in vivo *27*: 269-274 (2013)

Re-evaluation of Anti-inflammatory Potential of Eugenol in IL-1β-stimulated Gingival Fibroblast and Pulp Cells

TEHO KOH[1], YUKIO MURAKAMI[1], SHOJI TANAKA[1], MAMORU MACHINO[1] and HIROSHI SAKAGAMI[2]

Divisions of [1]Oral Diagnosis and [2]Pharmacology, Meikai University School of Dentistry, Sakado, Saitama, Japan

Abstract. *Background: We recently reported that eugenol exerted comparable cytotoxicity towards human normal and tumor cells. In the present study, we investigated the effect of eugenol on interleukin-8 (IL-8) production by IL-1β-stimulated oral cells. Materials and Methods: The viable cell number was determined by direct cell counting with a hemocytometer after trypsinization. IL-8 released into the culture medium was determined by enzyme-linked immunosorbent assay (ELISA). Results: IL-1β (5 ng/ml) induced two orders of magnitude higher production of IL-8 by human cultured cells than unstimulated cells. Upon IL-1β stimulation, both gingival fibroblasts (HGF) and periodontal ligament fibroblasts (HPLF) produced the greatest amounts of IL-8 (approximately 200-300 ng/ml), followed by pulp cells (HPCs) (approximately 40-50 ng/ml), whereas skin keratinocyte (HaCat) and oral squamous cell carcinoma cells (HSC-2, HSC-4) produced much less IL-8 (less than 15 ng/ml). The production of IL-8 depended on growth factor(s), since the omission of fetal bovine serum from the culture medium resulted in an approximately 90% decline of IL-8 production. Eugenol (5-500 μM) significantly stimulated IL-8 production in HGF cells, but had bi-modal effects on HPCs, causing slight stimulation at lower concentration (5 μM) and a significant inhibition at higher concentration (500 μM), regardless of the presence or absence of serum. Eugenol exerted similar effects on lipopolysaccharide-stimulated HGFs and HPCs. Conclusion: These results demonstrate that an anti-inflammatory effect of eugenol is observed in HPCs, but not in HGFs. The narrow therapeutic range of eugenol suggests the importance of careful usage of this compound for dental treatment.*

Correspondence to: Professor Hiroshi Sakagami, Division of Pharmacology, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: +81 492792758, Fax: +81 492855171, e-mail: sakagami@dent.meikai.ac.jp

Key Word: Eugenol, gingival firbroglast, pulp cells, interleukin-8.

Eugenol (Figure 1) is a component of dental cements, sealers and dental impression materials, and has antiseptic, analgesic and sedative actions. It has been reported that eugenol induced apoptosis of human promyelocytic leukemia (1), colon cancer (2) and breast cancer cells (3), possibly by elevating intracellular reactive oxygen species (ROS). However, studies on the cytotoxicity of eugenol against oral cells are limited (4-8). We recently reported that treatment of normal oral human cells (gingival fibroblast, HGF; pulp cells, HPC; periodontal ligament fibroblast, HPLF cells) and oral squamous cell carcinoma cell lines (HSC-2, HSC-4) with eugenol for more than 4 h, induced irreversible non-apoptotic cell death. Eugenol did not exhibit any apparent tumor specificity, nor hormetic growth stimulation, nor did it protect cells from UV-induced damage (9). For the safe use of eugenol in dentistry, it is crucial to investigate the effect of this dental compound on oral cells.

We have reported that treatment of HGFs with interleukin (IL)-1β resulted in two orders of magnitude higher production of IL-6, IL-8, Monocyte Chemoattractant Protein-1 (MCP-1) and prostaglandin (PG)E_2 compared with unstimulated cells , but not of tumor necrosis factor (TNF)-α and nitric oxide (NO) (10). Using this *in vitro* gingivatitis model, the anti-inflammatory effect of eugenol on IL-8 production was re-evaluated.

