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Quest for Anti-inflammatory Substances Using IL-1β-stimulated Gingival Fibroblasts

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Abstract. *Background: We have previously reported that azulene-related compounds, and alkaline extract of Sasa senanensis Rehder potently inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated mouse macrophages. We investigated here whether they can inhibit pro-inflammatory cytokine production, by activated human gingival fibroblast (HGF). Materials and Methods: HGF was established from the periodontal tissues of extracted tooth. Viable cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Production of Prostaglandin E_2 (PGE_2) and cytokines was determined by enzyme immunoassay, and enzyme-linked immunosorbent assay, respectively. Results: Interleukin (IL)-1β did not inhibit, but rather slightly stimulated the growth of HGF cells. IL-1β stimulated the production of PGE_2, IL-6, IL-8 and monocyte chemotactic protein-1 very potently, but not that of nitric oxide and tumor necrosis factor-α. Native LPS and synthetic lipid A from E. coli and P. gingivalis was much less stimulatory. Dexamethasone, not indomethacin, was an efficient inhibitor of IL-8 production. Among five azulene-related compounds, benzo[b]cyclohepta[e][1,4]thiazine most potently inhibited the IL-8 production by HGF cells, as well as NO production by activated RAW264.7 cells. The alkaline extract of Sasa senanensis Rehder significantly inhibited IL-8 production, without affecting the cell viability. Conclusion: The present system may be applicable for use in the search for anti-gingivitis substances.*

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Key Words: Anti-inflammation, human gingival fibroblast, cytokines, alkaline extract of the leaves, *Sasa senanensis* Rehder.

Chemotherapy and radiotherapy, while highly effective in the treatment of neoplast, adversely affect the epithelia of oral mucosa, causing severe inflammation, lesioning, ulceration and bleeding in the mouth (1). The cause of stomatitis is unknown and thought to be multifactorial with many triggers or precipitating factors. The risk factors include the poor oral hygiene, secondary infection of both Gram-positive and Gram-negative bacteria, low rates of salivary production, poor production of mucins, antimicrobial-, anti-fungal and anti-viral factors, impaired local or systemic immunity, and age/gender (2). Since some risk factors for stomatitis and those for oral cancer are common between the two diseases overlapped with each other (3), there is a possibility that stomatitis may trigger the carcinogenesis of oral cancer. It is therefore very important to establish an *in vitro* assay system for the measurement of anti-stomatitis activity. For this purpose, as the first part of the present study, we investigated here several stimulators for their ability to induce the production of pro-inflammatory substances in human gingival fibroblast (HGF) cells.

We have previously reported that benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]**, 6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine **[2]** (4), benzo[*b*]cyclohept[*e*][1,4]oxazin-6(11*H*)-one **[5]** (5), 3-methyl-1-trichloroacetylazulene **[2b]** and 3-ethyl-1-trichloroacetylazulene **[3b]** (6) (Figure 1) inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-activated mouse macrophage-like RAW264.7 cells (Table I). Similarly, alkaline extract of the leaves of *Sasa senanensis* Rehder or *Sasa albo-marginata* Makino et Shibata (SE) (SASA-Health®) inhibited NO and prostaglandin E_2 (PGE_2) production by activated macrophages *via* inhibition of inducible NO synthase and cyclooxygenase-2 expression at both protein and mRNA levels (7) (Table I). As the second part of our study, we investigated here whether these compounds and SE can effectively inhibit the production of pro-inflammatory cytokines by HGF.

