ORIGINAL ARTICLE

Effects of Platelet-derived Growth Factor-bb on Tissue Inhibitor of Metalloproteinase-1 Production in Human Gingival-derived Fibroblasts

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Synopsis

Matrix metalloproteinases degrade the extracellular matrix during remodeling, and their activity is regulated by tissue metalloproteinase inhibitors (TIMPs). Platelet-derived growth factor (PDGF) is produced in periodontal tissue and is an important regulator of wound healing and tissue repair. ERK1/2, members of the MAPK family, are intracellular signaling substance kinases involved in TIMP-1 production. To elucidate the mechanisms underlying connective tissue remodeling, the present study investigated the relationship between PDGF-bb-stimulated TIMP-1 production and ERK1/2 phosphorylation in human gingival fibroblasts (HGF). TIMP-1 production increased in a PDGF-bb concentration-dependent manner. PDGF-bb-induced increases in TIMP-1 were further enhanced by the p38α/β inhibitor SB239063 and p38α-siRNA. The PDGF-bb stimulation also induced the phosphorylation of ERK1/2. In contrast, the treatment with the p38-MAPK inhibitor SB203580 suppressed the phosphorylation of ERK1/2. These results suggest that PDGF-bb induced the production of TIMP-1 through the ERK1/2-activated signal transduction pathway in HGF. In addition, signaling cross-talk between p38α and ERK1/2 was involved in ERK1/2 activation, suggesting that ERK1/2 activation is coordinately regulated by p38α.

Key words*: PDGF-bb, TIMP-1, human gingival fibroblasts*

Introduction

Periodontal tissue remodeling is a very important process in the treatment of periodontal nflammation. Among periodontal tissues, the regenerative capacity of the gums is excellent. Although the gums are subjected to constant stress-induced stimuli (bacterial infection, mechanical forces, and physical and chemical injuries), resident cells (e.g. gingival fibroblasts) ensure the continuous repair of gingival tissue without scar formation [1,2]. Therefore, gingival fibroblasts are cells with a superior remodeling capacity in the gingiva. Collagen is the main component of gingival connective tissue, and gingival fibroblasts play a major role in remodeling.

The extracellular matrix (ECM) of the gingiva is composed of collagen fibers and is degraded by matrix metalloproteinases (MMPs) [3]. MMPs are proteases that play an important role in ECM remodeling. They are secreted as inactive pro-enzymes, which may be activated by proteolytic processing in the ECM [4]. Tissue metalloproteinase inhibitors (TIMPs) are endogenous inhibitors of MMPs and regulate their activities [5]. Interactions between MMPs and TIMPs play a crucial role in the physiological remodeling of the periodontium [6]. The balance between MMP-1 and TIMP-1 is considered to be important for maintaining tissue homeostasis. TIMPs are produced by the same types of cells that secrete MMPs, such as fibroblasts, epithelial

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cells, macrophages, and neutrophils [7, 8]. Gingival fibroblasts were previously shown to increase the release of TIMP-1 [9,10]. Platelet-derived growth factor (PDGF) is released by platelets, fibroblasts, endothelial cells, macrophages, and epithelial cells in the inflamed periodontium [11].

PDGF, a major serum polypeptide growth factor, is stored in platelets and is released during blood clotting [12]. It mediates normal tissue repair processes and stimulates wound healing in soft tissues [12]. PDGF is a cytokine with five isotypes (PDGF-aa, -bb, -ab, -cc, and -dd) that activate downstream PDGF receptor α or β in a paracrine or autocrine manner under physiological or pathological conditions [13]. PDGF-bb, a member of the PDGF family that was originally identified in platelets, has been recognized as a key regulator in wound healing and tissue repair [14]. PDGF-bb binds to all types of PDGF receptors.

A PDGF-bb stimulation of human osteoblasts was previously shown to affect the activation of ERK [15]. PDGF-bb binds to all types of PDGF receptors. A PDGF-bb stimulation of rat fibroblasts induced changes in MMP-1 expression and p38 activation. p38 is a subfamily of MAP kinases that plays roles in cell growth, prostanoid synthesis, and the production of MMPs [16]. We previously reported the involvement of p38 activation in MMP-1 production in PDGF-bb-stimulated human gingival fibroblasts (HGF) [17]. However, the relationships between a PDGF-bb stimulation and the expression of TIMP-1 and ERK 1/2 activity in HGF remain unclear.

