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Bioactive Collagen Membrane as a Carrier for Sustained Release of PDGF

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Abstract

The objective of this study was to evaluate the ability of a collagen membrane (CM) as a carrier to successfully deliver platelet-derived growth factor (PDGF) and to observe the subsequent effects of the factor on preosteoblasts *in vitro*. MC3T3-E1 mouse preosteoblasts were cultured with a commercially available CM containing PDGF. After a two-day cell culture, cell viability was investigated by the MTT assay and cell proliferation was assessed by the crystal violet proliferation assay. Expression levels of the following osteoblastic differentiation marker genes were measured by real-time PCR: runt-related transcription factor 2 (RUNX2), osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OCN). A cell proliferation assay was conducted, and osteoblastogenesis was determined by alkaline phosphatase (ALP) activity. A sustained release of PDGF from a CM was observed for ~3 weeks. Gene expression of all RUNX2, OPN, BSP, and OCN in CM with PDGF was significantly upregulated compared to those in CM without PDGF (all $p < 0.05$). Interestingly, CM without PDGF also significantly increased gene expression of RUNX2 and OPN in MC3T3-E1 cells compared to the cell control (both $p < 0.05$). Furthermore, it was observed that the PDGF released from CM significantly promoted ALP activity and cell proliferation with little cytotoxicity. These results suggest that a CM can be utilized for sustained delivery of PDGF. Also, released PDGF can promote MC3T3-E1 cell activities. This strategy may lead to an improvement in the current clinical treatment of bone defects in periodontal and implant therapy.

Keywords: Collagen membrane; Growth factor; Tissue regeneration; Delivery; Bone

Introduction

Regenerative procedures using barrier membrane technology are presently well established in implant dentistry and periodontal therapy. Collagen membranes (CMs) are frequently used in guided tissue regeneration (GTR) and guided bone regeneration (GBR), based on the premise that barrier membrane materials will promote selective cell re-population and subsequent reconstitution of the periodontal attachment apparatus as well as bone [1]. The adherence of connective tissue cells to the inside of a CM promotes periodontal regeneration [2], and an attachment can help to stabilize the blood clot and integrate the membrane into the tissue [3]. Collagen is suitable for GTR/GBR application because it is chemotactic for periodontal ligament fibroblasts, acts as a barrier in migrating epithelial cells, provides homeostasis, and serves as a fibrillar scaffold for early vascular and tissue ingrowth [4].

Platelet-derived growth factor (PDGF) is a potent mitogen that facilitates wound healing [5] and stimulates bone repair by expanding osteoblastic precursor cells [6,7]. Studies have shown that recombinant human PDGF treatment of rat periodontal ligament (PDL) fibroblasts induced a strong mitogenic and chemotactic cell response, which also stimulated collagen synthesis [8]. Furthermore, it was found that the PDGF-BB isoform is more effective than PDGF-AA and PDGF-AB in promoting PDL cell mitogenesis *in vitro* [9]. PDGF-BB is approved by United States Food and Drug Administration (US FDA) for use in the treatment of localized periodontal defects and diabetic ulcers [10-12]. When PDGF has been used as a clinical application, it was usually mixed with grafting materials and then the site was covered by CMs [13]. Limited information is available regarding how long PDGF stays in the local site. In addition, the influence of PDGF on the mechanical integrity of grafting materials is unknown. Also, the lack of effective delivery of PDGF and the efficient targeting specificity limits its clinical applications. We hypothesized that a CM can be utilized as a carrier for sustained delivery of PDGF to enhance bone regeneration. This is the first study to examine the use of a commercially available CM for the delivery of PDGF. The objective of this study was first to evaluate the ability of a CM as a carrier to successfully release PDGF-BB. The second aim was to determine the subsequent effects of the released factor on cell viability, cell proliferation, expression levels of differentiation marker genes, and osteoblastogenesis.

