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Original

High-Temperature Requirement Protein A1 (HtrA1) Gene Expression in Dog Mandible Bone by β-TCP

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Abstract: Beta-tricalcium phosphate (β-TCP) has been used for bone regeneration in a variety of surgical procedures including dental implant therapy with satisfactory clinical results. However, very little is known about the molecular basis mechanisms enhancing the bone formation by β-TCP. To understand the molecular basis mechanism of β-TCP in bone formation, β-TCP was implanted into bone defects of mandible bones in beagle dogs. After 4, 7 and 14 days, bone tissues during the healing process were recovered and gene expression profiles were examined using an Affymetrix GeneChip system. A significantly higher high-temperature requirement protein A1 (HtrA1) mRNA level was found in the 4-day samples after β-TCP implantation compared with the controls. The elevated HtrA1 gene expressions in β-TCP-implanted bone tissues were confirmed by RT-PCR and real-time PCR. Because HtrA1 is known as a key regulator of physiological and pathological matrix mineralization, the enhancement of HtrA1 gene expression in β-TCP-implanted mandible bone might be one of the molecular mechanisms for stimulating bone formation.

Keywords: High-temperature requirement protein A1 (HtrA1), β-TCP, Bone formation, Gene chip, Dog mandible

Introduction

Beta-tricalcium phosphate (β−TCP) has been used for bone regeneration with satisfactory clinical and histological results in both animal models [1], and human trials [2]. β-TCP is also known to be a suitable bone substitute that will biodegrade and can be replaced by newly mineralising bone tissue without fibrous tissue proliferation [3]. In recent years, the use of β-TCP as an alloplastic bone graft material for sinus grafting procedures has also received increasing attention in oral implant therapy [4, 5] . β-TCP particles in the extraction socket are clinically osteoconductive 6). However, very little is known about the molecular basis for mechanisms enhancing the bone formation by β-TCP.

In this study, to understand the usefulness and molecular basis mechanism of β-TCP for accelerating bone formation, β-TCP was implanted into dog mandible bone, and gene expression profiles in bone forming tissues were examined.

Materials and methods

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Implantation of β-TCP

Beagle dogs (body weight 13±2 kg) were purchased from Japan SLC (Shizuoka, Japan). The dogs were allowed free access to food and water *ad libitum* at all times and were maintained on a 12 h light/dark cycle (lights on 8:00 to 20:00) at 23±1℃, All beagle dogs were maintained and used in accordance with the guidelines of the care and use of Laboratory Animals of Kanagawa Dental College. Experimental groups were the β-TCP scaffold-implanted group and the no implanted control. The left and right mandible bones of beagle dogs were divided randomly into three time groups (4, 7 and 14 days).

All beagle dogs were injected with sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 35 mg/kg. Their premolar teeth were then extracted. After three months of bone healing, sockets of bone defects (4.5 mm diameter, 8 mm length) were made in mandible bone, using an implant drill with physiologic saline cooling under anaesthesia. β-TCP was purchased from Taihei Chemical Industry (TCP-100, more than 99% pure). The implant materials were filled randomly into the left or right mandible bone defects. After surgery, each beagle dog received an intramuscular injection of sodium ampicillin

(Viccillin®, Meiji, Tokyo, Japan) at a dose of 100 mg per kg body weight. All wounds healed gradually, and the beagle dogs were active with no complications after surgery. All specimens (4.5 mm diameter, 8 mm length) were taken out in cylinder types 4, 7 and 14 days after surgery, and immersed in an RNA stabilisation solution (RNA later®, Applied Biosystems, Ambion, USA).

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 Ltd.) according to the manufacturer's protocol. One piece of a biopsy (a 3 mm cube of tissue) was placed into 350 µl of lysis buffer (Buffer RLT, from the isolation kit) in a lysing matrix A tube (FastPrep® System, MP Biomedicals, Japan) and homogenised six times at 6.0 m/s for 45 s. Samples were processed using the RNeasy Isolation System following the manufacturer's instructions. The RNA quality was confirmed by calculating the $OD_{260/280}$ ratio via a spectrophotometer, and its integrity was verified by Agilent RNA 6000 nano kits (Agilent, Santa Clara, CA, USA).

