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Original Article

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

Cheng-Hsing Chu,[1,2] Toshiyuki Oshitani,[1] Tetsuji Takahashi,[1] Jian Zhao,[1] Sheng-Yang Lee,[2] Takao Watanabe,[3] and Yoshimitsu Abiko[1]

[1]Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan
[2]Graduate Institute of Oral Rehabilitation Sciences and School of Dentistry, Taipei Medical University, Taipei, Taiwan
[3]Department of Oral Anatomy, Kanagawa Dental College, 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 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.

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 β-TCP

Ten beagles (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 (Approval #: 08.12.TTH). 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 (dNTP) 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 Gene Chip Expression Analysis (Affymetrix) and GeneSpring (Silicone Genetics, Redwood, CA, USA) software packages. The reliability of gene expression level in the gene chip 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.

Ingenuity Pathways Analysis

IPA software version 4.0 (Ingenuity Systems, Mountain View, CA, USA) was used to search for possible biological processes, pathways, and networks. A detailed description of IPA can be found on www. Ingenuity. com. As previously described, lists of genes with significant changes in gene expression based on the GeneChip experiments were moved from GeneSpring GX software into IPA software. The functional analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network.

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.

The DNA primer sequences were 5'-ATGCAGTG CAGACTGTGGTC-3' (the forward primer for the PDGFRB gene); 5'-TCAGCACTAGGGATGTGCA 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'-ATCGACTGTGGTCATGAG-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 diaminobenzidine (Kirkegaard & Perry, Gaithersburg, MD, USA) as a chromogen using anti-PDGFRB polyclonal antibody (1 : 400; Sigma, [**city**], Japan). Peroxidase-conjugated rabbit anti-mouse 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 1. Molecules in network-23

ID	Day	Molecules in Network	Score	No
23	4d	Alpha Actinin, Calpain, ↓CDCP1, ↓CLCA2 (includes EG:9635), ↑COL1A1*, ↑FBLN2*, ↓FGR*, Filamin, ↑FN1, ↑HTRA1*, Integrin, Integrin alpha 4 beta 1, Integrinα, Integrinβ, ↑ITGA1, ↑ITGA9, ↓ITGB4*, ↓ITGB7*, ↑ITGB1BP1*, ↑KDR, ↓LAMA3*, ↑LAMA4*, ↓LAMB3*, ↓LAMC2*, ↑NID2*, ↑PARVA*, ↓PARVG, Pdgfr, ↑PDGFRB*, ↓PLAUR*, ↓RABGAP1, ↓SDC4*, ↓TNK2*, ↑TRAM2 (includes EG:9697)*, ↑YES1	25	27

