Inhibitory effect of a "Shoma" (rhizome of *Cimicifuga heracleifolia* KOMAROV) on the production of a neutrophil chemotactic factor, rat gro/CINC in cell cultures *in vitro* and inflammation models *in vivo*

Hideko KATO[a] Mari MITAMURA[a] Junji NITTA[a] Michiyo KACHI[a] Miki OHARA[a] Yukie TANAKA[a] Susumu TSURUFUJI[b] Masao HATTORI[c] and Hideo NAKAGAWA*[a]

[a] *Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University*
[b] *Institute of Cytosignal Research Inc.*
[c] *Department of Cell-Resources Engineering, Research Institute for WAKAN-YAKU, Toyama Medical and Pharmaceutical University*

(Received December 4, 1992. Accepted January 28, 1993.)

Abstract

Effect of the boiling water extract from a "Shoma (升麻), rhizome of *Cimicifuga heracleifolia* KOMAROV" on the production of rat gro/cytokine-induced neutrophil chemoattractant (CINC) was studied *in vitro* and *in vivo*. CINC concentrations in conditioned media of cell cultures and in exudates of inflammatory sites were measured by a sensitive enzyme-linked immunosorbent assay for CINC. The extract of "Shoma" inhibited dose-dependently the CINC production by interleukin-1β-stimulated rat fibroblasts (NRK-49F cell line), lipopolysaccharide (LPS)-stimulated rat epithelioid cells (NRK-52E cell line) and LPS-stimulated rat macrophages in cultures. In addition, oral administration of the extract significantly suppressed the CINC levels in the pouch fluids (exudates) at 6 hr after the injections of LPS or carrageenin into the preformed air-pouch in rats, resulting in the reduced infiltration of neutrophils into the inflammatory lesion. The results suggest that rhizome of *Cimicifuga heracleifolia* KOMAROV contains a component(s) having inhibitory activity on the rat gro/CINC production by activated inflammatory cells.

Key words *Cimicifuga heracleifolia* KOMAROV, *Shoma*, neutrophil infiltration, neutrophil chemotactic factor, interleukin-8, cytokine-induced neutrophil chemoattractant.

Abbreviations BSA, bovine serum albumin; *C. heracleifolia*, *Cimicifuga heracleifolia* KOMAROV; CINC, cytokine-induced neutrophil chemoattractant; CMC, carboxymethylcellulose; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; rhIL-1β, recombinant human interleukin-1β.

Introduction

"Shoma", the rhizomata of Cimicifuga species are used as antipyretic, analgesic, and anti-inflammatory drugs in Japanese Oriental (Kampo) medicines. Little is known, however, about the mechanism by which "Shoma" exerts its anti-inflammatory effects, though the effects of "Shoma" on some inflammatory reactions have been reported.[1,2] Neutrophil infiltration into the inflammatory lesion is an important characteristic of inflammation. Recently, novel neutrophil chemotactic factors, interleukin-8 (IL-8) family, have been found in culture media of cytokine-stimulated inflammatory cells including monocytes/macrophages, fibroblasts and endothelial cells. Generation of IL-8 can be expected upon diseases where the levels of cytokines such as IL-1β and tumor necrosis factor-α are ele-

*〒930-01 富山市杉谷2630
富山医科薬科大学薬学部 中川秀夫
Sugitani, Toyama 930-01, Japan

Journal of Medical and Pharmaceutical Society for WAKAN-YAKU **10**, 13－20, 1993

vated. IL-8 has been suggested as an important mediator in the pathogenesis of rheumatoid arthritis[3] ; IL-8 accumulates in the synovial fluid of arthritic joints[4,5] and is released by stimulated synovial cells[6] and chondrocytes,[7] suggesting that IL-8 is likely to be the main cause of the local accumulation of neutrophils, and activates the accumulated neutrophils. On the other hand, cytokine-indiced neutrophil chemoattractant (CINC) has been purified from conditioned medium of cytokine-stimulated normal rat kidney epithelioid cells.[8,9] CINC is the rat counterpart of the human gro protein which belongs to IL-8 family.[9] In the present studies, therefore, we have focused our attention upon the effect of *Cimicifuga heracleifolia* KOMAROV (*C. heracleifolia*) on the production of rat gro/CINC in cell cultures *in vitro* and inflammation models of rats *in vivo*.

