Early Effects of P-15 on Human Bone Marrow Stem Cells

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ABSTRACT

Objectives: Peptide-15 (P-15) is an analogue of the cell binding domain of collagen. P-15 has been shown to facilitate physiological to process in a way similar to collagen, to serve as anchorage for cells, and to promote the binding, migration and differentiation of cells. However, how P-15 alters osteoblast activity to promote bone formation is poorly understood. To study the osteoinductive properties of peptide P-15, we analyzed the expression levels of bone related genes in human mesenchymal stem cells treated with this biomaterial.
Material and methods: Using real time Reverse Transcription-Polymerase Chain Reaction the quantitative expression of specific genes, like transcriptional factors (RUNX2 and SP7), bone related genes (SPP1, COL1A1, COL3A1, BGLAP, ALPL, and FOSL1) and mesenchymal stem cells marker (ENG) were examined.
Results: P-15 causes a considerable induction of osteoblast transcriptional factor like osterix (SP7) and of the bone related genes osteopontin (SPP1) and osteocalcin (BGLAP). In contrast the expression of endoglin (ENG) was markedly decreased in stem cells treated with P-15 respect to untreated cells, indicating the differentiation effect of this biomaterial on stem cells.
Conclusion: The present study shows the effect of P-15 on mesenchymal stem cells in the early differentiation stages: P-15 is an inducer of osteogenesis on human stem cells as indicated by the activation of bone related markers SP7, SPP1 and BGLAP. The results may allow a better understanding of the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects.

Keywords: stem cells; biomaterial; P-15; differentiation; bone.

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INTRODUCTION

Several studies have been involved in the identification of factors that could help in the regeneration of missing tissue [1]. One avenue of research has been the identification of the specific cell-binding domain of type I collagen [1]. Type I collagen represents approximately one third of the total body proteins. Collagen, moreover, is a major determinant of the architecture and tensile strength of the tissues, and it modulates cell proliferation, migration, differentiation, and specific gene expression [2]. P-15 is a highly conserved linear peptide with a 15-amino acid sequence identical to the sequence contained in the residues 766-780 of the alpha chain of type I collagen [1]. P-15 from DENTSPLY Friadent (CeraMed, Lakewood, CO, USA) is an analogue of the cell-binding domain of collagen [2]. P-15 competes for cell surface sites for attachment of collagen and, when immobilized on surfaces, it promotes adhesion of cells [3]. P-15 has been shown to facilitate physiological processes in a way similar to collagen, to facilitate the exchange of mechanical signals, and to promote cell differentiation [4-6]. Like other bone augmentation materials, P-15 associated with anorganic-derived bone matrix (ABM), has been shown to be helpful in the treatment of periodontal defects, and sinus-lifting procedures [1,7-11].

In previous studies a genome wide screening of osteoblast-like cell line (MG-63) following treatment with P-15 was performed by using cDNA microarray [12]. Several genes covering a broad range of functional activities, like signalling transduction, differentiation, apoptosis, cell-cycle regulation, were significantly up- or down-regulated [12]. Then the genetic effect of P-15 was studied in the same cell system at post-transcriptional level, with microRNA microarray [13,14]. A miRNA that regulates the transduction of genes related to bone formation (TFIP11), skeletal development (HOXD13, AEBP1, SHOX EN1 COMP SUFU IGF1 MATN1) and cartilage remodelling (NOG) was identified.

Because few reports analyze the effects of P-15 on stem cells [15] and none focus on the genetic effects, the expression of genes related to the osteoblast differentiation were analyzed using cultures of bone marrow derived human mesenchymal stem cells (BM-hMSCs) treated with P-15 for seven days, in order to detect the early effects of the common path of BMPs on stem cells.

MATERIAL AND METHODS

The study was approved by the Ethics Committee of the Ferrara University.

Stem cell preparation

Bone marrow derived human mesenchymal stem cells (BM-hMSCs) were obtained from the three healthy adult volunteers. There were 2 males and 1 female (average age of volunteers was 45 years); experiments were done in duplicate.

Bone Marrow collected in heparinized tubes, was diluted 1 : 3 with phosphate buffered saline (PBS) (Lonza, Basel, Switzerland) and layered over a Ficoll-Histopaque gradient (1.077 g/ml; Sigma, St. Louis, MO, USA). The low-density mononuclear cells were washed twice in PBS, counted and plated at 106/cm^2 in cell culture flasks (BD Falcon, Bedford, MA, USA) in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 20% heat inactivated fetal bovine serum (FBS) (Lonza, Basel, Switzerland) and antibiotics (100 U/ml Penicillin, 100 µg/ml Streptomycin) (Sigma-Aldrich, Inc., St Louis, Mo, USA), and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 1 week, the non-adherent cells were removed by replacing the medium supplemented with 10% FBS. When the cultures were near confluence (after 2 weeks) the cells were recovered, by treatment with 1X trypsin/EDTA solution (Sigma-Aldrich, Inc., St Louis, Mo, USA), for cytometric analysis and functional assays. BM-hMSCs were maintained and subcultured for up to 10-15 passages.