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

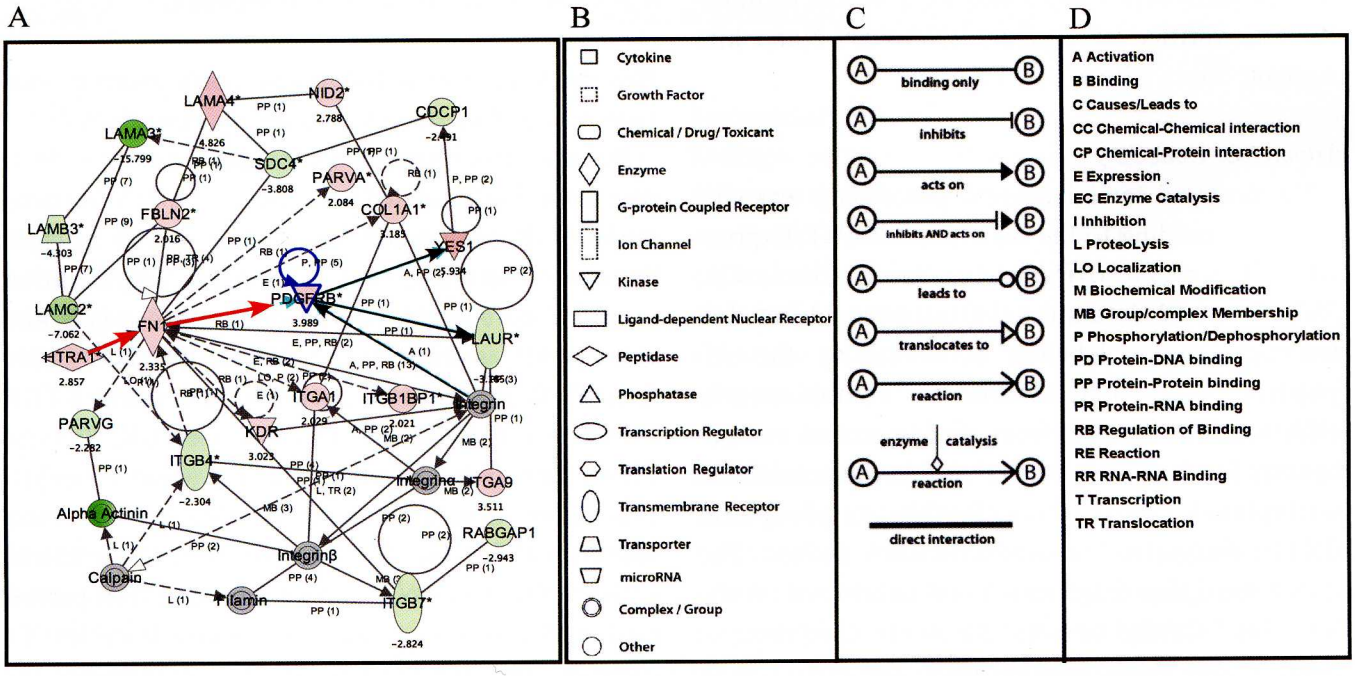

Fig. 1. IPA network analysis. A. Outlook of network-23. Molecules: red, up-regulated; green, down-regulated. The red arrow indicates the relation of HtrA1 and fibronectin to PDGFEB. B, Network shapes and characteristics of genes. C, Relationship of molecules. D, Relation labels.

dog mandible tissue by β-TCP using Affymetrix GeneChip and found that β-TCP enhanced the gene expression of high-temperature requirement protein A1 (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 β-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 β-TCP, RT-PCR was performed. As shown in Fig. 3, the mRNA level of β-TCP implanted into dog mandible tissue was higher than that of β-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 β-TCP-implanted sample and the control.

