Stimulation of Insulin-like Growth Factor Receptor 1 Expression and Its Phosphorylation in Dog Mandibles by β-TCP

TAKAHASHI Tetsuji, BHAWAL Ujjal Kumar, MIZUKAMI Tetsuya, WATANABE Takao, TAKAHASHI Tsuneo and ABIKO Yoshimitsu

β-TCPによるイヌ顎骨のインスリン成長因子受容体1の発現とそのリン酸化の促進

髙橋　徹次　　バワール ウジャール クマール　　水上　哲也
渡辺　孝夫　　髙橋　常男　　安孫子宜光

日本口腔インプラント学会誌　第25巻　第3号　別刷
（平 成24年9月30日　発 行）
Reprinted from
Journal of Japanese Society of Oral Implantology
Vol. 25, No. 3 (2012)

<原　著>

Stimulation of Insulin-like Growth Factor Receptor 1 Expression and Its Phosphorylation in Dog Mandibles by β-TCP

TAKAHASHI Tetsuji[1], BHAWAL Ujjal Kumar[1], MIZUKAMI Tetsuya[1], WATANABE Takao[2], TAKAHASHI Tsuneo[2] and ABIKO Yoshimitsu[1]

β-TCPによるイヌ顎骨のインスリン成長因子受容体1の発現とそのリン酸化の促進

髙橋　徹次[1]　バワール ウジャール クマール[1]　水上　哲也[1]
渡辺　孝夫[2]　髙橋　常男[2]　安孫子宜光[1]

It has been reported that β-TCP can stimulate bone formation, but the mechanisms are not well understood. The enhancement of bone formation and IGFs gene expression in bone defects of Beagle dog mandibles by β-TCP were reported. IGFs are the most abundant growth factors produced by osteoblasts, and exert important effects on the proliferation and differentiation of osteoblasts. However, the mechanism of activation of IGF signaling by β-TCP has not been elucidated. In this study, an implant drill was used to make bone defects in Beagle dog mandibles, and β-TCP was filled into the bone defects. The effect of β-TCP on IGF receptor 1 (IGF1R) gene expression was analyzed by RT-PCR and real-time PCR. Moreover, the gene products in mandibles were examined by immunohistochemistry. Increased mRNA levels of IGF1R gene were observed in β-TCP implanted samples compared with controls. The enhancement of IGF1R mRNA levels by β-TCP was confirmed by RT-PCR and real-time PCR. Immunohistochemical staining revealed increased IGF1R protein expression and phosphorylation in β-TCP-implanted bone tissue. Taken together, the stimulation of IGF1R expression and phosphorylation by β-TCP might be a part of the mechanism of accelerating bone formation.

Key words : *dog mandible, β-TCP, insulin-like growth factor receptor 1 (IGF1R), IGF signaling*

Introduction

The osteogenic potential of autogenous bone graft is due to the retention of osteogenic precursor cells with the ability to proliferate and differentiate to osteoblasts[1]. The number of clinicians performing endosseous dental implants, whether immediately following tooth extraction or after a period of time, is rapidly increasing. However, on many occasions, clinicians encounter insufficient bone due to a number of reasons including injury, eradicated tumor masses, or progressive periodontal diseases. To overcome this difficulty, many bone grafts have been developed

[1]Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo
[2]Department of Anatomy, Kanagawa Dental College
[1]日本大学松戸歯学部生化学・分子生物学講座
[2]神奈川歯科大学人体構造学講座肉眼解剖
平成24年4月25日受付

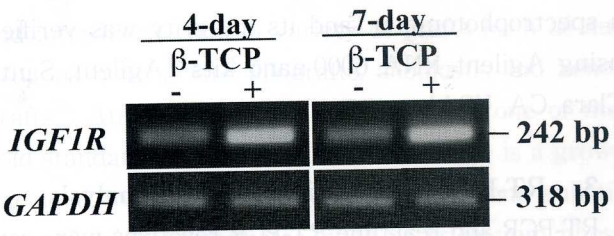

Fig. 1 RT-PCR analysis of IGFR1 gene expression
The amplification of each DNA band intensity of IGFR1 gene was greater in β-TCP implanted dog mandibles than in the control. Each GAPDH was used as an internal control.

