Investigative Study

ANORGANIC BOVINE BONE MATRIX TREATED WITH P15 EFFECTS IN OSTEOBLASTIC STEM CELL DIFFERENTIATION

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ABSTRACT

Molecular interaction between stem cells and grafting biomaterial is a key factor for the success of regenerative surgery. The covalent attachment of P15, a synthetic peptide mimicking the cell-binding site of collagen type I, to biomaterials could favor integrins binding and activation of multiple cell signaling pathways. In this investigation, we studied the effect of P15-coated anorganic bone matrix on bone marrow stem cells cultured *in vitro*. The expression level of a panel of differentiation markers was monitored to verify cell differentiation towards the osteoblast lineage. We observed some signs of osteoblast differentiation, such as the over-expression of osterix and under-expression of endoglin. However, most typical markers, osteonectin, osteopontin, osteocalcin, and alkaline phosphatase, were strongly under-expressed.

KEYWORDS: *p15, bone marrow stem cells, bone regeneration, gene expression*

INTRODUCTION

Bone replacement graft materials are used in orthopedic and oral surgery to encourage new bone formation. Experimental data demonstrated that different extracellular matrix proteins may improve cell attachment to synthetic and anorganic graft materials (1). Cells can bind extracellular matrix proteins with a family of transmembrane receptors named integrins. (2). Integrins can activate and modulate multiple signaling pathways. As a result, cell-substrate interaction may influence cell behavior throughout cell cycle regulation, directing cells to live or die, to proliferate, or to exit the cell cycle and differentiate (3). Specifically, integrins can play an important role in osteoblast differentiation as well as in bone remodeling (4).

Cells bind collagen type I, the main bone matrix protein, using the integrin α2β1 receptor. The specific binding site on type I collagen is the short aminoacidic sequence 766-GTPGPQGIAGQRGVV-780, (5, 6). A synthetic peptide mimicking the cell-binding site of collagen type I, named P15, has been frequently employed in attempting to enhance the regenerative potential of biomaterials. In osteoregenerative surgery, P15 has been conjugated with different materials to improve osteoblast and mesenchymal cell attachment and to promote cell activities essential for bone formation and regeneration (5, 7). The covalent attachment of P15 to titanium surfaces has been shown to improve cell adhesion, proliferation, and maturation, highlighting its efficacy in promoting tissue regeneration in orthopedic procedures (8).

A common source of bone allograft material is bovine or porcine bone. The bone is treated to obtain a scaffold for new bone growth that is essentially composed of its anorganic part, the hydroxyapatite, a crystalline form of calcium

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phosphate. This porous material is commonly used in periodontal surgery, dental implantology, and maxillofacial surgery to fill bone defects, support dental implants, and reconstruct jaw bones. To improve the osteoconductivity of anorganic bone and promote the natural healing process, this material was coated with the peptide P15. Some investigations were aimed to compare P15 treated and untreated hydroxyapatite with alternate results (9).

Bone marrow stem cells (BMSCs) are multipotent stromal cells able to differentiate into various cell lineages, including osteoblasts, adipocytes, chondrocytes, and myocytes (10). They contribute together with periosteal cells to bone fracture healing and play a central role in orthopedic and oral regenerative applications (10, 11). BMSCs can be isolated from bone marrow aspirates obtained from the iliac crest or other long bones through minimally invasive procedures or from peripheral blood (12, 13). The BMSCs have been extensively studied and represent one of the main sources of cells for several regenerative medicine applications (14).

In this investigation, we studied the effect of P15-coated anorganic bone matrix on BMSCs cultured *in vitro*. The expression level of different differentiation markers was monitored to verify cell differentiation towards the osteoblast lineage.

MATERIALS AND METHODS

Isolation of Bone Marrow Stem Cells (BMSCs)

Bone marrow was extracted from the iliac crest of subjects (operated for grafting rehabilitation after trauma) 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 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 remove debris and large cell aggregates. Isolated cells were cultivated in α-MEM culture medium (Sigma-Aldrich, St Louis, MO, U.S.A.) supplemented with 20% Fetal Bovine Serum, 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. Adherent cells cultured in this medium were identified as BMSCs.

Immunofluorescence staining revealed the presence of mesenchymal stem cell markers, such as CD90 and CD73, while the hemopoietic marker CD34 was not detected.

