-6 expression occurs at the post-transcriptional level and

[1] Unpublished data.

requires ongoing protein synthesis to mediate these effects. A possible mechanism consistent with these findings would be the induction of RNA-binding protein(s) to truncate the 3′-end of the IL-6 mRNA and target the message for degradation.

Other studies previous to this study have shown the potential of tetracyclines to regulate IL-6 in osteoporosis animal models. Minocycline was shown to decrease IL-6 in ovariectomized (OVX) rats and increase type I collagen expression without affecting osteocalcin or alkaline phosphatase expression [66]. We have had similar results in MC3T3-E1 cells ([28], and see footnote 1). Additional studies with CMT-8 have demonstrated that IL-6 is decreased in periodontal disease animal models where LPS-induced bone loss can be measured [67]. Recent data have been shown that low doses of doxycycline, where antibacterial actions are not observed, can reduce acute phase response protein, C-reactive protein, IL-6, MMP-2, and MMP-9 in patients with recent acute coronary syndrome [68]. Data presented herein provide additional support for the use of CMTs in the treatment of IL-6-mediated bone diseases. Bone-related diseases which may benefit from CMT therapeutics include osteoporosis, rheumatoid arthritis, periodontitis, and other metabolic bone diseases.

Acknowledgments

This work was supported, in part through the Moir P. Tanner Research Fund (K.L.K.), the ADA Health Foundation (K.L.K.), and CollaGenex, Inc. (K.L.K.).

