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Transcriptional Induction of Matrix Metalloproteinase-13 (Collagenase-3) by 1α,25-Dihydroxyvitamin D_3 in Mouse Osteoblastic MC3T3-E1 Cells

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ABSTRACT

The removal of unmineralized matrix from the bone surface is essential for the initiation of osteoclastic bone resorption because osteoclasts cannot attach to the unmineralized osteoid. Matrix metalloproteinases (MMPs) are known to digest bone matrix. We recently reported that among the MMPs expressed in mouse osteoblastic cells, MMP-13 (collagenase-3) was the one most predominantly up-regulated by bone resorbing factors including 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. In this study, we examined the mechanism of regulation of MMP-13 expression by 1α,25$(OH)_2D_3$ in mouse osteoblastic MC3T3-E1 cells. 1α,25$(OH)_2D_3$ increased steady-state messenger RNA (mRNA) and protein levels of MMP-13. De novo protein synthesis was essential for the induction because cycloheximide (CHX) decreased the effect of 1α,25$(OH)_2D_3$ on the MMP-13 mRNA level. 1α,25$(OH)_2D_3$ did not alter the decay of MMP-13 mRNA in transcriptionally arrested MC3T3-E1 cells; however, it increased the MMP-13 heterogeneous nuclear RNA (hnRNA) level and MMP-13 transcriptional rate. The binding activity of nuclear extracts to the AP-1 binding site, but not to the Cbfa1 binding site, in the MMP-13 promoter region was up-regulated by 1α,25$(OH)_2D_3$, suggesting the mediation of AP-1 in this transcriptional induction. To determine the contribution of MMPs to bone resorption by 1α,25$(OH)_2D_3$, the inhibitory effect of BB94, an MMP inhibitor, on resorbed pit formation by mouse crude osteoclastic cells was examined on either an uncoated or collagen-coated dentine slice. BB94 did not prevent resorbed pit formation on uncoated dentine whereas it did on collagen-coated dentine. We therefore propose that the transcriptional induction of MMP-13 in osteoblastic cells may contribute to the degradation of unmineralized matrix on the bone surface as an early step of bone resorption by 1α,25$(OH)_2D_3$. (J Bone Miner Res 2001;16:221–230)

Key words: matrix metalloproteinase, collagenase, 1α,25-dihydroxyvitamin D_3, osteoblast, bone

INTRODUCTION

1α,25-DIHYDROXYVITAMIN D_3 [1α,25$(OH)_2D_3$], the biologically active metabolite of vitamin D_3, not only plays an essential role in calcium homeostasis in bone, kidney, and intestine,[1,2] but also regulates cell growth and differentiation.[3] 1α,25$(OH)_2D_3$ is also one of the most potent bone resorbing agents[4] and is known to affect osteoclasts indirectly through osteoblastic cells in which its receptor (vitamin D receptor [VDR]) exists.[5] This action of 1α,25$(OH)_2D_3$ was reported recently to be mediated by the induction of the osteoclast differentiation factor (ODF/receptor activator of nuclear factor $\kappa\beta$ ligand [RANKL]) in osteoblastic cells.[6] However, because osteoclasts are not

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capable of attaching to the surface of unmineralized bone, digestion of unmineralized bone by proteinases is necessary for osteoclastic bone resorption to proceed.[7,8] Thus, $1\alpha,25(OH)_2D_3$ may possibly degrade matrix proteins by regulating proteinases in osteoblastic cells as one of its bone resorbing activities. In fact, previous studies indicated that $1\alpha,25(OH)_2D_3$ induced collagenase production in osteoblastic cells. [9–11]

Matrix metalloproteinases (MMPs) are a family of structurally and functionally related enzymes responsible for the proteolytic degradation of extracellular matrix components such as collagens, elastin, glycoproteins, proteoglycans, and glucosaminoglycans.[12,13] To date, at least 16 distinct members of the MMP family have been identified and classified into subgroups based on substrate specificity.[14,15] Among them, MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase), and MMP-13 (collagenase-3) are known to have collagenase activity and to be able to cleave the native helix of type I collagen, the major protein of bone matrix.[16,17] Recently, we have reported that MMP-2, -9, -11, -13, and -14 were expressed both in mouse osteoblastic MC3T3-E1 cells and in mouse primary osteoblastic cells, making MMP-13 the only collagenase expressed in mouse osteoblastic cells.[18] Furthermore, among the MMPs expressed in osteoblastic cells, MMP-13 was the one most predominantly up-regulated by systemic bone resorbing factors such as parathyroid hormone, prostaglandin E_2, and $1\alpha,25(OH)_2D_3$ and was the major MMP whose steady-state messenger RNA (mRNA) level was increased by $1\alpha,25(OH)_2D_3$. Hence, the present study was undertaken to determine the mechanism whereby $1\alpha,25(OH)_2D_3$ regulates MMP-13 expression in mouse osteoblastic MC3T3-E1 cells.

MATERIALS AND METHODS

Materials

$1\alpha,25(OH)_2D_3$, Denhardt's solution, dithiothreitol (DTT), DNAse I, and bacterial collagenase were purchased from Wako Pure Chemical (Osaka, Japan). 24*R*,25-dihydroxyvitamin D_3 [24*R*,$25(OH)_2D_3$] was synthesized at Kureha Chemical Industry Co., Ltd. (Tokyo, Japan). BB94 was the generous gift of Dr. M. Nakajima (Novaritis Pharma K. K., Hyogo, Japan). α-Modification of Eagle's minimal essential medium (α-MEM), fetal bovine serum (FBS), and Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) were purchased from Life Technologies, Inc. (Grand Island, NY, USA). Rediprime labeling kit, [α-^{32}P]deoxycytidine triphosphate (dCTP, specific activity of 3000 Ci/mmol), [α-^{32}P]uridine 5′-triphosphate (UTP, specific activity of 3000 Ci/mmol), [γ-^{32}P]deoxyadenosine 5′-triphosphate (dATP, specific activity of 3000 Ci/mmol), Sephadex G-25 column, Hybond N^+ Nylon membrane, and Hyperfilm were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). Recombinant *Taq* polymerase, T4 polynucleotide kinase, deoxynucleotide triphosphate mixture solution (dNTP, 2.5 mM), and SeaKem GTG agarose were obtained from Takara Shuzo (Shiga, Japan). Cycloheximide (CHX), 5,6-dichlorobenzimidazole riboside (DRB), NP-40, and dextran sulfate were purchased from Sigma (St. Louis, MO, USA). Polydeoxyinosine-deoxycytidilic acid [poly(dI-dC)] and Complete were purchased from Boehringer Mannheim (Mannheim, Germany)

