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Algipore effects on stem cells derived from peripheral blood

ABSTRACT

Aim Algipore is a marine derived highly porous carbonated red alga, chemically converted into hydroxyapatite. Due to its porosity, Algipore adsorbs physiologic fluids so that cytokines and growth factors permeate in full thickness the material, allowing bone forming cells to colonize and differentiate inside. However, how this material alters osteoblast activity to promote bone formation is currently under research.

To evaluate how Algipore can induce osteoblast differentiation in mesenchymal stem cells, the expression levels of bone related genes and mesenchymal stem cells marker were analyzed, using real time Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Materials and methods Quantitative real-time RT-PCR of SP7 and BGLAP showed a significant induction while RUNX2 showed a slight induction after treatment with Algipore. However, Algipore treatment did not affect the mRNA expression of ALPL that was similarly in both treated and untreated mesenchymal stem cells.

Results COL1A1, COL3A1, ENG and SPP1 were significantly decreased in the presence of Algipore at day 7. FOSL was slightly decreased in the presence of Algipore.

Conclusion These results could be relevant to better understand the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects.

Keywords Stem cell, hydroxyapatite, bone, gene expression.

INTRODUCTION

Bone grafting procedures are undergoing a major shift from autologous and allogenic bone grafts to synthetic bone graft substitutes (1). Biomaterials used in bone regeneration are designed to be gradually resorbed by the osteoclast and replaced by new bone formed through osteoblastic activity (2). Large bone defects still represent a major problem in orthopedics and maxillofacial surgery. Traditional bone-repair treatments are based on two principles: graft transplant and distraction osteogenesis, also known as Ilizarov technique. Thus far, none of these strategies have proven to be always resolving. As an alternative, a tissue engineering approach has been proposed where osteogenic cells, Hydroxyapatite (HA) scaffolds, growth factors and physical forces are involved in the bone defect repair. Different sources of osteoprogenitor cells have been suggested, bone marrow stromal cells being in most cases the first choice (3). For optimal bone regeneration, scaffolds need to fit anatomically into the bone defects and, ideally, augment cell growth and differentiation (4).

In our study we used Algipore, which is a marine derived highly porous carbonated

red alga that is chemically converted into HA (5). Thanks to its porosity, Algipore adsorbs physiologic fluids so that cytokines and growth factors permeate in full thickness the material allowing bone forming cells to colonize and differentiate inside. Algipore is therefore useful in conducting bone regeneration in all the situations in which bone defects filling is required.

Because few reports analyze the effects of Algipore on stem cells (6, 7) and none focus on the genetic effects, the expression of genes related to the osteoblast differentiation were analyzed using cultures of mesenchymal stem cells derived from peripheral blood (PB-hMSCs) treated with Algipore.

To investigate the osteogenic differentiation of PB-hMSCs, the quantitative expression of the mRNA of specific genes, like transcriptional factors (RUNX2 and SP7), bone related genes (SPP1, COL1A1, COL3A1, BGLAP, ALPL, and FOSL1) and mesenchymal stem cells marker (CD105) were examined by means of real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR).

MATERIALS AND METHODS

a) Stem preparation

PB-hMSCs were obtained for gradient centrifugation from peripheral blood of healthy volunteers, using the Acuspin System-Histopaque 1077 (Sigma Aldrich, Inc., St Louis, Mo, USA). Firstly, 30 ml of heparinized peripheral blood were added to the Acuspin System-Histopaque 1077 tube and centrifugated at 1000 x g for 10 minutes. After centrifugation the interface containing mononuclear cells was transferred in another tube, washed with PBS and centrifugated at 250 x g for 10 minutes. The enriched mononuclear pellets were resuspended in 10 ml of Alphamem medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with antibiotics (Penicillin 100 U/ml and Streptomycin 100 µm/ml - Sigma, Chemical Co., St Louis, Mo, USA) and amminoacids (L-Glutamine - Sigma, Chemical Co., St Louis, Mo, USA). Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C. Medium was changed after 24 hours. PB-hMSC were selected for adhesivity and characterized for staminality by immunofluorescence.

