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Medpor® Acts on Stem Cells Derived from Peripheral Blood

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ABSTRACT

Porous polyethylene (*PP or Medpor®*) *is an alloplastic material used worldwide for craniofacial reconstruction. Although several clinical studies are available, how this material alters osteoblast activity to promote bone formation is poorly understood. To study how PP 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. PP causes induction of osteoblast transcriptional factor RUNX*2 *and of the bone related genes osteocalcin* (*BGLAP*) *and alkaline phosphatase* (*ALPL*). *In contrast the expression of ENG was decreased in stem cells treated with PP respect to untreated cells, indicating the differentiation effect of this biomaterial on stem cells. The obtained results can be relevant to better understand the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects.*

Keywords: *Alloplastic Material, Porous Polyethylene, Gene Expression, Stem Cells*

1. Introduction

Craniofacial "skeletal" defects should be ideally corrected with autologous bone or cartilage. However, collecting an adequate amount of bone from other donor sites of the same patient is not always possible, it carries additional morbidity and it determines prolonged operation time. On the contrary, homologue bank tissues and animal derived products are not completely safe because unknown diseases can be potentially transferred. Consequently, alloplastic materials are used for craniofacial reconstruction. Among them, porous polyethylene (PP or Medpor®) has been extensively used since the nineties. Its properties make it an excellent choice for correcting cranial and facial defects. The implant is easy to shape, flexible, remarkably stable, and it exhibits rapid soft-tissue growth.

PP has been used to repair cranial defects [1,2], to restore facial deformities [3], to reconstruct both ear [4] and orbit [5], as spherical orbital prostheses [6], to correct lower eyelid retraction [7] and to restore nasal function and shape [8]. Reported complications are persistent pain and anesthesia, implant exposure, infection and subsequent graft removal.

Although several studies have analyzed applications and risk factors associated with the use of this synthetic graft, there is a lack as regards research into genetic effects.

In previous studies by using cDNA microarray containing 19,200 genes, we identified in osteoblast-like cell lines (*i.e.* MG-63) cultured on PP, several genes where expression were differentially regulated. The differentially expressed genes cover a broad range of functional activities: 1) signal transduction, 2) transcription, 3) translation, 4) cell cycle regulation, 5) vesicular transport, and 6) production of cytoskeletal elements, cell-adhesion molecules and extracellular matrix components [9]. We therefore investigated, by using microRNA microarray techniques, the translation regulation in osteoblasts exposed to PP. We identified miRNA that regulates the transduction of genes related to bone formation (OSTF1), skeletal development (CHRD, EN1, ADAMTS4, GHR-HR) and cartilage remodeling (MGP, NOG, LECT1, PTH) [10].

Since PP is always fixed onto bone and the mechanism by which PP acts on osteoblasts is incompletely known, we therefore attempted to get more inside by using human stem cells isolated from peripheral blood.

Because no reports analyze the genetic effects of PP on stem cells, the expression of genes related to the osteoblast differentiation were analyzed using cultures of human mesenchymal stem cells derived from peripheral blood (PB-hMSCs) treated with PP.

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

2. Materials and Methods

2.1 Stem Preparation

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

2.2 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℃ 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).

2.3 Cell Culture

PB-hMSCs at fourth 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 micrograms/ml - Sigma Aldrich, Inc., St Louis, Mo, USA) and amminoacids (L-Glutamine - Sigma Aldrich, Inc., St Louis, Mo, USA). The cells were maintained in a 5% CO_2 humidified atmosphere at 37℃. For the assay, cells were collected and seeded at a density of 1×10^5 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.

In one set of wells a sheet of Medpor® (Porex Corporation, Fairburn, Georgia, USA) was placed on the bottom leaving cells growing on it for 7 days.

Another set of wells containing untreated cells was used as control. The medium was changed every 3 days.

After seven days, when cultures were sub-confluent, cells were processed for RNA extraction.

2.4 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.

