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The Ionic Products of Bioactive Glass Particle Dissolution Enhance Periodontal Ligament Fibroblast Osteocalcin Expression and Enhance Early Mineralized Tissue Development

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Abstract

This study resulted in enhanced collagen type 1 and osteocalcin expression in human periodontal ligament fibroblasts (hPDLF) when exposed to glass conditioned media from bioactive glasses that subsequently may promote early mineralized tissue development. Commercial Bioglass™ (45S5) and experimental bioactive coating glass (6P53-b), were used to make a glass conditioned media (GCM) for comparison to control medium. ICP-MS analysis showed increased concentrations of Ca^{2+} , PO_4^{3-} , Si^{4+} , and Na^+ , for 45S5 GCM and Mg^{2+} , K^+ , Ca^{2+} , PO_4^{3-} , Si^{4+} , and Na^+ for 6P53-b GCM (relative to control medium). Differentiating hPDLF cultures exposed to 45S5 and 6P53-b GCM showed enhanced expression of collagen type 1 (Col1 α 1, Col1 α 2), osteocalcin, and alkaline phosphatase gene expression. These GCM also enhanced osteocalcin protein expression. After 16 days of culture, 45S5 and 6P53-b GCM treated cells showed regions of deep red Alizarin staining, indicating increased Ca within their respective extracellular matrices (ECM), while control-treated cells did not. SEM analysis showed more developed ECM in GCM treated cultures, indicated by multiple tissue layering and abundant collagen fiber bundle formation, while control treated cells did not. SEM analysis showed polygonal structures suggestive of CaP in 45S5 GCM treated cultures. These results indicate the osteogenic potential of bioactive coating glass in periodontal bone defect filling applications.

Keywords

bioactive glass; human periodontal ligament fibroblast; osteogenesis; mineralized tissue; gene expression; silicon

Introduction

Biomaterials for periodontal bone defect healing have been generally categorized as bioinert or bioactive. Bioinert materials (e.g., titanium (Ti)) do not adversely affect the surrounding living tissue when implanted; however, they form a mechanical bond to the surrounding

osseous tissue, which makes it difficult for direct bone bonding (chemical and mechanical attachment) [1, 2]. Bioactive materials for bone healing are categorized as osteoconductive and osteoinductive. Bioactive glasses, such as bioactive coating glass (50–59 wt.% SiO₂) [3], and Bioglass™ (45S5), have traditionally been considered as osteoconductive [2]. Such osteoconductive materials partially dissolve in the physiological environment, releasing ions (e.g., silicon, calcium, phosphate) that are used by cells to initiate the bone healing process [4]. This partial dissolution results in the formation of a surface hydroxyapatite layer that has a stiffness closely matching the mineral phase of bone [5]. In contrast, osteoinductive materials (e.g., growth factor, pharmaceuticals) alter the bone healing process by influencing osteoblast gene expression during differentiation [4].

Other studies suggest that bioactive glasses are osteoinductive, since the ionic products released by the bioactive glasses enhance the expression of osteogenic markers (e.g., collagen type 1, alkaline phosphatase, core binding factor α (Runx2), and osteocalcin) important for osteoblast differentiation [6, 7, 8]. Moreover, such enhanced marker expression yielded increased rate and density of mineralized tissue. This enhanced effect could also occur in other cell lines depending on the implant site. For example, these bioactive glasses are used in applications involving periodontal bone defect filling [9]; thus, it is important to determine if these materials will enhance the osteogenic potential of periodontal ligament cells.

Several key features of human periodontal ligament fibroblasts (hPDLFs) make them osteogenic. First, hPDLFs are partially responsible for producing, maintaining and remodeling the periodontal ligament, gingiva, cementum and bone because of their unique location. This heterogeneity has been suggested to be of fundamental importance for normal function and wound healing of connective tissue [10]. Second, hPDLFs can express osteoblast-like properties, such as mineralized nodule formation (e.g., calcium phosphate or hydroxyapatite crystals) and alkaline phosphatase synthesis, that gingival fibroblasts lack [11]. Third, hPDLF osteogenesis follows a timeline associated with its differentiation into a mineralizing phenotype that is similar to osteoblast differentiation. Starting from senescence (10–12 days), hPDLFs express collagen type 1 (0–2 d after senescence), followed by alkaline phosphatase expression (2–8 d after senescence), and then osteocalcin expression and mineralized tissue synthesis (6–10 d after senescence) [12, 13, 14].

