

Expression of Prostaglandin E Synthases in Periodontitis *Immunolocalization and Cellular Regulation*

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The inflammatory mediator prostaglandin E₂ (PGE₂) is implicated in the pathogenesis of chronic inflammatory diseases including periodontitis; it is synthesized by cyclooxygenases (COX) and the prostaglandin E synthases mPGES-1, mPGES-2, and cPGES. The distribution of PGES in gingival tissue of patients with periodontitis and the contribution of these enzymes to inflammation-induced PGE₂ synthesis in different cell types was investigated. In gingival biopsies, positive staining for PGES was observed in fibroblasts and endothelial, smooth muscle, epithelial, and immune cells. To further explore the contribution of PGES to inflammation-induced PGE₂ production, *in vitro* cell culture experiments were performed using fibroblasts and endothelial, smooth muscle, and mast cells. All cell types expressed PGES and COX-2, resulting in basal levels of PGE₂ synthesis. In response to tumor necrosis factor (TNF- α), IL-1 β , and cocultured lymphocytes, however, mPGES-1 and COX-2 protein expression increased in fibroblasts and smooth muscle cells, accompanied by increased PGE₂, whereas mPGES-2 and cPGES were unaffected. In endothelial cells, TNF- α increased PGE₂ production only via COX-2 expression, whereas in mast cells the cytokines did not affect PGE₂ enzyme expression or PGE₂ production. Furthermore, PGE₂ production was diminished in gingival fibroblasts derived from mPGES-1 knockout mice, compared with wild-type fibroblasts. These results suggest that fibroblasts and smooth muscle cells are important sources of mPGES-1, which may contribute to in-

creased PGE₂ production in the inflammatory condition periodontitis. (Am J Pathol 2011, 178:1676–1688; DOI: 10.1016/j.ajpath.2010.12.048)

Periodontitis is a chronic inflammatory disease involving interactions among bacterial products, host cells, and inflammatory mediators. The inflammatory response results in destruction of the tissue and alveolar bone supporting the teeth and can ultimately lead to tooth loss.¹ The inflammatory mediator prostaglandin E₂ (PGE₂) has been identified as a potent mediator in the pathogenesis of periodontitis. Levels of PGE₂ are elevated in the gingival tissue and gingival fluid of patients with periodontitis, compared with periodontally healthy subjects.^{2–5} It has also been reported that the inhibition of PGE₂ using selective or nonselective nonsteroidal anti-inflammatory drugs decreases periodontal disease progression and reduces alveolar bone resorption, which highlights the significance of PGE₂ in the pathogenesis of periodontal disease.^{6–8} In addition to these findings, bone resorption in lipopolysaccharide-treated mice has been shown to be dependent on PGE₂ synthesis, a finding that might be explained by the observation that PGE₂ stimulates the formation of osteoclasts.^{9,10}

Prostaglandin E₂ is produced via three different groups of enzymes, acting sequentially. The first group of enzymes, phospholipase A₂, converts membrane lipids to arachidonic acid.^{11,12} The second group of isoenzymes, cyclooxygenases (COX-1 and COX-2) convert arachidonic acid to prostaglandin H₂.¹³ Finally, the third and most recently identified group of isoenzymes, the prostaglandin E synthases (PGE synthases) catalyze the conversion of COX-derived prostaglandin H₂ to PGE₂ in the final step of PGE₂ biosynthesis.^{14,15} Three distinct PGE synthase isoenzymes have been characterized, and research is ongoing to further define the roles of these enzymes in different

Supported by grants from the Swedish Research Council, project no. 73XD-15005, the Swedish Patent Revenue Fund, the Swedish Dental Society, and the Karolinska Institute.

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Accepted for publication December 22, 2010.

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chronic inflammatory conditions, especially in light of the discovery of the adverse effects of COX-2 inhibitors.^{16–19} The microsomal membrane-associated and glutathione-dependent PGE synthase (mPGES-1) is induced by pro-inflammatory stimuli and involved in delayed PGE₂ synthesis.^{14,17,20} The cytosolic PGE synthase (cPGES) is reported to be involved in the immediate release of PGE₂,²¹ and the glutathione-independent mPGES-2 has been reported to contribute to immediate and delayed PGE₂ synthesis but is not essential for PGE₂ synthesis.^{22–24}

The enhanced biosynthesis and role of PGE₂ in periodontal tissue have been well established, although there are currently no data about the expression of the three PGE synthases in periodontitis. Furthermore, there are no reports addressing the contribution of the different cells in the connective tissue to PGE₂ production. In one study, however, Siegel et al²⁵ demonstrated the expression of mPGES-1 in gingival tissue of periodontally healthy subjects and in experimental gingivitis.

In contrast to the PGE synthases, the upstream enzyme COX-2 has been relatively widely studied. It has been reported that COX-2 expression is up-regulated in inflamed periodontal tissue, as well as in gingival tissue from subjects with chronic periodontitis, compared with gingival tissue obtained from healthy subjects.^{26–28}

In light of the lack of information on PGE synthases in periodontal tissue, the aim of the present study was to investigate the cellular localization of PGE₂-producing enzymes, focusing on the expression of PGE synthases in human gingival tissues collected from patients with periodontitis. An additional aim was to investigate the regulation of these enzymes with *in vitro* model systems mimicking an inflammatory situation. Here, we report novel findings on the localization of the PGE₂-synthesizing enzymes mPGES-1, mPGES-2, and cPGES in gingival tissue from patients with periodontitis. The expression of PGE synthases and the upstream enzyme COX-2 was observed in fibroblast-like cells, vessels, epithelial cells, and immune cells. Our *in vivo* results were further validated by *in vitro* experiments, using different cell types as model systems to investigate their contribution to cytokine-stimulated PGE₂ production via PGE synthases. The results suggest that inflammation-induced PGE₂ production is positively regulated by the enzymes mPGES-1 and COX-2 in fibroblasts and smooth muscle cells, which might contribute to the increased PGE₂ levels present in the gingival tissue of patients with periodontitis.

Materials and Methods

Gingival Tissue Collection

Eleven gingival tissue biopsies were obtained from otherwise healthy adult patients (age 35 to 68 years) diagnosed with periodontitis. For the definition of periodontitis, bone resorption resulting in a clinical attachment loss between 2 and ≥ 6 mm, and pocket probing depth (PPD) ranging from 3 mm to ≥ 6 mm have been suggested as clinical criteria.²⁹ The inclusion criteria

used in the present study were clinical signs of periodontitis at the site of biopsy collection, including radiographical bone resorption, PPD ≥ 6 mm, and bleeding on probing. The biopsies were taken during surgery as part of the normal course of periodontal therapy. The study was approved by the ethical committee at Karolinska Institutet. Each patient gave informed consent for the use of gingival tissue in the present study.

Histological and Immunohistochemical Analysis

Gingival tissues were either formaldehyde fixed (4% neutral buffered formaldehyde; Apoteket, Stockholm, Sweden), processed, and paraffin-embedded or immediately snap-frozen in liquid nitrogen. For the paraffin-embedded biopsies, deparaffinized serial sections (4 μ m) were prepared, and sections of each biopsy were stained with hematoxylin and eosin (H&E) and Giemsa to assess the orientation of the epithelium and the degree of cell infiltration. To investigate leukocyte infiltration in the tissues, sections were incubated with primary CD45 antibody (monoclonal mouse anti-CD45/LCA, 1:400; Novocastra Laboratories, Newcastle upon Tyne, UK), washed, and then incubated with a BOND polymer refine detection kit using BOND DAB enhancer (Leica Microsystems–Vision BioSystems Division, Wetzlar, Germany). The staining was performed using an automated BOND-MAX instrument (Leica Microsystems).

For the immunostaining of PGE synthase, COX-2, and IL-1 β expression in the biopsies, the sections were deparaffinized using xylene and then were rehydrated through an ethanol series. Immunohistochemical staining was performed using a cell and tissue staining kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The primary antibodies used for the staining included mPGES-1 (polyclonal rabbit, 1:100), mPGES-2 (polyclonal rabbit, 1:200), cPGES (polyclonal rabbit, 1:200), COX-2 (monoclonal mouse, 1:50) (all from Cayman Chemical, Ann Arbor, MI), or IL-1 β (monoclonal mouse, 1:400; Invitrogen, Carlsbad, CA). For negative controls, the primary antibody was substituted with isotype-matched control antibody. To identify mast cells in the tissue, sections were deparaffinized and stained either with 0.5% Toluidine Blue in McIlvaine's buffer (pH 4) or with anti-human mast cell tryptase, clone AA1 (monoclonal mouse, 1:2000; DakoCytomation, Glostrup, Denmark), using an automated BOND-MAX instrument (Leica Microsystems).

