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Protease-activated receptor type 1 (PAR1) increases CEMP1 gene expression through MAPK/ERK pathway

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Abstract: PAR1 is a G-coupled protein receptor that regulates several cellular metabolism processes, including differentiation and proliferation of osteogenic and cementogenic related cells and our group previously demonstrated the regenerative potential of PAR1 in human periodontal ligament stem cells (hPDLSCs). In this study, we hypothesized that PAR1 regulates the cementogenic differentiation of hPDLSCs. Our goal was to identify the intracellular signaling pathway underlying PAR1 activation in hPDSLC differentiation. hPDLSCs were isolated using the explant technique. Cells were cultured in an osteogenic medium (OST) (α-MEM, 15% fetal bovine serum, L-glutamine, penicillin, streptomycin, amphotericin B, dexamethasone, and beta-glycerophosphate). The hPDLSCs were treated with a specific activator of PAR1 (PAR1 agonist) and blockers of the MAPK/ERK and PI3K pathways for 2 and 7 days. The gene expression of CEMP1 was assessed by RT-qPCR. The activation of PAR1 by its agonist peptide led to an increase in CEMP1 gene expression when compared with OST control. MAPK/ERK blockage abrogated the upregulation of CEMP1 gene expression induced by PAR1 agonist ($p < 0.05$). PI3K blockage did not affect the gene expression of CEMP1 at any experimental time ($p > 0.05$). We concluded that CEMP1 gene expression increased by PAR1 activation is MAPK/ERK-dependent and PI3K independent, suggesting that PAR1 may regulate cementogenetic differentiation of hPDLSCs.

Keywords: Cementogenesis; Stem Cells; Gene Expression.

Introduction

The ultimate goal of periodontal therapy is to achieve complete regeneration of lost periodontal tissues. However, despite the effectiveness of periodontal treatment through scaling and root planning at disease progression control, it still fails to provide a significant regeneration of the periodontal tissues.[1] In these cases, a long junctional epithelium is developed, taking place as a tissue repair response, reducing the periodontal pocket depth.[2]

Periodontal regeneration is an organized and dynamic process initiated by migration, adhesion, proliferation, and ultimately differentiation of progenitor cells into osteoblasts and periodontal ligament cells, which actively synthesize the tissue components of the functional periodontal

insertion apparatus.[3,4] The success of all these steps relies on the availability of growth factors present in the extracellular matrix that controls the gene expression in these cells.[3,5] In the absence of these molecular and cellular components, regeneration can be compromised, leading to tissue repair rather than forming the specialized periodontal tissues.[5]

Cementogenesis is one of the most important processes in periodontal regeneration, where several molecules in the extracellular matrix play a major role in cementoblast proliferation and differentiation, resulting in the formation of a new cementum matrix.[4,6] CEMP1 regulates the protein expressed in periodontal ligament cells involved in the local metabolism in cementogenesis, mediating cementoblast differentiation and induction of cement deposition.[7,8] Human periodontal ligament stem cells (hPDLSCs) are well known for their pluripotency properties that enable them to differentiate into osteoblasts, fibroblasts, and cementoblasts.[9] hPDLSCs can regenerate periodontal ligament and trabecular bone tissues in a periodontal defect *in vivo* model, suggesting a potential role of hPDLSCs in cementogenesis.[10]

PAR1 is a G protein-coupled receptor that, once activated, triggers a series of intracellular signaling cascades that mediate cellular responses to a subset of extracellular proteins.[11] It can be activated by thrombin and some matrix metalloproteinases (MMPs), playing a major role in tissue repair and bone healing.[12] In fact, our previous study demonstrated that PAR1 activation in hPDLSCs leads to an increase in osteogenic activity.[13] PAR1 is expressed by human gingival fibroblasts, gingival epithelial cells, periodontal ligament cells, hPDLSCs, and in the musculoskeletal system on the surface of the cell membrane of osteoblasts, myoblasts, and chondroblasts.[12-16] Intracellular stimuli are usually mediated by the MAPK/ERK, p38, and PI3K/Akt pathways,[14,17] and the persistent stimulation and phosphorylation of the MAPK/ERK are considered to play a major role in cellular responses.[18]