Materials and Methods

Materials. The following chemicals and materials were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) from Gibco BRL, Grand Island, NY, USA; fetal bovine serum (FBS), eugenol (MW=164), dimethylsulfoxide (DMSO) from Wako Pure Chemical, Osaka, Japan; lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4) from Sigma Chem. Ind., St. Louis, MO, USA; IL-1β was purchased from R&D Systems, Minneapolis, MN, USA; 6-well plates from Becton Dickinson, Franklin Lakes, NJ, USA; HuMedia-KG2 from Kurabo, Osaka, Japan. Eugenol was dissolved in DMSO at 200 mM before use, and diluted with medium.

Cell culture. HaCat cells were provided by Deutsches Krebsforschungszentrum (German Cancer Research Center), Heidelberg, Germany. Human OSCC cell lines (HSC-2, HSC-4) were

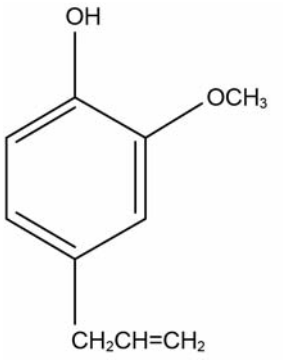

Figure 1. *Chemical structure of eugenol.*

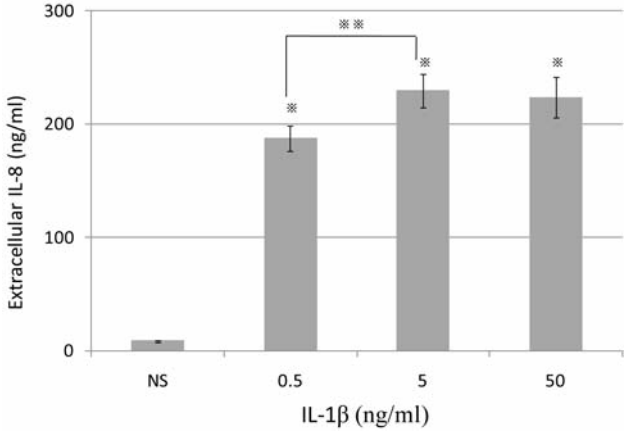

Figure 2. *Dose-dependent stimulation of interleukin-8 (IL-8) production by IL-1β in human gingival fibroblasts (HGFs). HGFs were incubated for 24 h with the indicated concentrations of IL-1β in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and the IL-8 in the culture supernatant was determined by enzyme-linked immunosorbent assay. Each value represents the mean±SD of triplicate assays. *Significant difference (p<0.01) from no stimulation(NS); **significant difference (p<0.01) between 0.5 and 5 ng/ml of IL-1β.*

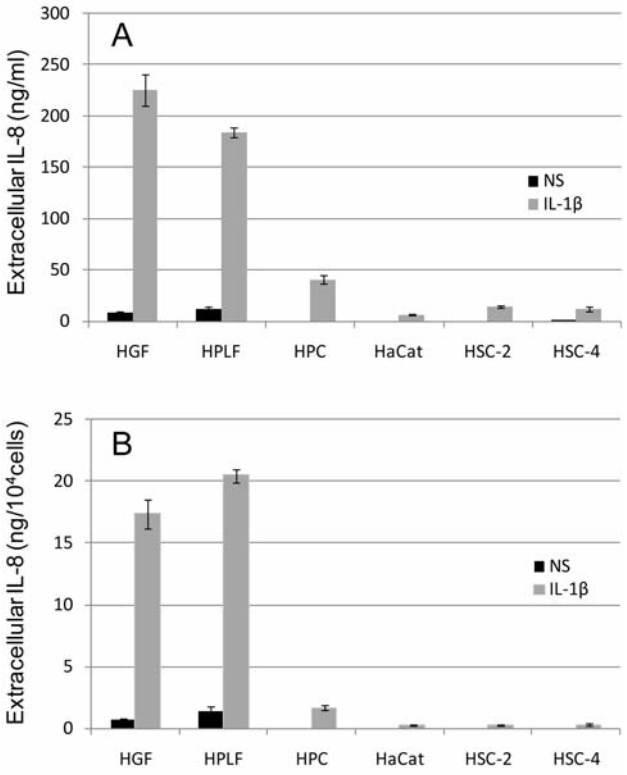

Figure 3. *Fibroblasts identified as one of the major sources of interleukin-8 (IL-8) production. Near-confluent cells were incubated for 24 h without (non stimulation, NS) or with 5 ng/ml IL-1β, and the IL-8 in the supernatant was determined by enzyme-linked immunosorbent assay. Each value represents the mean±SD of triplicate assays. The differences between the NS and IL-1β-treated groups were all significant (p<0.01).*