For immunofluorescence double staining, frozen biopsies were cryostat-sectioned (7 μ m). For staining for fibroblast marker anti-prolyl-4-hydroxylase (clone 5B5) (monoclonal mouse; DakoCytomation)³⁰ and mPGES-1, the sections were fixed and permeabilized with cold acetone and blocked with 1% bovine serum albumin in 0.25% Triton X-100 solution. For the endothelial cell marker von Willebrand factor (clone F8/86) (monoclonal mouse; DakoCytomation) and smooth muscle actin (monoclonal mouse; DakoCytomation), the sections were fixed in 2% formaldehyde, permeabilized with 0.1% saponin, and blocked with 10% human

serum, 1% bovine serum albumin, and 0.3 mol/L glycine (Sigma-Aldrich, St. Louis, MO). After blocking, the slides were incubated with a mixture of two primary antibodies consisting of mPGES-1 (1:100) and either the fibroblast marker anti-prolyl-4-hydroxylase (1:50),³⁰ the endothelial cell marker von Willebrand factor (1:50), or smooth muscle actin (1:50). After washing, the sections were incubated with a mixture of secondary antibodies (anti-rabbit conjugated with Alexa Fluor 594 and anti-mouse conjugated with Alexa Fluor 488, dilution 1:1000; Invitrogen). The slides were mounted and observed using a Nikon fluorescence microscope, and image analysis was performed with the NIS-Elements software package (version F 2.30; Nikon Instruments, Melville, NY). For all experiments, isotype-matched irrelevant antibodies were used as negative controls in the staining procedures.

Cell Cultures

Human gingival fibroblasts were established from gingival biopsies obtained from six healthy patients with no clinical signs of periodontal disease. The protocol, including the collection of gingival biopsies, was approved by the Ethical Committee at Huddinge University Hospital. Gingival fibroblasts, cultured as described previously,^{31,32} were seeded in 60-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), penicillin (50 units/mL), and streptomycin (50 μ g/mL) (all from Invitrogen) and then cultured. The cell layers were rinsed with serum-free DMEM, followed by the addition of DMEM containing the cytokines tumor necrosis factor α , TNF- α (20 ng/mL), or IL-1 β (0.5 ng/mL) (R&D Systems). Control cells were treated with medium only. After the incubation (24 hours), the medium was collected and stored at -20°C for PGE₂ determination. The cell monolayer was washed twice with PBS, and cells were collected for flow cytometric analysis or immediately lysed for Western blot analysis.

Gingival fibroblasts from mPGES-1 knockout mice³³ and wild-type mice were established from gingival biopsies collected from the palate of the mice. Mouse gingival fibroblasts were cultured using DMEM supplemented with 10% FCS, penicillin (50 units/mL), streptomycin (50 μ g/mL), and sodium pyruvate (100 mmol/L). The cells were seeded in four-well chamber slides (Lab-Tek; Nalge Nunc International, Naperville, IL), cultured for 24 hours at 37°C , and stimulated either with DMEM containing TNF- α or with DMEM alone (control cells). After an additional incubation period (24 hours), the medium was collected and stored at -20°C for PGE₂ analysis. The cell monolayers were fixed in 2% formaldehyde diluted in PBS for 15 minutes at 4°C and stained with polyclonal antibodies against mPGES-1 (diluted 1:5000) as described previously.^{34,35} Isotype-matched irrelevant polyclonal antibodies were used as negative controls.

The human umbilical vein endothelial cell (HUVEC) line CRL-1730 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in serum-free medium (SFM; Invitrogen) supplemented

with 10% FCS (Invitrogen), penicillin (100 units/mL), streptomycin (100 μ g/mL), and endothelial cell growth supplement (50 μ g/mL; BD Biosciences, San Jose, CA). The cells were routinely passaged using 0.025% trypsin (Invitrogen) in PBS containing 0.02% EDTA. Endothelial cells were seeded in 60-mm Petri dishes and were cultured for 24 hours. The cells were washed with medium containing all supplements except FCS, followed by addition of the same medium with or without IL-1 β or TNF- α . After 24 hours of incubation, the medium was collected and stored at -20°C for PGE₂ measurements; the cells were collected for flow cytometric analysis or lysed for Western blot analysis, as described above.

Primary cultures of human airway smooth muscle (HASM) cells were prepared from explants of human airway smooth muscle as previously reported³⁶ and cultured in DMEM containing 10% FCS, penicillin (50 units/mL), and streptomycin (50 g/mL) at 37°C . The cells were trypsinized and seeded in 60-mm Petri dishes in culture medium. The smooth muscle cells were rinsed with DMEM without FCS, followed by addition of serum-free culture medium with or without IL-1 β or TNF- α . After 24 hours of incubation, the medium was collected and stored at -20°C for PGE₂ analyses; the cells were collected for flow cytometric or Western blot analysis.

The human mast cell line HMC-1.2 (a subclone of the cell line HMC-1) and human primary mast cells (cord blood mast cells) were isolated and prepared as previously described.³⁷⁻³⁹ The HMC-1.2 cells were cultured in Iscove's modified Dulbecco's medium supplemented with penicillin (50 units/mL), streptomycin (50 μ g/mL), monothioglycerol (1.5 mmol/L), and 10% FCS. Primary mast cells were cultured in RPMI 1640 medium in the presence of recombinant human stem cell factor (100 ng/mL; kindly provided by Amgen, Thousand Oaks, CA) and rhIL-6 (10 ng/mL; kindly provided by Amgen) and supplemented with 10% FCS, 50 μ mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, and 50 μ g/mL streptomycin (Sigma-Aldrich). Before stimulation, cells were washed with complete growth medium without FCS. The cells were centrifuged and resuspended in serum-free medium with or without TNF- α or IL-1 β . After incubation (24 hours), the medium was collected for PGE₂ determination and the cells were collected for flow cytometric or Western blot analysis as described above.

Coculture experiments were performed with lymphocytes isolated from whole blood using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Piscataway, NJ) according to the manufacturer's instructions. Fibroblasts, endothelial cells, or smooth muscle cells were cultured and seeded (5×10^5 cells) in 60-mm Petri dishes as described above. The cells were rinsed and cultured either alone or in coculture with lymphocytes (2.5×10^6) resuspended in appropriate medium as previously described.⁴⁰ After 18 hours of incubation, the coculture medium containing lymphocytes was removed and centrifuged, after which the medium was collected and stored at -20°C for PGE₂ measurements. Fibroblasts, HUVECs, or HASM cells were trypsinized. All of the cell

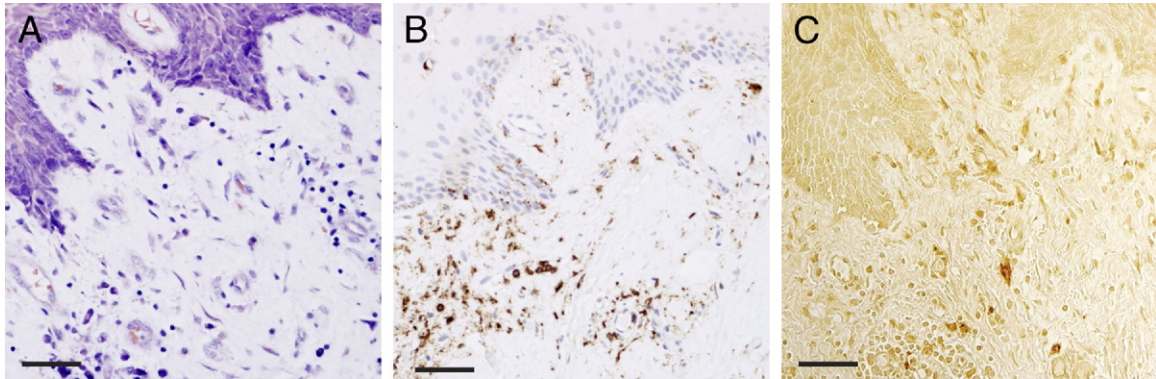


Figure 1. Contrast staining and immunohistochemical staining of CD45 and IL-1 β in gingival tissue of a representative biopsy from a patient with periodontitis. All images are taken from the same area. **A:** Giemsa contrast staining for tissue morphology. **B:** CD45 and H&E staining, showing immune cells. **C:** Immunohistochemical staining for the inflammatory cytokine IL-1 β . Scale bars = 50 μ m.

types, including lymphocytes, were collected and analyzed by flow cytometry.