Cell culture

The MC3T3-E1 clonal cell line was kindly provided by Dr. Kumegawa of Meikai University (Saitama, Japan).[19] MC3T3-E1 cells were plated at a density of 20,000 cells/cm^2 in 100-mm dishes and cultured in α-MEM containing 10% FBS. After 3 days of culture, cells were precultured in serum-free medium for 24 h and exposed to experimental medium.

For the study on MMP-13 stability, MC3T3-E1 cells were precultured in serum-free medium with or without $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 16 h and exposed to the experimental medium containing DRB (10^{-4} M) with or without $1\alpha,25(OH)_2D_3$ by completely changing the medium. At 0, 4, 8, 16, and 24 h after the medium change, total RNA from control or $1\alpha,25(OH)_2D_3$-treated cells was prepared as described in the following section.

Northern blot analysis

DNA probes for MMP-13, -2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated by RT-polymerase chain reaction (PCR) with the total RNA from MC3T3-E1 cells. For MMP-13 complementary DNA (cDNA), the PCR primers were 5′-CATTCAGCTATCCTGGCCACCTTC-3′ and 5′-CATCCACATGGTTGGGAAGTTCTG-3′, yielding a 1016-base pair (bp) fragment. For MMP-2 cDNA, the PCR primers were 5′-AAGGATGGACTCCTGGCACATGCCTTT-3′ and 5′-ACCTGTGGGCTTGTCACGTGGTGT-3′, yielding a 963-bp fragment. For GAPDH cDNA, the PCR primers were 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′, yielding a 983-bp fragment. Each PCR product was subcloned in a TA-cloning vector pCRII vector (Invitrogen, Carlsbad, CA, USA), as confirmed by sequencing. The cDNA fragments were radiolabeled with the Rediprime DNA labeling system and [α-^{32}P]dCTP.

Total RNA was isolated using ISOGEN following the manufacturer's instructions (Wako Pure Chemical). Samples of 20 μg of total RNA were separated by electrophoresis in 2.2 M formaldehyde–1.0% agarose gels and transferred to a Hybond N^+ nylon membrane. Hybridization was performed in 4× SSCP (0.48 M NaCl, 60 mM Na_3 citrate, 60 mM Na_2HPO_4, and 20 mM NaH_2PO_4), 10% dextran sulfate, 1× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml sheared salmon sperm DNA at 65°C overnight. The filter was washed in 1× SSC (0.15 M NaCl and 15 mM Na_3 citrate, pH 7.0)–0.1% SDS four times for 15 minutes at 65°C and then once for 15 minutes in 0.1× SSC–0.1% SDS at 65°C. Blottings were analyzed by autoradiography using Hyperfilm and the bands were quantitated using a Molecular Imager (Bio-Rad, Hercules, CA, USA). Quantitative mRNA levels of MMPs were expressed by the density of each blotting normalized to GAPDH. Each

Northern blot analysis experiment was performed using two or three independent cultures.

Western blot analysis

Culture supernatants were collected from MC3T3-E1 cells cultured for 48 h in the presence and absence of $1\alpha,25(OH)_2D_3$. The supernatants were heat-denatured in boiling water and subjected to 5–20% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins were transferred onto Immobilon P membranes (Millipore, Bedford, MA, USA). Western blot analysis for detection of MMP-13 was performed using specific anti-mouse MMP-13 primary antibody[20] and secondary anti-rabbit alkaline phosphatase–conjugated immunoglobulin G (IgG). The bound antibodies were detected with Western blotting detection reagents using as the alkaline phosphatase substrate CSPD (Clontech, Palo Alto, CA, USA). Western blot analysis was performed using two independent cultures.

Nuclear run-on assay

MC3T3-E1 cells were treated with $1\alpha,25(OH)_2D_3$ for 16 h, placed in a hypotonic lysis buffer (10 mM HEPES, pH 8.0, and 1.5 mM $MgCl_2$),l and kept on ice for 15 minutes. The cell membrane was disrupted by homogenization in a Dounce homogenizer and released nuclei were harvested by centrifugation. Nuclei were resuspended with a buffer comprised of 40% glycerol, 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, and 0.1 mM EDTA. Run-on transcription was performed by mixing 100 μl of nuclei with 100 μl of 2× reaction buffer (10 mM Tris-HCl; pH 8.0; 5 mM $MgCl_2$; 0.3 M KCl; 1 mM DTT; 0.2 mM EDTA; 4 mM ATP, GTP, and CTP; and 100 μCi [α-^{32}P]UTP; 3000 Ci/mmol) and incubated at 30°C for 30 minutes. Radiolabeled RNA from the reaction mixture was extracted with ISOGEN. Approximately 500,000 cpm was hybridized with equal amounts of heat-denatured and slot-blotted plasmids containing MMP-13 and GAPDH cDNAs. The pCRII vector served as a negative control.

