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Protease-activated receptor type 2 activation downregulates osteogenesis in periodontal ligament stem cells

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Abstract: Protease-activated receptor-2 (PAR2) is associated with the pathogenesis of many chronic diseases with inflammatory characteristics, including periodontitis. This study aimed to evaluate how the activation of PAR2 can affect the osteogenic activity of human periodontal ligament stem cells (PDLSCs) *in vitro*. PDLSCs collected from three subjects were treated in osteogenic medium for 2, 7, 14, and 21 days with trypsin (0.1 U/mL), PAR2 specific agonist peptide (SLIGRL-NH2) (100 nM), and PAR2 antagonist peptide (FSLLRY-NH2) (100 nM). Gene (RT-qPCR) expression and protein expression (ELISA) of osteogenic factors, bone metabolism, and inflammatory cytokines, cell proliferation, alkaline phosphatase (ALP) activity, alizarin red S staining, and supernatant concentration were assessed. Statistical analysis of the results with a significance level of 5% was performed. Activation of PAR2 led to decreases in cell proliferation and calcium deposition ($p < 0.05$), calcium concentration ($p < 0.05$), and ALP activity ($p < 0.05$). Additionally, PAR2 activation increased gene and protein expression of receptor activator of nuclear factor kappa-B ligand (RANKL) ($p < 0.05$) and significantly decreased the gene and protein expression of osteoprotegerin ($p < 0.05$). Considering the findings, the present study demonstrated PAR2 activation was able to decrease cell proliferation, decreased osteogenic activity of PDLSCs, and upregulated conditions for bone resorption. PAR2 may be considered a promising target in periodontal regenerative procedures.

Keywords: Stem Cells; Osteogenesis; Mesenchymal Stem Cells; Periodontal Ligament.

Introduction

The destruction of periodontal supporting tissues is the result of an inflammatory disease known as periodontitis.[1] The patient's quality of life can be severely affected by the destruction caused by periodontal disease.[2] Today, the main objective of periodontal therapy is the regeneration of lost periodontal tissues,[3] but this poses a great challenge. Stem cells appear as an interesting option in the search for periodontal regeneration thanks to their ability to differentiate and proliferate in multiple types of cell lines, such as osteoblasts, osteocytes, adipocytes, and chondrocytes, maintaining specific functions.[4] Periodontal ligament

stem cells (PDLSCs) are of great importance in the regeneration of periodontal tissues because of their potential to differentiate into important cell lines such as osteoblasts, cementoblasts, and fibroblasts,[5] and also because they have already shown a fundamental role in the regeneration of the periodontal ligament and trabecular bone.[6]

Inflammation plays a detrimental role on PDLSC osteogenesis for reducing mineralization and the gene expression of osteogenic markers, such as osteocalcin (OC), runt-related transcription factor 2 (RUNX-2), and alkaline phosphatase (ALP).[7] Li et al.[8] found that PDLSCs collected from resorbed primary teeth showed an important increase of receptor activator of nuclear factor kappa-B ligand (RANKL) and a significant decrease in the expression of osteoprotegerin (OPG). This imbalance between RANKL and OPG leads to greater osteoclast differentiation, possibly contributing to higher periodontal bone loss.[9-11]

Periodontal disease has already been proven to be associated with protease receptor type 2 (PAR2) by a series of *in vitro* and *in vivo* animal and human studies.[12-16] PAR2 activation triggers a proinflammatory response in periodontal tissues due to increased production of tumor necrosis factor alpha (TNF-alpha), matrix-2 metalloproteinase (MMP-2), matrix-8 metalloproteinase (MMP-8), prostaglandins E2 and F2 (PGE2 and PGF2), interleukins 6 and 8 (IL-6 and IL-8), and granulocyte colony-stimulating factor by fibroblasts, keratinocytes, osteoblasts, and neutrophils.[17-19] After periodontal treatment, levels of PAR2 expression decreased significantly. These data suggest a direct bidirectional relationship between periodontitis and PAR2 activation.[15,16]

The specific effects that PAR-2 activation can have on PDLSCs are unknown in the literature. There is an important gap in the literature considering the role of PAR2 in periodontal inflammation, so it is essential to understand the effects of PAR2 on PDLSCs, particularly on their potential to regenerate periodontal tissues, thus filling this gap. Therefore, the present study sought to evaluate how the activation of PAR2 can affect PDLSCs, especially osteogenic activity.

Methodology

The methodology of this study was based on a previous study by our group published by Rovai et al.[20]

Isolation and characterization of the periodontal ligament stem cells

This study was approved by the Research Ethics Committee of the School of Dentistry of the University of São Paulo (FO-USP) under protocol #803.811. A free and informed consent form was signed by all participants. Three patients were selected from the surgery outpatient clinic of the School of Dentistry of the University of São Paulo, and PDLSCs were extracted from healthy third molars following orthodontic recommendations. The patients were aged 20 to 35 years, with no history of systemic diseases. Exclusion criteria included patients who had been evaluated by a periodontist and presented third molars in the process of root formation, pericoronitis, and poor health status.

The explant technique was employed for the cell culture of the periodontal ligament removed from the middle third of the root. Cells collected from three different patients were utilized for cell passaging.[3-7]

The selected third molars and freshly extracted specimens were transported in an α-Modified Eagle Medium (α-MEM) (Gibco, Invitrogen Life Technologies, Carlsbad, USA) to the cell culture laboratory. Under aseptic conditions, the teeth whose gingival tissue was still attached were removed and properly discarded. Periodontal ligament tissue was obtained by scraping off the middle third of the root, thus preventing possible contamination of the gingival or pulpal tissues. The collected tissues were fragmented into specimens with approximately 1 mm in thickness. After fragmentation, the fragments were centrifuged at 800x g for 5 min at room temperature (RT) and resuspended. The suspension was then deposited into 25 cm2 culture flasks immersed in α-MEM supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, 100 µg/ mL streptomycin, 0.5 mg/mL amphotericin B (all reagents from Gibco, Invitrogen, Life Technologies, Carlsbad, USA) and collected into an incubator at 37°C, 5% CO2 , and 100% humidity.

Cells were cultured in α-MEM supplemented with 15% FBS, 100 µg/mL penicillin, 100 µg/mL streptomycin, 0.5 mg/mL amphotericin B (control medium (CM)) and incubated at 37°C with 5% CO_2 and 100% air humidity. Once subconfluent, the cells were trypsinized and subcultured. FBS, penicillin, streptomycin, and amphotericin B were all cultured using reagents from the same manufacturer (Gibco, Invitrogen, Life Technologies, Carlsbad, USA).

