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# Enamel matrix derivative induces the expression of tissue inhibitor of matrix metalloproteinase-3 in human gingival fibroblasts via extracellular signal-regulated kinase

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*Background and Objective:* Periodontal disease is characterized by increased expression and activity of matrix metalloproteinases (MMPs) and insufficient expression/activity of their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). This altered MMP–TIMP balance results in progressive destruction of gingival and periodontal extracellular matrix. Enamel matrix derivative (EMD), clinically used for periodontal regeneration in a device called Emdogain®, has been suggested to enhance gingival healing following periodontal procedures in humans. We previously showed that EMD increases the proliferation of human and rat gingival fibroblasts and protects them from tumor necrosis factor-induced apoptosis. In the present study, the modulation of MMP and TIMP expression by EMD was investigated.

*Material and Methods:* Primary human gingival fibroblasts were treated *in vitro* with tumor necrosis factor, EMD or both in serum-free conditions, and RNA was analyzed with an extracellular matrix-focused microarray and quantitative real-time polymerase chain reaction.

*Results:* Microarray analysis showed detectable expression of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-13, as well as TIMP-1 and TIMP-3 in untreated cells. There was no apparent regulation of the expression of MMP-2, MMP-7, MMP-13 and TIMP-1 by either tumor necrosis factor or EMD. In contrast, tumor necrosis factor significantly increased MMP-1 expression, and EMD reduced it when both agents were present. Also, EMD significantly induced TIMP-3 expression, an effect which was dependent on activation of extracellular signal-regulated kinase 1/2, since it was totally abolished by a selective extracellular signal-regulated kinase pathway inhibitor.

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*Conclusion:* These data suggest that EMD may affect gingival health by ways other than cell proliferation/survival, i.e. by stimulation of TIMP-3 production, which could improve the MMP–TIMP balance in gingival tissue and curb extracellular matrix destruction.

Matrix metalloproteinases (MMPs) are a family of $Zn^{2+}$-dependent endopeptidases capable of cleaving various components of extracellular matrix (ECM), an activity which is essential for normal physiological processes, such as embryonic development, cell migration, wound healing and tissue remodeling (1,2). However, their increased, uncontrolled activity participates in many pathological conditions, such as tumor metastasis and inflammation-induced tissue destruction (3,4). Currently, there are about 25 MMPs, possessing various catalytic properties (e.g. collagenases, gelatinases and stromelysin). Matrix metalloproteinase activity is, in turn, regulated by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs; 5,6).

The balance between MMPs and TIMPs is tightly regulated during tissue remodeling and wound healing. While MMP activity is necessary at the first step of wound healing to allow the locomotion and arrival of cells and growth factors to the injured area, TIMP activity is essential at the late stage of tissue repair to minimize tissue damage (7,8).

Periodontal disease is caused by an inflammatory process, which results from the interactions between a bacterial insult and host responses. Cells of the gingival connective tissue are targets for both the bacterial virulence factors and the inflammatory mediators (9), both of which stimulate gingival fibroblasts to produce other inflammatory mediators, such as cytokines [e.g. tumor necrosis factor (TNF) and interleukin-1] and prostanoids (e.g. prostaglandin $E_2$). These, in turn, induce MMP production and activation, leading to extracellular matrix degradation (10,11). Indeed, substantial dissolution of the connective tissue occurs in inflamed gingival sites (12), presumably due to the excessive and continuous activity of various MMPs (10). In support of this notion, high levels of MMPs were found in inflamed periodontal tissues (13–18). Notably, the ratios between MMP-1 and TIMP-2, MMP-3 and TIMP-2, and MMP-1 and TIMP-3 expression was significantly elevated in periodontal lesions compared with healthy sites (19).

The ability of gingival fibroblasts to produce MMP-1 and MMP-3 in response to inflammatory cytokines, such as TNF and interleukin-1, has been demonstrated in several studies (20–22). Furthermore, exposure of gingival fibroblasts *in vitro* to *Porphyromonas gingivalis* resulted in increased expression of MMP-1, MMP-2, MMP-3 and MMP-4 and decreased expression of TIMP-1 but not TIMP-2 (14). Studies in which periodontal tissue destruction was experimentally prevented by treatment with doxycycline, a broad-spectrum MMP inhibitor, support a role of MMPs in this process (23).

