Evaluation Study

TITANIUM MODULATES DENTAL PULP STEM CELL DIFFERENTIATION

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ABSTRACT

Titanium (Ti) is the most widely used material for dental, orthopedic, and maxillofacial applications because of its excellent biocompatibility and mechanical properties. Several studies have suggested that implant anchorage to bone and soft tissue can be modulated by surface characteristics. Therefore, we studied how titanium can induce osteoblast differentiation in stem cells derived from dental pulp by measuring the expression levels of bone-related genes and stem cell markers using real-time polymerase chain reaction. The results demonstrated that the upregulation of SP7 and MMP12 enhanced differentiation via the activation of stem cells. Titanium disks facilitate implant integration and promote cell activation.

KEYWORDS: *titanium, disks, osteoblasts, gene, expression*

INTRODUCTION

Titanium implants are renowned for their biocompatibility, which allows osseointegration, the direct structural and functional connection between the implant and the surrounding bone tissue. Additionally, titanium exhibits excellent corrosion resistance, ensuring long-term stability and durability of dental implants in the oral environment. Moreover, the mechanical properties of titanium, including its high strength make it an ideal material for dental implantation (1-3).

Titanium dental implants are used in various clinical scenarios, including single-tooth replacement, multiple-tooth restorations, and full-arch rehabilitation. They offer predictable outcomes and provide patients with functional and aesthetically pleasing solutions for missing teeth. Titanium implants are compatible with various prosthetic options ranging from single crowns to fixed bridges and implant-supported overdentures (4, 5).

Titanium dental implants offer several advantages, including high success rates, excellent long-term stability, and minimal invasiveness during placement. Their biocompatibility ensures a favorable tissue response, facilitates osseointegration, and promotes long-term implant survival. Titanium implants also provide clinicians with versatility in treatment planning, thereby enabling tailored solutions to meet individual patient needs (6, 7).

Titanium has revolutionized modern dentistry owing to its unique properties and versatile applications. This light, strong, and biocompatible metal is currently the material of choice for dental implants, prosthetics, and other dental devices. Its ability to bond with bone (osseointegration) and corrosion resistance make it an ideal material for long-term dental applications (2, 8).

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Titanium, particularly in its commercially pure form (cpTi) and titanium alloys, exhibits several properties that make it highly suitable for dental applications. Titanium is non-toxic and not rejected by the body, which minimizes the risk of adverse reactions and ensures patient safety.

Titanium dental implants have a high success rate, often exceeding 95%, which makes them a reliable choice for tooth replacement. The ability of titanium to integrate with the bone and tissue reduces discomfort and provides a natural look and feel, enhancing patient satisfaction. Ti can be tailored for various dental needs, from small orthodontic wires to robust implant posts, catering to a wide range of treatments (9, 10).

Integrating Ti with stem cell technology has led to innovative solutions in several medical fields (11-13). For this reason, we studied how titanium can induce osteoblast differentiation in stem cells derived from dental pulp (DPSCs) by measuring the expression levels of bone-related genes and mesenchymal stem cell markers by real-time RT-PCR. In addition, we investigated the same gene in the well-known cell line TE85. TE85 cells, derived from a human osteosarcoma cell line, are widely used in bone biology, cancer biology, and cell signaling pathways research.

MATERIALS AND METHODS

Dental Pulp Stem Cells (DPSCs) Isolation

Dental pulp was extracted from the third molars of healthy subjects and digested for 1 h at 37°C in a solution containing 1 mg/ml collagenase type I and 1 mg/ml dispase, dissolved in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 µg/ml clarithromycin. The solution was then filtered using 70 µm Falcon strainers (Sigma Aldrich, St Louis, Mo, U.S.A.) to separate mesenchymal stem cells from fibroblasts. Stem cells were cultivated in α-MEM culture medium (Sigma Aldrich, St Louis, Mo, U.S.A.) supplemented with 20% Fetal Bovine Serum (FBS), 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Aldrich, St Louis, Mo, U.S.A.). The flasks were incubated at 37 °C and 5% CO_2, and the medium was changed twice per week.

DPSCs were characterized by immunofluorescence for the cytoskeletal component vimentin, positive mesenchymal stem cell markers CD90 and CD73, and the negative marker CD34 as described in Sollazzo et al. (14).

TE85 cell culture

TE85 osteosarcoma cell lines were cultured in sterile wells containing DMEM supplemented with 10% FBS and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C.

Cell treatment

DPSCs and TE85 were seeded at a concentration of 1.0×10^5 cells/ml on titanium disks in 9 cm^2 (3 ml) wells containing DMEM supplemented with 10% serum and antibiotics. Another set of wells containing untreated cells was used as control. The treatment was carried out at two time points: 24 h and 4 days.

The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. At the end of the treatment period, the cells were lysed and processed for total RNA extraction.