Immunofluorescence

Cells were washed with PBS for three times and fixed with cold methanol for 5 min at room temperature. After washing with PBS, cells were blocked with bovine albumin 3% (Sigma-Aldrich, Inc., St Louis, Mo, USA) for 30 min at room temperature. The cells were incubated overnight sequentially at 4 °C with primary antibodies raised against CD105 1 : 200, mouse (BD Biosciences, San Jose, CA, USA), CD73 1 : 200, mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CD90 1:200, mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CD34 1 : 200, mouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). They were washed with PBS and incubated for 1 h at room temperature with secondary antibody conjugated-Rodamine goat anti-mouse 1:200 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Subsequently, cells were mounted with the Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under a fluorescence microscope (Eclipse TE 2000-E, Nikon

Instruments S.p.a., Florence, Italy).

Cell culture

BM-hMSCs at fourth passage were grown in medium (Alphamem-Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 10% fetal calf serum, antibiotics (Penicillin 100 U/ml and Streptomycin 100 µg/ml – Sigma-Aldrich, Inc., St Louis, Mo, USA) and amminoacids (L-Glutamine – Sigma-Aldrich, Inc., St Louis, Mo, USA). The cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

For the assay, cells were collected and seeded at a density of 1 x 105 cells/ml into 9 cm^2 (3ml) wells by using 0.1% trypsin, 0.02% EDTA in Ca++ - and Mg - free Eagle's buffer for cell release.

One set of wells were added with P-15 at the concentration of 10 µl/ml. Another set of wells containing untreated cells were used as control. The medium was changed every 3 days.

After seven days, when cultures were subconfluent, cells were processed for RNA extraction.

RNA processing

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMAn Gene Expression Cells-to-Ct Kit (Ambion Inc., Austin, TX, USA), following manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA released in this solution. Cell lysate were reverse transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc., Austin, TX, USA).

Finally the cDNA was amplified by real time PCR using the included TaqMan Gene Expression Master Mix and the specific assay designed for the investigated genes.

Real time PCR

Expression was quantified using real time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene RPL13A and were expressed as fold changes relative to the expression of the untreated BM-hMSCs. Quantification was done with the delta/delta calculation method [16].

Forward and reverse primers and probes for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) and are listed in Table 1.

All PCR reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer and 200 nM of

Table 1. Primer and probes used in real time PCR

Gene symbol	Gene name	Primer sequence (5' > 3')	Probe sequence (5' > 3')
SPP1	osteopontin	F-GCCAGTTGCAGCCTTCTCA R-AAAAGCAAATCACTGCAATTCTCA	CCAAACGCCGACCAAGGAAAACTCAC
COL1A1	collagen type I alpha1	F-TAGGGTCTAGACATGTTCAGCTTTGT R-GTGATTGGTGGGATGTCTTCGT	CCTCTTAGCGGCCACCGCCCT
RUNX2	runt-related transcription factor 2	F-TCTACCACCCCGCTGTCTTC R-TGGCAGTGTCATCATCTGAAATG	ACTGGGCTTCCTGCCATCACCGA
ALPL	alkaline phosphatase	F-CCGTGGCAACTCTATCTTTGG R-CAGGCCCATTGCCATACAG	CCATGCTGAGTGACACAGACAAGAAGCC
COL3A1	collagen, type III, alpha 1	F-CCCACTATTATTTTGGCACAACAG R-AACGGATCCTGAGTCACAGACA	ATGTTCCCATCTTGGTCAGTCCTATGCG
BGLAP	osteocalcin	F-CCCTCCTGCTTGGACACAAA R-CACACTCCTCGCCCTATTGG	CCTTTGCTGGACTCTGCACCGCTG
CD105	endoglin	F-TCATCACCACAGCGGAAAAA R-GGTAGAGGCCCAGCTGGAA	TGCACTGCCTCAACATGGACAGCCT
FOSL1	FOS-like antigen 1	F-CGCGAGCGGAACAAGCT R-GCAGCCCAGATTTCTCATCTTC	ACTTCCTGCAGGCGGAGACTGACAAAC
SP7	osterix	F-ACTCACACCCGGGAGAAGAA R-GGTGGTCGCTTCGGGTAAA	TCACCTGCCTGCTCTTGCTCCAAGC
RPL13A	ribosomal protein L13	F-AAAGCGGATGGTGGTTCCT R-GCCCCAGATAGGCAAACTTTC	CTGCCCTCAAGGTCGTGCGTCTG

the probe, and cDNA. The amplification profile was initiated by 10 minute incubation at 95 °C, followed by two-step amplification of 15 seconds at 95 °C and 60 seconds at 60 °C for 40 cycles. All experiments were performed including non-template controls to exclude reagents contamination. PCRs were performed with two biological replicates.