Emdogain®, a commercial preparation of enamel matrix derivative (EMD), is being widely used clinically for regeneration of periodontal tissues, such as periodontal ligament, cementum and bone. In contrast, the effects of EMD on gingival tissues have not been well studied. Several clinical observations suggest that the application of EMD onto root surfaces during periodontal therapy has beneficial effects on gingival tissue. For instance, application of EMD during coronally positioned flap procedures for correction of gingival recession resulted in an increase in the width (24–26) and density (27) of the keratinized gingiva compared with control treatment. This finding could suggest an effect of EMD on cells of the gingival connective tissue, separate from their effect on periodontal ligament cells. We have recently shown that EMD enhances the proliferation of rat (28) and human gingival fibroblasts (29) and protects these cells from TNF-induced apoptosis (30). However, EMD could promote gingival post-surgical healing by other means as well. In this study, we investigated whether EMD could modulate the proteolytic activity of gingival fibroblasts. The only study that examined the effect of EMD on the balance between MMPs and TIMPs in gingival tissue showed a decrease in TIMP-1 level and a greater decease in MMP-1 and MMP-8 activity in human gingival crevicular fluid following surgical treatment with EMD (31). These changes implied a reduction in the MMP/TIMP ratio following treatment.

Therefore, the purpose of the present study was to asses the effect of EMD on the expression of various MMPs and TIMPs in gingival fibroblasts exposed to an inflammatory stimulus simulated by the ubiquitous pro-inflammatory cytokine, TNF.

## Material and methods

### Materials

Enamel matrix derivative was generously donated by Institut Straumann AG (Basel, Switzerland). All chemicals and reagents for tissue culture were from Biological Industries (Beit Haemek, Israel). Tissue culture dishes were from Nunc (Rosekilde, Denmark). Human recombinant TNF was obtained from PeproTech (Rocky Hill, NJ, USA), and U0126 [mitogen-activated protein kinase (MAPK) inhibitor] was from ALEXIS Biochemicals (Lausen, Switzerland).

### Cell isolation and culture

The experiments were approved by the Helsinki committee of the Tel-Aviv University. Healthy, non-inflamed, gingival tissue was removed from patients during periodontal or implant

surgery procedures upon obtaining an informed consent, whenever tissue 'thinning' or elimination was necessary. The epithelium was removed, and connective tissue fragments were cut into small pieces and placed in culture medium [α-minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 12.5 U/mL nystatin, 0.11 mg/mL sodium pyruvate, and non-essential amino acids] at 37°C in an atmosphere of 5% $CO_2$ and 95% air in 25 $mm^2$ flasks to allow for cell outgrowth until confluence was reached. About 750,000 cells between the fourth and eighth passage, having a typical fibroblastic morphology, were seeded in 60 mm culture dishes with complete medium until they attached and were then serum-starved for 24 h and then treated in serum-free conditions with EMD (100 μg/mL), TNF (50 ng/mL) or both for 20 h before harvesting total RNA.

### Isolation of RNA and determination of mRNA by real-time polymerase chain reaction (PCR)

Total RNA was isolated with the perfect pure RNA cultured cell kit (5Prime, Gaithersburg, MD, USA) according to the manufacturer's instructions. Cells from six donors, between the second and fourth passage, having a typical fibroblastic morphology, were used. Integrity of the RNA was assessed by visualization in ethidium bromide-stained gels, and its quantity was estimated with a nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only samples with a ratio of opitical density at 260 nm/280 nm > 1.8 were used. Total RNA (1 μg) was then reverse transcribed by EZ-First Strand cDNA synthesis kit (Biological Industries) using random hexamer primers according to the manufacturer's instructions. Transcribed cDNA was then amplified using TaqMan gene expression assay (Hs00899658-M1 for MMP-1,Hs00165949-M1 for TIMP-3 and Hs999999901-S1 for the endogenous control 18S ribosomal RNA, all from Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using a Prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Calculations of relative gene expression (normalized to 18S reference gene) were performed according to the $2^{-\Delta\Delta C_T}$ method (32).

### Gene expression analysis by ECM-specific microarray

For analysis of the expression of genes of the MMP and TIMP family members, oligo GEArray Q Series Human ECM and Adhesion Molecules gene expression arrays (OHS-013) were purchased from SuperArray Bioscience Corporation (Frederick, MD, USA). Each array consists of 113 genes known to be involved in cell adhesion and ECM deposition or degradation, as well as control sequences [*PUC18* plasmid DNA as negative control; β-actin and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for loading]. Specifically, it represents seventeen MMPs (1–3, 7–17, 20, 24 and 26) and three TIMPs (1–3). Gingival fibroblasts from two different donors were analyzed. The microarrays were used according to the manufacturer's instructions. Briefly, cDNA was prepared from total RNA, using the TrueLabeling-AMP™ kit (SuperArray Bioscience Corp.), by reverse transcription with MMLV reverse transcriptase. Complementary RNA (cRNA) transcripts, labeled with Biotin-16-dUTP (Roche, Indianapolis, IN, USA), were generated and purified using the ArrayGrade™ cRNA cleanup kit (SuperArray Bioscience Corp.), and the amount of cRNA was assessed by nanodrop spectrophotometer. Four micrograms of cRNA were hybridized to a positively charged nylon membrane containing the arrayed DNA. Hybridized RNA was detected using the supplied chemiluminescence detection system and BioMax light film (Kodak, Rochester, NY, USA). Photoshop (Adobe) software was used to determine the mean optical density of each band after subtracting that of the empty film. Gene expression was calculated as the ratio between each band and that of *GAPDH* within the same membrane.