Bioactive glass treatment of hPDLFs may enhance osteogenic marker expression and mineralized tissue production. Improved osteogenic potential has been reported as a result of reduced particle size (from 1000–3000 μm to 100–300 μm) [15]. This is probably owed to increased surface area and relatively faster dissolution. Others have found that enhanced collagen type 1, alkaline phosphatase, and mineralized nodule formation occurred in the presence of bioactive glasses and vitamin D when they were added to differentiating hPDLF cells [16, 17, 18]. However, these encouraging results did not connect early mineralized tissue production, a product of Ca tissue incorporation, to osteocalcin expression, a key Ca-binding protein.

This study tests the hypothesis that enhanced hPDLF osteocalcin expression leads to early calcium incorporation in developing mineralized tissue. The aim of this study is to

demonstrate that bioactive coating glasses possess the same osteogenic potential as Bioglass™ and that the enhanced mineralized tissue formation is directly coincident with the enhanced expression of osteocalcin.

Materials and Methods

Study Design.

Bioactive glass (45S5 (Mo-Sci, Rolla, MO)) and 6P53-b particles (SEM-COM, Toledo, OH) were dissolved in cell culture medium (Dulbecco's modified Eagle's Medium (DMEM)) and measured for their ion concentrations using inductively coupled plasma mass spectrometry (ICP-MS) after 3 days of immersion. These glass conditioned media (GCM) were studied for their effect on hPDLF osteogenesis. Cells were introduced to glass conditioned media for a period of 16 days along with ascorbic acid (AA). Cells were lysed for total protein and total RNA. Total protein was assayed using enzyme-linked immunosorbent assay (ELISA) and mRNA was used for relative gene expression analysis via quantitative polymerase chain reaction (qPCR). Finally, cell layers were stained histologically (using Alizarin red staining) and by scanning electron microscopy with electron dispersive x-ray analysis (SEM) to visualize ECM as a result of GCM treatment. All comparisons were made to control treatments for statistical significance. All experiments were repeated. Triplicate sampling was used per experiment. For all assays, inter-well and internal control assays were conducted according to the manufacturer's protocol.

Glass Conditioned Media Preparation.

45S5 and 6P53-b glass powders were soaked in DMEM for 3 days to make 45S5 and 6P53-b ion extracts. This ion extract was filter-sterilized (0.2 µm) and supplemented with fetal bovine serum (FBS, 10% by volume), penicillin-streptomycin (pen-strep, 1% by volume), and fungizone (0.5% by volume) to provide the final glass conditioned medium (GCM). Control medium was prepared using D-MEM with the same supplements. Differentiation of the hPDLF into a mineralizing phenotype was carried out by addition of ascorbic acid (AA, 50 ppm). Samples (1 ml) were removed from each solution and analyzed for their ion concentrations using inductively coupled plasma mass spectrometry (ICP-MS).

ICP-MS Analysis.

At each time point listed above, 1 ml of 45S5 and 6P53-b ion extracts were removed for analysis with ICP-MS. Samples were diluted 1:100 in 3% nitric acid. Measurement of the ion concentrations within ion extracts was carried out at the Interdisciplinary Center for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at the University of California, at Davis (described previously [6]).