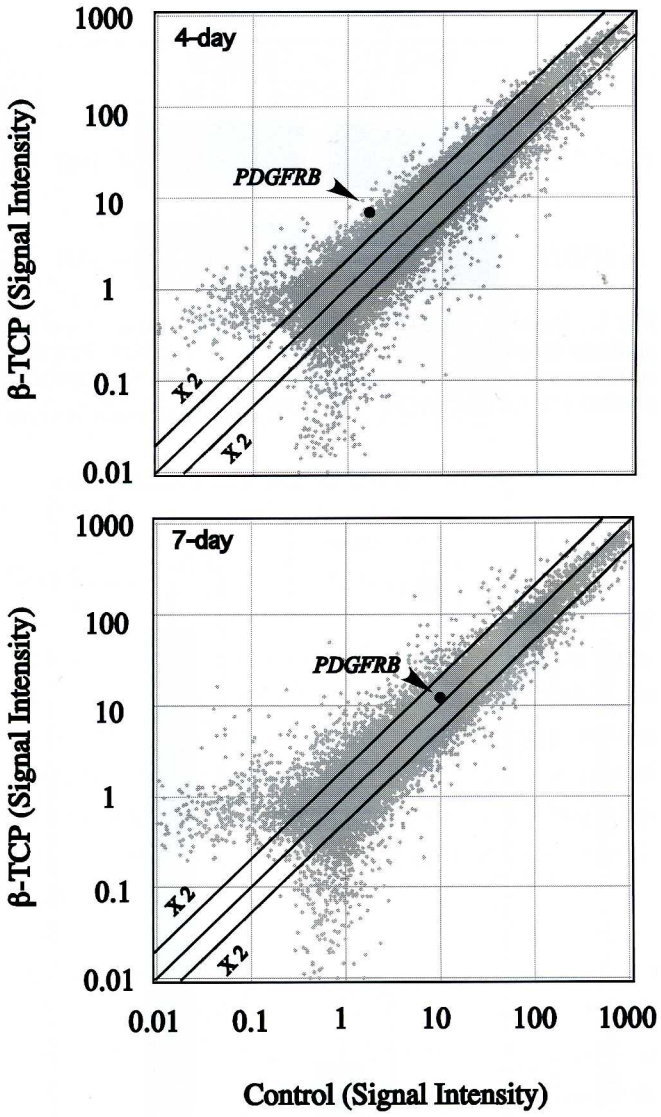

Fig. 2. A scatter plot of mRNA levels of PDGFRB expressed in 4- and 7-day samples after β-TCP implantation in dog mandible tissue.

Table 2. Raw intensity signals of PDGF gene

Gene	Genebank ID	4-day			7-day		
		β-TCP		Fold*	β-TCP		Fold*
		−	+		−	+	
PDGFA	XM_843505	1.41 (A)	0.80 (A)	−	0.46 (A)	0.79 (A)	−
PDGFB	NM_001003383	19.13 (P)	32.41 (P)	−	7.32 (A)	13.37 (A)	−
PDGFC	XM_539783	9.63 (A)	12.10 (A)	−	1.41 (A)	10.37 (A)	−
PDGFRA	XM_532374	50.44 (A)	64.58 (M)	−	42.08 (A)	96.92 (P)	−
PDGFRB	NM_001003382	26.04 (A)	118.95 (P)	4.0	139.09 (P)	231.28 (P)	1.3

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.

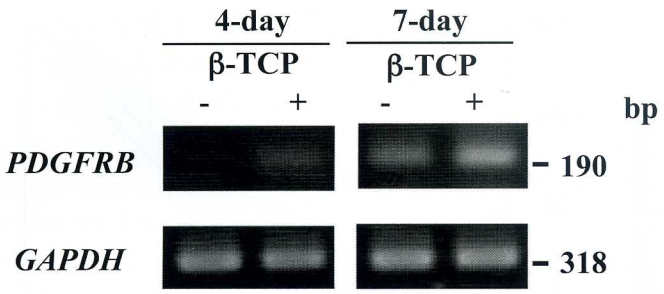

Fig. 3. Reverse transcription-polymerase chain reaction analysis of mRNA levels of PDGFRB. An ethidium bromide staining pattern of the amplified polymerase chain reaction products using agarose gel electrophoresis.

