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Matrix metalloproteinases regulate extracellular levels of SDF-1/CXCL12, IL-6 and VEGF in hydrogen peroxide-stimulated human periodontal ligament fibroblasts

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ABSTRACT

Periodontitis is a highly prevalent infectious disease characterized by the progressive inflammatory destruction of tooth-supporting structures, leading to tooth loss. The underling molecular mechanisms of the disease are incompletely understood, precluding the development of more efficient screening, diagnostic and therapeutic approaches. We investigated the interrelation of three known effector mechanisms of the cellular response to periodontal infection, namely reactive oxygen species (ROS), matrix metalloproteinases (MMPs) and cytokines in primary cell cultures of human periodontal ligament fibroblast (hPDLF). We demonstrated that ROS increase the activity/levels of gelatinolytic MMPs, and stimulate cytokine secretion in hPDLF. Additionally, we proved that MMPs possesses immune modulatory capacity, regulating the secreted levels of cytokines in ROS-stimulated hPDLF cultures. This evidence provides further insight in the molecular pathogenesis of periodontitis, contributing to the future development of more effective therapies.

1. Introduction

Inflammatory periodontal diseases are characterized by an uncontrolled chronic immune-inflammatory response to bacterial agents and/or their products, which ultimately leads to the loss of periodontal supporting tissues. Chronic inflammation uncouples the homeostatic mechanisms of tissue turnover, resulting in a net loss of soft and hard tooth-supportive structures. These inflammatory events include a series of complex interactions between enzymatic and non-enzymatic mechanisms [1], such as the synthesis of reactive oxygen species (ROS), pro-inflammatory cytokines and chemokines, and matrix metalloproteinases (MMPs) [2].

During periodontal inflammation ROS are produced in large quantities by activated polymorphonuclear neutrophils (PMN) and macrophages with the aim to eliminate bacterial agents [3]. Sublethal doses of ROS are proposed to play a role as second messengers activating redox-sensitive transcription factors, such as nuclear factor kappa B (NFκB), generating an inflammatory positive feedback loop [4]. ROS have also been involved in bone resorbing activity, down-regulating osteoblast differentiation and stimulating osteoclastogenesis [5].

MMPs are recognized as key effectors of hard and soft periodontal tissue destruction [6]. Evidence of an oxidative regulation of MMPs is also emerging [7,8], as ROS have been involved in increased expression and activation of bone-derived MMPs -13, -2 and -9 [9,10]. It is noteworthy that MMPs, in addition to their classical collagen cleaving properties, can process various components of the extracellular matrix through limited proteolysis. Thus, MMPs can modulate the inflammatory response by liberating bioactive molecules from their reservoirs or by directly modifying

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their biologic activity [11]. These roles make them interesting targets for pharmacological modulation in the treatment of locally destructive inflammatory pathologies, such as periodontitis.

The complex network of cytokines released to the extracellular milieu during periodontal inflammation, along with their redundant regulatory mechanisms hamper the understanding of their individual and collective roles in the context of clinical studies [12]. In this regard, *in vitro* models provide a useful and straightforward mean for testing their interactions. Pro inflammatory cytokines, chemokines and growth factors are known to induce MMPs synthesis, playing a pivotal role in the development of periodontal lesions and are, in turn, subjected to complex regulatory loops [13]. Even after being released to the extracellular environment, cytokine levels and their biological activities might be modulated by MMP-mediated proteolysis [14]. Although there is a wide body of evidence supporting that MMP synthesis in periodontal ligament might be induced by specific pro inflammatory cytokines, few studies address the regulatory role of MMPs over cytokines and other potentially non matrix-bioactive substrates of MMPs.

Human periodontal ligament fibroblasts (hPDLF) represent the most abundant cell type in the periodontal ligament, and are responsible for its turnover and adaptive capabilities. The hPDLF exhibit osteoblast-like features and play key roles in periodontal tissue homeostasis. Under pro-inflammatory stimuli, such as TNF-α and IL-1β, they are able to synthesize pro-inflammatory cytokines and MMPs [15]; however, the effects of non-toxic exposure to ROS over hPDLF MMP and cytokine secretion, and the interrelation between these inflammatory molecules are mostly unknown. Altogether, these mechanisms might act synergistically in the disruption of periodontal homeostasis during inflammatory periodontal diseases, promoting tooth-supporting tissue destruction.

In this study, we examined the effect of MMPs' inhibition on the extracellular levels of the pro-inflammatory mediators SDF-1/CXCL12, IL-6, VEGF, ENA-78 and IL-8/CXCL8, as well as its functional effects over cell migration in an *in vitro* hPDLF oxidative-stress model.

2. Material and methods

2.1. Primary hPDLF cultures

The hPDLF were isolated from 4 patients (3 females and 1 male, mean age of 24.5 ± 5.2 years) that attended the clinic of surgery at the Faculty of Dentistry University of Chile, having indication of extraction of fully erupted third molars with complete root formation and no inflammatory or infectious complication.

Pregnant women, nursing mothers, diabetics, and patients who underwent antibiotic or non-steroidal anti-inflammatory treatment in the last three months were screened and excluded. The study protocol was approved by the Ethical Committee of the Faculty of Dentistry University of Chile in full accordance with the guidelines of the World Medical Association's Declaration of Helsinki [16]. A complete clinical examination was performed in all participants, clinical data were recorded and a written consent was obtained from each subject.