Materials and Methods

Cell culture

MC3T3-E1 mouse preosteoblasts (donated by Dr. Mani Alikhani, New York University College of Dentistry) were cultured in alpha minimal essential medium (αMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 5,000 U/ml penicillin (Invitrogen) and 5,000 μg/ml streptomycin (Invitrogen) at 37°C in a 5% CO_2 humidified atmosphere.

Preparation of CM containing PDGF

A commercially available CM, OsseoGuard (noncross-linked bovine type I collagen, Biomet 3i, Warsaw, Indiana, USA), samples was prepared to a size of 7 mm diameter each. Recombinant PDGF-BB solution (R&D Systems, Minneapolis, MN, USA) was dropped onto the CMs and incubated at room temperature for 1 h. The amount of PDGF applied to each CM disc for each study was the following: (1) release

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kinetics, 1.248 ng; (2) ALP assay, 16.25, 32.5, or 65 pg; (3) proliferation assay, 32.5 pg; (4) gene expression, 32.5 pg; (5) MTT assay, 32.5 pg.

Release kinetics of PDGF from CMs

CMs containing 1.248 ng of PDGF were incubated in 1 ml of pH 7.4 PBS buffer at 37°C. As a control, the same amount of PDGF without CM was added to 1 ml of PBS buffer. At pre-determined time intervals (0, 1, 2, 3, 4, 9, 10, 12, 15, 17, 18, and 19 days), these samples were collected from the buffer and assayed for PDGF concentration as determined by a commercial enzyme-linked immunosorbent assay (ELISA) for PDGF-BB (R&D Systems). The lower limit of detection was 5 pg/ml. Cumulative release profiles were generated for each test CM and controls by summing the total PDGF recovered up to and including each time point and plotting the data against time. The percent recoveries were normalized to recoveries of control PDGF, based on ELISA data, which were considered to represent 100% recovery of the protein.

ALP activity assay

ALP activity was measured by SensoLyte pNPP ALP Assay Kit (AnaSpec Inc, San Jose, CA, USA). MC3T3-E1 cells (2×10^4 cells/well) were prepared in 48-well culture plates. After cell culture with CMs carrying PDGF for 2 days, the cells were washed twice with 1× lysis buffer. Then, 70 ml of a mixed buffer, containing 20 µl of Triton X-100 and 10 ml of 1× lysis buffer, was added to the cells. After the adherent cells were scraped off, the cells were collected in a microcentrifuge tube and incubated for 10 min at 4°C under agitation. Then, the supernatant was corrected after the cell suspension was centrifuged for 10 min at 2,500 *g* at 4°C. Fifty µl of the supernatant or ALP standard and 50 µl of pNPP reaction mixture, containing 100× diluted pNPP stock solution with 2× assay buffer, were applied each well (96-well plate). After the plate was gently shaken to mix the reagent well for 30 sec and incubated for 30 min at 37°C, 50 µl of stop solution was added to each well. The optical density was quantified in a multi-detection microplate reader, Synergy™ HT (BioTek Instruments Inc, Winooski, VT, USA) at 405 nm wavelengths after the plate was gently shaken for 1 min.

Cell proliferation assay

MC3T3-E1 cells (5×10^3 cells/well) were prepared in 48-well culture plate. After cell culture with CMs carrying PDGF for 2, 4, and 6 days, CMs and medium were discarded. Two hundred µl of 1% glutaraldehyde was applied the well and it was aspirated after 15 min incubation. Cells were washed with 300 µl of 1× PBS. After 200 µl of 0.02% crystal violet aqueous solution was added to the well, cells were incubated for 30 min. Then, cells were washed with H_2O for 15 min to wash away excess dye. After washing, cells were dissolved in 360 µl of 70% ethanol with shaking for 3 h. The absorbance was read by a multi-detection microplate reader, Synergy™ HT (BioTek Instruments Inc) at 578 nm wavelength. The effects of CM or CM with PDGF on cell proliferation were calculated as follows: [%] $T/C = (T–C_0)/(C–C_0) \times 100$ with T representing the mean absorbance of the treated cells, C representing the mean absorbance of the controls, and C_0 representing the mean absorbance of the cells at time zero [14].

