

Original

Differential Gene Expression of Matrix Metalloproteinase-3 and -13 during Mineralization of MC3T3-E1 Cells Cultured on Titanium Implant Material

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Abstract: Successful dental implant requires optimal tissue repair after implant insertion, which involves formation of new bone directly bonded to the device. The objective of this study was to use real-time PCR to investigate the temporal mRNA expression patterns of matrix metalloproteinases (MMP-2, -3, -9, -13, and -14) and tissue inhibitors of metalloproteinases (TIMP-1, -2, and -3) that contribute to tissue repair, during mineralization of MC3T3-E1 cells cultured on titanium (Ti). Lysates of the cells cultured on Ti and plastic wells (PI) for 10 to 50 days were used for calcium and mRNA quantification. Although the onset of calcium accumulation in the cultures on Ti (30-40 days) was slower than cultures on PI (20-30 days), the gene expression patterns during mineralization were similar in both cultures. The mRNA expression patterns of MMP-3 and -13 were distinctly different during the period from just before mineralization onset to mineralization development. The level of MMP-13 was substantial until just before the onset of mineralization but declined as mineralization developed, while MMP-3 level increased once mineralization proceeded. MMP-2, -9, and -14 and TIMP-1, -2, and -3 showed constant levels throughout. This report presents the gene expression of MMP-2, -3, -9, -13, and -14 and TIMP-1, -2, and -3 during mineralization by MC3T3-E1 cells cultured on titanium implant material, and suggest temporally distinct roles of MMP-3 and -13 in new bone formation around the implant.

Keywords: implant; matrix metalloproteinases, mineralization, osteoblasts, tissue inhibitors of metalloproteinases

Introduction

The final goal of endosseous dental implant treatment is to obtain a direct bone-to-implant structure defined as "osseointegration" with functional ankylosis provided by the device in the newly formed bone. The process to obtain osseointegration can be regarded as analogous to the bone healing process after injury¹⁻³. The bone-implant interface is rapidly filled by a blood clot containing blood cells, inflammatory cells and degenerating cellular elements immediately after insertion of implants⁴⁻⁵; and is filled with fibrin in 3-5 days. Cuboidal cells cover the implant surface in 5-7 days, and new bone matrix formation develops with mineral deposition or mineralization progressing around the implant up to 28 days after surgery⁶. The bone matrix is synthesized by only one cell type, the osteoblast. Mature osteoblasts produce collagen, mainly type I collagen, and non-collagenous proteins including proteoglycans. The collagen networks produced by osteoblasts become a template for hydroxyapatite deposition and mineralization⁷. In mineralized tissues such as bone and dentin, structural organization (intermolecular cross-linkage) of collagen fibrils and the expression and distribution of proteoglycans change dramatically

from the matrix formation stage to the mineralization stage^{7,8}, implying that mineralization requires, at least, a minor rather than a major rearrangement of matrix organization. The molecular mechanisms of mineralization, however, remain to be elucidated.

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are capable of degrading all extracellular matrix proteins and are essential for normal and pathological remodeling processes such as embryonic development, tissue repair, inflammation, tumor invasion, and metastasis^{9,10}. The MMPs can be classified into collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25), minimal-domain MMPs (MMP-7 and -26) and others (MMP-12, -19, -20, -21, -22, -23, -27, and -28). In mouse calvarial cultures and osteoblastic cells, at least MMP-2, -3, -9 -13, and -14 have been shown to be important for both bone resorption and bone formation¹¹⁻¹³. The endopeptidase activities of the MMPs are specifically controlled by a combination of zymogen activation and inhibition by endogenous inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs)¹⁴. Like MMPs, the expression of TIMPs in the tissue is also controlled during tissue remodeling and physiological conditions to maintain a balance in the metabolism of the extracellular matrix^{15,16}. Four TIMPs (TIMP-1, -2, -3, and -4) have been identified so far. Among the

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TIMPs, TIMP-1, -2, and -3 are expressed by osteoblasts^{12,17-19}. Thus, MMP-2, -3, -9 -13 and -14 and TIMP-1, -2 and -3 secreted by osteoblasts may be involved in the rearrangement of bone matrix organization before and after the onset of mineralization around the implant.