The extraction and isolation of hPDLF from the periodontal ligament was performed by the explant method [17]. Briefly, after tooth extraction, samples were washed with sterile biopsy medium, high glucose and glutamine Dubecco's Modified Eagle Medium (DMEM) (GIBCO Invitrogen Co, Carlsbad CA, USA), supplemented with 100 UI/mL penicillin, 100 mg/mL streptomycin, 250 mg/mL gentamicin, 5 mg/L amphotericin B and 10% fetal bovine serum (FBS) (HyClone Laboratories Inc., South Logan UT, USA) for 30 s and stored in 4 mL of biopsy medium. Later, the periodontal ligament located 3 mm below the cementum–enamel junction and 1 mm above the apical area was extracted in a sterile environment and attached to a 25 mm coverslip inside a 35 mm petri dish, and incubated in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was replaced three times a week (DMEM supplemented with 100 UI/mL penicillin, 100 mg/mL streptomycin, and 10% FBS). After reaching confluence, cell cultures were digested (0.08% trypsin and 0.04% ethylenediaminetetraacetic acid [EDTA]) (GIBCO Invitrogen Co), suspended, counted in a Neubauer chamber, and plated (which was regarded as passage 1). The cells were seeded at 2×10^5 cell density in 100 or 35 mm petri dishes. All the experiments described in the sequence were repeated three times and performed in triplicates using the primary cultures between passages 4 and 6.

The supernatants of all experimental groups were collected, centrifuged at 1000*g* for 15 min at 4 °C, the sediment was discarded, and the liquid phase was frozen at −80 °C for later analysis.

Aliquots of the supernatants were dialyzed through a SnakeSkin® membrane 3500 Da MWCO (ThermoScientific, Waltham MA USA), freeze-dried (FreeZone Freeze Dry Systems, Labconco, USA) and diluted in a fixed volume to obtain salt-free highly concentrated samples (100×).

2.2. Hydrogen peroxide stimulation

Cell-cycle synchronized hPDLF cultures in passage 4–6 were serum-deprived for 24 h and subsequently stimulated with previously determined sublethal concentration of 5 μM H_2O_2 for 24 h. After 24 h of stimulation the supernatants were collected, handled and stored as previously described. To inhibit the MMP activity the culture medium of the experimental groups was supplemented with Ilomastat GM6001 100 μM (USBiological, Swampscott MA USA) following the manufacturer's recommendations. All culture media were supplemented with an EDTA-free protease inhibitory cocktail (10 μM Bestatin, 10 μM E-64, 10 μM Pepstatin, and 1.5 μg/mL Aprotinin) (Sigma–Aldrich, Germany) to isolate the effect of MMPs on the levels of soluble cytokines.

2.3. Anti-oxidant defense system

In order to assess the pro-oxidant effect of our experimental protocol we measured the relative levels of the anti-oxidant defense system enzymes catalase, peroxiredoxin 2 (PRX2), thioredoxin 1 (TRX1), superoxide dismutase 1 (SOD1) and 2 (SOD2) in lysate samples from stimulated and control hPDLF cultures. After recovering the supernatants, the culture dishes were washed twice with cold phosphate buffered saline (PBS) and incubated with 400 μL per dish of RIPA buffer (ThermoScientific, Waltham MA USA) and 40 μL of a protease inhibitor cocktail (Complete Mini protease inhibitor cocktail, Roche, Germany) in ice for 10 min according to the manufacturer's recommendations. The cells were scrapped and collected in a 1.5 mL Eppendorf tube, centrifuged at 1000*g* 4 °C for 5 min, and the liquid phase was transferred to a new tube.

The relative concentration of the anti-oxidant defense system enzymes was measured with a Milliplex® assay (EMD Millipore Corp, Billerica MA USA) for the Luminex MAGPIX® platform (Luminex Corp, Austin TX USA). The results were analyzed with the xPONENT® software (Luminex Corp, Austin TX USA) and expressed as arbitrary fluorescence units. The measurement was performed with samples obtained from three independent experiments in triplicates, standardized by total protein content.

2.4. Zymographic evaluation of gelatinolytic activity

To assess the direct inhibitory effect of Ilomastat on MMP activity, 20 μL of supernatants from each experimental condition were

separated by electrophoresis in 0.75 mm width 10% polyacrylamide gels copolymerized with 1 mg/mL of gelatin in denaturizing non-reducing conditions. The gels were sequentially washed in 2.5% Triton X-100 (US Biological, USA) and deionized distilled water for 20 min. Afterwards, each gel was incubated in buffer 20 mMTrispH 7.4 and 5 mM $CaCl_2$ at37 °C for 17 h; washed in deionized distilled water, stained with Coomassie Brillant Blue® R-250 (ThermoScientific, USA) and distained with 70% (v/v) methanol, 10% (v/v) acetic acid. Gelatinolytic activity was evidenced as white bands in a blue background. The gels were photographed in standardized conditions in a Carestream Gel Logic 220 pro™ imaging system (Carestream, USA) and densitometric analysis was performed in the Carestream MI software® (Carestream, USA). Results were expressed in arbitrary units.