In the present study, Western blotting was performed to confirm the effects of PDGF-bb on TIMP-1 production. Western blotting was also conducted using the p38α/β inhibitor SB203580 and p38α-small interfering RNA (siRNA) to examine the effects of p38α on TIMP-1 production. Furthermore, we investigated the phosphorylation of ERK1/2 using Western blotting to confirm signaling cross-talk between p38α and ERK1/2.

Materials and Methods

1. Cell culture

HGF were grown from explants of the healthy marginal gingiva of healthy donors (regardless of age, gender, or tooth type). Primary cultures were grown in α-modified Minimum Essential Medium (α-MEM; FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (Cosmo Bio, Tokyo, Japan), 2 mM L-glutamine (FUJIFILM Wako PureChemical), 100 μg/mL penicillin (FUJIFILM Wako Pure Chemical), and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemical) at 37°C in an atmosphere of 5% CO_2-95% air. The first subcultures were obtained 20 to 30 days later, maintained at 37°C in an atmosphere of 5% CO_2-95% air, and routinely subcultured after the addition of trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA·4Na (Nacalai Tesque, Kyoto, Japan)) for cell release. Experiments with HGF were performed between passages 3 and 10. The present study was approved by the Ethical Review Board of Osaka Dental University (Approval No.110326). Informed consent was obtained from all participants and the study was conducted according to the principles of the Declaration of Helsinki.

2. Reagent

Recombinant human PDGF-bb was purchased from R&D System, MN, USA. Selective p38 MAPK inhibitor SB203580 and potent and selective p38 MAPKα/β inhibitor 239063 were obtained from Merck KGaA, Darmstadt, Germany.

3. Western blot analysis of TIMP-1

HGF were seeded on a 12-well plate at a density of 1.0×10^5 cells/well and incubated in serum-free α-MEM containing PDGF-bb (0, 1, 10, 20, 50, or 100 ng/mL) for 24 hours. Western blotting was performed to investigate the production of TIMP-1 in HGF upon a stimulation with PDGF-bb. To investigate the effects of p38α/β on TIMP-1 production in HGF, we treated HGF with the potent and selective p38α/β inhibitor SB239063 and performed Western blotting. Cells were preincubated with SB239063 for 1 hour before the PDGF-bb stimulation. To confirm the effects of p38α on TIMP1 production induced by the PDGF-bb stimulation, p38α expression in HGF was knocked down by siRNA transfection. Conditioned medium was collected, centrifuged to remove debris, concentrated up to 30-fold with Amicon Ultra (Merck KGaA), and proteins were visualized by Western blotting. Equal amounts of each sample were separated on 8% SDS/PAGE. Following their separation, proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Merck KGaA) and blocked with 10% Blocking One (Nacalai) in 50 mM Tris–HCl at pH 7.5 and 150 mM NaCl overnight. Mem-

branes were treated with TIMP-1 antibodies (Cell Signaling Technology, Danvers, MA, USA) used at a 1:1000 dilution at room temperature for 1 hour. A horseradish peroxidase (HRP)-conjugated secondary antibody, goat anti-rabbit IgG-HRP (Jackson ImmunoResearch, West Grove, PA, USA), was used at a 1:2000 dilution and the chemiluminescent reagent, Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA) was employed to visualize immunoreactive bands. Blot images were acquired using ChemiDoc MP (Bio-Rad, Hercules, CA, USA).

4. p38α siRNA transfection

SignalSilence Control siRNA and p38α siRNA were purchased from Thermo Fisher Scientific, Waltham, MA, USA. SignalSilence Control siRNA (non-silencing siRNA, 30 pM) and p38α siRNA (30 pM) were transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. SignalSilence Control siRNA was used as a negative control. Twenty-four hours after transfection, cells were used in experiments. To confirm the effects of p38α on TIMP1 production induced by the PDGF-bb stimulation, p38α expression in HGF was knocked down by siRNA transfection.

