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PRODUCTION OF AN INTERLEUKIN-8-LIKE CHEMOKINE BY CYTOKINE-STIMULATED RAT NRK-49F FIBROBLASTS AND ITS SUPPRESSION BY ANTI-INFLAMMATORY STEROIDS

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Abstract—Normal rat kidney fibroblasts (NRK-49F cells) stimulated with interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) produced mainly cytokine-induced neutrophil chemoattractant (CINC) which is the rat counterpart of human gro/melanoma growth stimulatory activity. In addition, the cytokine-stimulated cells produced two minor neutrophil chemoattractants which are highly related to murine macrophage inflammatory protein-2 in their NH_2-terminal amino acid sequences. IL-1β was a stronger stimulator than TNF-α, and addition of both the cytokines into the NRK-49F cell culture caused an additive stimulation for rat gro/CINC production. The anti-inflammatory steroids (dexamethasone, prednisolone and hydrocortisone) at 10^{-9}–10^{-6} M significantly suppressed the production of rat gro/CINC by the IL-1β-stimulated NRK-49F cells in a dose-dependent manner. The relative potencies of the inhibitory activity of the steroids on the rat gro/CINC production were dexamethasone > prednisolone > hydrocortisone. On the other hand, the non-steroidal anti-inflammatory drugs (indomethacin and piroxicam) at 10^{-7}–10^{-5} M showed no apparent inhibitory effect on rat gro/CINC production by NRK-49F cells stimulated with IL-1β.

Infiltration of neutrophils into inflammatory lesions is brought about through chemical stimulation by chemotactic substances. Over the last few years there has been increasing knowledge of novel chemokines for neutrophils, the interleukin (IL‡)-8 family, which are produced by cytokine-stimulated inflammatory cells including macrophages, fibroblasts and endothelial cells [1–7]. We have recently purified and characterized a novel neutrophil chemotactic factor, named CINC, which is produced by the cytokine-stimulated normal rat kidney epitheloid cell line, NRK-52E [8, 9]. CINC consists of 72 amino acids having a similar amino acid sequence to human gro/melanoma growth stimulatory activities (MGSA), indicating that rat gro/CINC belongs to the IL-8 superfamily [9]. In the present studies we have demonstrated that IL-1β and tumor necrosis factor-α (TNF-α) stimulate rat fibroblasts (NRK-49F cells) to produce rat gro/CINC as a major chemokine, together with two minor chemokines whose NH_2-terminal amino acid sequences are highly homologous to that of murine macrophage inflammatory protein 2 (MIP-2), and demonstrated that anti-inflammatory steroids suppress rat gro/CINC production by recombinant human IL-1β (rhIL-1β)-stimulated rat fibroblasts in a dose-dependent manner.

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‡ Abbreviations: BSA, bovine serum albumin; CINC, cytokine-induced neutrophil chemoattractant; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IL, interleukin; MGSA, melanoma growth stimulatory activity; MIP-2, murine macrophage inflammatory protein 2; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; rhIL-1β, recombinant human interleukin-1β; rhTNF-α, recombinant human tumor necrosis factor-α; RP-HPLC, reverse-phase HPLC; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Cell culture. Normal rat kidney fibroblasts (NRK-49F cell line) were cultured in Dulbecco's modified Eagle's medium, supplemented with 5% (v/v) fetal calf serum, 25 mM HEPES, penicillin 0.1 mg/mL and streptomycin 0.1 mg/mL, in multiwell dishes (96 wells, Falcon, CA, U.S.A.). When the NRK-49F cells reached a confluent monolayer, the dish was washed three times with the culture medium, and cultured with serum-free culture medium containing 0.1% (w/v) bovine serum albumin (BSA) and various concentrations of cytokines. The effects of anti-inflammatory drugs on the production of rat gro/CINC by rhIL-1β-stimulated NRK-49F cells were studied by addition of drugs into the serum-free culture medium containing 10^{-11} M rhIL-1β and 0.1% (w/v) BSA. After 24 hr culture, cell-free conditioned medium was obtained by centrifugation at 700 *g* for 20 min at 4° and used for the sandwich enzyme-linked immunosorbent assay (ELISA) to rat gro/CINC.