kindly provided by Professor Nagumo, Showa University, Japan. Normal human oral cells, HGF, HPC and HPLF, were prepared from periodontal tissues, as previously reported (9), and used at 8-15 population doubling levels (PDL). All these adherent cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. Human skin keratinocytes (HEK-a) were purchased from Kurabo Ind. Ltd., Osaka, Japan and cultured in HuMedia-KG2 supplemented with insulin, human recombinant epidermal growth factor (EGF), hydrocortisone, gentamicin, amphotericin B and bovine pituitary extract (BPE), as instructed by the supplier. DMSO used at concentrations below 0.25% did not affect the viability of the cells.

Assay for cytotoxic activity. All of the cells were inoculated at 6×10^3 cells/well in 6-well plates (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 48 h, the medium was removed by suction with an aspirator, and replaced with 2 ml of fresh medium containing different concentrations of eugenol. After 30 min, IL-1β (final, 5 ng/mL) was added. The cells were incubated for 24 h, and the relative viable cell number was then determined by direct cell counting with a hemocytometer, after trypsinization

IL-8 determination. The IL-8 in the culture medium was determined by ELISA, according to the manufacturer's instruction (Quantikine ELISA kit; R&D Systems) (10).

Statistical analysis. The difference between two groups was evaluated by Student's *t*-test. The value of statistical significance was set at the 0.01 level.

Results

Optimal conditions for IL-8 production. IL-1β dose-dependently and significantly ($p<0.01$) stimulated the IL-8 production by HGFs, reaching a plateau level at 5 ng/ml (Figure 2). A higher concentration of IL-1β rather slightly reduced IL-8 production. Based on these data, 5 ng/ml of IL-1β were used for subsequent experiments.