Western Blot Analysis

Cells were seeded and cultured as described above. To isolate the total protein, the cells were resuspended in PBS and the pellet was resuspended in lysis buffer consisting of 10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, and protease inhibitors [1 mmol/L phenylmethylsulfonyl fluoride, pepstatin, aprotinin, and leupeptin at 1 μ g/mL (all from Sigma-Aldrich)]. Protein concentration was determined, and equal amounts of the protein were separated by electrophoresis on a 4%–15% linear gradient polyacrylamide Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) and immunoblotted as previously described.^{31,32}

Flow Cytometric Analysis

Cells were seeded and grown as described above. After treatment, the cells were collected by trypsinization and/or centrifugation of culture medium, depending on cell type, and washed with PBS. The cells were then fixed in 2% paraformaldehyde and washed with PBS before permeabilization with PBS containing 0.1% saponin and 0.01 M HEPES and flow cytometric analysis was performed as previously described.³¹ For each sample, 10,000 events were acquired. A gated area for the particular cell type was determined using forward versus side scatter parameters, and the gated cells were analyzed for expression of mPGES-1, mPGES-2, cPGES, or COX-2. The results obtained are either shown as histograms of cell counts drawn using the R programming environment (version 2.4.0, R Development Core Team; <http://www.r-project.org>) together with the R software package RFlowCyt, or are presented as mean fluorescence intensity (MFI).⁴¹

Prostaglandin E₂ Determination

The amount of PGE₂ in the culture medium collected from control, cytokine-stimulated, or cocultured cells was de-

termined by enzyme immunoassay using a Luminex PGE₂ kit (Cayman Chemical) and Bio-Plex suspension array system (Bio-Rad Laboratories) and a conventional EIA kit (Cayman Chemical).

Statistical Analysis

Cell culture experiments were analyzed in triplicate, and reproducible data representing one of at least three independent experiments is demonstrated. Results are expressed as the mean \pm SD. Student's *t*-test (two-tailed) was used in the statistical analysis, and *P* values of <0.05 were considered statistically significant.

Results

Presence of CD45 and IL-1 β in Gingival Tissue of Patients with Periodontitis

Serial paraffin sections of gingival biopsies obtained from patients with periodontitis were stained with Giemsa and H&E histological methods, as well as for CD45 and IL-1 β as markers of inflammation.^{42,43} A representative photomicrograph of gingival tissue stained with Giemsa, demonstrating the presence of immune cells in the tissue, is presented in Figure 1A. The leukocyte marker CD45 (LCA, leukocyte common antigen) was used to assess the histological grade of inflammation, shown in double staining with H&E (Figure 1B). In addition, the biopsies were also stained for the pro-inflammatory cytokine IL-1 β , demonstrating ubiquitous expression of this mediator in the gingival tissue (Figure 1C). Altogether, the immunostaining of gingival biopsies showed an infiltrate of immune cells, as well as detection of IL-1 β , demonstrating the presence of inflammation of the gingival tissue obtained from patients with periodontitis (Figure 1).

PGE Synthases Are Expressed in Gingival Tissue of Patients with Periodontitis

We first investigated the expression and localization of PGE synthases in 11 gingival biopsies collected from

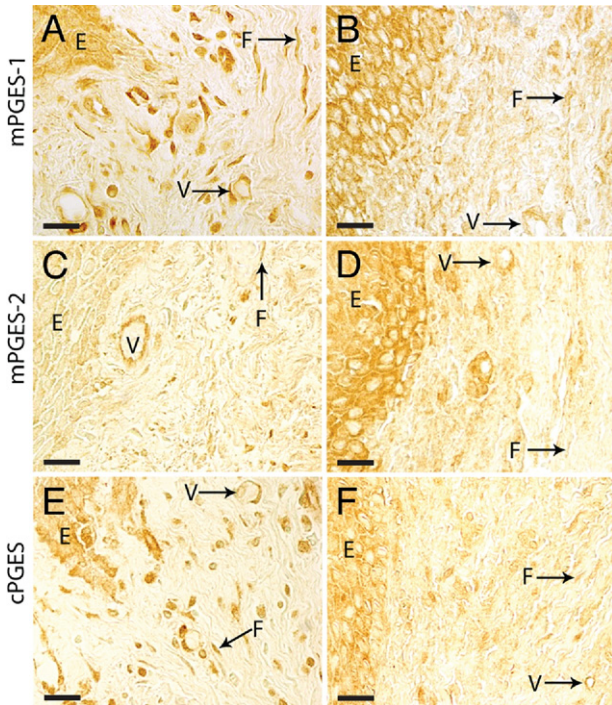


Figure 2. Immunohistochemical localization of PGE synthases in gingival tissue from two representative patients with periodontitis, stained against mPGES-1 (**A** and **B**), mPGES-2 (**C** and **D**), or cPGES (**E** and **F**). **A**, **C**, and **E**: One biopsy. **B**, **D**, and **F**: The other biopsy. E, epithelium, F, fibroblast-like cell; V, vessel. Scale bars = 20 μ m.

patients with periodontitis. Immunohistochemical staining of mPGES-1, mPGES-2, and cPGES produced positive cells in the gingival tissues from subjects with periodontitis, as seen from biopsies from two representative patients with periodontitis (Figure 2). Microscopic analysis of the sections showed that the expression of the three PGE synthases was localized to fibroblast-like cells, vessels (endothelial cells and smooth muscle cells), epithelial cells, and immune cells (Figure 2). Immunohistochemical staining of the upstream enzyme COX-2, used as a control in this study, also resulted in positively stained fibroblast-like cells, vessels, epithelial cells, and immune cells in the gingival tissue (Figure 3). Control stainings with the isotype-matched irrelevant antibodies were negative (data not shown).

mPGES-1 Protein Expression Is Increased by TNF- α and IL-1 β in Gingival Fibroblasts

To further explore the cellular source of inflammatory increased PGE₂ production, and to clarify the involvement of the PGE synthases, we performed cell culture experiments using cell types that were positively stained for PGE₂-synthesizing enzymes in gingival connective tissue. To mimic an *in vivo* inflammation *in vitro*, we stimulated cell cultures of fibroblasts, endothelial cells, smooth muscle cells, and mast cells with the inflammatory cytokines TNF- α and IL-1 β and then investigated the expression of mPGES-1, mPGES-2,

and cPGES in parallel with COX-2 expression and PGE₂ production. We focused first on human gingival fibroblasts, which are the most abundant cells in gingival connective tissue.

In immunohistochemical staining of gingival biopsies for PGE synthases, fibroblast-like cells were positively stained, as demonstrated by mPGES-1 staining (Figure 4A). Co-immunostaining of mPGES-1 and the fibroblast marker prolyl-4-hydroxylase (5B5) showed colocalization of mPGES-1 and prolyl-4-hydroxylase in fibroblasts of gingival tissue (Figure 4, B–D). For *in vitro* experiments, gingival fibroblasts were treated with the inflammatory cytokines TNF- α and IL-1 β (as described under *Materials and Methods*). In accord with our previous findings,³¹ the production of PGE₂ was markedly increased by the cytokines (Figure 4E). Furthermore, Western blot analysis revealed that expression of both mPGES-1 and COX-2 was concomitantly increased by the cytokines TNF- α and IL-1 β , whereas mPGES-2 and cPGES expression was constitutive and unaffected by cytokine treatment (Figure 4F). The up-regulation of mPGES-1 and COX-2, as well as the lack of induction of mPGES-2 and cPGES, in gingival fibroblasts was also confirmed by flow cytometric analysis (Figure 4G).