Immunostaining and flow cytometry were used to examine cell surface markers, $5x10^5$ cells were isolated, washed in phosphate-buffered saline (PBS) and incubated for 30 min at 4°C with the following monoclonal antibodies: CD90-FITC (eBioscience, San Diego, USA), CD34-FITC, CD31-PE, CD-44-PE, and CD146-PE (Biolegend, San DiegoUSA). The cell suspension was rinsed twice with PBS and analyzed using the FACS Diva flow cytometer (Becton Dickinson, São Paulo, Brazil). The registered events were evaluated using the Cell Quest software (Becton Dickinson, São Paulo, Brazil).

Experimental groups

Twenty-four (24)-well plates were used at a density of 25,000 cells/cm^2 to seed the PDLSCs, with a control medium (CM) or osteogenic medium (OM) (CM + 0.1 mM dexamethasone (Invitrogen, Carlsbad, USA), 180 mM $KH_2PO_4$50 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis, USA)) and treated with trypsin (Sigma-Aldrich, St. Louis, USA) 1 U/mL for different experimental time periods. To test whether the effect induced by trypsin is specifically mediated by PAR2, another experimental group was treated with LRGILS-NH2†† PAR2 control peptide, 100 µMol/L. In addition, the cells were exposed to the synthetic agonist peptide of PAR2, SLIGRL-NH2 (Tocris Bioscience Inc, Bristol, UK), 100 µMol/L concentration and to the synthetic antagonist FSLLRY-NH2 (Tocris Bioscience Inc, Bristol, UK) peptide, 100 µMol/L. As experimental control, the cells were cultured with plain medium only or vehicle only. The culture medium and the cells were collected for further analysis. The culture medium and the specific treatments were changed every day.

Cell proliferation

The Quick Cell Proliferation Assay kit (Abcam, Cambridge, USA) was used to measure cell proliferation. The assay was performed in triplicate at 48 and 96 h in CM and OM after each of the five treatments in accordance to the manufacturer's instructions. The cells were prepared with a dye working solution and were then incubated at 37°C for 10-30 min. The dye working solution was removed and the sample was analyzed with a flow cytometer at Ex/Em = 511/525 nm.

Mineralized nodule formation (alizarin red S staining)

The measurement of mineralized nodule formation was assessed as reported by Gregory et al.[21] with aliquots (150 mL) of the supernatant measured in triplicate at 405 nm and 550 nm in 96-well format with the use of a spectrophotometer.

All reactions were made in triplicate. The *in vitro* mineralization was performed at 7, 14, and 21 days by alizarin red S staining. The cold PBS was used to wash the cell culture and they were then fixed in 10% formaldehyde for 30 min at room temperature. Thereafter, distilled water was used to wash the cells twice and then at room temperature exposed to 1 mL of alizarin red S solution 40 mM (Sigma-Aldrich, St. Louis, USA) (pH 4.1) per well for 30 min. Once again, distilled water was used for washing; and for easier removal of excess water, the plates were tilted. Digital images of the mineral deposits were taken using an inverted microscope.

Calcium concentration and alkaline phosphatase activity

The samples were evaluated using commercial colorimetric kits according to the manufacturer's guidelines (Abcam, Cambridge, USA) and all reactions were performed in triplicate. The calcium concentration in the culture medium (supernatant) and the ALP activity were evaluated in 2, 7, and 14 days.

Gene expression of bone metabolism

Reverse transcription followed by quantitative PCR (RT-qPCR) was used to evaluate the gene expression of Runx2, OPG, kappa-B ligand (RANKL), and

osteocalcin (OC) in samples taken at 2, 7, 14, and 21 days after the specific treatments.

Using 1 mL TRIzol® Reagent (Thermo Fisher Scientific Inc, Waltham, USA) per well, total RNA was extracted according to the manufacturer's instructions. By means of a reverse transcription reaction, complementary DNA (cDNA) was synthesized using RNA samples as templates in conjunction with a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc, Waltham, USA). Finally, cDNA was subjected to qPCR reactions using Taqman mastermix (Applied Biosystems, Foster City, USA) along with Taqman assays with the following standard PCR conditions: 37ºC (60 min) and then 40 cycles of 95ºC (15 s) and a final cycle at 4ºC (20 min). The following GenBank accession numbers for the oligonucleotide sequences used for cDNA amplification were applied: OPG (NM_002546.4), Runx2 (NM_001015051.4), RANKL (NM_003701.4), OC (NM_199173.6), and GAPDH (NM_001256799.3). The reactions were performed in triplicate and the levels of gene expression were based on a reference sample (untreated control) standardized for the housekeeping gene (GAPDH). In addition, RNA-free and reverse transcriptase-free samples were used as negative controls. The samples were taken at 2, 7, 14, and 21 days.

Protein expression of osteogenic factors, bone metabolism and inflammatory cytokines

Commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, USA) were used to evaluate the levels of Runx2, OPG, OC, IL-10, IL-6, CCL2, MCSF, and RANKL in culture medium samples (supernatant), in accordance with the manufacturer's instructions. Reactions were performed in triplicate and the results were reported in pg/mL. Samples were collected and analyzed at 2, 7, 14, and 21 days.

Statistical analysis

The GraphPad Prism 5.01 software (GraphPad Software, La Jolla, USA) was used to perform the statistical analysis, with a 5% significance level. The data obtained were representative of three independent experiments conducted with cells derived from three different donors. ANOVA and the Kruskal Wallis test were used for multiple comparisons with the Brown-Forsythe test and the Bartlett test.

Results

Cell characterization

In order to identify the phenotype of mesenchymal cells and to evaluate cell purity and homogeneity, immunostaining and flow cytometry analysis were performed. The results showed that the cells were CD-146-, CD-44-, and CD-90-positive. In addition, the cells were CD34- and CD31-negative (Figure 1).

PAR2 activation and cell proliferation

PAR2 activation modified cell proliferation. In the growth medium, the cells showed a significantly decreased proliferation when exposed to the specific PAR2 agonist in the first 48 h compared to the control group ($p < 0.05$) (Figure 2).

At 96 h, the cells exposed to the PAR2 agonist peptide maintained decreased cell proliferation compared to the control group. On the other hand, trypsin increased cell proliferation (Figure 2). A similar result was observed for the osteogenic medium at 48 h, when those cells exposed to PAR2 agonist peptide exhibited lower proliferation compared to the control group (CM) (Figure 2).

At 96 h, the cells cultured with trypsin in the osteogenic medium maintained increased proliferation compared to the control group ($p < 0.05$). The proliferation rate of those cells exposed to the PAR2 agonist peptide, however, remained similar to that of the control group. This finding shows that the specific activation of PAR2 through its agonist may have an important impact by reducing cell proliferation in its initial phases (48 h), but this effect appears to decrease over time (Figure 2).