2.5. Scratch wound healing assay

To assess the functional effect of H_2O_2 stimulation and MMP inhibition in hPDLF cell migration we performed a scratch wound healing assay. We produced a longitudinal scratch with a sterile pipette tip across the larger diameter of confluent hPDLF cultures in 35 mm Petri dishes at passage 5. The cultures were then washed three times with PBS to eliminate all debris and detached cells and incubated in serum-free culture medium for 24 h. Experimental groups were stimulated with 5 μM H_2O_2 and/or 100 μM Ilomastat. Positive control cultures were supplemented with 10% FBS. The cultures were photographed in three standardized positions at baseline and at 24 h with a digital camera (Carl Zeiss AxioCam ERc5S, Germany) mounted in an inverted phase contrast microscope (Carl Zeiss Primo Vert, Germany). The standardized photographs were digitally subtracted in an image editing software (Adobe Photoshop Elements 8™, USA) and migrating hPDLF reaching the central quadrant of the scratch were accounted for.

2.6. Secreted cytokine levels

Supernatants standardized by total protein content of the H_2O_2-stimulated cultures and their controls, with and without Ilomastat were analyzed for the levels of SDF-1/CXCL12, IL-6, VEGF, ENA-78 and IL-8/CXCL8 with a Milliplex® assay (EMD Millipore Corp, Billerica MA USA) for the Luminex MAGPIX® platform (Luminex Corp, Austin TX USA). The results were analyzed with the xPONENT® software (Luminex Corp, Austin TX USA) and expressed as pg/mL in accordance to the linear interpolation to the assay's standard curve. The measurement was performed with samples obtained from three independent experiments in triplicates.

2.7. Statistical analysis

Normal distribution of data was tested by Shapiro–Wilk test. Statistical differences among groups were tested by one-way ANOVA and *post hoc* Bonferroni or Kruskall–Wallis and *post hoc* Dunn's test, according to data distribution. Tests were performed with Stata 11 (StataCorp, College Station TX USA) or GraphPad Prism 5 (GraphPad Inc. La Jolla CA USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. H_2O_2 at 5 μM elicited the enzymatic anti-oxidant defense system in PDLF

H_2O_2 stimulation significantly increased the relative levels of the anti-oxidant defense system enzymes catalase ($p = 0.018$), SOD1 ($p = 0.004$) and SOD2 ($p = 0.001$), demonstrating a shift in the intracellular redox state that triggered an enzymatic defensive response. On the other hand, TRX1 and SRX2 relative levels remained mostly unaffected (Fig. 1). Ilomastat had no measurable effect in the relative levels of any of the anti-oxidant defense system enzymes.

3.2. Ilomastat abrogated H_2O_2-induced MMP gelatinolytic activity

The gelatinolytic activity of proMMP-9 was significantly upregulated by the H_2O_2 stimulation protocol ($p < 0.001$), while Ilomastat completely reversed the peroxide-induced effect, resulting in proMMP-9 activity equivalent to controls. Similarly, the gelatinolytic activity of proMMP-2 was significantly stimulated by H_2O_2 ($p < 0.001$), while Ilomastat significantly inhibited the proMMP-2 activity even below control levels ($p < 0.001$). Further, active MMP-2 (aMMP-2) gelatinolytic activity was significantly stimulated by H_2O_2 and Ilomastat reversed its activity to control levels ($p < 0.001$) (Fig. 2).

3.3. Ilomastat abrogated H_2O_2-induced and basal hPDLF migratory capacity

In the functional wound healing scratch assay the peroxide stimulation protocol generated a boost in the migratory capacity of hPDLF, significantly increasing the number of fibroblasts that migrated to the central quadrant of the scratch, while Ilomastat reversed the stimulatory effect of the peroxide to control levels ($p < 0.001$). In the control group (10%FBS), Ilomastat significantly reduced the migratory capacity of hPDLF ($p < 0.05$) (Fig. 3).

3.4. Ilomastat reduced H_2O_2-induced soluble levels of SDF-1 and VEGF and increased IL-6 and VEGF in non H_2O_2-stimulated PDLF

The H_2O_2 stimulation protocol significantly augmented SDF-1/CXCL12 levels in hPDLF supernatants ($p < 0.05$). Pharmacological MMP inhibition by Ilomastat in H_2O_2-stimulated cultures resulted in a reduction of soluble SDF-1/CXCL12 to levels equivalent with non-stimulated cultures ($p < 0.05$). IL-6 levels increased significantly in the peroxide-stimulated cultures ($p < 0.01$), whereas the addition of Ilomastat resulted in an increase of IL-6 levels, both in the stimulated and non-stimulated cultures, although significant only for the non-stimulated group ($p < 0.05$). In the latter case, MMP inhibition by Ilomastat augmented IL-6 to a level comparable to H_2O_2 group. Further, VEGF levels were significantly augmented by the peroxide treatment, while Ilomastat significantly increased VEGF levels in the control cultures and significantly reduced them in the stimulated group ($p < 0.01$) (Fig. 4).

On the other hand, neither IL-8 nor ENA78/CXCL5 levels were affected by the peroxide treatment or by the addition of Ilomastat to the culture system. It is noteworthy that even though all the samples were positively quantified for IL-8 and ENA78/CXCL5, their levels were slightly under the assay's detection limit as established by the manufacturer (30 and 3 pg/mL, respectively) (Fig. 4).