PGE Synthases Are Not Affected by TNF- α and IL-1 β in Endothelial Cells

Our immunohistochemical analysis of the *in vivo* expression of PGE synthases revealed positive staining of vessels, including endothelial cells, as seen in a representative staining of mPGES-1 (Figure 5A). Immunostaining of mPGES-1 and the endothelial cell marker von Willebrand factor showed colocalization of mPGES-1 and von Willebrand factor in endothelial cells of gingival tissue (Figure 5, B–D). We further investigated the expression of PGE synthases and COX-2 in relation to PGE₂ production, using a human umbilical vein endothelial cell line (HUVEC) as an *in vitro* model. PGE₂ production in HUVECs was significantly increased only by the inflammatory cytokine TNF- α (Figure 5E). The protein expression of mPGES-1, mPGES-2, and cPGES remained unaffected by the cytokine treatment, as demonstrated by Western blot analysis (Figure 5F); however, an increase of COX-2 protein

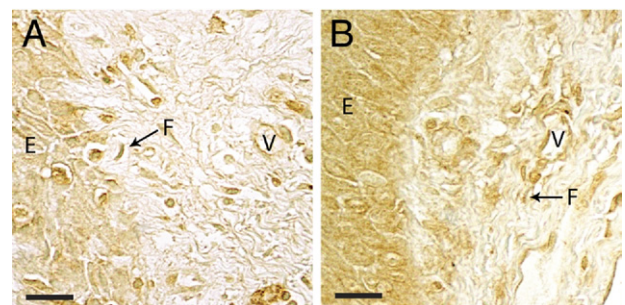


Figure 3. Immunohistochemical localization of COX-2 in gingival tissue from representative biopsies from two different patients (**A** and **B**) with periodontitis. E, epithelium, F, fibroblast-like cell; V, vessel. Scale bars = 20 μ m.

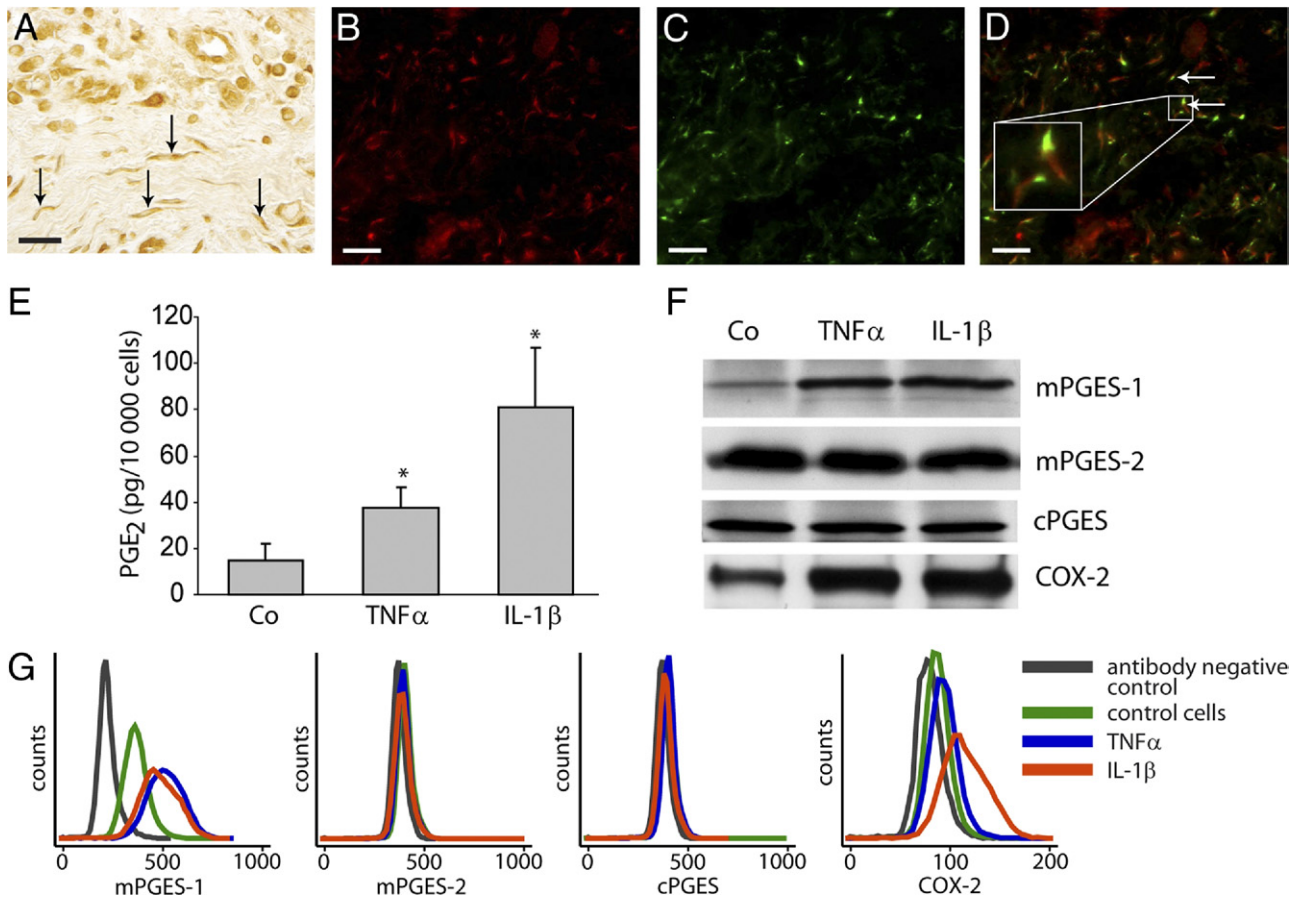


Figure 4. Production of PGE₂ and expression of PGE synthases and COX-2 after treatment with TNF- α (20 ng/mL) or IL-1 β (0.5 ng/mL) in gingival fibroblasts. **A:** Representative immunohistochemical mPGES-1 staining of a gingival biopsy from a patient with periodontitis, showing positively stained fibroblast-like cells (arrows). **B:** Fluorescent staining of mPGES-1 using Alexa Fluor 594-conjugated secondary antibody (red). **C:** Fluorescent staining of fibroblasts using fibroblast antibody prolyl-4-hydroxylase (5B5) and Alexa Fluor 488-conjugated secondary antibody (green). **D:** Merged image of mPGES-1 and prolyl-4-hydroxylase staining showing mPGES-1 expression in fibroblasts (arrows). **E:** *In vitro* production of PGE₂ in gingival fibroblasts; control cells (Co) 15 \pm 7 pg PGE₂/10,000 cells, TNF- α -stimulated 38 \pm 9 pg PGE₂/10,000 cells, and IL-1 β -stimulated 81 \pm 26 pg PGE₂/10,000 cells. **P* < 0.05 compared with control cells. **F:** Expression of PGE synthases and COX-2 in gingival fibroblasts as detected by Western blot. **G:** Expression of PGE synthases and COX-2 in gingival fibroblasts as analyzed by flow cytometry. All analyses were performed in triplicate. Results shown are representative from at least three independent experiments. Scale bars = 20 μ m.

expression in response to TNF- α was observed, as demonstrated by Western blot analysis (Figure 5C). The increased COX-2 expression in TNF- α -stimulated cells was also confirmed by flow cytometric analysis (Figure 5G).

mPGES-1 Protein Expression Is Increased by TNF- α and IL-1 β in Smooth Muscle Cells

Vessels consisting of smooth muscle cells and endothelial cells were positively stained for the PGE₂-synthesizing enzymes mPGES-1, mPGES-2, and cPGES in gingival biopsies from representative patients with periodontitis. A representative gingival biopsy demonstrating positive staining of mPGES-1 in vascular smooth muscle cells is presented in Figure 6A. In addition, co-immunostaining of mPGES-1 and smooth muscle actin demonstrated colocalization of mPGES-1 and actin in vascular smooth muscle cells of gingival tissue (Figure 6, B–D). To study the regulation of PGE₂-synthesizing enzymes and subsequent PGE₂ production in smooth muscle cells, we used HASM cells as an *in vitro* model. When HASM cells were treated with the pro-inflammatory cytokines TNF- α or IL-

1 β , the production of PGE₂ increased markedly (Figure 6E). Similarly, the protein expression of the enzymes mPGES-1 and COX-2 was also up-regulated by the cytokines, as demonstrated by Western blot (Figure 6F). Flow cytometric analyses, in accord with the Western blot results, showed an up-regulation of both mPGES-1 and COX-2 in response to TNF- α and IL-1 β , whereas mPGES-2 and cPGES remained unaffected (Figure 6, F and G).