Materials and Methods

Preparation of C. heracleifolia extract : The dried rhizomata of "Shoma (升麻), *Cimicifuga heracleifolia* KOMAROV (Heilong Jiang Prov., 黑龍江省 ; China)" were cut into small pieces. The pieces (45 g) were suspended in 400 ml of distilled water and gently boiled for 50 min, filtered while warm, and then the filtrate (extract) was adjusted to 300 ml with distilled water. The extract was stored at -20℃ until use. Just before experiments, the frozen extract was thawed in running tap-water and centrifuged at 800×g for 20 min at 4℃, and the supernatant was used as the *C. heracleifolia* extract for *in vitro* and *in vivo* experiments. One ml of the supernatant was 62 mg after lyophilization.

Cell cultures : Normal rat kidney fibroblasts (NRK-49F cell line) and epithelioid cells (NRK-52E cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 25 mM HEPES, penicillin 0.1 mg/ml, and streptomycin 0.1 mg/ml in multiwell dishes (96 wells, Falcon, CA, USA). When the cells reached a confluent monolayer, the dishes were washed with serum-free culture medium, and the effects of *C. heracleifolia* extract on the CINC production by the cells were studied ; NRK-49F cells or NRK-52E cells were cultured for 24 hr with rhIL-1β (10^{-10}M) or LPS 2 μg/ml), respectively, in the presence of various concentrations of the *C. heracleifolia* extract in serum-free medium containing 0.1% (w/v) bovine serum albumin (BSA).

Rat macrophages were collected by peritoneal lavage with phosphate-buffered saline (PBS) 3 days after intraperitoneal injection of 10% (w/v) proteose peptone/0.9% (w/v) NaCl solution (35 ml/kg body wt. of male Wistar rats). The peritoneal cells were washed twice with PBS and once with 10% (v/v) fetal calf serum/DMEM, and then centrifuged at 500×g for 5 min at 4℃. The cells were suspended in DMEM supplemented with 10% fatal calf serum, 10 mM HEPES, penicillin 0.1 mg/ml, and streptomycin 0.1 mg/ml. The cell suspension (5×10^5 cells/ 0.2 ml/well) was added to 96-multiwell dishes and incubated for 24 hr at 37℃ under 5% CO_2/95% air. The non-adherent cells were removed by washing with serum-free DMEM, and the adherent cells (macrophages) were cultured for 48 hr with LPS (5 μg/ml) and various concentrations of the *C. heracleifolia* extract in a serum-free medium containing 0.1% (w/v) BSA.

After cultures of NRK-49F cells, NRK-52E cells and macrophages, the conditioned media were centrifuged at 900×g for 30 min at 4℃ and the supernatants were used for the sandwich enzyme-linked immunosorbent assay (ELISA) to CINC.

Air-pouch/LPS- or carrageenin-induced inflammation : An inflammation was induced by a modification of the procedure of Fukuhara and Tsurufuji.[10] Briefly, an air-pouch was formed on the back of male Wistar rats (weighing 160-190 g) by the subcutaneous injection of air (10 ml), and 3 days later additional air (10 ml) was injected into the preformed air-pouch to maintain a large air-pouch. On day 6 after the first air-injection, the *C. heracleifolia* extract (10 ml/kg body wt.) was orally administered 1 hr before the injections of 7 ml of LPS solution (LPS 0.1 μg/ml of 1%, w/v, carboxymethylcellulose, CMC) or 4ml of 2% (w/v) solution of carrageenin (Seakem 202 ; Marine

