Osteomodulin Gene Expression in Beagle Dog Mandible by Beta-Tricalcium Phosphate

Kyoichiro Imai,¹ Tetsuya Mizukami,¹ Tetsuji Takahashi,¹ Takao Watanabe,² and Yoshimitsu Abiko¹,³

Department of ¹Biochemistry and Molecular Biology, and ³Research Institute of Oral Sciences, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan
²Department of Anatomy, Kanagawa Dental College, 82 Inaoka, Yokosuka, Kanagawa 238-8580, Japan

Correspondence to:
Yoshimitsu Abiko
E-mail: abiko.yoshimitsu@nihon-u.ac.jp

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Abstract
Beta-tricalcium phosphate (β-TCP) has been used for bone regeneration with satisfactory clinical results in accelerating bone formation. However, little is known about the molecular mechanisms enhancing the bone formation by β-TCP. To understand this mechanism, β-TCP was implanted into bone defects of the mandible in beagles and gene expression profiles were examined using DNA microarray technology. β-TCP altered many gene expressions; among those genes, a significantly higher mRNA level of osteomodulin (OMD) was observed. The enhanced OMD gene expression level was successfully confirmed by reverse transcription-polymerase chain reaction and real-time polymerase chain reaction. Because OMD is a potent regulator of mesenchymal cell function of wound healing and osteogenic differentiation, the enhancement of the OMD gene expression by β-TCP may be an important mechanism in accelerating bone formation.

Introduction
Technologies to repair orofacial skeletal defects take place at the same time with surgical therapies using bone grafts (1) and synthetic materials (2). Beta-tricalcium phosphate (β-TCP) has been used for bone regeneration in a variety of surgical procedures with satisfactory clinical results (3). β-TCP is thought to be a suitable bone substitute that will biodegrade and be replaced by newly mineralizing bone tissue without fibrous tissue proliferation. Furthermore, the use of β-TCP as alloplastic bone graft material for sinus grafting procedures has also received increasing attention in oral implant therapy (4, 5).

Multipotent mesenchymal stromal cells (MSCs) reside in bone marrow and play key roles in bone homeostasis because they proliferate, migrate, and undergo osteogenic differentiation in response to different stimuli (6). Osteogenesis of MSCs in orthopedic sites occurs by a direct conversion of mesenchymal cells into osteoblasts rather than by an endochondral sequence (7). However, very little is known about the molecular basis for the mechanisms that enhance bone formation by β-TCP.

In the present study, to understand the mechanisms and usefulness of β-TCP in accelerating bone formation, we used DNA microarray analysis coupled with a signal pathway database (Ingenuity Pathway Analysis; IPA).

Materials and Methods
Implantation of β-TCP
Ten beagle dogs (body weight 13±2 kg) were purchased from Japan SLC (Shizuoka, Japan). All beagles were maintained and used following the Guidelines of the Care and Use of Laboratory Animals of Kanagawa Dental College. The implantation of β-TCP was described in previous reports (8, 9).

RNA preparation
Total RNA was extracted from each bone biopsy using an optimised RNA extraction protocol based
on the RNeasy® Fibrous Tissue Midi Kit Isolation System (Qiagen, CA, USA) according to the manufacturer’s protocol. One piece of a bone biopsy was placed into 350 μl of lysis buffer (Buffer RLT), in a Lysing Matrix A tube (FastPrep® System, MP Biomedicals, Tokyo, Japan) and homogenized at 6.0 m/s, 45 s, six times. Total RNA was extracted from the bone using an optimized RNA extraction protocol based on the RNeasy® and Fibrous Tissue Midi Kit Isolation System (Qiagen, Valencia, CA, USA). The RNA quality was confirmed by calculating the OD_{260/280} ratio with a spectrophotometer and its integrity was verified by Agilent RNA 6000 Nano kits (Agilent, Santa Clara, CA, USA).