Protein Expression of PGE Synthases and PGE₂ Production Is Not Affected by Cytokines in Mast Cells

In immunohistochemical staining of gingival biopsies for PGE synthases and COX-2, immune cells were positively stained, as seen in a representative mPGES-1 staining (Figure 7A). Using the metachromatic interaction between mast cell granules and Toluidine Blue as a method of identifying mast cells, we confirmed that mast cells were present among the immune cells in the gingival tissue, as seen from a representative example of a gin-

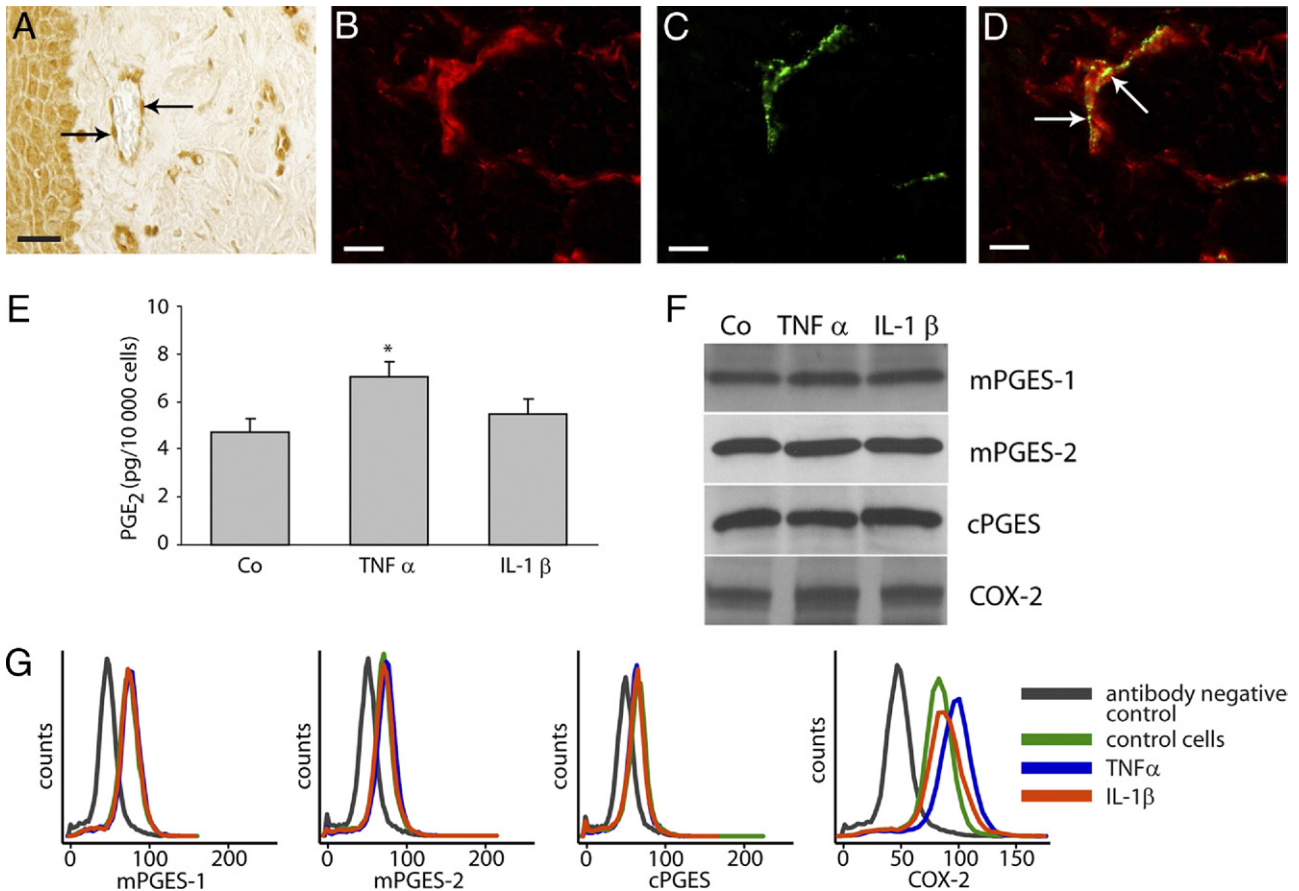


Figure 5. Production of PGE₂ and expression of PGE synthases and COX-2 after stimulation with TNF- α (20 ng/mL) or IL-1 β (0.5 ng/mL) in HUVECs. **A:** Representative immunohistochemical mPGES-1 staining of a gingival biopsy, showing positively stained endothelial cells (arrows). **B:** Fluorescent staining of mPGES-1 using Alexa Fluor 594-conjugated secondary (red). **C:** Fluorescent staining of endothelial cells using anti-human von Willebrand factor (green). **D:** Merged image of mPGES-1 and von Willebrand factor staining showing mPGES-1 expression in endothelial cells (arrows). **E:** *In vitro* production of PGE₂ in HUVECs; control cells (Co) 4.7 \pm 0.6 pg PGE₂/10,000 cells, TNF- α -treated cells 7.1 \pm 0.6 pg PGE₂/10,000 cells, and IL-1 β -treated cells 5.5 \pm 0.6 pg PGE₂/10,000 cells. **P* < 0.05 compared with control cells. **F:** Expression of PGE synthases and COX-2 in HUVECs as detected by Western blot. **G:** Expression of PGE synthases and COX-2 in HUVECs as analyzed by flow cytometry. All analyses were performed in triplicate. Results shown are representative from at least three independent experiments. Scale bars = 20 μ m.

gingival biopsy (Figure 7B). Staining for the mast cell marker tryptase further confirmed the identity of mast cells in the tissue (Figure 7C). Because the expression of PGE synthases has not, to our knowledge, been investigated previously in mast cells, this particular type of immune cell was included in the *in vitro* analyses.

To investigate the *in vitro* expression of PGE synthases and production of PGE₂ in mast cells, we stimulated human mast cells of the cell line HMC-1.2 with the inflammatory cytokines TNF- α and IL-1 β . The results showed a basal production of PGE₂ by mast cells, but no changes in PGE₂ production were observed in response to the inflammatory cytokines (Figure 7D). All three PGE synthases and COX-2 were constitutively expressed in unstimulated mast cells, but neither PGE synthase nor COX-2 expression was affected by TNF- α or IL-1 β treatment, as assessed by Western blot analyses (Figure 7E). In addition to the mast cell line, primary cord blood mast cells were also included to investigate mPGES-1 and COX-2 expression. The flow cytometry results showed no up-regulation of either mPGES-1 or COX-2 expression in response to TNF- α or IL-1 β treatment (Figure 7F). Furthermore, these primary mast cells, in accordance with

our results on the cell line HMC-1.2, produced PGE₂ at a basal level, which did not increase by TNF- α or IL-1 β treatment (data not shown).

Increased Protein Expression of mPGES-1 and COX-2, and Production of PGE₂, in Cocultures of Lymphocytes with Fibroblasts or Smooth Muscle Cells

To investigate the interaction between lymphocytes and the cell types expressing mPGES-1 and COX-2 enzymes, coculture experiments were performed. Coculture of fibroblasts or HASM cells with lymphocytes resulted in increased mPGES-1 and COX-2 expression in fibroblasts and in HASM cells (Table 1). Similarly, PGE₂ production increased in fibroblasts and HASM cells when cocultured with lymphocytes (Table 1). Furthermore, when fibroblasts, the most ubiquitous cell type in gingival connective tissue, were cocultured with lymphocytes in the presence of the cytokines IL-1 β or TNF- α , no additional increase of PGE₂-synthesizing enzymes or PGE₂ production was observed (data not shown). In the HUVEC cell