Colloid, NJ, USA). The pouch fluids (0.2 ml) were collected at 2, 4, 6 and 8 hr after the injections of LPS or carrageenin solutions. In the case of air-pouch/ LPS-induced inflammation, a part (0.1 ml) of the pouch fluids was diluted with PBS (0.9 ml) and centrifuged at 2,000×g for 20 min at 4℃, and the supernatant was used for ELISA to CINC. Another part (0.1 ml) was diluted with PBS (0.9 ml) containing 0.25% (w/v) EDTA, and the diluted solution was mixed with 1 ml of cellulase solution (0.2 mg/ml of PBS) and incubated for 40 min at 37℃. After incubation, the reaction mixture was diluted with 8 ml (for the 2- and 4-hr pouch fluids) or 18 ml (for the 6- and 8-hr pouch fluids) of 0.25% EDTA/PBS solution, and the total number of neutrophils was then counted by a Coulter counter. After cell counting, the cell suspension was centrifuged at 2,000×g for 30 min at 4℃, and protein concentration in the supernatant was determined by a dye-binding microassay (Bio-Rad, Richmond, CA, USA). On the other hand, in the case of air-pouch/carrageenin-induced inflammation, a part (0.1 ml) of the pouch fluids was diluted 10-20 times with 0.1 % (w/v) trypan blue/PBS solution and the number of neutrophils was counted microscopically. Another part (0.1 ml) of the pouch fluid was diluted with PBS (0.9 ml) and centrifuged at 100,000×g for 30 min at 4℃, and then the supernatant was used for the measurements of CINC and protein concentrations.

Gel filtration : The *C. heracleifolia* extract (30 ml) was fractionated with $(NH_4)_2SO_4$; the precipitate between 25% and 70% (w/v) saturation with $(NH_4)_2SO_4$ was obtained by centrifugation at 10,000×g for 30 min at 4℃. The precipitate was dissolved in 2 ml of PBS and dialyzed against PBS using Spectra/Por membrane (molecular-mass cutoff 3,500) at 4℃. A part (1ml) of the non-dialyzable sample was applied to a Sephadex G-75 column (1.5×85 cm) and eluted with PBS at a flow rate of 6.7 ml/hr. Effluent fractions of 3.7 ml were collected and the inhibitory activity of fractions on the CINC production by LPS-stimulated NRK-52E cells was determined by the method described above.

CINC assay : Samples were diluted 5-10 times with 0.1% (w/v) BSA/PBS solution, and CINC concentrations were determined by a sensitive biotin-streptavidin sandwich ELISA reported by Nakagawa *et al*.[11] in which rabbit anti-CINC antibody and biotinylated anti-CINC antibody were used as the capture antibody and the second layer antibody, respectively. Color development proceeded for 5 to 15 min by addition of peroxidase-coupled streptavidin and substrate before terminating the reaction with 2 M H_2SO_4. The absorbance was then measured at 492 nm on a microplate reader, and the concentration of CINC was determined by reference to a CINC standard curve.

The results were subjected to statistical analysis according to the Student's *t* test.

Results

Inhibitory effect of the C. heracleifolia extract on CINC production in vitro

Normal rat kidney fibroblasts (NRK-49F cells), epithelioid cells (NRK-52E cells) and rat macrophages were stimulated with rhIL-1β or LPS in the presence of various concentrations of the *C. heracleifolia* extract. As shown in Fig. 1 (A, B and C), the extract suppressed in a dose-dependent manner the CINC production by rhIL-1β-stimulated NRK-49F cells (A), LPS-stimulated NRK-52E cells (B) and rat macrophages (C) in cultures. Addition of distilled water (final 5%, v/v) to culture medium had no effect on the CINC production by LPS-stimulated NRK-52E cells (data not shown), and no direct influence of the *C. heracleifolia* extract on the ELISA for CINC was found in the present experimental conditions (data not shown). Our results indicate that the extract contains a component(s) having inhibitory activity on the CINC production by the stimulated cells. To estimate the molecular mass of an inhibitory component(s) in the *C. heracleifolia* extract, the extract was dialyzed against distilled water, and then the inhibitory effect of the non-dialyzable sample on the CINC production by the stimulated cells was determined. Figure 2 shows that the non-dialyzable fraction of the extract dose-dependently suppressed the CINC produc-

