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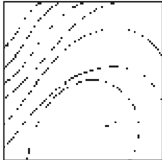
Effects of PDGF-BB and OP-1 on Mesenchymal Stem Cells in a Porous Mineral Block



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The aim of this study was to evaluate the effects of an osteogenic medium supplemented with platelet-derived growth factor (PDGF) BB and osteogenic protein (OP) 1 on the proliferation and differentiation of mesenchymal stem cells (MSCs) in an anorganic bovine cancellous bone scaffold. At day 7, there was a statistically significant increase in the number of cells in the scaffolds in the group treated with the medium supplemented with both PDGF-BB and OP-1 when compared with the control groups. The highest alkaline phosphate levels, at 14 and 21 days, were recorded for the samples in medium supplemented with OP-1 alone reflecting osteogenic differentiation. The results commend OP-1, as well as PDGF-BB, for incorporation into porous mineral scaffolds for vertical ridge augmentation. (Int J Periodontics Restorative Dent 2013;33:e72–e78. doi: 10.11607/prd.1670)

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Vertical ridge augmentation continues to present a challenge for bone reconstruction in the mandible and maxilla. A prior canine investigation demonstrated the promise of a porous mineral block infused with recombinant human platelet-derived growth factor (rhPDGF)BB.[1] In that study, two threaded titanium dental implants were inserted into the block prior to its implantation. Holes were drilled into the mandible prior to implantation to allow the mesenchymal stem cells (MSCs) of the marrow access to the pores of the scaffold. The results demonstrated the ingrowth of new bone into the scaffold, the attendant resorption of a portion of the scaffold, and partial osseointegration of the dental implants. The promising results raise questions for how the procedure can be improved and what role other growth factors may be able to play. To answer these and other questions, MSCs were grown in the mineral scaffold in vitro and the effects of osteogenic protein (OP) 1 (also known as bone morphogenetic protein, BMP-7) as well as PDGF-BB on the attachment, proliferation, and differentiation of MSCs were evaluated.

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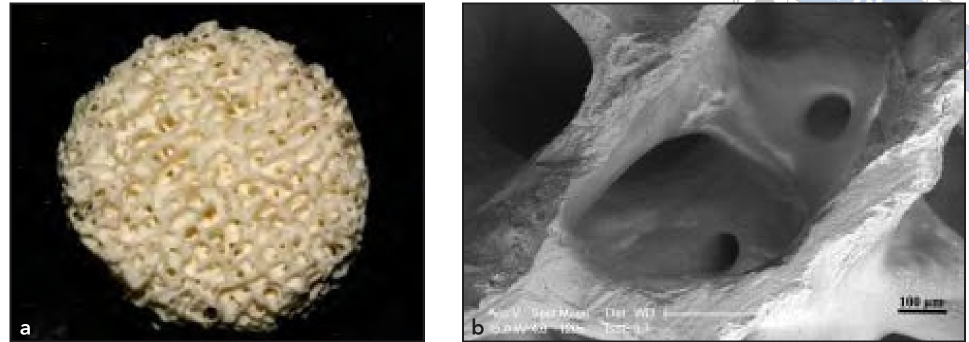


Fig 1 (a) *Porous mineral block.* (b) *SEM of the typical surface of the walls of the porous scaffold.*

In addition to being commended for this application by its mitogenic activity,[1] its role in bone regeneration,[2,3] and its demonstrated effect in causing the proliferation of osteoblastic cells in an anorganic bovine bone scaffold,[4] prior research[5] has shown that PDGF-BB can stimulate the migration of MSCs at concentrations of up to 100 ng/mL. A recent report that evaluated the effects of 26 growth factors/cytokines on the migration activity of rabbit and human MSCs in a migration chamber in vitro[6] found that PDGF-BB had the greatest effect on migration. Another recent study using the Boyden chamber[7] found that PDGF induced the most significant increase in migration of human bone marrow–derived MSCs compared with a host of other growth factors. OP-1 has also been proven to induce the growth and differentiation of osteoblasts in bone marrow–derived MSC cultures at levels ranging from 0.3 to 3 nmol/L.[8]

