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 (χ2) 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.

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 bone4 whereas in ovariecotmized 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 defects6 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
template for the formation of bioengineered bone tissue.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 TGFβ-related proteins).\textsuperscript{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).\textsuperscript{14}

Because few reports analyze the genetic effects of PG on stem cells,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsubscript{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., Burlington, 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\textsubscript{2} in air.

For the assay, cells were collected and seeded at a density of 1 x 10\textsuperscript{5} cells/ml into 9 cm\textsuperscript{2} (3 ml) wells
by using 0.1% trypsin, 0.02% EDTA in Ca\(^{2+}\) and Mg-free Eagle’s buffer for cell release.

One set of wells were added with PerioGlas\textsuperscript{®} (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.\textsuperscript{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-GCCAGTTGGAGCCTTTCTCA R-AAAGGCAAACCTGCAATATTCTCA | CCAGCGGAGGAAAGGAAAACCTCAC |
| COL1A1 | collagen type I alpha1 | F-TAGGGTCTGAGCATGTTCACTTTCTG R-GTGATTTGTGGAGGTGTTCTCTTGT | CCACTTACGCGCCACCAGCGCC |
| RUNX2 | runt-related transcription factor 2 | F-TCTACACACCCCGGCTGTTCTC R-TGGGACGTGTCATCTCGAATAG | ACTGGGCTTCTCTGCCATCAGGA |
| ALPL | alkaline phosphatase | F-CCATGGCCAGAAGCCTATTATGG R-CAGGGCCATTTGCCAGACACAG | TCCGAGGAGGACACAGGAAGG |
| COL3A1 | collagen, type III, alpha 1 | F-CCCACTATATTATTTGGAACAGAG R-AACGGATCCCTGAGTCAAGACAGAC | ATGTGTTCCATTTGGAAGATTTCTT |
| BGLAP | osteocalcin | F-CCCTTCCTGCTGAGACACAAA R-CAGACCTCCTGCCCTATTGG | CTGGTTGTCAGTCCTGACCAGCT |
| CD105 | endoglin | F-TGTATCCACACAGCAGAGAAAAA R-GGTAGAGGGCCAGACGAGGA | TGCAGTCGCTCAGATGGAACGCT |
| FOSL1 | FOS-like antigen 1 | F-GGCGGAGGGAGGACCAAGCT R-GCAGGCCGAGAGGCTTTCATCAG | ACTTGCTAGCGAGGACAGACGCT |
| SP7 | osterix | F-ACTACACACCCCGGAGAAGAAA R-GGTGAGTGGTGCTTGGAGAA | TCAGACCTGGTTCTTGGCTT |
| RPL13A | ribosomal protein L13 | F-AAAGGAGGATGTTGTTGCCCT R-GGCCAGATAGGCAAACTTTC | CTGAGGTCAGTGGTGGTGCGGCTT |

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Statistical method
Pearson’s chi-square ($\chi^2$) 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, COL1A1, 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 upregulated in treated cells respected as control. At the contrary, the two collagens COL1A1 ($P < 0.01$), 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).
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. In clinical trials, PG was effective as an adjunct to conventional surgery in the treatment of intrabony defects as well as in the treatment of dental extraction sites. In order to get more insight into how PG acts on PB-hMSCs, changes in expression of bone related marker genes (RUNX2, SP7, SPP1, COLIA1, COL3A1, BGLAP, ALPL and ENG) 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, 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. 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. SPP1 encodes osteopontin, which is a phosphoglycoprotein of bone matrix and it is the most representative non-collagenic component of extracellular bone matrix. Osteopontin is actively involved in bone resorptive processes directly by osteoclasts. Osteopontin produced by osteoblasts, show high affinity to the molecules of hydroxyapatite in extracellular matrix and it is chemo-attractant to osteoclasts.

ENG (CD105), a surface marker used to define a bone marrow stromal cell population capable of multilineage differentiation, 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. 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 collagen extracellular matrix proteins like collagen type Iα1 (COL1A1) and SPP1.

Figure 2. Gene expression analysis of PB-hMSCs after 7 days of treatment with PG.
COL3A1. COL3A1 encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues. Collagen type 1 is the most abundant in the human organism. 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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References