RESULTS

BM-hMSCs were characterized by immunofluorescence. The cell surfaces were positive for mesenchymal stem cell marker, CD105, CD90 and CD73 and negative for markers of haematopoietic origin, CD34 (Figure 1).

Transcriptional expressions of several osteoblast-related genes (RUNX2, SP7, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were examined after 7 days of supplement treatment with P-15 (10 µl/ml).

Quantitative real time RT–PCR of SP7, SPP1, BGLAP showed a considerable induction after treatment with P-15. However, P-15 treatment did not affect the mRNA expression of RUNX2 and ALPL that were similarly in both treated and untreated BM-hMSCs. COL1A1, COL3A1 and FOSL1 were decreased in the presence of P-15 at day 7 like the stem cell marker ENG (Figure 2).

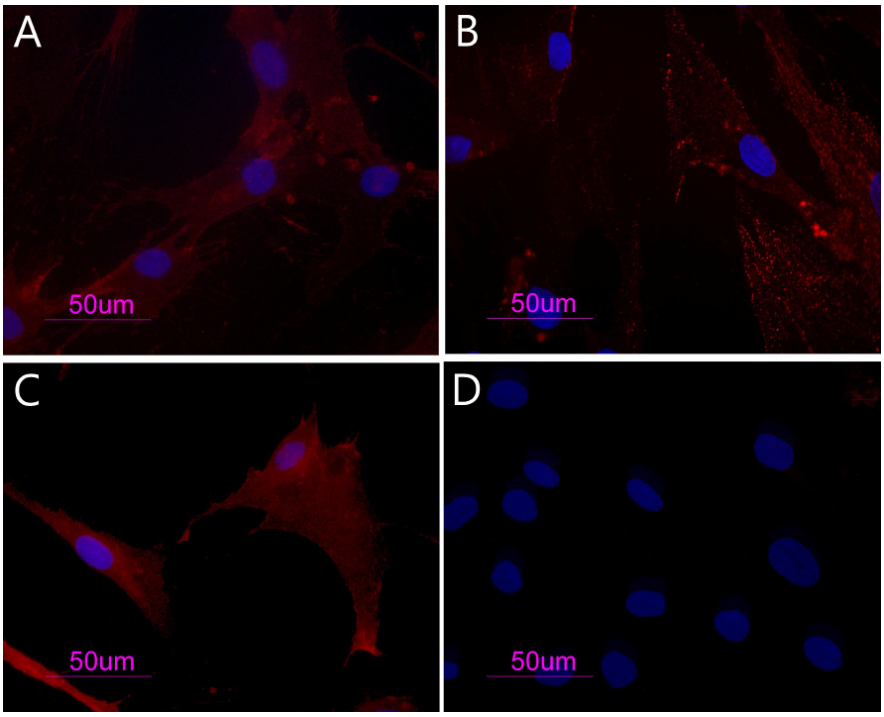

Figure 1. BM-hMSCs with indirect immunofluorescence (Rodamine). Cultured cells were positive for the mesenchymal stem cell marker CD73 (A), CD90 (B), CD105 (C) and negative for the hematopoietic markers CD34 (D). Nuclei were stained with DAPI. Original magnification x40.

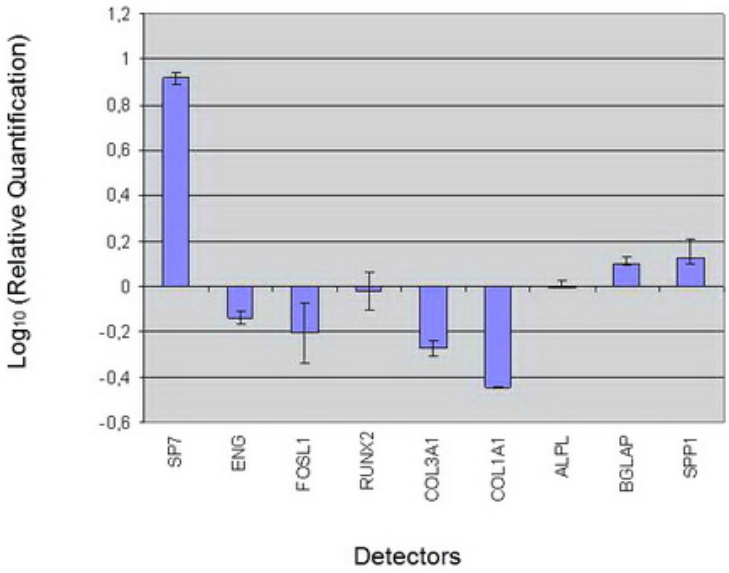

Figure 2. Gene expression analysis of BM-hMSCs after 7 days of treatment with P-15.