b) 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 Biotecnology, Inc., Santa Cruz, CA, USA), CD90 1:200, mouse (Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA), CD34 1:200, mouse (Santa Cruz Biotecnology, 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 Biotecnology, 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).

c) Cell culture

PB-hMSCs at second passage were cultured in Alphamem medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 10%

fetal calf serum, antibiotics (Penicillin 100 U/ml and Streptomycin 100 µm/ml - Sigma Aldrich, Inc., St Louis, Mo, USA) and amminoacids (L-Glutamine - Sigma Aldrich, Inc., St Louis, Mo, USA). Cells were kept in a 5% CO_2 humidified atmosphere at 37 °C. For the assay, cells were collected and seeded at a density of 1x105 cells/ml into 9 cm^2 (3 ml) 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 Algipore (FRIOS Algipore, DENTSPLY Friadent CeraMed, Lakewood, CO) at the concentration of 25 mg/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 sub-confluent, cells were processed for RNA extraction.

d) RNA processing

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMAn Gene Expressio 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.

Simbolo geni	Nome gene	Sequenza primer (5'>3')	Sequenza sonde (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 phospatasi	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

Table 1 Primer and probes used in real time PCR.

e) 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 SC-PB. Quantification was done with the delta/delta calculation method (8). 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 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

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

Transcriptional expressions of several osteoblast-related genes (RUNX2, SP7, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (CD105) were examined after 7 days of supplement