retrieval and endogeneous peroxidase blocking (30 min), and rinsed with phosphate-buffered saline (PBS). Anti-IGF1R (signal way antibody) and anti-IGF1R Phospho-Tyr1161 (abm) rabbit polyclonal antibodies were used, and anti-IGF1R Phospho-Tyr1161 (abm) rabbit polyclonal antibodies were used. After overnight incubation at 4C, the specimens were rinsed with PBS and incubated at room temperature for 1 h with secondary antibody conjugated to Alexa Flour 488 donkey anti-rabbit IgG (H+L) (Molecular Probes, Inc., OR, USA). After rinsing with PBS, all specimens were color-developed with ProLong Gold Antifade Reagent with DAPI (Molecular Probes, Inc., OR, USA). The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAPI exposure conditions. The cells were visualized using a confocal laser scanning microscope (BZ-8000, Keyence Corporation, Osaka, Japan) by DAPI nuclei staining. The merged image represents the localization of IGF1 and IGF2 in cells. Original magnification = ×20 ; Bar = 50 μm.

5. Statistical analyses

Significant differences were analyzed by Student's *t*-test. $P<0.05$ was considered to be statistically significant and $p<0.01$ highly significant.

Results

First, we examined the gene expression of IGF1R by RT-PCR in mandible bone tissues. An increase in

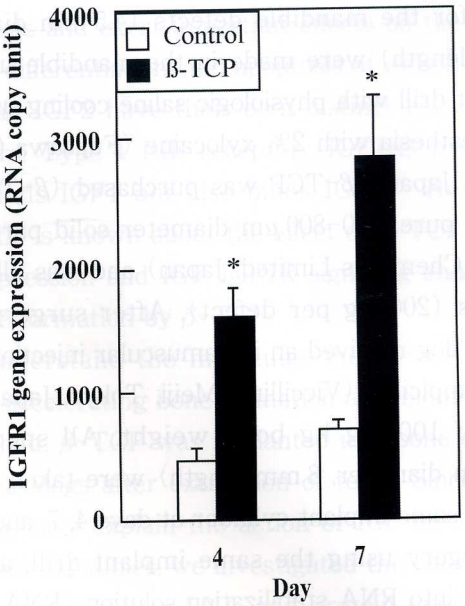

Fig. 2 Real-time PCR analysis of IGFR1 gene expression
The level of IGFR1 gene expression relative to the level of GAPDH mRNA expression was quanitified. The data was converted to mRNA copy unit. Differences between control and β-TCP group were determined using Student's *t*-test. *, $p<0.01$, n = 3.

the amplified DNA band intensity of IGF1R gene was seen in β-TCP implanted mandibles compared with the untreated control. In contrast, mRNA levels of GAPDH, the housekeeping gene control, showed no change between β-TCP and control groups.

To confirm the enhancement of IGF1R gene expression by β-TCP, RT-PCR analysis was carried out. Figure 2 shows that significantly higher levels of IGF1R DNA amplified bands were observed compared with the control. In contrast, mRNA levels of GAPDH, the housekeeping gene control, showed no change between β-TCP and control groups.

To examine the phenotypic expression of IGF1R, we performed immunofluorescence staining for IGF1R in Beagle mandible tissues. As shown in Fig. 3, the staining for IGF1R was found in granulation tissue-forming processes in the mandibles, which showed markedly positive immunostaining in the β-TCP implanted samples, whereas weak staining was

