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Irsogladine maleate abolishes the increase in interleukin-8 levels caused by outer membrane protein 29 from *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* through the ERK pathway in human gingival epithelial cells

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Background and Objective: Irsogladine maleate (IM) suppresses the increase in interleukin (IL)-8 production induced by outer membrane protein (OMP) 29 from *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* in cultures of human gingival epithelial cells (HGEC). However, how IM suppresses the OMP29-induced increase in IL-8 expression remains unknown. In this study, we focused on intracellular signaling pathways to elucidate the mechanism behind the suppression.

Material and Methods: HGEC, which had been pretreated with inhibitors of intracellular signaling molecules, were exposed to OMP29 (1 μg/mL) with or without IM (1 μM). IL-8 expression at the mRNA and protein levels was examined by real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Extracellular signal-regulated kinase (ERK) activity was measured with a p44/42 mitogen-activated protein kinase assay kit.

Results: An ERK inhibitor, PD98059, as well as IM, obviated the OMP29-induced increase in IL-8 levels in HGEC. A Jun kinase inhibitor, SP600125, and a nuclear factor κB inhibitor, PDTC, did not influence the OMP29-induced increase in IL-8 mRNA expression. The OMP29 stimulated phosphorylation of ERK in HGEC. Irsogladine maleate inhibited the phosphorylation.

Conclusion: The suppression of the phosphorylation of ERK by IM in HGEC culminates in inhibition of the OMP29-induced increase in IL-8.

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Periodontitis is an inflammatory lesion caused by the colonization of periodontopathogenic bacteria in the gingival sulcus. The gingival epithelium is the primary barrier facing the bacterial challenge. Epithelial cells function as a mechanical barrier through cell–cell junction complexes, for example, tight junctions and gap junctions, against invasion from pathogenic organisms (1–5). Bacterial attack on epithelial cells induces the production of inflammatory cytokines such as interleukin (IL)-8 and IL-6 (1). Thus, the interaction between epithelial cells and bacteria plays an important role in the initial stage of inflammation.

Cyclic AMP stimulates gap junction intercellular communication by stimulating connexin protein levels and phosphorylating connexins (6). Irsogladine maleate (IM) enhances gap junction intercellular communication in cultured rabbit gastric epithelial cells and pancreatic cancer cells through augmentation of the cyclic AMP (7,8). In addition, IM increases levels of connexin 32, which is related to the repair of gastric ulcers (9). IM has been used clinically as an anti-gastric ulcer agent. We have investigated the effect of IM on the interaction between human gingival epithelial cells (HGEC) and periodontopathogenic bacteria to clarify whether IM could help to prevent periodontal disease. Exposure of HGEC to outer membrane protein (OMP) 29 from *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* or whole live *A. actinomycetemcomitans* increases IL-8 secretion and inhibits gap junctional intercellular communication (4). IM obviates the increase in IL-8 levels and recovers the reduction of gap junctional intercellular communication in HGEC stimulated by OMP29 or *A. actinomycetemcomitans* (4). Furthermore, regulation of IL-8 levels by IM causes abrogation of the reduction of gap junctional intercellular communication (5). Thus, IL-8 is a key regulator for inflammation and the cell–cell junctional complex. However, the mechanism whereby IM suppresses the OMP29-induced increase in IL-8 expression remains unknown.

Mitogen-activated protein (MAP) kinases play a central role in mediating intracellular signal transduction and regulating cell functions, for example, the expression of human β-defensin or plasminogen activator, in response to various extracellular stimuli in HGEC (10–12). Three distinct mammalian MAP kinases have been indentified: extracellular signal-regulated kinase (ERK or p44/42 MAP kinase), c-Jun kinase or the stress-activated protein kinase (c-JNK or SAP kinase), and p38 MAP kinase. Each kinase apparently signals to activate cell functions (13). Cyclic AMP and protein kinase A are known to be intracellular signaling molecules regulated by IM (7). In the present study, to find the intracellular signaling molecules that IM acts on, in addition to cyclic AMP and protein kinase A, we examined the involvement of MAP kinase in the inhibition of the OMP29-induced increase in IL-8 levels by IM.