1. Fetner AE, Hartigan MS, Low SB. Periodontal repair using PerioGlas in nonhuman primates: clinical and histologic observations. Compendium 1994; 15(7): 932.
2. Karatzas S, Zavras A, Greenspan D, Amar S. Histologic observations of periodontal wound healing after treatment with PerioGlas in nonhuman primates. Int J Periodontics Restorative Dent 1999; 19(5): 489-99.
3. Cancian DC, Hochuli-Vieira E, Marcantonio RA, Garcia J, I. Utilization of autogenous bone, bioactive glasses, and calcium phosphate cement in surgical mandibular bone defects in Cebus apella monkeys. Int J Oral Maxillofac Implants 2004; 19(1): 73-9.
4. Johnson MW, Sullivan SM, Rohrer M, Collier M. Regeneration of peri-implant infrabony defects using PerioGlas: a pilot study in rabbits. Int J Oral Maxillofac Implants 1997; 12(6): 835-9.
5. Teofilo JM, Brentegani LG, Lamanol-Carvalho TL. Bone healing in osteoporotic female rats following intra-alveolar grafting of bioactive glass. Arch Oral Biol 2004; 49(9): 755-62.
6. Zamat JS, Darbar UR, Griffiths GS, Buhman JS, Bragger U, Burgin W, et al. Particulate bioglass as a grafting material in the treatment of periodontal infrabony defects. J Clin Periodontol 1997; 24(6): 410-8.
7. Gatti AM, Simonetti LA, Monari E, Guidi S, Greenspan D. Bone augmentation with bioactive glass in three cases of dental implant placement. J Biomater Appl 2006; 20(4): 325-39.
8. Nevins ML, Camel M, Nevins M, King CJ, Oringer RJ, Schenk RK, et al. Human histologic evaluation of bioactive ceramic in the treatment of periodontal osseous defects. Int J Periodontics Restorative Dent 2000; 20(5): 458-67.
9. Price N, Bendall SP, Frondoza C, Jinnah RH, Hungerford DS. Human osteoblast-like cells (MG63) proliferate on a bioactive glass surface. J Biomed Mater Res 1997; 37(3): 394-400.
10. Xynos ID, Batten JJ, Butterly LD, Hench LL, Polak JM. Bioglass 45S5 stimulates osteoblast turnover and enhances bone formation In vitro: implications and applications for bone tissue engineering. Calcif Tissue Int 2000; 67(4): 321-9.
11. Xynos ID, Edgar AJ, Butterly LD, Hench LL, Polak JM. Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein synthesis. Biochem Biophys Res Commun 2000; 276(2): 461-5.
12. Xynos ID, Edgar AJ, Butterly LD, Hench LL, Polak JM. Gene-expression profiling of human osteoblasts following treatment with the ionic products of Bioglass 45S5 dissolution. J Biomed Mater Res 2001; 55(2): 151-7.
13. Carinci F, Palmieri A, Martinelli M, Petrovita V, Piattelli A, Brunelli G, et al. Genetic portrait of osteoblast-like cells cultured on PerioGlas. J Oral Implantol 2007; 33(6): 327-33.
14. Palmieri A, Pezzetti F, Spinelli G, Arlotti M, Avantaggiato A, Scarano A, et al. PerioGlas regulates osteoblast RNA interfering. J Prosthodont 2008; 17(7): 522-6.
15. Tsikou O, Jones JR, Polak JM, Stevens MM. Differentiation of fetal osteoblasts and formation of mineralized bone nodules by 45S5 Bioglass conditioned medium in the absence of osteogenic supplements. Biomaterials 2009; 30(21): 3542-50.
16. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential
of adult human mesenchymal stem cells. Science 1999; 284(5411): 143-7.
17. Valenti MT, Dalle CL, Donatelli L, Bertoldo F, Zanatta M, Lo C, V. Gene expression analysis in osteoblastic differentiation from peripheral blood mesenchymal stem cells. Bone 2008; 43(6): 1084-92.
18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4): 402-8.
19. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997; 89(5): 747-54.
20. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc-finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002; 108(1): 17-29.
21. Bilezikian JP, Raisz LG, Rodan GA. Principles of Bone Biology. 2nd ed. California: Academic Press; 2002.
22. McKee MD, Farach-Carson MC, Butler WT, Hauschka PV, Nanci A. Ultrastructural immunolocalization of noncollagenous (osteopontin and osteocalcin) and plasma (albumin and alpha 2HS-glycoprotein) proteins in rat bone. J Bone Miner Res 1993; 8(4): 485-96.
23. Dodds RA, Connor JR, James IE, Rykaczewski EL, Appelbaum E, Dul E, et al. Human osteoclasts, not osteoblasts, deposit osteopontin onto resorption surfaces: an in vitro and ex vivo study of remodeling bone. J Bone Miner Res 1995; 10(11): 1666-80.
24. Ohtsuki C, Kamitakahara M, Miyazaki T. Bioactive ceramic-based materials with designed reactivity for bone tissue regeneration. J R Soc Interface 2009; 6(Suppl 3): S349-S360.
25. McCabe LR, Banerjee C, Kundu R, Harrison RJ, Dobner PR, Stein JL, et al. Developmental expression and activities of specific fos and jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation. Endocrinology 1996; 137(10): 4398-408.
26. Chan TF, Poon A, Basu A, Addleman NR, Chen J, Phong A, et al. Natural variation in four human collagen genes across an ethnically diverse population. Genomics 2008; 91(4): 307-14.
27. Suuriniemi M, Kovanen V, Mahonen A, Alen M, Wang Q, Lyytikainen A, et al. COL1A1 Sp1 polymorphism associates with bone density in early puberty. Bone 2006; 39(3): 591-7.