RT-PCR

MMP-13 heterogenous nuclear RNA (hnRNA) was analyzed by RT-PCR using sense primer 5′-GTGTTCTG-CTGCATATACAGCCAC-3′ (hnMMP13S), which spans intron 2 of the mouse MMP-13 gene, and antisense primer 5′-CCTTCTCCACTTCAGAATGGGAC-3′ (hnMMP13A), which spans exon 3, yielding a 280-bp product. GAPDH mRNA was analyzed by RT-PCR using sense primer 5′-GTCTTCACCACCATGGAGAAG-3′ (position 341–361 of mouse GAPDH) and antisense primer 5′-CATGTAGGC-CATGAGGTCCACCAC-3′ (position 1010–1033 of mouse GAPDH). RNA samples, extracted as described for Northern analysis, were treated with DNAse I. RNA (1 μg) was copied into cDNA using the antisense primer and MMLV RT. The cDNA was amplified by PCR with 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 minute in the presence of recombinant *Taq* polymerase, 0.2 μM sense and antisense primers, and 5 μCi [α-^{32}P]dCTP. PCR products were resolved on a 5–20% polyacrylamide gel containing 8 M urea, 100 mM Tris-borate, and 1 mM EDTA and then visualized by autoradiography. The amplification protocol yielded products that were within the linear range for both the MMP-13 hnRNA and the GAPDH mRNA.

Nuclear extract preparation

Nuclear extracts were prepared from MC3T3-E1 cells treated with or without $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 2 h using a modified version of the protocol described by Dignam et al.[21] In brief, MC3T3-E1 cells (about 2×10^7 cells) were harvested in 5 ml of ice-cold phosphate-buffered saline by centrifugation at 150*g* for 5 minutes. The pellets were resuspended into 500 μl of Dignam buffer A containing 0.2% NP-40, 1 mM DTT, and 1× Complete (Boehringer Mannheim). The lysate was centrifuged at 2200*g* for 1 minute to pellet the NP-40–insoluble material, and the supernatant was removed. The pellet was resuspended in 50 μl of Dignam buffer C, and incubated on ice for 10 minutes with occasional vortexing to disrupt the nuclear membranes. Extracts were centrifuged for 1 minute at 20,000*g*, the supernatants were removed, and the pellets were discarded. The protein content of the nuclear extracts was determined using the Bio-Rad DC assay kit (Bio-Rad).

Electrophoretic mobility shift assay

Mobility shift assay was performed using a 22-oligomer ATAAGTGATGACTCACCATTGC and a 27-oligomer GATTCTGCCACAAACCACACTTAGGAA containing the putative AP-1 and Cbfa1 binding sites, respectively, in the 5′-upstream region of the mouse MMP-13 gene. Oligonucleotides were annealed, end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase, and further purified by Sephadex G-25 column chromatography. Five micrograms of nuclear extracts was incubated with binding buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM EDTA, 0.05% NP-40, 5 mM DTT, 5% glycerol, and 1 μg of poly(dI-dC) for 10 minutes. Labeled probe (40 fmol) was then added and incubated for an additional 20 minutes. The samples were subjected to electrophoresis at room temperature on 5% polyacrylamide gel in 89 mM Tris, 89 mM boric acid, and 2 mM EDTA buffer at 80 V for 1 h. In competitive experiments, competitor oligos (2 pmol) were incubated with nuclear extracts during the first 10 minutes before addition of the labeled probe. After electrophoresis the gel was dried and exposed for autoradiography.

Resorbed pit formation assay

Mouse osteoblastic cells and bone marrow cells were cocultured as described previously.[22] Briefly, primary osteoblastic cells were prepared from calvariae of neonatal ddY mice (Shizuoka Laboratories Animal Center, Shizuoka, Japan). Osteoblastic cells (1×10^6 cells/well) and bone marrow cells (2×10^7 cells/well) prepared from 8-week-old ddY mice were cocultured on a 0.24% collagen gel (Nitta Gelatin, Tokyo, Japan) coated on 100-mm tissue culture dishes with α-MEM containing 10% FBS and 10^{-8} M

$1\alpha,25(OH)_2D_3$. The medium was changed every 2 days. After culture for 6 days, each dish was treated with 4 ml of 0.2% collagenase solution for 20 minutes at 37°C. The cells released from the dish were collected by centrifugation twice at 1000 rpm for 5 minutes and suspended in 5 ml of α-MEM containing 10% FBS. Dentine slices (diameter, 6 mm; thickness, 0.15 mm) were distributed in a 96-well plate. A collagen solution prepared as described previously was diluted to 1.5 mg/ml with α-MEM and used to coat dentine slices (10 μl/slice). The collagen was allowed to polymerize by incubating it for 30 minutes at 37°C.[23] An aliquot of the crude osteoclast preparation (0.1 ml) was then seeded onto either uncoated or collagen-coated dentine slices and cultured with α-MEM containing 10% FBS in the presence and absence of 10^{-8} M $1\alpha,25(OH)_2D_3$, 10^{-5} M BB94, or 10^{-8} M salmon calcitonin (CT; Peninsula Laboratories, Inc., Belmont, CA, USA). After 48 h of culture, cells on dentine slices were removed in 1N NH_4OH solution and stained with 0.5% toluidine blue for 1 minute. The total area of pits on the dentine slice was estimated under a light microscope with a micrometer using an image analyzer (System Supply Co., Nagano, Japan) and expressed as a percentage of the whole area of dentine.

Statistical analyses

Means of groups were compared by analysis of variance (ANOVA) and significance of differences was determined by post hoc testing using Bonferroni's method.