Material and methods

Preparation of cells

Healthy gingival tissues, which had been surgically dissected during the extraction of wisdom teeth and are usually discarded, were collected with the patients' informed consent. Informed consent was obtained under a protocol approved by the Ethics Committee of the Hiroshima University (Hiroshima, Japan) Faculty of Dentistry. HGEC were isolated as previously described (1,3). Briefly, the gingiva was treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C and divided into the epithelium and connective tissues. The HGEC suspension was centrifuged at 120 *g* for 5 min, and the pellet was suspended in Humedia-KB2 medium (pH 7.4; Kurabo, Osaka, Japan) containing 10 μg/mL insulin, 5 μg/mL transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol, 10 nM sodium selenite, 50 μg/mL bovine pituitary extract, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 ng/mL amphotericin B (medium A). The cells were seeded in 60 mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO_2–95% air at 37°C. When the cells reached subconfluence, they were harvested and subcultured.

Preparation of OMP29

The OMP29 was purified using a disk preparative electrophoresis apparatus (NA-1800 type; Nippon Eido, Tokyo, Japan) according to the manufacturer's protocol. The OMPs were prepared by methods described elsewhere (14). Sarcosyl-insoluble OMPs were finally solubilized with 1% sodium dodecyl sulphate (SDS) in 10 mM sodium phosphate buffer (pH 6.8). The OMPs were resolved on a 12% polyacrylamide gel by electrophoresis with a constant voltage (80 V). After the blue dye reached the bottom of the gel, sequen-

tial volumes of every 10 drops (200 μL) were collected with a fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). A small portion of each fraction (total ca. 100 fractions) was electrophoresed on a 12% polyacrylamide gel and then stained with Coomassie brilliant blue. Fractions showing a single band of OMP29 with the Coomassie brilliant blue stain were collected and concentrated with Centriprep (Millipore, Bedford, MA, USA) to obtain a final concentration of 1 mg/mL as a stock solution.

Determination of IL-8 mRNA

Our preliminary studies revealed that OMP29 at 1 μg/mL increases IL-8 in a time-dependent manner until 24 h. In a dose–response experiment with IM at concentrations of 0.01–10 μM, 1 μM IM the most strongly abolished the increase in IL-8 induced by 1 μg/mL of OMP29 in HGEC (4). Therefore, in the following experiments, we have examined the effect of various concentrations of inhibitors or 1 μM of IM on the IL-8 expression induced by 1 μg/mL of OMP29 at 6 and 12 h for mRNA levels and at 12 and 24 h for protein levels. HGEC in cultures at the fourth passage were harvested, seeded at a density of 10×10^4 cells per 60 mm plastic tissue culture plate coated with type I collagen, and maintained in 5 mL of medium A. After 10 days of culture, these cells were washed three times with phenol red-free Hank's solution (pH 7.4). To study the involvement of MAP kinase and nuclear factor κB (NF-κB), SB203580 (p38 MAP kinase inhibitor; Calbiochem, La Jolla, CA, USA), PD98059 (ERK inhibitor; Calbiochem), SP600125 (JNK inhibitor; Calbiochem) and PDTC (NF-κB inhibitor; Sigma, St Louis, MO, USA) were used. Confluent HGEC were pretreated with SB203580 (1 μM), PD98059 (2 μM), SP600125 (2 μM) or PDTC (10 μM) for 1 h and then exposed to OMP29 (1 μg/mL) for 6 or 12 h before the end of incubation on day 11 in 5 mL of Humedia-KB2 medium containing 10 μg/mL insulin, 5 μg/mL transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 nM sodium selenite (medium B). Total RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) and quantified by spectrometry at 260 and 280 nm. First standard cDNA synthesis was performed with 1 μg of total RNA extract in a total volume of 20 μL (Roche, Tokyo, Japan). Real time polymerase chain reaction (PCR) was performed with a Lightcycler system using SYBER green (Roche). The sense primers and anti-sense primers for human IL-8 mRNA are listed in Table 1.