2.5 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 PB-hMSCs. Quantification was done with the delta/ delta calculation method [11].

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℃, followed by two-step amplification of 15 seconds at 95℃ and 60 seconds at 60℃ for 40 cycles. All experiments were performed including non-template controls to exclude reagents contamination. PCRs were performed with two biological replicates.

3. 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 (**Figure 1**).

Transcriptional expressions of several osteoblast-related genes (RUNX2, SPP1, COLIA1, COL3A1, BGLAP, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were examined after 7 days of treatment with PP.

Quantitative real-time RT–PCR of RUNX2, ALPL and BGLAP showed a significant induction after treatment with PP.

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 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
RPL13A	ribosomal protein L13	F-AAAGCGGATGGTGGTTCCT R-GCCCCAGATAGGCAAACTTTC	CTGCCCTCAAGGTCGTGCGTCTG

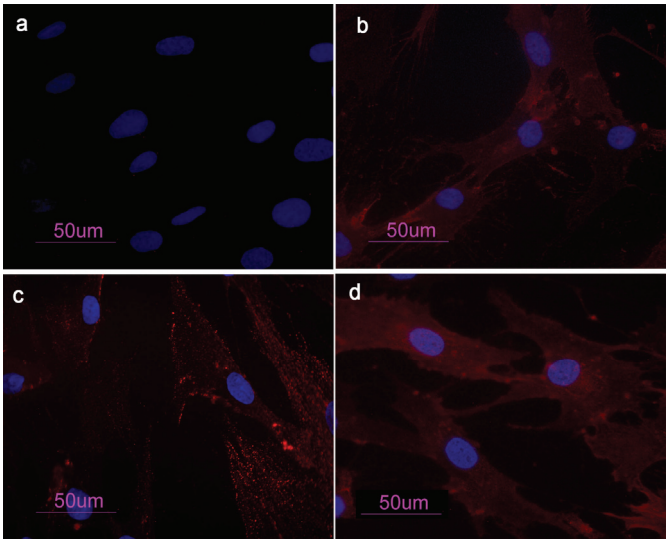

Figure 1. PB-hMSCs by indirect immunofluorescence (Rodamine). Cultured cells were positive for the mesenchymal stem cell marker CD73 (b), CD90 (c), CD105 (d) and negative for the hematopoietic markers CD34 (a). Nucleuses were stained with DAPI. Original magnification × 40

However, PP treatment did not affect the mRNA expression of SPP1 and FOSL1 that were similarly in both treated and untreated PB-hMSCs. COL1A1 and COL3A1 were decreased in the presence of PP at day 7 like the stem cell marker ENG (**Figure 2**).

4. Discussion

Although autologous bone and cartilage grafts are the gold standard for craniofacial reconstruction, they carry additional morbidity related to the second operation field and to prolonged operation time. Moreover, bone grafts resorb in a way that is not predictable. Consequently, alloplastic materials have a specific role in craniofacial reconstruction especially because they are safer with respect to homologue bank tissues and animal derived products which can potentially transfer diseases of unknown etiology.

PP is a pure polyethylene with a unique manufacturing process and pore size. Technically, it is easy to work with; it can be carved, contoured, adapted, and fixed to obtain a precise three-dimensional shape. Physically, it is a pure, biocompatible, strong substance that does not resorb or degenerate. It demonstrates long-term stability, high tensile strength, and a virtual lack of surrounding soft-tissue reaction.

Several papers have shown PP effectiveness in restoring craniofacial defects [1-8]. Few complications have been reported, such as: persistent pain, paresthesia, implant exposure, infection and subsequent graft removal.

Although several studies have analyzed the applications and risk factors associated with the use of this synthetic graft, there is a lack as regards its genetic effects.

