Experimental Study

GENE EXPRESSION OF STEM CELLS TREATED WITH MARINE-DERIVED POROUS CARBONATED ALGA IN VITRO

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ABSTRACT

Marine-derived porous carbonated red algae (MDPCRA) is a bone substitute for bone regeneration in dentistry. It is manufactured through a controlled process, resulting in a porous scaffold with interconnected pores and a high surface area conducive to cell attachment, proliferation, and tissue ingrowth. MDPCRA exhibits excellent biocompatibility, osteoconductivity, and resorbability, making it an ideal substrate for bone regeneration and remodeling. Dental follicle stem cells (DFSCs) are a population of mesenchymal stem cells found within the dental follicle surrounding developing teeth. DFSCs exhibit self-renewal capacity and multipotent differentiation potential, allowing them to differentiate into various cell types, including osteoblasts, cementoblasts, adipocytes, and periodontal ligament fibroblasts. To verify how MDPCRA stimulates bone regeneration, we treated dental DFSCs with MDPCRA to obtain information regarding the expression of genes related to osteoblast differentiation. In DFSCs, after 24 h of treatment, MMP15 was up-regulated. After 4 days of treatment, MMP15 still increased, and IL6 and SP7 were also upregulated. In conclusion, MDPCRA can stimulate several genes in DFSCs involved in osteoblast differentiation.

KEYWORDS: *marine-derived porous carbonated red alga, dental follicle, stem cells, bone regeneration, gene expression*

INTRODUCTION

Among various bone substitutes for bone regeneration in dentistry is a marine-derived porous carbonated red alga (MDPCRA). It is manufactured through a controlled process, resulting in a porous scaffold with interconnected pores and a high surface area conducive to cell attachment, proliferation, and tissue ingrowth (1, 2). MDPCRA exhibits excellent biocompatibility, osteoconductivity, and resorbability, making it an ideal substrate for bone regeneration and remodeling. It has been used in various oral surgical procedures for bone augmentation. It is a bone graft substitute, providing structural support and promoting new bone formation (3, 4). Unlike autografts, MDPCRA eliminates the need for additional donor-site surgery. Its porous structure and interconnected pore network facilitate vascularization and tissue integration, promoting faster healing and improved clinical outcomes. Moreover, it resorbs gradually over time, allowing for replacement by host bone tissue (5, 6).

Dental follicle stem cells (DFSCs) are a population of mesenchymal stem cells found within the dental follicle surrounding developing teeth. DFSCs exhibit self-renewal capacity and multipotent differentiation potential, allowing

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them to differentiate into various cell types, including osteoblasts, cementoblasts, adipocytes, and periodontal ligament fibroblasts. DFSCs can be isolated from the dental follicle tissue of impacted third molars or extracted teeth undergoing orthodontic treatment. Various isolation techniques, such as enzymatic digestion and explant culture, have obtained a heterogeneous population of DFSCs from dental follicle tissues (7, 8). DFSCs are promising for various therapeutic applications in regenerative dentistry and oral maxillofacial surgery. They have been investigated for their potential to regenerate periodontal tissues, including the cementum, periodontal ligament, and alveolar bone, in cases of periodontal disease, dental trauma, and tooth loss. Additionally, DFSCs have shown potential for use in dental implantology, root canal therapy, and craniofacial bone defects (9, 10). Their immunomodulatory properties render them attractive candidates for treating inflammatory and autoimmune oral diseases.

To verify how MDPCRA stimulates bone regeneration, we treated dental DFSCs with MDPCRA to get information regarding gene expression related to osteoblast differentiation.

MATERIALS AND METHODS

Dental Follicle Stem Cells (DFSCs) Isolation

A dental follicle was collected during third molar extraction 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 weekly (6).

DFSCs 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. (11).

Cell treatment

DFSCs were seeded at a concentration of 1.0×10^5 cells/ml Algipore (Dentspay Italia SRL, Roma, Italy) at the concentration of 10 mg/ml 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 a 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 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.