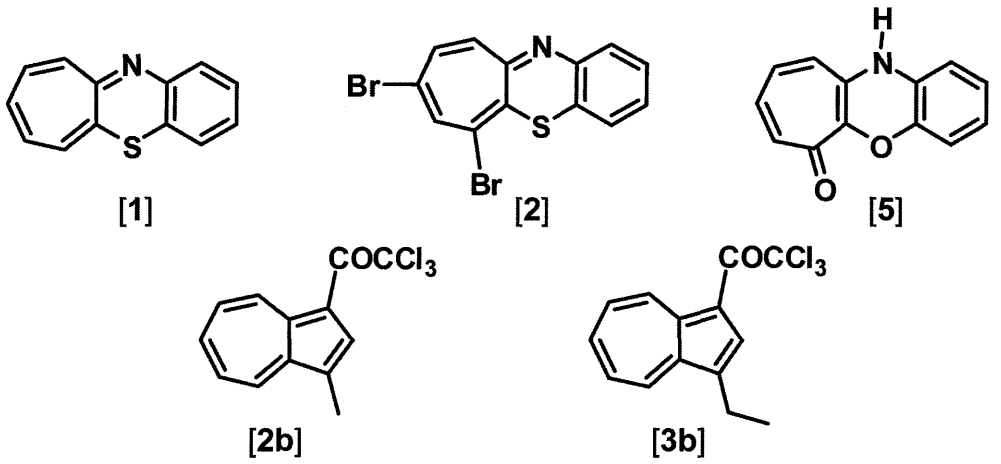

Figure 1. *Chemical structure of group II compounds: benzo[b]cyclohepta[e][1,4]thiazine [1], 6,8-dibromobenzo[b]cyclohepta[e][1,4]thiazine [2], benzo[b]cyclohepta[e][1,4]oxazin-6(11H)-one [5], 3-methyl-1-trichloroacetylazulene [2b], 3-ethyl-1-trichloroacetylazulene [3b].*

Table I. *Anti-inflammatory activity of test samples as judged by two different assay systems.*

	[a]Inhibition of LPS-stimulated NO production by macrophages (SI)			[b]Inhibition of IL-1β-stimulated IL-8 production by HGF cells		
	Sample →LPS	Sample →LPS	LPS →Sample	CC_{50}	IC_{50}	SI
				(μM)	(μM)	
Benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]**	>463.0	28.2	11.8	>200	7.3	>27.5
6,8-Dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine **[2]**	18.4	6.6	8.7	>4	0.37	>10.9
Benzo[*b*]cyclohept[*e*][1,4]oxazin-6(11*H*)-one **[5]**	74.9	43.9	8.6	>200	>200	><1.0
3-Methyl-1-trichloroacetylazulene **[2b]**	>37.7	>5.6	>2.9	>200	>200	><1.0
3-Ethyl-1-trichloroacetylazulene **[3b]**	>36.1 (ref. 10)	>6.8	3.6	>200	>200	>2.8
				(μg/ml)	(μg/ml)	
SE	10.3 (ref. 7)			>1164 (μM)	246 (μM)	>4.7
Dexamethasone				>1	0.000386	>2590.6
Indomethacin				>10	>10	><1.0

[a]RAW264.7 cells were incubated for 24 hours with various concentrations of sample, and the NO released into the culture supernatant, and the cell viability were determined to yield the selectivity index (SI) value. [b]HGF cells were incubated for 48 h with various concentrations of sample in the presence of 1 ng/ml IL-1β, and CC_{50} and IC_{50} for IL-8 production were determined to yield the SI value.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS from *Escherichia coli* (serotype 0111:B4) (Sigma-Aldrich, St. Louis, MO, USA); dexamethasone, indomethacin, dimethyl sulfoxide (DMSO) (Wako Pure Chemical Ltd., Osaka, Japan); interleukin (IL)-1α, IL-1β (R&D systems, Minneapolis, MN, USA).

Preparations of Porphyromonas gingivalis LPS, lipid A and synthetic SU63 lipid A. The procedures for preparation of *P. gingivalis* Su63 LPS and lipid A were described previously (8, 9). Briefly, the LPS was extracted from acetone-dried cells with phenol-water, digested with RNase A, DNase I, and proteinase K, and then purified by repeated ultracentrifugation (105,000 ×g, 12 h, 6 times). The LPS was washed successively with phenol/chloroform/petroleum ether (2:5:8, v/v) and acetone and then lyophilized.

Free lipid A was recovered from hydrolysates (1% acetic acid, 100°C, 1.5 hours) of LPS as described previously (9). It was purified by passage through a Dowex 50 (H^+) column with chloroform/

Table II. *Effect of various stimulators on the production of pro-inflammatory substances by HGF. HGF cells were incubated for 24, 72 or 120 h without (control) or with LPS (100 ng/ml), IL-1β (1 ng/ml) or IL-1β (1 ng/ml) and LPS (derived from E. coli) (100 ng/ml), and the extracellular concentrations of NO, TNF, PGE_2, IL-6, IL-8 and MCP-1 were determined. Each value represents the mean of duplicate determinations.*