We have shown previously that collagen-binding small leucine-rich proteoglycans (decorin, biglycan, fibromodulin, and lumican); potential regulators of collagen fibril formation and mineralization, and lysyl hydroxylase (LH) isoforms (LH-1, -2, and -3); enzymes crucial for determining collagen intermolecular cross-linking pathway/patterns, are differentially expressed during mineralization of mouse calvarial osteoblastic cells (MC3T3-E1) cultured on titanium (Ti) material⁸. Using this culture system, although the timing of the cell differentiation, onset of mineralization, and mineralization development are different in cells cultured on Ti and plastic tissue culture plate, the mRNA levels and patterns of the proteoglycans and LHs during mineralization are identical in cultures on the two materials, indicating the usefulness of this culture system for investigating the *in vitro* expression patterns of target molecules during mineralization on implant materials. We used this *in vitro* culture system to define the temporal gene expression patterns of MMP-2, -3, -9 -13, and -14 and TIMP-1, -2, and -3 during the mineralization process (at cell differentiation, just before onset of mineralization, and at mineralization development) of osteoblasts cultured on a Ti implant material.

Materials and Methods

Materials

The MC3T3-E1 cell line was purchased from Riken Cell Bank (Osaka, Japan). Titanium (purity >99.6%, JIS type I) disks (Ti, 10 x 10 x 2 mm³) obtained from Shinei Kiko Co. (Osaka, Japan) was used as an implant material, and 12-well tissue culture plates (PI; Becton Dickinson, Franklin Lakes, NJ, USA) were used as control.

Cell culture

Cells were cultured (5% CO₂ at 37°C) on 10-cm petri dishes (Iwaki, Chiba, Japan) in alpha modified minimal essential medium containing 10% heat-inactivated fetal bovine serum and supplemented with 50 U/ml of penicillin and 50 µg/ml of streptomycin (ICN Biomedicals, Aurora, OH, USA). Cells were detached from culture dishes by treatment with trypsin-EDTA (Dainippon Pharmaceutical, Osaka, Japan), centrifuged at 500g, and suspended in the same medium supplemented with 50 µg/ml of ascorbic acid (Gibco BRL Life Technologies, Grand Island, NY, USA) and 10 mM beta-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA). The cells were then plated onto PI and Ti at a density of 800,000 cells/cm² to reach confluence the next day. This procedure minimizes the difference in the rate of cell proliferation between cultures on PI and Ti. Culture was continued

for 10, 20, 30, 40, and 50 days under these conditions. The cells and the matrices cultured for indicated time intervals were used for mineralization analysis and mRNA quantification by real-time polymerase chain reaction (PCR) amplification.

Mineralization analysis

To visualize the mineral deposition of cells cultured on PI, cells were fixed in 4% paraformaldehyde, washed, and stained with Alizarin Red solution. Calcium content was determined by a commercial kit (kit #437-58201, Wako, Osaka, Japan). Briefly, the cultures on PI and Ti were washed with phosphate-buffered saline (PBS) and dissolved in 0.5% Igepal CA-630 (Sigma-Aldrich). Two milliliters of the buffer for color development were added to 50 µl of the culture suspension, followed by 1 ml of the color development reagent. The concentration of calcium was determined by measuring the absorbance at 650 nm using a spectrophotometer (GeneQuant Pro; Amersham Pharmacia Biotech, Cambridge, UK). Parallel to the assay, the amount of DNA obtained from matching samples was measured using TRIzol reagent (Gibco BRL Life Technologies, Grand Island, NY, USA). The calcium content normalized to the amount of DNA in each sample was presented as the mean of 4 independent measurements.