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

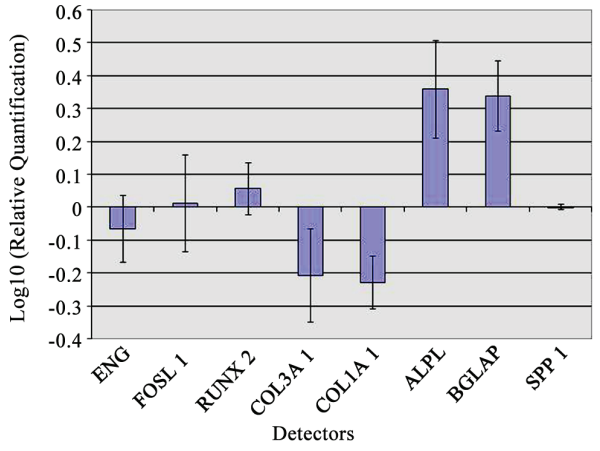

Figure 2. Gene expression analysis of PB-hMSCs after 7 days of treatment with PP

Mesenchymal stem cells are defined as self-renewable, multipotent progenitor's cells with the ability to differentiate, under adequate stimuli, into several mesenchymal lineages, including osteoblasts [12].

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 MSC surface molecules (CD90, CD105, CD73) and negative for markers of haematopoietic progenitors (CD34).

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

ENG (CD105), a surface markers used to define a bone marrow stromal cell population capable of multilineage differentiation [13], was down-regulated in treated PB-hMSCs respect to control, indicating the differentiation effect of this biomaterial on stem cells. There is an inverse correlation between CD105 expression and the differentiation status of MSC [14]. This gene is a receptor for TGF-β1 and -β3 [15] and modulates TGF-β signaling 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 [16]. 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 [17].

Expression of transcription factor RUNX2 was up-regulated in treated cells respect to control after 7 days of treatment with PP. RUNX2 is the most specific osteoblast transcription factor and is a prerequisite for osteoblast differentiation and consequently mineralization.

PB-hMSCs treated with PP showed a high expression of ALPL compared to untreated cells. Thus we demonstrated that PP increases the activity of ALPL gene, which is a key point in osteodifferentiation.

BGLAP, a bone specific protein involved in mineralization and bone resorption, that is generally express by osteoblast in the early stage of their differentiation [18] was significantly up-regulate in treated PB-hMSCs.

Another investigated gene was 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.

McCabe *et al.* [19] 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 signifi-

cant for osteoblast differentiation.

In our study FOSL1 was down-regulated, probably because cells were at early stage of differentiation. Kim *et al.* [20] 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.

SSP1 encodes osteopontin, which is a phosphoglycoprotein of bone matrix and it is the most representative non collagenic component of extracellular bone matrix [21].

Osteopontin is actively involved in bone resorbitive processes directly by ostoclasts [22]. Osteopontin produced by osteoblasts, show high affinity to the molecules of hydroxylapatite in extracellular matrix and it is chemo-attractant to osteoclasts [23]. However, in our experimental model, SPP1 expression during osteogenic induction was similar in both treated and control because this gene regulates the later stages of osteoblast differentiation and bone development [22]. This result is not in contrast with those previously reported as we investigated stem cells treated for 7 days.

PP also modulates the expression of genes encoding for collagenic extracellular matrix proteins like collagen type 1α1 (COL1A1). Collagen type1 is the most abundant in the human organism [24].

COL1A1 and COL3A1 were significantly down expressed as compared to the control when exposed to PP probably because these genes are activated in the late stage of differentiation and are related to extracellular matrix synthesis.

COL3A1 encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues [25].

The present study shows the effect of PP on PB-hMSCs in the early differentiation stages: PP is an inducer of osteogenesis on human stem cells, as demonstrated by the expression of bone related genes like RUNX2, ALPL and BGLAP. Moreover, we have chosen to perform the experiment after 7 days in order to get information on the early stages of stimulation. It is our understanding, therefore, that more investigations with different time points are needed in order to get a global comprehension of the molecular events related to PP action. The reported model is useful to investigate the effects of different substances on stem cells.

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