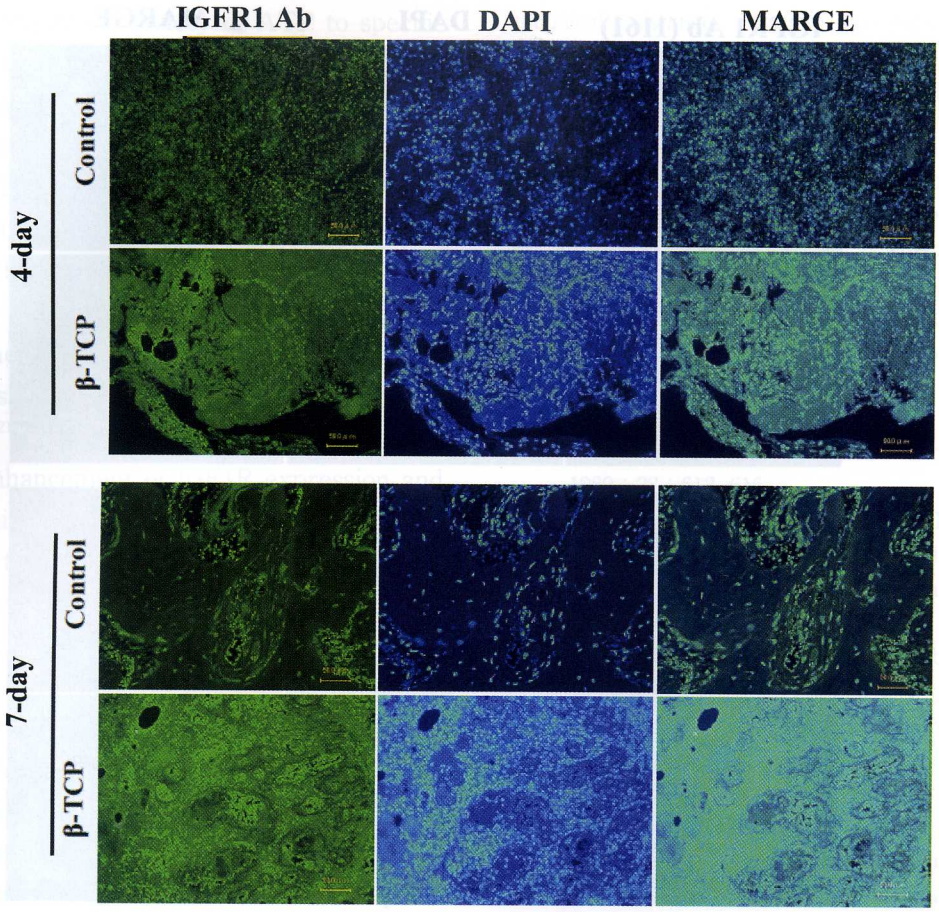

Fig. 3 Immunostaining for IGFR1 at day 4 and day 7 after surgery
The nuclei were counter-stained with ProLong *A* Gold antifade reagent with DAPI. The cells were visualized using confocal laser scanning microscopy. The merged image represents the localization of IGF1 and IGF2 in cells. Original magnification = ×20 ; Bar = 50 μm.

observed in the control group. Enhanced staining around the cells presumably represents deposits of IGF1R in the β-TCP implanted specimen. When parallel non-treated specimens were stained as controls, a barely visible fluorescence was observed.

Next, we examined IGF1R phosphorylation in the β-TCP. As shown in Fig. 4, the staining for phosphorylated IGF1R was markedly positive in the β-TCP implanted samples compared with the control group. After β-TCP implantation, the osteoblastic niche contained accumulated tyrosine-phosphorylated IGF1R protein. These observations suggested that the osteoblastic niche mediates the IGF1-receptor signal to its intracellular signaling molecules.

Discussion

IGF1 and IGF2 are important regulators of bone formation and promote both the proliferation and differentiation of osteoblast precursor. The expression level of IGF1 and IGF2 was also increased at a relatively early stage in the β-TCP-implanted group compared with the control[17]. IGFs are the most abundant growth factors and exert important effects on the proliferation, differentiation, and apoptosis of osteoblasts[18]. In this study, we demonstrated that β-TCP enhanced IGF1R mRNA and protein expression followed by phosphorylation of IGF1R in dog mandibles.