Effect of PAR2 activation on mineralized nodule formation (alizarin red S staining)

Mineralized nodule formation was evaluated at 7, 14, and 21 days (Figure 3). Trypsin and PAR2 agonists decreased mineralization *in vitro* at 21 days ($p < 0.05$) in comparison to osteogenic medium control (Figure 3).

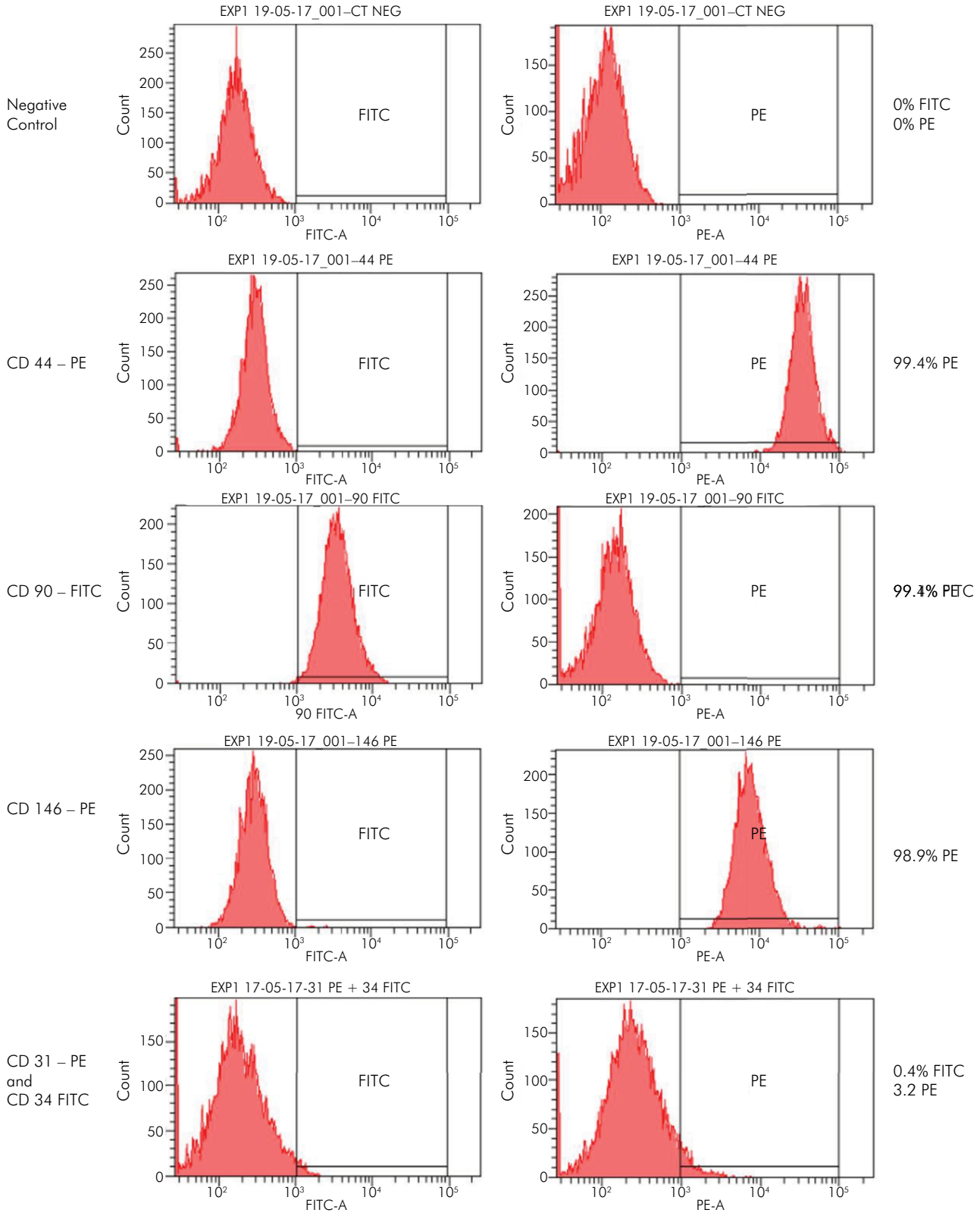
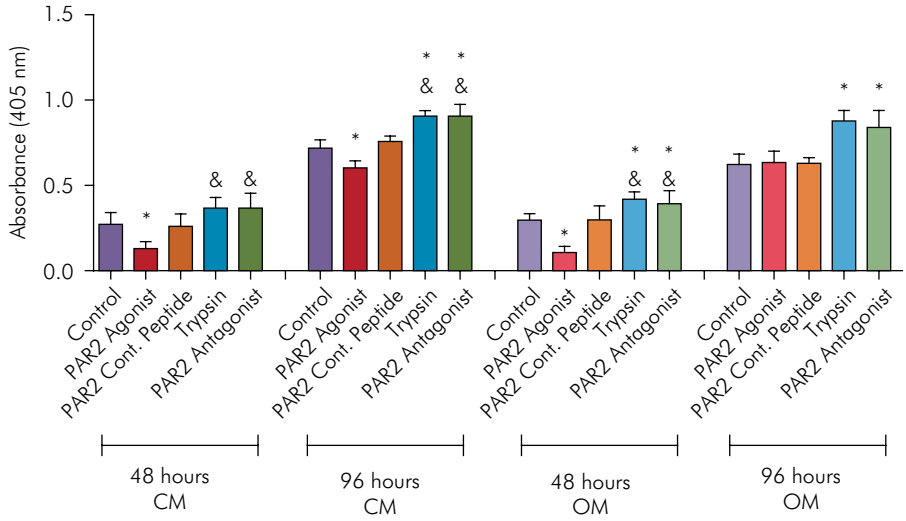
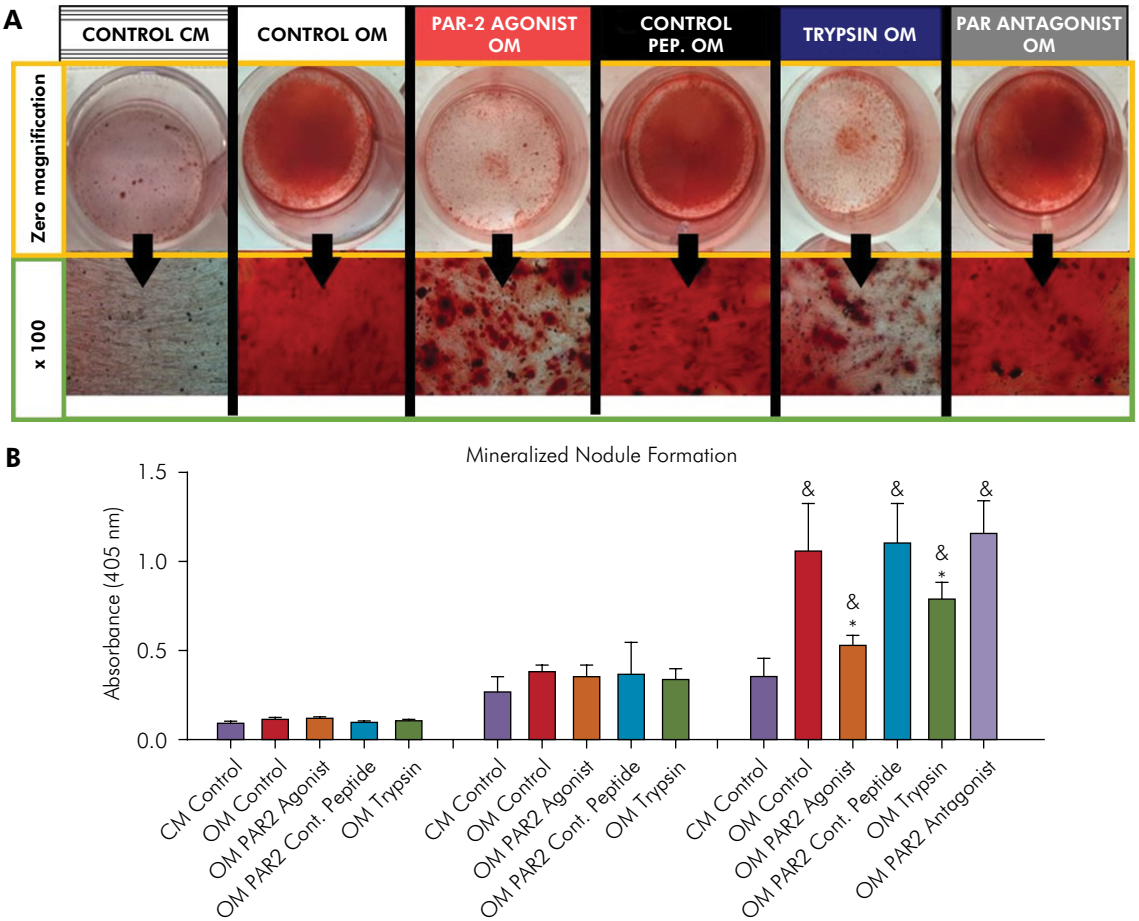


