Original Article

PerioGlas® Acts on Human Stem Cells Isolated from Peripheral Blood

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ABSTRACT

***Background*:** PerioGlas® (PG) is an alloplastic material used for grafting periodontal osseous defects since 1995. In animal models, it has been proven that PG achieves histologically good repair of surgically created defects. In clinical trials, PG was effective as an adjunct to conventional surgery in the treatment of intrabony defects. Because the molecular events due to PG that are able to alter osteoblast activity to promote bone formation are poorly understood, we investigated the expression of osteoblastic related genes in mesenchymal stem cells exposed to PG.

***Methods*:** The expression levels of bone related genes like RUNX2, SP7, SPP1, COL1A1, COL3A1, BGLAP, ALPL, and FOSL1 and mesenchymal stem cells marker (CD105) were analyzed, using real time reverse transcription-polymerase chain reaction. Pearson's chi-square (χ^2) test was used to detect markers with significant differences in gene expression.

***Results*:** PG caused induction of osteoblast transcriptional factor (like RUNX2), bone related genes osteopontin (SPP1), osteocalcin (BGLAP) and alkaline phosphatase (ALPL). All had statistical significant P values (< 0.05).

***Conclusion*:** PG has a differentiation effect on mesenchymal stem cells derived from peripheral blood. The obtained results can be relevant to better understanding of the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects.

***Keywords*:** Allograft, Alloplastic material, Bone, Stem cell.

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Introduction

PerioGlas® (PG) is an alloplastic material used for grafting periodontal osseous defects since 1995. In animal models, it achieved histologically good repair of surgically created defects.[1-2] In monkey, PG demonstrated biocompatibility and osteoconductive activities.[1-3] It was mostly resorbed and replaced by bone and the remaining granules were in close contact with bone. In rabbit model, PG was able to improve bone healing at the interface between titanium dental implants and bone[4] whereas in ovariectomized rats, a neoformation of bone trabeculae into extraction sockets was improved.[5]

In a clinical trial, bioactive glass was effective as an adjunct to conventional surgery in the treatment of intrabony defects[6] as well in the treatment of dental extraction sites before dental implant placement, to implement bone regeneration and to augment early fixation of implant.[7] However, PG had no regenerative properties as regard to cementum and periodontal ligament.[8] In *in vitro* studies on human cells, the osteoblast cell line MG63 was used as a prototype of human bone cells to test bioglass with favorable results.[9] Human primary osteoblasts were used to investigate the osteogenic potential of a melt-derived bioactive glass (BG). It was shown that the BG induces osteoblast proliferation and augments osteoblast commitment thus, it was hypothesized that BG could be used as a

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template for the formation of bioengineered bone tissue.[10]

From a molecular point of view, it has been shown that ionic products of BG dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein synthesis.[11] In addition, a gene-expression profiling of human osteoblasts following treatment with the ionic products of BG dissolution was performed by using cDNA microarray containing 1,176 genes.[12]

In previous studies, we carried out a genome wide screen of osteoblast-like cell line (MG-63) following treatment with PG, using cDNA microarray. PG acted on bone formation by determining both osteoconduction (as demonstrated by the reduced cell adhesion) and osteogenesis (as shown by TGFB-related proteins).[13] Then, the genetic effect of PG was studied in the same cell system at post-transcriptional level, with microRNA microarray. PG acted on microRNAs that regulate several messengers related to bone formation (like NOG, EN1, CHRD) and cartilage development (COMP, NOG).[14]

Because few reports analyze the genetic effects of PG on stem cells,[15] 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 PG. Mesenchymal stem cells (MSCs) are defined as self-renewable, multipotent progenitor cells with the ability to differentiate, under adequate stimuli, into several mesenchymal lineages, including osteoblasts.[16] The principal source of stem cells is bone marrow; in addition, MSCs are obtained from other tissues such as fat, umbilical cord blood, fetal tissue and peripheral blood.[17]

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

Stem preparation

PB-hMSCs were obtained for gradient centrifugation from peripheral blood of healthy anonymous volunteers, using the Accuspin System-Histopaque 1077 (Sigma Aldrich, Inc., St Louis, MO, USA). Firstly, 30 ml of heparinized peripheral blood were added to the Accuspin System-Histopaque 1077 tube and centrifuged at 1000 x g for 10 minutes. After centrifugation, the interface containing mononuclear cells was transferred to another tube, washed with PBS and centrifuged at 250 x g per 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 micrograms/ml) (Sigma Aldrich, Inc., St Louis, MO, USA) and aminoacids (L-Glutamine) (Sigma Aldrich, Inc., St Louis, MO, USA). The cells were maintained at 37°C in a fully humidified atmosphere at 5% CO_2 in air. Medium was changed after 24 hours. PB-hMSCs were selected for adhesiveness and characterized for staminality by immunofluorescence.