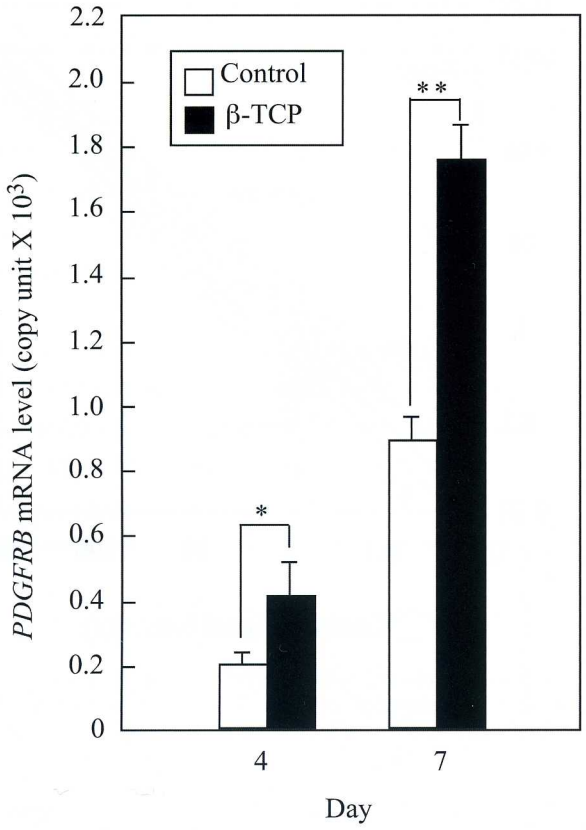

Fig. 4. Real-time polymerase chain reaction analysis of PDGFRB mRNA levels. Results were expressed as mRNA copy unit by normalization to the housekeeping gene GAPDH. Differences between β-TCP implantation and the control were determined using the Student's t-test. $*p<0.05$ $**p<0.001$, n=3.

In additional experiments to confirm PDGFRB mRNA levels quantitatively, we performed real-time PCR. As a result, the increased PDGFRB gene expression levels were confirmed to be significantly different between the β-TCP implanted sample and the control, as shown in Fig. 4. The quantities of 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.

Table 3. Summary of *PDGFRB* gene expression

Gene	Day	β-TCP	GeneChip (Fold)	Real-time PCR	
				mRNA copy unit	Fold
PDGFRB	4	−	4.0	199.67± 27.21	2.1
		+		411.44± 96.18*	
	7	−	1.3	892.00± 58.13	2.0
		+		1,756.84±103.07**	

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

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 in accelerating 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

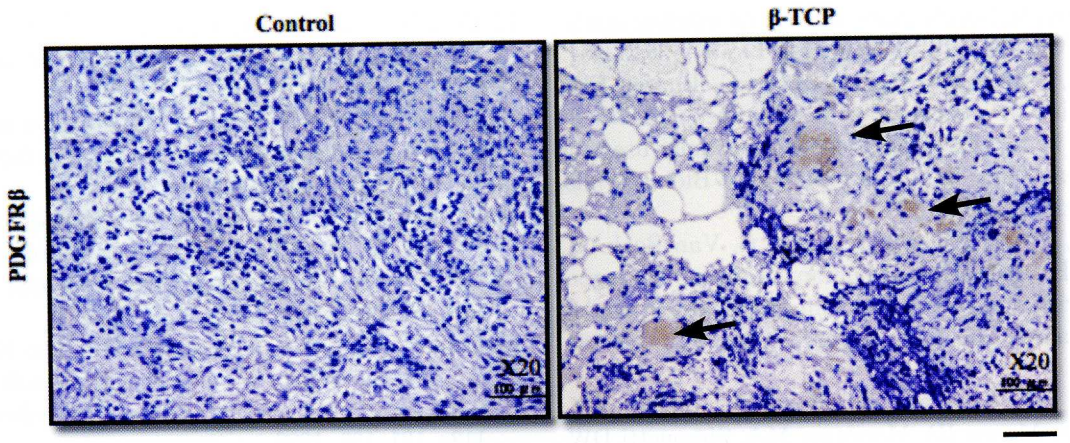

Fig. 5. Immunostaining of PDGFRB. Representative photomicrographs of sections of dog mandible tissue stained with polyclonal anti-fibronectin antibody 7 days after surgery. Arrows indicate positive staining of PDGFRB. Bar=100 μm.

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 (PDGFRA and PDGFRB) 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 PDGFR 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.

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References

1. Tallgren A: The continuing reduction of the residual alveolar ridges in complete denture wearers: a mixed -longitudinal study covering 25 years. J Prosthet Dent, 89: 427-435, 2003.
2. Araújo MG, Sukekava F, Wennström, JL, Lindhe J: Ridge alterations following implant placement in fresh extraction sockets: an experimental study in the dog. J Clin Periodontol, 32: 645-652, 2005.
3. Artzi Z, Weinreb M, Givol N, Rohrer MD, Nemcovsky CE, Prasad HS, Tal H: Biomaterial resorption rate and healing site morphology of inorganic bovine bone and β-tricalcium phosphate in the canine: a 24-month