Purification of chemokines. NRK-49F cells were maintained in 9-cm plastic culture dishes containing 10 mL of the culture medium. When the fibroblasts reached a confluence, the cells were cultured for 2

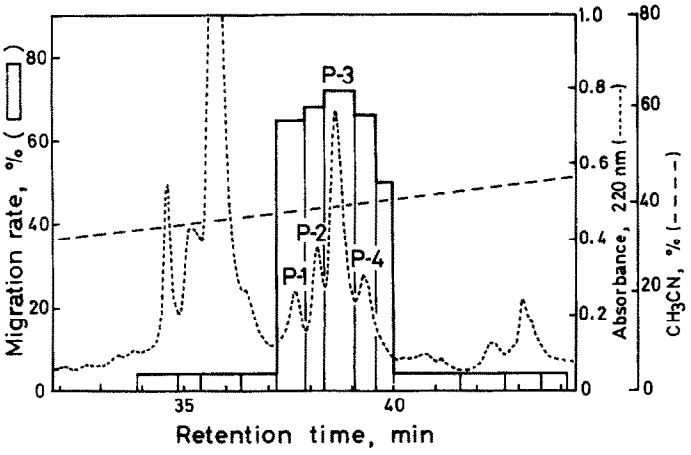

Fig. 1. RP-HPLC of chemokines produced by cytokine-stimulated rat fibroblasts (NRK-49F cells). Chemokines were eluted from a C-18 reverse-phase column with a linear concentration gradient of acetonitrile from 0% (v/v) to 80% (v/v) in 0.05% (v/v) trifluoroacetic acid at a flow rate of 0.8 mL/min. Chemotactic activity of each fraction was assayed at a concentration of 3×10^{-9}–2×10^{-8} M in duplicate, and expressed as migration rate (%).

days in serum-free culture medium supplemented with rhIL-1β (3×10^{-11} M), recombinant human TNF-α (rhTNF-α) (3×10^{-11} M) and 0.1% (w/v) BSA. Cell-free conditioned medium was obtained by centrifugation and frozen at −30° until use.

Chemokines were purified by similar procedures previously described for the purification of rat gro/CINC [8, 9]; the conditioned medium (19.4 L) was chromatographed sequentially on heparin-Sepharose, CM-Sephadex, and finally loaded on a C-18 reverse-column (0.45 × 25 cm; Tosoh Co., Japan).

The purity and M_r of chemokines were determined by SDS–PAGE on a slab gel of a discontinuous 10.0%/16.5% acrylamide in tricine buffer system [10]. For M_r calibration, M_r standards (Pharmacia LKB low-M_r calibration kit supplemented with aprotinin, 6.4 kDa) were run simultaneously.

Sandwich ELISA for rat gro/CINC. The concentration of rat gro/CINC in the conditioned medium was determined by the biotin-streptavidin sandwich ELISA for rat gro/CINC using polyclonal rabbit anti-rat gro/CINC antiserum as described previously [11]. Briefly, assay plates (flexible assay plates, Falcon, CA, U.S.A) were coated with 0.4 μg/well of polyclonal anti-rat gro/CINC immunoglobulin G (IgG) and incubated at 37° for 3 hr, and the plates were washed with phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween-20. PBS containing 1% (w/v) BSA (200 μL) was added, and the plates were left overnight at 4°. After the plates were washed with PBS/Tween-20, rat gro/CINC standards or samples (80 μL/well) which had been diluted with PBS containing 0.1% (w/v) BSA were added. The plates were incubated at 37° for 60 min and washed with PBS/Tween-20. Biotinylated polyclonal rabbit anti-rat gro/CINC IgG solution (80 μL/well of the IgG, 1 mg/mL) was added and incubated at 37° for 60 min. After the plates were washed with PBS/Tween-20, 80 μL of a 1/10,000 dilution of streptavidin–horseradish peroxidase (CALTAG Laboratories, CA, U.S.A.) was added. After 30 min at 37°, the plates were washed and 100 μL of substrate solution (0.1% *o*-phenylenediamine–0.02% H_2O_2–50 mM citrate–100 mM phosphate buffer, pH 5.0) was added. After optimal color development, the reaction was stopped by the addition of 100 μL of 2 M H_2SO_4. The absorbance at 492 nm was then measured on a microplate reader (model 450, Bio-Rad Laboratories, Richmond, CA, U.S.A.), and the concentration of rat gro/CINC was calculated based on the standard curve of rat gro/CINC.

