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Original Article

Alterations of Gene Expression by Beta-tricalcium Phosphate in Osteoblast-like MG63 Cells

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Abstract

Purpose: Beta-tricalcium phosphate (β-TCP) is a synthetic calcium phosphate ceramic that has widely been used as a bone material to repair bone defects. Despite many clinical studies, the molecular mechanism whereby this biomaterial alters the gene expression in osteoblasts to promote bone formation is poorly understood. Thus, we attempted to address this question by using microarray techniques to identify the genes that are differentially regulated in osteoblasts exposed to β-TCP.
Methods: By using DNA microarrays, we identified several genes whose expression levels were significantly up- or down-regulated in osteoblast-likeMG-63cells cultured with β-TCP at a concentration of 100 mg/10 ml for 24 hours.
Results: The differentially expressed genes covered a broad range of functional activities: signal transduction, transcription, cell cycle regulation, vesicular transport, apoptosis, immunity, cytoskeletal elements and cell proliferation and differentiation.
Conclusion: The gene expression changes related to cell proliferation and differentiation, vesicle transport, immunity and defense could affect the osteogenic activities of osteoblasts for bone regeneration. However, further studies will be required to verify the relative importance of these genes in bone formation, their temporal and spatial expression patterns and their interactions with each other.

Key words: Beta-tricalcium phosphate, Bone regeneration, Microarray analysis, Osteoblasts

Introduction

Many graft materials have been used for the repair and restoration of bone defects in oral and maxillofacial surgery. Autogenous bone is accepted as the gold standard for graft material, but it needs to be harvested from other sites of the body. Various kinds of bone materials have been introduced and developed to replace the harvesting of autogenous bone.

Beta-tricalcium phosphate (β-TCP) is a kind of synthetic calcium phosphate ceramic that is widely being used as a bone material to repair bone defects[1]. β-TCP is biocompatible and absorbable, and the mechanism of bone formation by β-TCP is mainly osteoconduction since TCP is composed of calcium and phosphate ions, the most common elements found in bone[1]. β-TCP acts as a space maker and scaffold for bone ingrowth[1], is completely resorbed coinciding with bone formation, and is replaced by vital host bone without residue within 6～12 months[2,3]. β-TCP is completely replaced by newly formed bone because it dissolves in the presence of acids released by cells, such as osteoclasts or macrophages[3]. In a comparative

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histomorphometric study of various graft materials in pigs, TCP showed superior degradation and substitution, as compared to other graft materials, such as hydroxyapatite and demineralized freeze-dried bone allografts[4,5].

Cerasorb (Curasan AG, Kleinostheim, Germany), a synthetic pure-phase β-TCP, has been widely used for bone generation. It is multi-porous and has a polygonal structure[2]. The porosity of the granules contributes to increased surface area, which is available for wetting by tissue fluids and cells, thus promoting its resorption[2]. In addition, growth factors for osteogenesis or osteoconduction can easily penetrate through surface pores[3]. Macropores permit the growth of blood vessels into the matrix to supply blood, cellular, and fibrimental factors, as well as newly formed bone[2,3]. On the other hand, the disadvantage of β-TCP is relatively early resorption independent of new bone formation, resulting in insufficient bone formation, replacement, and the induction of ectopic bone formation due to residues remaining in regenerating tissue[4,6,7].

DNA microarray methodology enables the molecular analysis of gene expression of a very large number of genes spanning a significant fraction of the human genome, in parallel[8]. The analysis is both qualitative and quantitative, since the sensitivity is high enough to detect changes in expression levels in perturbed, as compared to normal cells[9]. The overall result is the generation of a so-called genetic portrait, corresponding to up-regulated or down-regulated genes in the investigated cell system[8-10].

β-TCP's properties and structure have been the subject of many basic and clinical studies. However, there is insufficient information on the direct effects of β-TCP on gene regulation in osteoblasts. Therefore, in this study, we investigated alterations in gene expression in osteoblast-like MG63 cells by β-TCP using DNA microarrays and examined the potential role of β-TCP in bone regeneration.

Materials and Methods

1. Cell culture

Osteoblast-like MG63 cells were cultured in Eagle's minimum essential medium (MEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma) and antibiotics (penicillin 100 U/ml and streptomycin 100 mg/ml, Sigma). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C.