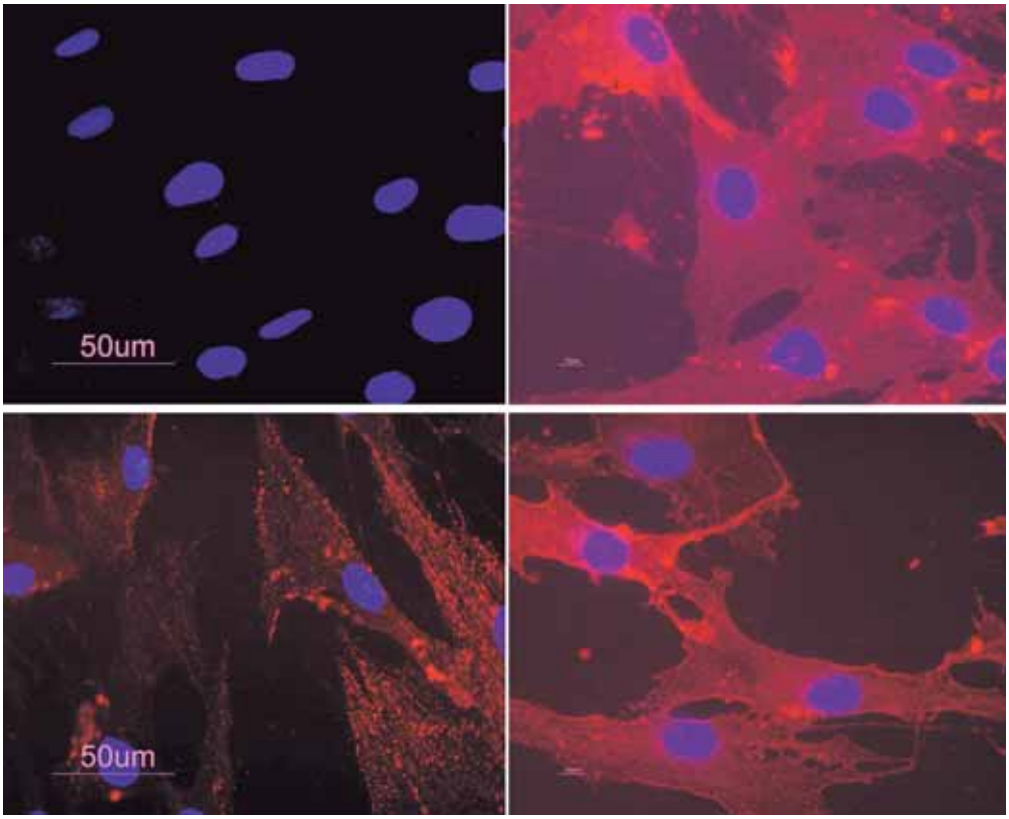

Fig. 1 Immunofluorescence of cultured PB-hMSCs were positive for mesenchymal markers CD73 (b), CD90 (c), CD105 (d) and negative for hemopoietic markers CD34 (a). Nuclei are showed with DAPI. (40x).

treatment with Algipore (25 mg/ml).
Quantitative real-time RT–PCR of SP7 and BGLAP showed a significant induction while RUNX2 showed a slight induction after treatment with Algipore. However, Algipore treatment did not affect the mRNA expression of ALPL that was similarly in both treated and untreated PB-hMSCs. COL1A1, COL3A1, ENG and SPP1 were significantly decreased in the presence of Algipore at day 7. FOSL1 was slightly decreased in the presence of Algipore (Fig. 2).

DISCUSSION

Although bone autograft continues to be considered the gold standard for sinus grafting (5), tissue engineering may represent an alternative (6).
Bone tissue engineering entails the successful interplay between osteoinductive factors, osteogenic cells, their extracellular environment, and an osteoconductive biomaterial scaffold.
Research in regenerative medicine is developing at a quick pace. Cell-based bone and cartilage replacement is an evolving therapy aiming at the treatment of patients who suffer from limb amputation, damaged tissues and various bone and cartilage-related disorders and dental and maxillofacial reconstructive surgery. Stem cells are undifferentiated cells with the capability to regenerate into one or more committed cell lineages. Stem cells isolated from multiple sources are widely used to advance the field of tissue repair (9). Bone regeneration for the correction of defects in revision surgery of joint replacement is an increasingly important issue. To repair bone defects, bone cell activation by growth factors using synthetic resorbable scaffold is a useful and safe option (10). Synthetic and biological materials are increasingly used to provide temporary or permanent scaffolds for bone regeneration (11). Bone regeneration by autologous cell transplantation in combination with a biodegradable scaffold is one of the most promising techniques being developed in craniofacial and orthopedic surgery (12). Tissue engineering approaches attempt to create tissue replacement by culturing autologous cells onto three-dimensional matrixes that help cell progenitor migration, proliferation and differentiation (13).
Naturally produced ceramics, such as HA calcified from red algae, are the most promising materials for use as scaffolds in this field (7). Maxillary sinus augmentation is frequently necessary before placement of dental implants in the posterior maxilla. Beside bone autograft, various bone substitutes have been used, with favorable results (14). In a retrospective study of over 14 years Ewers showed that the sinus lift procedure with grafting of the sinus floor and subsequent implant placement is a proven method and he also showed that the marine derived HA material ACA in a mixture with approximately 10% autogenous bone and blood or platelet rich plasma is able to enhance enough new bone in 6 months AND to allow implant osseointegration after 6

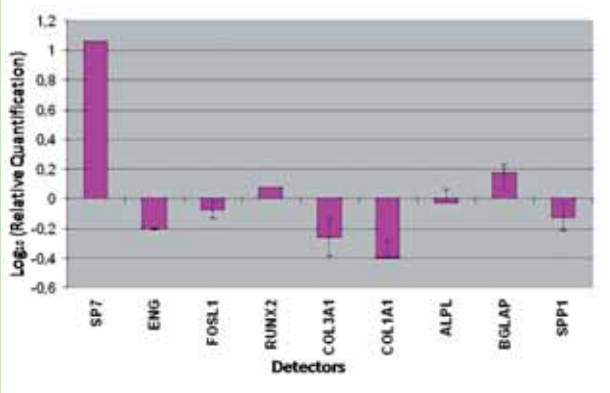

Fig. 2 Analysis of genic expression in PB-hMSCs after 7 days of treatment with Algipore.

more months with a high implant survival rate (5).