GeneChip analysis

Gene expression profiling was performed for 4-day RNA samples using a GeneChip® Canine Genome 2.0 Array (Affymetrix, 38,000 genes). The protocol for microarray processing was carried out according to the GeneChip® 3' IVT express kit user manual. After hybridisation, the arrays were scanned using the GeneChip® Scanner 3000, and the scanned images were analysed using GeneChip Operating Software (Affymetrix, Santa Clara, CA, USA). The data were imported into GeneSpring GX software (Agilent) to select the induced and repressed genes in each experiment. Values below 0.01 were set to 0.01. Each time point was normalised against the median of the corresponding control sample. Each measurement for each gene in those , β-TCP samples was divided by the median of that gene's measurements in the corresponding control samples.

RT-PCR and real time PCR

RT-PCR and real-time PCR reactions were carried out using a real-time DNA thermal analyser (Rotor-Gene™ 6000; Corbett Life Science, Sydney, Australia). For RT-PCR, PCR amplified 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, Japan) and a green PCR kit (Qiagen GmbH, Dusseldorf, Germany). Amplification by PCR was started with an initial incubation at 95℃ for 15 seconds to activate the Taq DNA polymerase, and then performed at 95℃ for five seconds and 56 ℃ for 15 seconds for 40 cycles. To calculate the gene expression fold changes, 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 as follows: 5'-TGTACGCAGTGTGCTTTTCC-3' (the forward primer for the high-temperature requirement protein A1 (HtrA1) gene); 5'-CCTCCTCTGGGTGCTGACTA-3' (the reverse primer for HtrA1 gene; predicted size=198 bp); 5'-ATCACCATCTTCCAGGAG-3' (the forward primer for GAPDH); and 5'-ATCGACTGTGGTCATGAG-3' (the reverse primer for GAPDH gene; predicted size=318 bp). Values were calculated as means ± standard deviation (SD). Comparisons were made between two groups using a student's *t* test.

Results

By raw signal intensity and flag analysis using the GeneChip operating system, the HtrA1 mRNA level for 4-day samples showed a present (P) flag (data not shown). A scatter plot of mRNA levels of HtrA1 after being normalised with GAPDH mRNA levels for 4-day samples is shown in Figure 1. Interestingly, a 2.9-fold higher level of HtrA1 gene expression in 4-day samples was found in β-TCP-implanted tissues compared with controls.

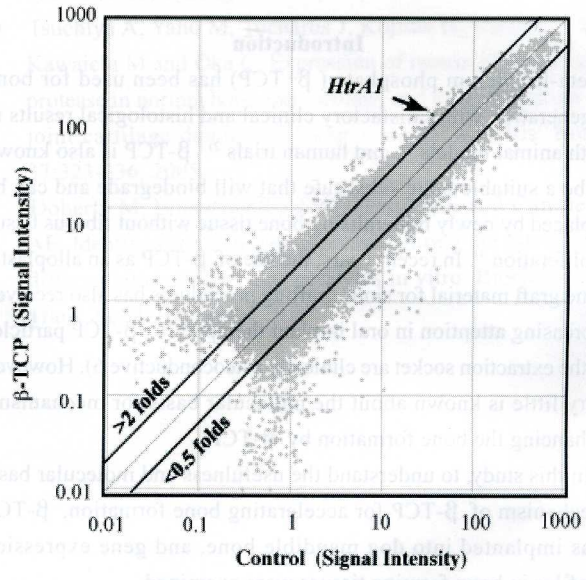

Figure 1: A scatter plot of the HtrA1 gene expression. The raw intensities of HtrA1 mRNA levels were normalised with GAPDH mRNA levels after four days.

To further investigate the elevated mRNA level of the HtrA1

genes, RT-PCR was performed on 4-, 7- and 14-day samples. As shown in Figure 2, the mRNA level from dog mandible bone tissue-implanted β-TCP was higher than the control for 4-, 7- and 14-day samples. By contrast, mRNA levels of GAPDH, the housekeeping control, showed no differences between each β-TCP-implanted sample and control.

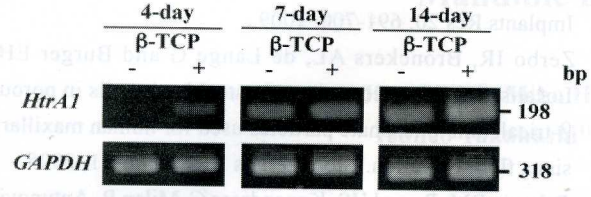

Figure 2: RT-PCR analysis of the HtrA1 mRNA level. An ethidium bromide-staining pattern of the amplified PCR products using agarose gel electrophoresis is shown.