Treatment	Time (hours)	Extracellular concentration (% of control)					
		NO (λM)	TNF (pg/ml)	PGE_2 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	MCP-1 (pg/ml)
Control	24	<1.6	<1.6	8 (100)	18 (100)	77 (100)	10 (100)
	72	<1.6	<1.6	4 (100)	160 (100)	72 (100)	86 (100)
	120	<1.6	<1.6	6 (100)	310 (100)	133 (100)	129 (100)
LPS	24	<1.6	<1.6	12 (139)	32 (178)	53 (69)	12 (111)
	72	<1.6	<1.6	7 (187)	155 (97)	119 (165)	122 (141)
	120	<1.6	<1.6	6 (102)	359 (116)	193 (145)	198 (153)
IL-1β	24	<1.6	<1.6	407 (4914)	262 (1458)	1785 (2323)	139 (1334)
	72	<1.6	<1.6	881 (23742)	742 (464)	3081 (4280)	287 (332)
	120	<1.6	<1.6	713 (12657)	748 (241)	3206 (2405)	336 (260)
IL-1β+LPS	24	<1.6	<1.6	684 (8266)	275 (1533)	1883 (2451)	165 (1582)
	72	<1.6	<1.6	2054 (55361)	693 (433)	3160 (4389)	277 (321)
	120	<1.6	<1.6	1467 (26063)	694 (224)	3423 (2568)	320 (248)

methanol (3:1, v/v) as the eluent and gel permeation chromatography with a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column with the same solvent as the eluent.

The synthetic SU63 lipid A analog for *P. gingivalis* was prepared basically according to the procedure previously reported (9). The synthetic analog consisted of a β(1-6)-linked D-glucosamine disaccharide 1,4'-bisphosphate backbone acylated with (*R*)-3-hydroxy-15-methylhexadecanoic acid, (*R*)-3-hydroxyhexadecanoic acid, (*R*)-3-*O*-(hexadecanoyl)-15-methylhexadecanoic acid and (*R*)-3-hydroxy-13-methyltetradecanoic acid at positions 2, 3, 2' and 3' of a hydrophilic backbone.

Preparation of azulene-related compounds. Benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]**, 6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine **[2]**, benzo[*b*]cyclohept[*e*][1,4]oxazin-6(11*H*)-one **[5]**, 3-methyl-1-trichloroacetylazulene **[2b]** and 3-ethyl-1-trichloroacetylazulene **[3b]** were prepared as described previously (4-6).

Preparation of SE. SE was prepared and supplied by Daiwa Biological Research Institute Co., Ltd., Kawasaki, Kanagawa, Japan. SE (21 ml) was freeze-dried to produce the powder (1.1 g).

Cell culture. HGF cells were established from the periodontal tissues of the first premolar extracted tooth in the lower jaw, as described previously (10, 11). The life-span of HGF cells was about 40 population doubling level (PDL), and the cells at 10-20 PDL were used for the present study. HGF cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate under humidified 5% CO_2 atmosphere.

Assay for cytotoxic activity. Cells were inoculated at 1×10^3 cells/0.1 ml in the inner 60 wells of a 96-microwell plate (Falcon Becton Dickinson, Franklin Lakes, NJ, USA). The surrounding 36 exterior wells were filled with 0.1 ml of PBS(–) to minimize the evaporation of water from the culture medium. After 48 h, the medium was removed by suction with aspirator, and replaced with 0.1 ml of fresh medium containing different concentrations of sample. Cells were incubated for 24, 48 or 72 h, and the relative viable cell number was then determined by MTT method (10, 11).

Assay for pro-inflammatory substances. The concentration of PGE_2 released into the culture medium was determined by enzyme immunoassay (EIA) (Cayman Chemical Co., Ann Arbor, MI, USA). Tumor necrosis factor (TNF)-α, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) released into the culture medium were determined by enzyme-linked immunosorbent assay (ELISA) (Quantikine ELISA kit; R&D systems).

Effect on NO production by macrophages. RAW264.7 cells (6×10^4/ml) were inoculated into 96-microwell plates, and incubated for 24 h. The medium was then replaced with phenol red-free DMEM medium containing 10% FBS and the indicated concentrations of stimulators. After incubation for 24 h, the NO released into the culture supernatant was measured by the Griess method. From the dose response curve, the 50% inhibitory concentration (IC_{50}) was calculated. The attached cells were stained with MTT reagent to determine the CC_{50}, as described above. The selectivity index (SI) for the inhibition of NO production was determined by the following equation: $SI=CC_{50}/IC_{50}$ (5).

Statistical analysis. The mean values and standard deviations were calculated. The average values were compared by paired *t*-test. The value of statistical significance was set at the 0.01 level.