DISCUSSION

In order to understand the action of P-15 on BM-hMSCs, changes in expression of bone related marker genes (RUNX2, SP7, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were investigated by real time RT–PCR.

Mesenchymal stem cells are defined as self-renewable, multipotent progenitor cells with the ability to differentiate, under adequate stimuli, into several mesenchymal lineages, including osteoblasts [17].

In present study, mesenchymal stem cells from human bone marrow were isolated and characterized by morphology and immunophenotype. Isolated BM-hMSCs showed fibroblast-like morphology and were positive for MSC surface molecules (CD90, CD105, CD73) and negative for markers of haematopoietic progenitors (CD34).

Two osteoblast-specific genes, SPP1 and BGLAP, that are generally expressed by osteoblast in the early stage of their differentiation [18], were up-regulate in treated BM-hMSCs.

Another up-regulated gene was SP7, a zinc

finger transcription factor that regulates bone formation and osteoblast differentiation *in vitro* and *in vivo* and that is expressed in the early stage of osteogenic differentiation.

ENG (CD105), a surface markers used to define a bone marrow stromal cell population capable of multilineage differentiation [19], was down-regulated in treated BM-hMSCs respect to control. There is an inverse correlation between CD105 expression and the differentiation status of MSC [20]. This gene is a receptor for TGF-β1 and -β3 [21] and modulates TGF-β signalling by interacting with related molecules, such as TGF-β1, -β3, BMP-2, -7, and activin A. It is speculated that these members of the TFG-β superfamily are mediators of cell proliferation and differentiation and play regulatory roles in cartilage and bone formation [22]. The disappearance of the CD105 antigen during osteogenesis suggests that this protein, like others in the TFG-β superfamily, is involved in the regulation of osteogenesis [23].

Expression of RUNX2 and ALPL didn't have significant change in treated cells respect to control after 7 day of treatment with P-15. RUNX2 is the most specific osteoblast transcription factor and is a prerequisite for osteoblast differentiation and consequently mineralization. This result is comparable with data reported by Kim et al. [24]. They showed that there was no BMP-2-mediated up-regulation of RUNX2 mRNA expression at days 3 or 7, but BMP-2 treatment induced a significant and time dependent increase in SP7 mRNA expression [24].

Alkaline phosphatase regulates mineralization of bone matrix. Several studies demonstrated that the potency of individual substances to induce alkaline phosphatase varies in a species-dependent manner. Glucocorticoids such as dexamethasone are potent inducers in human and rat stromal cells, but they have no effect on alkaline phosphatase activity in mouse stromal cells [25,26]. Instead bone morphogenetic proteins (BMPs) are potent inducers of osteogenesis in both mouse and rat bone marrow stromal cells [27]. However, Diefenderfer et al. [28] showed that BMP-2 alone is a poor osteoblast inducer in human marrow derived stromal cells.

P-15 also modulates the expression of FOSL1 that encodes for Fra-1, a component of the dimeric transcription factor activator protein-1 (Ap-1), which is composed mainly of Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun proteins (c-Jun, JunB and JunD).

AP-1 sites are present in the promoters of many developmentally regulated osteoblast genes, including alkaline phosphatase, collagen I, osteocalcin (OC).

McCabe et al. [29] demonstrated that differential expression of Fos and Jun family members could play a role in the developmental regulation of bone-specific gene expression and, as a result, may be functionally significant for osteoblast differentiation.

In our study FOSL1 was down-regulated, probably because cells were at early stage of differentiation. Kim et al. [30] studying the effect of a new anabolic agents that stimulate bone formation, found that this gene was activated in the late stage of differentiation, during the calcium deposition.

P15 also modulates the expression of genes encoding for collagenic extracellular matrix proteins like collagen type 1α1 (COL1A1) and collagen type 3α1 (COL3A1). COL1A1, and COL3A1 were considerably down expressed as compared to the control when exposed to P15, probably because this gene are activated in the late stage of differentiation and are related to extracellular matrix synthesis.

The present study shows the effect of P-15 on BM-hMSCs in the early differentiation stages: P-15 is an inducer of osteogenesis on human stem cells but RUNX2 is not immediately activated. Moreover, we have chosen to perform the experiment after 7 days in order to get information on the early stages of stimulation.

More investigations with several different time points are needed in order to understand the molecular events related to P-15 action. This model is useful to investigate the effects of a variety of substances on stem cells.

CONCLUSIONS

The results obtained showed that P-15 participates in the initial process of differentiation of BM-hMSCs into osteoblasts, inducing expression of factors related to the differentiation like SP7, SPP1 and BGLAP.

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The authors report no conflicts of interest related to this study.

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