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Cyclooxygenase-2-derived prostaglandin E_2 is involved in vascular endothelial growth factor production in interleukin-1α-stimulated human periodontal ligament cells

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Background and Objective: Prostaglandin E_2, which exerts its actions via EP receptors (EP1, EP2, EP3 and EP4), is a bioactive metabolite of arachidonic acid produced by cyclooxygenase-1 and/or cyclooxygenase-2. Interleukin-1α induces prostaglandin E_2 production via cyclooxygenase-2 in human periodontal ligament cells. Vascular endothelial growth factor is a key regulator of physiologic as well as pathologic angiogenesis and has been indicated to be involved in the pathology of periodontal diseases. In the present study, we investigated whether interleukin-1α induced vascular endothelial growth factor production in human periodontal ligament cells and whether cyclooxygenase-2-derived prostaglandin E_2 regulated interleukin-1α-induced vascular endothelial growth factor production.

Material and Methods: Human periodontal ligament cells were obtained from extracted teeth of periodontally healthy subjects. After pre-incubation with a nonselective cyclooxygenase-1/2 inhibitor, indomethacin or a selective cyclooxygenase-2 inhibitor (NS-398), periodontal ligament cells were treated with or without interleukin-1α, prostaglandin E_2, various EP receptor agonists and dibutyryl cAMP (a cAMP analogue). The levels of vascular endothelial growth factor and prostaglandin E_2 in the culture supernatant were measured by enzyme-linked immunosorbent assay. The vascular endothelial growth factor mRNA expression was evaluated by semiquantitative reverse transcription–polymerase chain reaction.

Results: Interleukin-1α induced vascular endothelial growth factor production in a dose-dependent and time-dependent manner. The interleukin-1α-induced vascular endothelial growth factor mRNA and protein expression was inhibited to the same extent by indomethacin and NS-398. Indomethacin and NS-398 completely inhibited interleukin-1α-induced prostaglandin E_2 production. Exogenous prostaglandin E_2, butaprost (an EP2 receptor agonist) and dibutyryl cAMP

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abolished the inhibitory effect of indomethacin on interleukin-1α-induced vascular endothelial growth factor production.

Conclusion: We suggest that interleukin-1α induced vascular endothelial growth factor production via cyclooxygenase-2-derived prostaglandin E_2 in human periodontal ligament cells. The interleukin-1α/prostaglandin E_2 pathway might regulate vascular endothelial growth factor production in periodontal lesions.

Prostaglandins, including prostaglandin E_2, play an important role in regulating diverse cellular functions in physiological and pathological conditions (1,2). Prostaglandin E_2 exerts actions including vasodilation, the inhibition of lymphocyte proliferation and bone resorption (1–4). In response to various stimuli, arachidonic acid released from membrane phospholipids is metabolized to prostaglandins by cyclooxygenase. There are two types of cyclooxygenase: cyclooxygenase-1 and cyclooxygenase-2 (1,5). Cyclooxygenase-1 is expressed constitutively in many tissues and cells, and it supports the prostaglandin biosynthesis that is required for maintaining organ and tissue homeostasis. Cyclooxygenase-2 is induced after stimulation with pro-inflammatory molecules, such as interleukin-1, tumor necrosis factor-α and lipopolysaccharides, and is up-regulated during inflammation (2,5–7). The cellular effects of prostaglandin E_2 are mediated by the activation of multiple, functionally distinct, subtypes of prostaglandin E_2 receptors, which are designated EP1, EP2, EP3 and EP4 (3,8). EP1 receptors mediate an increase in the intracellular calcium levels. EP2 and EP4 receptors activate adenylate cyclase via a cholera toxin-sensitive, stimulatory G protein and elevate intracellular cAMP levels. EP2 receptors are sensitive to butaprost, an agent that selectively binds prostaglandin E_2 receptors, whereas EP4 receptors are not. Multiple isoforms of EP3 receptors with different C-terminal tails are generated by alternative mRNA splicing. EP3 receptor variants mediate several signaling pathways, including the inhibition and stimulation of adenylate cyclase, the activation of phospholipase C and the mobilization of intracellular calcium.