### Statistical analysis

All real-time PCR assays were performed at least in quadruplicate for each set of conditions, and each experiment was repeated at least twice. The results are presented as means and standard deviation (SD). Statistical analysis was performed by Student's unpaired *t*-test, and the value of $p < 0.05$ was the cut-off for statistical significance.

## Results

Analysis of the microarrays revealed substantial expression of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-13, as well as TIMP-1 and TIMP-3 in serum-starved, untreated gingival fibroblasts in serum-free conditions (Fig. 1). Many additional ECM-related genes, such as collagens, laminins and integrins, were strongly detectable as well, but their analysis is outside the scope of the present study. Negative controls (e.g. PUC18) did not yield any detectable signal. Densitometric quantitation of the arrays indicated that among the MMP and TIMP members, MMP-2 and TIMP-1 were expressed at the highest levels, MMP-1, MMP-13 and TIMP-3 were expressed at intermediate levels, and MMP-3 and MMP-7 were expressed at much lower levels (Fig. 2). These MMPs and TIMPs were previously reported to be expressed by unstimulated human gingival fibroblasts (33–35). There was no apparent regulation of the expression of MMP-2, MMP-7, MMP-13 and TIMP-1 by either TNF or EMD, and these molecules were therefore not further analyzed. In contrast, MMP-1 was markedly induced by TNF and not by EMD, MMP-3 seemed to be induced by both treatments, and TIMP-3 was induced by EMD only. The expression of these genes was further analyzed by quantitative real-time PCR. As can be seen in Fig. 3, TNF treatment of human gingival fibroblasts significantly induced the expression of MMP-1 several-fold, while EMD alone had no effect. However, when cells were treated simultaneously with TNF and EMD, the MMP-1 mRNA levels were lower than with TNF alone. This effect of

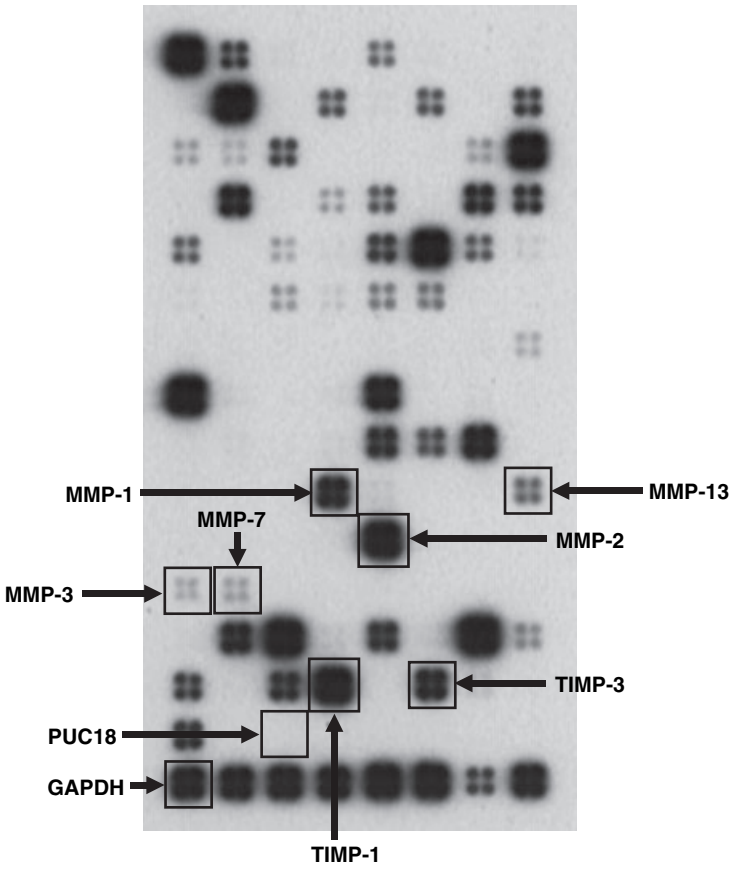


*Fig. 1.* Photograph of the microarray used in this study with the positions of the genes analyzed.