Custom primers belonging to the “extracellular matrix, adhesion molecule” pathway, “osteoblast differentiation,” and “inflammation” pathway were purchased from Sigma Aldrich. The selected genes grouped by functional pathways are as follows: osteoblast differentiation [SPP1 (Osteopontin), SPARC (Osteonectin), RUNX2 (Runt-related transcription factor 2), ALP (Alkaline phosphatase), BGLAP (Osteocalcin), FOSL1 (FOS-like antigen 1), SP7 (Osterix), ENG (Endoglin)], extracellular matrix, adhesion molecule [COL1A1 (Collagen type I alpha1), COL4A1 (Collagen type IV alpha 1), MMP14 (Matrix Metallopeptidase 12), MMP15 (Matrix Metallopeptidase 15)], inflammation [IL6 (Interleukin 6), IL6R (Interleukin 6 Receptor)] and RPL13 (Ribosomal protein L13) as reference gene. 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 (12).

RESULTS

DFCSs were phenotypically characterized using immunofluorescence. Fig. 1a shows a cytoskeletal filament 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).

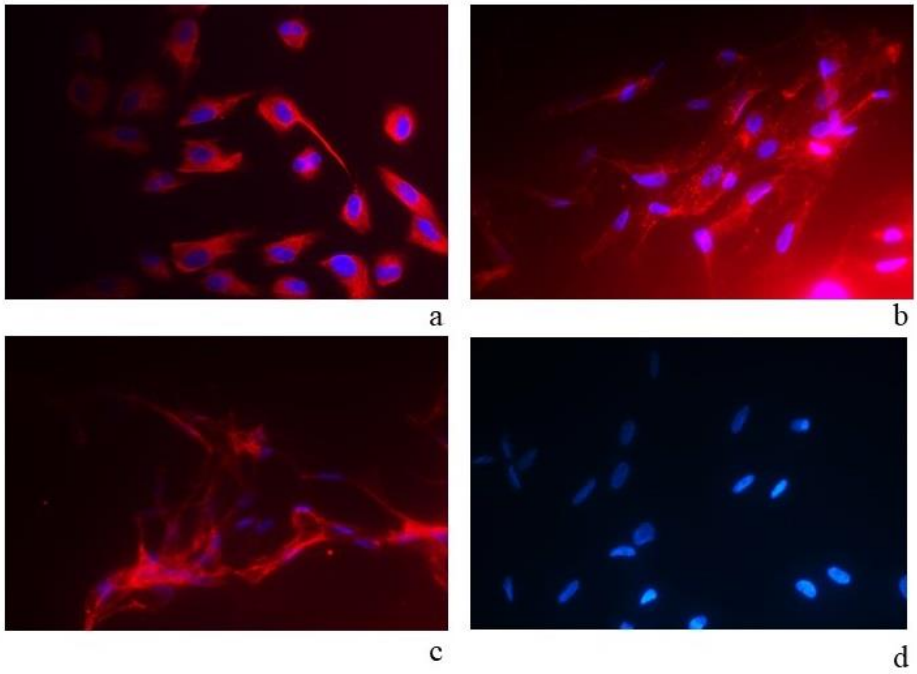

Fig. 1. *DFCSs by indirect immunofluorescence (Rhodamine). Immunofluorescence staining of vimentin (a), mesenchymal stem cell marker CD90 (b), CD73 (c), and hematopoietic markers CD34 (d). Nuclei were stained with DAPI. Original magnification x40.*

MDPCRA treatment of DFSCs was analyzed by 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. Table I reports the significant fold-change obtained after 24 h and 4 days for DFCSs (bold).

Significantly up-regulated genes showed ≥ 2-fold change in expression (P value ≤ 0.05) while significantly down-regulated genes showed ≤ 0.5-fold change in expression (P value ≤ 0.05). In DFSCs, after 24 h of treatment, MMP15 was up-regulated (Table I). After 4 days of treatment, MMP15 was still increased and IL6 and SP7 were up-regulated.

Table I. *Gene expression in DFCSs 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.*

	24 h	**4 gg**
SPP1	nd	nd
SPARC	0.3	0.3
RUNX2	0.4	0.5
ALP	0.3	0
BGLAP	nd	nd
FOSL1	0.6	1.3
SP7	0.8	**2.6**
ENG	0.6	1
COL1A1	0.3	0.1
COL4A1	0.6	0.5
MMP14	**1.5**	1.3
MMP15	**2**	**2.4**
IL6	1.6	**2.1**
IL6 R	1.2	1.2

DISCUSSION

Matrix Metalloproteinase 15 (MMP 15), also known as membrane-type matrix metalloproteinase 2 (MT2-MMP), is a member of the matrix metalloproteinase (MMP) family of enzymes that play crucial roles in the degradation and remodeling of the extracellular matrix (ECM). MMP XV is unique among MMPs because of its localization to the cell membrane and its ability to activate other MMPs and process various ECM components (13-16).