5. Western blot analysis of ERK1/2

Various MAPK pathways may be co-activated by a single stimulus [18]. Therefore, we investigated the effects of p38 on ERK1/2 phosphorylation in PDGF-bb-stimulated HGF. After a pretreatment with various concentrations of the p38 MAPK inhibitor SB203580 for 1 hour, HGF were stimulated with PDGF-bb for 30 minutes. After the stimulation, samples were prepared by separating total protein in sample buffer (containing 0.25 M Tris HCl pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue, and 4% 2-mercaptoethanol) supplemented with Halt protease and a phosphatase inhibitor cocktail (Thermo Fisher Scientific). Western blotting was performed in the same manner as TIMP-1. Western blotting with PVDF was similarly performed using an anti-phospho ERK1/2 antibody (Cell Signaling Technology) at 1000:1. Both membranes were treated with secondary antibodies and phosphorylated bands were visualized using ChemiDoc MP. The same membrane was stripped and reprobed with an anti-ERK1/2 antibody (Cell Signaling Technology) at 1:1000. Blot images were acquired using ChemiDoc MP.

Results

1. The PDGF-bb stimulation promotes TIMP-1 production in HGF.

PDGF-bb increased the production of TIMP-1 in a dose-dependent manner (Fig. 1A). The dose-dependent production of TIMP-1 was observed in HGF cultured in the presence of 1, 10, 20, 50, and 100 ng/mL PDGF-bb, with a peak at 50 ng/mL PDGF-bb (Fig. 1A).

2. The p38 α/β inhibitor SB239063 promotes TIMP-1 production in PDGF-bb-stimulated HGF.

The PDGF-bb stimulation enhanced the production of TIMP-1. PDGF-bb-induced increases in TIMP-1 were further enhanced by the treatment with SB239063, potent and selective p38α/β inhibitor.

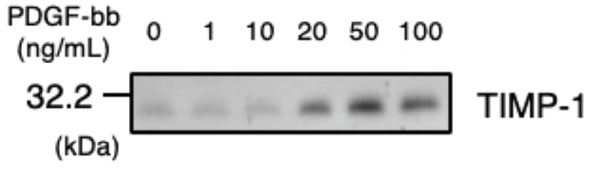

Fig. 1 PDGF-bb stimulation enhances TIMP-1 production in HGF

HGF (1×10^5cells/well) were incubated in a 12-well plate at 37°C for 24 hours. Cells were then incubated in serum-free α-MEM for 24 hours. After the incubation, HGF were cultured in serum-free α-MEM containing PDGF-bb (0, 1, 10, 20, 50, or 100 ng/mL) for 24 hours. Western blotting was performed to evaluate TIMP-1 production. The data presented are representative of four independent experiments. HGF, human gingival fibroblasts; α-MEM, alpha-modified Minimum Essential Medium; PDGF, platelet-derived growth factor; TIMP -1, tissue metalloproteinase inhibitor-1.

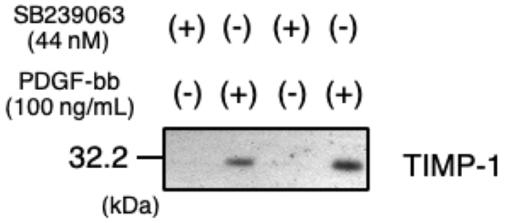

Fig. 2 The p38 α/β inhibitor SB239063 enhances TIMP-1 production in PDGF-bb-stimulated HGF.

HGF (1×10^5cells/well) were incubated in a 12-well plate at 37°C for 24 hours. Cells were then incubated in serum-free α-MEM for 24 hours.

After the incubation, HGF were pretreated with 44 nM SB239063 at 37°C for 1 hour before the incubation with PDGF-bb (100 ng/mL) for 24 hours. Western blotting was performed to evaluate TIMP-1 production. Data presented are representative of four independent experiments. HGF, human gingival fibroblasts; α-MEM, alpha-modified Minimum Essential Medium; PDGF, platelet-derived growth factor; TIMP -1, tissue metalloproteinase inhibitor-1

3. p38α siRNA HGF increases PDGF-bb-induced TIMP-1 production.

A stimulation with 50 ng/mL PDGF-bb confirmed that TIMP-1 production with p38α siRNA was greater than that with control non-silencing siRNA (Fig. 3, upper panel). The total amount of actin was not affected by the PDGF-bb stimulation or siRNA (Fig. 3, middle panel). p38α siRNA significantly reduced the protein level of p38α in HGF (Fig. 3, lower panel).

4. The p38 MAPK inhibitor SB203580 is involved in suppressing ERK1/2 phosphorylation.

ERK1/2 was constitutively phosphorylated even without the stimulation. ERK1/2 phosphorylation levels were slightly higher in PDGF-bb-stimulated HGF than in unstimulated HGF; however, ERK1/2 phosphorylation was suppressed by 20 μM SB203580 (Fig. 4, upper panel). To ensure that equal amounts of ERK1/2 were obtained from the lysates, membranes were stripped and reprobed with the anti-ERK1/2 antibody. The results obtained revealed that equal amounts of ERK1/2 were produced by lysates obtained from each sample (Fig. 4, lower panel).