In this study, the DNA content of porous mineral scaffolds seeded with goat MSCs was determined after 1, 7, 14, and 21 days. The alkaline phosphatase (ALP) content of the scaffolds was also determined at these time points as a measure of osteogenic differentiation. At termination of the cultures (28 days), constructs in select groups were analyzed for their uptake of technetium-99m-methylene diphosphonate (Tc-99m-MDP) based on the authors' prior work that demonstrated the utility of this radionuclide method for quantifying the mineralization in cultures of osteoblast-like cells.[9]

Method and materials

Harvesting and culture of MSCs

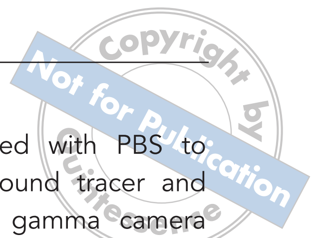
Bone marrow aspirates (approximately 2 mL) were obtained from the posterior iliac crests of six female Spanish goats, and MSCs were isolated as described previously[10]; cells from the six goats were grown separately. The cells were grown in an expansion medium (EXP): low-glucose Dulbecco modified Eagle medium (DMEM-LG) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cells were plated in tissue culture flasks at a density of approximately 4,000 cells/cm^2. Upon reaching 80% to 90% confluence, the cells were trypsinized and frozen. Cells were subsequently thawed in the EXP. After thawing, 250,000 cells of each goat were seeded in T-150 flasks (passage 1, P1), and cultured in EXP for 10 days.

Scaffold preparation

Scaffolds were fabricated by shaping a porous block of anorganic cancellous bovine bone (Bio-Oss Block, Geistlich Pharma) to samples 8 mm in diameter by 2 mm in thickness using a diamond disk (Fig 1a). Scaffolds were autoclaved and packed in a sterilized bag. The dry weights of the scaffolds ranged from 83 to 111 mg.

Cell seeding of the mineral scaffolds

Upon reaching 80% to 90% confluence, the cells were trypsinized and seeded into the mineral scaffolds. Cells were counted, and viability was assessed via trypan blue staining.



Scaffolds were placed in 12-well culture plates. Each scaffold was seeded with 1 × 10^6 cells by pipetting 100 μL of the cell suspension onto the block. Seeded mineral blocks were incubated for 2 hours in a humidified atmosphere of 37°C and 5% carbon dioxide (CO_2). Next, 2 mL of DMEM containing 10% FBS, 100 U/mL penicillin, and 100 mg/L streptomycin was added to each well. The mineral blocks were incubated overnight in EXP to allow the cells to attach. One of the following media was then added to the wells: *(1)* EXP; *(2)* osteogenic medium (OST): EXP plus 100 nmol/L dexamethasone; 10 mM β-glycerol phosphate, and 0.05 mM ascorbate; *(3)* OST plus rhPDGF-BB (100 ng/mL), selected based on prior work that demonstrated the PDGF-BB chemoattraction of MSCs at values of 50 to 100 ng/mL[5,6]; *(4)* OST plus rhOP-1 (100 ng/mL), selected based on prior work that demonstrated that OP-1 enhances osteoblast activity[2] and MSC differentiation[8] around 100 ng/mL; or *(5)* OST plus rhPDGF-BB/rhOP-1 (each at 100 ng/mL).

Total DNA and ALP quantification

On days 1, 7, 14, and 21, four scaffolds were used for DNA quantification and ALP activity measurement. DNA was extracted from scaffolds in the following manner. After washing three times with phosphate-buffered saline (PBS), cell-scaffold constructs were ground with a cell lysis reagent (0.2% TritonX-100, Sigma), and aliquots of lysate, which were sonicated for 30 seconds and centrifuged for 5 minutes to remove scaffold debris, were divided for DNA and ALP assay. On day 28, scaffolds were allocated for Tc labeling and SEM investigation.