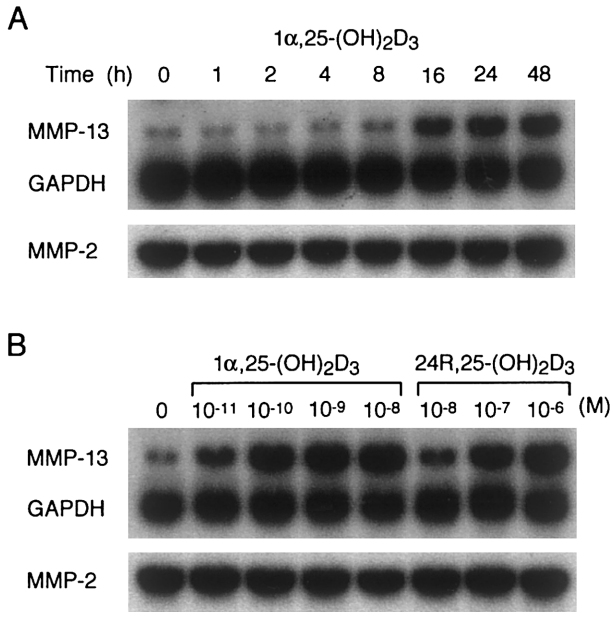

FIG. 1. (A) Time course and (B) dose response of steady-state mRNA levels of MMP-13 and MMP-2 stimulated by $1\alpha,25(OH)_2D_3$ in MC3T3-E1 cells. (A) MC3T3-E1 cells were treated with 10^{-8} M $1\alpha,25(OH)_2D_3$ for the indicated periods. (B) MC3T3-E1 cells were treated with $1\alpha,25(OH)_2D_3$ or $24R,25(OH)_2D_3$ at the indicated concentrations for 16 h. Northern blot analysis was performed using 20 μg of total RNA to determine MMP-13 and MMP-2 mRNA levels with GAPDH as a control. The size of the hybridizing signals was 2.9 kb and 3.1 kb with MMP-13 and MMP-2 probe, respectively. Representative autoradiograms from duplicate experiments that yielded similar results are shown.

RESULTS

Effect of $1\alpha,25(OH)_2D_3$ on steady-state mRNA and protein levels of MMP-13

Figures 1A and 1B show the time course and dose response of the effects of $1\alpha,25(OH)_2D_3$, respectively, on the steady-state MMP-13 mRNA level in cultured MC3T3-E1 cells as determined by Northern blot analysis. $1\alpha,25(OH)_2D_3$ caused a time-dependent increase in this level: 6.0-fold at 16 h and approximately 10-fold at 24 and 48 h (Fig. 1A). At 16 h of culture, $1\alpha,25(OH)_2D_3$ (10^{-11}–10^{-8} M) and $24R,25(OH)_2D_3$ (10^{-8}–10^{-6} M), a less active vitamin D_3 metabolite, stimulated the MMP-13 mRNA level dose dependently with maximal effects of 16-fold at 10^{-8} M and 15-fold at 10^{-6} M, respectively (Fig. 1B). Neither $1\alpha,25(OH)_2D_3$ nor $24R,25(OH)_2D_3$ regulated the MMP-2 mRNA level.

To determine whether the MMP-13 mRNA stimulated by $1\alpha,25(OH)_2D_3$ was translated into protein, Western blot analysis was conducted at 48 h of culture (Fig. 2). Immunoreactive MMP-13 protein could not be detected in the control culture medium; however, it was induced by $1\alpha,25(OH)_2D_3$ (10^{-8} M).

Involvement of de novo protein synthesis in MMP-13 induction by $1\alpha,25(OH)_2D_3$

The involvement of de novo protein synthesis in $1\alpha,25(OH)_2D_3$ stimulation of the MMP-13 mRNA level was investigated by Northern blot analysis in the presence and absence of CHX (10 μM; Fig. 3). CHX inhibited not only basal MMP-13 mRNA expression, but also $1\alpha,25(OH)_2D_3$-induced MMP-13 mRNA expression to a level similar to that of the control. These inhibitions by CHX were greater than those seen for MMP-2 mRNA. Thus, de novo protein synthesis was assumed to be requisite for the induction of MMP-13 by $1\alpha,25(OH)_2D_3$.

Effect of $1\alpha,25(OH)_2D_3$ on MMP-13 mRNA stability

The effect of $1\alpha,25(OH)_2D_3$ on the stability of MMP-13 mRNA in MC3T3-E1 cells was determined using DRB (10^{-4} M), an RNA polymerase II inhibitor, to arrest transcription, and the decay of MMP-13 mRNA was monitored by Northern blot analysis. In the presence and absence of $1\alpha,25(OH)_2D_3$, MC3T3-E1 cells were cultured with DRB for up to 24 h after 16 h of preculture without DRB (Fig. 4). No difference of MMP-13 mRNA degradation was observed between control and $1\alpha,25(OH)_2D_3$-treated cultures, indicating that $1\alpha,25(OH)_2D_3$ does not affect MMP-13 mRNA stability.

Effect of $1\alpha,25(OH)_2D_3$ on transcriptional activation of MMP-13

We further investigated the transcriptional activation of MMP-13 by $1\alpha,25(OH)_2D_3$ using RT-PCR for hnRNA (Fig.

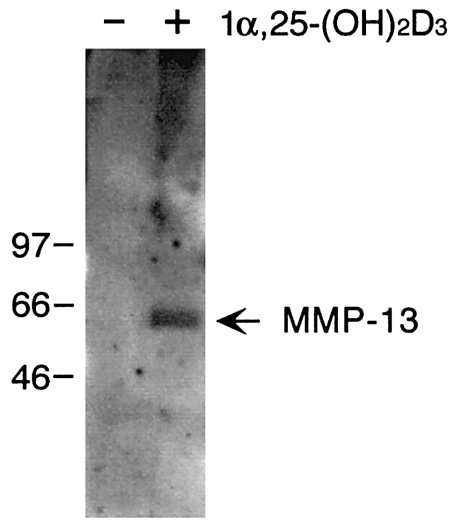

FIG. 2. Immunoreactive MMP-13 protein level in the presence and absence of 1α,25(OH)$_2$D$_3$ in the culture media of MC3T3-E1 cells. The media in which MC3T3-E1 cells were cultured with or without 1α,25(OH)$_2$D$_3$ (10^{-8} M) for 48 h were collected. Western blot analysis was performed using a polyclonal antibody originally raised against mouse MMP-13. A representative picture from duplicate experiments that yielded similar results is shown.