Quantitative real-time polymerase chain reaction with reverse transcription (QRT-PCR)

The cell-seeded CMs were incubated for 2 days. After cell culture with CMs carrying PDGF in 24-well culture plates for 2 days, total RNA in the harvested cells were isolated with RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The cells were first homogenized for 30 sec in a lysis buffer. The lysis buffer containing the homogenate was centrifuged for 1 min at 13,000 *g* at 4°C. The supernatant was applied to RNeasy column, rinsed and eluted. RNAs were measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and treated with DNase I. Mouse Universal ProbeLibrary probes and target-specific PCR primers for runt-related transcription factor 2 (RUNX-2), osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OC), and GAPDH, a housekeeping gene, were selected using the ProbeFinder assay design software (Table 1). cDNAs were synthesized from 1 µg of total RNA for each sample using reverse transcriptase (Roche, Nutley, NJ, USA). Reactions for the 480 LightCycler (Roche) were performed in 20 µl reaction volumes for the genes encoding RUNX2, OPN, BSP, OC, and GAPDH using 1 µl of cDNA under the following conditions: 95°C for 5 min, 50 cycles for 95°C for 10 sec, 60°C for 15 sec, and 72°C for one sec. The method used for obtaining quantitative data of relative gene expression was the comparative Ct method (also as known the $2^{-\Delta\Delta Ct}$ method). QRT-PCR for experimental genes was normalized against the internal control, GAPDH. Quadruplicates of each data were averaged, and the mean values were used for statistical analysis.

MTT assay

Cytotoxicity of CM, PDGF, and CM carrying PDGF was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, MTT assay. MC3T3-E1 cells (1×10^6/ml) in 200 µl of αMEM (Invitrogen) supplemented with 10% FBS were seeded in 48-well plates and incubated overnight. After incubation, 300 µl of medium was added and cells were incubated with CM with/without PDGF for 24 h. Then, CMs and medium were discarded after incubation. Two hundred µl of medium and 20 µl of MTT reagent were added into the plates and incubated 4 h. After incubation, the medium was aspirated and dimethyl sulfoxide (200 µl/well) was added to stop the reaction. The optical density was quantified in a multi-detection microplate reader, Synergy™ HT (BioTek Instruments Inc) at 570 nm wavelength. The percentage of cell viability was calculated by comparing the appropriate optical density to the control cells.

Statistical methods

Descriptive statistics were computed for each experimental condition to summarize the mean expression levels. Bivariate comparisons (ANOVA and *t*-test) were computed to compare different experimental conditions. For all statistical analyses, p values less than 0.05 were considered significant.

Results

PDGF release from CMs

To evaluate the ability of a CM to deliver a growth factor, cumulative PDGF release from the CM in PBS was measured (Figure 1). Initially, approximately 60% of the incorporated PDGF was released within the first 3 days. After that, a sustained release of PDGF from CM was observed for ~3 weeks.

Biological effects of released PDGF from CM on cellular ALP and proliferation activity

To evaluate the biological effect of PDGF from CM, osteoblastogenesis was determined by ALP activity measured in MC3T3-E1 cells (Figure 2). CM carrying 16.25 or 32.5 pg of PDGF significantly promoted an ALP activity compared to the control, CM without PDGF (both $p < 0.05$). Based on the results 32.5 pg of

PDGF was used for all subsequent studies. In addition to ALP activity, cell proliferation activity was measured to evaluate the proliferative effect of PDGF released from CM (Figure 3). Although there was no significant difference in cell proliferation activity between CM carrying PDGF and CM alone for up to 4 days, CM carrying PDGF significantly induced cell proliferation at 6 days compared to the control CM alone ($p < 0.05$).