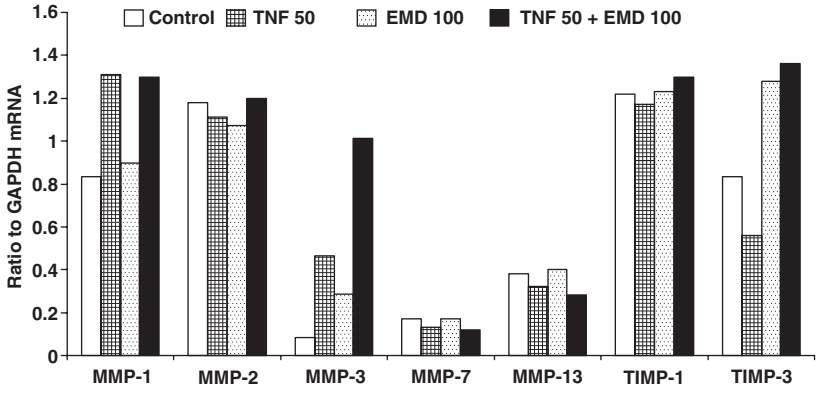


*Fig. 2.* Densitometric analysis of mRNA levels of the relevant array genes. The ratio between the optical density of each spot and that of *GAPDH*, a housekeeping gene, within the same array is depicted. TNF 50, TNF 50 ng/mL; EMD 100, EMD 100 μg/mL.

TNF on MMP-1 expression in gingival fibroblasts was previously reported by others (21,22,36). Repeated analysis of the expression of MMP-3 in response to these two agents did not yield reproducible results (not shown). In contrast, EMD significantly induced the expression of TIMP-3, while TNF had no effect (Fig. 4), so that TIMP-3 mRNA levels were elevated after the combined (TNF + EMD) treatment, in agreement with the microarray data. Since this finding is a novel one, with possible beneficial clinical relevance, we investigated a potential intracellular mechanism for this induction of TIMP-3 by EMD. The marked induction of TIMP-3 expression in gingival fibroblasts by EMD was completely prevented when the cells were incubated with the specific MEK (MAPK/ERK kinase) 1/2 inhibitor, U0126 (Fig. 5), indicating that activation of the MAPK extracellular signal-regulated kinase 1/2 (ERK1/2) in gingival fibroblasts is essential for the EMD-induced TIMP-3 expression, as it is essential for EMD-induced gingival fibroblast mitogenesis (29).

## Discussion

There is significant evidence that the balance between the activity of MMPs and their endogenous inhibitors (TIMPs) is shifted toward the former in sites inflicted with periodontal disease, leading to excessive proteolytic activity (13,17,19,37,38). Many reports indicate that the abundance of several distinct MMPs is increased in diseased periodontal tissues [e.g. MMP-8 and MMP-13 (13), MMP-1 (15,19), MMPs 1, 3, 8 and 9 (16,37,38), and MMPs 1 and 3 (17,22)]. Moreover, most studies found that the total collagenolytic/gelatinolytic activity present in these tissues and in the gingival crevicular fluid is greatly increased. It is believed that this uncontrolled proteolytic activity is responsible for most of the gingival and periodontal tissue ECM destruction. The major cells responsible for MMP production and secretion in gingival tissues are fibroblasts, sulcular epithelial cells, monocytes/macrophages and neutrophils (9,13,37). These cells, found at the dentogingival junction area, are targets for both the bacterial virulence factors and the primary inflammatory mediators and react by production and secretion of additional pro-inflammatory cytokines such as TNF, interleukin-1 and interleukin-6, prostanoids such as prostagladin $E_2$, histamine and other mediators (9), as well as proteolytic enzymes. TNF, one of the major driving forces in periodontal inflammation, has been shown many times to induce the expression of MMP-1 and MMP-3 in gingival (22,36) as well as dermal (39,40), dental pulp (41), lung (42) and synovial (43) fibroblasts. Our microarray and quantitative real-time