Isolation of hPDLF Cells.

hPDLF cell preparation and culture was modified slightly from the technique by Somerman *et al.* [19]. Premolar teeth that were to be extracted for orthodontic reasons were used. A sulcular incision was made around the teeth and the gum flapped; the tooth was extracted using elevators and forceps. The teeth were rinsed and placed in D-MEM. To minimize contamination with gingival and apical tissue only PDL tissues attached to the middle third

of the root were curetted. Moreover, PDL was removed by cutting it away from the tooth root. Cells were obtained from the PDL by additional slicing of the tissue and incubating in trypsin (0.25%, 10 minutes) to alleviate contact inhibition. The PDL cells while suspended in trypsin were then pelleted and allowed to culture in basal medium (D-MEM, 1% pen-strep, 10% FBS) and incubated (37°C, 5% CO₂, 100% RH). The next day, the medium was replaced with fresh basal medium. When cells surrounding the tissue explants were confluent, they were transferred to 150 cm² tissue culture flasks.

Cell Culture.

hPDLFs (passage 5) from flasks were seeded (40,000 cells cm⁻²) and allowed to attach and proliferate through their doubling time (24 hours) in triplicate in 6-well tissue culture plastic plates in control media. Cells were counted using a standard hemacytometer and an optical microscope (Nikon TE300, Nikon Incorporated, Tokyo, Japan) and were synchronized with D-MEM, 1% FBS, and 1% pen-strep. Media was then exchanged for control + AA and GCM+AA treatments. Cultures were maintained for 16 days with replenishment of media at 2 day intervals. Experiments were carried out in triplicate for each treatment. hPDLF cells were then allowed to mineralize by the introduction of mineralization media, which consisted of their respective GCM with ascorbic acid. Experiments were carried out in triplicate for 16 days, with specimens generated at days 2, 4, 8, and 16.

Cell Proliferation Assay.

Cells were assayed for proliferation in each glass conditioned medium and control medium. Cells were seeded at 50 000 cells cm⁻² in 96-well plates. The cells were allowed to grow in each medium for a period of 16 days (the assay was described previously [6]). At the specified time point (1, 2, 4, 8, and 16 d), cells were removed from culture and their media was changed for assay medium. Cell number was then assayed using a spectrophotometer with absorbance measured at 540 nm.

Quantitative RT-PCR.

Details describing the qPCR method used were given previously [6] and briefly described here. At each time point (2, 4, 8, 16 d) cells were disrupted for their total cellular RNA using the RNEasy Mini kit (Qiagen, Valencia, CA) and the lysed products were collected in microcentrifuge tubes. A DNase digestion kit (RNase-free DNase set (50), Qiagen, Valencia, CA) was used to improve cDNA quality (recommended by RNEasy Mini Kit protocol). Total RNA was converted to cDNA using reverse transcriptase (RTS, Promega, Madison, WI) with total RNA and cDNA quantified for their concentration using a full-spectrum nanodrop spectrophotometric analyzer (ND-1000, Nanodrop products, Wilmington, DE). Total cDNA was diluted to a final concentration of 100 ng µL⁻¹. Total RNA and cDNA quality was measured using the optical density ratio at A_{260}/A_{280} , with a ratio of 1.8–2.1 used to ensure quality and A260 for measurement of concentration.

Quantitative PCR was used to measure gene expression. The cDNA was amplified using the following reagent mixture (all final concentrations): FastStart TaqMan® Probe Master (Roche Applied Science, Mannheim, Germany) at 2× concentration, TaqMan assay kit (forward primer 900 nM, reverse primer 900 nM, probe 250 nM), RNase/DNase free water

(35% by volume), and 10% sample by volume in a 10 μ L total reaction volume. Standard curves were generated by amplification of serially diluted samples for each gene measured for its expression in this study.

PCR amplification was measured using the *SDS software package* (Applied Biosystems). The amplification curves were regressed using a sigmoidal curve fitting method according to the method by Qiu *et al.* [20] using the *SigmaPlot software package* (Systat Inc., San Jose, CA) to estimate the threshold cycle (C_T). The delta-delta C_T method was used to report relative gene expression (GAPDH reference gene).

Protein expression assays.