Periodontal disease is characterized by gingival inflammation and the loss of the periodontal attachment apparatus, including bone and periodontal ligament. Prostaglandin E_2 is believed to be involved in the pathogenesis of periodontal disease because it is a potent stimulator of bone resorption and is associated with attachment loss (4,9,10). It is very likely that cyclooxygenase-2 plays an important role in prostaglandin E_2 production in periodontal lesions (6). Cyclooxygenase-2 is responsible for prostaglandin E_2 production in human periodontal ligament cells challenged with interleukin-1α (7).

Angiogenesis contributes to an inflammatory pathology and it can maintain the inflammatory state by facilitating the migration of inflammatory cells to the inflammatory sites and supplying nutrients and oxygen to the granulation tissue (11). Vascular endothelial growth factor induces endothelial cell proliferation, angiogenesis and capillary permeability (12,13). Human vascular endothelial growth factor is a basic, heparin-binding and homodimeric 45-kDa glycoprotein that includes at least five different splicing variants: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (12,14). Among these proteins, $VEGF_{165}$ is the most abundant and widespread isoform *in vivo* and has the most potent biological activity, in contrast to $VEGF_{121}$, which is considered to be a soluble protein, and $VEGF_{189}$ and $VEGF_{206}$, which have been shown to be almost completely sequestered in the extracellular matrix (15).

Interleukin-1 (interleukin-1α and interleukin-1β), a pro-inflammatory cytokine, is thought to be involved in the pathogenesis of periodontal diseases (16). Interleukin-1 can stimulate the production of pro-inflammatory mediators (including prostaglandin E_2), enhance osteoclast formation and induce matrix metalloproteinase expression. The levels of interleukin-1α in the gingival crevicular fluid of periodontally diseased patients are highly elevated compared with those in periodontally healthy patients (17,18). Furthermore, interleukin-1α mRNA is expressed in macrophages of human inflamed gingival tissues (19).

Vascular endothelial growth factor has been found to be present at higher levels in inflamed gingiva than in normal gingiva (20–22). However, the mechanism by which vascular endothelial growth factor production is regulated in periodontal lesions remains unclear. It has been reported that vascular endothelial growth factor is produced by human periodontal ligament cells stimulated with mechanical stress and tumor necrosis factor-α (23,24). Interleukin-1 is a potent stimulator of vascular endothelial growth factor production in several types of cells, including synovial fibroblasts, gastric fibroblasts and mesangial cells, and prostaglandin E_2 is involved in interleukin-1-induced vascular endothelial growth factor generation in gastric fibroblasts and synovial fibroblasts, but not in mesangial cells (25–27). Therefore, the involvement of prostaglandin E_2 in vascular endothelial growth factor production may be dependent on cell types. The present study examined whether interleukin-1α induced vascular endothelial growth factor in cultured human periodontal ligament cells, and whether cyclooxygenase-2-dependent prostaglandin E_2 was involved in interleukin-1α -induced vascular endothelial growth factor production.

Material and methods

Cell culture

Human periodontal ligament cells were established from explants of periodontal ligaments that were obtained from premolars or third molars extracted from periodontally healthy subjects with no clinical attachment loss and no radiographic bone loss, and who had no systemic diseases. The study protocol satisfied the ethical standards of Tokyo Medical and Dental University, and informed consent was obtained from all the subjects. The periodontal ligament tissues were scraped from the middle third of the root surface using a sterile scalpel. The tissue was minced and transferred to sterile culture dishes. The explants were cultured in α-minimum essential medium containing 10% fetal bovine serum (Bioserum, Canterbury, Vic., Australia) in the presence of 100 U/mL of penicillin (Sigma Chemical Co., St Louis, MO, USA) and 100 μg/mL of streptomycin (Sigma Chemical Co.) in a humidified atmosphere of 5% CO_2 in air at 37°C. When the cells that grew out of the explants reached confluence, they were subcultured after treatment with 0.05% trypsin and 0.02% EDTA solution. The cells were used for the experiments between the fifth and tenth passages.