**Affymetrix GeneChip analysis**

The protocol for microarray processing was carried out according to the GeneChip® 3’IVT Express Kit user manual (Affymetrix, Santa Clara, CA, USA). Total RNA samples (100 ng) were subjected to two-cycle target labeling according to the Affymetrix instructions. Antisense complimentary RNA (cRNA) derived from double-strand complimentary DNA (cDNA) was labeled in the presence of biotinylated deoxyribonucleotide triphosphate (dTTP) derivatives to produce cRNA probes. The probes were then fragmented and hybridized on the GeneChip® Canine Genome 2.0 Array (Affymetrix; 38,000 genes). After hybridization, the arrays were scanned using the GeneChip® Scanner 3000 and the scanned images were analyzed using GeneChip Operating Software (Affymetrix). The data were imported into GeneSpring GX software (Agilent Technologies) for selection of induced and repressed genes in each experiment. Data analysis was performed using the GeneChip Expression Analysis (Affymetrix) and GeneSpring (Silicone Genetics, Redwood, CA, USA) software packages. The reliability of gene expression level in the GeneChip was demonstrated as present (P), marginal (M), or absent (A). Fold change was shown by normalization against the median of the corresponding control following the Affymetrix GeneChip manual.

**Reverse transcription–polymerase chain reaction and real–time polymerase chain reaction analysis**

Reverse transcription–PCR and real–time PCR reactions were carried out using a DNA thermal analyser (Rotor–Gene™ 6000; Corbett Life Science, Sydney, Australia). For RT–PCR, amplification products were electrophoresed on agarose gels and subsequently stained with ethidium bromide. Real–time PCR reactions were performed using SYBR Premix Ex Taq™ (Perfect Real–Time PCR, Takara, Ohtsu, Japan) and a green PCR kit (Qiagen GmbH, Dusseldorf, Germany). Amplification by PCR was started with an initial incubation at 95 °C for 15 s to activate the Taq DNA polymerase, and then performed at 95 °C for 5 s and 56 °C for 15 s for 40 cycles. To calculate the fold changes in gene expression, the initial template concentration was derived from the cycle number at which the fluorescent signal crossed the threshold in the exponential phase of the real–time PCR reaction. The mRNA copy unit was given by the cycle threshold value from the fluorescent signal of all the samples, including the standard curve and target genes, following the method provided by Corbett Life Science using RCx43–Gene™ 6000 software. Details were described in an operation manual, version 1.7.40, 2006.

![Table 1. Raw intensity signals shown in GeneChip.](image)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genebank ID</th>
<th>4-day</th>
<th>7-day</th>
<th>14-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>β·TCP</td>
<td>Fold*</td>
<td>β·TCP</td>
</tr>
<tr>
<td>OMD</td>
<td>XM 848235</td>
<td>103.0 (P)</td>
<td>729.8 (P)</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Flag (P) indicates the reliability of the data according to present (P) of gene expression in GeneChip. *Fold, fold change by normalization against the median of the corresponding to control.
The DNA primer sequences were 5′-ccacccaattttc
catcatc-3′ (the forward primer for OMD gene); 5′-
ttttggtggtggtggt-3′; (the reverse primer for
OMD gene), (expected product size=175 bp); 5′-
caccatcttcaggag-3′ (the forward primer for
GAPDH); 5′-atgcagctggtcatgag-3′ (the reverse
primer for GAPDH gene), (expected product size=
318 bp). Values were calculated as means±standard
deviation (SD). Comparisons were made between two
groups using a Student’s t-test.

Results

Table 1 shows the raw intensity signals of
osteomodulin (OMD) mRNA level in dog mandibular
bone with or without β-TCP implantation from
Affymetrix GeneChip analysis results. OMD gene
show “Present” in analysis results in 4-, 7-, 14-days
were shown in Table 1.

A scatter plot of mRNA levels of OMD expressed
in β-TCP implanted tissues in 4-, 7-, 14-days were
shown in Fig. 1. OMD mRNA levels show 6.2-, 2.9-
and 1.2-fold higher level of OMD in β-TCP implant-
ed tissues as compared to control at 4-, 7-, 14-days,
respectively.

