Expression of Platelet-Derived Growth Factor Receptor Beta in Dog Mandible by Beta-Tricalcium Phosphate

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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 platelet-derived growth factor receptor beta (PDGFRB) was observed. The enhanced PDGFRB gene expression level was successfully confirmed by reverse transcription-polymerase chain reaction and real-time polymerase chain reaction. Immunohistochemical study using antibody against PDGFRB also demonstrated that PDGFRB protein expression was enhanced by β-TCP. Because PDGFRB is a potent regulator of mesenchymal cell function of wound healing and osteogenic differentiation, the enhancement of the PDGFRB gene expression by β-TCP may be an important mechanism in accelerating bone formation.

Keywords:
β-TCP, dog mandible, platelet-derived growth factor receptor beta, gene chip

Introduction
After tooth extraction, the residual alveolar ridge generally provides limited bone volume because of ongoing, progressive bone resorption (1). Healing events within a post-extraction socket generally reduce the dimensions of the socket over time (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 may 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 our previous study, we found that β-TCP enhanced high-temperature requirement protein A1 (HtrA1) (8) and fibronectin (9) gene expressions in dog mandible tissues. 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 \( \beta \)-TCP

Ten beagles (body weight \( 13 \pm 2 \) 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 (Approval #: 08.12.TTH). The implantation of \( \beta \)-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\textsuperscript{®} 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 \( \mu \)l of lysis buffer (Buffer RLT), in a Lysing Matrix A tube (FastPrep\textsuperscript{®} 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 optimised RNA extraction protocol based on the RNeasy\textsuperscript{®} 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\textsuperscript{®} 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 deoxyribo nucleotide triphosphate (dNTP) derivatives to produce cRNA probes. The probes were then fragmented and hybridized on the GeneChip\textsuperscript{®} Canine Genome 2.0 Array (Affymetrix; 38,000 genes). After hybridization, the arrays were scanned using the GeneChip\textsuperscript{®} Scanner 3000 and the scanned images were analyzed using GeneChip Oper-
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.

The DNA primer sequences were 5'-ATGCAGTG CAGACTTGGTGGTC-3' (the forward primer for the PDGFRB gene); 5'-TCAGCAGTACGGATGTCGA G-3' (the reverse primer for the PDGFRB gene; predicted size=190 bp); 5'-ATCACCATCTTCCAG GAG-3' (the forward primer for the GAPDH gene); and 5'-ATCGACTTGGTGTACATGAG-3' (the reverse primer for the GAPDH gene; predicted size=318 bp). Values were calculated as mean±standard deviation. Comparisons were made between groups using the Student’s t-test.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded specimens were subjected to antigen retrieval and endogenous peroxidase blocking (30 min), and rinsed with phosphate-buffered saline. Immunostaining was performed using Elite ABC kits (Vector, Burlingame, CA, USA) and dianminobenzidine (Kirkegaard & Perry, Gaithersburg, MD, USA) as a chromogen using anti-PDGFRB polyclonal antibody (1: 400; Sigma, [city], Japan). Peroxidase-conjugated rabbit antimuscle immunoglobulin diluted 1: 10 in phosphate-buffered saline supplemented with 2-vol% heat-inactivated normal human serum was used. Peroxidase activity was visualized with 0.06% diaminobenzidine (Walter, Kiel, Germany) and 0.01 vol% hydrogen peroxide.

**Results**

In previous studies, we employed gene profiling in

<table>
<thead>
<tr>
<th>Table 1. Molecules in network-23</th>
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<tbody>
<tr>
<td><strong>ID</strong></td>
</tr>
<tr>
<td>23</td>
</tr>
</tbody>
</table>

Red underlines, FN1, HTRA1; PDGFRB was marked by yellow box.

Fig. 1. IPA network analysis. A. Outlook of network-23. Molecules: red, up-regulated; green, down-regulated. The red arrow indicates the relation of HtrAl and fibronectin to PDGFEB. B. Network shapes and characteristics of genes. C. Relationship of molecules. D. Relation labels.

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dog mandible tissue by $\beta$-TCP using Affymetrix GeneChip and found that $\beta$-TCP enhanced the gene expression of high-temperature requirement protein Al (HtrA1) (8) and fibronectin (9). In this study, we searched the gene network that included fibronectin and HtrA1 gene expressions using an IPA database. As a result, the network analysis showed that network-23 contained fibronectin and HtrA1 with increasing gene expression. Interestingly, network-23 also contained PDGFRB with increased gene expression, as shown in Table 1. The relation of genes in network-23 is shown in Fig. 1.

Next, we searched the raw intensity signals of mRNA levels of the PDGF gene family from Affymetrix GeneChip analysis results with “present” and “absent” flags in 4- and 7-day samples. Among the genes analyzed, PDGFRB was present with higher levels of PDGFRB mRNA found in 4- and 7-day samples after $\beta$-TCP implantation; the level in 4-day samples was 4.0-fold higher and the level in 7-day samples was 1.3-fold higher (Table 2). Fig. 2 displays the scatter plot of the mRNA level of PDGFRB expression after normalization by the median of the corresponding control.

To further investigate the elevated mRNA level of the PDGFRB gene by $\beta$-TCP, RT-PCR was performed. As shown in Fig. 3, the mRNA level of $\beta$-TCP implanted into dog mandible tissue was higher than that of $\beta$-TCP that was not implanted as a control in 4- and 7-day samples. In contrast, mRNA levels of GAPDH, a housekeeping gene, showed no differences between the $\beta$-TCP-implanted sample and the control.