Table 1. Primers and probes for real-time PCR used in this study

IL-8
Sense: 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′
Anti-sense: 5′-TCT CAG CCC TCT TCA AAA ACT TCT C-3′
GAPDH
Sense: 5′-AAC GTG TCA GTG GTG GAC CTG-3′
Anti-sense: 5′-AGT GGG TGT CGC TGT TGA AGT-3′

ELISA for IL-8

HGEC were cultured as described in the subsection entitled *Determination of IL-8 mRNA*. Confluent HGEC, which had been pretreated with PD98059 (2 μM) or IM (Nippon Sinyaku, Kyoto, Japan) at 1 μM for 1 h were exposed to OMP29 (1 μg/mL) for 12 or 24 h before the end of incubation on day 11 in 5 mL of medium B. The concentration of IL-8 in the medium was determined using an IL-8 ELISA kit (Biosource International, Camarillo, CA, USA).

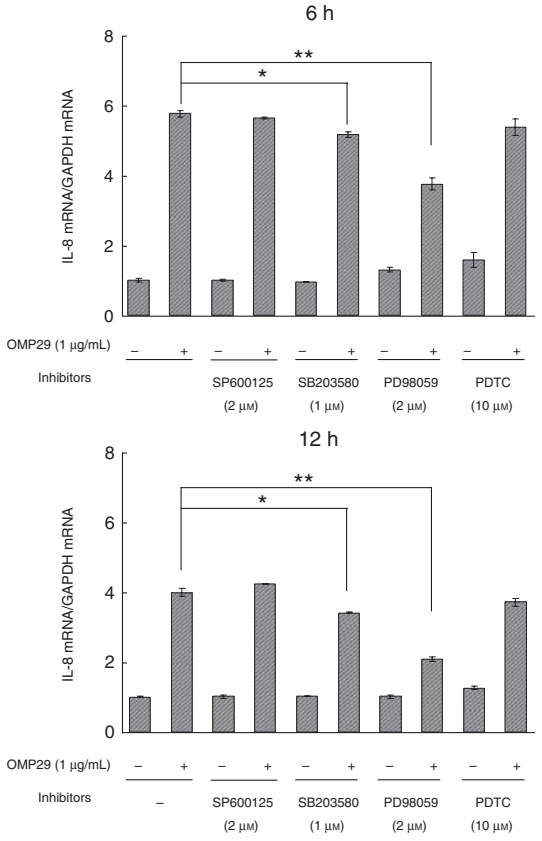

Fig. 1. Effects of MAP kinase inhibitors or a NF-κB inhibitor on IL-8 mRNA expression in HGEC stimulated by OMP29. Confluent HGEC were pretreated with SB203580 (1 μM), PD98059 (2 μM), SP600125 (2 μM) or PDTC (10 μM) for 1 h and subsequently exposed to OMP29 at 1 μg/mL for 6 or 12 h before the end of incubation on day 11 in 5 mL of medium B. IL-8 mRNA levels were analyzed by real-time PCR. Values are means ± SD for three cultures. $*p < 0.05$; $**p < 0.01$.

