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Inhibitory Effects of Triterpenoids on Interleukin-8/CINC-1 Induction in LPS-Stimulated Rat Peritoneal Macrophages

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Abstract – The CINC-1 is a member of rat interleukin-8 with chemotactic and activating properties to neutrophils. The CINC-1 induction in LPS-stimulated rat peritoneal macrophages was analyzed using a sensitive enzyme-linked immunosorbent assay. The peritoneal macrophages contained about 3 ng/ml as a basal level, and induced to maximal 18 ng/ml of CINC-1 by stimulation with 5 μg/ml of LPS. Antiinflammatory steroids of dexamethasone and triamcinolon significantly suppressed the CINC-1 induction, where as aspirin and idomethacin did not show suppression. Inhibitory effects on the CINC-1 induction by natural triterpenoids having steroidal structures were analyzed. Among the 39 kinds of triterpenoids isolated from herbal medicines, acacigenin B and nigaichigoside F1 exhibited the highest suppression on the CINC-1 induction.

Key words – Interleukin-8, CINC-1 induction, rat macrophages, triterpenoids.

Introduction

Interleukin-8 (IL-8) is a proinflammatory cytokine with biological effects similar to known chemotactic substances such as *N*-formylmethionyl peptides, complement fragment C5a, leukotriene B4, and platelet-activating factor (Baggiolini *et al.*, 1992; Van Damme, 1994). Apart from the *N*-formylmethionyl peptides that originate from bacteria, other chemotactic substances are largely derived as main elements of the inflammatory process, the complement system extravasating with plasma, and the phagocytes accumulating in the affected tissue. IL-8 was identified on the basis of two biological effects on neutrophils, namely chemotaxis and activation (Baggiolini *et al.*, 1992). Compared to other chemotactic substances mentioned, chemotaxis and activation of neutrophils by IL-8 seem to be more selective in that monocytes, and eosinophils were not responsive (Schroder *et al.*, 1987; Yoshimura *et al.*, 1987b). IL-8 is also chemotactic for activated T lymphocytes and basophils (Larson *et al.*, 1989; Leonard *et al.*, 1990). IL-8 activates neutrophils to release their granule constituents including gelatinase, elastase, myeloperoxidase, and β-glucuronidase (Baggiolini and Dewald, 1984; Peveri *et al.*, 1988). In addition, IL-8 activates neutrophils to elicit the respiratory burst through formation of superoxide and hydrogen peroxide (Thelen *et al.*, 1988; Walz *et al.*, 1991; Wymann *et al.*, 1987). Intradermal injection of human IL-8 into rabbits induces plasma leakage and neutrophil accumulation in the skin (Colditz *et al.*, 1989; Rampart *et al.*, 1989). The skin reactivity by IL-8 is enhanced by the presence of a vaso-

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dilator substance PGE_2, and dependent on the presence of neutrophils (Colditz *et al.*, 1990).

IL-8 was originally discovered as a product of lipopolysaccharide (LPS)-activated monocytes and subsequently found to be induced in a variety of tissue cells by various stimuli (Baggiolini *et al.*, 1992; Van Damme, 1994; Yoshimura *et al.*, 1987). As the member of IL-8 family, several proteins including GRO, NAP, MIP, and CINC were identified and their cDNA and/or genomic structures were analyzed (Baggiolini *et al.*, 1992; Van Damme, 1994). The CINC was designated as an abbreviation of cytokine-induced neutrophil chemoattractant that was originally identified from normal rat kidney cell line, NRK-52E, stimulated with IL-1, tumor necrosis factor (TNF) or LPS (Watanabe *et al.*, 1989a and 1989b). Recently three chemokines related to the CINC were purified from conditioned medium of granulation tissue obtained from carrageenin-induced inflammation in rats (Nakagawa *et al.*, 1994). After analysis of cDNA sequence, the three chemokines were termed as CINC-2α, CINC-2β and CINC-3, and the original CINC was renamed as CINC-1 (Shibata *et al.*, 1995).

Accumulation and activation of leukocytes in inflammed tissue are central events in the inflammatory process. CINC-1, a member of rat IL-8 family, has a potent chemotactic and activating properties to neutrophils *in vitro* and *in vivo* (Watanabe *et al.*, 1991). In this study, inhibitory effects of antiinflammatory drugs and triterpenoids on CINC-1 induction in LPS-stimulated rat peritoneal macrophages were analyzed by a sensitive ELISA.