RNA isolation, reverse transcription, and quantitative real-time RT-PCR

According to the manufacturer's instructions, total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Hilden, Germany). The pure RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA synthesis was performed starting from 500 ng of total RNA using the PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan). The reaction mixture was incubated at 37 °C for 15 min and inactivated by heating at 70 °C for 10 s. cDNA was amplified by real-time quantitative PCR using an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). All PCR reactions were performed in a 20 µL volume. Each reaction contained 10 µl of 2x qPCRBIO SYGreen Mix Lo-ROX (PCR Biosystems, Ltd., London, UK), 400 nM of each primer, and cDNA.

Sigma Aldrich purchased custom primers belonging to the “extracellular matrix, adhesion molecule” pathway, “osteoblast differentiation” pathway, and “inflammation” pathway. Table I lists the selected genes grouped by functional pathways.

Table I. *Selected genes used in Real Time PCR grouped by functional pathway.*

Pathway	Gene
Osteoblast differentiation	SPP1 (Osteopontin) SPARC (Osteonectin) RUNX2 (Runt-related transcription factor 2) ALP (Alkaline phospatasi) BGLAP (Osteocalcin) FOSL1 (FOS-like antigen 1) SP7 (Osterix) ENG (Endoglin)
Extracellular matrix, adhesion molecule	COL1A1 (Collagen type I alpha1) COL3A1 (Collagen, type III, alpha 1) MMP7 (Matrix Metallopeptidase 7) MMP12 (Matrix Metallopeptidase 12)
Inflammation	IL1α (Interleukin 1 Alpha) IL1R (Interleukin 1 Receptor Type 1)
Reference gene	RPL13 (Ribosomal protein L13)

All experiments were performed using non-template controls to exclude reagent contamination. PCR was performed using two analytical replicates. The amplification profile was initiated by incubation for 10 min at 95°C, followed by a two-step amplification for 15 s at 95°C and 60 s at 60°C for 40 cycles. In the final step, melt curve dissociation analysis was performed.

Statistical analysis

The gene expression levels were normalized to the expression of the reference gene (RPL13) and expressed as fold changes relative to the expression in untreated cells. Quantification was performed using the delta-delta Ct method (10).

RESULTS

The DPCSs were phenotypically characterized using immunofluorescence. Fig. 1a shows cytoskeletal filaments stained with vimentin. The cell surfaces were positive for mesenchymal stem cell markers CD90 (Fig. 1b) and CD73 (Fig. 1c), and negative for markers of hematopoietic origin CD34 (Fig. 1d).

Titanium treatment in DPSCs was analyzed using quantitative real-time PCR after 24 h and 4 days of treatment, and the expression levels of osteoblast-related genes, extracellular matrix, and inflammation pathways were measured. Tables II and III report the significant fold changes obtained after 24 h and 4 days, respectively, for each cell type (DPSCs and TE85).

Significantly upregulated genes showed ≥ 2-fold change in expression (P value ≤ 0.05), while significantly downregulated genes showed ≤ 0.5 -fold change in expression (P value ≤ 0.05). After 24 h of treatment, SP7, MMP7, MMP12, and IL1α were strongly upregulated in DPSCs (Table II). MMP7 and MMP 12 levels decreased after four days. SP7, MMP7, and MMP12 were overexpressed (Table III).

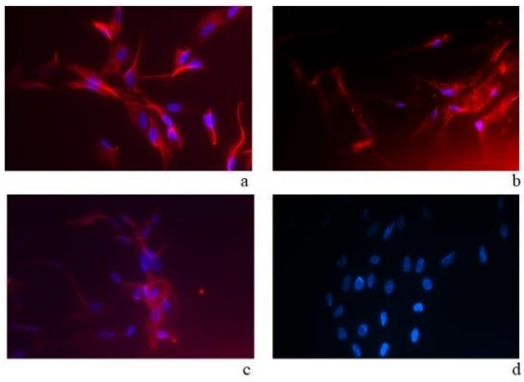

Fig. 1. *DPCSs by indirect immunofluorescence (Rhodamine). Immunofluorescence staining of vimentin (**a**), mesenchymal stem cell marker CD73 (**b**), CD90 (**c**), and hematopoietic markers CD34 (**d**). Nuclei were stained with DAPI. Original magnification x40.*

Table II. *Gene expression in DPSCs after 24h and 4 days of treatment. Numbers express the fold changes of the de-regulated genes in treated cells vs. untreated cells. ND – not determined. In bold significant gene expression level.*

Gene	24 h	4 days
SPP1	nd	nd
SPARC	0.8	0.6
RUNX2	0.7	0.7
ALP	0.7	0.3
BGLAP	nd	nd
FOSL1	nd	0.7
SP7	**8.3**	0.9
ENG	0.8	1.2
COL1A1	0.4	0.5
COL3A1	0.9	0.8
MMP7	**15.8**	**3.1**
MMP12	**6.8**	**2**
IL1α	**7.3**	1.2
IL1R	0.7	0.7

Table III. *Gene expression in TE85 after 24h and 4 days of treatment. Numbers express the fold changes of the de-regulated genes in treated cells vs. untreated cells. ND – not determined. In bold significant gene expression level.*

Gene	24 h	4 days
SPP1	0,59	0,86
SPARC	0,61	1,6
RUNX2	0,45	1,07
ALP	0,54	1,27
BGLAP	nd	nd
FOSL1	0,6	1
SP7	**9,5**	0
ENG	0,8	1,3
COL1A1	0,43	1,43
COL3A1	0,3	0,8
MMP7	**7,3**	0,5
MMP12	**7,1**	1,1
IL1α	1,4	1,8
IL1R	0,4	0,7

DISCUSSION

Since SP7 and MMP12 were upregulated after 24 h and 4 days, respectively, these genes seem to play a prominent role in the titanium-cell interaction.