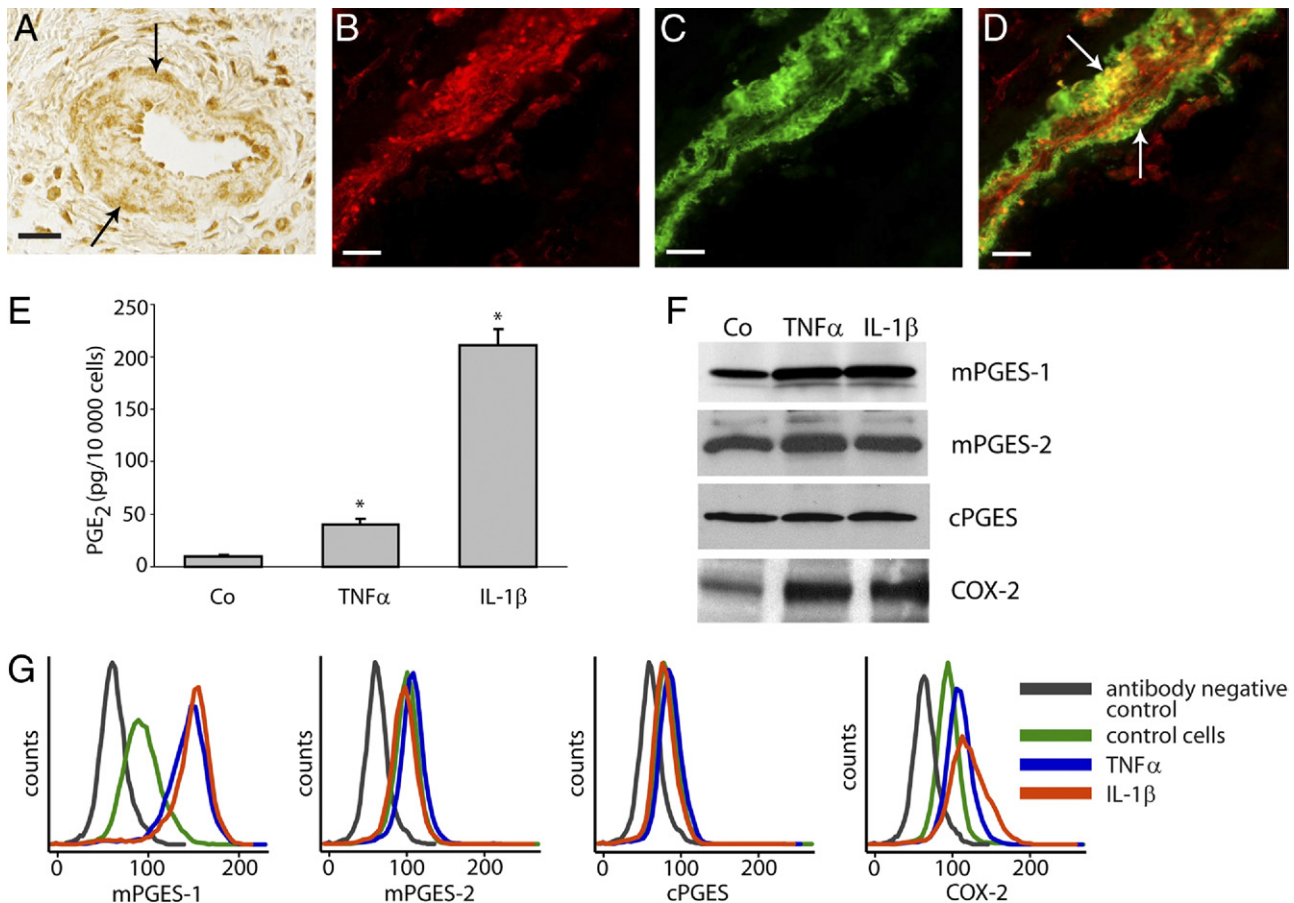


Figure 6. Production of PGE₂ and expression of PGE synthases and COX-2 after stimulation with TNF- α (20 ng/mL) or IL-1 β (0.5 ng/mL) in HASM cells. **A:** Representative immunohistochemical mPGES-1 staining of a gingival biopsy, showing positive vascular smooth muscle cells (arrows). **B:** Fluorescent staining of mPGES-1 using Alexa Fluor 594-conjugated secondary antibody (red). **C:** Fluorescent staining of smooth muscle cells using anti-human smooth muscle actin and Alexa Fluor 488-conjugated secondary antibody (green). **D:** Merged image of mPGES-1 and smooth muscle actin showing mPGES-1 expression in smooth muscle cells (arrows). **E:** *In vitro* production of PGE₂ in HASM cells; control cells (Co) 10 \pm 0.6 pg PGE₂/10,000 cells, TNF- α -stimulated 40 \pm 6 pg PGE₂/10,000 cells, and IL-1 β -stimulated 211 \pm 16 pg PGE₂/10,000 cells. **P* < 0.05 compared with control cells. **F:** Expression of PGE synthases and COX-2 in HASM cells as detected by Western blot. **G:** Expression of PGE synthases and COX-2 in HASM cells as analyzed by flow cytometry. All analyses were performed in triplicate. Results shown are representative from at least three independent experiments. Scale bars = 20 μ m.

line, however, neither the expression of mPGES-1 and COX-2 enzymes nor the production of PGE₂ was affected when cocultured with lymphocytes (Table 1).

mPGES-1 Protein Expression and PGE₂ Production Are Reduced in Gingival Fibroblasts Isolated from mPGES-1 Knockout Mice

To explore the role of mPGES-1 in the regulation of PGE₂ in gingival tissue, we also used mPGES-1 null gingival fibroblasts derived from mPGES-1 knockout mice.⁴⁴ The mPGES-1 knockout cells produced significantly lower basal levels of PGE₂ (88% decrease), compared with PGE₂ synthesis in gingival fibroblasts derived from wild-type mice (Figure 8A). Similarly, the TNF- α -stimulated PGE₂ production was strongly reduced (80% decrease) in mPGES-1 null gingival fibroblasts, compared with wild-type cells (Figure 8A). Immunostaining of mPGES-1 revealed no induction of mPGES-1 expression in TNF- α -stimulated mPGES-1 null gingival fibroblasts (Figure 8I), compared with un-

treated control cells (Figure 8H). In contrast, wild-type fibroblasts showed increased expression of mPGES-1 in response to TNF- α (Figure 8E), compared with untreated control cells (Figure 8D).

Discussion

The PGE synthases mPGES-1, mPGES-2, and cPGES are the terminal enzymes in the synthesis of PGE₂, a potent mediator associated with the chronic inflammatory disease periodontitis. As a novel outcome of the present study, we have demonstrated the cellular localization of all three PGE synthases in gingival tissue from patients with periodontitis. Immunohistochemical analysis showed the expression of the PGE synthases mPGES-1, mPGES-2, and cPGES, as well as of the upstream enzyme COX-2, to be localized to vessels, fibroblast-like cells, immune cells, and epithelium. Co-immunostaining using cell type-specific markers confirmed *in vivo* mPGES-1 expression in fibroblasts, smooth muscle cells, and endothelial cells of inflamed gingival tissue. To in-