In order to better understand how Algipore acts on PB-hMSCs, changes in expression of bone related marker genes (RUNX2, SP7, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (CD105) were investigated by real-time RT–PCR.

Mesenchymal stem cells (MSCs) are defined as self-renewable, multipotent progenitors cells with the ability to differentiate, under adequate stimuli, into several mesenchymal lineages, including osteoblasts (15).

In our study, mesenchymal stem cells from human peripheral blood were isolated and characterized by morphology and immunophenotype. Isolated PB-hMSCs showed fibroblast-like morphology and were positive for MSCs surface molecules (CD90, CD105, CD73) and negative for markers of haematopoietic progenitors (CD34).

After 7 days of treatment with Algipore the expression levels of osseodifferentiation genes were measured by relative quantification methods using real-time RT–PCR.

Two osteoblast-specific genes, RUNX2 and BGLAP, that are generally expressed by osteoblast in the early stage of their differentiation (16), were up-regulated in treated PB-hMSCs. RUNX2 is the most specific osteoblast transcription factor and is a prerequisite for osteoblast differentiation and subsequent mineralization.

Another up-regulated gene is 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 marker used to define a bone marrow stromal cell population capable of multilineage differentiation (17), is down-expressed in treated PB-hMSCs respect to control at 7 days.

COL3A1 encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues (9).

Algipore also modulates the expression of genes encoding for collagenic extracellular matrix proteins such as collagen type 1·1 (COL1A1). Collagen type1 is the most abundant in the human organism (10). In our study COL1A1, ENG and COL3A1 are significantly down-expressed as compared to the control when exposed to Algipore, probably because these genes are activated in the late stage of differentiation and are related to extracellular matrix synthesis.

Alkaline phosphatase regulates mineralization of bone matrix and in our study is not affected when exposed to Algipore when compared to control. 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 (18,19). On the contrary, bone morphogenetic proteins (BMPs) are potent inducers of osteogenesis in both mouse and rat bone marrow stromal cells (20), but Diefenderfer et al showed that BMP-2 alone is a poor osteoblast inducer in human marrow derived stromal cells (21). Perhaps ALPL is not up-regulated because while Algipore induces differentiation of mesenchymal stem cells, by the way they are at the first stages of differentiation and they are not already specialized in extracellular matrix secretion.

Another investigated gene is FOSL-1 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. McCabe et al. (22) 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 FOSL-1 was slightly down-regulated, probably because cells were at an early stage of differentiation. Kim et al. (23) studying the effect of a new anabolic agents that stimulate bone formation, find that this gene is activated in the late stage of differentiation, during the calcium deposition.
SPP1 encodes osteopontin, which is a phosphoglycoprotein of bone matrix and it is the most representative non collagenic component of extracellular bone matrix (11). Osteopontin is actively involved in bone resorbitive processes directly by ostoclasts (12). Osteopontin produced by osteoblasts shows high affinity to the molecules of hydroxylapatite in extracellular matrix and it is chemo-attractant to osteoclasts (24). In our study osteopontin is significantly down-expressed when exposed to Algipore. Therefore Algipore seems to act reducing bone resorption processes in the early stages of cell differentiation.

CONCLUSION

The present study shows the effects of Algipore on PB-hMSCs in the early differentiation stages: Algipore is an inducer of osteogenesis on human stem cells, as RUNX2 is immediately activated, while reducing osteoclastic activity, as SPP1 is down-expressed. Moreover, we have chosen to perform the experiment after 7 days in order to get information on the early stages of stimulation. The reported model is useful to investigate the effects of different substances on stem cells.

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