Cell stimulation

Periodontal ligament cells were seeded in 96-well plastic plates in 200 μL of α-minimum essential medium supplemented with 10% fetal bovine serum. After reaching confluence, the cells were stimulated with vehicle or human recombinant interleukin-1α (Sigma Chemical Co.), prostaglandin E_2, 17-phenyl-ω-trinor prostaglandin E_2 (an EP1 receptor agonist; Cayman Chemicals, Ann Arbor, MI, USA), butaprost (an EP2 receptor agonist; Cayman Chemicals), ONO-AP-324 (an EP3 receptor agonist; ONO Pharmaceuticals, Tokyo, Japan), ONO-AE1-329 (an EP4 receptor agonist; ONO Pharmaceuticals), dibutyryl cAMP (WAKO, Tokyo, Japan), indomethacin (WAKO) or NS-398 (Cayman Chemicals) in the combinations and concentrations indicated. Indomethacin and NS-398 were added 30 min before stimulation with interleukin-1α. Conditioned media were collected after the indicated incubation time and stored at −80°C until analysis. The supernatant was harvested at selected time-points after stimulation and then stored at −20°C until analysis.

Assay of vascular endothelial growth factor and prostaglandin E_2 levels

$VEGF_{165}$ and prostaglandin E_2 levels in the conditioned media collected from control or stimulated cells were determined using commercially available enzyme-linked immunosorbent assay kits (VEGF: BioSource International Camarillo, CA, USA; prostaglandin E_2: Amersham Pharmacia Biotech, Bucks., UK).

Semiquantitative reverse transcription–polymerase chain reaction

Six hours after stimulation, total RNA was extracted using RNeasy Kits (Qiagen, Sciences, Maryland, MD, USA), according to the manufacturer's instructions. A first-stand cDNA was synthesized by the reverse transcription of 5 μg of total RNA in the presence of oligo(dt) and reverse transcriptase with SuperScript™ III First-strand Super-Mix (Invitrogen, Carlsbad, CA, USA). The specific primer pairs used to amplify vascular endothelial growth factor isoforms (including $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$) and glyceraldehyde-3-phosphate dehydrogenase as an internal standard were selected according to previous reports (28,29). The primers were as follows: vascular endothelial growth factor sense primer, 5′-CTGCTGTCTTGGGTGCATTG-3′ and antisense primer, 5′-TCACCGCC-TCGGCTTGTCACA-3′; and glyceraldehyde-3-phosphate dehydrogenase sense primer, 5′-ACCACAGTCCAT-GCCATCAC-3′ and antisense primer, 5′-TCCACCACCCTGTTGCTGTA-3′. The 20-μL polymerase chain reaction (PCR) reaction mixture contained 1 μL of cDNA, 0.4 μL of dNTPs, 2 μL of primers and 0.4 μL of Taq DNA polymerase (Clontech, a TAKARA BIO company, Mountain View, CA, USA) in PCR buffer containing 40 mM Tricine-KOH, 15 mM KOAc and 3.5 mM $Mg(OAc)_2$. The PCR amplification for vascular endothelial growth factor consisted of 35 cycles of denaturation at 94°C for 20 s, annealing at 64°C for 30 s and extension at 72°C for 1 min, and the PCR amplification for glyceraldehyde-3-phosphate dehydrogenase consisted of 32 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. The PCR products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide. The intensities of the obtained bands were determined using an imaging system (SYGENE Bio Imaging System; SYGENE, Cambridge, UK).

Statistical analysis

Data are expressed as the mean ± standard deviation. Data were subjected to a one-way analysis of variance using the STATVIEW software program. Fisher's protected least significance test was used in the *post hoc* comparisons of specific groups.