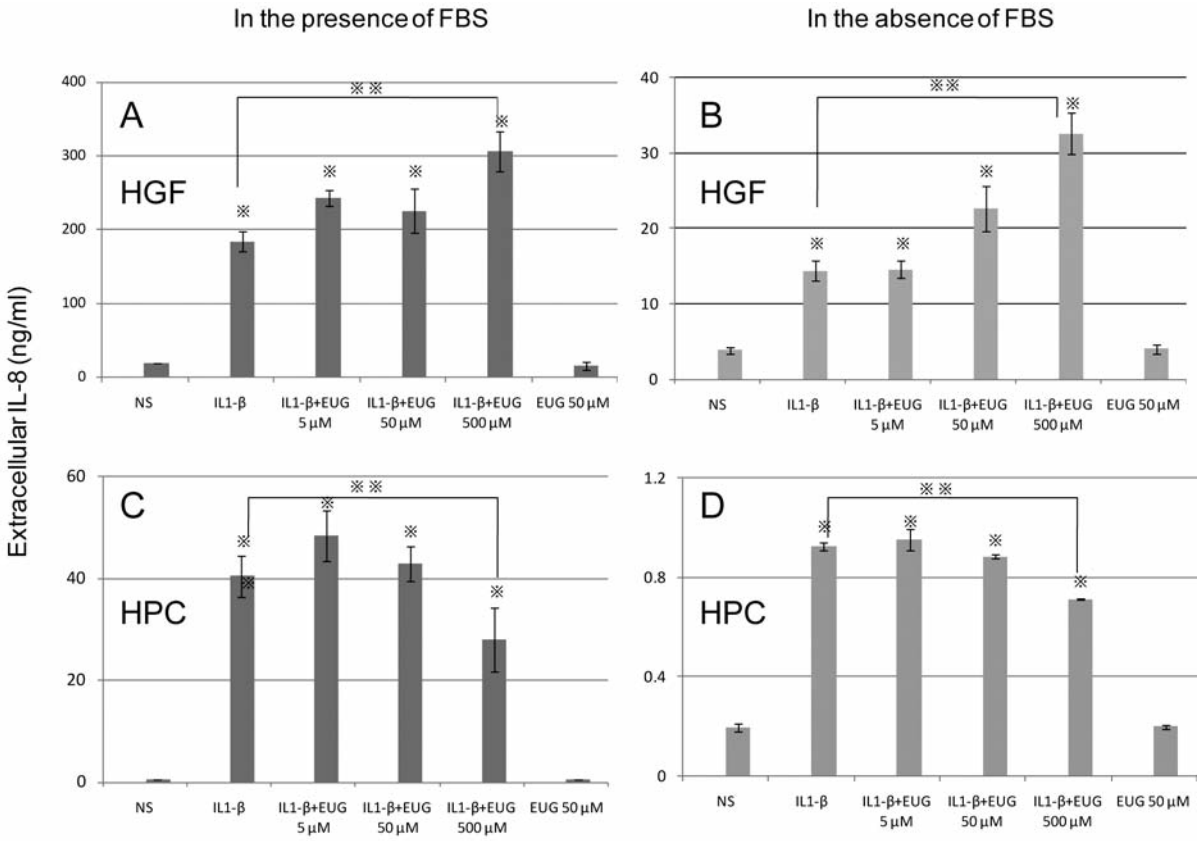

Figure 4. *Effect of eugenol on interleukin-8 (IL-8) production by IL-1β-stimulated human gingival fibroblasts (HGF) (A, B) and human pulp cells (HPC) (C, D) in the presence (A, C) or absence (B, D) of 10% fetal bovine serum in the culture medium. Near-confluent cells were treated for 24 h without (NS) or with 5 ng/ml IL-1β in the presence of 5, 50 or 500 μM eugenol, and the production of IL-8 in the culture medium was determined by enzyme-linked immunosorbent assay. Each value represents the mean±SD of triplicate assays. *Significant difference (p<0.01) from NS.,**significant difference (p<0.01) between IL-1β alone and IL-1β + 500 μM eugenol.*

Preferential IL-8 production by fibroblasts. Upon IL-1β stimulation, two fibroblast cell lines (HGF, HPLF) produced the greatest amounts of IL-8 (approximately 200-300 ng/ml), followed by HPCs (approximately 40-50 ng/ml), whereas epithelial cells such as human skin keratinocyte (HaCat) and oral squamous cell carcinoma cell lines (HSC-2, HSC-4) produced much less amounts (less than 15 ng/ml) (Figure 3A). We also found that human skin keratinocytes (HEKa) also had a low level of IL-8 production upon IL-1β stimulation (HaCaT<HEKa<HPC) (data not shown). Similar trends were observed when the IL-8 production was expressed on a per cell basis (Figure 3B), where the difference between fibroblasts (high IL-8 production) and non-fibroblasts (low IL-8 production) was more dramatic.

The production of IL-8 depended on growth factor(s), since the omission of FBS from the culture medium resulted in an approximately 90% decline of IL-8 production in all tested cells (comparison of A and B, C and D in Figure 4).

Evaluation of the anti-inflammatory effect of eugenol. Eugenol (5-500 μM) significantly ($p<0.01$) enhanced IL-1β-stimulated IL-8 production in HGFs, regardless of the presence or absence of FBS (Figure 4). At 500 μM, IL-8 production nearly doubled (Figure 4A, B). Eugenol (50 μM)-alone did not induce IL-8 production. These data suggest that eugenol may exert a pro-inflammatory, but not an anti-inflammatory effect on HGFs.

On the other hand, eugenol had bi-modal actions, depending on its concentration, on HPCs. Eugenol slightly stimulated IL-8 production at lower concentration (5 μM), but rather significantly ($p<0.01$) inhibited it at a higher concentration (500 μM), regardless of the presence or absence of FBS (Figure 4). Since this concentration was only slightly lower than its 50% cytotoxic concentration (763 μM), the apparent decline of IL-8 production at higher eugenol concentration may be due, at least in part, to its cytotoxicity (9).