Experimental

Chemicals and animals – Fetal calf serum and HEPES were purchased from GIBCO Lab. (USA), and *O*-phenylenediamine from Wako Chem. Inc. Ltd. (Japan). Dulbecco's modified Eagle's medium, RPMI, and N-hydroxysuccimidobiotin were obtained from Sigma Chem. Co. (USA), maleimide-activated keyhole limpet hemocyanin (KLH) from PIERCE (USA), and *E. coli* 026:B6 LPS and proteose peptone from DIFCO Lab. (USA). Sprague-Dawley rats and New Zealand white rabbits were purchased from Jeil Animal Center (Korea), and maintained in an animal room with 12-hr light cycle.

Preparation of polyclonal antibody against CINC-1 – The CINC-1 was purified from crude lysates prepared from *E. coli* harboring a recombinant plasmid with a functional CINC-1 gene according to the method reported elsewhere (Konishi *et al.*, 1993). The purified CINC-1 which was identified as a single band in SDS-PAGE was treated with 2-mercaptoethanol, and then conjugated to maleimide-activated KLH by following the specification of the supplier (PIERCE). The conjugated CINC-1 was repetitively treated to a rabbit by subcutaneous and intravenous injections. Polyclonal antibody was purified from the antiserum by a protein A chromatography. The antiserum was diluted with the same volume of PBS, dialyzed against PBS overnight, and then applied to a protein A column as follows. The column was extensively washed with PBS and then eluted the antibody with 10 mM glycine (pH 2). Half of the antibody was dialyzed against 50 mM carbonate buffer (pH 9.6), and stored at -20 °C until use. The other half of the antibody was dialyzed against 0.1 M bicarbonate buffer (pH 8), and then subjected to a biotinylation with *N*-hydroxysuccimidobiotin. The biotinylated antibody was dialyzed against PBS, added with BSA as a final concentration of 0.5%, and then stored at -20 °C until use.

Primary culture of peritoneal macrophages in rat – Macrophages were col-

lected from peritoneal lavages of Sprague-Dawley rats injected intraperitoneally with 10% proteose peptone 2 days beforehand. The collected macrophages were suspended in DMEM (13.4 mg/ml of Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM HEPES, 100 μg/ml of streptomycin, and 143 U/ml of penicillin G potassium, pH 7.1) containing 10% fetal calf serum, and diluted to 2×10^6 cells/ml with the same medium. The macrophages were dispensed into a 96-well culture plate (4×10^5 cells per well), and then incubated at 37 °C with 5% CO_2 for 24 hr. The primary culture of macrophages was washed with DMEM containing 0.1% BSA, and then treated with indicated dose of LPS (100 μl per well) and sample (100 ml per well), where control group was treated with LPS only, and blank group was treated with DMEM containing 0.1% BSA. The LPS was dissolved in DMEM containing 0.1% BSA, and the samples were dissolved in the same medium with dimethyl sulfoxide. The final concentration of dimethyl sulfoxide used for dissolution of the samples was less than 0.1% in the culture of macrophages. After incubation at 37 °C with 5% CO_2 for 48 hr, the culture of macrophages was centrifuged at $1,000\times g$ for 30 min to collect supernate.

ELISA of CINC-1 – CINC-1 in the supernate was quantitated by a sandwich ELISA as follows. Ninety six-well assay plate was coated with 0.4 μg of the anti-CINC-1 IgG per well, where the antibody was dissolved in 50 mM carbonate buffer (pH 9.6). After incubation at 37 °C for 3 hr, the assay plate was washed with PBS containing 0.05% Tween-20. PBS containing 1% BSA was added to the assay plate. After incubation at 4 °C overnight, the assay plate was washed with PBS containing 0.05% Tween-20. The macrophage-culture supernate was added to the assay plate (80 μg per well), incubated at 37 °C for 1 hr, and washed with PBS containing 0.05% Tween-20. The assay plate was added with biotinylated anti-CINC-1 IgG (0.4 μg per well), incubated at 37 °C for 1 hr, and washed with PBS containing 0.05% Tween-20. Streptavidin-horseradish peroxidase was 10,000 fold diluted with PBS containing 0.05% Tween-20, and then added to the assay plate (80 μl per well). The assay plate was incubated at 37 °C for 30 min, and then extensively washed with PBS containing 0.05% Tween-20. One hundred μl of a substrate solution (0.1% *O*-phenylenediamine, 0.02% hydrogen peroxide, 50 mM sodium citrate and 100 mM phosphate buffer, pH 5) was added to each well of the assay plate. After incubation at room temperature for 10 to 30 min, each well of the assay plate was added with 100 μl of 4 N H_2SO_4 and then measured the absorbance at 492 nm using a microplate reader. Inhibitory effect of each sample on CINC-1 induction was expressed as % of inhibition, $[1-(\text{sample } A_{492}/\text{control } A_{492})]\times100$.