MMP15 is synthesized as an inactive zymogen that undergoes post-translational modifications to become enzymatically active. It consists of several domains, including a signal peptide, prodomain, catalytic domain containing the zinc-binding motif essential for proteolytic activity, hemopexin-like domain, and transmembrane domain that anchors it to the cell membrane. The prodomain acts as an autoinhibitory module that prevents premature enzyme activation. The expression and activity of MMP15 are tightly regulated at multiple levels. Transcriptionally, MMP15 expression is induced by various stimuli, including growth factors, cytokines, and mechanical stress, through the activation of specific transcription factors, such as AP-1 and NF-κB. Post-translational modifications, including glycosylation and proteolytic cleavage, regulate MMP15 activity and localization. Moreover, tissue inhibitors of metalloproteinases (TIMPs) and other endogenous inhibitors modulate MMP15 activity, maintaining a balance between ECM degradation and synthesis (17, 18).

MMP15 is primarily localized in the cell membrane, where it functions in pericellular proteolysis and ECM remodeling. One of MMP15's unique functions is its ability to activate other MMPs such as MMP2 and MMP13, by cleaving their prodomains, thereby amplifying ECM degradation. MMP15 also cleaves various ECM components, including collagen type I, fibronectin, and laminin, thereby facilitating cell migration, invasion, and angiogenesis. Beyond its role in ECM remodeling, MMP15 participates in various physiological processes including embryonic development, tissue repair, and immune responses. MMP15 contributes to morphogenetic processes such as branching morphogenesis in the lung and mammary glands during development. In wound healing and tissue repair, MMP15 facilitates the clearance of damaged ECM and promotes the migration of fibroblasts and endothelial cells to the site of injury (19, 20).

Interleukin 6 (IL6) is a multifunctional cytokine that plays a pivotal role in immune regulation, inflammation, and tissue homeostasis. Beyond its classical role in the immune system, IL 6 has emerged as a critical regulator of bone metabolism, influencing both the bone formation and resorption processes.

IL6 exerts its effects through the IL6 receptor (IL6R) and signal-transducing component glycoprotein 130 (gp130), which form a complex upon IL6 binding. This IL6/IL6R/gp130 complex activates intracellular signaling pathways, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)/Akt pathways. These pathways regulate gene expression, cell proliferation, differentiation, and survival (21-23).

IL6 plays a dual role in regulating osteoblast functions. Under physiological conditions, low concentrations of IL6 stimulate osteoblast proliferation and differentiation and promote bone formation. IL6 enhances the expression of osteogenic genes such as runt-related transcription factor 2 (RUNX2) and osteocalcin, facilitating osteoblast maturation and mineralization. Additionally, IL6 promotes osteoblast survival by activating anti-apoptotic pathways. IL6 also influences osteoclast function, albeit indirectly, through its effects on other cell types. IL6 stimulates RANKL production by osteoblasts and stromal cells, thereby promoting osteoclast differentiation and activity. Additionally, IL6 enhances the macrophage colony-stimulating factor (M-CSF) expression, which is essential for osteoclast precursor proliferation and survival (23).

IL6 plays a complex role in bone metabolism and influences both the bone formation and resorption processes. Under physiological conditions, IL6 stimulates osteoblast activity and promotes bone formation, whereas dysregulated IL6 signaling in pathological conditions leads to bone loss and an increased risk of fractures.

Understanding the molecular mechanisms underlying IL6's effects on bone cells and its involvement in bone-related disorders is essential for developing targeted therapies to preserve bone health and to treat osteoporosis, rheumatoid arthritis, and other bone-related diseases. Ongoing research into IL6 biology and its therapeutic potential holds promise for improving treatment outcomes and addressing unmet medical needs in skeletal disorders (23).

CONCLUSIONS

In conclusion, MDPCRA is a bone graft substitute with various applications. Its biocompatibility, osteoconductivity, and resorbability make it an attractive option for promoting bone regeneration and tissue remodeling in a wide range of clinical scenarios. Here, we demonstrated that MDPCRA can stimulate several genes of DFSCs involved in osteoblast differentiation. However, we know that more experiments are needed to establish the global effect of MDPCRA on bone formation.

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