Immunofluorescence

Cells were three times washed with PBS 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), and 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-rhodamine 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

PB-hMSCs at second passage were grown 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 aminoacids (L-Glutamine) (Sigma Aldrich, Inc., St Louis, MO, USA). The cells were maintained at 37°C in a fully humidified atmosphere at 5% CO_2 in air.

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.

One set of wells were added with PerioGlas® (US Biomaterials Corp., Alachua, FL) at the concentration of 0.04 g/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.

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 lysates 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 PB-hMSCs. Quantification was done with the delta/delta calculation method.[18] 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.

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

Statistical method

Pearson's chi-square (χ^2) test was used to detect markers with significant differences in gene expression. P value less than 0.05 was considered significant.

Results

PB-hMSCs were characterized by immunofluorescence. The cell surfaces were positive for mesenchymal stem cell markers, CD105, CD90 and CD73 and negative for marker of hematopoietic 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 (CD105) were examined after 7 days of supplement treatment with PG (0.04 g/ml) (Figure 2). PG enhanced the expression of the transcriptional factor RUNX2 ($P < 0.01$), and of several bone related genes like FOSL1 (P was not significant), ALPL ($P < 0.049$), BGLAP ($P < 0.027$) and SPP1 ($P < 0.039$). The mesenchymal related marker CD105 ($P < 0.017$) was up regulated in treated cells respected as control. At the contrary, the two collagens COLIA1 ($P < 0.01$), COL3A1 ($P < 0.042$) and zinc finger transcription factor SP7 (p was not significant) were down-regulated.

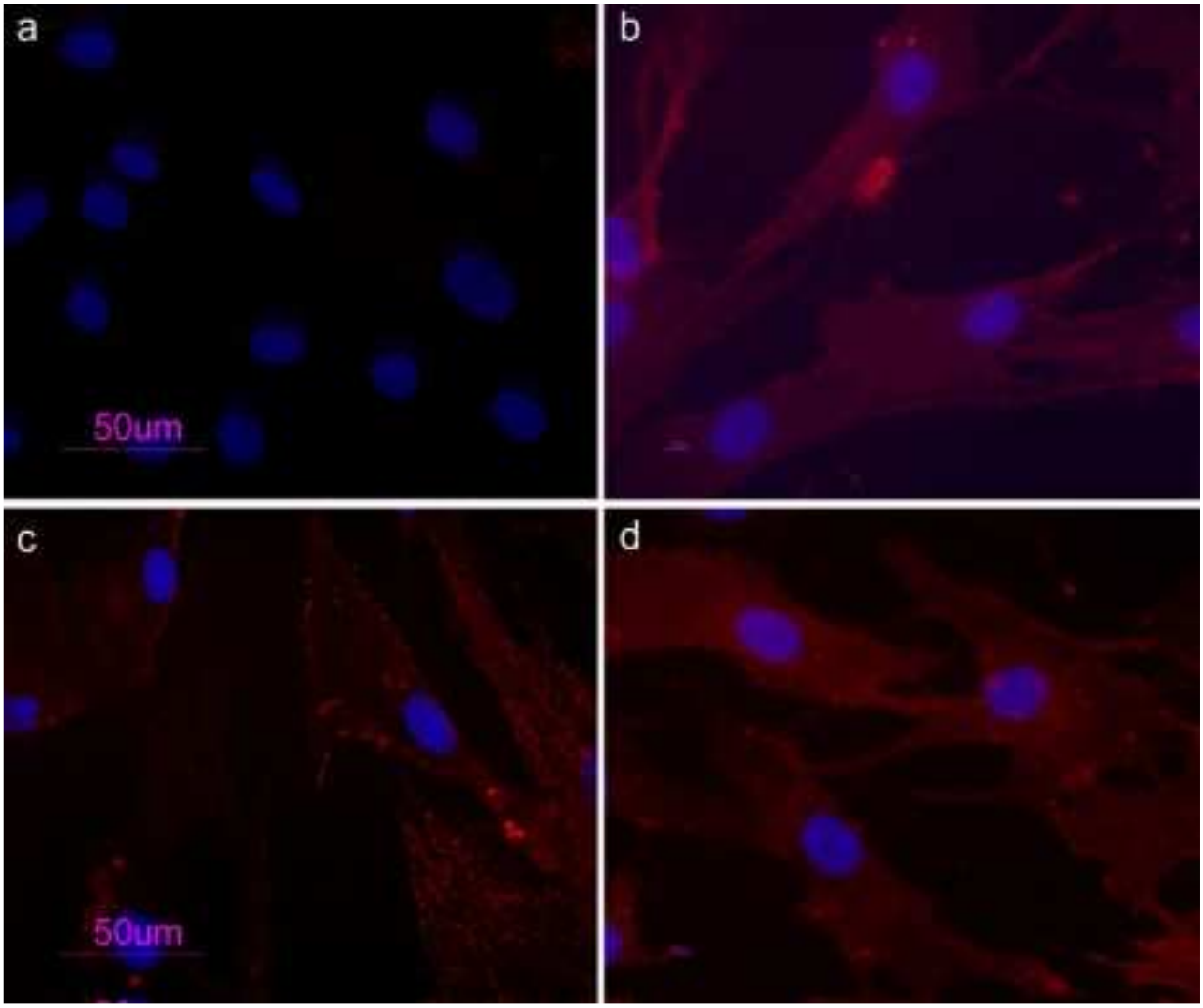

Figure 1. PB-hMSCs by indirect immunofluorescence (Rhodamine). Cultured cells were positive for the mesenchymal stem cell marker CD73 (a), CD90 (b), CD105 (c) and negative for the hematopoietic markers CD34 (d). Nuclei were stained with DAPI (Original magnification x40).