References

[1] Dayer JM, Fenner H. The role of cytokines and their inhibitors in arthritis. Baillieres Clin Rheumatol 1992;6(2):485–516.
[2] Vervoordeldonk MJ, Tak PP. Cytokines in rheumatoid arthritis. Curr Rheumatol Rep 2002;4(3):208–17.
[3] Yano K, Nakagawa N, Yasuda H, Tsuda E, Higashio K. Synovial cells from a patient with rheumatoid arthritis produce osteoclastogenesis inhibitory factor/osteoprotegerin: reciprocal regulation of the production by inflammatory cytokines and basic fibroblast growth factor. J Bone Miner Metab 2001;19(6):365–72.
[4] Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NFkappaB ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. Bone 2002;30(2):340–6.
[5] Carroll GJ, Bell MC. Leukaemia inhibitory factor stimulates proteoglycan resorption in porcine articular cartilage. Rheumatol Int 1993;13(1):5–8.
[6] van de Loo FA, van den Berg WB. Gene therapy for rheumatoid arthritis. Lessons from animal models, including studies on interleukin-4, interleukin-10, and interleukin-1 receptor antagonist as potential disease modulators. Rheum Dis Clin North Am 2002;28(1):127–49.
[7] Kawashima N, Stashenko P. Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. Arch Oral Biol 1999;44(1):55–66.
[8] Manolagas SC. Role of cytokines in bone resorption. Bone 1995;17(Suppl 2):63S–7S.
[9] Linkhart TA, Linkhart SG, MacCharles DC, Long DL, Strong DD. Interleukin-6 messenger RNA expression and interleukin-6 protein secretion in cells isolated from normal human bone: regulation by interleukin-1. J Bone Miner Res 1991;6(12):1285–94.
[10] Nagy Z, Radeff J, Stern PH. Stimulation of interleukin-6 promoter by parathyroid hormone, tumor necrosis factor alpha, and interleukin-1beta in UMR-106 osteoblastic cells is inhibited by protein kinase C antagonists. J Bone Miner Res 2001;16(7):1220–7.
[11] Atkins GJ, Haynes DR, Geary SM, Loric M, Crotti TN, Findlay DM. Coordinated cytokine expression by stromal and hematopoietic cells during human osteoclast formation. Bone 2000;26(6):653–61.
[12] Neale SD, Schulze E, Smith R, Athanasou NA. The influence of serum cytokines and growth factors on osteoclast formation in Paget's disease. QJM 2002;95(4):233–40.
[13] Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P. Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. J Biol Chem 2001;276(23):20659–72.
[14] Aubin JE, Bonnelye E. Osteoprotegerin and its ligand: a new paradigm for regulation of osteoclastogenesis and bone resorption. Medscape Womens Health 2000;5(2):5.
[15] Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA, Rifkin BR. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. Crit Rev Oral Biol Med 1991;2(3):297–321.
[16] Rifkin BR, Vernillo AT, Golub LM. Blocking periodontal disease progression by inhibiting tissue-destructive enzymes: a potential therapeutic role for tetracyclines and their chemically-modified analogs. J Periodontol 1993;64(Suppl 8):819–27.
[17] Chang KM, Ramamurthy NS, McNamara TF, Evans RT, Klausen B, Murray PA, Golub LM. Tetracyclines inhibit *Porphyromonas gingivalis*-induced alveolar bone loss in rats by a non-antimicrobial mechanism. J Periodontal Res 1994;29(4):242–9.
[18] Ryan ME, Usman A, Ramamurthy NS, Golub LM, Greenwald RA. Excessive matrix metalloproteinase activity in diabetes: inhibition by tetracycline analogues with zinc reactivity. Curr Med Chem 2001;8(3):305–16.
[19] Curci JA, Petrinec D, Liao S, Golub LM, Thompson RW. Pharmacologic suppression of experimental abdominal aortic aneurysms: a comparison of doxycycline and four chemically modified tetracyclines. J Vasc Surg 1998;28(6):1082–93.
[20] Golub LM, McNamara TF, D'Angelo G, Greenwald RA, Ramamurthy NS. A non-antibacterial chemically-modified tetracycline inhibits mammalian collagenase activity. J Dent Res 1987;66(8):1310–4.
[21] Greenwald RA, Golub LM, Ramamurthy NS, Chowdhury M, Moak SA, Sorsa T. In vitro sensitivity of the three mammalian collagenases to tetracycline inhibition: relationship to bone and cartilage degradation. Bone 1998;22(1):33–8.
[22] Hanemaaijer R, Visser H, Koolwijk P, Sorsa T, Salo T, Golub LM, van Hinsbergh VW. Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. Adv Dent Res 1998;12(2):114–8.
[23] Uitto VJ, Firth JD, Nip L, Golub LM. Doxycycline and chemically modified tetracyclines inhibit gelatinase A (MMP-2) gene expression in human skin keratinocytes. Ann NY Acad Sci 1994;732:140–51.
[24] Trachtman H, Futterweit S, Greenwald R, Moak S, Singhal P, Franki N, Amin AR. Chemically modified tetracyclines inhibit inducible nitric oxide synthase expression and nitric oxide production in cultured rat mesangial cells. Biochem Biophys Res Commun 1996;229(1):243–8.
[25] Amin AR, Patel RN, Thakker GD, Lowenstein CJ, Attur MG, Abramson SB. Post-transcriptional regulation of inducible nitric oxide synthase mRNA in murine macrophages by doxycycline and chemically modified tetracyclines. FEBS Lett 1997;410(2/3):259–64.