4. Discussion

Here we present the first study to investigate the interaction between redox-induced MMPs and secreted pro-inflammatory cytokines/chemokines in hPDLF. Additionally, we assessed the effect of sublethal doses of H_2O_2 and MMP inhibition in the enzymatic anti-oxidant defense system and in the migratory capacity of hPDLF.

We established that our stimulation protocol with a single sublethal dose of 5 μM H_2O_2 triggered a significant increase in intracellular levels of catalase, SOD1 and SOD2. Our results are in line

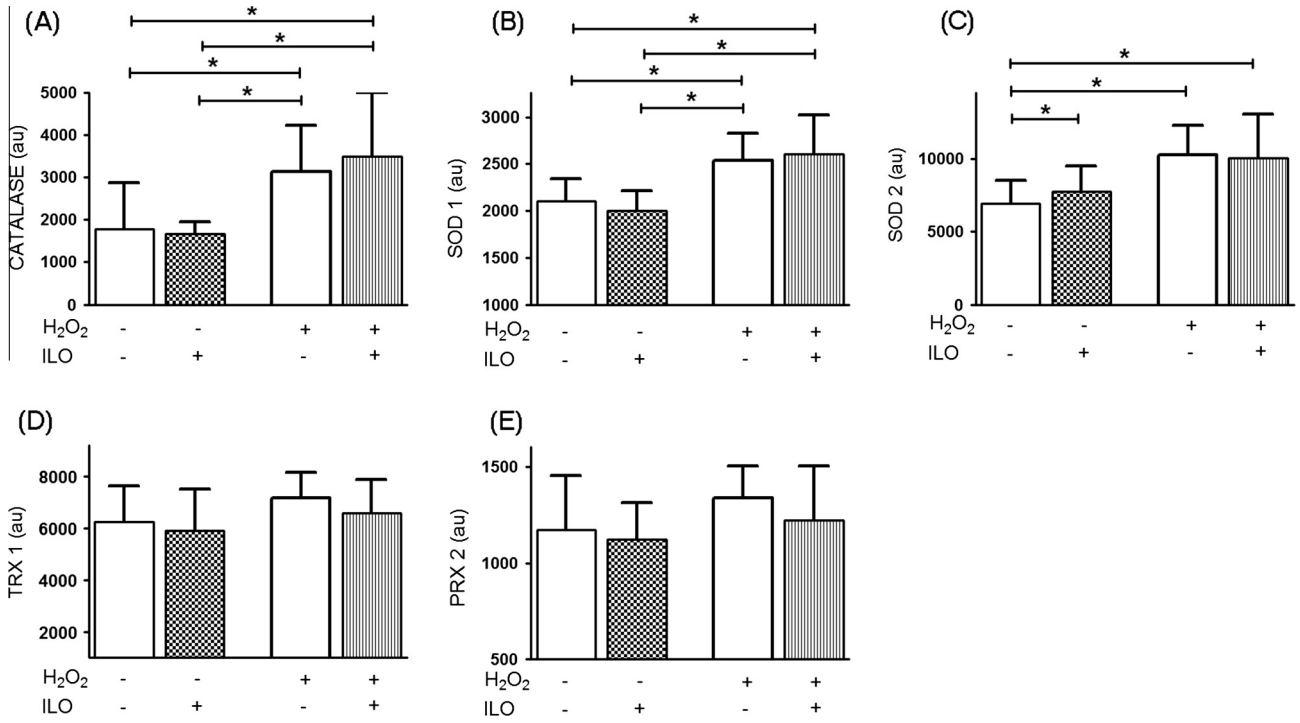

Fig. 1. Relative levels of anti-oxidant defense system enzymes in hPDLF under H_2O_2 stimulation and/or MMP inhibition. (A) Catalase; (B) SOD1; (C) SOD2; (D) TRX1; (E) PRX2. Brackets/asterisks represent statistical difference between groups ($p < 0.05$). au = arbitrary units.