Discussion

In the treatment of inflammation in periodontal tissue, it is important to pay attention to cellular or intracellular changes that occur in the periodontal ligament and alveolar bone. The main component of the periodontal ligament and gingival connective tissue is collagen, and its production and degradation (remodeling) are mainly performed by fibroblasts, which are present in large numbers in these tissues [19]. Since TIMPs regulate their enzymatic activity by binding to MMPs, TIMPs and MMPs cooperate to play an important role in remodeling [20]. Further studies are needed to elucidate the remodeling mechanism, and the findings obtained will contribute to the treatment and prevention of inflammation.

Gingival fibroblasts have been shown to release TIMP-1 [9, 10]. The present study investigated the effects of PDGF-bb on TIMP1 production in HGF. The results obtained showed that the PDGF-bb stimulation increased TIMP1 production. MMPs are a family of proteases that play an important role in ECM remodeling and are secreted as inactive pro-enzymes, which are activated by proteolytic processing in the

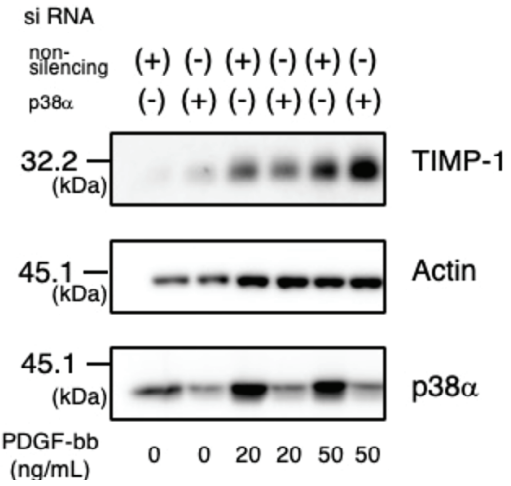

Fig. 3 p38α siRNA HGF increases PDGF-bb-stimulated TIMP-1 production.

The siRNA of p38 and non-silencing siRNA were transfected into HGF according to the manufacturer's instructions described in the Materials and Methods. HGF were cultured in serum-free a-MEM containing PDGF-bb (0, 20, or 50 ng/mL) for 24 hours. Western blotting was performed to evaluate TIMP-1 production (upper). The amounts of actin (middle) and p38a (lower) were evaluated by Western blotting. Data presented are representative of four independent experiments. siRNA, small interfering RNA; HGF, human gingival fibroblasts; α-MEM, alpha-modified Minimum Essential Medium; PDGF, platelet-derived growth factor; TIMP-1, tissue metalloproteinase inhibitor-1

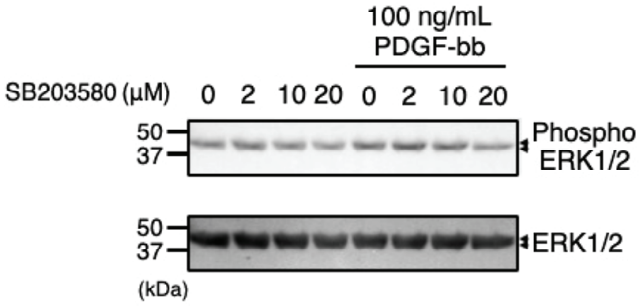

Fig. 4 The p38-MAPK inhibitor SB203580 is involved in suppressing ERK1/2 phosphorylation

HGF were pretreated with various concentrations of SB203580 at 37°C for 1 hour before being incubated with PDGF-bb (100 ng/mL) for 30 min. Cell lysate samples were prepared. The phosphorylation level of ERK1/2 was examined by Western blotting (upper). To ensure that equal amounts of ERK1/2 were obtained from lysates, membranes were stripped and reprobed with anti-ERK1/2 antibodies (lower). Four samples were analyzed and a typical image is shown for each. HGF, human gingival fibroblasts; PDGF, Platelet- derived growth factor

ECM [4]. TIMPs are endogenous inhibitors of MMPs and are produced by the same types of cells that secrete MMPs, such as macrophages, neutrophils, fibroblasts, keratinocytes, monocytes, and epithelial cells [7,8]. TIMPs and MMPs are essential for physiological remodeling of the periodontium [6]. We showed that TIMP-1 was spontaneously produced in unstimulated HGF and also that TIMP-1 production was enhanced in the presence of PDGF-bb. We previously reported that the PDGF-bb stimulation dose- dependently increased MMP-1 production in HGF [17]. Tissue remodeling is the result of a significant change in the local ECM [21] and modified fibroblast proliferation[22]. The amount of collagen in tissue remodeling is maintained by regulating the balance between TIMPs and MMPs. ECM turnover is characterized by a unique balance between the degradative activity of MMPs and its suppression by TIMP-1, a natural inhibitor of MMP-1.