As compared with IL-1β, LPS had a much lower ability to induce IL-8 production by HGFs and HPCs, confirming

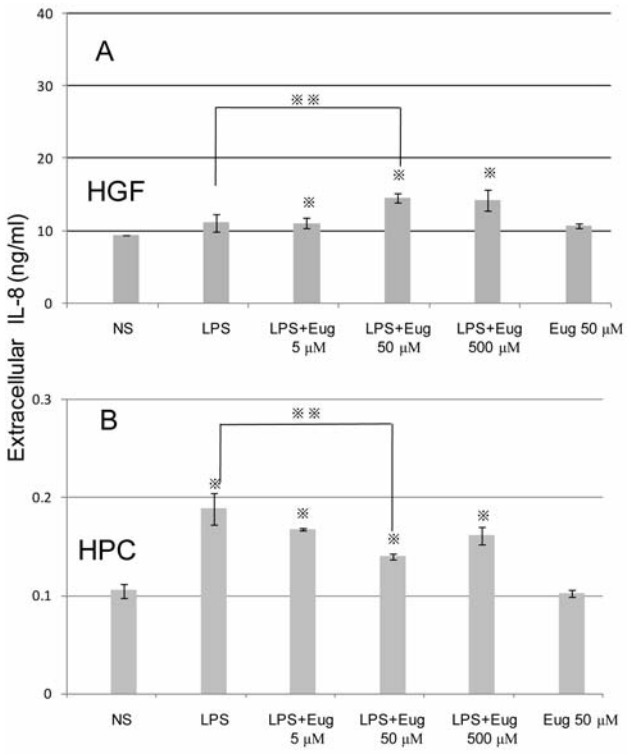

Figure 5. *Effect of eugenol on IL-8 production by LPS-stimulated HGF (A) and HPC (B) in the absence of FBS in the culture medium. Near-confluent cells were treated for 24 h without (NS) or with 100 ng/ml LPS in the presence of 5, 50 or 500 μM eugenol, and the production of IL-8 in the culture medium was determined by ELISA. Each value represents the mean±SD of triplicate assays. *Significant difference (p<0.01) from no stimulation. **Significant difference (p<0.01) between LPS-alone and LPS+50 μM eugenol.*

our previous findings (10). Eugenol slightly enhanced LPS-stimulated IL-8 production in HGFs (A), but rather inhibited IL-8 production in HPCs (Figure 5).

Discussion

The present study demonstrated, to our knowledge for the first time, that eugenol did not inhibit, but rather enhanced IL-1β-stimulated IL-8 production by HGFs. This suggests that eugenol may in fact aggravate gingivatitis. However, the chance that this happens may not be that great, considering that eugenol is not treated directly to gingival tissue. On the other hand, we found that eugenol showed bi-modal actions, by stimulating or inhibiting the IL-8 production at lower and higher concentrations. The reason for this bi-modal action of eugenol is not clear; however, it may be due to the heterogeneous populations of HPCs that are comprised of fibroblasts, undifferentiated mesenchymal cells and defense cells (macrophages and lymphocytes). In the early stages of culture, fibroblasts, which are most reactive to IL-1β, comprise a significant proportion of HPCs, but with subculturing, the proportion of fibroblasts declines, accompanied by the decline of IL-8 production (data not shown). We found that immortalized HPCs that lack fibroblasts lost the ability to produce IL-8 upon IL-1β stimulation (data not shown), further confirming that fibroblasts are the major producer of IL-8. At present, it is not clear whethert the slight decline of IL-8 production at higher concentrations of eugenol is due to its anti-inflammatory effects, which have been reported in other systems (12, 13), or is a secondary result of growth inhibition. There is another possibility that this activity of eugenol may be due to its antioxidant action, since this compound exhibited one order higher superoxide anion scavenging activity than 2-*t*-butyl-4-methoxyphenol and 2-methoxy-4-methylphenol (5). Further study is needed to test these possibilities. At present, whether eugenol acts extracellularly or intracellularly is not clear. Metabolomic analysis may be useful to identify the target molecules of eugenol.