Results

Effect of interleukin-1α on vascular endothelial growth factor and prostaglandin E_2 production

The production of $VEGF_{165}$ from periodontal ligament cells stimulated with interleukin-1α was examined. As shown in Fig. 1A, periodontal ligament cells were treated with various doses of interleukin-1α of between 0.02 and 2 ng/mL. The level of vascular endothelial growth factor was highest when cells were incubated with 0.2 ng/mL of interleukin-1α for 48 h. Figure 1B shows that periodontal ligament cells stimulated with 0.2 ng/mL of interleukin-1α produced vascular endothelial growth factor in a time-dependent manner.

To examine the involvement of endogenous prostaglandins on vascular endothelial growth factor secretion

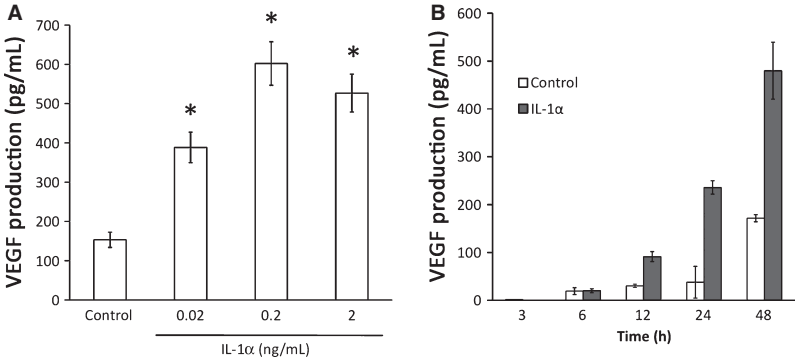

Fig. 1. Dose dependency (A) and time dependency (B) of the effect of interleukin-1α on vascular endothelial growth factor production in human periodontal ligament cells. Periodontal ligament cells were stimulated with various doses of interleukin-1α for 48 h (A) or stimulated with 0.2 ng/mL of interleukin-1α for the indicated time-periods (B). The vascular endothelial growth factor levels in the culture media were measured using enzyme-linked immunosorbent assay. Data are reported as the means ± standard deviation ($n = 4$). *Significantly different from the control ($p < 0.0001$). IL-1 α, interleukin-1α; VEGF, vascular endothelial growth factor.