To further investigate the elevated mRNA level
of the OMD gene, RT-PCR was performed. As shown
in Fig. 2, the mRNA level from dog mandible tissue
implanted β-TCP were higher than non-implanta-
tion as a control in all of 4-, 7-, 14-days samples. In
contrast, mRNA levels of GAPDH, the housekeeping
control, showed no differences between each β-TCP
implanted sample and control.

Another experiment to determine the exact rates
of enhancement of OMD gene expression levels were
performed using real-time PCR. The results were converted to mRNA copy units and the increase in OMD gene expressions were shown to be statistically different between each β-TCP implanted sample and control.

The quantities of mRNA levels of OMD and fold changes by β-TCP implantation in dog mandible normalized by GAPDH from the GeneChip analysis and real-time RT-PCR were summarized in Table 2.

**Discussion**

β-TCP particles in the extraction socket are clinically osteoconductive (10). When particles of β-TCP are mixed with the blood clot and surrounded by the bony walls of the alveolar socket, osteogenic cells including mesenchymal stem cells start migrating from bone surface and over the surface of the β-TCP particles.

In bone, most of the organic matrix is collagen, which provides the framework for the deposition of apatite crystals. The extracellular matrix of osteoblasts is dominated by mineral in the form of hydroxyapatite, and the mineral crystals are aligned along the fibrils of type I collagen (11). However, the mineralization process involves not only collagen as substrate for crystal formation but also non-collagenous acidic proteins (12). Member of proteoglycan, leucine–rich repeats (LRR) proteins have been shown to bind components of the extracellular matrix collagen (13). Interestingly, knock-out mice of LRR protein, have been generated and displayed abnormal collagen fibrillogenesis and developed a variety of bone diseases (14–16).

Recently, a new member of LRR protein family, osteoadherin has been isolated as a minor, leucine- and aspartic acid–rich keratan sulfate proteoglycan found in the mineralized matrix of bone (17). Osteoadherin also binds well to hydroxyapatite and thought to involve in mineral deposition. In fact, expression of osteoadherin mRNA has been detected by in situ hybridization in mature osteoblasts located superficially on trabecular bovine bone (18).

More recently, Buchaille et al (19) identified that human and mouse OMD was corresponding to bovine osteoadherin using genome database search, and concluded that osteoadherin and OMD represent the same protein in different species. On the other hand, to understanding the molecular targets of BMP-mediated gene transcription during the process of osteoblast differentiation, BMP-2 dependent differentiation of premyoblasts into the osteogenic lineage was identified using DNA microarray. Commitment to the osteogenic phenotype, most myogenic–related genes were downregulated, whereas Type I, VI col-

![Fig. 3. Real-time PCR of OMD mRNA levels. Results were expressed as mRNA copy unit by normalization to the house-keeping gene GAPDH. Differences between β-TCP implantation and the control were determined using the Student’s t-test. *p<0.01 **p<0.001, n=3.](image)

**Table 2. Summary of OMD gene expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>β-TCP</th>
<th>GeneChip (Fold)</th>
<th>Real-time PCR mRNA copy unit</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMD</td>
<td>4</td>
<td>–</td>
<td>140.7 ± 3.2</td>
<td>6.1</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>858.3 ± 33.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>+</td>
<td>1023.0 ± 27.5</td>
<td>8.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>9993.7 ± 217.7*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>667.7 ± 31.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1319.9 ± 36.5*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

β-TCP vs Control, *p<0.01 (n=3)
lagens and OMD gene expressions were induced (20). Furthermore, OMD gene overexpression in MC3T3-E1 cells resulted in an increase of osteoblastic differentiation features, such as increased alkaline phosphatase activity and increased in vitro mineralization (21). These investigations clearly demonstrated that OMD plays an important role in biomineralization in process of bone formation.

Taking together with these reports with our findings presented here, the enhancement of OMD gene expressions in β-TCP implanted dog mandible is one of mechanism in the stimulation of osteogenesis and mineralization in bone tissues.

Acknowledgments
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References
18. Sommarin Y, Wendel M, Shen Z, Hellman U and Heinegård D: Osteoadherin, a cell-binding keratan sulfate proteoglycan in bone, belongs to the family of