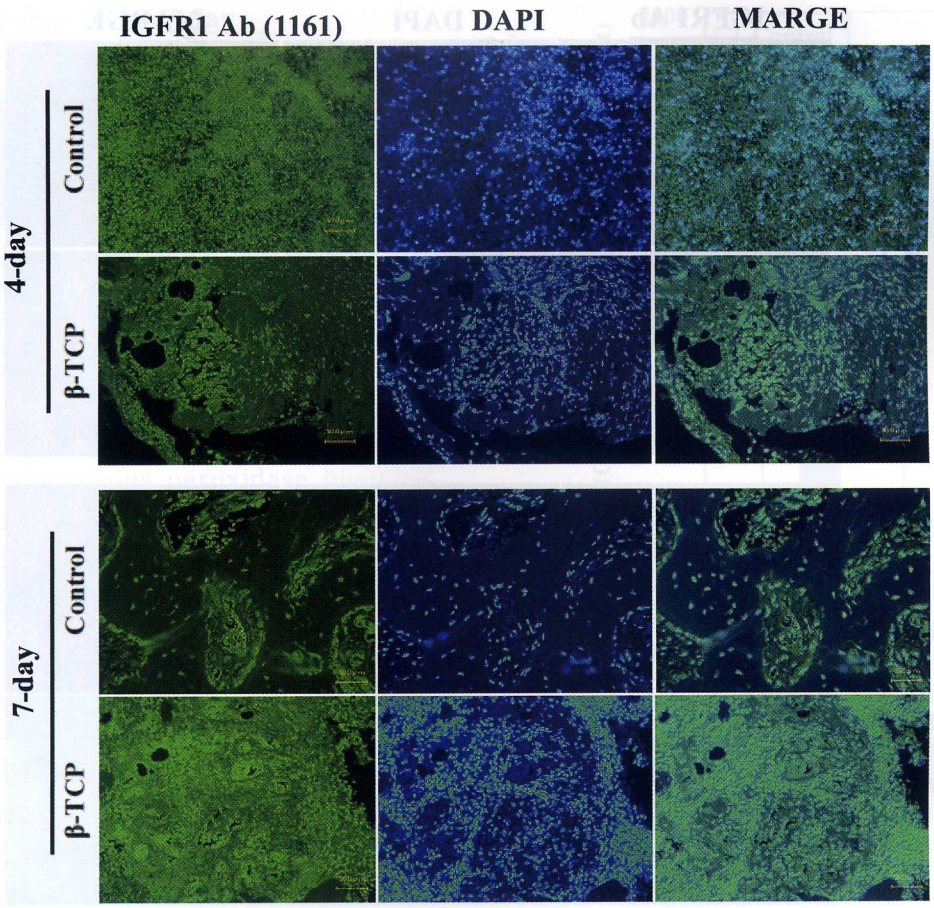

Fig. 4 Immunostaining for phosphorylated IGFR1 at day 4 and day 7 after surgery
The nuclei were counter-stained with ProLong *A* Gold antifade reagent with DAPI in accordance with the manufacturer's instructions. The cells were visualized using confocal laser scanning microscopy (BZ-8000, Keyence Corporation, Osaka, Japan). The merged image represents the localization of IGF1 and IGF2 in cells. Original magnification = ×20 ; Yellow bar = 50 μm.

When β−TCP is mixed with the blood clot and surrounded by the bony walls of the alveolar socket, osteogenic cells, including undifferentiated mesenchymal cells, start migrating from the bone surface between and over the surface of β−TCP. The β−TCP particles attract osteoprogenitor cells that migrate into the interconnecting micropores of the bone substitute material during 6 months[20], and β−TCP particles in the extraction sockets are clinically osteoconductive. The β−TCP implanted group showed a higher bone formation rate at 4 and 7 days, although this difference between the β−TCP and control groups seemed to disappear by day 14[16]. These findings suggest that tissue healing and bone formation occurred at a relatively early stage, and the gene expression of COL1A1, ALP, OPN and TGF-β_2 was increased and RANKL and IFN expression decreased in the β−TCP-implanted group[16].