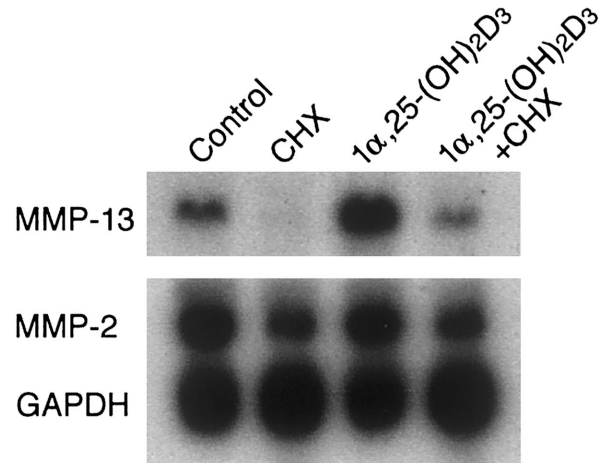

FIG. 3. Effect of CHX on mRNA levels of MMP-13 and MMP-2 in the presence and absence of 1α,25(OH)$_2$D$_3$ in MC3T3-E1 cell culture. MC3T3-E1 cells were cultured with or without 1α,25(OH)$_2$D$_3$ (10^{-8} M) for 16 h in the presence or absence of CHX (10^{-4} M). CHX was added 1 h before and during the treatment with 1α,25(OH)$_2$D$_3$. Total RNA (20 μg/lane) was analyzed by Northern blotting. A representative picture from three independent experiments that yielded similar results is shown.

5A). 1α,25(OH)$_2$D$_3$ induced MMP-13 hnRNA expression after 8 h of culture. This time course of the effect of 1α,25(OH)$_2$D$_3$ on the hnRNA level corresponded well with that on mRNA levels shown in Fig. 1A. To confirm the transcriptional induction of MMP-13 by 1α,25(OH)$_2$D$_3$, a nuclear run-on assay was performed at 16 h of culture (Fig. 5B). 1α,25(OH)$_2$D$_3$ potently stimulated the transcription rate for MMP-13, and the effect was much stronger than that

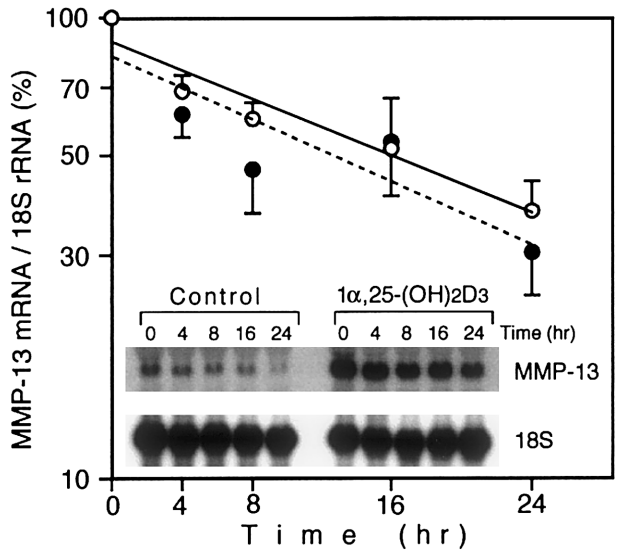

FIG. 4. Effect of 1α,25(OH)$_2$D$_3$ on MMP-13 mRNA stability in MC3T3-E1 cell culture. After the preculture in serum-free medium with or without 1α,25(OH)$_2$D$_3$ (10^{-8} M) for 16 h, cells were treated with DRB (10^{-4} M, open circles for each time point and the straight line for regression) or DRB + 1α,25(OH)$_2$D$_3$ (closed circles for each time point and the dotted line for regression) by completely changing the medium. Cells were harvested at 0, 4, 8, 16, and 24 h after treatment, and MMP-13 mRNA was analyzed by Northern blotting. Data are expressed as means (symbols) ± SEMs (error bars) of three independent experiments. The values in each experiment were calculated as the percentage of the intensity of each band normalized to that of GAPDH measured by densitometry as compared with that of time 0 (100%). The autoradiogram in this figure is representative of three experiments that yielded similar results.

for MMP-2. Thus, it was concluded that 1α,25(OH)$_2$D$_3$ up-regulated MMP-13 expression at the transcriptional level, but not the posttranscriptional level.

Effect of 1α,25(OH)$_2$D$_3$ on the binding activity of nuclear extracts to AP-1 and Cbfa1 binding sites of the MMP-13 promoter region

After 1α,25(OH)$_2$D$_3$ binds to the VDR, the ligand-receptor complex is known to form a heterodimer with a retinoid X receptor and to interact directly with a specific DNA element, the vitamin D responsive element (VDRE).[24] However, the VDRE-like sequence has not been identified in the mouse MMP-13 promoter region. Instead, binding domains of several transcription factors including AP-1 and Cbfa1 have been detected in this region.[25–27] We therefore examined the binding activity of nuclear extracts from MC3T3-E1 cells treated with or without 1α,25(OH)$_2$D$_3$ to the AP-1 and Cbfa1 binding sites in the mouse MMP-13 promoter region (Fig. 6). Binding to the AP-1 site was already detectable in nuclear extracts from untreated cells and was enhanced on stimulation by 1α,25(OH)$_2$D$_3$. The specificity of proteins binding to the AP-1 site was confirmed by the competition with unlabeled homologous AP-1 site probes. Although specific binding to