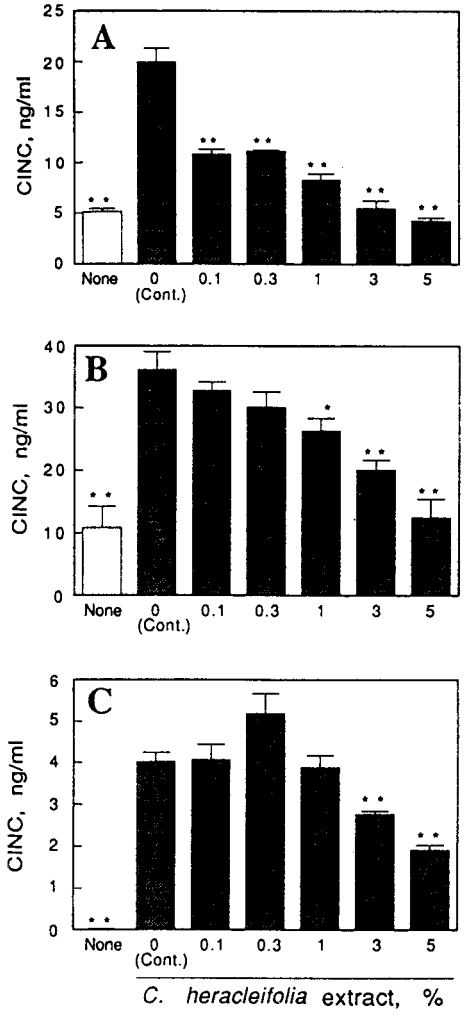

Fig. 1 Effect of the *C. heracleifolia* extraxt on the CINC production by rhIL-1β-stimulated NRK-49F cells (A), LPS-stimulated NRK-52E cells (B) and LPS-stimulated rat macrophages (C).

The stimulated cells were cultured with the *C. heracleifolia* extract at the indicated concentration (%, v/v). In the blank group (None), the cells were cultured without both the stimulant and the extract. The CINC concentrations in conditioned media were determined by the sandwich ELISA for CINC. Experimental details are described under Materials and Methods. Data sre shown as the means with horizontal bars representing SEM of 6 determinations. Values are statistically significant difference from the control group (Cont.) which was cultured without the extract; $*p<0.05$ and $**p<0.01$.

tion by the stimulated NRK-49F cells and NRK-52E cells. In addition, the precipitate from the *C. heracleifolia* extract between 25% and 70% (w/v) saturation with $(NH_4)_2SO_4$ had a strong inhibi-

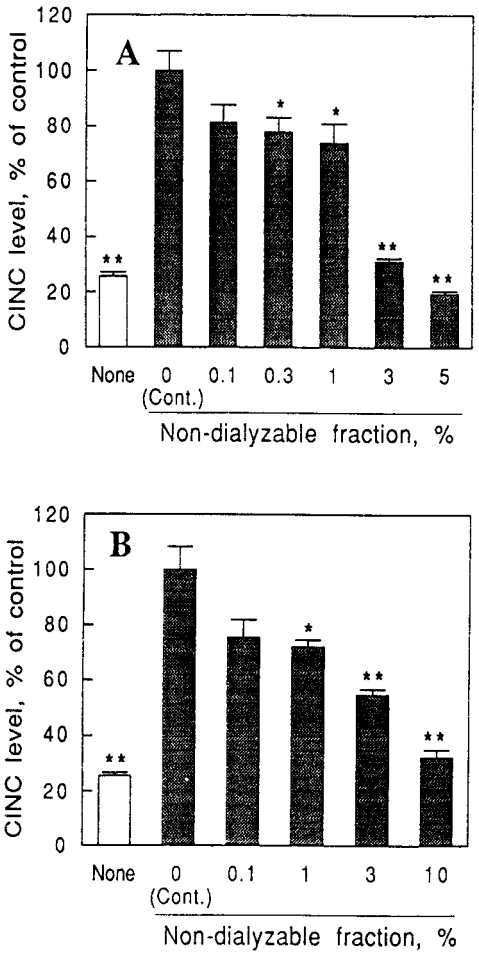

Fig. 2 Effect of the non-dialyzable fraction of *C. heracleifolia* extract on the CINC production by rhIL-1β- or LPS-stimulated cells.