### Table 2. Raw intensity signals of PDGF gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genebank ID</th>
<th>4-day</th>
<th>7-day</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$\beta$-TCP</td>
<td>Fold*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$-$</td>
<td>+</td>
</tr>
<tr>
<td>PDGFA</td>
<td>XM_843505</td>
<td>1.41 (A)</td>
<td>0.80 (A)</td>
</tr>
<tr>
<td>PDGFB</td>
<td>NM_001003383</td>
<td>19.13 (P)</td>
<td>32.41 (P)</td>
</tr>
<tr>
<td>PDGFC</td>
<td>XM_539783</td>
<td>9.63 (A)</td>
<td>12.10 (A)</td>
</tr>
<tr>
<td>PDGFR</td>
<td>XM_532374</td>
<td>50.44 (A)</td>
<td>64.58 (M)</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>NM_001003382</td>
<td>26.04 (A)</td>
<td>118.95 (P)</td>
</tr>
</tbody>
</table>

Flag (P), (M) or (A) indicate the reliability of the data according to present (P), median (M) or absent (A) of gene expression in GeneChip. *Fold, fold change by normalization against the median of the corresponding to control.
Table 3. Summary of PDGFRB gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>β-TCP (Fold)</th>
<th>GeneChip mRNA copy unit</th>
<th>Real-time PCR Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFRB</td>
<td>4</td>
<td>-</td>
<td>196.67 ± 27.21</td>
<td>411.44 ± 96.18**</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>+</td>
<td>892.00 ± 58.13</td>
<td>1,756.84 ± 103.07**</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01, n=3, β-TCP vs Control.

mRNA levels of PDGFRB and the fold changes by β-TCP implantation in dog mandible normalized by GAPDH from the GeneChip analysis and real-time RT-PCR are summarized in Table 3.

Finally, to examine the phenotypic expression of PDGFRB, we performed immunostaining of PDGFRB in dog mandible tissue. The experimental group of β-TCP implants showed more PDGFRB-positive cells than the control group in 7-day samples.

**Discussion**

In this study, we demonstrated that β-TCP implantation enhanced PDGFRB gene expression in dog mandible tissue. Immunohistochemical study also showed that β-TCP enhanced PDGFRB protein expression. PDGF is a potent mitogen that facilitates wound healing and stimulates bone repair by expanding osteoblastic precursor cells (10). So far, limited information is available regarding the potential of PDGFRB to promote osseointegration of oral implants. This study appears to be the first report to show the possibility of PDGFRB to accelerate bone formation by β-TCP.

When particles of β-TCP are mixed with the blood clot and surrounded by the bony walls of the alveolar socket, osteogenic cells including MSCs start migrating from the bone surface and over the surface of the β-TCP particles. It has been pointed out that PDGF gene therapy accelerates bone engineering and oral implant osseointegration (11). The biology of MSCs is a focus of interest in clinical medicine for the treatment of various bone diseases (12). Although PDGF has been reported to be involved in the osteogenic differentiation of MSCs, the role remains controversial and the network of PDGF signaling
Fig. 5. Immunostaining of PDGFRB. Representative photomicrographs of sections of dog mandible tissue stained with polyclonal anti-fibroblast growth factor receptor-2 antibody 7 days after surgery. Arrows indicate positive staining of PDGFRB. Bar = 100 μm.

has not been clarified. PDGFs are potent mitogens for mesenchymal cells and are involved in the wound healing process (13). PDGF-BB strongly induces the proliferation, migration, and differentiation of MSCs (14). PDGF ligands and receptors are upregulated during tissue remodeling in bone fractures and PDGF-A and -B synthesized by osteoblasts, chondrocytes, and MSCs are assumed to recruit MSCs to lesion sites to accelerate the repair process (15, 16). Two types of PDGFRs (PDGFRα and PDGFRβ) were found to be expressed in MSCs (17) and produced similar but distinctive signals (18). It is noteworthy that inhibition of PDGFRB suppresses proliferation and alters differentiation of human MSCs (19). More recently, Tokunaga et al. (20) studied the role of PDGF in the osteogenic differentiation process in MSCs; interestingly, the depletion of PDGFR gene expression decreased the mitogenic and migratory responses in MSCs. These findings suggest that PDGFRB is important for mediating PDGF signaling to regulate osteogenic differentiation in MSCs, and PDGFRB could represent an important target for guided tissue regeneration or tissue engineering of bone. From these investigations together with our findings, the enhancement of PDGFRB gene expression by β-TCP implanted in dog mandible tissue may be one of the mechanisms in the stimulation of osteogenesis and mineralization in bone tissues.

Conclusion

β-TCP enhances PDGFRB gene expression in dog mandible tissue. Because PDGFRB is a potent regulator of mesenchymal stromal cell function in osteogenic differentiation, the enhancement of the PDGFRB gene expression by β-TCP may be one of the important molecular mechanisms in accelerating the bone formation through the activation of PDGF signaling.

Acknowledgment

This study was supported in part by the “Academic Frontier” Project for Private Universities: a matching fund subsidy from Ministry of Education, Culture, Sports, Science and Technology, 2007–2011, and by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (B21390497).

References