The PicoGreen dsDNA Quantitation Kit (Invitrogen) was used for DNA quantification for photometric evaluation of MSC attachment (cell count after 1 day) and proliferation (increase in cell count from day 1 to day 7). Aliquots of scaffold digest were diluted 1:10 in Tris–EDTA buffer (TE; 10 mmol/L Tris, 1 mmol/L EDTA; pH, 8.0) and processed according to the kit protocol with fluorescence read at 485 nm/535 nm for 1 second against a lambda DNA standard on a microplate reader (PerkinElmer Life and Analytical Sciences). Because of possible variations in the exact dimensions of the scaffolds, the DNA data were expressed as ng/mL DNA per mg scaffold weight. The cell number in each of the constructs could be estimated based on the mean value for the DNA content of the MSCs, determined to be 5.6 pg DNA/cell.[10]

Radionuclide imaging

Radionuclide imaging was performed using a method previously described.[9] An aliquot of 150μCi Tc-99m-MDP was added to each culture of EXP and OST at day 28 (n = 6) for 2 hours at room temperature. The activity of Tc-99m-MDP was assayed with a dose calibrator (CRC-15R, Capintec). The dishes were then washed with PBS to remove the unbound tracer and placed under a gamma camera (ECAM Variable, Siemens Medical Solutions) using the two-detector setting. The radionuclide counts were acquired for 3 minutes, and the images were analyzed using Siemens software (ESOFT 2.0).

Scanning electron microscopy

Samples of the unseeded and seeded scaffolds were examined in an environmental scanning electron microscope (SEM). Sawed sections through plastic-embedded constructs were also examined in the SEM operating in the backscattered electron imaging (BEI) mode. Energy dispersive x-ray microanalysis (EDX) was performed on select regions in the samples to detect the elements present.

Statistical analysis

One and two-factor analysis of variance (ANOVA) with Fisher protected least squares difference (PLSD) and Bonferroni post hoc testing were performed using Statview (SAS Institute) and GraphPad Prism (GraphPad Software, version 5) to determine the significance of the effects of time in culture and the medium on DNA and ALP activity. One-factor ANOVA was used to determine the significance of the effects of medium (EXP versus OST) on the total gamma counts. The criterion for statistical significance was $P < .05$.

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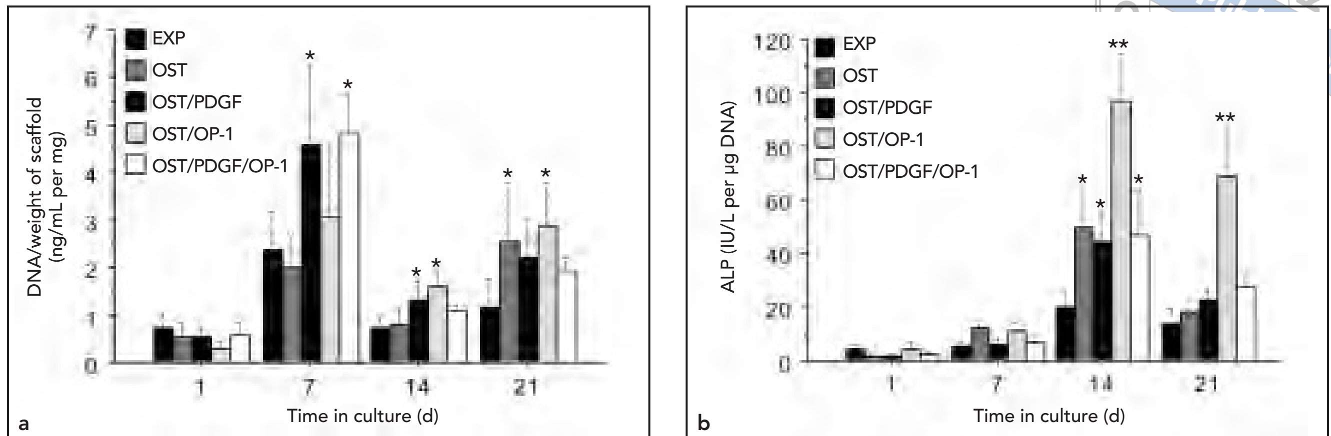