rhIL-1β-stimulated NRK-49F cells (A) and LPS-stimulated NRK-52E cells (B) were cultured with various concentrations (%, v/v) of the non-dialyzable fraction under the conditions similar to those described in Fig. 1. The CINC concentrations in conditioned media were expressed as percentage (%) of the value of control group which was cultured without the non-dialyzable fraction. Data are shown as the means with horizontal bars representing SEM of 6 determinations. Values are statistically significant difference from the control group (Cont.) which was cultured without the non-dialyzable fraction; $*p<0.05$ and $**p<0.01$.

tory activity on the CINC production by LPS-stimulated NRK - 52E cells (data not shown). The precipitate was dissolved in PBS and applied to a Sephadex G-75 column in order to estimate the molecular mass of a component(s) which inhibits the CINC production by LPS-stimulated NRK-52E cells. As shown in Fig. 3, the inhibitory activity was found in a wide range of molecular masses (2-6 kDa) with a maximum inhibitory activity of about 3 kDa on the gel filtration.

Inhibitory effect of the C. heracleifolia extract on CINC levels and neutrophil infiltration in vivo

The *C. heracleifolia* extract (10 ml/kg body wt.) was orally administered 1 hr before the induction of inflammation by the injection of LPS or carrageenin into the preformed air-pouch on the back of rats. The extract significantly suppressed neutrophil infiltration into the pouch (inflammatory site) at 4-8 hr after LPS injection, while the treatment with the extract showed a tendency to suppress the CINC levels in the pouch fluids at 2 and 4 hr and significantly suppressed at 6 hr, but the CINC level decreased to the level of the unstimulated group at 8 hr after LPS injection (Fig. 4). On the other hand, in the air-pouch/carrageenin-induced inflammation of rats, the *C. heracleifolia* extract showed a weak but significant inhibition of both CINC level and neutrophil infiltration; the CINC concentrations and the number of neutrophils in the pouch fluids were significantly suppressed at 6 and 8 hr, respective-

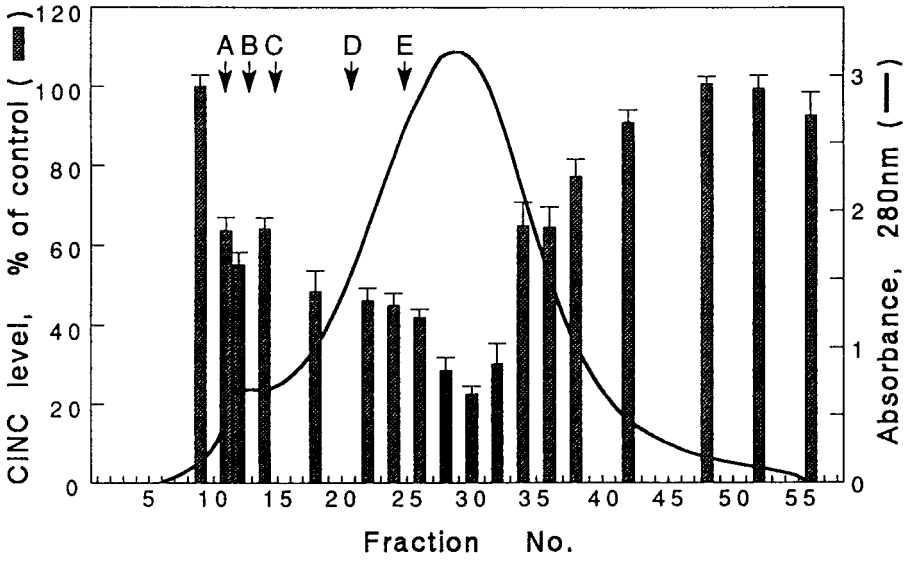

Fig. 3 Sephadex G-75 gel filtration of the ammonium sulfate precipitate from the *C. heracleifolia* extract.