We hypothesized that PAR1 regulates the cementogenic differentiation of hPDLSCs. Our goal was to identify the intracellular signaling pathway underlying PAR1 activation in hPDSLC differentiation. In this study, we evaluated how MAPK/ERK and PI3K/Akt signaling pathways may influence the gene-related expression of CEMP1 after PAR1 activation. Understanding the downstream signaling pathways of extracellular activation by PAR1 may help us to better understand how cellular cementogenic responses are regulated in hPDLSCs.

Methodology

Ethics statement

The study was approved by the Ethics Committee of the School of Dentistry of the University of São Paulo (FO-USP) under the protocol # 803.811. Informed consent from donors was acquired prior to the teeth extraction and procedures were performed according to the Helsinki Declaration.

hPDLSC isolation, culture and phenotyping

Three partially or totally erupted human third molars removed from systemically healthy patients (18 to 30 years old) without periodontal disease were used. hPDLSCs were isolated using the explant technique as described.[19] Briefly, teeth were washed with a phosphate-buffered saline (PBS) right after the extraction procedure and transported in alpha-modified Eagle's medium (α-MEM) (Gibco, Carlsbad, USA) to the laboratory. The removal of the periodontal tissue attached to the middle third of the root was carried out with a curette as described.[19]

To establish the cultures, hPDLSCs were harvested in a 25cm^2 culture flask (Corning, New York, USA) with clonogenic medium (CM) (α-MEM + 100 μg/mL penicillin, 15% fetal bovine serum, 100 μg/mL streptomycin, 0.5 mg/mL amphotericin B - Gibco, Invitrogen, Carlsbad, USA) in anaerobic chamber (37° C, 5% CO_2 and 95% humidity). Then, the culture medium was changed every 48 hours until the culture reached 80% of confluence when cells were trypsinized (Gibco, Life Technologies, city, USA) and subcultures were established. All the experiments were carried out with cells at passage 3-7.

The characterization of the stemness biomarkers was performed by means of immunostaining followed by flow cytometry for the following surface antigens: CD34-FITC CD14-FITC, CD31-PE, CD90-FITC, CD45-PE, CD146-PE and CD44-PE (all from

eBioscience, city, USA). Briefly, 5 × 10^5 cells were washed in PBS and incubated with the conjugated antibodies for 30 minutes at 4°C. Flow cytometry was performed using compensation beads for each specific antibody and negative control with unstained cells was used (FACSort, Becton Dickinson, Brazil). CellQuest software was used to further analysis (Becton Dickinson, City, Brazil).

Experimental design

hPDLSCs were seeded using a density of 2x10^5 per well in 24-well plates. Experiments were divided in 4 groups, as follows: a) osteogenic medium (OST) containing CM + 0.1 mM dexamethasone, 2 mM β-glycerophosphate, and 50 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, USA); b) osteogenic medium containing a PAR1 selective agonist peptide (OST + PAR1) TFLLR-NH2 (100 nM) (Tocris Bioscience Inc., Bristol, UK);[20] c) MAPK/ERK blockage (PD98059 -10μM) prior to OST + PAR1 - (ERK blockage + PAR1); d) PI3K blockage (LY294002 - 1.4 μM) prior to OST + PAR1 (PI3K blockage + PAR1). To ensure the full pharmacological effect, MAPK/ERK and PI3K pathway blocking drugs were administered into the cultures 30 min before the PAR1-selective activation by the agonist peptide. The culture medium was changed every two days.

CEMP1 quantitative gene expression

For the CEMP1 gene expression in hPDLSCs, samples were collected at 2 and 7 days for all groups: OST, OST + PAR1, ERK blockage + PAR1, and PI3K blockage + PAR1 and the RNA extraction was performed using Trizol (1 mL/well) (Invitrogen, Carlsbad, USA). The extracted RNA quality and concentration were evaluated using a NanoDrop™ One Spectrophotometer (Thermo Scientific, Foster City, USA) with an absorbance wavelength of 260 and 280 nm. DNA treatment was performed. Then, a reverse transcription reaction under controlled conditions (25°C for 10 min, 42°C for 60min and 85°C for 5min) was carried out in order to obtain complementary cDNA strand by using High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA).