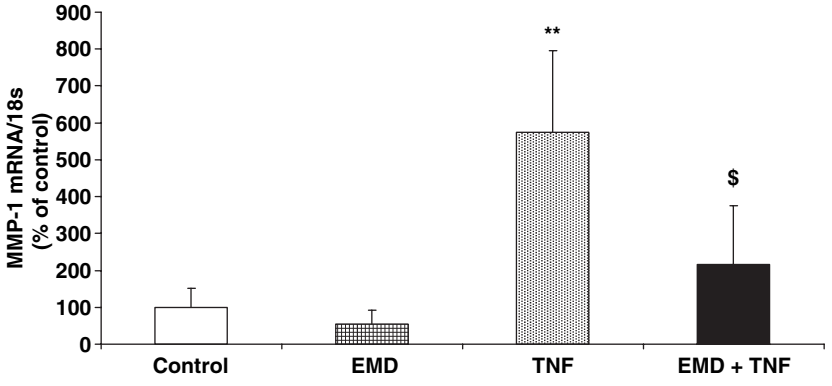


*Fig. 3*. The effect of EMD and TNF on MMP-1 expression in gingival fibroblasts. The mRNA levels of MMP-1 were measured by quantitative real-time PCR and normalized to the level of the endogenous control 18S ribosomal RNA. Data are presented as the means + SD of four independent cultures and expressed as a percentage of the mRNA level in untreated cultures. $^{**}p < 0.01$, control vs. TNF treatment; and $\$p < 0.05$, TNF vs. combined treatment with TNF and EMD.

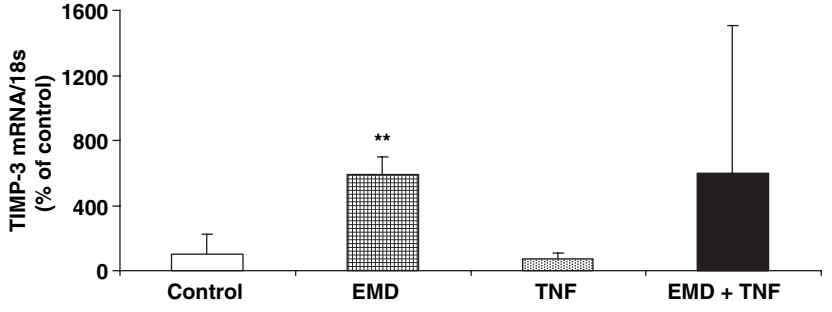


*Fig. 4*. The effect of EMD and TNF on TIMP-3 expression in gingival fibroblasts. The mRNA levels of TIMP-3 were measured by quantitative real-time PCR and normalized to the level of the endogenous control 18S ribosomal RNA. Data are presented as the means + SD of four independent cultures and expressed as a percentage of the mRNA level in untreated cultures. $^{**}p < 0.01$, control vs. EMD treatment.

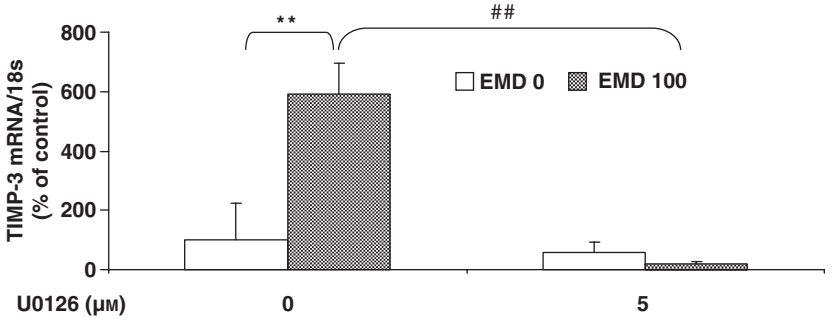


*Fig. 5*. The role of extracellular signal-regulated kinase (ERK) activation in EMD-induced TIMP-3 expression. Quiescent cells were pretreated with U0126, a specific inhibitor of the ERK pathway, for 45 min before the addition of EMD. The abundance of TIMP-3 mRNA was measured by quantitative real-time PCR and normalized to that of 18S ribosomal RNA. Data are presented as the means + SD of four independent cultures and expressed as a percentage of the mRNA level in untreated cultures. $^{**}p < 0.01$, control vs. EMD treatment; $\#\#p < 0.01$, effect of U0126. EMD 0, control; EMD 100, EMD 100 μg/mL.

PCR data support these findings. In this respect, TNF acts to upregulate MMP-1 together with other inflammatory cytokines, such as interleukin-1 (44).