Chemotaxis assay – Neutrophils were collected from peritoneal lavages of Sprague-Dawley rats injected intraperitoneally with 1% casein. The neutrophils were suspended in RPMI containing 0.1% BSA, and diluted to 1×10^7 cells/ml with the same medium. Three hundred ml of the neutrophils were loaded in each well of the upper chamber, and culture supernate as the sample in the lower chamber of a Boyden's microchemotaxis apparatus where the upper and lower chambers are divided by a polycarbonate membrane filter with 5 μm in pore size. The chemotaxis assembly was incubated at 37 °C with 5% CO_2 for 80 min. The upper chamber was washed with distilled water, and 200 μl of PBS containing 0.25% trypsin and 0.02% EDTA was added to the same chamber. After incubation at 37 °C with 5% CO_2 for 30 min, the chemotaxis assembly was centrifuged at $1,000\times g$ for 10 min. The cell pellet in each well of the lower chamber was resuspended in 200 μl of PBS containing 0.25% EDTA, and then optical density at wavelength of 655 nm was measured using

a microplate reader. The neutrophils migrated from the upper chamber to the lower chamber were expressed as % of chemoattracted cells, (sample OD_{655}/control OD_{655})× 100, where the control OD_{655} was the optical density from 3×10^6 neutrophils.

Results and Discussion

The CINC-1 is a member of chemokine family which is usually found as basal level in normal cells, but its synthesis is amplified in a variety of cells by treatment with inflammatory stimuli (Baggiolini *et al.*, 1992). The CINC-1 is known to be induced in LPS, IL-1 or TNF-stimulated rat kidney eiptheloid NRK-52 cells (Watanabe *et al.*, 1989a and 1989b), IL-1 or TNF-stimulated rat fibroblast NRK-49F cells (Nakagawa *et al.*, 1993), and LPS-stimulated rat peritoneal macrophages (Lee *et al.*, 1995).

In order to analyze the CINC-1 induction, a sandwich ELISA was established (Fig. 1). A good titer of polyclonal antibodies against CINC-1 were obtained by repetitive injections with CINC-1 conjugated with KLH into rabbits. However, rabbit antisera obtained from repetitive injections with intact CINC-1 did not have the high titer of polyclonal antibodies for ELISA. This result would be attributed to the globular shape of low molecular weight of CINC-1 (Clore *et al.*, 1990). A batch of isolated CINC-1 serially diluted to have 1 ng/ml to 30 ng/ml as the final concentrations was incubated with four different concentrations of rabbit antibodies against CINC-1 followed by biotin-labelled rabbit antibodies against CINC-1, and finally by streptavidin-horseradish peroxidase for chromogenic detection as described in the experimental section. The polyclonal antibodies with 250 μg/ml as protein concentration were 20, 50, 100, and 200-fold diluted with carbonate buffer. With the serially diluted polyclonal antibodies, dose-response curves were obtained when the resulting absorbances of wells were plotted against the dilutions of purified CINC-1. The detection limit of the assay procedure, defined as the minimal concentration of purified CINC-1 which produced well aligned linearity of the dose-response curves, was determined to be less than 3 ng/ml. The 50-fold diluted polyclonal antibodies with 5 mg/ml as protein concentration were used in later experiments.

Macrophages obtained from peritoneal lavages of rats were stimulated with different concentrations of LPS, and then CINC-1 induction was analyzed using the ELISA (Fig. 2). About 3 ng/ml of CINC-1 was contained as a basal level in the macrophages. LPS significantly increased the CINC-1 production of macrophages. When macrophages were