Figure 1. Cell characterization by flow cytometry.



Values are presented as mean and SEM; n = 3. *Mean significant difference when compared to the control of the same medium and experimental period (p < 0 05). &Mean significant difference when compared to PAR2 agonist treatment of the same medium and experimental period (p < 0 05).

Figure 2. Cell proliferation in different groups and experimental periods.



(M) Quantitative alizarin red staining analysis after 21 days. Data are shown as mean and SD; n = 3. *Mean significant difference when compared to OM + control (p < 0 05). & Mean significant difference when compared to CM control (p < 0 05).

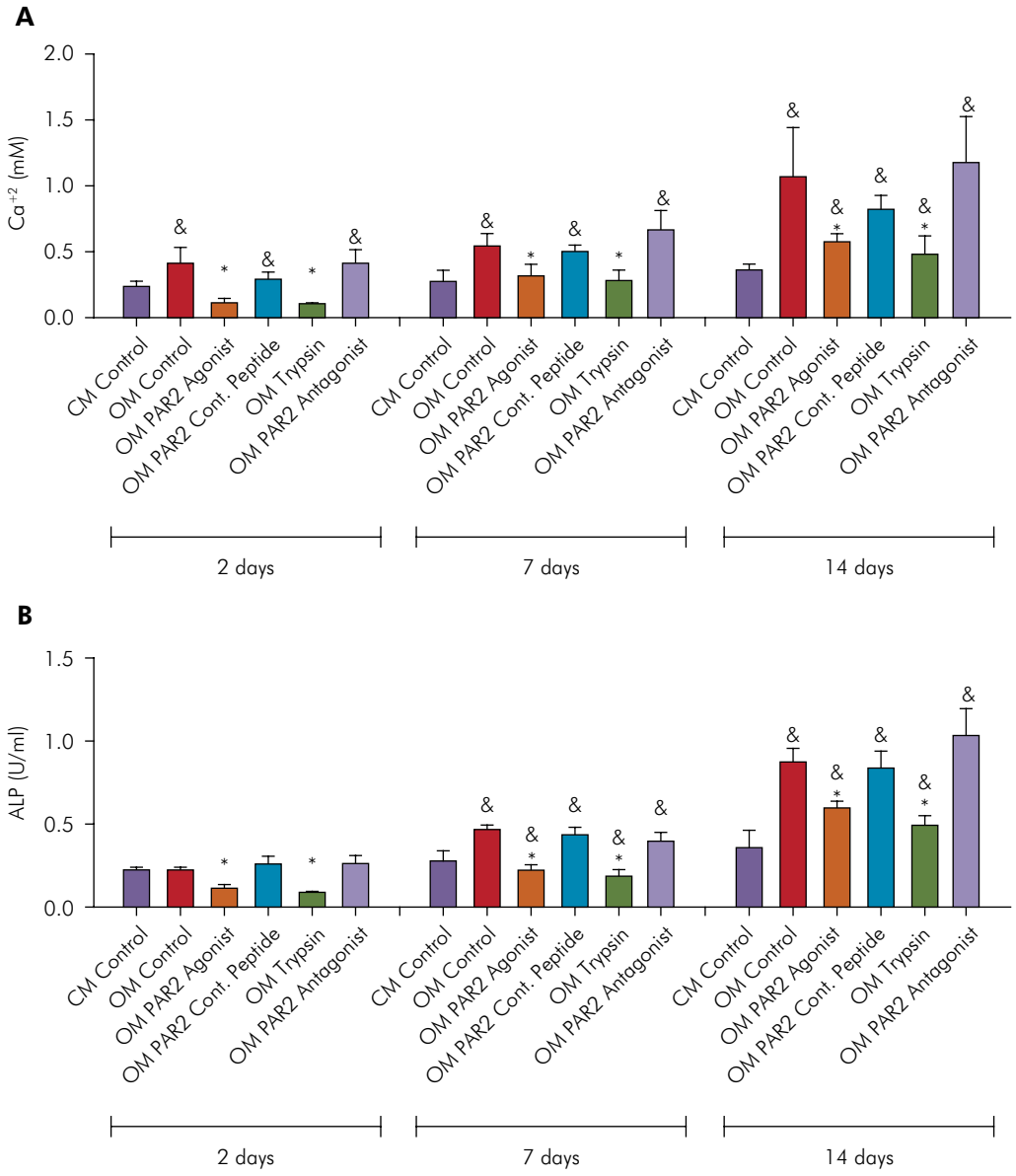
Figure 3. Effects of PAR2 on mineral deposition using alizarin red staining after 21 days (a–f: zero magnification; g–l: magnification ×100).