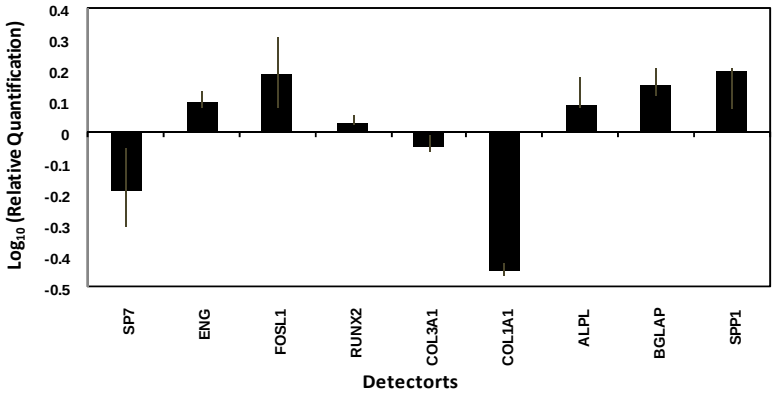

Figure 2. Gene expression analysis of PB-hMSCs after 7 days of treatment with PG

Discussion

PG is a silicate-based synthetic bone augmentation material that has been used to fill periodontal defects with bonding and integration to both soft tissue and bone. Previous studies in animal models have shown that PG achieves histologically good repair of surgically created defects.[1-5] In clinical trials, PG was effective as an adjunct to conventional surgery in the treatment of intrabony defects[6] as well in the treatment of dental extraction sites.[7] In order to get more inside how PG 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.

In our study, mesenchymal stem cells from peripheral blood were isolated by gradient and characterized by morphology and immunophenotype. Isolated PB-hMSCs showed fibroblast-like morphology and were positive for MSCs surface molecules (CD90, CD105, and CD73) and negative for markers of haematopoietic progenitors (CD34). After 7 days of treatment with PG, the expression levels of osteodifferentiation genes were measured by relative quantification methods using real-time RT–PCR. Two transcriptional factors had an opposite expression. RUNX2 was up-regulated in treated PB-hMSCs in respect to control while SP7 was down-expressed. RUNX2 is a prerequisite for osteoblast differentiation and consequently mineralization. It is expressed in the earlier stages as SP7,[19] a zinc finger transcription factor that regulates bone formation, is downstream of RUNX2 during bone development and is expressed in the later stage of osteoblast differentiation.[20] RUNX2 induce the expression of two bone related genes BGLAP and SPP1 that are up-regulated in PB-hMSCs after treatment. BGLAP, the most abundant protein in bone, is a mature osteoblastic marker and its expression correlates with bone formation.[21] SPP1 encodes osteopontin, which is a phosphoglycoprotein of bone matrix and it is the most representative non-collagenic component of extracellular bone matrix.[22] Osteopontin is actively involved in bone resorptive processes directly by ostoclasts.[23] Osteopontin produced by osteoblasts, show high affinity to the molecules of hydroxylapatite in extracellular matrix and it is chemoattractant to osteoclasts.[24]

ENG (CD105), a surface marker used to define a bone marrow stromal cell population capable of multilineage differentiation,[16] is up-regulated in treated PB-hMSCs in respect to control at 7 days. Another investigated gene was 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). McCabe et al.[25] 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. AP-1 sites are present in the promoters of many developmentally regulated osteoblast genes, up-regulated in the present study, including alkaline phosphatase (ALPL) and osteocalcin (BGLAP). PG also modulates the expression of genes encoding for collagenic extracellular matrix proteins like collagen type 1α1 (COL1A1) and

COL3A1. COL3A1 encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues.[26] Collagen type1 is the most abundant in the human organism.[27] In our study, they were down expressed as compared to the control when exposed to PG probably because these genes are activated in the late stage of differentiation and are related to extracellular matrix synthesis. The present study showed the effect of PG on PB-hMSCs in the early differentiation stages. PG was an inducer of osteogenesis on human stem cells as demonstrated by the activation of osteoblast transcriptional factor RUNX2 and bone related genes, osteopontin (SPP1), osteocalcin (BGLAP) and alkaline phosphatase (ALPL).

Moreover, we chose to perform the experiment after 7 days in order to get information on the early stages of stimulation.

Conclusion

PG had a differentiation effect on mesenchymal stem cells derived from peripheral blood. The obtained results can be relevant to better understanding of the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects.

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