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THE PRODUCTION OF CINC/GRO, A MEMBER OF THE INTERLEUKIN-8 FAMILY, IN RAT ANTERIOR PITUITARY GLAND

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We investigated the possibility of detection of CINC/gro, which is a IL-8-like neutrophil chemoattractant, immunoreactivity in rat normal anterior pituitary gland by immunohistochemistry, western blot analysis and an ELISA. We first ascertained the possibility of detection of CINC/gro immunoreactivity in the anterior pituitary gland by immunocytochemistry. In the anterior lobe of the pituitary gland, a few CINC/gro-like immunoreactive cells were observed (1-3% of all cells in the anterior pituitary). The positive cells were middle or large in size and looked angular in shape. Intense immunoreactivity was observed in the cytoplasm but not in the nucleus. Analysis by immunoblotting with anti-CINC/gro antiserum gave a characteristic single CINC/gro band with a molecular weight of 6.3 kDa. CINC/gro immunoreactivity was also detected in 3-h conditioned medium of normal anterior pituitary cells by an ELISA, and that immunoreactivity increased significantly in a time-dependent manner during 24-h incubation. This immunoreactivity could be induced by TNF-α in a dose-dependent manner. These findings indicate that CINC/gro is produced in pituitary gland and also suggests the possibility that CINC/gro may play some role asa modulator of anterior pituitary function, especially in the cross-talk mechanism between the immune and neuroendocrine systems.

There is growing evidence that the neuroendocrine and immune systems are engaged in a functionally relevant cross-talk with each other. Neuroendocrine hormones such as PRL and GH, both of whose receptors belong to the cytokine family (1), have recently been shown to stimulate immune functions both *in vivo* and *in vitro* (2-3). It has also been reported that GH acts as an important immune modulator (4). On the other hand, cytokines, such as

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Abbreviations: CINC/gro, cytokine-induced neutrophil chemoattractant/ growth-related oncogene; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay.

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interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and IL-6, influence pituitary hormone release (5-8).

Among the cytokines, interleukin-8 (IL-8) is a key mediator in the migration of neutrophils from the circulation to sites of inflammation in the tissues (9,10). IL-8 is secreted by many cell types, such as immunocompetent cells, fibroblasts and endothelial cells, in response to inflammatory stimuli (11). IL-8 is structurally and functionally related to several members of the macrophage inflammatory protein-2 (MIP-2) family of cytokines, which bind to specific G protein-coupled receptors on neutrophils (12) and appear to share a receptor on neutrophils with IL-8 (13-15).

Recently, a cytokine-induced neutrophil chemoattractant (CINC/gro), an IL-8-like neutrophil chemoattractant, was purified and cloned from the culture fluid of normal rat kidney epithelial cells (16). The primary structure of CINC/gro indicates that CINC/gro is the rat counterpart of human GRO (17), and therefore belongs to the IL-8 family. Although there have been many studies focusing on the biochemistry of the IL-8 family (13,18), the roles of the IL-8 family in the pituitary gland have not yet been clarified. Recently, we have found that CINC/gro stimulated the secretion of PRL, GH and ACTH but suppressed the secretion of LH and FSH from rat anterior pituitary cells (submitted for publication). In the present study, we examined the possibility of detection of CINC/gro immunoreactivity in rat normal anterior pituitary gland by immunohistochemistry, western blot analysis and a recently-developed ELISA.

Materials & Methods

Cell culture

Normal anterior ptuitary cells were obtained from female Wistar rats (200-250 g) and dispersed enzymatically as described previously (19). The dispersed cells were seeded into Falcon 24-well plates at a density of 0.7 X 10^6 viable cells/well and allowed to attach for at least 4 days in a humidified 37°C atmosphere of 5% CO_2 and 95% air. For the experiments on CINC/gro secretion, the cells were washed twice and cultured in serum-free RPMI-1640 medium (Handai Biken, Osaka, Japan) or specially designed media containing various concentrations of TNF-α. After designated incubation times, the conditioned media was collected from the wells and stored at -20°C until CINC/gro assay. Throughout the experiments, the cell morphology was observed by phase-contrast optics. TNF-α had no effects on the cell viability, as determined by the trypan blue exclusion test, the cell morphology or the attachment of the cells to the plastic culture dish.

Immunocytochemistry

Three female Wistar rats weighing about 150 g were used for the immunochemistry study. The animals were perfused intracardially with 50 ml of saline and then with 450 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The pituitary gland was removed and post-fixed with the same fixative overnight, and then the sample was immersed in 30% sucrose in 0.1 M PBS for about 40 hours. Sections of 10 μm in thickness were cut on a cryostat. They were immersed in a preincubation solution containing 1% bovine serum albumin (BSA) and 0.3% Trion X-100 in 0.1 M PBS for 1 hour and then incubated with

antibody against CINC/gro at a concentration of 2 μg/ml for 24 hours at 4°C. The subsequent procedures for the visualizations followed by Vector's protocol (Vectastain HRP ABC kit,Vector, Burlingame, CA) except that the incubation was done overnight at 4°C.