Effect of PAR2 activation on calcium concentration and alkaline phosphatase activity

Treatments with PAR2 and trypsin agonists led to a significant decrease in calcium concentration compared to the other groups ($p < 0.05$) (Figure 4). In addition, PAR2 agonist decreased ALP activity compared to the control group ($p < 0.05$) (Figure 4).

Production of bone metabolism and osteogenic factors at the gene and protein levels

The gene expression and protein production of molecules involved in bone metabolism (Runx2, OPG, OC, and RANKL) were assessed by RT-qPCR and ELISA, respectively. PAR2 specific agonist and trypsin increased the gene expression of RANKL at 2, 7, 14, and 21 days ($p < 0.05$) (Figure 5). Similar



Values are presented as mean and SEM n = 3. *Mean significant difference when compared to OM + control ($p < 0\ 05$). & Mean significant difference when compared to CM control ($p < 0\ 05$).

Figure 4. Calcium concentration in the supernatant (A) and ALP activity (B) in different groups and experimental periods.

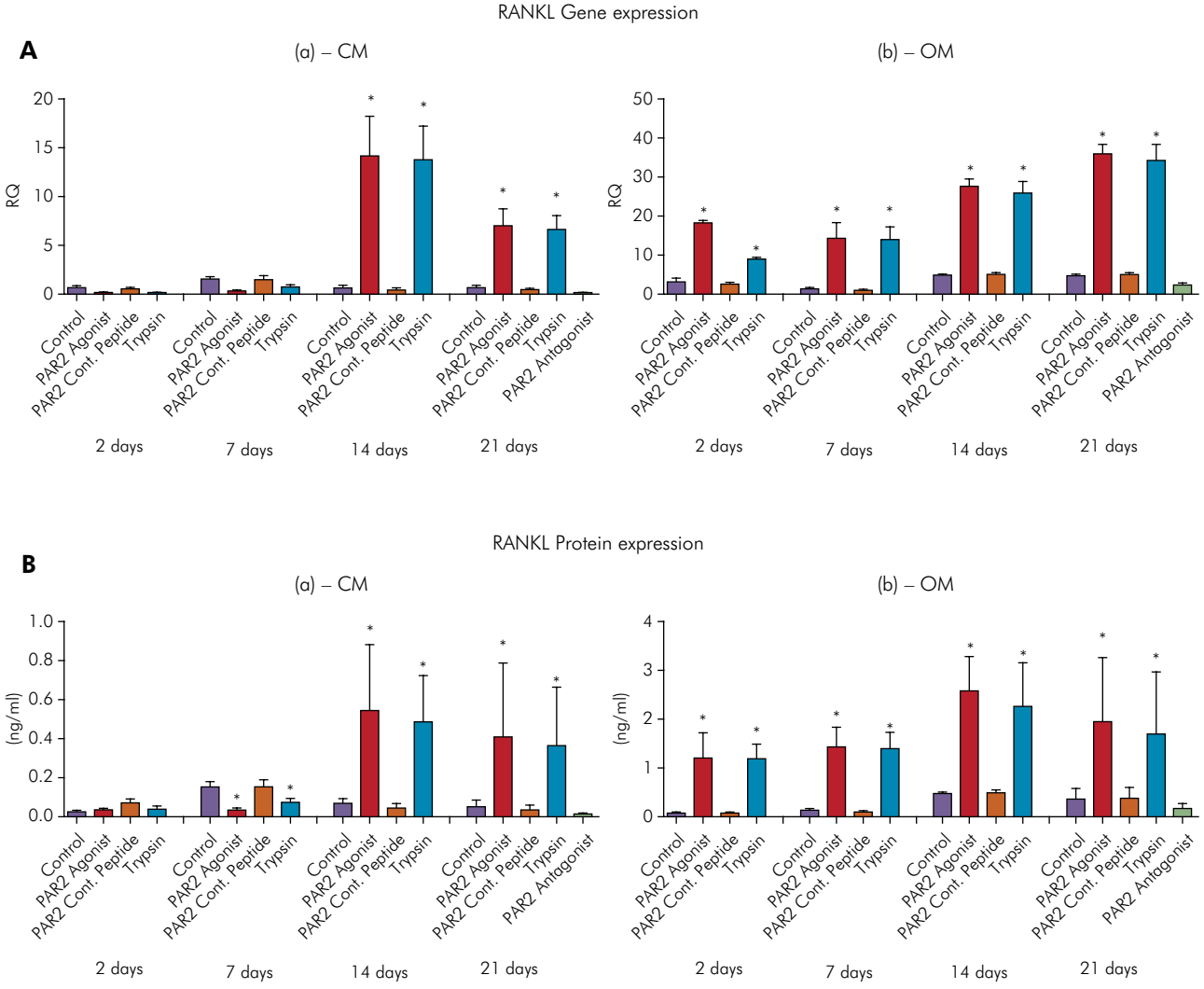
findings were obtained at the protein level with the increase in RANKL by PAR2 agonist and trypsin (Figure 5). Interestingly, trypsin and PAR2 agonist peptide also decreased in OPG gene expression ($p < 0.05$) (Figure 6) and protein levels at 2, 7, 14, and 21 days ($p < 0.05$) (Figure 6).

Simultaneously, Runx2 was reduced in terms of gene expression and protein levels by PAR2 agonist- and trypsin-treated cells at 7, 14, and 21 days ($p < 0.05$) (Figure 7). In addition, there was no statistically significant difference in the gene expression and levels of OC between the groups analyzed in any experimental period (Figure 7).

The levels of M-CSF, CCL2, IL-6, and IL-10 were also assessed by ELISA. M-CSF and IL-10 levels were reduced by PAR2 agonist- and trypsin-activated cells at 2,7,14, and 21 days ($p < 0.05$) (Figure 8). Production of IL-6 was downregulated by PAR2 agonist and trypsin at 2 and 7 days, but upregulated at 21 days. PAR2 agonist and trypsin also increased CCL2 levels in all experimental periods ($p < 0.05$) (Figure 8).