Chemotaxis assay. Rat neutrophils were collected by peritoneal lavage 16 hr after intraperitoneal injection of 1% (w/v) casein in Krebs–Ringer bicarbonate solution. Chemotactic activity for rat neutrophils was assayed in multiwell chambers as described previously [12]. As an index of chemotaxis, the number of neutrophils migrated into the lower chamber during 80 min incubation was expressed as percentage (migration rate) of that of neutrophils applied in the upper chamber.

NH_2-terminal amino acid sequencing analysis. The purified chemotactic factors were reduced and carboxymethylated as described previously [9]. NH_2-terminal amino acid sequences of the samples were determined by automated Edman degradation on a gas-phase protein sequencer (model 470A, Applied Biosystems, CA, U.S.A.) with on-line phenylthiohydantoin amino acid analysis (model 120A HPLC system).

RESULTS

Rat gro/CINC species produced by cytokine-stimulated NRK-49F cells

The chemokines were purified from the conditioned medium of NRK-49F cells stimulated with 3×10^{-11} M rhIL-1β and rhTNF-α. Neutrophil chemoattractants were finally eluted from a reverse-

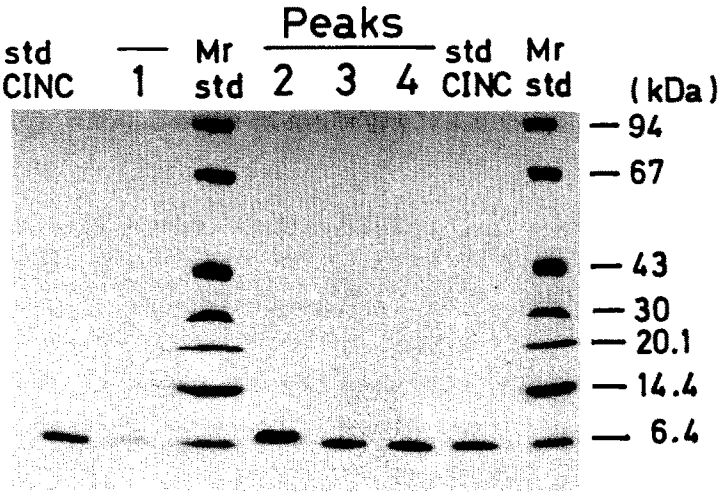

Fig. 2. SDS–PAGE of the purified chemokines. The samples (P-1–P-4 in Fig. 1) were reduced with 25% 2-mercaptoethanol and subjected to SDS–PAGE. Standard rat gro/CINC (std CINC) and M_r standards (M_r std) were run simultaneously.