Enamel matrix derivative is used clinically for periodontal regeneration, but several studies have suggested that it also enhances gingival healing when applied surgically (24–27) or non-surgically (45). Owing to the paucity of studies investigating the effects of EMD on gingival tissue and cells, the mechanisms whereby these are affected by EMD are largely unknown. We have recently conducted several studies to explore the cellular effects of EMD on primary human gingival fibroblasts. We have demonstrated that EMD stimulates human gingival fibroblast proliferation in an ERK-dependent manner that involves transactivation of the epidermal growth factor receptor (29,46), and that EMD protects human gingival fibroblasts from TNF-induced apoptosis by upregulating cellular FLICE-like inhibitory protein and preventing caspase activity (30). These combined actions of EMD may increase the number of gingival fibroblasts and counteract the documented fibroblast cell death that occurs in inflamed gingiva (47–49). However, EMD can potentially promote gingival healing in other ways. Several studies found that EMD possesses anti-inflammatory properties, i.e. it limits the release of pro-inflammatory cytokines (TNF and interleukin-8) from blood cells or monocytes (50,51). Also, EMD was shown to moderately inhibit the growth of periopathogenic bacteria, such as *Porphyromonas gingivalis* (52) and *Aggregatibacter actinomycetemcomitans* (53). Our study adds another way in which EMD can promote gingival healing, i.e. by induction of TIMP-3 expression, which would diminish the MMP/TIMP ratio and reduce ECM degradation. Since the ratio of MMP-1 to TIMP-3 is significantly increased in diseased gingiva (19), the finding that EMD reduces the abundance of MMP-1 mRNA and increases that of TIMP-3 should mitigate ECM breakdown. This hypothesis is in line with the ability of EMD to reduce MMP-1 mRNA in human osteoblastic cells (54). Notably, the effect of EMD on the

expression/activity of MMPs/TIMPs may vary between different cells and different MMPs/TIMPs, since our study found that EMD stimulates, in human gingival fibroblasts, the expression of TIMP-3 and not other TIMPs, while it has been reported that EMD stimulates the production of MMP-2 and MMP-9 in human tongue squamous cell carcinoma (HSC-3) cells (55).

Increased TIMP-3 expression in gingival fibroblasts in response to EMD is a novel finding, and we investigated a possible intracellular mechanism of this effect. We found that ERK phosphorylation, a hallmark of MAPK pathway activation, is crucial to TIMP-3 induction by EMD. In support of this conclusion, TIMP-3 expression is also induced in chondrocytes by transforming growth factor beta in an ERK-dependent manner (56). Our previous studies showed unequivocally that EMD treatment of human gingival fibroblasts results in a rapid and substantial activation of the ERK cascade (29), and that this activation of the MAPK pathway is crucial for the mitogenic effect of EMD in these cells. Therefore, EMD-induced ERK activation results in several cellular consequences, some of which we have elucidated (induction of DNA synthesis and TIMP-3 expression).

In conclusion, mRNA analysis of human gingival fibroblasts indicated that the expression of MMP-1 is increased in response to TNF while that of TIMP-3 is increased in response to EMD. These data imply that, while TNF and other inflammatory mediators increase MMP expression and activity in gingival tissue, EMD counteracts their action by increasing TIMP expression with a potential to curb ECM degradation.