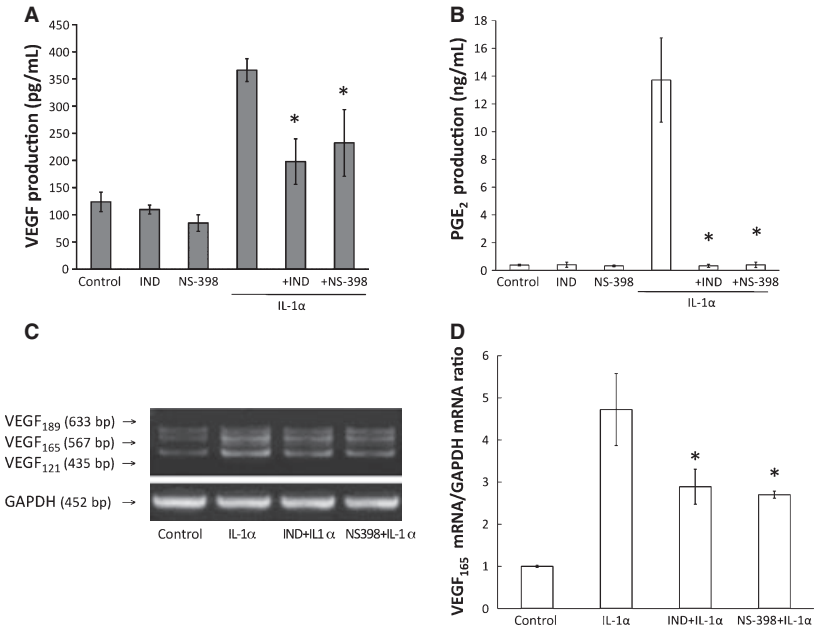

Fig. 2. The effect of indomethacin and NS-398 on prostaglandin E_2 production (A) and on vascular endothelial growth factor protein (B) and mRNA (C, D) expression in interleukin-1α-stimulated human periodontal ligament cells. (A, B) The periodontal ligament cells were stimulated with 0.2 ng/mL of interleukin-1α in the presence or absence of 1 μM NS-398 and 1 μM indomethacin for 48 h. The vascular endothelial growth factor and prostaglandin E_2 levels in the culture media were measured using enzyme-linked immunosorbent assay. The values are the means ± standard deviation ($n = 4$). *Significantly different from interleukin-1α ($p < 0.0001$). (C, D) The periodontal ligament cells were stimulated with 0.2 ng/mL of interleukin-1α in the presence or absence of 1 μM indomethacin and 1 μM NS-398 for 6 h. After incubation, total RNA was extracted and vascular endothelial growth factor mRNA expression was examined by reverse transcription–polymerase chain reaction. The ratio of vascular endothelial growth factor mRNA expression to glyceraldehyde-3-phosphate dehydrogenase mRNA expression was calculated using an imaging system. The ratio of control was normalized to 1. The experiment was performed three times. Data are depicted as the means ± standard deviation ($n = 3$). *Significantly different from interleukin-1α ($p < 0.0001$). IL-1 α, interleukin-1α; IND, indomethacin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor.

in periodontal ligament cells stimulated with interleukin-1α, the effect of indomethacin, a nonselective cyclooxygenase1/cyclooxygenase-2 inhibitor, and NS-398, a selective cyclooxygenase-2 inhibitor, on vascular endothelial growth factor and prostaglandin E_2 production was investigated by interleukin-1α-stimulated periodontal ligament cells. Figure 2A shows that 1 μM NS-398 reduced interleukin-1α-induced vascular endothelial growth factor production about 50% as effectively as 1 μM indomethacin. However, indomethacin and NS-398 completely inhibited the production of prostaglandin E_2 by periodontal ligament cells (Fig. 2B). A reverse transcription-

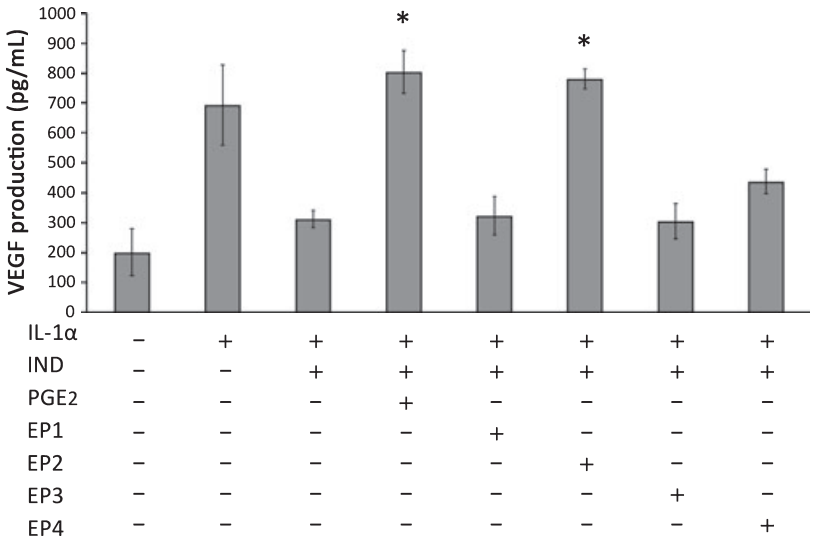

Fig. 3. The effect of various EP receptor agonists on interleukin-1α-induced vascular endothelial growth factor production in human periodontal ligament cells. The cells were stimulated with 0.2 ng/mL of interleukin-1α in the presence of 1 μM indomethacin and 1 μM prostaglandin E_2, 1 μM 17-phenyl-ω-trinor prostaglandin E_2 (EP1), 1 μM butaprost (EP2), 1 μM ONO-AP-324 (EP3), or 1 μM ONO-AE1-329 (EP4). After 48 h, the vascular endothelial growth factor levels in the culture media were measured using enzyme-linked immunosorbent assay. The values are the means ± standard deviation ($n = 4$). *Significantly different from indomethacin + interleukin-1α ($p < 0.0001$). IL-1 α, interleukin-1α; IND, indomethacin; PGE_2, prostaglandin E_2, VEGF, vascular endothelial growth factor.