Effect of released PDGF from CM on gene expression of osteogenic markers

The biological effect of released PDGF on gene expression levels of osteoblastic differentiation markers, RUNX2, OPN, BSP, and OCN, in MC3T3-E1 cells was evaluated by QRT-PCR. Three experimental groups, (1) cell only, (2) CM only, and (3) CM with PDGF, were assessed. Gene expression of RUNX2 and OPN in CM with PDGF was significantly upregulated compared to expression in CM without PDGF ($p < 0.001$ and $p < 0.01$, respectively) (Figure 4A & B). Interestingly, the CM without PDGF group also significantly increased gene expression of RUNX2 and OPN compared to the cell control group ($p < 0.001$ and $p < 0.01$, respectively). However, the increase was significant less than the CM with PDGF group. CM without PDGF group significantly decreased gene expression of BSP and OCN compared to the cell control group (both $p < 0.001$) (Figure 4C & D). Despite this CM-mediated decrease of BSP and OCN gene expression, CM with PDGF produced a significant increase in expression of these factors compared to CM without PDGF (both $p < 0.001$).

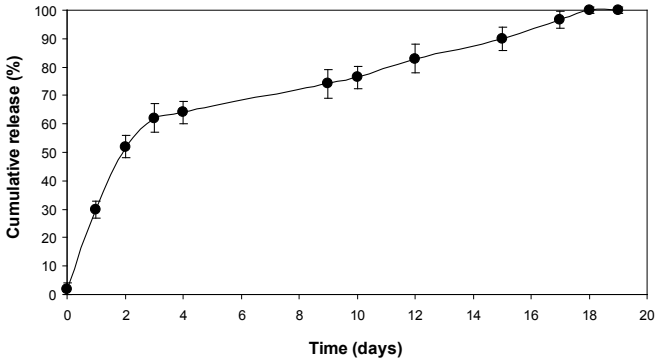

Figure 1: Cumulative release of Platelet-derived growth factor (PDGF) from a collagen membrane (CM). Data were calculated by detected PDGF divided by total PDGF as % release of PDGF from the CM.

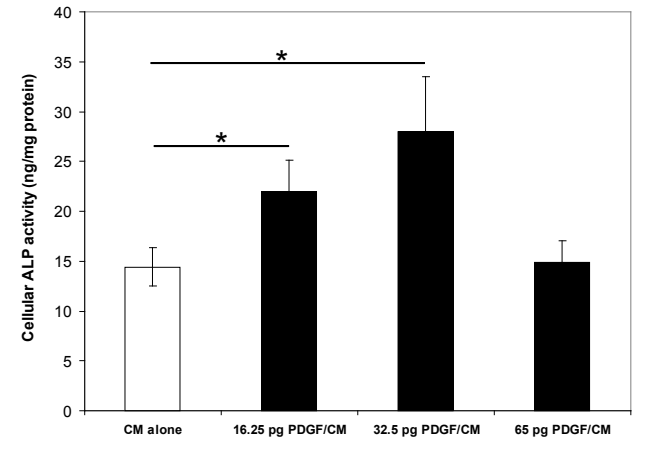

Figure 2: Alkaline phosphatase (ALP) activity in MC3T3-E1 cells after culture with a collagen membrane (CM) with/without platelet-derived growth factor (PDGF) (16.25, 32.5, or 65 pg). Data are expressed as mean value ng/mg protein ± standard deviation from quadruplicates. * $p < 0.05$.

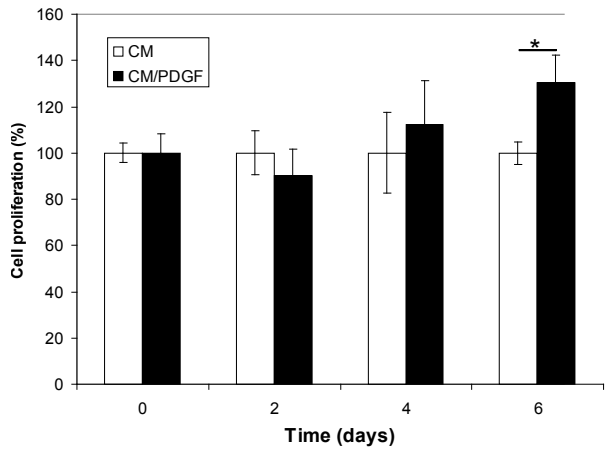

Figure 3: Cell proliferation activity in MC3T3-E1 cells after culture (2, 4, and 6 days) with a collagen membrane (CM) with/without platelet-derived growth factor (PDGF). The percentage of cell proliferation was calculated by comparing the appropriate luminescent signal to the signal obtained with the control cells. Each value represents the mean ± standard deviation from quadruplicates (white bars, CM; and black bars, CM with PDGF). As a control, non-treated cells were used. * $p < 0.05$.