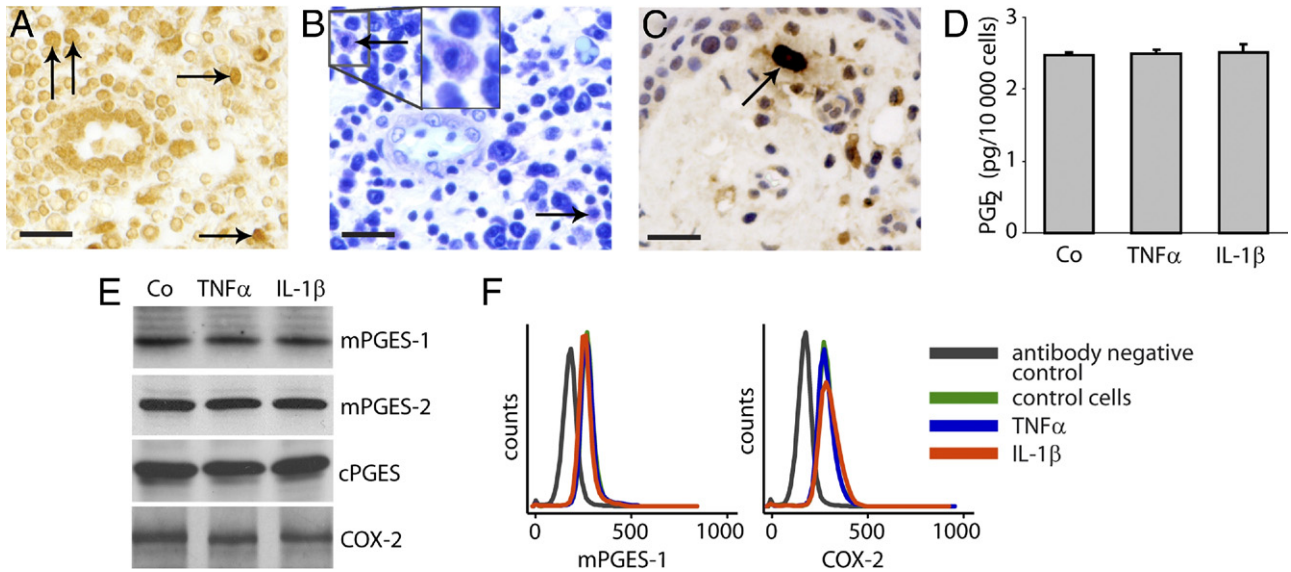


Figure 7. Localization in gingival tissue, with PGE₂ production and expression of PGE synthases and COX-2 in cultured cells stimulated with TNF- α (20 ng/mL) or IL-1 β (0.5 ng/mL) in human mast cells. **A:** Representative immunohistochemical mPGES-1 staining of a gingival biopsy, showing positive immune cells (arrows). Toluidine Blue staining (**B**) and immunostaining using the mast cell marker tryptase (**C**) of gingival tissues from patients with periodontitis showing the presence of mast cells (arrows). **A** and **B** are from the same biopsy, and **C** is from another. **D:** *In vitro* production of PGE₂ in the mast cell line HMC-1.2; control cells (Co) 2.5 \pm 0.02 pg PGE₂/10,000 cells, TNF- α -treated cells 2.5 \pm 0.06 pg PGE₂/10,000 cells, and IL-1 β -treated cells 2.5 \pm 0.12 pg PGE₂/10,000 cells. **E:** Expression of PGE synthase and COX-2 in the mast cell line HMC-1.2 as detected by Western blot. **F:** Expression of mPGES-1 and COX-2 in primary mast cells as detected by flow cytometry. All analyses were performed in triplicate. Results shown are representative from at least three independent experiments. Scale bars = 20 μ m.

investigate the expression of the PGE₂-synthesizing enzymes and their contribution to inflammation-induced PGE₂ production, *in vitro* experiments using various cell culture model systems were performed. The results showed protein expression of mPGES-1, mPGES-2, cPGES, and COX-2 in cultured fibroblasts, endothelial cells, and, as a novel finding, also in smooth muscle cells and mast cells. Moreover, mPGES-1 and COX-2 protein expression was increased by both TNF- α and IL-1 β treatment in fibroblasts and smooth muscle cells, whereas in endothelial cells only COX-2 was induced in response to TNF- α treatment.

The immunohistochemical results showed that the expression of PGE synthases and the upstream enzyme COX-2 was localized to vessels, fibroblast-like cells, and immune cells in gingival connective tissue from patients with periodontitis. The *in vivo* expression of all three PGE synthase isoforms has not been previously investigated in gingival tissue, although mPGES-1 and COX-2 have been shown to be expressed in gingivitis.²⁵ Our results concerning the lo-

calization of mPGES-1 and COX-2 are consistent with previously published results showing positive staining of mPGES-1 and COX-2 in epithelial, endothelial, and fibroblast-like connective tissue cells of the gingiva.^{25,27} In regard to nonoral chronic inflammatory conditions, the isoenzymes mPGES-1 and cPGES are reported to be expressed in synovial tissue sections from patients with the chronic inflammatory disease rheumatoid arthritis.³⁵ Furthermore, the expression of all three PGE synthases has been reported in gastric ulcer tissue, demonstrating strong mPGES-1 expression in fibroblasts and macrophages of the ulcer bed.⁴⁵

Given that levels of PGE₂ have been shown to be increased in gingival crevicular fluid from patients with periodontitis, compared with healthy controls, it is important to investigate the cellular sources of this inflammatory mediator in gingival tissue.^{46,47} To gain further insight into the contribution and regulation of PGE₂ production, we investigated the *in vitro* protein expression of PGE synthases in the cell types that were positively stained in gingival connective tissue. The cytokines

Table 1. Effect of Lymphocytes on mPGES-1 and COX-2 Expression and PGE₂ Production In Cocultures of Gingival Fibroblasts, HUVECs, and HASM Cells

	mPGES-1, MFI (SD)		COX-2, MFI (SD)		PGE ₂ (pg/10,000 adherent cells)	
	-	+	-	+	-	+
Fibroblasts	15.3 (7.3)	88.1 (35.3)*	12.0 (5.7)	139.2 (71.5)*	5.4 (0.1)	1141.4 (124.6)*
HUVECs	22.5 (8.9)	21.5 (8.6)	16.5 (5.5)	16.1 (5.5)	0.6 (0.2)	1.3 (0.4)
HASM cells	72.7 (36.5)	205.9 (93.8)*	30.4 (12.0)	161.7 (65.5)*	10.1 (0.9)	953.8 (30.1)*

HUVEC, human umbilical vein endothelial cell; MFI, mean fluorescence intensity (arbitrary units); +, cocultured with lymphocytes; -, not cocultured with lymphocytes.

**P* < 0.05, compared with cells not cocultured with lymphocytes.

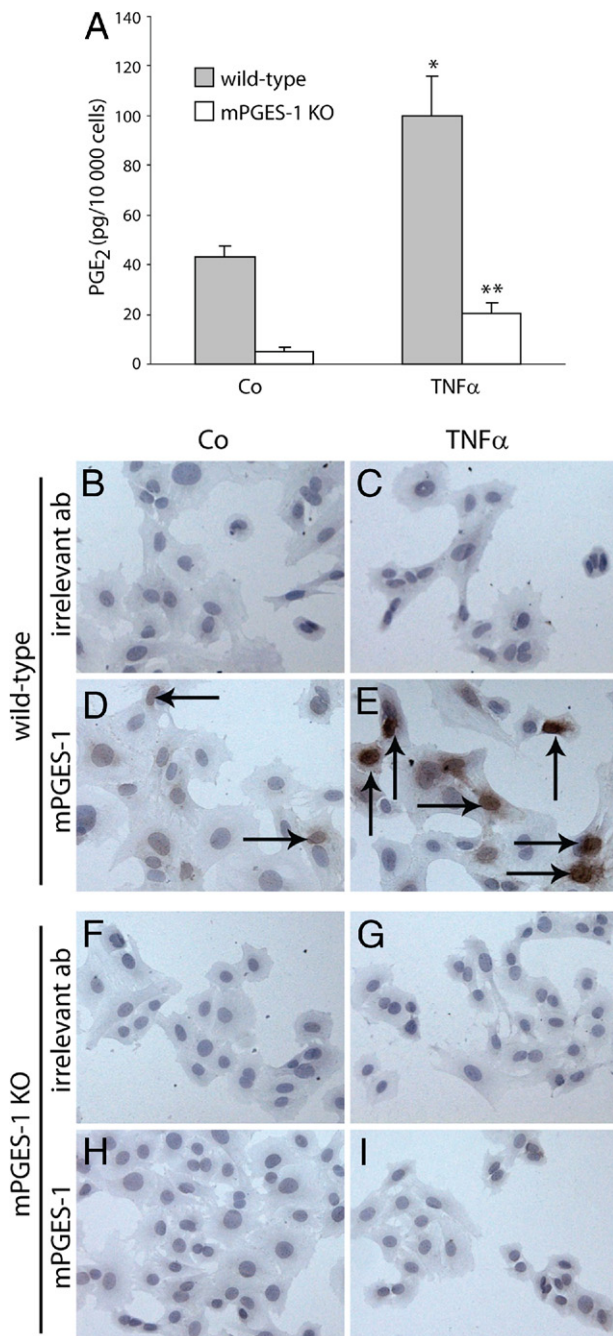


Figure 8. Production of PGE₂ and expression of mPGES-1 in gingival fibroblasts isolated from mPGES-1 knockout (KO) mice and wild-type mice. The cells were treated either with medium alone (control) or with TNF- α (20 ng/mL). **A:** *In vitro* production of PGE₂ in gingival fibroblasts isolated from mPGES-1 KO and wild-type mice. **P* < 0.05 compared with wild-type control cells not treated with TNF- α ; ***P* < 0.05 compared with mPGES-1 KO control cells not treated with TNF- α . **D** and **H:** Expression of mPGES-1 in untreated gingival fibroblasts (control cells, Co) derived from wild-type mice and mPGES KO mice, respectively, as detected by immunohistochemistry. **E** and **I:** Expression of mPGES-1 in TNF- α stimulated gingival fibroblasts derived from wild-type mice and mPGES-1 KO mice, respectively. **B, C, F,** and **G:** Immunohistochemistry staining of the cells with irrelevant polyclonal antibodies (irrelevant ab). Intracellular positive staining is revealed as dark-brown deposit (arrows). Original magnification, $\times 500$ (all images).