Results

Establishment of assay conditions. Treatment of HGF cells with IL-1β (1 ng/ml) resulted in one to two orders higher production of PGE_2, IL-6, IL-8 and MCP-1, but induced no detectable amount of NO and TNF production (Table II). Addition of IL-1α (1 ng/ml) alone or in combination with LPS or LPS with IL-1β failed to induce NO production into

Table III. *Effect of LPS and lipid A on the production of pro-inflammatory substances by HGF. HGF cells were incubated for 48 h without (control) or with 100 ng/ml of each sample or IL-1β (1 ng/ml) and the extracellular concentrations of IL-6, IL-8 and MCP-1 were then determined. Each value represents the mean of duplicate determinations.*

	Extracellular concentration (pg/ml) (% of control)		
	IL-6	IL-8	MCP-1
None (control)	186 (100)	78 (100)	95 (100)
E. coli LPS	206 (109)	162 (208)	106 (112)
P. gingivalis ATCC33277 LPS	222 (117)	133 (170)	115 (121)
P. gingivalis Su63 LPS (batch 1)	177 (94)	98 (125)	93 (98)
P. gingivalis Su63 LPS (batch 2)	186 (98)	129 (165)	101 (107)
E. coli synthetic lipid A (506)	179 (94)	105 (135)	96 (101)
P. gingivalis Su63 native lipid A	165 (87)	115 (148)	95 (100)
P. gingivalis synthetic lipid A	162 (85)	77 (98)	75 (79)
IL-1β	1713 (904)	2507 (3214)	777 (820)

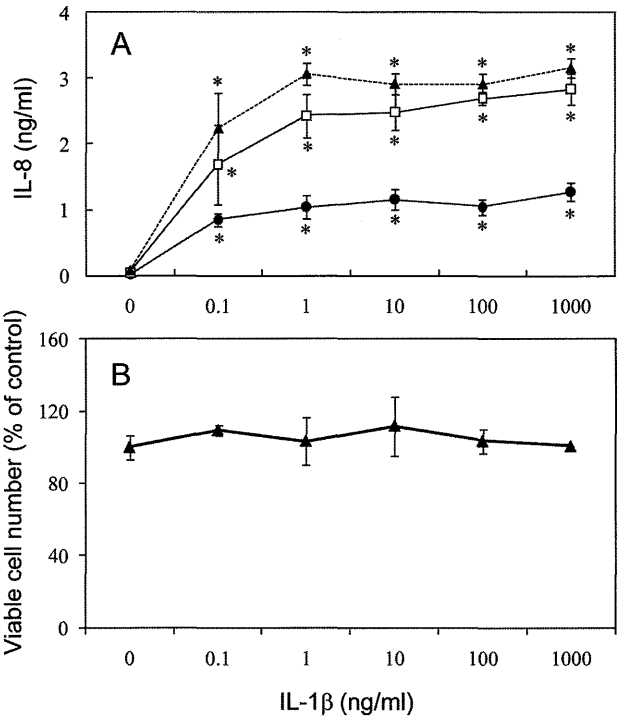

Figure 2. *Effect of IL-1β on the IL-8 production (A) and growth (B) of HGF cells. Near confluent HGF cells (10 PDL) were treated for 24 (●), 48 (□) or 72 (▲) h without (control) or with the indicated concentrations of IL-1β and the extracellular concentrations of IL-8 (A) and viable cell number (only at 72 h) (B) were then determined. Each value represents mean±S.D. of triplicate assays. *p<0.01 compared to without IL-1β.*

the culture medium (data not shown). LPS derived from *E. coli* and *P. gingivalis*, and native and synthetic lipid A were substantially inactive (Table III). However, addition of LPS further enhanced the IL-1β-induced PGE_2 production, but not that of IL-6, IL-8 and MCP-1 (Table II).

IL-1β significantly ($p<0.01$) stimulated the IL-8 production, reaching a plateau level after 48 h (Figure 2A). The production of IL-8 was increased with the concentration of IL-1β, reaching a plateau level above 1 ng/ml (Figure 2A). IL-1β (0.1-1000 ng/ml) did not inhibit growth of HGF cells (Figure 2B). Based on these data, we have adopted IL-1β at 1 ng/ml as stimulator, and 48 h as incubation time, for the subsequent experiments.

Effect of anti-inflammatory substances. The efficacy of two popular anti-inflammatory drugs, dexamethasone and indomethacin (Figure 3), was tested first. Dexamethasone potently inhibited IL-8 production in IL-1β-stimulated HGF cells. The IC_{50} of dexamethasone was 0.000386 μM (Figure 3B), and the 50% cytotoxic concentration (CC_{50}) was above 1 μM (Figure 3E), yielding the selectivity index ($SI=IC_{50}/CC_{50}$) of >2590.6 (Table I). Indomethacin did not significantly affect the production of IL-8 production (SI ><1.0) (Figure 3C and F).