[26] Patel RN, Attur MG, Dave MN, Patel IV, Stuchin SA, Abramson SB, Amin AR. A novel mechanism of action of chemically modified tetracyclines: inhibition of COX-2-mediated prostaglandin E2 production. J Immunol 1999;163(6):3459–67.
[27] D'Agostino P, Ferlazzo V, Milano S, La Rosa M, Di Bella G, Caruso R, Barbera C, Grimaudo S, Tolomeo M, Feo S, Cillari E. Anti-inflammatory effects of chemically modified tetracyclines by the inhibition of nitric oxide and interleukin-12 synthesis in J774 cell line. Int Immunopharmacol 2001;1(9/10):1765–76.
[28] Kirkwood KL, Golub LM, Bradford PG. Non-antimicrobial and antimicrobial tetracyclines inhibit IL-6 expression in murine osteoblasts. Ann NY Acad Sci 1999;878:667–70.
[29] Bradford PG, Maglich JM, Kirkwood KL. IL-1 beta increases type 1 inositol trisphosphate receptor expression and IL-6 secretory capacity in osteoblastic cell cultures. Mol Cell Biol Res Commun 2000; 3(2):73–5.
[30] Kirkwood KL, Dziak R, Bradford PG. Inositol trisphosphate receptor gene expression and hormonal regulation in osteoblast-like cell lines and primary osteoblastic cell cultures. J Bone Miner Res 1996;11(12): 1889–96.
[31] Kirkwood KL, Homick K, Dragon MB, Bradford PG. Cloning and characterization of the type I inositol 1,4,5-trisphosphate receptor gene promoter. Regulation by 17beta-estradiol in osteoblasts. J Biol Chem 1997;272(36):22425–31.
[32] Asahina I, Sampath TK, Nishimura I, Hauschka PV. Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol 1993;123(4):921–33.
[33] Luben RA, Wong GL, Cohn DV. Biochemical characterization with parathormone and calcitonin of isolated bone cells: provisional identification of osteoclasts and osteoblasts. Endocrinology 1976;99(2): 526–34.
[34] Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiki S, Matsuda T, Hirano T, Kishimoto T, Suda T. IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990;145(10):3297–303.
[35] Ragheb JA, Deen M, Schwartz RH. The destabilization of IL-2 mRNA by a premature stop codon and its differential stabilization by *trans*-acting inhibitors of protein synthesis do not support a role for active translation in mRNA stability. J Immunol 1999;163(6):3321–30.
[36] Ng SB, Tan YH, Guy GR. Differential induction of the interleukin-6 gene by tumor necrosis factor and interleukin-1. J Biol Chem 1994; 269(29):19021–7.
[37] Dani C, Piechaczyk M, Audigier Y, El Sabouty S, Cathala G, Marty L, Fort P, Blanchard JM, Jeanteur P. Characterization of the transcription products of glyceraldehyde 3-phosphate-dehydrogenase gene in HeLa cells. Eur J Biochem 1984;145(2):299–304.
[38] Winzen R, Kracht M, Ritter B, Wilhelm A, Chen CY, Shyu AB, Muller M, Gaestel M, Resch K, Holtmann H. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. EMBO J 1999;18(18):4969–80.
[39] Shimada M, Andoh A, Hata K, Tasaki K, Araki Y, Fujiyama Y, Bamba T. IL-6 secretion by human pancreatic periacinar myofibroblasts in response to inflammatory mediators. J Immunol 2002;168(2):861–8.
[40] Xu K, Robida AM, Murphy TJ. Immediate-early MEK-1-dependent stabilization of rat smooth muscle cell cyclooxygenase-2 mRNA by Galpha(q)-coupled receptor signaling. J Biol Chem 2000;275(30): 23012–9.
[41] Golub LM, Lee HM, Ryan ME, Giannobile WV, Payne J, Sorsa T. Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. Adv Dent Res 1998;12(2):12–26.
[42] Pruzanski W, Stefanski E, Vadas P, McNamara TF, Ramamurthy N, Golub LM. Chemically modified non-antimicrobial tetracyclines inhibit activity of phospholipases A2. J Rheumatol 1998;25(9): 1807–12.
[43] Eklund KK, Sorsa T. Tetracycline derivative CMT-3 inhibits cytokine production, degranulation, and proliferation in cultured mouse and human mast cells. Ann NY Acad Sci 1999;878:689–91.
[44] Choi JY, Lee BH, Song KB, Park RW, Kim IS, Sohn KY, Jo JS, Ryoo HM. Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells. J Cell Biochem 1996;61(4): 609–18.
[45] Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res 1999;14(6):893–903.
[46] Miyazawa K, Mori A, Miyata H, Akahane M, Ajisawa Y, Okudaira H. Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. J Biol Chem 1998;273(38):24832–8.
[47] Stoecklin G, Stoeckle P, Lu M, Muehlemann O, Moroni C. Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. RNA 2001;7(11):1578–88.
[48] Craig RG, Yu Z, Xu L, Barr R, Ramamurthy N, Boland J, Schneir M, Golub LM. A chemically modified tetracycline inhibits streptozotocin-induced diabetic depression of skin collagen synthesis and steady-state type I procollagen mRNA. Biochim Biophys Acta 1998;1402(3): 250–60.
[49] Ramamurthy N, Bain S, Liang CT, Barnes J, Llavaneras A, Liu Y, Puerner D, Strachan MJ, Golub LM. A combination of subtherapeutic doses of chemically modified doxycycline (CMT-8) and a bisphosphonate (clodronate) inhibits bone loss in the ovariectomized rat: a dynamic histomorphometric and gene expression study. Curr Med Chem 2001;8(3):295–303.
[50] Aoyagi M, Sasaki T, Ramamurthy NS, Golub LM. Tetracycline/flurbiprofen combination therapy modulates bone remodeling in ovariectomized rats: preliminary observations. Bone 1996;19(6): 629–35.
[51] Sachs AB. Messenger RNA degradation in eukaryotes. Cell 1993;74(3):413–21.
[52] Chen CY, Xu N, Shyu AB. mRNA decay mediated by two distinct AU-rich elements from c-*fos* and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation. Mol Cell Biol 1995;15(10):5777–88.
[53] Shaw G, Kamen R. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 1986;46(5):659–67.
[54] Iwai Y, Bickel M, Pluznik DH, Cohen RB. Identification of sequences within the murine granulocyte-macrophage colony-stimulating factor mRNA 3′-untranslated region that mediate mRNA stabilization induced by mitogen treatment of EL-4 thymoma cells. J Biol Chem 1991;266(27):17959–65.
[55] Stoecklin G, Hahn S, Moroni C. Functional hierarchy of AUUUA motifs in mediating rapid interleukin-3 mRNA decay. J Biol Chem 1994;269(46):28591–7.
[56] Marshall CJ. MAP kinase kinase kinase. Curr Opin Genet Dev 1994;4(1):82–9.
[57] Blenis J. Signal transduction via the MAP kinases: proceed at your own RSK. Proc Natl Acad Sci USA 1993;90(13):5889–92.
[58] Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Biol Chem 1995;270(25):14843–6.
[59] Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995;270(13):7420–6.
[60] Lee JC, Badger AM, Griswold DE, Dunnington D, Truneh A, Votta B, White JR, Young PR, Bender PE. Bicyclic imidazoles as a novel class of cytokine biosynthesis inhibitors. Ann NY Acad Sci 1993;696:149–70.
[61] Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL. Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 2000;47(2/3):185–201.