SP7, also known as osterix, is a zinc-finger-containing transcription factor that plays a critical role in bone formation and osteoblast differentiation (15). Identified as a key regulator of the genetic network controlling osteogenesis, SP7 functions downstream of RUNX2, which is another essential transcription factor in bone development. The study of SP7 is vital for understanding the molecular mechanisms underlying bone formation, maintenance of bone health, and the pathogenesis of bone-related diseases (16).

SP7 is indispensable for osteoblast differentiation and bone formation. It acts primarily by binding to specific promoter regions of osteoblast-related genes, thereby activating their transcription. Key target genes include those encoding bone matrix proteins, such as collagen type I, osteocalcin, and bone sialoprotein, which are essential components of the extracellular matrix in the bone (15).

During skeletal development, SP7 expression was first detected in pre-osteoblasts and continues in mature osteoblasts, highlighting its role throughout the osteogenic lineage. The absence of SP7 results in a complete lack of bone formation, as observed in SP7 knockout mice, which exhibit severe skeletal defects and perinatal lethality. This underscores SP7's essential role in the transition of mesenchymal stem cells into fully differentiated osteoblasts.

In addition to its role in osteoblasts, SP7 influences the differentiation of chondrocytes, which are responsible for cartilage formation. Although primarily known for its osteogenic functions, emerging evidence suggests that SP7 may play a role in regulating the balance between osteogenesis and chondrogenesis, which is crucial for endochondral ossification, the process by which long bones are formed (17).

Given its pivotal role in bone formation, mutations in or dysregulation of SP7 have been associated with various bone disorders. Osteogenesis imperfecta, a genetic disorder characterized by brittle bone, has been linked to mutations in SP7. Patients with these mutations exhibit symptoms such as frequent fractures, bone deformities, and growth deficiencies, reflecting impaired osteoblast function and bone matrix production (18).

SP7 has also been implicated in osteoporosis, a condition characterized by reduced bone mass and an increased fracture risk. Dysregulation of SP7 expression or activity can disrupt the balance between bone formation and resorption, contributing to the development of osteoporosis (19). Inflammatory conditions such as rheumatoid arthritis also affect SP7 function, where chronic inflammation impairs osteoblast differentiation and activity, leading to bone erosion and joint damage.

Understanding SP7's role in bone biology opens new avenues for therapeutic interventions to enhance bone formation and treat bone-related diseases. SP7 is a target for developing biomaterials and scaffolds for bone tissue engineering in regenerative medicine. Incorporating SP7 or its modulators into scaffolds can enhance osteoblast differentiation and bone regeneration, thereby providing effective treatment for bone defects and fractures (20). Our study showed SP7 was up-regulated, demonstrating its important role in bone formation in implant dentistry.

Metalloproteinase 12 (MMP12), also known as macrophage elastase, is a member of the matrix metalloproteinase (MMP) family, which consists of enzymes responsible for the degradation of extracellular matrix (ECM) components. Macrophages primarily produce MMP12 which is notable for its ability to degrade elastin, a key ECM protein that provides tissue elasticity. This enzyme plays a significant role in tissue remodeling, inflammation, and various pathological conditions, including chronic obstructive pulmonary disease, atherosclerosis, and cancer.

The primary physiological role of MMP12 is the degradation and remodeling of the ECM, with a particular affinity for elastin. This elastolytic activity is crucial for maintaining tissue elasticity and integrity, particularly in tissues such as the lungs, skin, and blood vessels. MMP12 is involved in the turnover and repair of elastic fibers in the lungs, contributing to normal respiratory function (21).

MMP12 is also involved in the modulation of the inflammatory response. MMP12 facilitates the migration of immune cells to sites of injury or infection by degrading extracellular matrix components. MMP12 can process and activate other bioactive molecules, such as cytokines and chemokines, thereby influencing the inflammatory milieu (22).

The involvement of MMP12 in various diseases makes it a potential diagnostic and prognostic biomarker. Elevated MMP12 levels in tissues and bodily fluids can indicate disease activity and progression. Given its role in tissue destruction and inflammation, MMP12 is an attractive therapeutic target. MMP12 inhibitors have been explored for their potential in the treatment of diseases such as atherosclerosis and cancer. In our study, MMP12 expression was upregulated, demonstrating its important role in bone formation in implant dentistry.

CONCLUSIONS

SP7 and MMP12 are involved in the regulation of bone formation and osteoblast differentiation. Their essential roles in skeletal development and maintenance, combined with their involvement in various bone diseases, underscore their importance in bone biology.

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