At the described time points (2,4,8,16 days), cell cultures were scraped and collected in phosphate buffered saline, centrifuged, and collected as a cell pellet. Pellets were treated with the appropriate lysis buffer (Celytic M, Sigma Aldrich, St. Louis, MO). Protease inhibitors (Sigma Aldrich) were used to prevent protein denaturation during their isolation and characterization. A portion of the lysed cells was separated and was not treated with protease inhibitor for analysis of alkaline phosphatase.. The concentration of total protein was assayed (BCA protocol, Pierce Biosciences, Rockford, IL) and the expression level of osteocalcin (Biomedical Technology Inc. Stoughton, MA) were evaluated. A standard curve was developed for the total protein, alkaline phosphatase, and osteocalcin assay. Measurement of alkaline phosphatase activity and osteocalcin expression (Human Osteocalcin ELISA kit, Biomedical Technologies, Springfield, MA) have been described [6].

Mineralized Tissue Staining and Analysis.

Cell plates obtained at selected time points (2,4,8 days) were visualized for collagen or ECM??? formation using Alizarin staining (Direct Red 80 Sigma Aldrich, St. Louis, MO). Cells on glass cover slips were removed from culture and fixed in 10% formalin (in PBS, pH = 7.4) for 30 minutes at room temperature.

Alizarin staining solution was prepared by dissolving Alizarin reagent (sigma Inc., St. Louis, MO) at 1% in deionized water. The solution was then titrated (using TRIS buffer and 0.1 N HCl) to bring the pH to 4.0–4.5. The stain was then applied by fully immersing specimens?? on cover slips for 1 hour. Excess stain was removed using deionized water, then xylene-acetone (1:1) for 30 seconds. Samples were then dried overnight prior to imaging. This protocol was used to remove Ca that was not bound to the ECM.

Specimens were imaged using optical light microscopy to first image mineralized tissue layers. Counterstaining was accomplished by staining the nuclei of hPDLF (and the plasma membranes) on glass cover slips with Weigert's haematoxylin for 8 minutes, and then washed for 10 minutes in deionized water. Samples were then sequentially alcohol dehydrated (80–100% ethanol). Imaging of cell layers was accomplished under polarized light microscopy (BX51, Olympus Inc., Tokyo, Japan). With this staining procedure, the stain appears pink to dark-red, with pink indicating cell and tissue layer and more intense red indicating calcium incorporation.

Specimens were also analyzed using scanning electron microscopy with electron dispersive x-ray analysis (SEM) (Hitachi S4300, 10 keV, high vacuum, Hitachi Inc., Tokyo, Japan, 10 keV). Samples were Au-coated using a sputter coater (Hummer VII, Anatech, LTD, Alexandria, VA) while mounted on sample holders and attached using carbon tape.

Statistical Analysis.

Statistical comparisons ($p < 0.05$ for statistical significance) were made between GCM-treated cells and control-treated cells using two-way analysis of variance (ANOVA, independent variables: time, treatment; dependent variables: gene expression, protein expression). All experiments were repeated with triplicate sampling per experiment. Internal standards and inter-well variation was also incorporated into assay measurements.

Results

Ion extracts were collected after 72 hours of soaking each bioactive glass in D-MEM. The ion concentrations for DMEM, 45S5 ion extract, and 6P53-b ion extract are reported in Table 1, with DMEM ion concentrations subtracted from 45S5 and 6P53-b ion concentrations. All ion concentrations were observed to increase after 72 hours for each GCM relative to D-MEM. Increased Mg and K was only observed for 6P53-b ion extracts.

Cell proliferation experiments showed no statistically significant differences between each GCM treatment and control treated cells (results not shown). This means that gene expression and protein expression results did not vary with cell number for group comparisons.

Results of gene expression analysis showed that the ionic products of bioactive glass dissolution enhanced hPDLF osteogenic gene expression. For example, both GCM and AA treatments increased *Col1 α 1* expression 6 times that of control and AA treatments (Figure 1), while 45S5 and 6P53-b GCM and AA treatments increased *Col1 α 2* expression 6 and 10 times that of control and AA treatments, respectively, after 2 days (Figure 2). After 16 days, 45S5 and 6P53-b GCM and AA treatments increased *Col1 α 1* expression 38 and 3 times that of control and AA treatments, respectively (Figure 1), while *Col1 α 2* was increased 42 and 4 times that of control and AA treatments, respectively (Figure 2). These results confirm previous work in which we showed each GCM enhanced osteoblast gene and ECM collagen matrix formation [6].