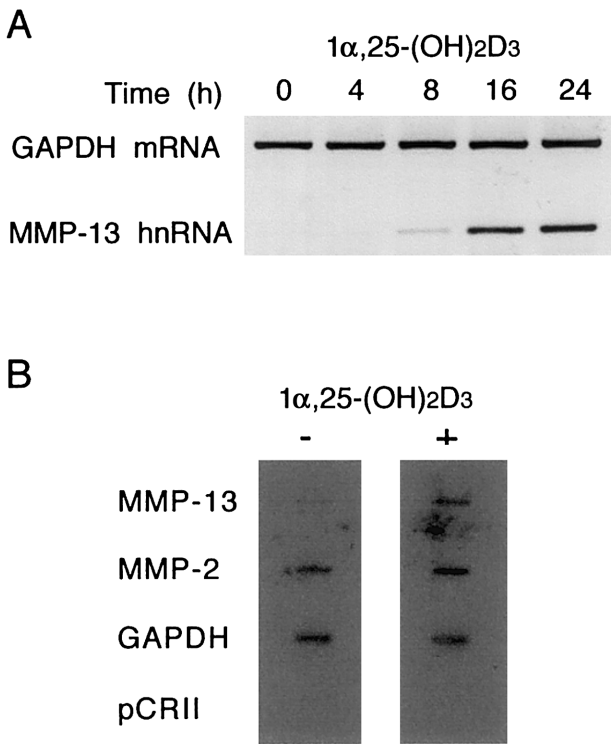

FIG. 5. Transcriptional activation of MMP-13 by $1\alpha,25(OH)_2D_3$ in MC3T3-E1 cells. (A) RT-PCR for hnRNA. Total RNA extracted from MC3T3-E1 cells treated with $1\alpha,25(OH)_2D_3$ (10^{-8} M) for the indicated periods was reverse-transcribed, and signals specific for GAPDH mRNA and MMP-13 hnRNA were amplified by PCR in the presence of 5 μCi of [α-^{32}P]dCTP. PCR products were visualized by autoradiography. (B) Transcriptional rate of MMP-13. Nuclei isolated from subconfluent MC3T3-E1 cultures treated with or without $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 16 h were analyzed by nuclear run-on assay for MMP-13, MMP-2, and GAPDH gene transcription. Transcripts were labeled by an in vitro elongation reaction in the presence of [α-^{32}P]UTP. Labeled RNAs (5×10^5 cpm) were hybridized to cDNAs immobilized on nylon filters. These cDNAs were those for MMP-13, MMP-2, and GAPDH cloned into the pCRII vector and the pCRII vector plasmid itself.

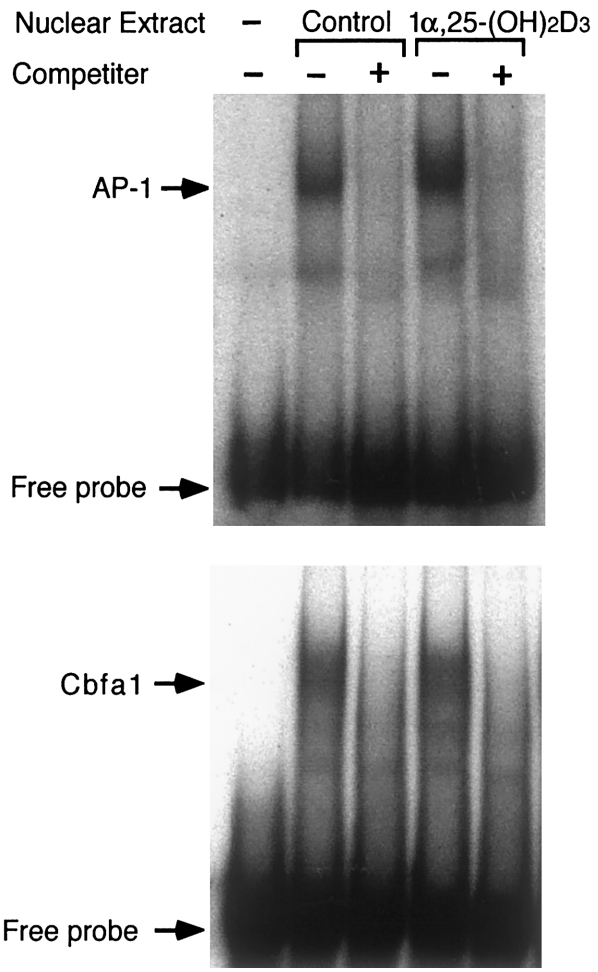

FIG. 6. Effect of $1\alpha,25(OH)_2D_3$ on the binding activity of nuclear extracts from cultured MC3T3-E1 cells to AP-1 and Cbfa1 binding sites in MMP-13 promoter region. Electrophoretic mobility shift assay was performed using a 22-oligomer ATAAGTGATGACTCACCATTGC and a 27-oligomer GATTCTGCCACAAACCACACTTAGGAA containing the putative AP-1 and Cbfa1 binding sites in the 5′-upstream region of the mouse MMP-13 gene. Nuclear extracts from MC3T3-E1 cells treated with or without $1\alpha,25(OH)_2D_3$ (10^{-8} M, 2 h) were incubated with these labeled probes. To show the specificity of these bindings, 50-fold molar excesses of unlabeled oligonucleotide probes were used as competitors.

the Cbfa1 binding element also was detectable in untreated cells, it was not altered by $1\alpha,25(OH)_2D_3$. These results imply that $1\alpha,25(OH)_2D_3$ might possibly regulate the transcriptional activation of MMP-13 through enhancement of DNA binding activity of AP-1 subunits.

Functional relevance of MMPs to bone resorptive effect of $1\alpha,25(OH)_2D_3$

The contribution of MMPs to bone resorption by $1\alpha,25(OH)_2D_3$ was determined by examining the inhibitory effects of BB94, an MMP inhibitor, using cultures of crude osteoclastic cells formed by the coculture of osteoblastic and bone marrow cells (Fig. 7). Resorbed pit formation by crude osteoclastic cells was compared between cultures on uncoated and collagen-coated dentine slices. When cultured on uncoated slices, BB94 did not decrease resorbed pit formation either in control or in $1\alpha,25(OH)_2D_3$-treated cultures whereas CT inhibited it in both cultures. This result indicates that the stimulation of osteoclast bone resorptive function on mineralized matrix by $1\alpha,25(OH)_2D_3$ is not mediated by MMPs. However, when cultured on collagen-coated dentine, BB94, as well as CT, inhibited resorbed pit formation induced by $1\alpha,25(OH)_2D_3$. Hence, $1\alpha,25(OH)_2D_3$ appears to stimulate bone resorption at least in part through the degradation of unmineralized matrix on bone surface by MMPs and thus initiate osteoclastic bone resorption.