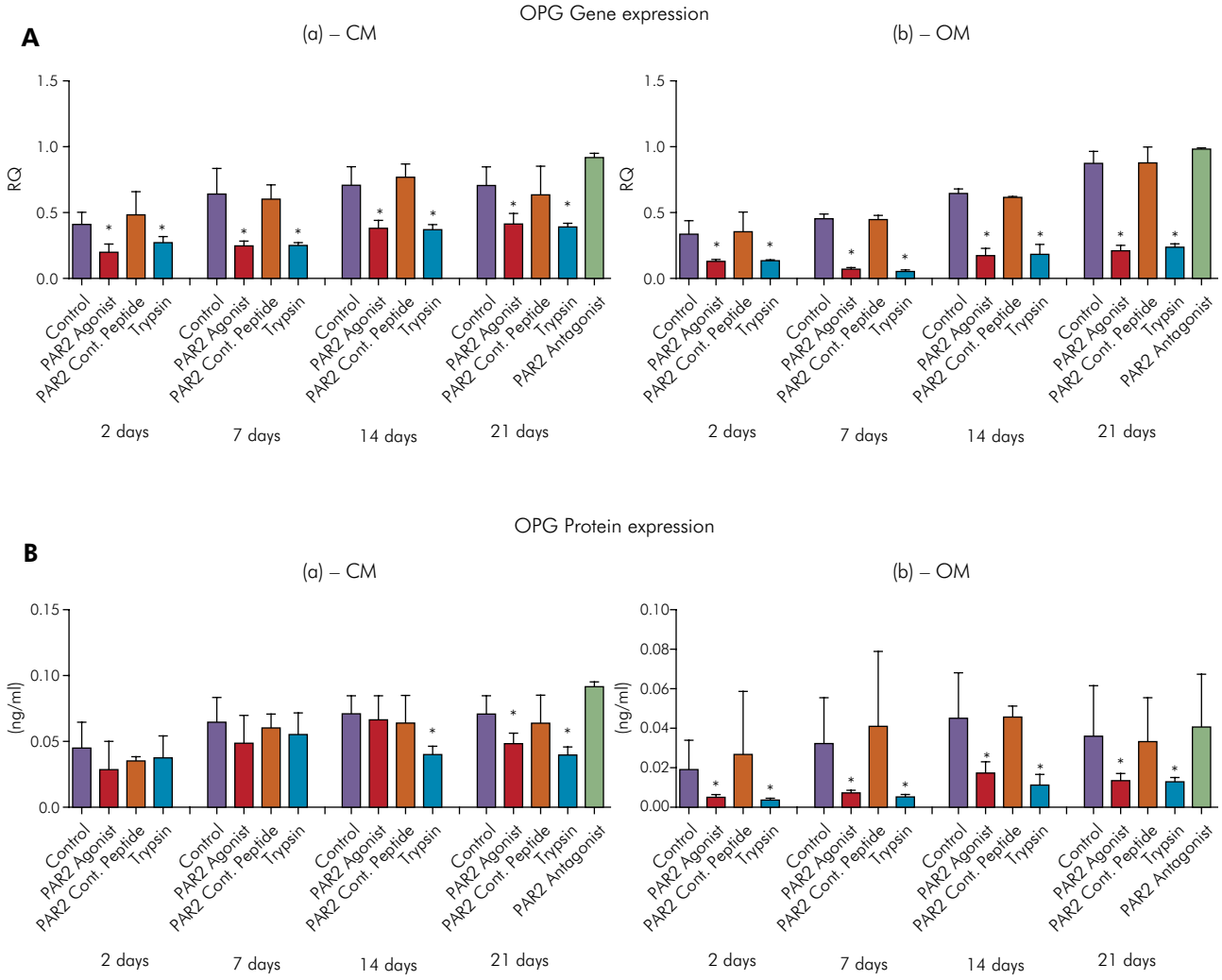
Discussion

Four types of receptors have been identified: PAR-1, PAR-3, and PAR-4, which can be activated



Values are presented as mean and SEM; n = 3. *Mean significant difference when compared to control of the same medium and experimental period ($p < 0\ 05$). (a) Control medium. (b) Osteogenic medium.

Figure 5. Effects of PAR2 activation on RANKL gene (A) and protein (B) expression at 2, 7, 14, and 21 days.