[62] Lee JC, Kassis S, Kumar S, Badger A, Adams JL. p38 mitogen-activated protein kinase inhibitors—mechanisms and therapeutic potentials. Pharmacol Ther 1999;82(2/3):389–97.

[63] Chae HJ, Chae SW, Chin HY, Bang BG, Cho SB, Han KS, Kim SC, Tae KC, Lee KH, Kim DE, Im MK, Lee SJ, Chang JY, Lee YM, Kim HM, Kim HH, Lee ZH, Kim HR. The p38 mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumor necrosis factor in osteoblasts. Bone 2001;28(1):45–53.

[64] Miwa M, Kozawa O, Tokuda H, Uematsu T. Mitogen-activated protein (MAP) kinases are involved in interleukin-1 (IL-1)-induced IL-6 synthesis in osteoblasts: modulation not of p38 MAP kinase, but of p42/p44 MAP kinase by IL-1-activated protein kinase C. Endocrinology 1999;140(11):5120–5.

[65] Gouble A, Morello D. Synchronous and regulated expression of two AU-binding proteins, AUF1 and HuR, throughout murine development. Oncogene 2000;19(47):5377–84.

[66] Williams S, Barnes J, Wakisaka A, Ogasa H, Liang CT. Treatment of osteoporosis with MMP inhibitors. Ann NY Acad Sci 1999;878:191–200.

[67] Ramamurthy NS, Rifkin BR, Greenwald RA, Xu JW, Liu Y, Turner G, Golub LM, Vernillo AT. Inhibition of matrix metalloproteinase-mediated periodontal bone loss in rats: a comparison of 6 chemically modified tetracyclines. J Periodontol 2002;73(7):726–34.

[68] Brown DL, Desaik K, Vakili BA, Lee H-M, Golub LM. Clinical and biochemical results of the metalloproteinase inhibition with low-dose doxycycline to prevent acute coronary syndromes (MIDAS) pilot trial [abstract]. American Heart Association Scientific Sessions; 2002.