DISCUSSION

This study showed that $1\alpha,25(OH)_2D_3$ time and dose dependently stimulated MMP-13 production by increasing

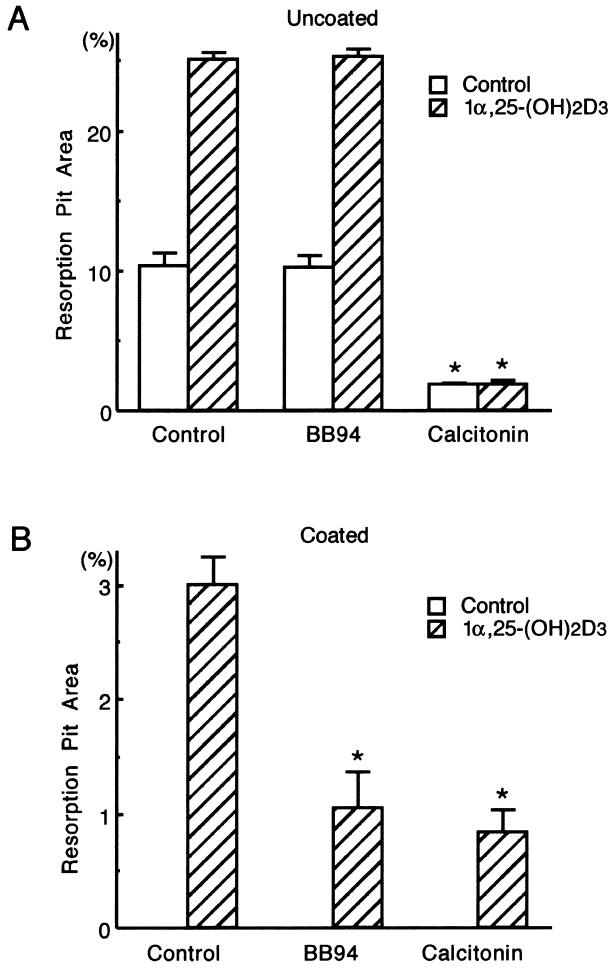

FIG. 7. Effect of BB94, an MMP inhibitor, on resorbed pit formation by crude osteoclastic cells on uncoated and collagen-coated dentine slices. Osteoblastic cells from neonatal mouse calvariae and bone marrow cells from 8-week-old mice were cocultured on a collagen gel for 6 days to form osteoclastic cells. Crude osteoclastic cells were then released from the dish and further cultured either on (A) uncoated or (B) collagen-coated dentine slices for 48 h with or without $1\alpha,25(OH)_2D_3$ (10^{-8} M), BB94 (10^{-5} M), and CT (10^{-8} M). The total area of pits on the dentine slice was estimated under a light microscope and expressed as a percentage of the whole area of dentine. No resorbed pit was seen without $1\alpha,25(OH)_2D_3$ on collagen-coated dentine (B). Data are expressed as means (bars) ± SEMs (error bars) for 8 cultures/group. *$p < 0.01$, significant inhibition versus cultures without inhibitors.

the hnRNA level and transcriptional rate without changing mRNA stability in MC3T3-E1 cells. Resorbed pit formation assay disclosed an essential role of MMPs in the bone resorptive effect of $1\alpha,25(OH)_2D_3$ to degrade unmineralized matrix on bone surface for the initiation of osteoclastic bone resorption. Because our previous study revealed that MMP-13 is the MMP whose expression was most predominantly up-regulated by $1\alpha,25(OH)_2D_3$ in osteoblastic cells,[18] it is proposed that this transcriptional induction of MMP-13 in osteoblastic cells may play an important role in the initial step of bone resorption by $1\alpha,25(OH)_2D_3$.

Three MMPs, MMP-1, MMP-8, and MMP-13, currently are known to be able to digest type I collagen.[16,17] The expression of MMP-8, which has been cloned recently, could not be detected by RT-PCR in MC3T3-E1 cells or mouse primary osteoblastic cells (M. Uchida, unpublished observation, 1998). Because the mouse MMP-1 gene has not been identified yet, we examined human MMP-1 and -13 expressions in cultured human primary osteoblasts by RT-PCR and found that only MMP-13 mRNA could be detected (M. Uchida, unpublished observation, 1998). Johansson et al. also reported that MMP-13, but not MMP-1, was expressed in periosteal cells and osteoblasts as well as hypertrophic chondrocytes during human fetal bone development.[28] Furthermore, in mice, MMP-13 has been shown to be expressed in osteoblasts localized along the newly formed bone trabeculae[29] and adjacent to osteoclasts.[30] These results suggest that among these three collagenases MMP-13 is the main MMP expressed in osteoblasts.

$1\alpha,25(OH)_2D_3$ is known to exert its pleiotropic effects by binding to its specific receptor (VDR), a member of the steroid hormone receptor superfamily.[31] To confirm the involvement of VDR in the stimulation of MMP-13 expression, we examined the effect of $24R,25(OH)_2D_3$ on the steady-state mRNA level of MMP-13 because we previously reported that this vitamin D analogue also bound to VDR (Fig. 1B).[32,33] $24R,25(OH)_2D_3$ is known to exhibit an approximately 1000-fold lower affinity for VDR and a 100-fold higher affinity for vitamin D binding protein in serum as compared with $1\alpha,25(OH)_2D_3$.[34] We also reported that $24R,25(OH)_2D_3$ induced an activation of human osteocalcin promoter through a VDR-VDRE–dependent mechanism in medium containing 0.1% FBS, but did not induce it in medium containing 5% FBS.[32] In the present study using serum-free medium, $24R,25(OH)_2D_3$ stimulated the steady-state mRNA expression of MMP-13 at 1000-fold higher concentrations of $1\alpha,25(OH)_2D_3$. Thus, the effects of both vitamin D_3 metabolites on the MMP-13 expression in osteoblastic cells may be mediated by a VDR-dependent mechanism.