The precipitate from the *C. heracleifolia* extract between 25% and 70% (w/v) saturation with $(NH_4)_2SO_4$ was dissolved and dialyzed against PBS. A part (1 ml) of the non-dialyzable sample was applied to a Sephadex G-75 column (1.5×85 cm). Experimental details are described under Materials and Methods. The effect of some fractions on the CINC production by LPS-stimulated NRK-52E cells was determined, and the CINC concentrations in conditioned media were expressed as percentage (%) of the value of control group which was cultured without fractions. Data are shown as the means with horizontal bars representing SEM of 6 determinations. The following molecular-mass markers were used: blue dextran (A), BSA (B; 66 kDa), ovalbumin (C; 43 kDa), cytochrome C (D; 12.4 kDa) and aprotinin (E; 6.4 kDa).

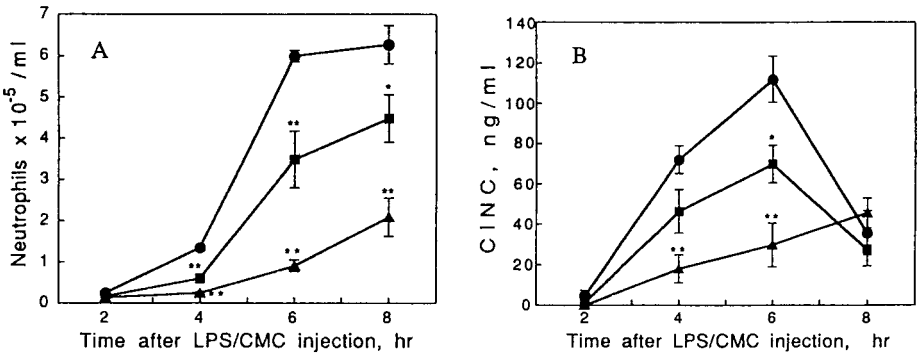

Fig. 4 Effects of the *C. heracleifolia* extract on the neutrophil infiltration and the CINC levels in the pouch fluids of air-pouch/LPS-induced inflammation in rats.

The *C. heracleifolia* extract (■) or distilled water (● ; control group) were administered orally in a dosage of 10 ml/kg body weight 1 hr before the injection of 7 ml of LPS/CMC solution (LPS 0.1μg/ml of 1 % CMC). In the reference group (▲), distilled water was orally administered 1 hr before the injection of CMC solution (1 % CMC alone). The pouch fluids were obtained at 2, 4, 6, and 8 hr after the injection of LPS/CMC solution. The number of neutrophils (A) and the concentration of CINC (B) in each pouch fluid were determined as described under Materials and Methods. Each point represents the mean with horizontal bar representing SEM of 5 rats. Values are statistically significant difference from the control group; $*p<0.05$ and $**p<0.01$.

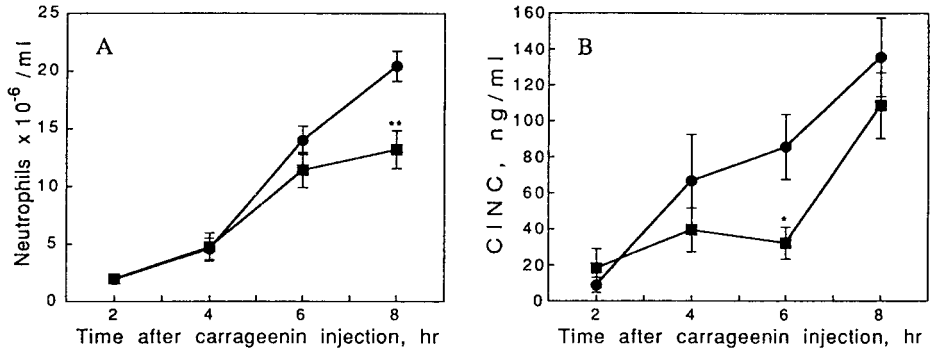

Fig. 5 Effects of the *C. heracleifolia* extract on the neutrophil infiltration and the CINC levels in the pouch fluids of air-pouch/carrageenin-induced inflammation in rats.

The *C. heracleifolia* extract (■) or distilled water (● ; control group) were administered orally in a dosage of 10 ml/kg body weight 1 hr before the injection of 4 ml of 2 % carrageenin solution. The pouch fluids were obtained at 2, 4, 6 and 8 hr after the injection of carrageenin solution. The number of neutrophils (A) and the concentration of CINC (B) in each pouch fluid were determined as described under Materials and Methods. Each point represents the mean with horizontal bar repersenting SEM of 7 rats. Values are statistically significant difference from the control group; $*p<0.05$ and $**p<0.01$.

ly, after carrageenin injection (Fig. 5).