MG63 cells were released with 0.1% trypsin, 0.02% EDTA in Ca^{2+} and Mg^{2+}-free Eagle's buffer (Sigma), collected and seeded at a density of 1×10^5 cells/ml into 9 cm^2 (3 ml) wells. In 6 sets of wells, Cerasorb was added at a concentration of 100 mg/10 ml. After 24 hours, when cultures were sub-confluent, cells were processed for RNA extraction.

2. RNA preparation and hybridization

Total RNA was extracted from cells with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and purified with RNeasy columns (Qiagen, Gaithersburg, MD, USA). After DNase digestion RNA samples were quantified, aliquoted and stored at −80°C until use. RNA purity and integrity were evaluated by denaturing gel electrophoresis, measuring the optical density 260/280 nm ratio, and analysis on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion Biosystems, Foster City, CA, USA) to yield biotinylated cDNA. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA by using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, in vitro transcribed, and labeled with biotin-nucleoside triphosphate. After purification, the cDNA was quantified by using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, NC, USA).

3. Microarray

Seven hundred fifty nanograms of labeled cRNA samples of each group were hybridized to each Human-8 expression bead array (Illumina Inc., San Diego, CA, USA) for 16~18 hours at 58°C. A total of 6 BeadChips were used in this study. Array signals were detected by using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK). Arrays were scanned with an Illumina bead array reader confocal scanner (Illumina Inc.). Export processing and analysis of the array data were conducted by using Illumina BeadStudio Gene Expression Module v. 3.3.8 (Illumina Inc.).

4. Statistical analysis

The array data were filtered by detection *P* values of less than .05 for at least 50% samples. The selected gene signal values were transformed by logarithms and were normalized by the quantile method (n=6). A comparative analysis between each sample was carried out by using fold change data. The fold value meant the fold difference between the test and control groups (test group value/control group value). All data analyses and visualization procedures of differentially expressed genes were conducted by using ArrayAssist (Stratagene, La Jolla, CA, USA) and R statistical language v. 2.4.1. Biological ontology-based analyses were conducted by using the Panther database (http://www.pantherdb.org).

Results

The genes that showed changes in fold values more than 2-fold were found at 24 hours and one hundred fifty-seven genes of the 24,546 genes expressed in BeadChip showed differences compared with those of the control group. Of these, 103 genes were up-regulated, and 54 were down-regulated. The genes that showed differences of more than 3-fold, and were considered to be related to mineralization out of genes that showed a difference of more than 3-fold were listed. Positive fold changes mean up-regulation (Table 1), and negative fold changes mean down-regulation of the gene (Table 2).

Of the 157 genes, the regulated genes were mainly related to transcriptional regulation, transduction, cell cycle control, vesicular transport, cytoskeletal elements, immunity, protein metabolism, and cell proliferation and differentiation.

Discussion

In oral and maxillofacial reconstruction, synthetic materials are widely used[4]. Despite numerous basic and clinical results[1-7], the direct effects of β-TCP on gene expression for osteoblast activity involved in bone regeneration are poorly understood. By using DNA microarrays[8-10], we

Table 1. Up-regulated genes expressed in osteoblast-like MG63 cells incubated with β-TCP at a concentration of 100 mg/10 ml for 24 hours