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## References

1. Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999; **274:** 21491–21494.
2. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 2007;**8:**221–233.
3. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003;**200:**448–464.
4. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001;**17:**463–516.
5. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta* 2000;**1477:**267–283.
6. Wojtowicz-Praga SM, Dickson RB, Hawkins MJ. Matrix metalloproteinase inhibitors. *Invest New Drugs* 1997;**15:**61–75.
7. Trengove NJ, Stacey MC, MacAuley S *et al*. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen* 1999;**7:**442–452.
8. Gill SE, Parks WC. Metalloproteinases and their inhibitors: regulators of would healing. *Int J Biochem Cell Biol*. 2008;**40:** 1334–1347.
9. Madianos PN, Bobetsis YA, Kinane DF. Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. *J Clin Periodontol* 2005;**32:**57–71.
10. Graves DT, Cochran D. The contribution of IL-1 and TNF to periodontal tissue destruction. *J Periodontol* 2003;**74:**391–401.
11. Reynolds JJ, Hembry RM, Meikle MC. Connective tissue degradation in health and periodontal disease and the roles of matrix metalloproteinases and their natural inhibitors. *Adv Dent Res* 1994;**8:**312–319.
12. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest* 1976;**34:**235–249.
13. Tervahartiala T, Pirilä E, Ceponis A *et al*. The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 2000;**79:**1969–1977.
14. Zhou J, Windsor LJ. *Porphyromonas gingivalis* affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases. *J Periodontal Res* 2006;**41:**47–54.
15. Aiba T, Akeno N, Kawane T, Okamoto H, Horiuchi N. Matrix metalloproteinases-1 and -8 and TIMP-1 mRNA levels in normal and diseased human gingiva. *Eur J Oral Sci* 1996;**104:**562–569.
16. Soell M, Elkaim R, Tenenbaum H. Cathepsin C, matrix metalloproteinases, and their tissue inhibitors in gingiva and gingival crevicular fluid from periodontitis-affected patients. *J Dent Res* 2002; **81:**174–178.
17. Kim DM, Ramoni MF, Nevins M, Fiorellini JP. The gene expression profile in refractory periodontitis patients. *J Periodontol* 2006;**77:**1043–1050.
18. Dahan M, Nawrocki B, Elkaïm R *et al*. Expression of MMPs in healthy and diseased human gingiva. *J Clin Periodontol* 2001;**28:**128–136.
19. Kubota T, Itagaki M, Hoshino C *et al*. Altered gene expression levels of matrix metalloproteinases and their inhibitors in periodontitis-affected gingival tissue. *J Periodontol* 2008;**79:**166–173.
20. Meikle MC, Atkinson SJ, Ward RV, Murphy G, Reynolds JJ. Gingival fibroblasts degrade type I collagen films when stimulated with TNF and IL-1: evidence that breakdown is mediated by metalloproteinases. *J Periodontal Res* 1989; **24:** 207–213.
21. Domeij H, Yucel-Lindberg T, Modeer T. Signal pathways involved in the production of MMP-1 and MMP-3 in human gingival fibroblasts. *Eur J Oral Sci* 2002; **110:**302–306.
22. Beklen A, Ainola M, Hukkanen M, Gürgan C, Sorsa T, Konttinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. *J Dent Res* 2007;**86:**347–351.
23. Reddy MS, Geurs NC, Gunsolley JC. Periodontal host modulation with anti-proteinase, anti-inflammatory, and bone-sparing agents. A systematic review. *Ann Periodontol* 2003;**8:**12–37.
24. Nemcovsky CE, Artzi Z, Tal H, Kozlovsky A, Moses O. A multicenter comparative study of 2 root coverage procedures: coronally advanced flap with the addition of enamel matrix proteins and subpedicle connective tissue graft. *J Periodontol* 2004;**75:**608–615.
25. Cueva MA, Boltchi FE, Halmon WW, Nunn ME, Rivera-Hidalgo F, Rees T. A comparative study of coronally advanced flaps with and without the addition of enamel matrix derivative in the treatment of marginal tissue recession. *J Periodontol* 2004;**75:**949–956.
26. Hägewald S, Spahr A, Rompola E, Haller B, Heijl L, Bernimoulin J-P. Comparative study of Emdogain and coronally advanced flap technique in the treatment of human gingival recessions. A prospective controlled clinical study. *J Clin Periodontol* 2002;**29:**35–41.
27. Tonetti MS, Fourmousis I, Suvan J, Cortellini P, Bragger U, Lang NP. Healing, post-operative morbidity and patient perception of outcomes following regenerative therapy of deep intrabony defects. *J Clin Periodontol* 2004;**31:**1092–1098.
28. Keila S, Nemcovsky CE, Moses O, Artzi Z, Weinreb M. In vitro effect of enamel matrix proteins on rat bone marrow cells