Quantification of mRNA by real-time PCR amplification

Total cellular RNA was isolated from the cultures for each culture period using TRIzol reagent. The RNA concentration was determined spectrophotometrically. To create first-strand cDNAs, 1 µg of the total RNA extract was used for reverse transcription. Reactions were performed using Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany) according to the manufacturer's protocol, in a final volume of 20 µl. After incubation for 1 h at 37°C, the cDNAs were heated to 93°C for 5 min to denature the RNA-cDNA duplex and to inactivate the reverse transcriptase. Amplification reactions were conducted for the following cDNAs: alkaline phosphatase (ALP)²⁰ MMP-2, -3, -9, -13, and -14, TIMP-1, -2 and -3, and the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH)²⁰. The primer sequences are as shown in Table 1. Before performing quantitative real-time PCR, the PCR products generated by the same PCR conditions as shown below were sequenced to confirm their identities. Real-time PCR was performed using a Smart Cycler system (Cepheid, Sunnyvale, CA, USA). The reaction volume was 25 µl, containing 1.25 units of TaKaRa Ex Taq R-PCR (Takara Biomedicals, Tokyo, Japan), R-PCR buffer (Takara), 3 mM MgCl₂, 0.3 mM dNTP mixture (Takara), diluted SYBR Green I (1:30,000; Takara), 0.3 µM of each primer, and 5 µl of cDNA sample. Initial denaturation was carried out at 95°C for 10 s, with denaturation at 94°C for 10 s, annealing at 50-55°C for 10 s, followed by 35 cycles of elongation at 72°C for 10 s. To verify the amplification specificity, we performed melting curve analyses by heating the samples from 60°C to 95°C at a slow rate of 0.2°C/s with continuous fluorescence

Table 1. Polymerase chain reaction primer sequences

Target	Primers equences (nucleotide coordinates)	Product size(bp)	GenBank accession number
GAPDH	F CACCATGGAGAAGGCCGGGG (349-368)	418	M32599
	R GACGGACACATTGGGGGTAG (766-747)		
ALP	F GCCCTCTCCAAGACATATA (468-486)	373	J02980
	R CCATGATCACGTCGATATCC (840-821)		
MMP-2	F AAGGACCGGTTTATTTGGCGG (1514-1534)	207	NM_008610
	R GGTCAGTGGCTTGGGGTATC (1720-1701)		
MMP-3	F TTGGCCATCTCTTCCATCCAACATGG (1078-1103)	229	NM_010809
	R GTATTTGTCCTCTACAAAGAAGTAGG (1306-1281)		
MMP-9	F ACTACTCTGAAGACTTGCCG (386-405)	419	NM_013599
	R GCCGTCCTTATCGTAGTCAG (804-785)		
MMP-13	F CATGGTGACTTCTACCCATTTG (527-548)	366	NM_008607
	R AATGGCATCAAGGGATAGGG (892-873)		
MMP-14	F TGTTCCTGATGACGATCGC (1055-1074)	456	NM_008608
	R TCCAGAAGAGAGCTGCATCG (1510-1490)		
TIMP-1	F ACAAGTCCCAGAACCGCAGTGA (296-317)	228	NM_011593
	R CCGTCCACAAACAGTGAGTGTCA (523-501)		
TIMP-2	F CCGGGAATGACATCTATGGC (388-407)	311	NM_011594
	R GGGACAGCGAGTGATCTTGC (698-679)		
TIMP-3	F CTCGGACTGTAGCATCAGCG (236-255)	525	NM_011595
	R CTTGATCTTGCAATTGCAAC (760-741)		

GAPDH:glyceraldehyde-3-phosphate dehydrogenase ALP:phosphatase.
MMP:matrix metalloprotenase. TIMP:tissue inhibitor of metalloprotenase

detection. Reaction products were quantified using an amplified series of dilutions of each sequence of known concentration to generate a standard curve. The mRNA content of each molecule was normalized to the amount of GAPDH mRNA in each sample.

Determination of cell stages

The cell stages during culture were determined by the pattern of ALP mRNA expression and calcium accumulation (Fig. 1). In cells cultured on PI, no calcium was detected at 10 or 20 days while a small amount of calcium was detected at 30 days, and the level of calcium detected increased in a time-dependent manner (Fig 1C). Similar observations were obtained by alzarin red staining (data not shown). ALP mRNA was expressed substantially at 10 days and peaked at 20 days, followed by a decline at 30 days and thereafter, corresponding to onset and development of mineralization (Fig. 1A). In cells cultured on Ti, the times of decline in ALP mRNA expression and calcium accumulation were delayed by around 10 days (Fig.1B and D). To focus on the expression during mineralization, mRNA data were used for statistical evaluation at the following time-points; 10 days on PI or 20 days on Ti considered to be the differentiation stage (D), 20 days on PI or 30 days on TI considered to be just before onset of mineralization (BM), 30 days on PI or 40 days on Ti considered to be the early mineralization stage (EM), and 40 days on PI or 50 days on Ti considered to be the late mineralization stage (LM).