RUNX2	Forward	CGAAATGCCTCCGCTGTTAT
	Reverse	CGCTCCGGCCCACAA
OPN	Forward	CATGAAGAGCGGTGAGTCTAAGG
	Reverse	CTTTCCGTTGTTGTCCTGATCA
BSP	Forward	AGGACTGCCGAAAGGAAGGT
	Reverse	ATGGAGACGGCGATAGTTCC
OCN	Forward	TGCTTGTGACGAGCTATCAG
	Reverse	GAGGACAGGGAGGATCAAGT
GAPDH	Forward	AACGACCCCTTCATTGAC
	Reverse	TCCACGACATACTCAGCAC

Table 1: Primer sequences for QRT-PCR.

Cytotoxicity of PDGF, CM, and CM carrying PDGF

For the determination of cytotoxicity of CM or CM carrying PDGF by MTT assay, composition and preparation of conditions were the same as described above (32.5 pg of PDGF). A high amount of viable cells was found in the PDGF alone, CM alone, CM carrying PDGF, and cell control groups, and there was no significance difference among them ($p = 0.943$) (Figure 5). Also, no obvious visible changes were noted in cell morphology in any of the groups.

Discussion

This is the first study to examine the use of a commercially available CM for the delivery of PDGF. The results of the present study demonstrate the achievement of a sustained release profile for PDGF and the subsequent effects of the released factor on cell functions *in vitro* using a commercially available CM as a carrier for bone tissue regeneration applications. When PDGF has been used in clinical applications, it is usually mixed with grafting materials or GEM 21S (Osteohealth, Shirley, NY, USA) which contains PDGF-BB and β-TCP