No difference between control group and the group treated with the extract was found in protein concentrations of the pouch fluids of both LPS- and carrageenin-induced inflammation in rats (data not shown), suggesting that vascular permeability in the inflammatory sites is not influenced by the treatment with *C. heracleifolia* extract.

Discussion

The boiling water extract from *C. heracleifolia* inhibited dose-dependently the CINC production by LPS- or IL-1β-stimulated rat cell lines and rat macrophages in cultures (Fig. 1). This inhibitory activity of the extract (0.1-5%, v/v) is roughly equivalent to that of dexamethasone (10^{-10}M-10^{-7}M) (submitted). The inhibitory activity was found in the non-dialyzable fraction of the extract (Fig. 2) and its molecular mass was about 2-6 kDa as determined by gel filtration on Sephadex G-75 (Fig. 3). However, our results do not rule out the possibility that a small organic compound has inhibitory activity on the CINC production, because the inhibitory activity was found not only in the non-dialyzable fraction but also in the dialysate when the *C. heracleifolia* extract was dialyzed against distilled water by using the membrane having molecular-mass cut-off 3,500 (data not shown). Shibata *et al.*[1,2] demonstrated that isoferuric acid is a constituent of Cimicifuga species and has an inhibitory effect on the dextran-and carrageenin-induced edemas of rat hind paw. In addition, we have found that isoferuric acid has a weak inhibitory activity on the CINC production by LPS-stimulated NRK-52E cells (data not shown). However, the anti-inflammatory activity of small compounds in the extract remains unclear, because the present studies were undertaken to ascertain whether a higher molecular - mass compound has anti - inflammatory activity, with particular reference to the suppression of CINC production.

In a previous paper,[11] we have demonstrated that CINC contributes, at least in part, to the migration of neutrophils into the inflammatory lesion of the carrageenin-induced inflammation in rats. Similarly, Iida *et al.*[12] reported that CINC is a functional neutrophil chemoattractant in LPS-induced inflammation in rats. The *C. heracleifolia* extract significantly suppressed both CINC level and neutrophil infiltration in LPS-or carrageenin-induced inflammation in rats (Figs. 4 and 5). From our results it should be pointed out that there is a time lag of 1-2 hr between the CINC level and the number of infiltrated neutrophils; CINC concentrations of the pouch fluids in the control rats reached a high level at 4 hr, whereas the number of neutrophils is small at 4 hr but large at 6 hr after injections of LPS or carrageenin. Similarly, the reduced CINC level at 6 hr probably resulted in the suppression of neutrophil infiltration at 8 hr after carrageenin injection in the rats treated with the *C. heracleifolia* extract (Fig. 5).[5]

In summary, our results demonstrate that the *C. heracleifolia* extract suppressed the production of rat gro/CINC by the stimulated inflammatory cells including macrophages and fibroblasts in cultures *in vitro* and in inflammation models *in vivo*. The purification of a component having inhibitory activity from the extract is currently under investigation.

Acknowledgements

We thank Otsuka Pharmaceutical Co. Ltd. (Tokushima) and Japanese Cancer Research Resources Bank (Tokyo) for their generous gifts of rhIL-1β and NRK-49F cells (JCRB9067), respectively. This work was supported in part by a Grant-in-Aid for Scientific Research from Toyama Prefecture.

和文抄録

IL-8 ファミリーに属するラット gro/CINC (cytokine-induced neutrophil chemoattractant) の産生に対する“升麻, *Cimicifuga heracleifolia* KOMAROV の根茎”の水煎液の影響を *in vitro* および *in vivo* 実験系において検討した。細胞培養液や炎症局所の滲出液中に存在する CINC 濃度は, CINC に対する感度の高い ELISA 法によって測定