Statistical evaluation

Descriptive statistics are presented as mean \pm standard error

(SE) of 4 independent measurements. One-way analysis of variance (ANOVA) was performed to compare mRNA levels among the cell stages, and then Tukey-Kramer post hoc tests were performed to compare those at BM and the other stages, using computer software (Statview 4.5; Abacus Concepts, Berkeley, CA, USA). Results were considered significant if *P* values were less than 0.05.

Results

Patterns of mRNA expression of MMPs

All MMPs tested in this study were expressed by MC3T3-E1 cells throughout all the stages (Figs. 2-6). The level of expression at each stage and the pattern of expression during all stages for each MMP were similar in PI and Ti cultures. MMP-13 was expressed most abundantly (Fig. 5), while MMP-3 was expressed most scarcely (Fig. 3). Although the other MMPs (MMP-2, -9, and -14) showed no significant changes in mRNA level throughout all stages (Figs. 2, 4 and 6), MMP-13 and MMP-3 exhibited distinct gene expression patterns (Figs. 3 and 5). The mRNA level of MMP-13 were substantial at BM, but declined at EM and LM in both PI and Ti cultures, with significant differences at EM in PI culture and at LM in Ti culture (Fig. 5). Conversely, the mRNA level of MMP-3 increased at EM and LM compared with BM, with significant differences at EM and LM in PI culture and at EM in Ti culture (Fig. 3).

Patterns of mRNA expression of TIMPs

Similar to MMPs, all TIMPs tested in this study were expressed

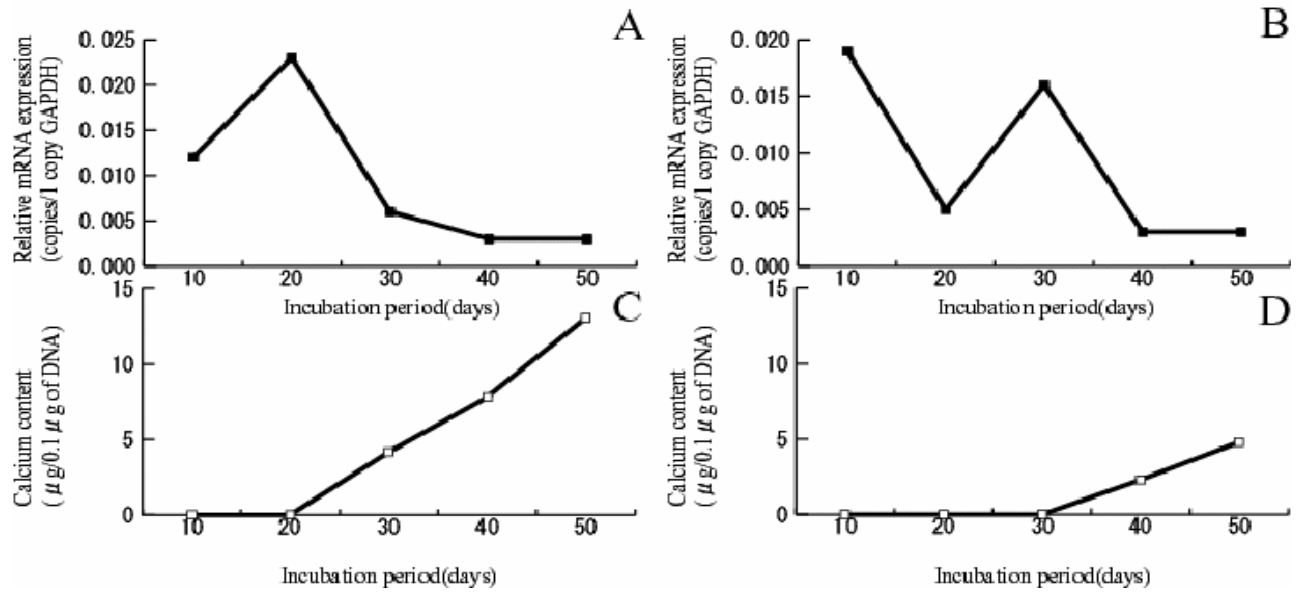


Fig. 1. Temporal mRNA expression of ALP (A and B) and calcium content (C and D) by MC3T3-E1 cells cultured on PI (A and C) and Ti (B and D) for 10, 20, 30, 40, and 50 days. Each value is presented as the mean of 4 independent measurements.