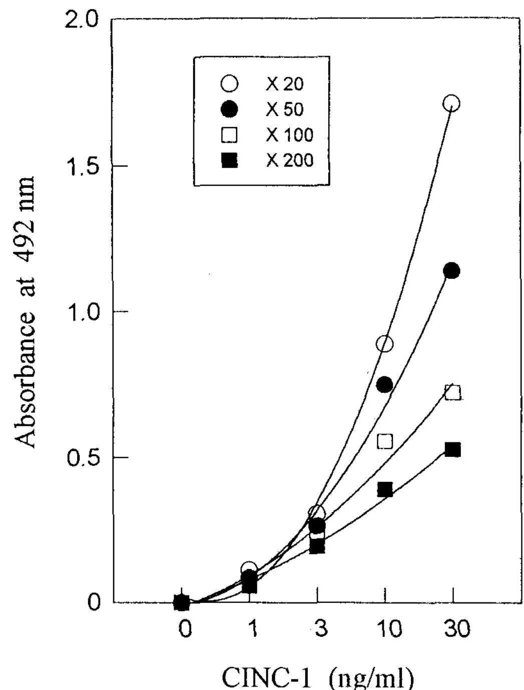

Fig. 1. Optimal dilution of anti-CINC-1 IgG for ELISA. The CINC-1 was serially diluted with PBS buffer. The polyclonal antibodies against CINC-1 were 250 mg/ml as protein concentration. The antibodies were serially diluted to 20 fold (X20), 50 fold (X50), 100 fold (X100) and 200 fold (X200) and then used for ELISA.

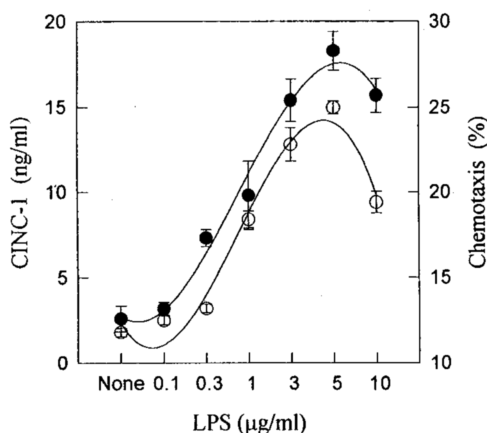

Fig. 2. CINC-1 induction in LPS-stimulated macrophages and its chemotactic effect on leukocytes. Macrophages collected from rat peritoneal cavity were stimulated with LPS. With the culture supernates of macrophages as samples, CINC-1 amount (soild circle) was analyzed by an ELISA, and chemotactic activity (open circle) to leukocytes by a modified Boyden's method.

stimulated with 0.1 μg/ml of LPS, CINC-1 production was not significantly increased. However, macrophages stimulated with 0.3 μg/ml of LPS produced CINC-1 with about 8 ng/ml, more than 2-fold amount of CINC-1 when compared to the basal level. The CINC-1 production in macrophages was dose dependent to LPS treated. Maximal CINC-1 induction with 18 ng/ml was obtained when macrophages were stimulated with 5 μg/ml of LPS. Compared to macrophages stimulated with 5 μg/ml of LPS, CINC-1 induction seems to be decreased when the cells were stimulated with 10 μg/ml of LPS. This result would be speculated as a kind of desensitization by excess amout of LPS. Therefore, 5 μg/ml of LPS was treated to macrophages for maximal CINC-1 induction in later experiments. CINC-1 is a member of rat IL-8 family with potent chemotactic activity to neutrophils. As shown in Fig. 2, chemotactic effect on leukocytes of CINC-1 produced in macrophages was estimated. About 12% of leukocytes were chemoattracted, micrograted from upper chamber to lower chamber of a Boyden's microchemotaxis apparatus, by culture supernates of macropahges with basal level of CINC-1. The highest chemotactic effect on leukocytes were exhibited in the culture supernates of macrophages with maximal CINC-1 induction when stimulated with 5 μg/ml of LPS. As the CINC-1 production of macrophages was increased by stimulation with LPS in a dose-dependent manner, chemotactic effects to leukocytes by the cell-culture supernates were proportionally increased. Thus, chemotactic effects of the macrophage-culture supernates are at least dependent on the CINC-1 amount induced in the cells by stimulation with LPS.