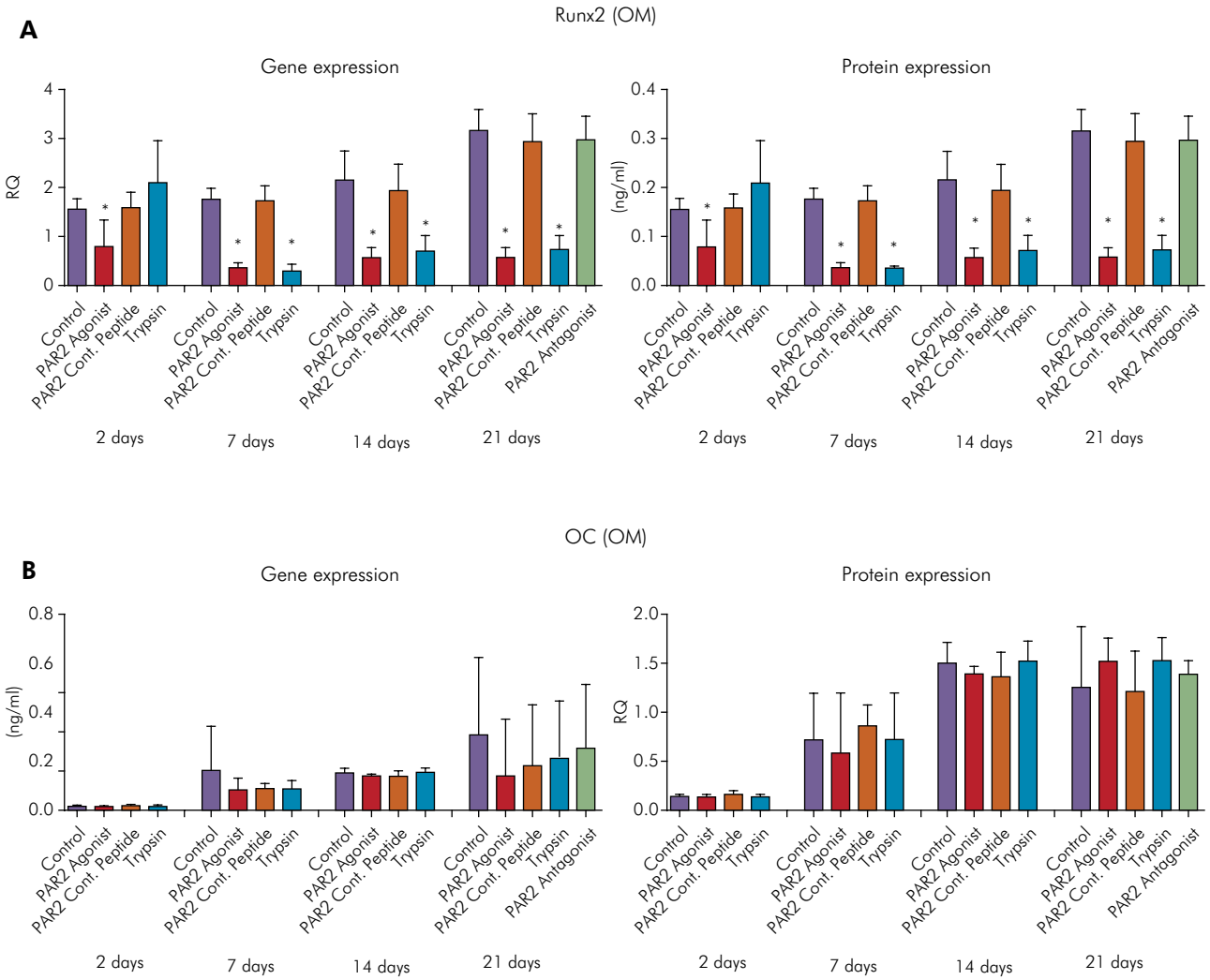


Values are presented as mean and SEM; n = 3. *Mean significant difference when compared to control of the same medium and experimental period (p < 0 05). (a) Control medium. (b) Osteogenic medium.

Figure 6. Effects of PAR2 activation on OPG gene (A) and protein (B) expression at 2, 7, 14, and 21 days.

by thrombin; and PAR-2, which can be activated by pancreatic, extrapancreatic (endothelial and epithelial) trypsins, mast cell tryptase, neutrophil type 3 proteinase, coagulation factors (VIIa/Xa), membrane-bound serine protease 1, granzymes, and gingipain (a bacterial protease), which is produced by the periodontal pathogen *P. gingivalis*.[12] PARs are innate immune receptors that recognize specific bacterial or endogenous serine proteases and initiate defensive immune responses. PAR2 is expressed by epithelial cells, fibroblasts, osteoblasts, endothelial cells, myocytes, neurons, mast cells, lymphocytes, astrocytes, and neutrophils.[12] Cells present in the periodontal ligament (PDLSCs) express PAR-2.[19] However, the specific effects PAR-2 activation can have on PDLSCs are unknown in the literature. As previously shown, the cells marked positively for CD 90, CD 146, and CD 44 are important mesenchymal cell markers. This indicates that the cells used in our study correspond to the profile of PDLSCs.

PAR activation is irreversible and to prevent overstimulation of the mechanism, this receptor undergoes rapid internalization, being destroyed within the lysosomes. There is a constant renewal of these receptors in the cell membrane through the synthesis carried out in the Golgi complex. New sensitization of PARs will only occur with re-availability on the cell surface.[17]



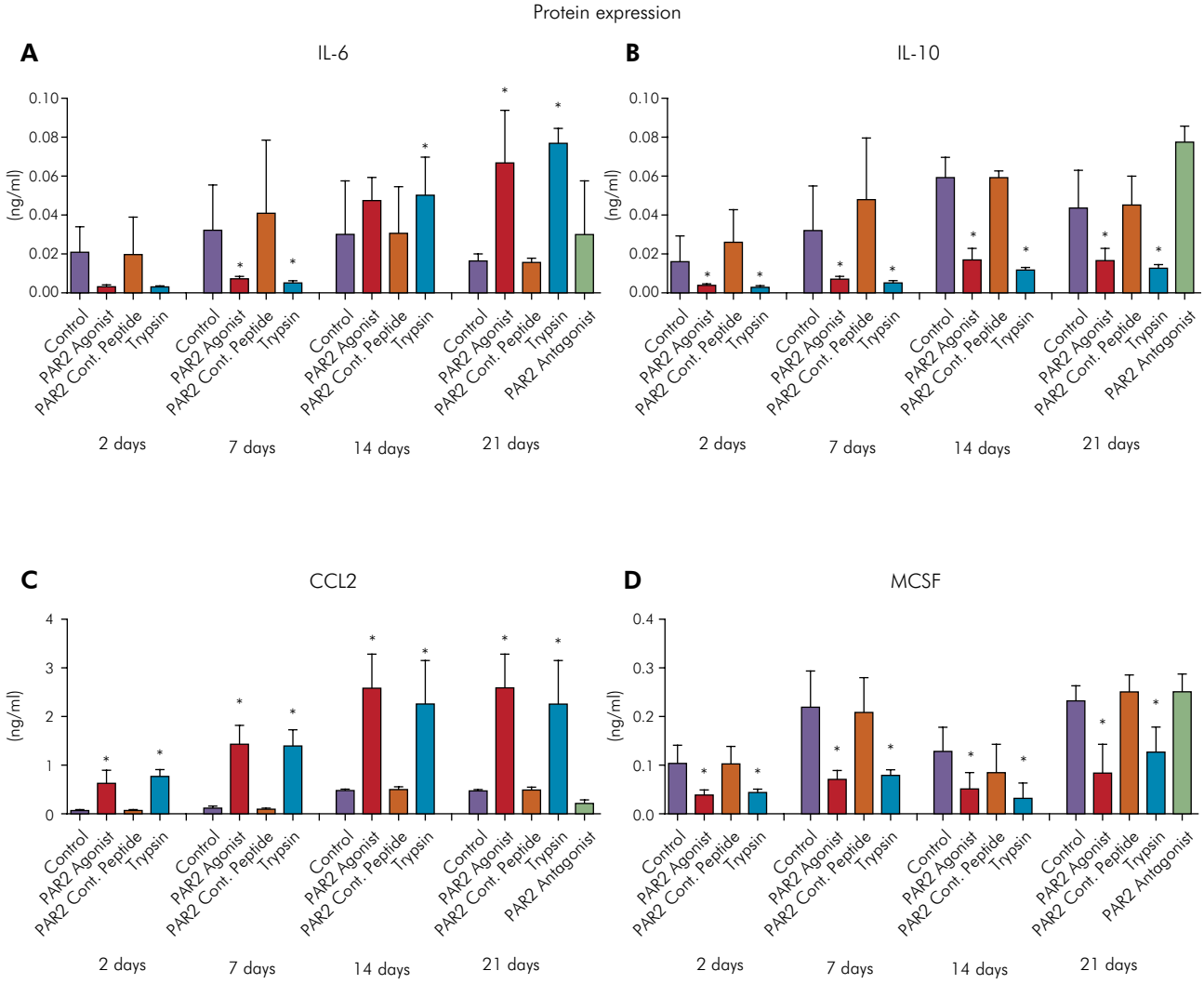
Values are presented as mean and SEM; n = 3. *Mean significant difference when compared to control of the same medium and experimental period (p < 0 05).

Figure 7. Effects of PAR2 activation on Runx2 (A) and OC (B) protein and gene expression at 2, 7, 14, and 21 days in osteogenic medium (OM).