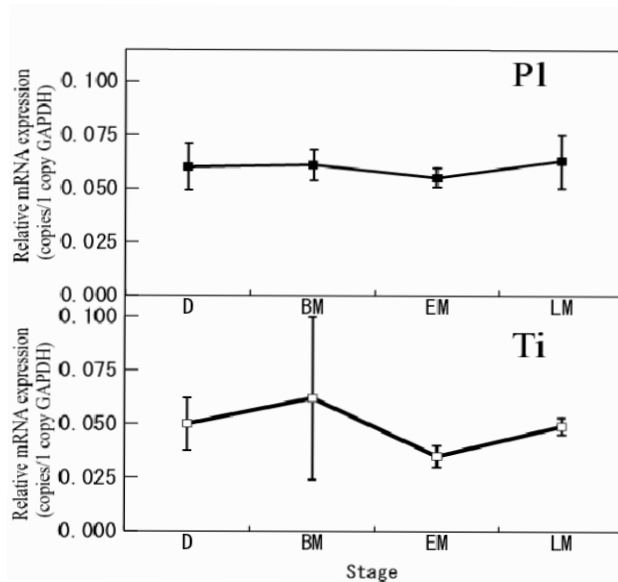


Fig. 2. Temporal mRNA expression of MMP-2 by MC3T3-E1 cells cultured on PI (upper) and Ti (lower). Each value is the mean of 4 independent measurements and the bar indicates standard error. D, differentiation stage; BM, just before onset of mineralization; EM, early mineralization stage; LM, late mineralization stage.

by MC3T3-E1 cells throughout all the stages, and the level of expression at each stage and the pattern of expression during all stages for each TIMP were similar in PI and Ti cultures (Figs. 7-9). TIMP-1, -2, and -3 showed no significant changes in mRNA levels throughout all stages.

Discussion

The present study for the first time shows the temporal gene expression profiles of MMPs and TIMPs during the mineralization

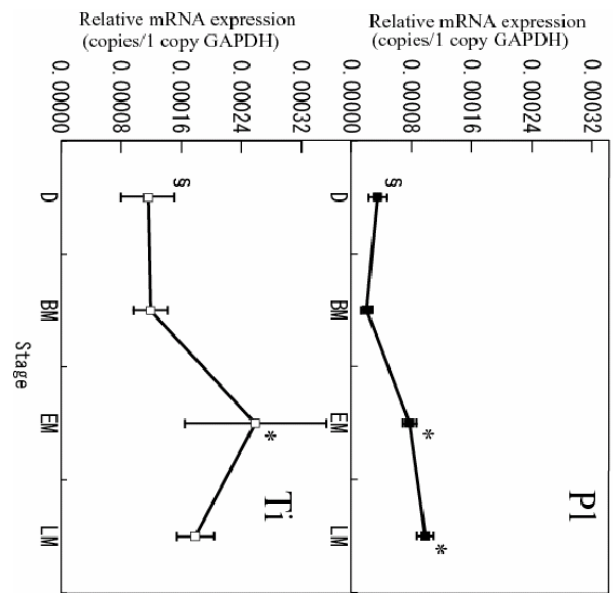


Fig. 3. Temporal mRNA expression of MMP-3 by MC3T3-E1 cells cultured on PI (upper) and Ti (lower). Each value is the mean of 4 independent measurements and the bar indicates standard error. D, differentiation stage; BM, just before onset of mineralization; EM, early mineralization stage; LM, late mineralization stage. §, $p < 0.05$ (ANOVA); *, $p < 0.05$ (Tukey-Kramer post hoc tests).

process (at cell differentiation, just before onset of mineralization, and at mineralization development) in osteoblasts cultured on a Ti implant material. The Ti and PI cultures showed identical or similar levels of mRNA expression at each stage and same pattern of expression in all stages for all the MMPs and TIMPs studied, as well as for the osteoblastic development marker ALP, consistent with our previous results for proteoglycans and LHs⁸). These findings suggest that the expression of MMPs and TIMPs tested