Expression of alkaline phosphatase was also enhanced in the presence of bioactive glass ions. For example, 45S5 and 6P53-b GCM and AA treatments increased alkaline phosphatase expression 13 and 6 times that of control and AA treatments after the first day, respectively (Figure 3) and 6 and 18 times that of control and AA treatments after the eighth day, respectively, Figure 3). Alkaline phosphatase is an important marker indicating early differentiation of hPDLFs into a mineralizing phenotype.

Osteocalcin expression was also enhanced in the presence of bioactive glass ions. 45S5 and 6P53-b GCM and AA treatments marked increased osteocalcin gene expression 14 and 11 times that of control and AA treatments after 8 days of culture, respectively (Figure 4).

Similarly, both GCM and AA treatments increased osteocalcin protein synthesis nearly 6 times that of control and AA treatments (Figure 5). Osteocalcin is a key, non-collagenous protein involved in the binding of calcium to the extracellular matrix.

Besides the enhanced expression of these biomarkers, a more distinct mineralized tissue formation occurred for cells cultured in GCM with AA as compared to control with AA. For example, 45S5 GCM and 6P53-b GCM and AA treatments (Figure 6A and B, respectively) had a deeper Alizarin red stain (c, Figure 6A and B) than that of control and AA treatment (Figure 6C). Regions with high numbers of hPDLF cells appeared to be interspersed with regions of ECM formation from tissue regions (a, Figure 6A, B, and C).

SEM analysis showed differences in extracellular matrix structure produced by GCM and AA treated cells as compared to control and AA treated cells (Figure 7). For 6P53-b (Figure 7A and B) and 45S5 GCM (Figure 7C and D) and AA treatments, extracellular matrix appeared to have multiple layers of tissue formation with each layer nearly orthogonally oriented. Control and AA treatments resulted in dense hPDLF cultures (Figure 7E and F), however, no complex ECM development was observed. Interestingly, 45S5 GCM and AA treated cells had polygonal structures within their ECM, which may indicate the presence of Ca-P structures. Control and 6P53-b GCM and AA treated cells did not appear to show such polygonal structures in their respective ECM.

Discussion

This study showed the effect that commercial Bioglass™ and bioactive coating glass had on hPDLF osteogenesis. Both bioactive glasses released ions that enhanced the expression of several key osteogenic markers: collagen type 1 (Col1 α 1, Col1 α 2, alkaline phosphatase (Akp2), and osteocalcin (Bglap). The ECM resulting from GCM and AA treatments showing increased layering of tissue and the appearance of collagen fiber bundles.

Our results showed evidence of Ca in GCM and AA treated cells. The amount of Ca that accumulated in GCM and AA treated cells was not high since only a few locations within these ECM showed its presence (note that at this early stage of ECM development little mineral formation or calcification was expected). This ECM incorporation of Ca (as shown by Alizarin staining) was probably owed to the increased Ca ion concentration in each GCM, which shifts the equilibrium in the media towards the precipitation of Ca since these media are nearly super-saturated. Ca was not observed in the ECM of control and AA treated cells. Thus, we can suggest that increased osteocalcin expression by GCM and AA treated hPDLFs may have led to increased Ca presence within hPDLF ECM. This process may have been delayed in the control and AA treated cells.