PCR analysis showed $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ mRNA expression in human periodontal ligament cells (Fig. 2C). The expression of $VEGF_{165}$ mRNA was enhanced in interleukin-1α-stimulated cells in comparison to that in unstimulated cells, and treatment of interleukin-1α-stimulated cells with indomethacin and NS-398 reduced the expression of $VEGF_{165}$ mRNA (Fig. 2D).

Effect of EP receptor agonists on interleukin-1α-induced vascular endothelial growth factor production

Prostaglandin E_2 acts through the activation of EP receptors. We examined the effect of various EP receptor agonists on interleukin-α-induced vascular endothelial growth factor production, in order to test which EP receptors were involved in prostaglandin E_2 regulation of interleukin-1α-induced vascular endothelial growth factor production. Figure 3 demonstrates that butaprost, a selective EP2 receptor agonist, significantly increased the expression of vascular endothelial growth factor. However, 17-phenyl-ω-trinor prostaglandin E_2 (a selective EP1 agonist), ONO-AP-324 (a selective EP3 agonist) and ONO-AE1-329 (a selective EP4 agonists) did not significantly affect the production of vascular endothelial growth factor (Fig. 3).

Effect of dibutyryl cAMP on vascular endothelial growth factor production

The effect of cAMP on vascular endothelial growth factor production in periodontal ligament cells stimulated with interleukin-α was investigated because EP2 receptors signal via cAMP. The addition of dibutyryl cAMP, a cAMP analogue, enhanced vascular endothelial growth factor production by periodontal ligament cells treated with interleukin-1α and indomethacin (Fig. 4). Dibutyryl cAMP alone did not significantly affect

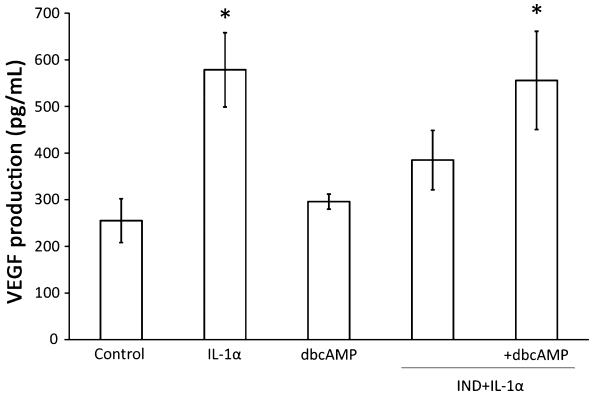

Fig. 4. The effect of dibutyryl cAMP on vascular endothelial growth factor production in human periodontal ligament cells. Cells were stimulated with vehicle or with 0.2 ng/mL of interleukin-1α and 1 μM indomethacin in the presence or absence of 10 μM dibutyryl cAMP for 48 h. The vascular endothelial growth factor levels in the culture media were measured using enzyme-linked immunosorbent assay. The values are the means ± standard deviation ($n = 4$). *Significantly different from indomethacin + interleukin-1α ($p < 0.005$). dbcAMP, dibutyryl cAMP; IL-1 α, interleukin-1α; IND, indomethacin; VEGF, vascular endothelial growth factor.

the production of vascular endothelial growth factor (Fig. 4).