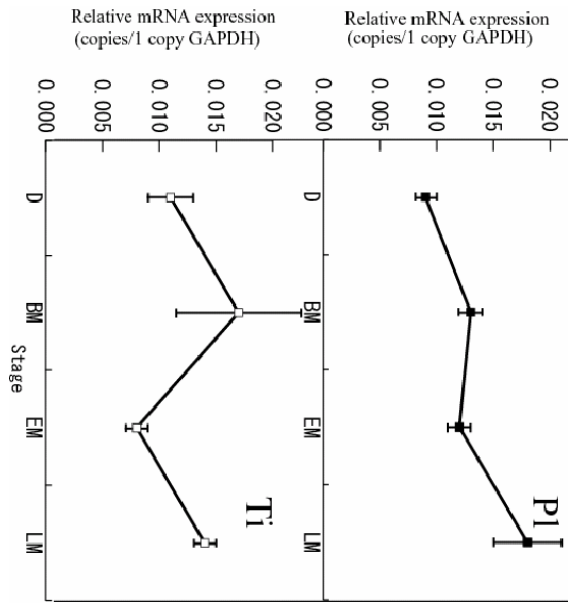


Fig. 4. Temporal mRNA expression of MMP-9 by MC3T3-E1 cells cultured on P1 (upper) and Ti (lower). Each value is the mean of 4 independent measurements and the bar indicates standard error. D, differentiation stage; BM, just before onset of mineralization; EM, early mineralization stage; LM, late mineralization stage.

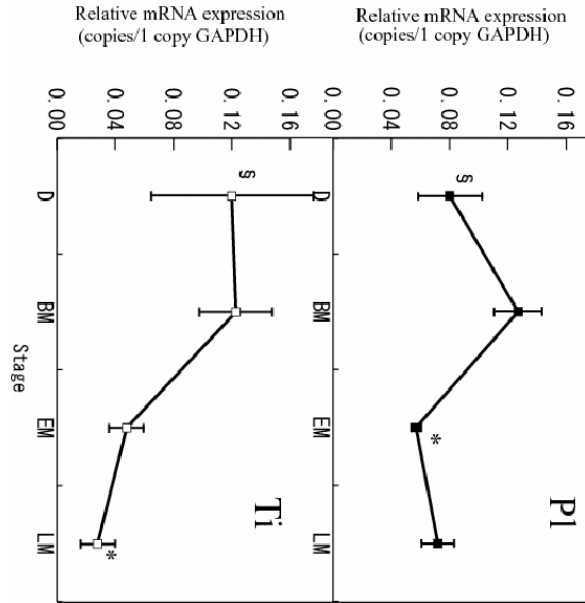


Fig.5. Temporal mRNA expression of MMP-13 by MC3T3-E1 cells cultured on P1 (upper) and Ti (lower). Each value is the mean of 4 independent measurements and the bar indicates standard error. D, differentiation stage; BM, just before onset of mineralization; EM, early mineralization stage; LM, late mineralization stage. §, $p < 0.05$ (ANOVA); *, $p < 0.05$ (Tukey-Kramer post hoc tests).

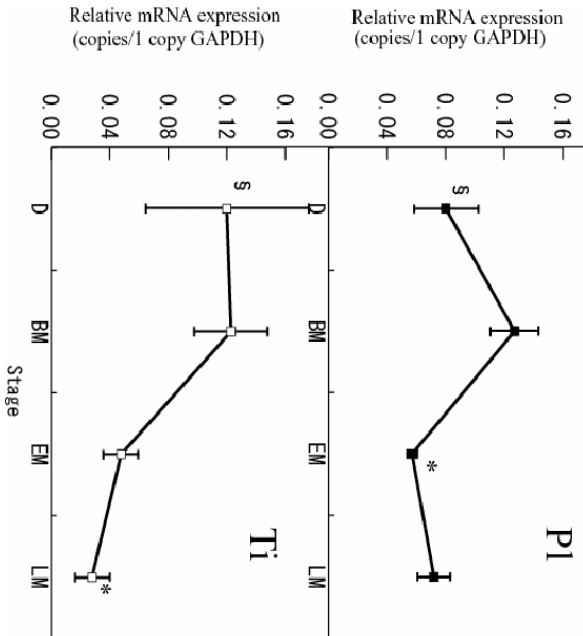


Fig. 6. Temporal mRNA expression of MMP-14 by MC3T3-E1 cells cultured on P1 (upper) and Ti (lower). Each value is presented as the mean of 4 independent measurements and the bar indicates standard error. D, the differentiation stage; BM, just before the onset of mineralization; EM, the early mineralization stage; LM, the late mineralization stage.