The lack of stimulatory effect on day 4 was expected for collagen type 1 and osteocalcin expression based on the timeline (given above), however, the lack of enhanced expression for alkaline phosphatase was unexpected. hPDLF alkaline phosphatase expression was shown to be maximally enhanced at the start of calcification (16–21 days) [12, 14], owed to an increase in phosphoric acid concentration (dissociates to PO₄³⁻ *in vitro*) and the elimination of calcification inhibitors [13] The increased total phosphate ion concentration

in GCM treatments may have caused an initial stimulatory effect on alkaline phosphatase expression after 2 days in the senescent hPDLFs. The lack of a stimulatory effect on alkaline phosphatase expression on day 4 may have been owed to hPDLF down-regulation of alkaline phosphatase as they differentiate into an osteogenic phenotype [13], suggesting, that calcification inhibitors may have not been eliminated at this time point. Another unexpected result related to the enhanced expression of collagen type 1 for 45S5 GCM and AA treated cells on day 16. This enhanced collagen expression could have been owed to the possible initiation of calcification (as indicated by Ca ECM incorporation and the presence of polygonal structures [13]) and the enhanced expression of osteocalcin (in this work).

Since collagen and osteocalcin expression were enhanced by these ions, they may play a role in intracellular and extracellular pathways important to osteogenesis. Of these ions, calcium is known to play a key role in hPDLF function. Calcium acts as a cell signaling agent during all phases of the cell cycle in mesenchymal cell biology. Most mesenchymal cells have a calcium receptor on their plasma membranes. For differentiation, calcium signaling is considered a secondary messenger signaling molecule that acts on G-protein coupled receptors, which in turn activate various MAPKs for regulation of differentiation. It was found that extracellular Ca is implicated in osteogenesis by increasing collagen type 1 and osteocalcin expression [21]. For hPDLFs, intracellular calcium is important in the expression of prostaglandin E₂ (PGE₂), a cell signal involved in hPDLF response to tooth movement or chemical stimuli. PGE₂ is implicated in bone mineralization and remodeling and its levels increase during bone inflammation and repair [22]. It is reasonable to investigate the effect of these ions on osteopontin (Spp1), bone sialoprotein (Bsp1), osteonectin, Cbfa1/Runx2, and Wnt signaling since these are also regulators of osteogenic differentiation. All of these markers affect hPDLF differentiation.

Silicon may also play an essential role in osteogenesis. We have shown in a previous study that silicon may directly be involved in osteocalcin-mediated mechanisms [6]. This was evident by the sensitivity of osteoblasts to increased concentrations of silicon. In addition, it was found that osteocalcin expression in primary human osteoblasts increased 1.4 times that of control treatments when treated with additional Si (50 µM) [23]. When treated with concentrations close to that for bioactive glass corrosion (~ 1 mM) [6, 24], we found markedly enhanced expression (40–70 times that of control treatments). Moreover, individual and synergistic roles in osteogenesis have been explored in osteoblasts [24], and, may be important for hPDLF osteogenesis. Thus, Si and Ca may play key roles in enhanced hPDLF osteogenesis.

Some clinical uses of bioactive glass in regenerative periodontal treatment have been demonstrated. Wilson and Low [9] showed that bioactive glass materials were found to be haemostatic and osteo-productive, allowing restoration of both alveolar bone and periodontal ligament in a monkey model. In humans, bioactive glass treatment of intra-osseous defects by means of bone grafting has shown statistically significant improvement of the bony lesion in surgical periodontal treatment [25, 26, 27, 28, 29, 30].

Both bioactive glasses studied had important effects which elevated levels of key osteogenic markers and increased calcium incorporation in extracellular matrix formation. At this point

an optimized bioactive glass composition has not been defined that would provide the best osteogenic response. The experimental bioactive glass provides an increased range of possible applications as compared to commercial Bioglass™. Future efforts to further chemically identify ECM structures and mechanically identify resultant tissue stiffness will help optimize such glass compositions. Several applications of both glasses could be investigated and include powder bone defect fillers, polymer-bioactive glass composite bone defect filler (larger defects), and post-wisdom tooth extraction pocket liner to enhance mineralized tissue formation. An advantage to the family of bioactive glasses that were developed from our laboratory have additional applications as coatings for dental and orthopaedic implants. Still, several application and molecular issues must be addressed and they include the impact of tooth movement on the interaction of bioactive glass ions and hPDLFs, the impact of implant movement on local adhesion of ligaments to the implant coating surface (if ankylosis has not occurred), the impact that these ions may have on ligament healing or other tissue types. These larger questions will be addressed in future work.