Fig 2 (a) *Quantification of DNA extracted from goat MSCs grown in the mineral block expressed per the weight of the respective scaffold samples (n = 5).* (b) *ALP activity of goat MSCs per scaffold normalized by μg DNA per scaffold (n=5); mean ± standard error;* *P < .01, **P < .001.

Results

Scaffold characterization

SEM revealed a structure similar to that previously reported,[11] with pores approximately 300 to 500 μm in diameter (Fig 1b). The scaffold walls were covered with needle- and plate-like crystallites of apatite, as previously reported.[12]

DNA quantification and cell number

Converting the total amount of DNA to the number of cells revealed that approximately 17% of the 1.0×10^6 cells initially seeded into the scaffold attached at day 1. There was a comparable amount of DNA in the scaffolds in the five groups after the first day of culture (Fig 2a). One-factor ANOVA demonstrated that there was no significant effect of medium group on the DNA content of the scaffold ($P = .76$).

From day 1 to day 7, there was a four- to eightfold increase in the number of cells in each group. This was followed by a decrease at day 14 and then a gradual increase to day 21 (Fig 2a). The largest number of cells was found in the two PDGF-BB–supplemented groups: OST/PDGF and OST/PDGF/OP-1 at 7 days. Two-factor ANOVA showed that there was a significant effect of time in culture ($P < .0001$; power = 1), but not medium group ($P = .32$; power = 0.35), on the amount of DNA (ie, number of cells) in the scaffolds. Fisher PLSD post hoc testing demonstrated that differences in the DNA content when comparing the two time periods were all significant except for the comparison of the data obtained at day 1 versus day 14.

Considering data for day 7 only, to determine the effects of the growth factors (ie, PDGF-BB and OP-1) supplementing the osteogenic medium on cell proliferation, separate comparisons of the OST/PDGF and OST/OP-1 groups versus the OST control group showed no statistical significance in DNA content ($P = .19$ and $P = .40$, respectively). There was, however, a statistically significant increase in the number of cells in the scaffolds in the group treated with the osteogenic medium supplemented with both PDGF-BB and OP-1 (OST/PDGF/OP-1) when compared with the control (OST) group ($P = .03$), albeit with a low power (0.62).

ALP activity

Only a small amount of ALP was assayed at day 1, with no notable differences among groups (Fig 2b). One-factor ANOVA of the day 1 data demonstrated no signifi-

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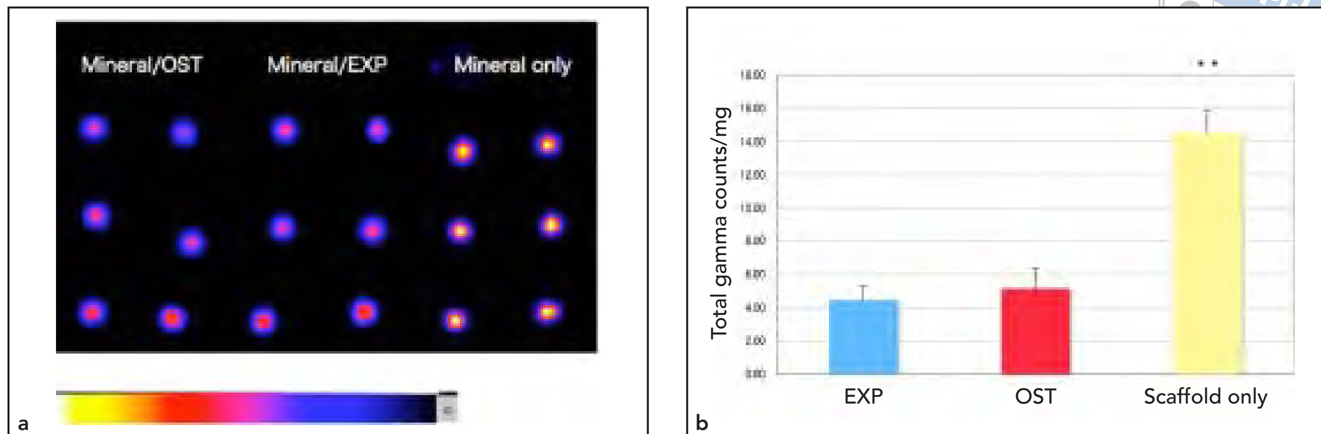