した。升麻水煎液は，IL-1β刺激したラット線維芽細胞（NRK-49F細胞株），LPS刺激したラット上皮様細胞（NRK-52E細胞株）およびラットマクロファージの培養系におけるCINC産生を用量依存的に阻害した。さらに升麻水煎液の経口投与は，ラットに予め作成した空気嚢内にLPS又はカラゲニン注射の6時間後の嚢内液（滲出液）中のCINCレベルを有意に抑制し，結果として炎症部位への好中球浸潤が減少した。これらの結果は，*Cimicifuga heracleifolia* KOMAROVの根茎が活性化した炎症細胞によるラットgro/CINC産生に対する抑制活性を有する成分を含んでいることを示唆している。

References

1) Shibata, M., Yamatake, Y., Amagaya, Y. and Fukushima, M.: Pharmacological studies on the Chinese crude drug "Shôma". I. Acute toxicity and anti-inflammatory action of Cimicifuga Rhizoma, *Cimicifuga dahurica* MAXIM. *Yakugaku Zasshi* **95**, 539-546, 1957.

2) Shibata, M., Sakurai, N. and Onoda, M.: Pharmacological studies on the Chinese crude drug "Shôma". II. Anti-inflammatory action of Cimiciguga Rhizoma, *Cimicifuga simplex* WORMSK. *Yakugaku Zasshi* **97**, 911-915, 1977.

3) Elford, P.R. and Cooper P.H.: Induction of neutrophil-mediated cartilage degradation by interleukin-8. *Arth. Rheum.* **34**, 325-332, 1991.

4) Seiz, M., Dewald, B., Gerber, N. and Baggiolini, M.: Enhanced production of neutrophil-activating peptide-1/interleukin-8 in rheumatoid arthritis. *J. Clin. Invest.* **87**, 463-469, 1991.

5) Brennan, F.M., Zachariae, C.O.C., Chantry, D., Larsen C.G., Turner, M., Maini, M., Maini, R.M., Matsushima, K. and Feldmann, M.: Detection of interleukin 8 biological activity in synovial fluids from patients with rheumatoid arthritis and production of interleukin 8 mRNA by isolated synovial cells. *Eur. J. Immunol.* **20**, 2141-2144, 1990.

6) Golds, E.E., Mason, P. and Nyirkos, P.: Inflammatory cytokines induce synthesis and secretion of *gro* protein and a neutrophil chemotactic factor but not B2-microglobulin in human synovial cells and fibroblasts. *Biochem. J.* **259**, 585-588, 1989.

7) Van Damme, J., Bunning, R.A.D., Conings, R., Graham, R., Rusell, G. and Opdenakker, G.: Characterization of granulocyte chemotactic activity from human cytokine-stimulated chondrocytes as interleukin 8. *Cytokine* **2**, 106-111, 1990.

8) Watanabe, K., Kinoshita, S. and Nakagawa, H.: Purification and characterization of cytokine-induced neutrophil chemoattractant produced by epithelioid cell of normal rat kidney (NRK-52E cell). *Biochem. Biophys. Res. Commun.* **161**, 1093-1099, 1989.

9) Watanabe, K., Konishi, K., Fujioka, M., Kinoshita, S. and Nakagawa, H.: The neutrophil chemoattractant produced by the rat kidney epithelioid cell line NRK-52E is a protein related to the KC/gro protein. *J. Biol. Chem.* **264**, 19559-19563, 1989.

10) Fukuhara, M. and Tsurufuji, S.: The effect of locally injected anti-inflammatory drugs on the carraggeenin granuloma in rats. *Biochem. Pharmacol.* **18**, 475-484, 1969.

11) Nakagawa, H., Ikesue, A., Kato, H., Debuchi, H., Watanabe, K., Tsurufuji, S., Naganawa, M. and Mitamura, M.: Changes in the levels of rat interleukin 8/CINC and gelatinase in the exudate of carrageenin-induced inflammation in rats. *J. Pharmacobio-Dyn.* **15**, 461-466, 1992.

12) Iida, M., Watanabe, K., Tsurufuli, M., Takahashi, K., Iizuka, Y. and Tsurufuji, S.: Level of neutrophil chemotactic factor CINC/gro, a member of the interleukin-8 family, associated with lipopolysaccharide-induced inflammation in rats. *Infection Immunity* **60**, 1268-1272, 1992.