The quantitative Polymerase Chain Reaction PCR (qPCR) was performed using a TaqMan Universal Master Mix II kit (Applied Biosystems, Foster City, USA) and TaqMan assays (ThermoFisher, Foster City, USA) for the following oligonucleotide sequences according to the GeneBank: GAPDH (NM_002046) and CEMP1 (Hs04185363_s1). The entire qPCR procedure was performed using the StepOne Plus™ System (Applied Biosciences, Foster City, USA) and divided in the following cycles: 95°C for 10 min, 40 (15 sec) cycles of 95° C, 60°C for 1 min and final cycle starting on 60°C and finishing in 95° C with 20 min duration. Quantification of the relative expression was calculated by using $2^{-\Delta\Delta^{CT}}$ method using GAPDH as the endogenous control and one sample from OST group as a reference sample.[21]

Statistical analysis

All the experiments were performed in triplicate. Statistical analysis was carried out using the GraphPad Prism 5.01 program (GraphPad Software, La Jolla, USA). Data were expressed as mean ±SD of the four independent experiments performed with hPDLSCs derived from three different patients. ANOVA with post hoc Tukey test was used to analyze statistical differences between groups considering a significance level of 5%.

Results

Phenotypic characterization of PDLSCs

Flow cytometry was used to examine cell surface markers. The cells presented a low expression of CD14, CD34, CD31 and a high expression of the surface markers CD90, CD146 and CD44, in agreement with the literature to be considered an hPDLSC lineage[22] (Figure 1).

PAR1 activation increased CEMP1 gene expression in hPDLSCs

At 2-day time-point, treatment with PAR1 agonist peptide significantly enhanced CEMP1 gene expression in the OST + PAR1 group when compared with the osteogenic control (OST; $p < 0.05$; Figures 2A and 3A). The same result was found at 7 days of experiment, where CEMP1 gene expression levels were statistically increased when compared with OST ($p < 0.05$; Figures 2B and 3B).