Conclusions

This study tested the hypothesis that enhanced hPDLF osteocalcin expression leads to early calcium incorporation in developing mineralized tissue. The glass ions *in vitro* concentration increased expression of collagen type 1 (Col1 α 1, Col1 α 2), alkaline phosphatase, and osteocalcin. The enhanced gene expression coincided with enhanced protein expression of osteocalcin. Thus, the earlier presence and increased osteocalcin concentration should lead to earlier incorporation of calcium within GCM treated extracellular matrices as suggested by the increased Alizarin staining. ”

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Figure 1.

Col1α1 gene expression by hPLDF cells in the presence of 45S5 GCM + AA, 6P53-b GCM + AA, and control medium + AA. GCM + AA treated cells induced increased expression of Col1a1 as compared to control (ANOVA, $p < 0.05$, * indicates statistical significance).

Figure 2.

Col1 α 2 gene expression by hPLDF cells in the presence of 45S5 GCM + AA, 6P53-b GCM + AA, and control medium + AA. GCM + AA treated cells induced increased expression of Col1 α 2 as compared to control (ANOVA, $p < 0.05$, * indicates statistical significance).

Figure 3. Alkaline phosphatase gene expression by hPLDF cells in the presence of 45S5 GCM + AA, 6P53-b GCM + AA, and control medium + AA. GCM + AA treated cells induced increased expression of alkaline phosphatase as compared to control (ANOVA, $p < 0.05$, * indicates statistical significance).

Figure 4. Osteocalcin gene expression by hPLDF cells in the presence of 45S5 GCM + AA, 6P53-b GCM + AA, and control medium + AA. GCM + AA treated cells induced increased expression of osteocalcin as compared to control (ANOVA, $p < 0.05$, * indicates statistical significance).

Figure 5. Osteocalcin protein expression by hPLDF cells in the presence of 45S5 GCM + AA, 6P53-b GCM + AA, and control medium + AA. GCM + AA treated cells induced increased expression of osteocalcin as compared to control (ANOVA, $p < 0.05$, * indicates statistical significance).

Figure 6.

Optical micrograph of Alizarin red staining of hPDLF cells exposed to glass conditioned media (GCM) + AA (A) 6P53-b + AA; (B), 45S5 GCM + AA ;, and (C) control media + AA (C) after 16 days of treatment *in vitro*. Areas of light pink stain (a) indicate PDLF cells. Areas of pink-red (b) stain indicate regions of extracellular matrix. Areas of deep red to red-black stain (c) indicate Ca-rich areas. Control treatments were not observed to have deep red to red-black staining.

Figure 7.

Scanning electron micrographs depicting areas of mineralized tissue formation for 6P53-b GCM treated cells (A and B), 45S5 GCM treated cells (C and D), and control-treated cells (E and F). Both 45S5 and 6P53-b GCM treated cells resulted in the formation of complex tissue structures. 45S5 GCM treatments showed the appearance of polygonal structures (a) that suggests incorporation of calcium and phosphate (Ca^{2+} and PO_4^{3-}), collagen fiber bundles (b), and hPDLFs (c). 6P53-b GCM treated cells showed the presence of similar collagen fiber bundle formation. Control treated cells showed only differentiated hPDLF dense cultures.

Table 1.

Ion Extract Concentration (ppm) after 72 h

	Si ⁴⁺	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	P0 ₄ ³⁻
D-MEM	2.536	3498	255.1	103.5	22.19	32.53
45S5-D-MEM	56.8 ±2.7	978.0 ±218.4		53.3 ±8.7		15.4 ±3.1
6P53b-D-MEM	65.0±1.2	767.0 ±43.1	230.3 ±3.5	57.0±3.6	40.3±0.2	13.1±1.4

45S5-DMEM, 6P53-b-DMEM represents the concentration of each ion with the DMEM concentration subtracted from the 45S5 and 6P53-b soaked DMEM ion concentrations.