Inhibitory effects of antiinflamatory drugs on CINC-1 induction in LPS-stimulated macrophages were analyzed (Table 1). The nonsteroidal antiinflammatory drugs used in this study were aspirin and indomethacin, and steroidal antiinflammatory drugs used were dexamethasone and triamcinolon. The steroidal antiinflammatory drugs exhibited about 50% of inhibition at 1 μM of the drugs as the final concentration, on the CINC-1 induction, but nonsteroidal drugs did not show inhibition. Aspirin as a nonsteroidal antiinflammatory drug seems to slightly increase the CINC-1 induction. The nonsteroidal drugs are known to be mainly implicated in the inhibition of enzyme activities for arachidonate metabolism, especially cyclooxygenase activity of prostaglandin H_2 synthase, as their antiinflammation mechanism (Meade *et al.*, 1993). However, steroidal antiinflammatory drugs are known to have regulatory effects on expression of certain genes through interaction with glucocorticoid-response DNA motif. As the regulatory effects on the cytokine network, glucocorticoids suppress the production of a variety of cytokines including IL-2, IL-3, IL-6, IL-8, TNF, and interferon-g, but increase the number of receptors for IL-1 and IL-6 (Goldstein *et al.*, 1992). The dexamethasone

is known to inhibit the expression of IL-8 gene of IL-1-activated human fibrosarcoma 8387 at transcription level (Mukaida *et al.*, 1992). Therefore, the effects on CINC-1 production of LPS-activated macrophages by dexamethasone and triamcinolon in this study would be attributed to their suppressive activities on the expression of CINC-1 gene.

Table 1. Inhibition on CINC-1 induction.

Conpound	% of Inhibition[a]
Antiinflammatory drugs	
Aspirin	<0
Indomethacin	2±3
Dexamethasone	54±5
Triamcinolon	56±3
Triterpenoids from plants	
Acacigenin B	40±5
Acetylaleuritolic acid	16±11
Aleuritolic acid	<0
β-Amyrin	21±3
β-Amyrin acetate	1±4
Araloside A	13±6
Azukisaponin V	30±7
Betulic acid	34±3
Dioscin	<0
Diosgenin	31±9
Erythrodiol	<0
Glycyrrhetic acid	13±8
Gracillin	32±8
α-Hederin	13±2
Jaligonic acid	28±6
Methyl protogracillin	20±7
Myricadiol 3-acetate	<0
Nigaichigoside F1	40±4
Oleanolic acid	<0
Oleanolic acid glucopyranosyl ester	11±3
Oleanolic acid 3-*O*-arabinose	8±3
Oleanolic acid acetate	9±5
Oleanolic acid methyl ester	<0
β-Peltoboykinolic acid	<0
Phytolaccagenic acid	19±9
Phytolaccagenin	32±9
Phytolaccoside B	13±2
Phytolaccoside E	3±5
Phytolaccoside F	<0
Phytolaccoside I	32±7
Pulsatilla saponin A	<0
Pulsatilla saponin F	<0
Pulsatilla saponin H	<0
(25*S*)-Ruscogenin	17±8
Stipuleanoside R1	9±7
Stipuleanoside R2	7±6
Suavissimoside R1	<0
Trillin	<0
Ursolic acid	14±5

[a]Data are represented as mean ± S.E. (n=4) where each sample treated was 1 μM as the final concentration.

As shown in Table 1, inhibitory effects on CINC-1 induction by triterpenoids were estimated. The triterpenoids were isolated from herbal medicines. Among the 39 kinds of triterpenoids, acacigenin B and nigaichigoside F1 exhibited the highest inhibition on CINC-1 induction in LPS-stimulated macrophages. Acacigenin B was isolated from *Albizzia julibrissin* and nigaichigoside F1 from *Rubus coreanus* (Kang and Woo, 1981; Kim and Kang, 1993). The suppressive effects of acacigenin B and nigaichigoside F1 on CINC-1 induction were about 40% of inhibition when LPS-stimulated macrophages were treated with 1 μM of each of the compounds as the final concentration. Azukisaponin V, betulic acid, diosgenin, gracillin, phytolaccagenin, and phytolaccoside I exhibited 30% to 34% inhibition on the CINC-1 induction, and β-amyrin, jaligonic acid, and methyl protogracillin displayed 20% to 28% inhibition compared to the control. Aleuritolic acid, β-amyrin acetate, myricadiol 3-acetate, oleanolic acid, oleanolic acid methyl ester, β-peltoboykinolic acid, phytolaccosides E and F, pulsatilla saponins A, F, and H, suavissimoside R1, and trillin did not inhibit the CINC-1 induction at all. Unfortunately we could not correlate the structures of triterpenoids to suppression on CINC-1 induction. Possible mechanisms of inhibitory effects on CINC-1 induction by some triterpenoids could be speculated in two ways, transcriptional downregulation of CINC-1 gene by interaction with glucocorticoid-response DNA motif and/or interference of LPS-mediated signal transduction pathway

by amplifying CINC-1 production.

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