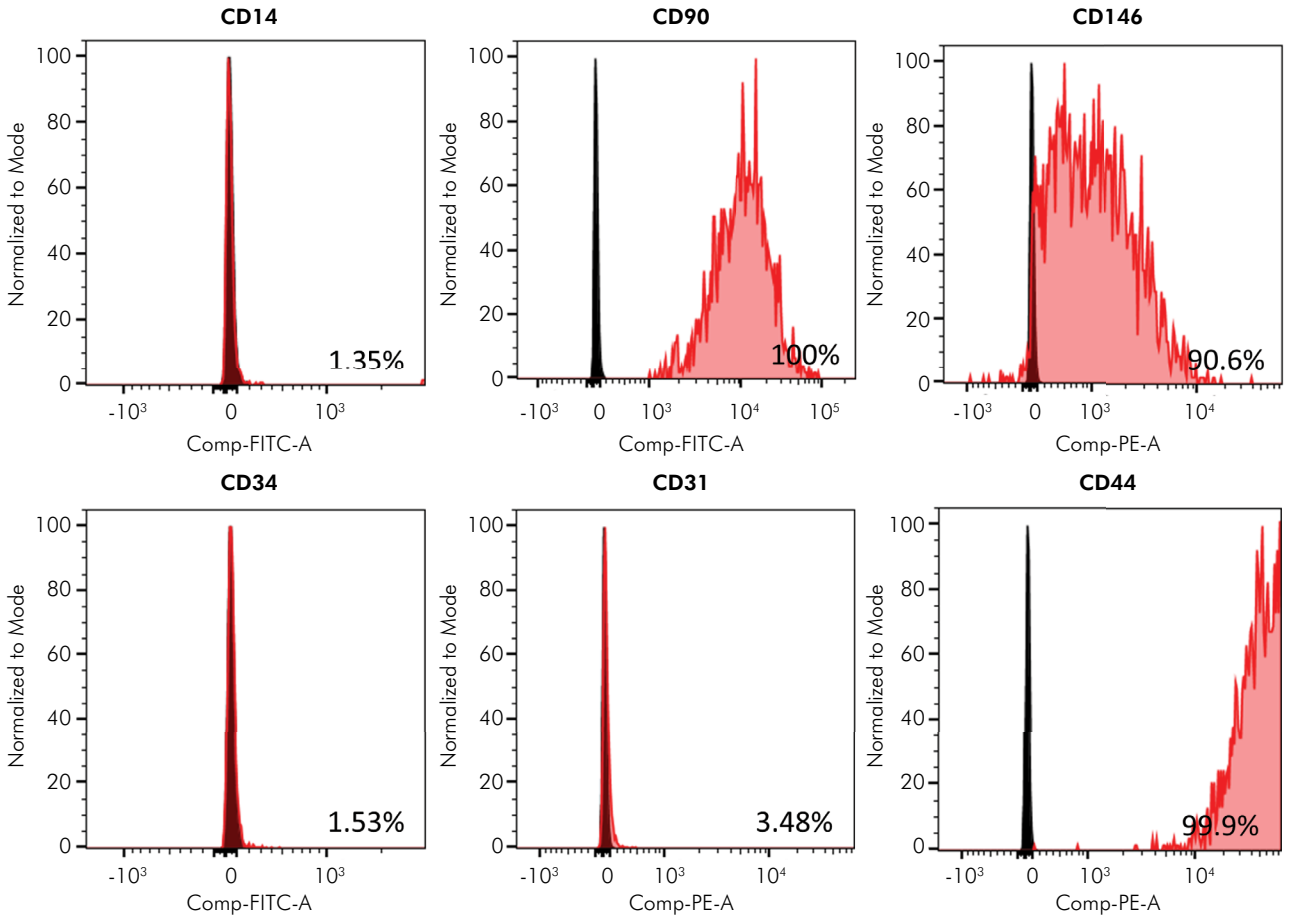

Figure 1. Flow cytometry analysis showing the percentage of hPDLSCs for each surface stemness and pluripotent embryonic biomarkers (CD14, CD34, CD90, CD31, CD146, CD44). Unstained cells were used to set positive cell populations.