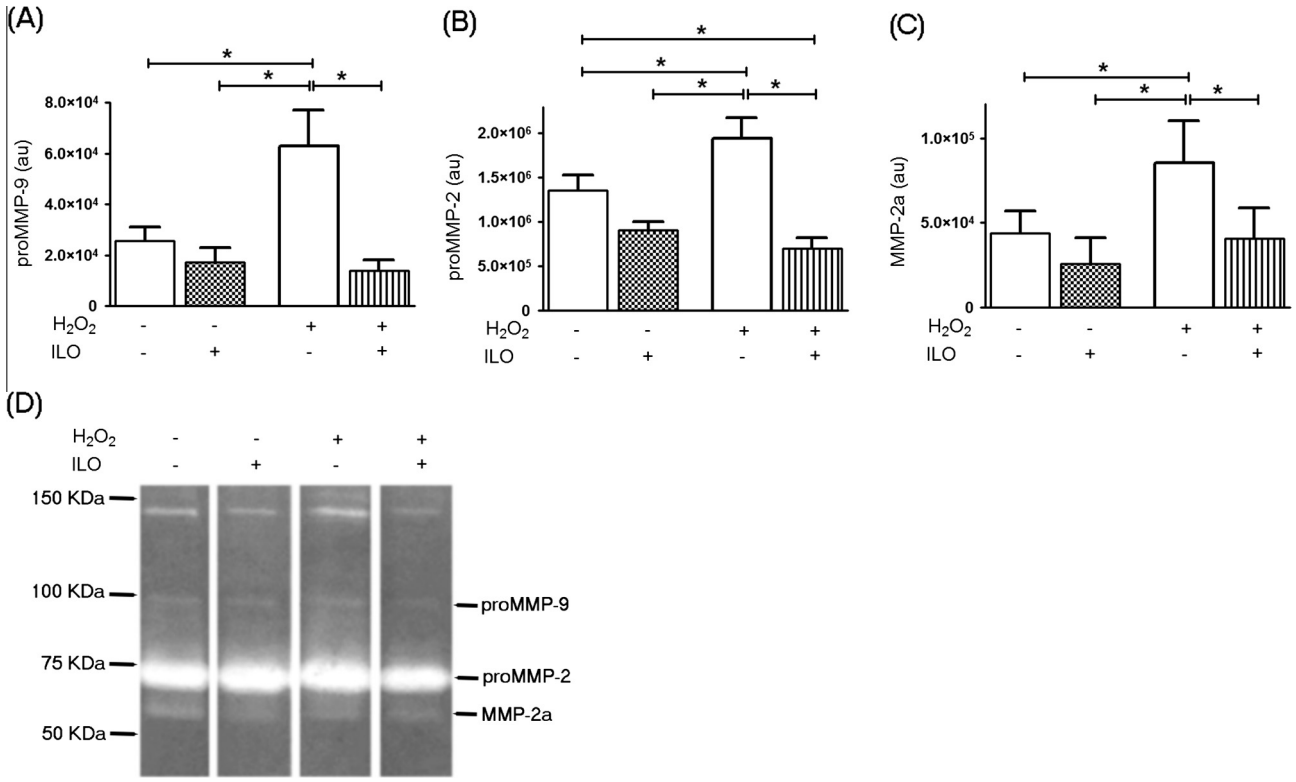

Fig. 2. Gelatinolytic activity of MMPs in PDLF under H_2O_2 stimulation and/or MMP inhibition. Densitometric analysis of (A) proMMP-9; (B) proMMP-2; (C) aMMP-2. (D) Representative figure showing the identified gelatinolytic bands under the different conditions. Brackets/asterisks represent statistical difference between groups ($p < 0.05$). au = arbitrary units.

with previous reports demonstrating that skin fibroblasts lacking SOD1 suffer from extensive free radical-induced damage, impaired proliferation and induced apoptosis [18], confirming the essential role of the enzymatic anti-oxidant defense system in intracellular redox equilibrium. It is noteworthy that the H_2O_2 concentration selected for our stimulation protocol in within the physiologic

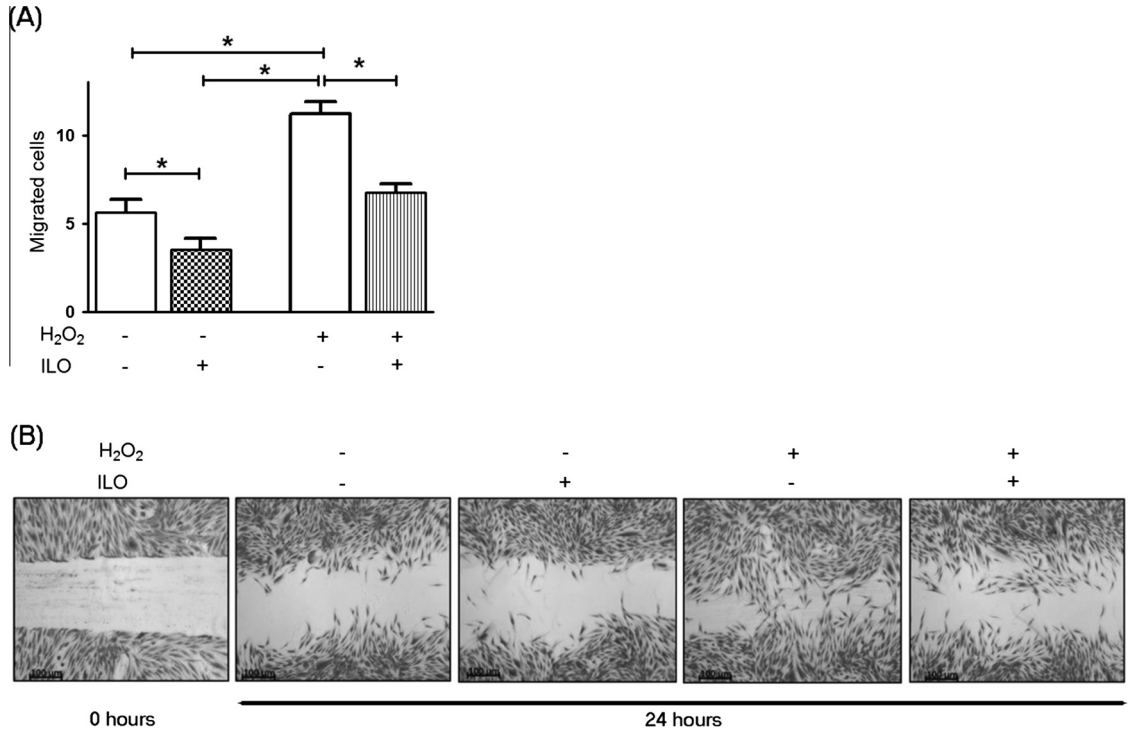

Fig. 3. Migratory capacity hPDLF under H_2O_2 stimulation and/or MMP inhibition. (A) Absolute number of hPDLF migrated to the central quadrant of the scratch wound. (B) Representative figure of the migratory response pattern under the different conditions. Effect Brackets/asterisks represent statistical difference between groups ($p < 0.05$).