We previously reported that benzo[*b*]cyclohepta[*e*][1,4]thiazine **[1]** inhibited the NO-production by LPS-activated RAW264.7 cells (SI≥463.0), more efficiently than 6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine **[2]** (SI=18.4), benzo[*b*]cyclohept[*e*][1,4]oxazin-6(11*H*)-one **[5]** (SI=74.9), 3-methyl-1-trichloroacetylazulene **[2b]** (SI≥37.7) and 3-ethyl-1-trichloroacetylazulene **[3b]** (>36.1) (4-6, 10) (Table I). We confirmed the potency of **[1]** to inhibit the NO production by activated macrophages, regardless of the order of administration (whether sample→LPS or LPS→sample) (Table I). Furthermore, **[1]** significantly ($p<0.01$) inhibited IL-1β-induced IL-8 production of HGF cells (Figure 3A, D), and the inhibitory activity of **[1]** (SI>27.5) exceeded that of other related compounds **[2, 5, 2b, 3b]** (SI=1.0-10.9) (Table I).

We previously reported that SE inhibited NO and PGE_2 production by activated macrophages [SI=10.4 (Table I) and 4.9, respectively)] (7). Here we found that SE significantly ($p<0.01$) inhibited IL-1β-stimulated IL-8 production at concentrations above 50 μg/ml (Figure 4A). The IC_{50} was calculated to be 246 μg/ml. SE slightly, but not significantly stimulated the growth of IL-1β-treated HGF cells (CC_{50} >1164 μg/ml) (B), yielding an SI value of >4.7 (Table I).

Discussion

We have established an *in vitro* gingivitis model, using cultured HGF cells stimulated by popular pro-inflammatory cytokine IL-1β. The extent of anti-gingivitis activity of sample can be monitored by the decrease of IL-8 production. Using this assay system, we found that dexamethasone (steroidal anti-inflammatory drug) inhibited IL-8 production

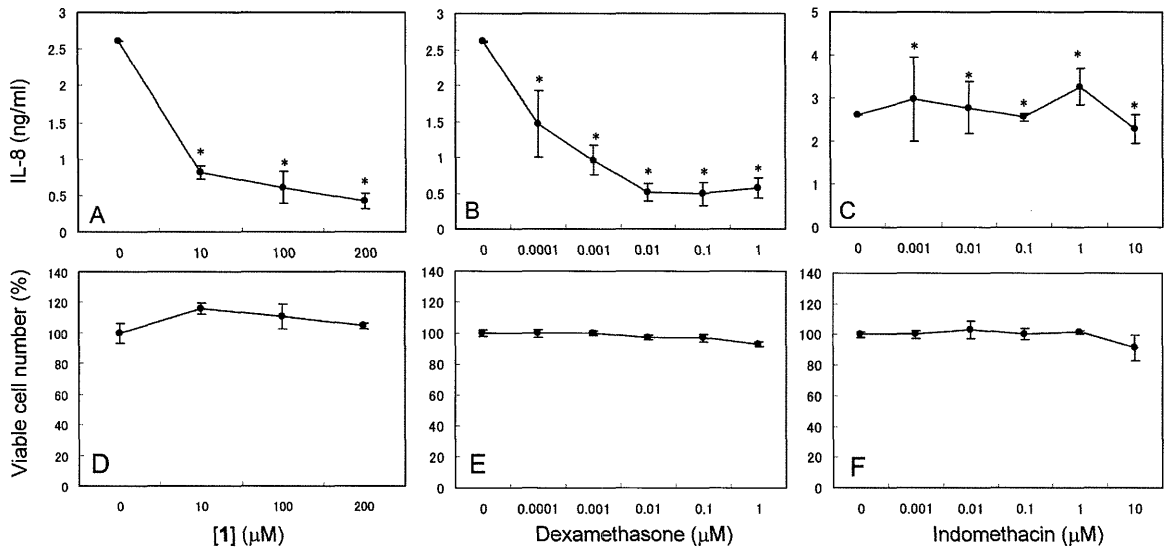

Figure 3. *Effect of [1], dexamethasone and indomethacin on IL-8 production and viability of HGF cells. Near-confluent HGF cells (16 PDL) were treated for 48 h without (control) or with the indicated concentrations of [1] (A, D), dexamethasone (B, E) and indomethacin (C, F) together with IL-1β (1 ng/ml), and the extracellular concentrations of IL-8 (A, B, C) and viable cell number (D, E, F) were then determined. Each value represents mean±S.D. of triplicate assays. *p<0.01 compared to control (without sample).*