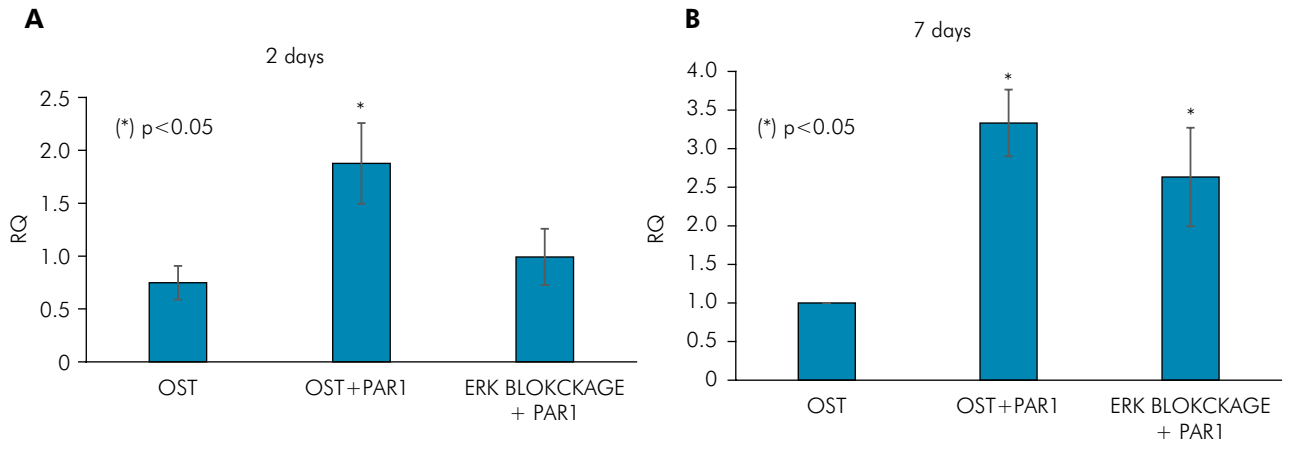

Figure 2. Cementogenic biomarker (CEMP1) relative gene expression in hPDLSCs evaluated by RT-qPCR using GAPDH as endogenous control after 2-days (A) and 7-days (B) of treatment with the following groups: osteogenic medium (OST), osteogenic + PAR1 activation by selective agonist peptide (OST + PAR1) and osteogenic medium containing the blocker of the MAPK/ERK pathway (ERK blockage + PAR1). (*) = $p < 0.05$ versus control (non-stimulated). Results are given as the mean ± SD.

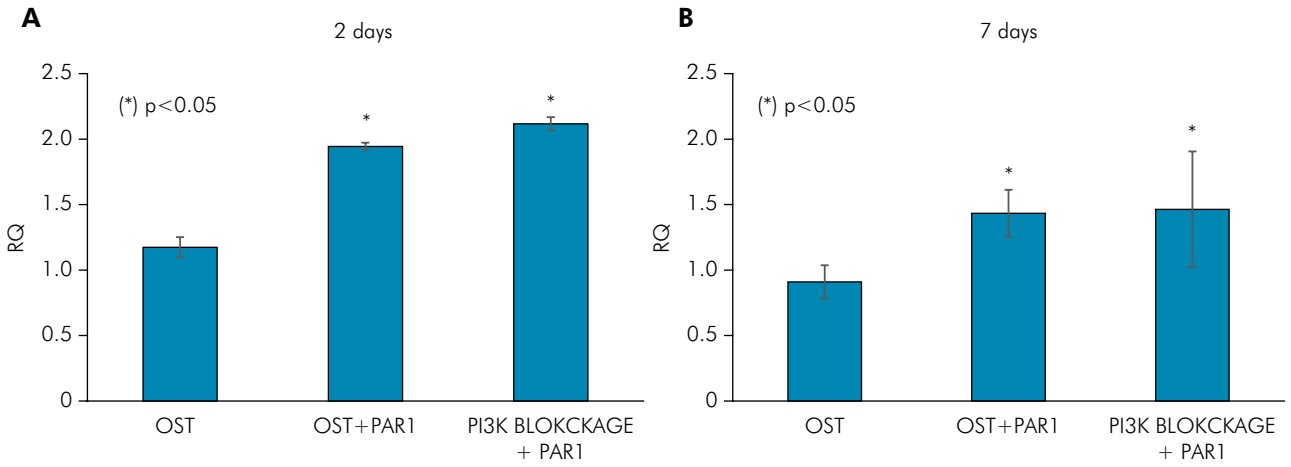

Figure 3. Cementogenic biomarker (CEMP1) relative gene expression in hPDLSCs evaluated by RT-qPCR using GAPDH as endogenous control after 2-days (A) and 7-days (B) of treatment with the following groups: osteogenic medium (OST), osteogenic + PAR1 activation by selective agonist peptide (OST + PAR1) and osteogenic medium containing the blocker of the PI3K pathway (PI3K blockage + PAR1). (*) = $p < 0.05$ versus control (non-stimulated). Results are given as the mean ± SD.

MAPK/ERK pathway blockage downregulated CEMP1 gene expression in hPDLSCs under PAR1 Activation

MAPK/ERK phosphorylation blockade (ERK blockage + PAR1) abrogated the increase in CEMP1 gene expression induced by PAR1 (OST + PAR1) at 2-day time-point ($p < 0.05$; Figure 2A). After 7 days, no statistical difference was detected between the OST + PAR1 and ERK blockage + PAR1 groups regarding CEMP1 gene expression (OST; $p > 0.05$; Figure 2B).