Fig 3 (a) *Radionuclide image of the cultures at 28 days (n = 6).* (b) *Graph of the mean values (± standard error) for the total counts from each group (n = 6), averaging the data from the two detectors (**$P < .001$).*

cant effect of group on ALP levels ($P = .54$). There was an increase in the ALP in all groups from 1 to 7 days in culture. This was followed by a substantial increase in the ALP levels in all groups from 7 to 14 days (Fig 2b). The ALP in all groups then declined from 14 to 21 days (Fig 2b). The highest ALP levels, at 14 and 21 days, which were more than twice that measured in the other groups, were recorded for the OST/OP-1 group (Fig 2b).

Two-factor ANOVA demonstrated that there were significant effects of time in culture and medium group on the ALP content of the scaffolds ($P < .0001$ and power = 1 for both effects). Fisher PLSD post hoc testing showed that the ALP difference for the following group comparisons was statistically significant: EXP vs OST/OP-1, OST vs OST/OP-1, OST/PDGF vs OST/OP-1, and OST/OP-1 vs OST/PDGF/OP-1. The difference in ALP when comparing groups at two time points was statistically significant, except for day 1 vs day 7.

Tc MDP labeling and total gamma count

The appearance of the radionuclide images for the OST and EXP groups was similar (Fig 3a) but also different from the scaffold alone (Fig 3b), which displayed a higher uptake of the Tc-99m-MDP. There was more than a threefold greater radionuclide uptake in the non-MSC seeded scaffold groups compared with the cell-seeded groups cultured in EXP and OST. One-factor ANOVA revealed a significant effect of group on the total gamma counts ($P < .001$), showing the uptake in the scaffold alone group to be significantly greater than in the cell-seeded groups (EXP and OST).

SEM and BEI evaluation

SEM demonstrated cells spread on the walls of the scaffold in all groups. BEI evaluation of all groups confirmed the presence of de novo mineral deposits on the walls of the scaffolds and in the pores of all of the cell-seeded scaffolds grown in osteogenic medium (Figs 4a and 4c to 4f), but not in the MSC-seeded scaffolds grown in the EXP control medium (Fig 4b). EDX demonstrated the presence of calcium and phosphorus in these deposits (such as those shown in Fig 4a). Qualitatively, there appeared to be a large number of such deposits in the OST/OP-1 group compared with the other groups. Interestingly, many small islands of mineral deposits within the pores of the scaffolds were often seen in the OST/PDGF/OP-1 group.