Concerning the IGF signaling system, the mature heterotetrameric IGF1R conformation, structurally a and b subunits of the receptors, reside entirely extracellularly and include a cysteine-rich region and potential N-linked glycosylation sites. The cysteine-rich domain of the IGF1R is known to be important for high-affinity IGF1 binding[19]. The b subunit constitutes the transmembrane domain and the cytoplasmic portion contains a tyrosine kinase domain. The inside of this catalytic region participates in the

transfer of the phosphate moiety of ATP to specific substrates, and upon ligand activation, the receptors undergo a conformational change that leads to ATP binding and autophosphorylation of the tyrosine kinase domain[20]. This event enhances the kinase activity of the receptors and leads to downstream signal transduction and engaging in biological activity such as proliferation and differentiation[20]. Interestingly, mice lacking the IGF1R exhibited delayed appearance of their ossification centers and delayed epiphyseal maturation[21]. Taken together, our findings suggest that the enhancement of IGF1R expression and its phosphorylation is a part of the mechanism of accelerating bone formation by β-TCP through activation of IGF signaling. The activation of IGF signaling by β-TCP may act either in an autocrine and/or paracrine fashion to promote an increase of bone formation. The present findings suggest the therapeutic potential of IGF signaling for bone regeneration in dental implant therapy. We consider that IGF/IGF1R signaling may be used in future, not only for the improvement of new bone formation in dental implant therapy, but also for the development of diagnostic screening in a wide array of biomaterials.

Conclusion

β-TCP enhanced IGF1R expression and its phosphorylation in dog mandible tissues. This finding suggests that the induction of IGF signaling through the enhancement of IGF1R expression and its phosphorylation in mandible tissues might be a part of the mechanism of accelerating bone formation by β-TCP.

Acknowledgements

This study was supported in part by the "Academic Frontier" Project for Private Universities : a matching fund subsidy from the 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). We thank Dr. J Zaou for her excellent technical assistance.

References

1) Matsushita N, Terai H, Okada T, Nozaki K, Inoue H, Miyamoto S, Takaoka K. A new bone-inducing biodegradable porous beta-tricalcium phosphate. J Biomed Mater Res A 2004 ; 70 : 450-458.
2) John HD, Wenz B. Histomorphometric analysis of natural bone mineral for maxillary sinus augmentation. Int J Oral Maxillofac Implants 2004 ; 19 : 199-207.
3) Boyne PJ, James RA. Grafting of the maxillary sinus floor with autogenous marrow and bone. J Oral Surg 1980 ; 38 : 613-616.
4) Moy PK, Lundgren S, Holmes RE. Maxillary sinus augmentation : Histomorphometric analysis of graft materials for maxillary sinus floor augmentation. J Oral Maxillofac Surg 1993 ; 51 : 857-862.
5) Younger EM, Chapman MW. Morbidity at bone graft donor sites. J Orthop Trauma 1989 ; 3 : 192-195.
6) Yuan H, Yang Z, De Bruij JD, De Groot K, Zhang X. Material-dependent bone induction by calcium phosphate ceramics : A 2.5-year study in dog. Biomaterials 2001 ; 22 : 2617-2623.
7) Santos FA, Pochapski MT, Martins MC, Zenobio EG, Spolidoro LC, Marcantonio E. Comparison of biomaterial implants in the dental socket : Histological analysis in dogs. Clin Implant Dent Relat Res 2010 ; 12 : 18-25.
8) Alcaide M, Serrano MC, Pagani R, Sanchez-Salcedo S, Vallet-Rehi M, Portoles MT. Biocompatibility markers for the study of interactions between osteoblasts and composite biomaterials. Biomaterials 2009 ; 30 : 45-51.
9) Kihara H, Shiota M, Yamashita Y, Kasugai S. Biodegradation process of alpha-TCP particles and new bone formation in a rabbit cranial defect model. J Biomed Mater Res B Appl Biomater 2006 ; 79 : 284-291.
10) Yamaguchi K, Hirano T, Yoshida G, Iwasaki K. Degradation-resistant character of synthetic hydroxyapatite blocks filled in bone defects. Biomaterials 1995 ; 16 : 983-985.
11) Ozawa M. Experimental study on bone conductivity and absorbability of the pure β-TCP. J Jpn Soc Biomater 1995 : 13 : 167-175.
12) Yoneda M, Terai H, Imai Y, Okada T, Nozaki K, Inoue H, Miyamoto S, Takaoka K. Repair of an intercalated long bone defect with a synthetic biodegradable bone-inducing implant. Biomaterials 2005 ; 26 : 5145-5152.
13) Horch HH, Sader R, Pautke C, Neff A, Deppe H, Kolk A. Synthetic, pure-phase beta-tricalcium phosphate ceramic granules (Cerasorb) for bone regeneration in the re-