Since the PDGF-bb-stimulated production of TIMP1 may play an important role in ECM remodeling, we investigated the intracellular signaling pathway of PDGF-bb-stimulated HGF for TIMP1 production. The activation of p38 MAPK is involved in TIMP1 production in human hepatic stellate cells [23]. p38 is a proline-directed serine/threonine protein kinase that transduces signals from an inflammatory stimulus[24]. The p38 MAPK family has four members, p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12/ERK-6), and p38δ (MAPK13/SAPK4), and the four isoforms share more than 60% homology [25, 26, 27]. The expression of p38 isoforms is dependent on tissue and cell types, suggesting that p38 isoforms exert different functions [28]. SB203580, a widely used pyridinylimidazole inhibitor of p38, is nearly equally potent against p38α and p38β, but does not inhibit p38γ or p38δ [28.29]. We showed that the p38 MAPK inhibitor SB203580 enhanced TIMP1 production in PDGF-bb-stimulated HGF cells. This result suggests the involvement of p38 in TIMP1 production. Among members of the p38 MAPK family, the pro-inflammatory effects of p38α have been extensively examined, whereas those of other isoforms remain unclear [25]. We herein showed that the transfection of p38α siRNA enhanced PDGF-bb-stimulated TIMP1 production (Fig. 3). These results suggest that the PDGF-bb-stimulated production of TIMP-1 in HGF was regulated by p38α MAP kinase signaling pathways.

To investigate the relationship between p38α inhibition and enhanced TIMP-1 production, we focused on ERK1/2. The concept that signaling cross-talk occurs between different MAPKs is now widely recognized [18]. Therefore, we performed Western blotting using SB203580, a p38-MAPK inhibitor, to examine the signaling cross-talk between p38α/β and ERK1/2. As shown in Fig. 4, the p38 MAPK inhibitor SB203580 suppressed the phosphorylation of ERK1/2 at a concentration of 20 μM. This suggests the involvement of signaling cross-talk between p38 and ERK1/2 in TIMP1 production in PDGF-bb-stimulated HGF cells. The present results demonstrated that p38α interferes with ERK1/2 in HGF. Lewthwaite *et al*. [30] reported that articular surface cells and osteoblasts cultured in the presence of SB202190 suppressed ERK1/2 phosphorylation. Moreover, the p38-dependent activation of ERK has been demonstrated in HEK293 and PC12 cell lines, supporting the complementary and reciprocal regulation of MEK-ERK signaling by p38[31]. The present results are consistent with these findings, suggesting signaling cross-talk between the p38α and ERK1/2 pathways in HGF, which plays a role in the regulation of PDGF-bb-induced TIMP1 production. The signaling events responsible for signaling cross-talk between the p38 and ERK1/2 pathways currently remain unknown. Although the mechanisms underlying the production of TIMP-1 from PDGF-bb-stimulated HGFs have not yet been characterized and currently remain unclear, the present results suggest that the induction of TIMP-1 is one of the key steps regulating matrix turnover in gingival tissue.

Conclusion

The present results indicate that PDGF-bb induced TIMP-1 production in HGF through the ERK1/2-activated signaling pathway. In addition, signaling cross-talk between p38α and ERK1/2 was involved in ERK1/2 activation, suggesting that ERK1/2 phosphorylation was coordinately regulated by p38α. The present study proposes a model in which TIMP-1, produced by HGF following a stimulation with PDGF-bb, plays a role in gingival tissue remodeling in inflamed periodontal tissue. Since TIMPs and MMPs form a complex network, further research is needed to clarify the remodeling control mechanism of the

PDGF-bb response in gingival epithelial cells. In the future, the combination of the present results with nanodrug delivery systems may lead to the development of drugs that are useful for treating oral inflammation.

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Conflict of Interest

The authors declare that they have no competing interests.

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