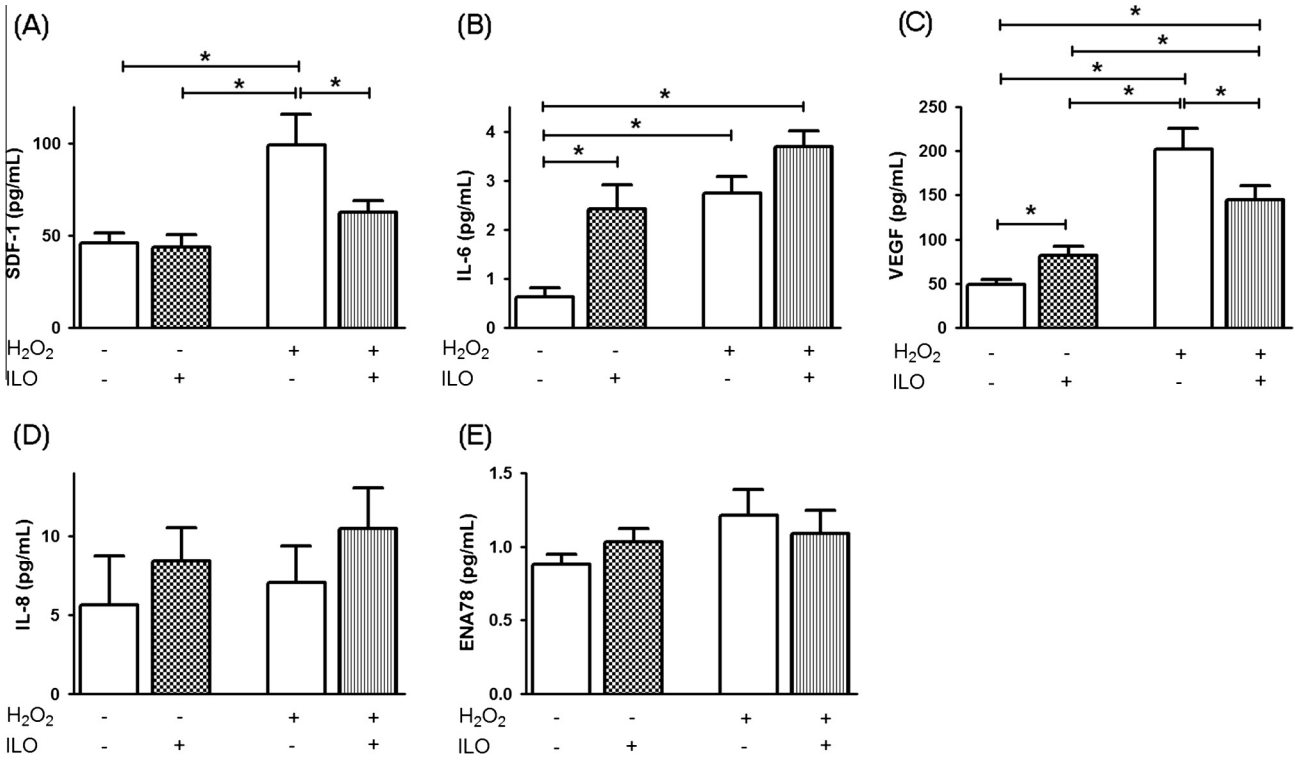

Fig. 4. Supernatant levels of cytokines/chemokines in H_2O_2-stimulated hPDLF cultures and controls with/without MMP inhibition. (A) SDF-1/CXCL12; (B) IL-6; (C) VEGF; (D) IL-8; (E) ENA-78/CXCL5. Brackets/asterisks represent statistical difference between groups ($p < 0.05$). Note the scale differences due to the broad range of cytokine-specific biological activity.

range reported in inflammatory apical periodontitis in contrast to most reports that range within mM concentrations [19–21], supporting that the cellular responses observed in our *in vitro* model might be similar to those for periodontal inflammation *in vivo*. Interestingly, in addition to their primary protective activity, anti-oxidant enzymes can be involved in cell signal transduction.

The oxidative state of anti-oxidant enzymes is a candidate pathway for intracellular signaling, and anti-oxidant enzymes are putative linking points acting as H_2O_2 sensors in the internal cell environment [22].