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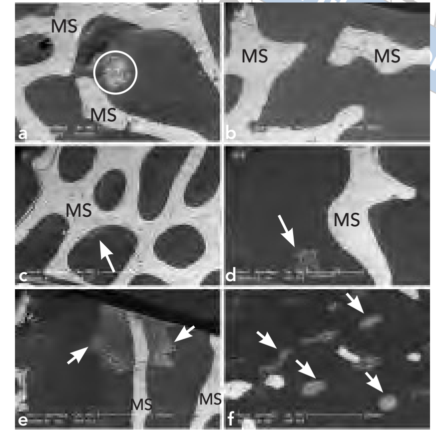
Fig 4 *Backscattered electron images.* (a) *The white circled area is likely the mineralized matrix–produced by the cultured cells. EDX analysis of the material in the circled area revealed the presence of calcium and phosphate ions (OST group). MS = mineral scaffold.* (b) *Noncalcified extracellular matrix was observed in the specimen (EXP group).* (c) *A mineralized deposit was observed on the wall of the scaffold inside a pore* (arrow) *(OST group).* (d) *An island of calcified ECM observed inside a pore next to the scaffold* (arrow) *(OST/PDGF group).* (e) *Relatively thick bone ECM matrix formation observed along the scaffold* (arrows) *(OST/OP-1 group).* (f) *Islands of calcified ECM observed among the struts of the scaffold; arrow shows a representative mineral deposit. The calcified ECM formation appeared along the surface of the walls of the scaffold and within the pores, apart from the surface of the scaffold (OST/PDGF/OP-1 group).*



Discussion

Prior work has already established that osteoblast-like cells can attach to anorganic bovine bone material.[13] As expected, there were no effects of the various media on the number of cells attached to the mineral scaffolds after only 1 day. However, the results of the current study demonstrated that select growth factors could be employed to increase the proliferation and osteogenic behavior of MSCs in the porous mineral scaffold. After 7 days, supplementation of OST with PDGF-BB and OP-1 (OST/PDGF/OP-1) doubled the cell number when compared with the control OST medium. Interestingly, neither PDGF-BB nor OP-1 alone resulted in the same cell increase. Another notable finding was the substantial increase (doubling) in the ALP level in the MSC cultures after 14 and 21 days achieved by the supplementation of the OST medium with OP-1. The findings suggest that OP-1, in addition to PDGF-BB, might be considered for incorporation into porous mineral blocks used for vertical ridge augmentation.[1,14,15]

Several studies have previously reported various aspects of the behavior of osteoblast-like cells[11,16–20] on the same type of mineral material used in the present work. The collagen in the cell layer on the natural bone mineral was found to have the same electrophoretic pattern as the collagen observed in human bone.[16] In a previous study, the osteoblast-like cells were first pretreated with 150 ng/mL OP-1 prior to being grown in the porous anorganic mineral block.[17] OP-1 was found to increase the collagen cross-link residues hydroxylysylpyridinoline and lysylpyridinoline compared to nonstimulated samples.[17] In another prior investigation, PDGF-BB incorporated into an anorganic bovine bone material was found to stimulate proliferation of osteoblastic cells.[4] The only prior study investigating the differentiation of stem cells in an anorganic bovine mineral scaffold in vitro[21] demonstrated that the natural bone mineral supported osteogenic differentiation of stem (follicular) cells.

Radionuclide imaging of Tc-99m-MDP uptake in the samples proved to have limitations in quantifying the mineral deposits resulting from the MSC activity in the scaffolds. The highest uptake was recorded for the non–cell-seeded control samples, likely due to the predilection of the Tc-99m-MDP to bind to the bone apatite crystallites comprising the scaffold. Cell-seeded samples demonstrated less radionuclide uptake, likely because the cells shielded the surface from the Tc-99m-MDP binding to the natural apatite. The radionuclide method will have to be modified in order for it to be of value in quantifying mineralization of the mineral scaffolds.[9]

ALP, a cell membrane-associated enzyme, is a widely recognized marker of osteoblast phenotype and differentiation. ALP catalyzes the hydrolysis of phosphate esters

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at alkaline pH providing a free phosphate pool and is a prerequisite for normal mineralization. The amount of mineral deposited in the cell layer is dependent on the dose of and exposure time to the phosphate source. In the present study, a qualitative assessment of the mineral deposited in the scaffolds was made using BEI. Given that the radionuclide method has not yet been developed for these types of mineralized scaffolds, the challenge remains to find methods to augment ALP determination for the accurate quantification of the mineralization process.

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