longitudinal histologic study and morphometric analysis. Int J Oral Maxillfac Implants, 19 : 357-368, 2004.
4. Zerbo IR, Bronckers AL, De Lange G, Burger EH : Localisation of osteogenic cells and osteoplastic cells in porous beta-tricalcium phosphate particles used for human maxillary sinus floor elevation. Biomaterials, 26 : 1445-1451, 2005.
5. Zerbo IR, Bronckers AL, De Lange G, Van Beek GJ, Burger EH : Histology of human alveolar bone regeneration with a porous tricalcium phosphate. A report of two cases. Clin Oral Implants Res, 12 : 379-384, 2001.
6. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR : Multilineage potential of adult human mesenchymal stem cells. Science, 284 : 143-147, 1999.
7. Chaudhary LR, Hofmeister AM, Hruska KA : Differential growth factor control of bone formation through osteoprogenitor differentiation. Bone, 34 : 402-411, 1999.
8. Watanabe T, Zhao J, Hayashi M, Huang MY, Imai K, Takahashi T, Abiko Y : High-temperature requirement protein A1 (HtrA1) gene expression in dog mandible bone by β-TCP. J Hard Tissue Biol, 19 : 23-26, 2010.
9. Yoshitake K, Zhao J, Takahashi T, Watanabe T, Abiko Y : Fibronectin and focal adhesion kinase gene expression in dog mandible implanted by β-TCP. J Oral Tissue Engineer, 8 : 1-8, 2010.
10. Canalis E, McCarthy TL, Centrella M : Effects of platelet-derived growth factor on bone formation in vitro. J Cell Physiol, 140 : 530-537, 1989.
11. Chang PC, Seol YJ, Cirelli JA, Pellegrini G, Jin Q, Franco LM, Goldstein SA, Chandler LA, Sosnowski B, Giannobile WV. PDGF-B gene therapy accelerates bone engineering and oral implant osseointegration. Gene Therapy, 17 : 95-104, 2009.
12. Uccelli A, Moretta L, Pistoia V : Mesenchymal stem cells in health and disease. Nat Rev Immunol, 8 : 726-736, 2008.
13. Heldin CH, Westermark B : Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev, 79 : 1283-1316, 1999.
14. Kratchmarova I, Blagoev B, Haack-Sorensen M, Kassem M, Mann M : Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science, 308 : 1472-1477, 2005.
15. Fujii H, Kitazawa R, Maeda S, Mizuno K, Kitazawa S : Expression of platelet-derived growth factor proteins and their 13 normal mouse. Histochem Cell Biol, 112 : 131-138, 1999.
16. Rasubala L, Yoshikawa H, Nagata K, Iijima T, Ohishi M : Platelet-derived growth factor and bone morphogenetic protein in the healing of mandibular fractures in rats. Br J Oral Maxillofac Surg, 41 : 173-178, 1999.
17. Minguell JJ, Erices A, Conget P : Mesenchymal stem cells. Exp Biol Med, 226 : 507-520, 2001.
18. Gao Z, Sasaoka T, Fujimori T, Oya T, Ishii Y, Sabit H, Kawaguchi M, Kurotaki Y, Naito M, Wada T, Ishizawa S, Kobayashi M, Nabeshima Y, Sasahara M : Deletion of the PDGFR-beta gene affects key fibroblast functions important for wound healing. J Biol Chem, 280 : 9375-9389, 2005.
19. Fierro F, Illmer T, Jing D, Schleyer E, Ehninger G, Boxberger S, Bornhäuser M : Inhibition of platelet-derived growth factor receptor beta by imatinib mesylate suppresses proliferation and alters differentiation of human mesenchymal stem cells in vitro. Cell Prolif, 40 : 355-366, 2007.
20. Tokunaga A, Oya T, Ishii Y, Motomura H, Nakamura C, Ishizawa S, Fujimori T, Nabeshima Y, Umezawa A, Kanamori M, Kimura T : PDGF receptor is a potent regulator of mesenchymal stromal cell function. J Bone Miner Res, 23 : 1519-1528, 2008.