TNF- α and IL-1 β were used as inflammatory stimuli, because both have been reported to stimulate PGE₂ production in different cell types, and have been implicated in the

pathogenesis of chronic inflammatory diseases, including periodontitis.^{48–51} Furthermore, because CD45⁺ cells were present in the tissues, we also investigated the effect of CD45-expressing cells (lymphocytes⁵²) on the production of PGE₂ and expression of mPGES-1 and COX-2 in the adherent cells, using cocultures.

We first investigated the most ubiquitous cells in gingival connective tissue, gingival fibroblasts, which were positively stained for mPGES-1, mPGES-2, cPGES, and COX-2 in immunohistochemical analysis of gingival tissue. We found that all three PGE synthases were also expressed in cultured primary gingival fibroblasts. Furthermore, in accord with our previous findings, the inducible enzymes mPGES-1 and COX-2 were up-regulated by the inflammatory cytokines TNF- α and IL-1 β in parallel with PGE₂ production.^{32,53} The increased PGE₂ production via enhanced expression of mPGES-1 and COX-2 observed in gingival fibroblasts is in accord with data on synovial, orbital, and gastric fibroblasts, as well as with our previous data on gingival fibroblasts.^{48,54–56} The present findings that gingival fibroblasts express increased amounts of mPGES-1 and COX-2, leading to enhanced PGE₂ production, together with reports on the significance of fibroblasts in chronic inflammatory conditions, highlight the importance of these cells in contributing to the pathogenesis of periodontitis.⁵⁷

The *in vitro* experiments using HUVECs as a model system revealed a basal expression of PGE synthases and COX-2, accompanied by PGE₂ production. Neither mPGES-1 nor mPGES-2 or cPGES were affected by cytokine treatment, in contrast to COX-2 increasing in parallel with PGE₂ production after TNF- α stimulation. Our results from this cell model system suggest that the inflammation-induced up-regulation of PGE₂ production in HUVECs may be dependent on increased expression of COX-2, rather than mPGES-1, as has also been reported for TNF- α -increased COX-2 expression in primary HUVECs.⁵⁸ Moreover, neither mPGES-1 nor COX-2 expression, nor PGE₂ production, increased in response to IL-1 β in a hybrid cell line between HUVECs and A549 cells, which is also in accord with our results.⁵⁹

Because vessels include both endothelial and smooth muscle cells, we also studied the expression of PGE synthases in relation to PGE₂ production in cultures of smooth muscle cells, using HASM cells as an *in vitro* model system. We have shown here that the expression of mPGES-1 and COX-2, as well as PGE₂ production, increased in response to TNF- α and IL-1 β stimulation in HASM cells. The expression of PGE synthases has not been previously reported in HASM cells, although COX-2 expression and PGE₂ production have been shown to be increased by IL-1 β , which is in accord with our results.⁶⁰ Similar to the present results, the expression of PGE synthases and the up-regulation of mPGES-1, COX-2, and PGE₂ in response to IL-1 β and TNF- α have also been demonstrated in primary human vascular smooth muscle cells.^{61,62} In one of those studies,⁶² the authors also demonstrated the lack of mPGES-1 induction in HUVEC cells, which is in accord with our results obtained from HUVEC cells. The substantial increase in expression of PGE synthases and subsequently increased PGE₂ pro-

duction observed in smooth muscle cells in the present study highlights these cells as potentially an important cell type contributing to the elevated levels of PGE₂ in gingival tissue.

In connective tissue, we showed that immune cells were positively stained for PGE synthases and COX-2. Monocytic cells have been previously studied in this regard, demonstrating that TNF- α and IL-1 β increase expression of mPGES-1 and COX-2 as well as PGE₂ production in the monocytic cell line THP-1.⁶³ Mast cells, on the other hand, constitute a part of the inflammatory response that has been poorly studied in regard to the expression of PGE synthases and PGE₂ production. Cultured human mast cells revealed a basal expression of PGE synthases and COX-2, as well as a basal production of PGE₂, but no increase in response to TNF- α or IL-1 β . Nonetheless, the basal PGE₂ production of these cells may still contribute to enhanced total PGE₂ production through an additive effect, because an increased number of mast cells has been reported in inflamed gingival tissue, compared with healthy gingival tissue.^{64,65} To our knowledge, there are a very limited number of studies investigating the expression of PGE₂-synthesizing enzymes or PGE₂ production in mast cells; however, COX-2 expression and PGE₂ production are increased by antigen stimulation and by the phospholipase A₂ activator melittin, respectively.^{66,67}

In addition to the PGE₂-synthesizing enzymes, the cytokine IL-1 β , apparently localized to CD45⁺ cells, was positively stained in the gingival tissue biopsies of patients with periodontitis. Our results demonstrating that the expression of mPGES-1 and COX-2 increased in fibroblasts and smooth muscle cells in response to the cytokines indicate that CD45⁺ cells, by producing cytokines such as IL-1 β ,⁶⁸ may regulate the production of PGE₂ in inflamed gingival tissue. These results are also in accord with our previous findings that fibroblasts cocultured with CD45-expressing lymphocytes⁵² increased their COX-2 expression as well as PGE₂ production,⁴⁰ suggesting a regulatory interplay among these cells contributing to the up-regulation of PGE₂ synthesis. The regulation of PGE₂ production may also include a network of signal transduction pathways within the cells involved. For instance, the c-jun N-terminal protein kinase and nuclear factor κ B signaling pathways are reported to play an important role in the regulation of mPGES-1 and COX-2 expression in response to the cytokines IL-1 β and TNF- α in gingival fibroblasts.^{69–71} These intracellular pathways have also been shown to play a significant role in smooth muscle cells, mast cells, and endothelial cells.^{72–74} Hence, the expression of PGE₂-synthesizing enzymes is most likely regulated by a complex network of cytokines and signal transduction pathways, as well as by cross-talk interactions among cells.

Whereas the mPGES-1 isoform has been more extensively studied, the roles of the PGE synthase isoforms mPGES-2 and cPGES have been debated. It has been suggested that mPGES-2 may substitute for mPGES-1 in case of nonfunctioning mPGES-1.⁷⁵ However, no up-regulation of mPGES-2 expression was observed under inflammatory conditions in murine mPGES-1 null fibroblasts

or macrophages.^{76,77} Moreover, we have previously reported that neither small interfering RNA (siRNA) knock-down nor chemical mPGES-1 inhibition affects the expression of cPGES or mPGES-2 in human gingival fibroblasts.³¹ Our experiments with mPGES-1 null gingival fibroblasts clearly demonstrated that mPGES-1 is important for basal production of PGE₂ and critical for PGE₂ production under inflammatory condition. The observed low but significant PGE₂ production in TNF- α -induced mPGES-1 null gingival fibroblasts might be due to COX-2 induction, as has been demonstrated for mouse mPGES-1 null macrophages.⁷⁶

Numerous cell types present in the gingival connective tissue may contribute to the increased PGE₂ production in gingival tissue and gingival fluid from patients with periodontitis. The cell types included in the present *in vitro* studies were shown to contribute to PGE₂ synthesis; however, the levels of PGE₂ released per cell varied among cell types (Figures 4–7). Of the cells included in the *in vitro* analyses, gingival fibroblasts and smooth muscle cells exhibited a higher basal PGE₂ production, and these two cell types also considerably increased PGE₂ production in response to cytokines, via the inflammation-induced enzymes mPGES-1 and COX-2. HUVECs and mast cells produced lower amounts of PGE₂, which in HUVECs was increased only in response to TNF- α , whereas PGE₂ production in mast cells was not stimulated by the cytokines. Our results suggest that, of the cells tested, the most prominent cellular sources of inflammation-induced PGE₂ are fibroblasts and smooth muscle cells. Furthermore, both endothelial cells (via COX-2) and mast cells may contribute to elevated PGE₂ levels. Nonetheless, one must keep in mind that the *in vitro* findings are not directly transferrable to the *in vivo* situation (an indication of the need for further studies).

In conclusion, the results presented here suggest that inflammation-induced PGE₂ production is regulated by mPGES-1 and COX-2 in fibroblasts and smooth muscle cells, which may contribute to the increased PGE₂ levels in the inflamed gingival tissue of patients with periodontitis. The present work contributes an initial step toward revealing the cellular source and regulation of increased PGE₂ in the inflammatory disease periodontitis.

Acknowledgments

We thank Dr. Per-Rune Wretling, D.D.S., for contributing to the collection of gingival biopsies, Ms. Caroline Nguyen for valuable help with cell cultures, Mr. Pierre Georgsson for contributing to illustrations, and Mr. Hernan Concha Quezada for help with flow cytometric analyses.

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