GeneID	Name	Symbol	Score (folds)	Chromosome
9774	BCL2-associated transcription factor 1	BCLAF1	4.21	6
80205	Chromodomain helicase DNA binding protein 9	CHD9	4.07	16
9867	Praja 2, RING-H2 motif containing	PJA2	3.73	
5707	Proteasome 26S subunit, non-ATPase, 1	PSMD1	3.69	2
10395	Deleted in liver cancer 1	DLC1	3.67	8
2058	Glutamyl-prolyl-tRNAsynthetase	EPRS	3.65	1
7707	Zinc finger protein 148	ZNF148	3.60	3
9652	Tetratricopeptide repeat domain 37	TTC37	3.56	5
5108	Pericentriolar material 1	PCM1	3.55	8
9879	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46	DDX46	3.51	5
57045	Twisted gastrulation homolog 1 (Drosophila)	TWSG1	3.47	18
11031	RAB31, member RAS oncogene family	RAB31	3.46	18
23517	Superkillerviralicidic activity 2-like 2 (S. cerevisiae)	SKIV2L2	3.42	5
23167	EFR3 homolog A (S. cerevisiae)	EFR3A	3.42	8
25963	Transmembrane protein 87A	TMEM87A	3.41	15
51520	Leucyl-tRNAsynthetase	LARS	3.40	5
9790	BMS1 homolog, ribosome assembly protein (yeast)	BMS1	3.39	10
10059	Dynamin 1-like	DNM1L	3.39	12
5597	Mitogen-activated protein kinase 6	MAPK6	3.37	15
150468	Cytoskeleton associated protein 2-like	CKAP2L	3.37	2
7905	Receptor accessory protein 5	REEP5	3.36	5
9276	Coatomer protein complex, subunit beta 2	COPB2	3.35	3
6491	SCL/TAL1 interrupting locus	STIL	3.34	1
23499	Microtubule-actin crosslinking factor 1	MACF1	3.32	1
8826	IQ motif containing GTPase activating protein 1	IQGAP1	3.32	15
3920	Lysosomal-associated membrane protein 2	LAMP2	3.32	
10730	YME1-like 1, encoding mitochondrial protein	YME1L1	3.32	10
10778	Zinc finger protein 271	ZNF271	3.30	18
55133	S1 RNA binding domain 1	SRBD1	3.29	2
51719	Calcium binding protein 39	CAB39	3.28	2

Table 2. Down-regulated genes expressed in osteoblast-like MG63 cells incubated with β-TCP at a concentration of 100 mg/10 ml for 24 hours

GeneID	Name	Symbol	Score (folds)	Chromosome
5062	p21 (CDKN1A)-activated kinase 2	PAK2	−4.72	
10379	Interferon-stimulated transcription factor 3, gamma	IRF9	−4.21	14
55775	Tyrosyl-DNA phosphodiesterase 1	TDP1	−4.10	14
4938	2',5'-oligoadenylate synthetase 1, 40/46 kDa	OAS1	−4.06	12
10534	Sjögren's syndrome/scleroderma autoantigen 1	SSSCA1	−4.04	11
29796	Ubiquinol-cytochrome c reductase complex	UCRC	−3.93	22
8862	Apelin, AGTRL1 ligand	APLN	−3.89	
4501	Metallothionein 1X	MT1X	−3.83	16
30834	Zinc ribbon domain containing 1	ZNRD1	−3.81	6
3860	Keratin 13	KRT13	−3.80	17
644314	Metallothionein E	MTE	−3.79	
1385	cAMP responsive element binding protein 1	CREB1	−3.78	2
4803	Nerve growth factor, beta polypeptide	NGF	−3.77	1
4938	2',5'-oligoadenylate synthetase 1, 40/46 kDa	OAS1	−3.75	12
6590	Secretory leukocyte peptidase inhibitor	SLPI	−3.74	20
4358	MpV17 mitochondrial inner membrane protein	MPV17	−3.73	2
55294	F-box and WD-40 domain protein 7 (Drosophila)	FBXW7	−3.73	4
3665	Interferon regulatory factor 7	IRF7	−3.72	11
468	Activating transcription factor 4	ATF4	−3.72	22
3885	Keratin 34	KRT34	−3.72	17
54434	Slingshot homolog 1 (Drosophila)	SSH1	−3.71	12
4599	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	−3.70	21
55937	Apolipoprotein M	APOM	−3.70	6

identified several genes whose expression was clearly up-regulated and down-regulated when osteoblast-like MG63 cells were treated with β-TCP.

1. Up-regulated genes

β-TCP appeared to up-regulate some functional activities of osteoblast-like MG63 cells. BCLAF1 is an up-regulated transcriptional regulatory gene that induces the apoptotic response to DNA damage for cellular replacement[11], whereas ZNF271 is associated with cell proliferation and differentiation, and is involved in almost all cellular reactions required to maintain genomic stability[12]. The up-regulated activators of cell cycle control (PSMD1, MACF1 and IQGAP1) mediate multi-protein complex assembly and regulate multiple physiological cellular processes, such as cell-cell adhesion, cell polarization, cell migration, and transcription[13,14].