PI3K pathway blockage did not alter CEMP1 gene expression patterns in hPDLSCs under PAR1 activation

Conversely to data obtained by MAPK/ERK blockade, no difference was found at CEMP1 gene expression under PI3K pathway blockage at both 2- and 7-day time-points between the experimental groups (OST + PAR1 and PI3K blockage + PAR1; $p > 0.05$; Figures 3 A and 3 B).

Discussion

Despite the great advance in knowledge about cellular and molecular events involved in the regeneration of periodontal tissues, achieving complete bone, periodontal ligament, and cementum regeneration is still considered a challenge.(1,16-18) Cementogenesis represents one of the main events in periodontal regeneration, once the new fibers of the periodontal ligament will be inserted through.[1,24] In this context, hPDLSCs have shown a great potential for regeneration of the periodontal tissues, including cementum.[10,25] A previous study conducted by our group showed that PAR1 activation in hPDLSCs leads to an increase in osteogenic activity.[13] In the present study, we provided evidence for the first time that increases in CEMP1 gene expression induced by PAR1 activation is ERK, but not PI3K, dependent. These data suggest that PAR1 may have a potential role in cementogenesis.

CEMP1 is the main regulatory protein for cementogenesis. It plays an important role in local metabolism, regulating cementoblastic differentiation and induction of cement deposition.[7] Through the regulation of specific genes, CEMP1 has the ability to change the phenotype from non-mineralizing cells to mineralizing ones (cementoblasts/osteoblasts), resulting in the formation of an extracellular mineralized matrix similar to cementum.[1] In this study, activation of PAR1 resulted in increased expression of CEMP1 in hPDLCs treated with osteogenic medium, suggesting that PAR1 may increase cementogenic activity.

Literature suggests an important role of PAR1 in periodontal regeneration and bone metabolism.[12,13,16,26-28] However, despite the fact that PAR1 activation by its selective agonist or thrombin has already been shown to result in increased expression of Runx2 and OPG in hPDLCs,[13,29] transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF) by fibroblasts[30] and increased expression of TGF-β, fibroblast growth factor type 1 (FGF-1) and type 2 (FGF-2) in osteoblasts,[16,26] the present study was the first to demonstrate a PAR1 relevant role in another process associated with periodontal regeneration.

ERK1/2 and PI3K/Akt are considered the two major PAR1 downstream signaling pathways.[29,31,32] In the PI3K/Akt pathway investigation, we found that the PI3K/Akt inhibitor had no impact on the increased CEMP1 gene expression mediated through PAR1 activation, suggesting that this pathway has little or no role in the results observed. Conversely, the above-mentioned pathway has already been implicated in increased OPG expression via PAR1 activation in periodontal ligament cells.[29]

When the ERK1/2 pathway inhibitor was used, we observed that the PAR1 effect on increased CEMP1 gene expression was abrogated, suggesting that PAR1 activation can lead to an increase in CEMP1 gene expression in an ERK1/2 dependent manner. The ERK1/2 pathway plays a pivotal role in cell differentiation and proliferation.[25] In fact, in an ERK-dependent manner, PAR1 activation is associated with an increased proliferation of dental pulp fibroblasts,[34] astrocytes,[35] and vascular smooth muscle cells.[28,29] In addition, PAR1 may increase CCL2 expression in human osteoblastos,[36] and prostaglandin E_2 in mouse osteoblastic cells[37] via ERK pathway. Regarding the relation of CEMP1 and the ERK pathway, a previous study found that treatment with calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of mesenchymal periodontal ligament cells in a CEMP1 and ERK-dependent manner,[38] which corroborates with the findings of the present study, which shows a role of the ERK1/2 pathway on CEMP1 expression.

Understanding some of the key regulators associated with cementogenesis is of great interest for further developing molecular therapies for cementum regeneration. Therefore, the present study provides evidence that PAR1 and ERK1/2 may serve as potential targets for the application of therapies of periodontal regeneration through PDLSCs.

Conclusion

PAR1 activation of hPDLSCs enhanced CEMP1 gene expression *in vitro*. This response was found to be ERK-dependent and PI3K independent. These data provide insights about the role of PAR1 and ERK pathway at cementogenesis by hPDLSCs.

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