Another experiment to determine the exact rates of enhancement of the HtrA1 gene expression levels was performed using real-time PCR. The results were converted to mRNA copy units, and the increase in HtrA1 gene expressions were shown to be significantly different between β-TCP-implanted samples and controls for 4-, 7- and 14-day samples. As shown in Figure 3,

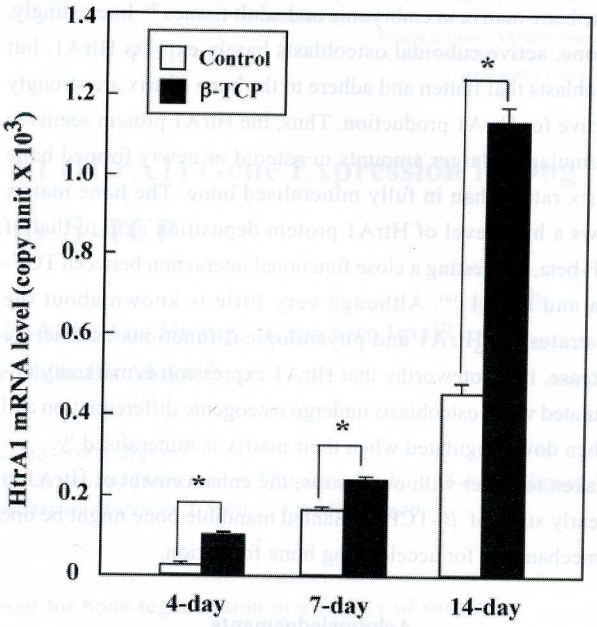

Figure 3: Real-time analysis of HtrA1 mRNA levels. Results were expressed as mRNA copy units by normalising the housekeeping gene GAPDH. The differences between β-TCP implantation and control were determined using a student's *t* test. * $p<0.001$, n=3.

Table 1 Summary of HtrA1 gene expression

Genes	Time (-day)	β-TCP	GeneChip (Fold)	Real-time PCR mRNA copy unit	Fold
HtrA1	4	-	2.9	270.67 ± 8.50	3.8
		+		1019.99 ± 17.70*	
	7	-	ND	1667.00 ± 18.08	1.4
		+		2410.79 ± 27.71*	
	14	-	ND	4594.33 ± 200.50	2.5
		+		11395.86 ± 322.61*	

β-TCP vs Control, * $p < 0.001$, n=3. ND, not determined.

HtrA1 gene expressions were significantly enhanced in 4-, 7- and 14-day samples compared with each control.

The quantities of mRNA levels of HtrA1 and fold changes by β-TCP implantation in dog mandible bone normalised by GAPDH from the GeneChip analysis and real-time RT-PCR are summarised in Table 1.

Discussion

β -TCP has been used for bone regeneration including dental implant therapy. When β -TCP particles are mixed with the blood clot and surrounded by the bony walls of the alveolar socket, osteogenic cells, including undifferentiated mesenchymal stem cells, start migrating from the bone surface between and over the surface of the particles. β -TCP particles in the extraction socket are clinically osteoconductive; however, the molecular basis mechanism for accelerating bone formation is unclear. In this study, we attempted to search the candidate genes that play a role of osteoinductive activity for β -TCP using a dog mandible bone socket model and a GeneChip system, and found the enhancement of HtrA1 gene expression at an early stage.

HtrA1 was originally identified as a gene down-regulated in transformed fibroblasts [7]. Mammalian HtrA1 proteins contain a trypsin-like serine protease domain an IGF-binding domain at the N-terminal end, which might modulate the activity of the associated serine protease with the assumption that HtrA1 represents one of the proteases that regulate the availability of IGFs by cleaving IGF-binding proteins [8]. Mice studies have shown that the HtrA1 gene is primarily expressed by osteoblasts adherent

to the bone matrix in embryonic and adult tissues [9]. Interestingly, in bone, active cuboidal osteoblasts barely express HtrA1, but osteoblasts that flatten and adhere to the bone matrix are strongly positive for HtrA1 production. Thus, the HtrA1 protein seems to accumulate in larger amounts in osteoid or newly formed bone matrix rather than in fully mineralised bone. The bone matrix shows a high level of HtrA1 protein deposition akin to that of TGF-beta, suggesting a close functional interaction between TGF-beta and HtrA1 [10]. Although very little is known about the substrates for HtrA1 and physiological functions as a serine protease, it is noteworthy that HtrA1 expression is markedly up-regulated when osteoblasts undergo osteogenic differentiation and is then down-regulated when their matrix is mineralised [11].

Taken together with our results, the enhancement of HtrA1 at an early stage of β-TCP-implanted mandible bone might be one of mechanisms for accelerating bone formation.

Acknowledgements

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