phase HPLC (RP-HPLC) system as four peaks (P-1–P-4 in Fig. 1). The four chemoattractants were purified to homogeneity by rechromatography of RP-HPLC. P-3 was the major chemokine produced by cytokine-stimulated NRK-49F fibroblasts, while other peaks (P-1, P-2 and P-4) were minor ones. Each purified chemoattractant gave a single band on SDS–PAGE under reducing conditions (Fig. 2). P-1, P-3 and P-4 gave the same M_r (6.4 kDa) as standard rat gro/CINC which had been purified from the conditioned medium of rhIL-1β-stimulated NRK-52E epithelioid cell culture [8], whereas the M_r (6.6 kDa) of P-2 was slightly higher than that of standard rat gro/CINC (Fig. 2). Agreeing with the results, the M_r (about 15 kDa) of P-2 was higher than that (about 12 kDa) of standard rat gro/CINC on high pressure gel filtration, while P-1, P-3 and P-4 gave the same retention time as standard rat gro/CINC (data not shown). These results suggest that each chemokine exists as a dimer form in solution.

NH_2-terminal amino acid sequences of the reduced and carboxymethylated P-1–P-4 are summarized in Fig. 3. The sequence of P-3 was identical to that of rat gro/CINC. In addition, the sequences of fragments isolated from proteinase V8-treated P-3 corresponded to the amino acid sequence of rat gro/CINC, and COOH-terminal amino acid, Lys was also identical to that of rat gro/CINC (data not shown). The results indicate that P-3 is rat gro/CINC. The NH_2-terminal amino acid sequence of P-4 was also identical to that of rat gro/CINC, suggesting that P-4 may be a modified rat gro/CINC. However, there was not enough purified P-4 to analyse its complete amino acid sequence, and future investigation is required to clarify the differences between rat gro/CINC (P-3) and P-4. On the other hand, the NH_2-terminal amino acid sequences of P-1 and P-2 were very similar to each other (86% homology of NH_2-terminal 14 residues). Although the homology of NH_2-terminal 24 residues between P-2 and rat gro/CINC was high (63%), rabbit anti-rat gro/CINC antiserum could hardly neutralize the chemotactic activity of P-2 whose chemotactic potency for rat neutrophils was similar to that of rat gro/CINC (Fig. 4). In addition, P-2 was first detected in the sandwich ELISA for rat gro/CINC when the concentration of P-2 was 100 times higher than that of rat gro/CINC (P-3) (data not shown), suggesting that P-2 differs from rat gro/CINC in its antigenicity.

Effects of anti-inflammatory drugs on rat gro/CINC production

The concentrations of rat gro/CINC in the conditioned media of cytokine-stimulated NRK-49F cells were determined by the biotin–streptavidin sandwich ELISA for rat gro/CINC. Rat gro/CINC production by NRK-49F cells stimulated with rhIL-1β or/and rhTNF-α was summarized in Fig. 5. rhIL-1β exhibited a much more potent stimulating activity for the rat gro/CINC production than rhTNF-α, and an additive stimulation was found by addition of rhIL-1β in a combination with rhTNF-α. In the present studies, therefore, rhIL-1β was used to stimulate NRK-49F cells. As shown in Fig. 6, steroidal anti-inflammatory drugs, dexamethasone, prednisolone and hydrocortisone, at concentrations of 10^{-10}–10^{-6} M suppressed rat gro/CINC production by 10^{-11} M rhIL-1β-stimulated NRK-49F cells in a dose-dependent manner, whereas non-steroidal anti-inflammatory drugs, indomethacin and piroxicam, were without effect.

DISCUSSION

Cytokine-stimulated rat fibroblasts (NRK-49F cells) produced mainly rat gro/CINC. Rat gro/CINC (P-3) accounted for more than 50% of total chemokines when the amount of each chemokine

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P-1 :          1   5   10
               SELRCQCLTTLPRV

P-2 :          1   5   10   15   20
               RELRCQCLKTLPRVDFENIQSLTV

MIP-2: 1   5   10   15   20   25   30   35   40
       AVVASELRCQCLKTLPRVDFKNIQSLSVTPPGPHCAQTEV · ·

P-3 :  1   5   10   15   20   25   30
       APVANELRCQCLQTVAGIHFKNIQSLKVMPPGP

P-4 :  1   5   10   15   20   25   30
       APVANELRCQCLQTVAGIHFKNIQSLKVMPPGP

CINC : 1   5   10   15   20   25   30   35   40
       APVANELRCQCLQTVAGIHFKNIQSLKVMPPGPHCTQTEV · ·
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Fig. 3. NH_2-terminal amino acid sequences of the reduced and carboxymethylated chemokines (P-1–P-4). The arterial sequences of rat gro/CINC (CINC) and MIP-2 are also shown.