PARs are characterized by a unique activation mechanism that allows them to act as cellular sensors of proteolytic activity. The activation of PARs occurs through the cleavage of the extracellular N-terminal domain, generating a new sequence, consisting of a linker peptide with five or six amino acids. This terminal portion will then connect to the fourth transmembrane loop of the receiver itself, activating it and initiating cell signaling.[17]

The relationship between PAR2 and periodontitis can be seen in the study conducted by Holzhausen et al.,[15] in which they found that patients with chronic periodontitis had, at baseline, higher levels of PAR-2 expression correlated with higher levels of IL-1ß, IL-6, IL-8, and TNF-alpha in the gingival crevicular fluid. After periodontal treatment, PAR-2 expression levels decreased significantly. In our study, the production of IL-6 was downregulated by PAR2 agonist and trypsin at 2 and 7 days, but upregulated at 21 days.

Fagundes et al.[14] showed in 2011 that patients with chronic periodontitis have a greater expression of PAR-2 in their periodontal pockets and a greater concentration of pro-inflammatory cytokines when infected with *P. gingivalis*. Furthermore, Alves et al.[16] showed that PAR-2 plays an important role in the



Values are presented as mean and SEM; n = 3. *Mean significant difference when compared to control of the same medium and experimental period (p < 0 05).

Figure 8. Effects of PAR2 activation on IL-6 (A), IL-10 (B), CCL2 (C), and MCSF (D) protein expression at 2, 7, 14, and 21 days in osteogenic medium.

inflammatory process and is associated with the increase of important inflammatory mediators associated with the destruction of the periodontium, such as IL-6, IL -8, TNF-α, MMP- 2, and MMP-8, among others. Alves et al.[16] also showed that non-surgical periodontal treatment led to decreased gene and protein expression of PAR-2 and also of pro-inflammatory mediators, thus suggesting that PAR-2 expression is associated with the presence of periodontal inflammation.

One of the most important findings of the present study concerns the negative impact of PAR-2 activation on the potential for mineralization and formation of calcium deposits at 21 days in the alizarin red mineralization assay, suggesting that PAR-2 activation downregulates osteogenic activity. This effect seems to be associated with the initial decrease in cell proliferation, decreased ALP activity, and imbalance between OPG and RANKL, with the increase of RANKL and decrease of OPG at both gene expression and protein levels.

The above-mentioned findings are consistent with the literature, which shows that PAR-2 activation elevates RANKL expression in osteoblasts and this,

in turn, may be associated with increased osteoclastic differentiation and increased bone resorption. [22]

In addition, there was a decrease in gene expression and Runx2 levels when PAR-2 was activated in PDLSCs. Runx2 participates in osteoblast differentiation, control of osteogenic capacity, and bone growth. [23]

Another important molecule that can influence bone metabolism is CCL2, which can be found at dental eruption sites and bone degradation, which is expressed by mature osteoclasts and osteoblasts[24] and is a key factor for monocyte recruitment. In the present study, CCL2 levels also increased in the PAR-2 and trypsin specific agonist groups at 7, 14, and 21 days, showing the influence of PAR-2 activation on leukocyte chemotaxis and bone resorption.

Accordingly, in a periodontal regeneration process, bone formation is desirable and very important, and osteoblast differentiation is critical. The balance between OPG and RANKL is an important basis for this process; and besides, reduced levels of CCL2 are expected for bone formation, and this process can be completely altered through PAR2 activation, as shown by our data. As it is a complex mechanism, periodontal regeneration involves several cellular, molecular, and genetic factors and several studies need to be performed to better elucidate these mechanisms and their possible impacts on periodontal regeneration.

Tang et al.[7] showed that the inflammatory process in PDLSCs collected from sites with gingival inflammation makes PDLSCs dysfunctional in their capacity for osteogenic differentiation, including their ability to mineralize nodules and expression levels of osteogenic genes, as also observed in our study. This dysfunctionality observed in PDLSCs caused by inflammation may also be a negative factor for periodontal regeneration.

Another factor that can be affected by the inflammatory process related to PAR2 activation is ALP activity. ALP is an important marker of osteoblast differentiation.[25] It is known that PDLSCs decrease ALP activity during the inflammatory process,[8] a finding similar to that of our study, in which PAR-2 activation by trypsin and PAR-2 agonist resulted in weaker ALP activity. Considering a possible application of PDLSCs in periodontal regeneration therapy, ALP activity can be an important modulating factor for bone formation, and our findings show that PAR2 activation can exert a modulating factor on it.

IL-10 has potent anti-inflammatory properties that play an important role in limiting host immune response to pathogens.[26] Our data show a decrease in IL-10 expression with PAR-2 activation, thus supporting the pro-inflammatory role of PAR2. In 2002, Cenac et al.[26] showed that IL-10 expression is not altered in rat intestines with the specific activation of PAR-2. In addition, Steven et al.[27] showed that PAR-2 activation has a complex role in macrophage biology and may have implications for macrophage-driven disease (an alteration of the MCSF), as well as reduced IL-10, as observed in our data.

PAR-2 activation and its influence on the proliferation of cancer cells have already been defined in several types of cancer, such as pancreatic cancer, melanoma, and liver cancer, among others;[28-31] however, the role of PAR-2 activation in PDLSCs is not yet completely clear. Trypsin acts as a non-specific PAR-2 activator[27] and also acts in other pathways, leading to increased cell proliferation. The specific PAR-2 activator (SLIGRL-NH2) showed an important reduction in cell proliferation in the first 48 h and a return to similar levels in the control group at 96 h, suggesting that specific PAR-2 activation may impair initial cell proliferation and may have a compensatory effect over time. This effect of PAR-2 specific activation on decreasing cell proliferation in mesenchymal cells of the periodontal ligament is not yet clear in the literature and our findings demonstrate that more research is needed to elucidate this mechanism.

The findings of the present study strongly suggest that the metabolism of PDLSCs may be influenced by PAR-2 activation, which reduces osteogenesis through altered cell proliferation, decreased ALP activity, and imbalance between RANKL and OPG. Considering the possible use of PDLSCs in periodontal regeneration therapy, PAR2 modulation may have a positive effect on osteoblast differentiation and allow for greater bone formation. Nonetheless, more studies are needed to clarify these issues.

Conclusions

In conclusion, PAR2 activation downregulates osteogenic activity in PDLSCs. In addition, PAR2 activation decreased cell proliferation, calcium deposition, and mineralized nodules formation by PDLSCs. Concurrently, PAR2 activation decreased the OPG to RANKL ratio. Taken together, our data point to PAR2 as a molecule that downregulates osteogenic activity and, at the same time, increases bone resorption. Thus, this study suggests that PAR-2 might be important in regenerative procedures that use mesenchymal cells of the periodontal ligament.

Acknowledgements

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