A number of VDREs have been identified in the promoter region of several genes: osteocalcin, osteopontin, 25-hydroxyvitamin D_3 24-hydroxylase, and β3-integrin.[35] These VDREs consist of a direct repeat structure of two hexanucleotides with a spacer of three nucleotides: AGGTCA NNN AGGTCA. Grumbles et al. reported that $1\alpha,25(OH)_2D_3$ increased 2.1-kilobase (kb) rat MMP-13 promoter activity in rat chondrocytes; however, they did not identify the VDRE-like sequence in this promoter region.[36] We also could not find a VDRE-like sequence in mouse MMP-13 promoter region approximately 2.2 kb upstream from the transcriptional starting site. Our functional study using this 2.2-kb mouse promoter construct ligated to the luciferase reporter gene failed to show the stimulation of the reporter activity of transfected MC3T3-E1 cells by $1\alpha,25(OH)_2D_3$ (data not shown). Because the increase in the MMP-13 mRNA level induced by $1\alpha,25(OH)_2D_3$ was shown to be dependent on de novo protein synthesis, $1\alpha,25(OH)_2D_3$ may act indirectly on mouse MMP-13 gene transcription by inducing the synthesis of one or more transcription factors. This might explain why the increase in

the hnRNA as well as steady-state mRNA level were seen at later time points (after 8 h of culture). It is speculated that the expression of the transcription factor(s) was sufficient for the activation of the endogenous MMP-13 promoter but might be insufficient for that of the exogenous promoter-reporter construct in MC3T3-E1 cells.

$1\alpha,25(OH)_2D_3$ increased the binding activity of nuclear extract to AP-1 binding site but not to Cbfa1 binding site in MC3T3-E1 cells. In fact, $1\alpha,25(OH)_2D_3$ has been reported to increase mRNA levels of the AP-1 family: c-*fos*, c-*jun*, and jun-B in MC3T3-E1 cells.[37] Among them, *c-fos* has been suggested to play an important role in cell growth, differentiation, transformation, and regulation of specific gene expression in osteoblastic cells.[38–40] The c-*fos* over-expressing transgenic mice that exhibit impaired development of long bones and developed osteosarcoma[40–42] have been shown to be associated with the increase in MMP-13 expression in bone.[43] Cbfa1 is another transcription factor that up-regulates the promoter activity of MMP-13.[26] However, it is reported that $1\alpha,25(OH)_2D_3$ down-regulates cbfa1 mRNA level in mouse primary osteoblasts.[44] This result and our finding in this study indicate that it is unlikely that $1\alpha,25(OH)_2D_3$ stimulates MMP-13 expression through Cbfa1.

Grumbles et al. reported that $1\alpha,25(OH)_2D_3$ up-regulated the MMP-13 mRNA level in primary chondrocytes derived from rachitic rats.[36] In their study $1\alpha,25(OH)_2D_3$ increased the transcriptional rate of MMP-13, and this stimulation was mediated by de novo protein synthesis, as we observed in this study. However, the concentration of $1\alpha,25(OH)_2D_3$ affecting MMP-13 was much higher in rat chondrocytes as compared with MC3T3-E1 cells in our study (10^{-8} M vs. 10^{-11} M). Because vitamin D_3 supplementation is known to restore cartilage growth function in rachitis,[45] the pharmacologic action of $1\alpha,25(OH)_2D_3$ at high concentrations may stimulate the resorption of the growth plate cartilage by inducing MMP-13. Although the physiological and pathological roles of $1\alpha,25(OH)_2D_3$ on bone metabolism have not been elucidated yet, this study infers a possible role for endogenous $1\alpha,25(OH)_2D_3$ on matrix degradation during bone remodeling. In addition, Grumbles et al. also indicated the involvement of a protein kinase C pathway in $1\alpha,25(OH)_2D_3$ action on MMP-13.[36] Because the mediation of this pathway also has been shown in $1\alpha,25(OH)_2D_3$ stimulation on 25-hydroxyvitamin D_3 24-hydroxylase,[46] such a nongenomic pathway might possibly contribute to MMP-13 induction in osteoblastic cells, although this study did not investigate that aspect.

Although BB94, a nonspecific MMP inhibitor, negatively affected the bone resorptive activity of $1\alpha,25(OH)_2D_3$ on collagen-coated dentine, the origin of MMPs mediating this activity cannot be ascribed only to osteoblastic cells in this study. It is believed that osteoblastic cells are the main source for MMPs in bone; however, MMPs such as MMP-9, -12, and -14 have been reported to be expressed in osteoclastic cells as well.[47–52] MMP-9, in fact, is known to be produced by osteoclastic cells in higher amounts than by osteoblastic cells.[47,48] Although our previous report showed that MMP-13 is the major MMP that is up-regulated by $1\alpha,25(OH)_2D_3$ in osteoblastic cells,[18] the possibility that $1\alpha,25(OH)_2D_3$ degrades unmineralized matrix through the induction of MMPs in cells of osteoclastic lineage cannot be denied. The establishment of specific inhibitors may further clarify the contribution of each MMP to bone resorption.

$1\alpha,25(OH)_2D_3$ is known to exhibit bone resorptive activity indirectly through the induction of ODF/RANKL in stromal/osteoblastic cells.[6] The stimulation of resorbed pit formation on uncoated dentine by $1\alpha,25(OH)_2D_3$ shown in Fig. 7A is assumed to be mediated by this action. In addition to this well-known function, we hereby propose that the transcriptional induction of MMP-13 by $1\alpha,25(OH)_2D_3$ in osteoblastic cells may contribute to the degradation of unmineralized matrix on bone surface as a step before osteoclastic bone resorption.

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