The ERK activity and total ERK expression

To examine ERK activity in HGEC stimulated by OMP29 in the presence or absence of IM, we used a kinase assay kit (Cell Signaling, Beverly, MA, USA). Briefly, HGEC were stimulated with OMP29 (1 μg/mL) in the presence or absence of IM at 1 μM. They were then lysed in 500 μL of cell lysis buffer [20 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and sonicated four times for 5 s each time on ice. The samples were microcentrifuged at 15,000 *g* for 10 min at 4°C, and the supernatant was transferred to a new tube. For the assay of ERK activity, the supernatants were immunoprecipitated with anti-p44/42 (Thr202/Tyr204) monoclonal antibody. After incubation overnight, beads were microcentrifuged for 30 s at 4°C. The collected beads were washed four times and suspended in 50 μL of kinase buffer supplemented with 200 μM ATP and 2 μg of ETs-like protein (Elk)-1 fusion protein for p44/42, before being incubated for 30 min at 30°C. The reaction was terminated by adding 3 × SDS sample buffer. From each sample, 30 μL was then loaded onto a 10% SDS-polyacrylamide gel. After transfer, polyvinylidene fluoride (PVDF) membranes were blocked in Tris-buffered saline (TBS)-Tween (TBS-T) with 5% skimmed milk for 3 h at room temperature. The membranes were then incubated with primary antibody (rabbit anti-phosphor-Elk-1 antibody; 1:1000) diluted in primary antibody buffer (TBS-T, 5% bovine serum albumin) overnight at 4°C. Membranes were washed and incubated with horseradish peroxide-conjugated anti-rabbit secondary antibody (1:2000 dilution) in blocking buffer. After washing, proteins were detected using an enhanced chemiluminescence system (Cell Signaling). For total ERK, the supernatant was mixed with 3 × SDS sample buffer, and total ERK expression was detected by Western blotting using rabbit polyclonal anti-p44/42 MAP kinase antibody (Cell Signaling). The band density was analyzed by using National Institutes of Health (NIH) image software.

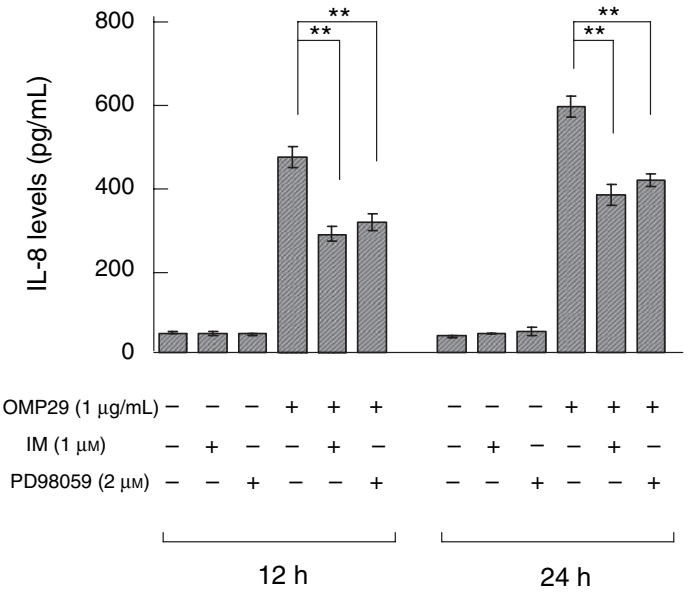

Fig. 2. Effect of MAP kinase inhibitor on IL-8 levels in HGEC stimulated by OMP29. Confluent HGEC were pretreated with PD98059 (2 μM) or IM (1 μM) for 1 h and exposed to OMP29 at 1 μg/mL for 12 or 24 h before the end of incubation on day 11 in 5 mL of medium B. IL-8 levels were determined using an IL-8 ELISA kit. Values are means ± SD for three cultures. $^{**}p < 0.01$.

Statistical analysis

Comparisons between groups in the experiments of an effect of each MAP kinase inhibitor on IL-8 expression at mRNA and protein levels in HGEC stimulated by OMP29 were analyzed with Student's *t*-test. The difference was considered significant if the *p*-value was < 0.05.

Results

The increase in IL-8 mRNA levels caused by OMP29 was suppressed by pretreatment with PD98059 at 2 μM for 6 h. At 1 μM, SB203580 also inhibited the increase. Although there was a significant difference in IL-8 mRNA levels between no pretreatment and pretreatment with SB203580, the decrease caused by SB203580 was much less pronounced than that induced by PD98059. In contrast, pretreatment with SP600125 and PDTC did not influence the IL-8 mRNA expression (Fig. 1). The incubation of HGEC with OMP29 for 12 h produced a similar result to that for 6 h. Furthermore, PD98059, as well as IM, diminished the OMP29-induced increase in IL-8 levels at 12 and 24 h, although PD98059 alone or IM alone did not affect the IL-8 levels (Fig. 2). The exposure of HGEC to OMP29 induced phosphorylation of ERK at 15 and 30 min, and the addition of IM to some extent inhibited the OMP29-induced phosphorylation of ERK. Neither OMP29 nor IM affected total ERK expression (Fig. 3).