Cell treatment

BMSCs were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were seeded at a concentration of 1.0×10^5 cells/ml with P15-coated anorganic bovine bone (Dentsply Friadent Ceramed, Lakewood CO) at the concentration of 10 mg/ml in 9 cm^2 (3 ml) wells containing DMEM supplemented with 10% serum and antibiotics.

The treatment was carried out at two time points: 24 h and 4 days. The treatments were triplicated in different wells. Another set of wells containing untreated cells was used as a control. 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

Total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 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 listed in Table I.

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.

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 phosphatase) 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	IL1A (Interleukin 1 alpha) IL1R1 (Interleukin 1 Receptor type 1)
Reference gene	RPL13 (Ribosomal protein L13)

Statistical analysis

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

RESULTS

The BMSCs 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).

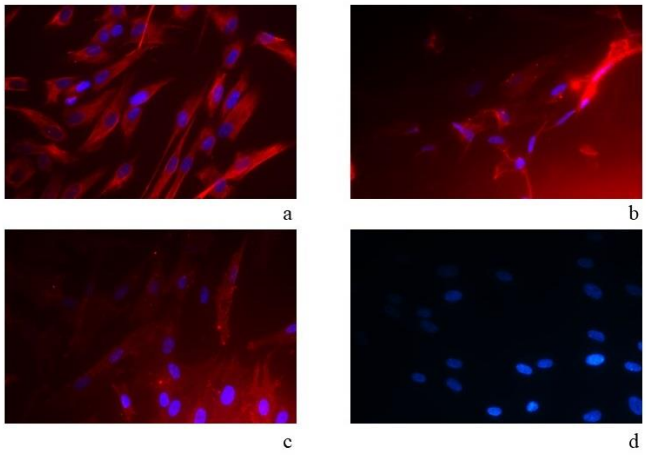

Fig. 1. *BMSCs by indirect immunofluorescence (Rhodamine). Immunofluorescence staining of vimentin (a), mesenchymal stem cell marker CD90 (b), CD73 (c), and hematopoietic markers CD34 (d). Nuclei were stained with DAPI. Original magnification x40.*

The effect of P15-coated anorganic bone matrix treatment in BMSCs 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 II reports expression fold change obtained after 24 h and 4 days. An expression level change equal to or greater than 2 or equal to or less than 0.5 was considered biologically relevant.

An up-regulation of the IL1A gene was observed after 24 h of treatment. After 4 days, SP7 was the only osteogenic marker upregulated, while all the other appeared under-expressed.

Table II. *Gene expression in DPSCs after 24 h 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 days**
SPP1	ND	ND
SPARC	0.7	**0.3**
RUNX2	0.8	**0.4**
ALP	0.6	**0.1**
BGLAP	ND	ND
FOSL1	0.9	**0.4**
SP7	1.5	**7.3**
ENG	1.3	**0.3**
COL1A1	0.9	**0.4**
COL3A1	0.7	**0.4**
MMP7	1.5	1.7
MMP12	1.7	1.4
IL1A	**2.6**	1
IL1R1	1.2	**0.2**

DISCUSSION

The peptide P15 coating has emerged as a common practice to enhance the performance of biomaterials used in bone regeneration processes. P15, a synthetic peptide that mimics the integrins binding site on collagen type I, should favor cell adhesion to biomaterials, thus activating signaling pathways for cell proliferation and differentiation. In this investigation, we tested the ability of bovine bone to stimulate BMSCs through osteoblastic differentiation. The expression profiles of several differentiation markers were monitored at 24 hours and 4 days of treatment. At 24 hours, the expression profile resulted unchanged, apart from a 2.6-fold increase of the IL1A gene. This is not considered a sign of cell differentiation but may represent a response to cell stress or damage.

The IL1A gene codes for interleukin-1 alpha (IL-1α), a pro-inflammatory cytokine belonging to the interleukin-1 family, which plays a crucial role in the regulation of immune responses, inflammation, and tissue homeostasis (15). IL1A is constitutively expressed in many cell types in healthy tissues at a steady state, and its expression can be increased in response to growth factors and proinflammatory or stress-associated stimuli, such as canonical proinflammatory mediators that activate toll-like receptors (16).