and gingival fibroblasts. *J Dent Res* 2004; **83:**134–138.
29. Zeldich E, Koren R, Nemcovsky C, Weinreb M. Enamel matrix derivative stimulates human gingival fibroblast proliferation via ERK. *J Dent Res* 2007; **86:**41–46.
30. Zeldich E, Koren R, Dard M, Nemcovsky C, Weinreb M. Enamel matrix derivative protects human gingival fibroblast from TNF-induced apoptosis by inhibiting caspase activation. *J Cell Physiol* 2007; **213:**750–758.
31. Okuda K, Miyazaki A, Momose M *et al*. Levels of tissue inhibitor of metalloproteinases-1 and matrix metalloproteinases-1 and -8 in gingival crevicular fluid following treatment with enamel matrix derivative (EMDOGAIN). *J Periodontal Res* 2001;**36:**309–316.
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitive PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001; **25:**402–408.
33. Cury PR, Araújo VC, Canavez F, Furuse C, Araújo NS. Hydrocortisone affects the expression of matrix metalloproteinases (MMP-1, -2, -3, -7, and -11) and tissue inhibitor of matrix metalloproteinases (TIMP-1) in human gingival fibroblasts. *J Periodontol* 2007;**78:**1309–1315.
34. Cury PR, Canavez F, de Araújo VC, Furuse C, de Araújo NS. Substance P regulates the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinase in cultured human gingival fibroblasts. *J Periodontal Res* 2008; **43:**255–260.
35. Zhou J, Olson BL, Windsor LJ. Nicotine increases the collagen-degrading ability of human gingival fibroblasts. *J Periodontal Res* 2007;**42:**228–235.
36. Kato T, Okahashi N, Ohno T, Inaba H, Kawai S, Amano A. Effect of phenytoin on collagen accumulation by human gingival fibroblasts exposed to TNF-alpha in vitro. *Oral Dis* 2006;**12:**156–162.
37. Sorsa T, Tjäderhane L, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. *Oral Dis* 2004;**10:**311–318.
38. Séguier S, Gogly B, Bodineau A, Godeau G, Brousse N. Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gingival tissue? *J Periodontol* 2001;**72:**1398–1406.
39. Lee J, Jung E, Lee J *et al*. Emodin inhibits TNF alpha-induced MMP-1 expression through suppression of activator protein-1 (AP-1). *Life Sci* 2006;**79:**2480–2485.
40. Dasu MR, Barrow RE, Spies M, Herndon DN. Matrix metalloproteinase expression in cytokine stimulated human dermal fibroblasts. *Burns* 2003;**29:**527–531.
41. Wisithphrom K, Winsdor LJ. The effects of tumor necrosis factor-alpha, interleukin-1beta, interleukin-6, and transforming growth factor-beta1 on pulp fibroblast mediated collagen degradation. *J Endod* 2006;**32:**853–861.
42. O'Kane CM, Elkington PT, Friedland JS. Monocyte-dependent oncostatin M and TNF-alpha synergize to stimulate unopposed matrix metalloproteinase-1/3 secretion from human lung fibroblasts in tuberculosis. *Eur J Immunol* 2008; **38:**1321–1330.
43. Tagoe CE, Marjanovic N, Park JY *et al*. Annexin-1 mediates TNF-alpha-stimulated matrix metalloproteinase secretion from rheumatoid arthritis synovial fibroblasts. *J Immunol* 2008;**181:**2813–2820.
44. Kida Y, Kobayashi M, Suzuki T *et al*. Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. *Cytokine* 2005;**29:**159–168.
45. Wennström JL, Lindhe J. Some effects of enamel matrix proteins on wound healing in the dento-gingival region. *J Clin Periodontol* 2002;**29:**9–14.
46. Zeldich E, Koren R, Dard M, Nemcovsky C, Weinreb M. The role of EGFR in EMD-induced gingival fibroblast mitogenesis. *J Dent Res* 2008;**87:**850–855.
47. Zappa U, Boretti G, Graf H, Case D. Numbers and vitality of leukocytes in pocket washings of untreated periodontitis lesions in humans utilizing a novel intracrevicular lavage technique. *J Periodontal Res* 1992;**27:**274–284.
48. Nemeth E, Kulkarni GW, McCulloch CA. Disturbances of gingival fibroblast population homeostasis due to experimentally induced inflammation in the cynomolgus monkey (*Macaca fascicularis*): potential mechanism of disease progression. *J Periodontal Res* 1993;**28:**180–190.
49. Tonetti MS, Cortellini D, Lang NP. In situ detection of apoptosis at sites of chronic bacterially induced inflammation in human gingiva. *Infect Immun* 1998;**66:** 5190–5195.
50. Myhre AE, Lyngstadaas SP, Dahle MK *et al*. Anti-inflammatory properties of enamel matrix derivative in human blood. *J Periodontal Res* 2006;**41:**208–213.
51. Sato S, Kitagawa M, Sakamoto K *et al*. Enamel matrix derivative exhibits anti-inflammatory properties in monocytes. *J Periodontol* 2008;**79:**535–540.
52. Walter C, Jawor P, Bernimoulin JP, Hägewald S. Moderate effect of enamel matrix derivative (Emdogain Gel) on *Porphyromonas gingivalis* growth in vitro. *Arch Oral Biol* 2006;**51:**171–176.
53. Spars A, Lyngstadaas SP, Boeckh C, Andersson C, Podbielski A, Haller B. Effect of the enamel matrix derivative Emdogain on the growth of periodontal pathogens in vitro. *J Clin Periodontol* 2002;**29:**62–72.
54. Mizutani S, Tsuboi T, Tazoe M, Koshihara Y, Goto S, Togari A. Involvement of FGF-2 in the action of Emdogain on normal human osteoblastic activity. *Oral Dis* 2003;**9:**210–217.
55. Laaksonen M, Suojanen J, Nurmenniemi S, Läärä E, Sorsa T, Salo T. The enamel matrix derivative (Emdogain) enhances human tongue carcinoma cells gelatinase production, migration and metastasis formation. *Oral Oncol* 2008;**44:**733–742.
56. Qureshi HY, Sylvester J, El Mabrouk M, Zafarullah M. TGF-beta-induced expression of tissue inhibitor of metalloproteinases-3 gene in chondrocytes is mediated by extracellular signal-regulated kinase pathway and Sp1 transcription factor. *J Cell Physiol* 2005;**203:**345–352.