DNM1L is involved in intracellular protein trafficking and endocytosis and belongs to the microtubule-associated protein (MAP) family, which is thought to regulate microtubule dynamics for cell polarization and movement[15]. Although the exact function of this protein has not been determined, there is evidence suggesting a role in mineral accumulation in bone healing, as well as microtubule stabilization[16].

DLC1 and IQGAP1 are up-regulated signal transduction genes. Moreover, COPB2 and TWSG1, which are involved in intracellular trafficking and transport, also have the unusual property of being able to enhance signaling for the bone morphogenetic protein (BMP) family[17]. Up-regulation of these genes might lead to a more rapid osteogenic process by enhancing BMP signaling in the area grafted with β-TCP.

Because osteoblasts conduct new bone formation, osteoconduction is always controlled by cell cycle proliferation and differentiation of osteoblasts. Carinci et al.[8,10] showed that anorganic bovine bone or zirconium oxide up-regulates genes of osteoblast-like MG63 cells involved in the regulation of vesicular transport and cell cycle, proliferation, and differentiation, thereby producing excellent biocompatibility through modifications in the turn-over of extracellular matrix. Similarly, ZNF271, MACF1 and IQGAP1 are considered to have positive effects on growth and differentiation of osteoblasts with higher rates of protein metabolism induced by β-TCP. LAMP2, YME1L1 and MAPK6 are closely regulated by protein kinases for almost all intracellular events, and are highly expressed in protein metabolism and modification[18].

2. Down-regulated genes

β-TCP mainly suppressed the expression of genes in-

volved in the immunological activities of osteoblast-like MG63 cells. OAS1 is known to synthesize intracellular 2',5'-oligoadenylate, which inhibits viral replication through general degradation of RNA[12]. MPV17 is associated with autolysis by encoding an unusual mitochondrial inner membrane protein[19]. The IRF family, such as IRF9 and IRF7, contains nine members to date, with diverse roles in host defense, cell cycle regulation, apoptosis, oncogenesis, and immune cell development[20]. In addition, IRF9, OAS1, and IRF7 are important in interferon-mediated immunity[20,21], and MPV17 functions as an antioxidant in the removal of free radicals for cellular homeostasis[4,19].

Several genes encoding transcriptional regulatory factors are also down-regulated. ZNRD1 encodes a protein consisting of two zinc ribbon domains and may function in multi-drug resistance, tumorigenesis, and the cell cycle[22]. ATF4, which belongs to the ATF/CREB protein family, is required to maintain endoplasmic reticulum homeostasis for calcium storage[23]. KRT13 and KRT34 are cytokeratins, and, thus, are reliable biochemical indicators of the epithelial differentiation process[24]. Transcriptionally induced by interferon, MX1 has antiviral activities against several RNA viruses[25]. Other down-regulated genes encoding for protein metabolism were PAK2, SLPI, FBXW7, and SSH1.

The down-regulation of some genes, with simultaneous up-regulation of others in osteoblast-like MG63 cells by β-TCP could explain the clinical rapid bone formation and biodegradation of β-TCP. For example, β-TCP may simultaneously increase BMP signaling for bone formation and decrease autolysis of osteoblasts around the graft. Also, β-TCP might increase microskeletal MAP signaling for rapid mineral accumulation while temporarily decreasing or holding off the inflammation process originating from the graft. The suppression of immunological response in osteoblasts might promote the early stabilization and settlement of grafted β-TCP while suggesting that more efforts for infection control may be needed after using β-TCP.

Conclusion

Our study showed that β-TCP up-regulated some osteoblast genes that function in mRNA transcriptional regulation, cell proliferation and differentiation, cell cycle control, vesicular transport, production of cytoskeletal elements, as well as protein metabolism and modification. In contrast, β-TCP primarily down-regulated osteoblast genes involved in immunity and in the production of cytoskeletal elements. Via β-TCP, the simultaneous up-regulation of cell proliferation and differentiation, the cell cycle, and vesicle transport, and down-regulation of early phase immunity could result in rapid proliferation, differentiation, and osteogenic functions of the osteoblasts in the grafted area. However, the relative importance for bone formation, temporal and spacial function, and interactions of these genes are not yet clear. Further investigations will be required to understand the molecular interactions between β-TCP and bone regeneration.

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