IL-1α plays a pivotal role in the early stages of inflammation by promoting the recruitment of immune cells to the site of injury or infection and stimulating the production of other inflammatory mediators like IL6 and TNFα (17). Dysregulation of IL-1α is associated with various inflammatory diseases, including autoimmunity, cancer, and infectious diseases. It is one of the key cytokines involved in the pathogenesis of graft-*versus*-host disease, a major limiting factor in transplant success (18).

At four days of treatment, the IL1A expression appears normalized, while other differentiation markers appear dysregulated. Indeed, a marked over-expression of osterix, coded by the SP7 gene was observed.

Osterix is a transcription factor that plays a critical role in osteoblast differentiation and bone formation (19, 20). Indeed, osterix is essential for the activation of osteoblast-specific genes, such as osteonectin, osteopontin, osteocalcin, and alkaline phosphatase, which are fundamental components of bone extracellular matrix and its mineralization (21, 22). Identified as a key regulator of the genetic network controlling osteogenesis, SP7 functions downstream of RUNX2, another fundamental transcription factor in bone development (22, 23). Other findings suggest that osterix is regulated via both RUNX2-dependent and -independent mechanisms and that osterix controls osteoblast differentiation, at least in part, by regulating the expression of genes not controlled by RUNX2 (24). Given its role in bone formation, mutations in or dysregulation of SP7 are associated with various bone disorders. Common SP7 polymorphisms are associated with bone mineral density and fracture risk, while rare SP7 mutations cause skeletal dysplasia, and SP7 may contribute to bone metastasis (25). Osteogenesis imperfecta, a genetic disorder characterized by brittle bones, has been linked to mutations

in SP7 (26). Patients with these mutations exhibit symptoms such as frequent fractures, bone deformities, and growth deficiencies, reflecting impaired osteoblast function and bone matrix production (27).

Osteoporosis is a condition characterized by reduced bone mass and increased fracture risk. Dysregulation of SP7 expression or activity can disrupt the balance between bone formation and resorption, contributing to the development of osteoporosis (25). In addition to its role in osteoblasts, SP7 also 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, a process by which long bones are formed (28).

At 4 days after treatment with P15-hydroxyapatite, osterix appeared overexpressed, while its typical activator, RUNX2, or their downstream targets resulted in under-expressed. These data need to be confirmed and seem difficult to explain. Possibly, additional time points may help to explain this anomaly. On the other hand, we observed endoglin under-expression, a potential sign of osteoblast differentiation (29, 30). Indeed, endoglin is normally expressed in human mesenchymal stem cells, while its expression decreases as cells differentiate into an osteogenic lineage (31).

Osteonectin, osteopontin, osteocalcin, and alkaline phosphatase are all considered markers of osteoblast differentiation because they are abundantly synthesized by active osteoblasts and play essential roles in bone development, remodeling, and mineralization (32). Osteonectin is a matrix glycoprotein that interacts with hydroxyapatite, calcium, and type I collagen (33). Osteopontin plays a crucial role in bone mineralization and the attachment of osteoclasts to the mineral matrix. Osteopontin is involved in various physiological and pathological processes, including bone remodeling, immune response, and inflammation (34). Osteocalcin is specifically expressed in osteoblasts and is the most abundant non-collagenous protein in bone (35). Osteonectin is not involved in the regulation of bone formation and bone quantity, but osteonectin regulates bone quality by aligning biological apatite (BAp) parallel to the collagen fibrils (36). Some evidence indicates that osteocalcin functions as a hormone that regulates insulin secretion in the pancreas, testosterone synthesis in the testis, and muscle mass based; however, this is still a matter of debate (37).

Alkaline phosphatase (ALP) is an enzyme crucial for bone mineralization by dephosphorylating organic phosphate groups, essential for the formation of hydroxyapatite crystals in bone tissue (38, 39). Alkaline phosphatase is considered a marker of early osteoblast differentiation, even if it is also expressed in other tissues (40).

CONCLUSIONS

P15 is considered a promising tool for increasing the osteointegration of biomaterials used in bone regeneration surgery. BMSCs represent a valuable resource in regenerative medicine and tissue engineering, offering versatile applications in musculoskeletal regeneration, hematopoietic transplantation, and immunomodulatory therapies. BMSCs are a good model for studying P15 because they have a strong role in bone repair. In our *in vitro* experiments, we observed some elements of osteoblast differentiation, such as overexpression of SP7 and underexpression of ENG. However, most typical markers, osteonectin, osteopontin, osteocalcin, and alkaline phosphatase, were strongly underexpressed.

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