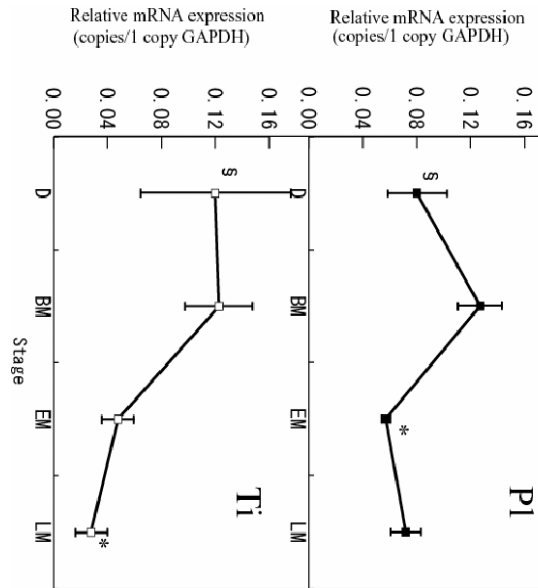


Fig. 7. Temporal mRNA expression of TIMP-1 by MC3T3-E1 cells cultured on P1 (upper) and Ti (lower). Each value is the mean of 4 independent measurements and the bar indicates standard error. D, differentiation stage; BM, just before onset of mineralization; EM, early mineralization stage; LM, late mineralization stage.

in this study are intimately related to the differentiation and matrix mineralization of MC3T3-E1 cells cultured on either material. It is noteworthy that the gene expression of MMP-3 and -13 during mineralization was different; the former was upregulated but the latter was downregulated at the stage of mineralization development, compared with just before onset of mineralization.

MMP-13 (collagenase-3) is a non-osteoclastic but osteoblastic collagenase and is thought to be a candidate for bone collagen solubilization, especially in situations such as cathepsin K deficiency or tumor-induced osteolysis²¹. MMP-13 cleaves the interstitial type I, II, and III collagens and gelatin, and its expression is upregulated by bone-resorbing agents such as

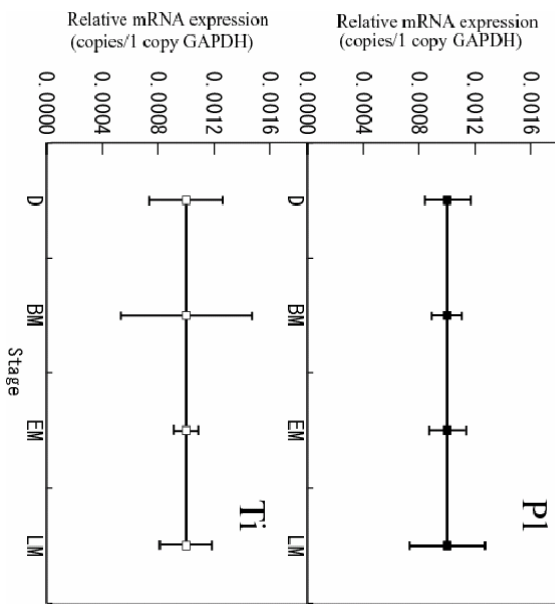


Fig. 8. Temporal mRNA expression of TIMP-2 by MC3T3-E1 cells cultured on P1 (upper) and Ti (lower). Each value is the mean of 4 independent measurements and the bar indicates standard error. D, differentiation stage; BM, just before onset of mineralization; EM, early mineralization stage; LM, late mineralization stage.

interleukin-1 and -6 but is downregulated by agents that induce bone formation such as bone morphogenetic proteins and insulin-like growth factors²²). MMP-13 was shown to be one of the late differentiation genes in a fetal rat calvarial osteoblast culture²³). A more detailed analysis showed that in mineralization medium, MMP-13 mRNA expression was low in the early differentiation stage, increased substantially with the onset of mineralization, and declined substantially thereafter, while this transcript was not increased in non-mineralization medium²⁴). These findings suggest that MMP-13 may be involved in the mineralization process, particularly just before and during onset of mineralization. The gene expression pattern of MMP-13 in the present study is consistent with these previous findings, although the expression pattern after onset of mineralization has not been studied. It is notable that the mRNA level of MMP-3 (stromelysin-1) was upregulated at the stage of mineralization development, although the level was lower than that of MMP-13 throughout the study period. MMP-3 degrades proteoglycans, fibronectin, laminin, gelatin, and other extracellular matrix proteins²⁵) and is expressed by osteoblasts at sites of bone formation and in osteoid²⁶). In osteoblast culture, MMP-3 is obviously expressed^{11,12}) but its expression at the mineralization stage has not been demonstrated before the present study. In dentin formation, MMP-3 is thought to be a candidate for degrading collagen-binding small proteoglycans^{27,28}) that appear to negatively regulate mineralization (for example, decorin)²⁹). These observations suggest that MMP-3 is involved as a positive regulator in the mineralization of predentin/dentin. This hypothesis could explain why MMP-3 is upregulated at the stage of bone mineralization development in