Discussion

In the present study, we have, for the first time, demonstrated that an ERK inhibitor blocked the OMP29-induced increase in IL-8 levels in HGEC, suggesting that OMP29 activates ERK to increase IL-8 levels in HGEC. The ERK inhibitor blocked the *A. actinomycetemcomitans* or *A. actinomycetemcomitans*-derived OMP100-induced increase in human β-defensin 2 mRNA levels in HGEC, whereas an inhibitor of NF-κB did not (15). Heat shock protein 60 purified from *A. actinomycetemcomitans* stimulated cell proliferation

through ERK phosphorylation in human skin epithelial cells (16). Thus, ERK has various functions in epithelial cells stimulated by *A. actinomycetemcomitans*.

IL-8 expression induced by various bacterial stimuli in epithelial cells also occurs through ERK. For example, an infection of *Helicobacter pylori* increased IL-8 production through the ERK signaling pathway in human gastric epithelial cells and human esophageal cells (17–19). In contrast, the p38 pathway was involved in IL-8 production induced by *H. pylori* in human gastric epithelial cells (20). Torok *et al*. have shown that *H. pylori* promotes the secretion of IL-8 in human embryonic kidney cells through a dual mechanism: a Toll-like receptor 2/5-independent component involving the activities of JNK and ERK and a Toll-like receptor 2/5-dependent component that requires p38 activity (21). The present and preliminary studies show that p38 MAP kinase inhibitor, as well as ERK inhibitor, suppressed the expression of IL-8 mRNA in HGEC exposed to OMP29 or heat-killed *A. actinomycetemcomitans*, although the effect of the p38 MAP kinase inhibitor was less than that of the ERK inhibitor. In addition, OMP29 enhanced the phosphorylation of the p38 MAP kinase in HGEC (unpublished data, Kishimoto, Fujita, Shiba). These findings demonstrate that p38 MAP kinase is also involved in the regulation of IL-8 expression induced by OMP29. Furthermore, ERK and p38 MAP kinase might be involved in the increase in production of IL-8 induced by other components from *A. actinomycetemcomitans* in HGEC.