In conclusion, the present study demonstrated that non-cytotoxic concentrations of eugenol (5-50 μM) do not inhibit IL-8 production by gingival fibroblast and pulp cells, whereas a near-cytotoxic concentration of eugenol slightly (only 30%) reduced IL-8 production by pulp cells. The narrow therapeutic range of eugenol suggests the importance of careful usage of this compound for dental treatment.

References

1 Atsumi T, Fujisawa S, Satoh K, Sakagami H, Iwakura I, Ueha T, Sugita Y and Yokoe I: Cytotoxicity and radical intensity of eugenol, isoeugenol or related dimmers. Anticancer Res *20*: 2519-2524, 2000.

2 Jaqanathan SK, Mazumdar A, Mondhe D and Mandal M: Apoptotic effect of eugenol in human colon cancer cell lines. Cell Biol Int *35*: 607-615, 2011.

3 Vidhya N and Devaraj SN: Induction of apoptosis by eugenol in human breast cancer cells. Indian J Exp Biol *49*: 871-878, 2011.

4 Okada N, Satoh K, Atsumi T, Tajima M, Ishihara M, Sugita Y, Yokoe I, Sakagami H and Fujisawa S: Radical modulating activity and cytotoxic activity of synthesized eugenol-related compounds. Anticancer Res *20*: 2955-2960, 2000.

5 Fujisawa S, Atsumi T, Satoh K, Kadoma Y, Ishihara M, Okada N, Kashiwagi Y, Yokoe I and Sakagami H: Radical generation, radical-scavenging activity and cytotoxicity of eugenol-related compounds. In Vitro Mol Toxicol *13*: 269-279, 2000.

6 Fujisawa S, Atsumi T, Kadoma Y and Sakagami H: Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. Forum "Phenolic compounds: Free radical mechanisms of toxicity, catalysis, and protection". Toxicology *177*: 39-54, 2002

7 Fujisawa S, Atsumi T, Satoh K and Sakagami H: Interaction between 2-ethoxybenzoic acid (EBA) and eugenol, and its cytotoxicity. J Dental Res *82*: 43-47, 2003.

8 Okada N, Hirata A, Murakami Y, Shoji M, Sakagami H and Fujisawa S: Induction of cytotoxicity and apoptosis and inhibition of cyclooxygenase-2 gene expression by eugenol-related compounds. Anticancer Res *25*: 3263-3270, 2005.
9 Koh T, Machino M, Murakami Y, Umemura N and Sakagami H: Cytotoxicity of dental compounds towards human oral squamous cell carcinoma and normal oral cells. In Vivo *27*: 85-96, 2013.
10 Ono M, Kantoh K, Ueki J, Shimada A, Wakabayashi H, Matsuta T, Sakagami H, Kumada H, Hamada N, Kitajima M, Oizumi H and Oizumi T: Quest for anti-inflammatory substances using IL-1β-stimulated gingival fibroblasts. In Vivo *25*: 763-768, 2011.
11 Okiji T: Pulp as a connective tissue. Chapter 4, *In*: Seltzer and Bender's Dental Pulp, Second Edition. Hargreave KM, Goodis HE and Tay RF (eds.). Quintessence Publishing Co., Inc., IL, USA, pp. 67-89, 2012.
12 Magalhães CB, Riva DR, DePaula LJ, Brando-Lima A, Koatz VL, Leal-Cardoso JH, Zin WA and Faffe DS: *In vivo* anti-inflammatory action of eugenol on lipopolysaccharide-induced lung injury. J Appl Physiol *108*: 845-851, 2010.
13 Bachiega TF, de Sousa JP, Bastos JK and Sforcin JM: Clove and eugenol in noncytotoxic concentrations exert immunomodulatory/anti-inflammatory action on cytokine production by murine macrophages. J Pharm Pharmacol *64*: 610-616, 2012.

Received November 29, 2012
Revised January 15, 2013
Accepted January 16, 2013