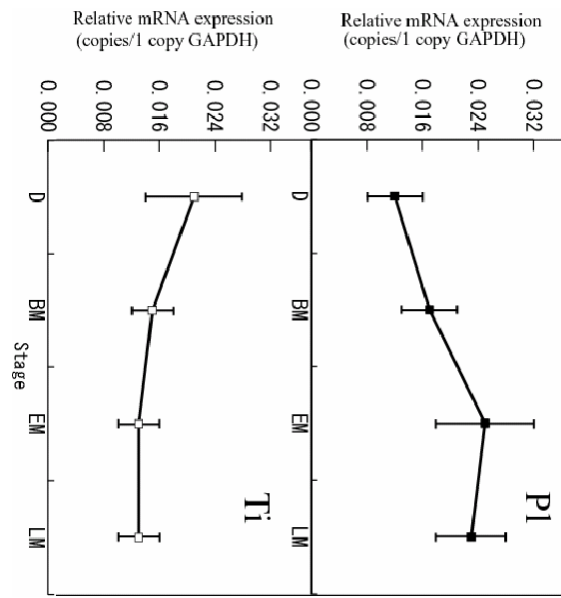


Fig. 9. Temporal mRNA expression of TIMP-3 by MC3T3-E1 cells cultured on P1 (upper) and Ti (lower). Each value is the mean of 4 independent measurements and the bar indicates standard error. D, differentiation stage; BM, just before onset of mineralization; EM, early mineralization stage; LM, late mineralization stage.

the present study. Until the onset of mineralization, the collagen-binding small proteoglycans bind to and reside at or near the gap zones of collagen fibrils³⁰⁻³²), which are the predominant hydroxyapatite nucleation sites³³⁻³⁵). MMP-3 degrades these proteoglycans and removes them from the gap zones, making space for hydroxyapatite nucleation and development. Collectively, it is possible that MMP-13 may be involved in the minor rearrangement of extracellular matrix (degradation of collagen) before and during onset of mineralization in Ti and P1 cultures, and then MMP-3 may be involved in the development of mineralization by degrading collagen-binding small leucine-rich proteoglycans that inhibit hydroxyapatite nucleation.

The activities of MMPs are controlled by TIMPs. TIMP-1 forms a specific complex with proMMP-9³⁶), although the significance of this complex remains unclear. TIMP-2 binds to proMMP-2, possibly playing a role in proMMP-9 activation, or forms complex with proMMP-2 and MMP-14 at the cell surface, resulting in proMMP-2 activation¹⁴). It is unclear whether the interaction between MMPs and TIMPs exists and/or plays a role during mineralization in this study, because their mRNA levels, except those of MMP-3 and -13, were not altered throughout. It is possible that MMP-3 is activated by osteopontin at the stage of mineralization development. Osteopontin is recently recognized as a member of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family³⁷). Osteopontin binds to and activates proMMP-3 or TIMP-inhibited MMP-3 without removing the propeptide³⁸). As osteopontin protein is substantially expressed in mature osteoblast and osteocytes³⁹) (although its gene expression remains to be investigated), the activity of MMP-3 may be

controlled during mineralization development by osteopontin simultaneously secreted by osteoblasts cultured on Ti and PL.

Elucidation of the roles of MMPs and TIMPs during mineralization on implant material requires the quantification of their protein levels and enzymatic activities. Nevertheless, during onset of mineralization of MC3T3-E1 cells cultured on Ti, the gene expressions of MMP-3 and -13 are dramatically altered over time and the patterns are distinct from each other. The temporally differential gene expression of MMP-3 and -13 implies a possibility of distinct roles of these enzymes at different stages of new bone formation around titanium implant material.

Acknowledgments

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