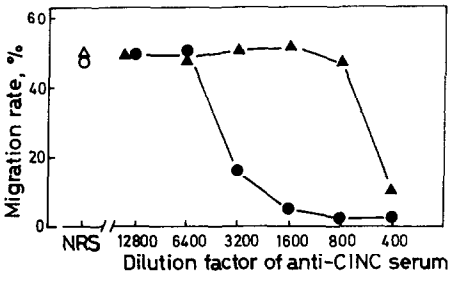

Fig. 4. Effect of polyclonal anti-rat gro/CINC antiserum on rat neutrophil chemotaxis induced by the purified chemokines (P-2 and P-3). Both P-2 (▲, △) and P-3 (●, ○) at a concentration of 1×10^{-8} M were preincubated at 37° for 60 min with various dilutions of polyclonal anti-rat gro/CINC rabbit antiserum (closed symbols) or with a 1:400 dilution of normal rabbit serum (NRS, open symbols). After preincubation, chemotactic activities of the samples were assayed by Boyden's method *in vitro* using multiwell chambers. Each point is shown as the mean of three determinations. Standard errors were within the area covered by symbols.

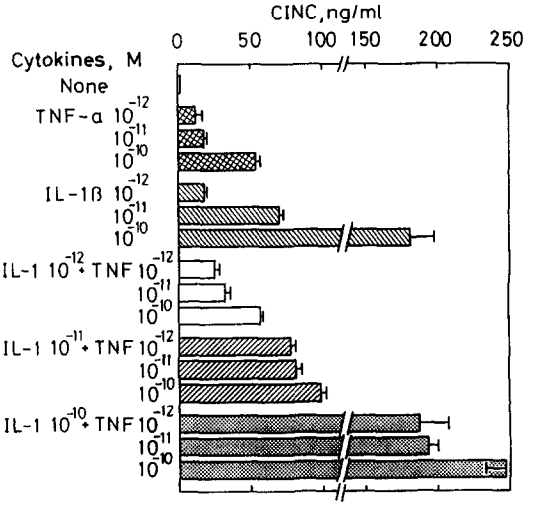

Fig. 5. Effects of rhIL-1β and rhTNF-α on rat gro/CINC production by rat NRK-49F fibroblasts. When NRK-49F cells reached confluence, the cells were cultured for 24 hr in serum-free medium containing 0.1% (v/v) BSA and various concentrations of rhIL-1β and/or rhTNF-α. The rat gro/CINC concentrations of conditioned media were determined by the sandwich ELISA for rat gro/CINC. Each value represents the mean of six determinations, and horizontal bars indicate the SEM.

was determined based on the area of its elution peak of RP-HPLC (P-1–P-4 in Fig. 1). P-2, a minor chemoattractant, had a similar neutrophil chemotactic activity to rat gro/CINC, but poorly bound rabbit anti-rat gro/CINC antibody (Fig. 4). The homology of NH_2-terminal 24 residues between P-2 and a member of the IL-8 family is as follows: 63% to rat gro/CINC [9]; 88% to MIP-2 [13]; 63% to human *gro*/MGSA-α [14, 15]; 46% to human IL-8/NAP-1 (neutrophil-activating peptide-1) [16, 17]. The results suggest that P-2 is the rat homologue of MIP-2 that was purified from the conditioned medium of lipopolysaccharide-stimulated mouse macrophage cell line (RAW 264.7 cells), though we know only about one-third of the amino acid sequence of P-2. In addition, the NH_2-terminal 14 residues of P-1 were also highly homologous to MIP-2, but the present knowledge concerning the amino acid sequence and properties of P-1 is very limited because of the small amount of purified P-1, and further studies will be required to evaluate P-1.