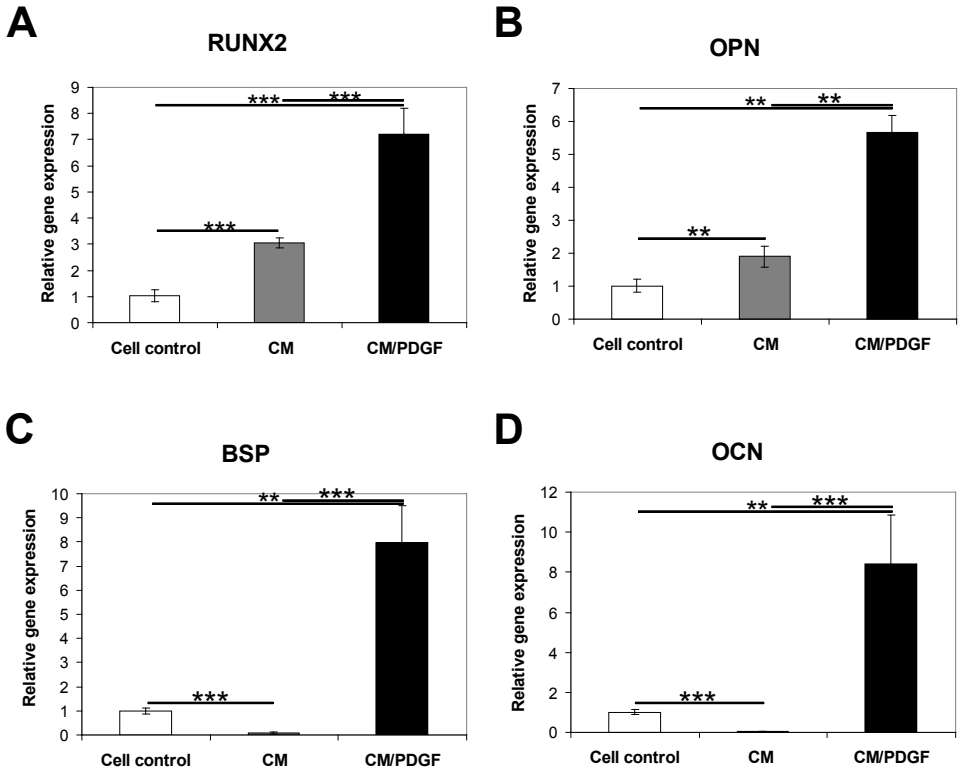

Figure 4: mRNA levels of differentiation maker genes, runt-related transcription factor 2 (RUNX2) (A), bone sialoprotein (BSP) (B), osteopontin (OPN) (C), and osteocalcin (OCN) (D) in MC3T3-E1 cells measured by real-time PCR after cell culture with a collagen membrane (CM) with/without platelet-derived growth factor (PDGF). Data represent relative expression to the level of the cell control, set at 1, and mean value ± standard deviation from quadruplicates (white bars, cell control; gray bars, CM; and black bars, CM with PDGF). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

particles approved by the US FDA and marketed for the treatment of periodontal bone defects. After the materials are packed, the surgical site is covered by CMs. It was reported about GEM 21S that almost 100% of the PDGF was rapidly released from β-TCP within 90 min *in vitro* and approximately 90% of the PDGF was depleted from calvarial defect sites within 72 h of implantation *in vivo* without any controlled release profile [15]. Although PDGF is one of the most important growth factors acting on all cells of mesenchymal derivation, its half-life is less than 2 min *in vivo* [16]. Ruskin et al. [17] demonstrated that the use of recombinant human transforming growth factor β1 (rhTGF-β1) in conjunction with a CM greatly enhanced bone regeneration in osseous oral defects *in vivo*. However, *in vivo* release kinetics was not measured in their study so no determination for the coral matrix as an effective carrier could be made. To achieve a successful release of growth factors in a tissue engineering approach, in general, tissues should be exposed for relatively long periods to these molecules to obtain the proposed effect [18]. Our *in vitro* results indicated that a sustained release of PDGF from CM was observed for ~3 weeks with 100% of PDGF delivered. The influence of an *in situ* environment is missing from an *in vitro* testing system and therefore may account for the differences between the *in vitro* and *in vivo* release profiles. Our delivery system may be more applicable to bone regeneration because it requires tissues be exposed to growth factors for a sustained period in order to complete regeneration [19].

In the present study, the biological functionality of PDGF released from CM was confirmed by real-time PCR, ALP assay, and cell proliferation assay using MC3T3-E1 cells. Our data showed that released PDGF could promote gene expression of the osteoblastic differentiation markers, RUNX2, OPN, BSP, and OCN, in MC3T3-E1 cells. Osteoblast differentiation is controlled by multiple transcription factors at various stages [20]. RUNX2 has been identified as zinc-finger containing proteins, the osteoblast-specific transcription factors acting as developmental regulators of cell differentiation and mineralization [21,22]. Loss of RUNX2 leads to severe impairment of bone formation. Hence, it is possible that the stimulatory actions of released PDGF on MC3T3-E1 cells in this study might be mediated by RUNX2. OPN has been implicated as an important factor in bone remodeling [23] and plays a role in anchoring osteoclasts to the mineral matrix of bones [24]. RUNX2 is required for the expression of OPN. RUNX2 binds promoters of osteoblast-specific genes such as type I collagen (COL I), BSP, and OPN and upregulate transcription [25]. It is possible that released PDGF first stimulated RUNX2 gene expression followed by upregulation of OPN and BSP gene expression in MC3T3-E1 cells. OCN is one of the molecular markers of late-stage differentiation. Therefore, our results indicate that released PDGF might affect late-stage differentiation of MC3T3-E1 cells. Interestingly, CM without PDGF significantly increased gene expression of RUNX2 and OPN in MC3T3-E1 cells, whereas CM without PDGF significantly decreased gene expression of BSP and OCN compared to the cell control. These results suggest that stimulated gene expression of RUNX2 and OPN might be mediated by COL I, the main component of the CM, though it is difficult to explain the reason of downregulation of BSP and OCN genes. While CMs have been widely used in implant dentistry, there are only a few reports, especially gene expression studies, regarding cellular responses to the commercially available resorbable CMs