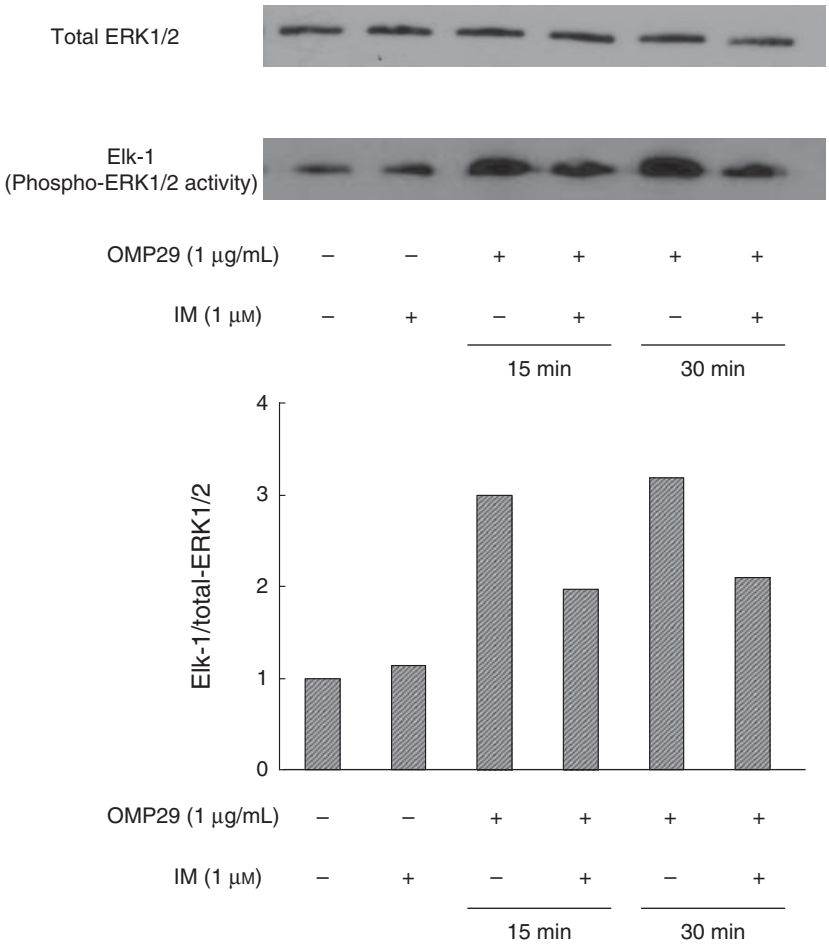

Fig. 3. Effect of IM on OMP29-induced phosphorylation of ERK in HGEC. Confluent HGEC were exposed to OMP29 at 1 μg/mL in the absence or presence of IM at 1 μM for 30 min before the end of incubation on day 11. HGEC were collected, resuspended in 500 μL of cell lysis buffer, and sonicated as described in the Materials and methods section. Total ERK expression was determined by Western blotting using anti-p44/42 MAP kinase antibody. The bands were detected with horseradish peroxidase-linked secondary antibody and enhanced chemiluminescent (ECL). To determine ERK activity, the sonicated samples were immunoprecipiated using immunobilized p44/42 (Thr202/Try204) monoclonal antibody. The immunoprecipitated samples were supplemented with Elk-1 fusion protein. Then, Western blotting was done using anti-phospho-Elk-1 antibody. The bands were detected with horseradish peroxidase-linked secondary antibody and ECL. The band density was analyzed by using NIH image software. Figure 3 is representative of three experiments.

Regarding the signal transduction during an IM-induced cellular response, the involvement of cyclic AMP and protein kinase A has been reported. IM enhanced gap junctional intercellular communication by increasing cyclic AMP levels in rabbit gastric epithelial cells (8). IM also upregulated gap junctional intercellular communication between human pancreatic cancer cells via protein kinase A (7). In addition, IM countered the reduction in OMP29-induced gap junctional intercellular communication in HGEC by upregulating the expression of cyclic AMP (4). In the present study, IM prevented the OMP29-induced increase in IL-8 by suppressing phosphorylation of ERK. Since some reports have shown that cyclic AMP stimulates phosphorylation of ERK in human epithelial cells (22–24), it is questionable whether cyclic AMP is involved in the OMP29-induced enhancement of ERK's phosphorylation in HGEC. However, our previous studies have shown that the increase in cyclic AMP caused by IM did not influence the suppression of the increase in IL-8 levels, although it caused a recovery of the OMP29-induced reduction in gap junctional intercellular communication (4). From these results, the abolition of the increase in IL-8 by IM is dependent on ERK but independent of cyclic AMP.

There is an argument about the contamination of lipopolysaccharide (LPS) or other factors in the purified OMP29. Our preliminary studies showed that commercially available

LPS from *E. coli* had much less effect than OMP29 from *A. actinomycetemcomitans* on the expression of IL-8 in HGEC (unpublished data, Kishimoto, Fujita, Shiba), suggesting that OMP29 is mainly involved in the increased effect on the IL-8 mRNA expression, even if the preparation contains LPS.

In conclusion, ERK, as well as cyclic AMP and protein kinase A, is an intracellular signaling molecule regulated by IM. Furthermore, IM inhibits the OMP29-induced promotion of IL-8 expression by suppressing the phosphorylation of ERK. From the present and previous studies, IM causes the enhancement of gap junctional intercellular communication through two pathways: suppression of OMP29-induced increase in IL-8 through ERK and recovery of OMP29-induced decrease in cyclic AMP levels (4,5). IM, which modulates gap junctional intercellular communication and IL-8 expression, may be a potential therapeutic agent to prevent periodontal disease. Further work, for example *in vivo* studies, would be required to render IM feasible as a medicant to prevent periodontal inflammation.

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