Discussion

The present study demonstrated that interleukin-1α induced $VEGF_{165}$ mRNA and protein expression via cyclooxygenase-2 in human periodontal ligament cells. The $VEGF_{165}$ isoform was studied because it is the most biologically active isoform, compared with $VEGF_{121}$ and $VEGF_{189}$ isoforms (15). Interleukin-1α-treated periodontal ligament cells generated vascular endothelial growth factor in a dose-dependent and time-dependent manner. The treatment of the cells with indomethacin, a nonspecific cyclooxygenase-1/cyclooxygenase-2 inhibitor, and NS-398, a cyclooxygenase-2 inhibitor, decreased, by about 50%, interleukin-1α-induced vascular endothelial growth factor mRNA and protein expression (Fig. 2), and the addition of exogenous prostaglandin E_2 restored the inhibitory effect of indomethacin on interleukin-1α-induced vascular endothelial growth factor production (Fig. 3), suggesting that interleukin-1α-induced vascular endothelial growth factor production is up-regulated by cyclooxygenase-2-derived prostaglandin E_2. The finding that the inhibition of interleukin-1α-induced vascular endothelial growth factor production by indomethacin and NS-398 was not complete, although interleukin-1α-induced prostaglandin E_2 generation was completely down-regulated, is consistent with the data reported in gastric fibroblasts and synovial fibroblasts (25,26). It suggests that regulatory mechanisms independent of prostaglandin E_2 may also be involved in the vascular endothelial growth factor release by interleukin-1α-stimulated human periodontal ligament cells.

Prostaglandin E_2 exerts its biological actions via specific prostaglandin E receptors on target cells and, based on ligand-binding selectivity and signaling pathway activation, prostaglandin E_2 receptors are classified as EP1, EP2, EP3 and EP4 subtypes (3,8). EP1, EP2 and EP4 receptors are expressed in human periodontal ligament cells (30). Butaprost, a selective EP2 agonist, restored indomethacin inhibition of interleukin-1α-induced vascular endothelial growth factor production, whereas EP1, EP3 and EP4 receptor agonists did not. The activation of EP2 receptors is linked to intracellular cAMP elevation (3,8) and the addition of dibutyryl cAMP abolished the inhibitory effect of indomethacin on interleukin-1α-induced vascular endothelial growth factor production (Fig. 4). Prostaglandin E_2 regulates interleukin-1α-induced vascular endothelial growth factor production in human synovial fibroblasts through EP2 and EP4 receptors (26). It is very likely that EP2 receptors participate in the regulation of interleukin-1α-induced vascular endothelial growth factor production via cAMP-dependent pathways in human periodontal ligament cells. However, the signaling pathways after cAMP accumulation, leading to the prostaglandin E_2 regulation of interleukin-1α-induced vascular endothelial growth factor production, remain to be examined.

Higher levels of vascular endothelial growth factor are detectable in periodontally diseased gingival tissue compared with normal gingival tissue, thus suggesting the possible contribution of vascular endothelial growth factor to the progression of periodontal disease (20). Furthermore, the gingival crevicular fluid vascular endothelial growth factor levels are higher in periodontal sites than in healthy sites (21,22). *In vitro* studies have shown that human periodontal ligament cells produce vascular endothelial growth factor in response to stimuli, including mechanical stress and tumor necrosis factor-α (23,24). Vascular endothelial growth factor is a potent stimulator of vascular permeability and angiogenesis (12,13). The present study showed that human periodontal ligament cells produced vascular endothelial growth factor in response to interleukin-1α. Although the role of vascular endothelial growth factor produced by periodontal ligament cells remains unclear, vascular endothelial growth factor produced by periodontal ligament cells challenged with pro-inflammatory stimuli, including interleukin-1α, may be involved in vascular permeability and angiogenesis in periodontal lesions. Furthermore, it has been shown that vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption (31), and it has been demonstrated that the local administration of vascular endothelial growth factor during tooth movement enhances the number of osteoclasts and the rate of tooth movement (32). Therefore, vascular endothelial growth factor produced by periodontal ligament cells may be involved in bone resorption.

In conclusion, we suggest that human periodontal ligament cells produce vascular endothelial growth factor in response to interleukin-1α, and that the vascular endothelial growth factor production is up-regulated via EP2 receptors by cyclooxygenase-2-dependent prostaglandin E_2. The interleukin-1α/prostaglandin E2 pathway might be involved in vascular endothelial growth factor production in periodontal lesions. Further *in vivo* studies are necessary to validate this hypothesis.

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