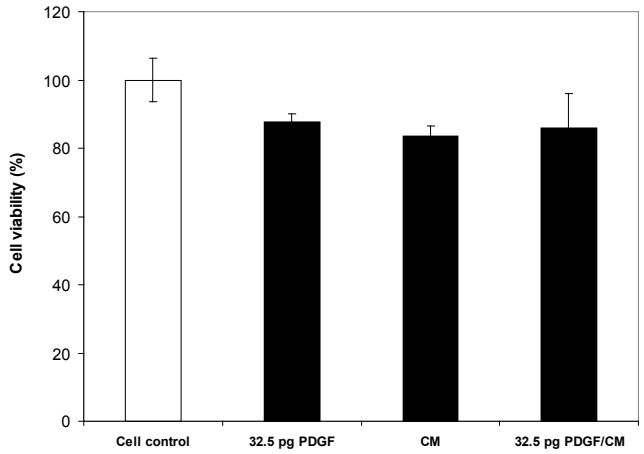

Figure 5: Cell viability with a collagen membrane (CM), platelet-derived growth factor (PDGF), and CM carrying PDGF evaluated by MTT assay. The percentage of cell viability was calculated by comparing the appropriate luminescent signal to the signal obtained with the control cells. Each value represents the mean ± standard deviation from quadruplicates. As a control (100% viability), the control cells were used. There was no significant difference among CM, PDGF, and CM carrying PDGF.

[26,27]. Changing membrane composition and structure, and culture conditions can also alter phenotypic expression of osteoblasts [28,29].

ALP activity is a marker of osteoblastic activity/differentiation, i.e., bone turnover and remodeling. Our results showed that PDGF released from CM significantly increased ALP activity in MC3T3-E1 cells compared to the control, CM without PDGF. Therefore, our data suggest that released PDGF might also affect late-stage differentiation of MC3T3-E1 cells. Also, our results showed that osteoblast cell proliferation activity was significantly increased in CM carrying PDGF compared to the control CM alone at 6 days in culture. This concurs with Strayhorn et al. [30] who demonstrated that recombinant PDGF treatment promoted proliferation of MC3T3-E1 cells. Therefore, together, our functional studies indicate that released PDGF affects both differentiation and proliferation of MC3T3-E1 cells. The concentration of PDGF used in our studies was 16.25 – 65 pg/CM. Interestingly, the amount of PDGF higher than 65 pg significantly reduced ALP activity of MC3T3-E1 cells. Optimal concentration of PDGF might be an important concern for clinical applications. In addition to functional activities, biocompatibility will be an important criterion for determining which device to choose for a clinical application. Toxicity, pyrogenic and hemolytic activity, and antigenicity must also be extensively examined under *in vitro* and *in vivo* conditions prior to clinical usage. Our *in vitro* study showed that no cytotoxicity was found in any of the conditions: PDGF alone, CM alone, or CM carrying PDGF. Therefore, this result suggests that the bioactive CM may be applicable for a variety of clinical applications. In addition, some commercially available CMs alone can induce enhanced cell proliferation [31]. While many studies have evaluated different collagen materials and cross-linking techniques for their effect on cell proliferation, the nordihydroguaiaretic acid and chitosan techniques are two promising approaches, both showing excellent cytocompatibility *in vitro* by enhancing cell proliferation to the same or even a higher extent as non-cross-linked controls [32,33].

In conclusion, this study demonstrates that PDGF can be incorporated into CM for sustained release over a prolonged period of time, ~3 weeks *in vitro*. The released PDGF significantly increased gene expression of osteoblast differentiation markers and ALP and cell proliferation activities with little cytotoxicity in MC3T3-E1 cells. This strategy may lead to an improvement in the current clinical treatment of bone defects for periodontal and implant therapy.

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