The up regulation of MMPs as a consequence of H_2O_2 stimulation has been demonstrated in other experimental cell models, such as HT1080 fibrosarcoma cell line [9] and mouse mammary epithelial cells (NMuMG) [7]. Collectively, the evidence suggests that the exposure to sublethal doses of H_2O_2 directly upregulates the expression and activity of MMP genes and pro-inflammatory pathways, which in turn favor MMP transcription and activation. Previous results from our laboratory demonstrated that similar sub lethal doses of H_2O_2 (2.5–5 μM) induced intracellular Ca^{2+} signaling and NFκB p65 subunit nuclear translocation in the same hPDLF model, which was inhibited by catalase, Ca^{2+} chelation and the specific NFκB inhibitory peptide SN50. In our model, peroxide induced a significant increase in MMP activity/activation rate that was abrogated by the inhibition of NFκB with SN50. Conversely, H_2O_2 at 10 μM did not induce p65 nuclear translocation, but its perinuclear accumulation, most probably representing an early sign of cell toxicity [23]. These previous results confirm that 5 μM H_2O_2 in our model is capable of activating NFκB, partially via intracellular calcium signals, inducing an increase in gelatinolytic activity, in agreement with previous reports in experimental cell models other than PDLF. This enables p65 subunit nuclear translocation facilitating its binding to the promoters of oxidizing enzymes, such as MMPs [7,9], and pro-inflammatory cytokine genes [24,25]. On the other hand, evidence also suggests that ROS can directly activate proMMP-2 and proMMP-9 [26,27]. Clinical studies have repeatedly reported increases in MMP active forms and/or activity during apical and marginal periodontal disease progression in association with ROS, suggesting that oxidative stress and MMP-mediated proteolysis might thus be cooperative in promoting the progression of periodontitis [28–31].

As a direct consequence of the H_2O_2-induced MMP upregulation, we demonstrated an induction of hPDLF's migratory capacity. In this particular model, the wound closure requires the coordinated action of matrix degrading enzymes (MMPs) to liberate the fibroblasts from their focal adhesion points, enabling them to "crawl" up to the central quadrant [32]. This MMP-dependency of cell migration has been previously demonstrated in human epithelial cells, were the treatment of the culture system with an anti-MMP-9 antibody significantly reduced cell migration [33,34]. Our results support the notion that oxidative stress exerts potent stimulatory effects in hPDLF that can be evidenced functionally. Interestingly, and further supporting the role of MMPs in cell migration, the enzymatic inhibition with Ilomastat totally reversed the stimulatory effect of H_2O_2, demonstrating a direct link between MMP levels and activity and the migration of the hPDLF in our scratch wound healing model. A similar response has been previously reported in human corneal epithelial cells [35,36] and rat cardiac fibroblasts [37], where the addition of Ilomastat to the culture system reversed the migratory stimulatory effect of HGF and IL-1β/TNF-α, respectively. In an analog fashion, it has been reported that Ilomastat reduces the contraction of airway smooth muscle cells seeded in collagen gels [38]. All these effects are mediated by the capacity of Ilomastat to inactivate MMPs by reversibly binding the Zn^{2+}-dependent active site [39]. Since the hPDLF were serum-starved during the assay it can be safely assumed that a great proportion of the cells were arrested in the G1 phase of the cell cycle [40]. Nevertheless, the possibility that cell proliferation might have contributed to the wound closure to a lesser extent cannot be totally ruled out.

The hPDLF stimulated with H_2O_2 demonstrated increased levels of soluble SDF-1/CXCL12, IL-6 and VEGF. It is widely accepted that H_2O_2 at micro molar concentrations can act as a second messenger, triggering inflammatory responses [41]. The recognition in the early 1990's of NFκB as a redox-sensitive transcription factor provided a direct molecular link between oxidative imbalance and inflammation [42]. Accordingly, the NFκB-induced over expression of pro-inflammatory cytokines and chemokines has widely been demonstrated in cultured fibroblasts [24,25,43,44]. In this regard, our results provide supportive new evidence of a regulatory axis linking oxidative stress-induced inflammation, MMPs and secreted cytokine/chemokines in cultured hPDLF.

For its part, Ilomastat produced cytokine-specific effects. MMP-inhibition in the unstimulated cultures significantly increased the secreted levels of both IL-6 and VEGF, but exerted no significant