constructive surgery of the jaws. Int J Oral Maxillofac Surg 2006 ; 35 : 708-713.
14) Brandoff JF, Silber JS, Vaccaro AR. Contemporary alternatives to synthetic bone grafts for spine surgery. Am J Orthop 2008 ; 37 : 410-414.
15) Szabó G, Huys L, Coulthard P, Maiorana C, Garagiola U, Barabás J, Németh Z, Hrabák K, Suba Z. A prospective multicenter randomized clinical trial of autogenous bone versus beta-tricalcium phosphate graft alone for bilateral sinus elevation : histologic and histomorphometric evaluation. Int J Oral Maxillofac Implants 2005 ; 20 : 371-386.
16) Zhao J, Watanabe T, Bhawal UK, Kubota E, Abiko Y. Transcriptome analysis of β-TCP implanted in dog mandible. Bone 2011 ; 48 : 864-877.
17) Chao KL, Bhawal UK, Watanabe T, Takahashi T, Chang WJ, Lee SY, Abiko Y. Induction of insulin-like growth factors expression in dog mandibles by β-TCP. J Hard Tissue Biol 2012 ; 1-8.
18) Schmid C. Insulin-like growth factors. Cell Biol Int 1995 ; 19 : 445-457.
19) Kjeldsen T, Andersen AS, Wiberg FC, Rasmussen JS, Schaffer L, Balschmidt P, Moller KB, Moller NPH. The ligand specificities of the insulin receptor and the insulin-like growth factor I receptor reside in different regions of a common binding site. Proc Natl Acad Sci USA 1991 ; 88 : 4404-4408.
20) Werner H, Weinstein D, Bentov I. Similarities and differences between insulin and IGF-I : structures, receptors, and signalling pathways. Arch Physiol Biochem 2008 ; 114 : 17-22.
21) Van Wyk JJ, Smith EP. Insulin-like growth factors and skeletal growth : possibilities for therapeutic interventions. J Clin Endocrinol Metab 1999 ; 84 : 4349-4354.

β-TCPは骨形成を促進すると報告されているが，その機序については不明な点が多い．ビーグル犬の下顎骨の骨欠損部にβ-TCPを填入することで骨形成を促進され，また，顎骨組織中のIGF遺伝子の発現増大が報告されている．IGFは，骨芽細胞で豊富に産生され，細胞増殖，分化に重要な役割を発揮する成長因子である．しかし，β-TCPによる顎骨のIGFシグナリングの活性化の機序については不明である．本研究では，インプラントドリルでビーグル犬下顎骨に骨欠損を作成してβ-TCPを填入した．そしてIGF受容体1（IGF1R）の遺伝子発現への影響についてRT-PCRおよびリアルタイムPCR法を応用して調べ，さらにタンパク質発現を免疫組織学的に検証した．

その結果，対照群に比べて，IGF1R遺伝子の発現がβ-TCPによって促進し，さらに，IGF1Rタンパクおよび IGF1Rリン酸化の促進が確認された．これらのことから，β-TCPによるIGF1R遺伝子発現およびリン酸化は骨形成促進の機序の一部であることが示唆される．