by the activated HGF cells more efficiently than did indomethacin (nonsteroidal anti-inflammatory drug). This supports the efficacy of corticosterones for treatment of stomatitis (1). We found that LPS enhanced the production of PGE_2 by approximately two-fold, whereas it did not affect the production of IL-6, IL-8 and MCP-1 in IL-1β-stimulated HGF cells (Table I). This suggests that the induction mechanism of PGE_2 upon stimulation with IL-1β in HGF cells may be different from that of IL-6, IL-8 and MCP-1.

Azulene-related compounds, which efficiently inhibited NO production by activated macrophages, only marginally inhibited IL-8 production by activated HGF cells. This suggests that the anti-inflammatory action of azulenes may be more pronounced in macrophages where higher levels of NO and PGE_2 generation were observed, but not in HGF cells where production of these pro-inflammatory substances were much less.

SE, available as an over-the-counter drug, is recognized as being effective in treating fatigue, low appetite, halitosis, body odour and stomatitis. However, there is no scientific evidence that demonstrates these phenomena. SE has been reported to show antiseptic (12), membrane stabilising (13), anti-inflammatory and phagocytic (14), radical-scavenging (15-17), antibacterial and anti-viral activities. The present study demonstrated that SE significantly inhibited IL-8 production by activated HGF cells, in addition to its potent inhibition of NO and PGE_2 production by activated macrophages, possibly due to the presence of multiple components in SE. Since SE has radical scavenging activity and anti-viral activity (7, 16, 17), its efficacy against

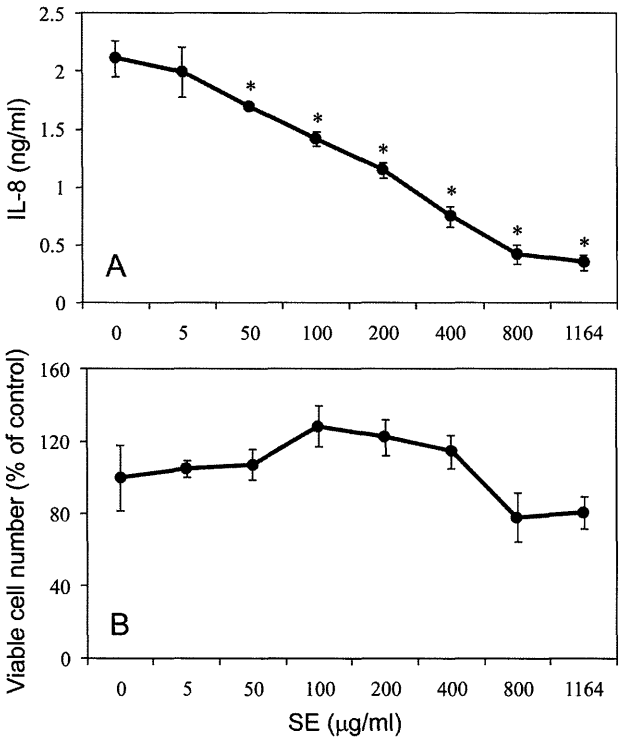

Figure 4. *Effect of SE on IL-1β-stimulated IL-8 production (A) and growth (B) of HGF cells. Near-confluent HGF cells (12 PDL) were treated for 48 h without (control) or with the indicated concentrations of SE together with IL-1β (1 ng/ml), and the extracellular concentrations of IL-8 (A) and viable cell number (B) were then determined. Each value represents the mean±S.D. of triplicate assays. *p<0.01 compared to without SE.*

stomatitis may be much enhanced when applied orally. However, clinical study with patients with stomatitis is crucial to confirm the anti-stomatitis effect of SE. Our recent study has suggested that multiple components of SE may be associated with each other in the native state or after extraction with alkaline solution (17). SE can be fractionated by gel filtration chromatography into four fractions: polysaccharide, lignin-carbohydrate complex (two peaks) and low molecular polyphenol fractions (18); it remains to be investigated which fraction is responsible for the anti-gingivitis activity.

The present assay system is applicable to the search for anti-stomatitis substances derived from natural products, as well as these synthesized chemically in the laboratory, using dexamethasone as positive control.

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