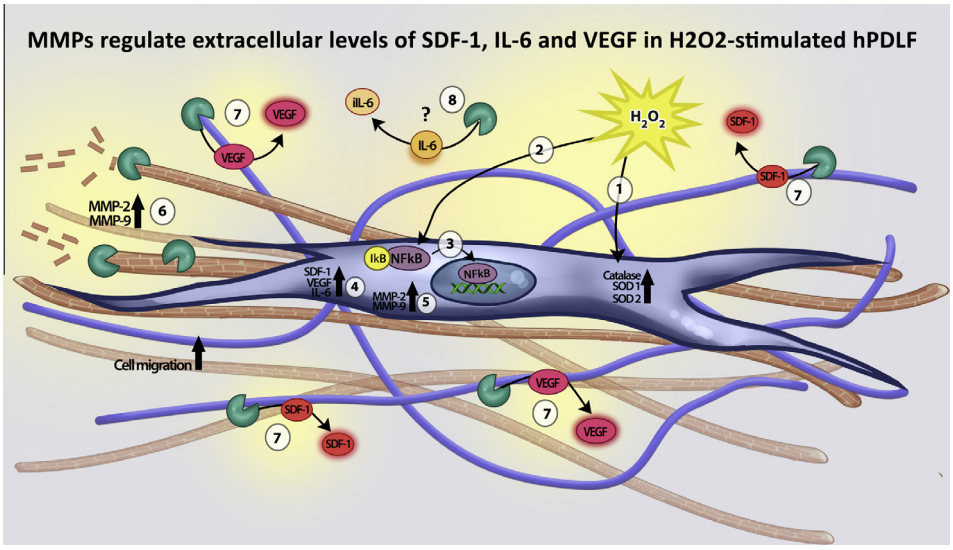

Fig. 5. Schematic representation of the proposed regulatory effect of MMPs over extracellular cytokine levels in H_2O_2-stimulated hPDLF. (1) Hydrogen peroxide triggers the activation of the enzymatic anti-oxidant defense system. (2) Redox sensitive transcription factors (such as NFκB) activates in response to intracellular changes in the redox state. (3) After its nuclear translocation, pro inflammatory transcription factors trigger the transcription of inflammatory cytokines and MMPs. (4) Oxidant stimulus increases the production of SDF-1/CXCL12, IL-6 and VEGF. (5) Oxidant stimulus increases the production of MMP-2 and MMP-9. (6) Increased levels/activity of MMPs stimulates collagen degradation, upregulating hPDLF migratory capacity. (7) Increased levels/activity of MMPs liberates SDF-1/CXCL12 and VEGF from their reservoirs in the ECM, augmenting their soluble levels. (8) Increased levels/activity of MMPs diminishes the soluble levels of IL-6.

effect on SDF-1/CXCL12 levels; while in the stimulated cultures, Ilomastat significantly diminished SDF-1/CXCL12 and VEGF levels; and increased IL-6 levels. The effect of MMPs on the secreted levels of cytokines could be explained by their capacity to cleave and process these bioactive substrates or to release them either from the cell surface or from their cryptic form in extracellular matrix reservoirs [45–47].

The ambiguous effect of Ilomastat over VEGF secreted levels is intriguing and requires further investigation to fully disclose its underlying mechanism. One possible explanation is that in steady-state conditions VEGF is secreted in low quantities and the majority of the molecules remain in a free unconjugated state in the supernatant; while in stimulated conditions the dramatic augment in VEGF secreted level increases the conjugation of VEGF with matrix proteins, from where they can only be liberated to soluble state by MMPs activity [48,49]. In this scenario, soluble/detectable VEGF is the net result of redox-induced transcriptional up regulation and MMP-dependent VEGF liberation from matrix reservoirs.

It is noteworthy that the absolute concentration of the assayed cytokines varied greatly (from the 1–2 pg/mL range for ENA-78 to the 50–250 pg/mL range for VEGF), reflecting one of the defining properties of these signaling molecules, which is the cytokine-specific biological activity dependent on their physical–chemical properties, half-life, bio availability, receptor affinity, affinity for ECM components, etc. These widely varying ranges of concentration have been extensively described in cell culture supernatants [50], gingival crevicular fluid [51], blood serum [52], and other biological samples [53].

In Fig. 5 we provide a schematic representation of our main results contextualized in the proposed mechanism of MMP-dependent regulation of cell migration and cytokine secretion in peroxide-stimulated hPDLF. This representation aims not to offer a complete picture of the ubiquitous cell regulatory functions of MMP, but to serve as a sketch of the complex and multifarious factors in which they contribute to cell function and tissue homeostasis.

Overall, we demonstrated that ROS increase the activity/levels of gelatinolytic MMPs, stimulate cell migration and cytokine secretion in hPDLF. Additionally, we proved that MMPs possesses immune modulatory capacity, regulating the secreted levels of cytokines in ROS-stimulated hPDLF cultures. This evidence provides further insight in the molecular pathogenesis of periodontitis, contributing to the future development of more effective therapies.

5. Conclusions

We investigated the effect of sublethal H_2O_2 doses on the migratory capacity, cytokine secretion pattern, MMPs activity/levels and anti-oxidant enzymatic defense system activity of hPDLF. We demonstrated that the migratory capacity of hPDLF is upregulated in pro-oxidant condition in a MMP-dependent manner, since MMP inhibition significantly affected the capacity of the cells to heal the wound in our *in vitro* scratch model.

We also demonstrated that SDF-1/CXCL12, IL-6 and VEGF levels are up regulated in pro-oxidant conditions, highlighting the importance of the redox state in the modulation of hPDLF responses. Further, we proved that MMP levels and activity are subjected to redox regulation, since hydrogen peroxide stimulation significantly increased MMP gelatinolytic activity.

Additionally, we provided suggestive evidence that the levels and activity of MMPs exert an influence in secreted levels of SDF-1/CXCL12, IL-6 and VEGF. This could be a putative enzymatic regulatory mechanism in inflammation. Further research including the selective blockade of pro-inflammatory pathways (such as NFκB) is required to elucidate the exact molecular mechanisms interrelating redox-state, cytokines and MMPs. The unveiling of the underlying molecular pathways regulating inflammatory destruction of periodontal tissue during the progression of periodontitis could provide new and refined therapeutic approaches to prevent tooth loss.

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The authors declare that they have no competing interests.

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