In the present studies the rat gro/CINC concentration was determined by using the biotin–streptavidin sandwich ELISA which is specific for rat gro/CINC, and no cross-reactivity was observed with C5a, IL-1β and TNF-α [11, 18]. P-2 cross-reacted weakly with polyclonal anti-rat gro/CINC antiserum (Fig. 4), suggesting that the sandwich

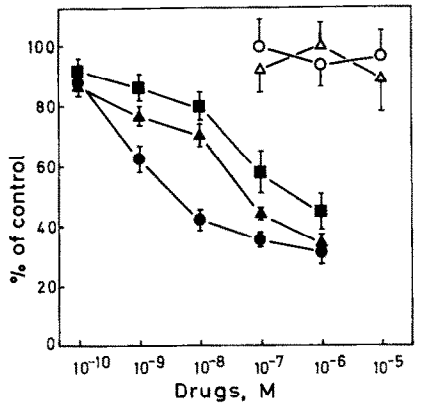

Fig. 6. Effects of anti-inflammatory drugs on rat gro/CINC production by rhIL-1β-stimulated rat NRK-49F fibroblasts. When NRK-49F cells reached confluence, the cells were cultured for 24 hr in serum-free medium containing 0.1% (v/v) BSA, 10^{-11} M rhIL-1β and various concentrations of dexamethasone (●), prednisolone (▲), hydrocortisone (■), indomethacin (○) or piroxicam (△). The rat gro/CINC concentrations of conditioned media were determined by the sandwich ELISA for rat gro/CINC, and the effects of the drugs on rat gro/CINC production are shown as percentage of control (without drugs). Each point represents the mean of six determinations. Vertical bars indicate the SEM.

ELISA selectively detected rat gro/CINC in the conditioned medium of cytokine-stimulated NRK-49F cell culture. The anti-inflammatory steroids suppressed rat gro/CINC production by the stimulated NRK-49F fibroblasts in a dose-dependent manner (Fig. 6), though maximum inhibition by the steroids was only about 60%. This incomplete inhibition is probably due to the lack of preincubation of the fibroblasts with the steroids in the present studies, since it has been demonstrated that a time lag of 2–4 hr is required before the steroids exert their anti-inflammatory activities through *de novo* syntheses of proteins including lipocortin [19–21]. The relative potencies of the inhibitory effect of the steroids on rat gro/CINC production were dexamethasone > prednisolone > hydrocortisone. These relative potencies are consistent with the anti-inflammatory activity of the steroids.

Mukaida *et al.* [22] reported that a potent synthetic glucocorticoid, dexamethasone, inhibited IL-8 mRNA induction in LPS-activated human peritoneal blood mononuclear cells, and suggested that repression of the IL-8 gene by dexamethasone probably occurs through interaction of the glucocorticoid receptor with the glucocorticoid response element in the 5′-flanking region [23]. However, the expression of IL-8 mRNA is not always linked to the secretion of IL-8 polypeptide; T lymphocytes stimulated with phorbol myristate acetate and ionomycin expressed IL-8 mRNA, but the T cells were not induced to secrete significant levels of IL-8 polypeptide [24]. Our results obtained by measuring rat gro/CINC polypeptide levels support the findings obtained by measuring IL-8 mRNA levels, and show that the inhibitory activity of anti-inflammatory steroids on the production of rat gro/CINC by IL-1β-stimulated NRK-49F cells is in good agreement with their anti-inflammatory activities, whereas the non-steroidal anti-inflammatory drugs, indomethacin and piroxicam, have no inhibitory activity on rat gro/CINC production.

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