Western blot analysis

The dispersed anterior pituitary cells were grown in 24-well dishes. 0.7 X 10^6 cells were lysed with Laemmli SDS sample buffer (20) and electrophoresed on a 12% sodium dodecyl sulfate(SDS)-polyacrylamide gel. The proteins were transferred to nitrocellulose filters and immunoblotted with anti-CINC/gro antiserum as described (21).

Measurement of rat CINC/gro

An enzyme-linked immunosorbent assay (ELISA) for CINC/gro was developed as reported by DeForge and Remick (22). Briefly, microplates were coated with 100 μl of anti-CINC/gro IgG at 10 μg/ml in 50 mM bicarbonate buffer (pH 9.0) and incubated for 4 h at room temperature. The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and then blocked overnight with 200 μl of 0.05% BSA in PBS-T at 4°C. These were then incubated for 2 h at room temperature with 100 μl of diluted samples. The wells were washed as before and incubated for 2 h at room temperature in 100 μl of 0.05% BSA-PBS-T containing horseradish peroxidase-conjugated anti-CINC/gro rabbit IgG at 10 μg/ml and then incubated with 100 μl of enzyme substrate solution (0.05% *o*-phenylenediamine in 50 mM Na_2HPO_4-24 mM citric acid buffer (pH 5.0) containing 0.0034% H_2O_2) for 5 min. The reaction was stopped with 50 μl of 6 N H_2SO_4, and the optical density at 492 nm was recorded. This ELISA was sensitive enough to detect 30 pg to 10 ng of CINC/gro per ml. The standard curve of CINC/gro was not influenced by normal rat serum, and no cross-reactivity was observed with C5a, human(h)IL-1β, hIL-8 or hTNF-α. The intra- and interassay variations were less than 8% and 10%, respectively.

Materials

Rat CINC/gro (Peptide Institute, Inc., Osaka, Japan) and TNF-α (a generous gift from Dainippon Pharmaceutical Co., Tokyo) were dissolved directly in RPMI-1640 medium to the desired concentrations. A rabbit polyclonal antibody to CINC/gro was raised as described previously (23), and IgG was isolated from the antisera using a protein A-agarose column (MASS 1 mg Protein A, Dainihon Co., Ltd.; Osaka). All other chemicals were commercial materials of the highest purity available and were used without further purification.

Statistical analysis

In this study, each data point represents the mean ± SEM of independent experiments. All data were subjected to analysis of variance, and differences between groups were assessed using the multiple range test of Duncan. A p value of less than 0.05 was considered to represent a statistically significant difference.

Results

We first ascertained the possibility of detection of CINC/gro immunoreactivity in the anterior pituitary gland by immunocytochemistry and western blot analysis. In the anterior lobe of the pituitary gland, a few CINC/gro-like immunoreactive cells were observed (1-3% of all cells in the anterior pituitary). The positive cells were middle or large in size and looked angular in shape. Intense immunoreactivity was observed in the cytoplasm but not in the nucleus (Figure 1). In the present study, the identification of the cell species was difficult, and further immunocytochemical double-labeling will be needed. Analysis of the whole cell lysate of anterior pituitary cells by SDS-PAGE followed by immunoblotting with anti-CINC/gro antiserum gave a characteristic

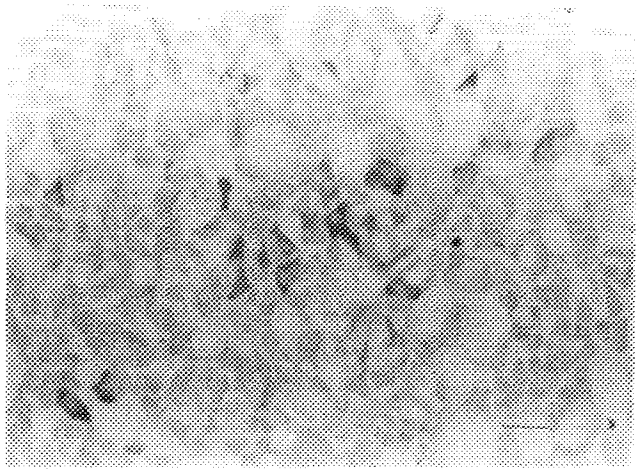

Figure 1. CINC/gro-like immunoreactive cells in the anterior pituitary gland. (scale bar = 25 μm.)

single CINC/gro band with a molecular weight of 6.3 kDa (Figure 2), as described previously (15), and the presence of CINC/gro in the anterior pituitary cells was thus confirmed. normal anterior pituitary cells. CINC/gro immunoreactivity was already detected in 3-h conditioned medium of normal anterior pituitary cells, and it increased significantly in a time-dependent manner during the first 24 h of culture (Figure 3). Since TNF-α was reported to stimulate CINC/gro secretion (16), we next tested whether TNFα could induce CINC/gro secretion by normal anterior pituitary cells. 100 ng/ml of TNF-α significantly increased the secretion of CINC/gro within 3 h of incubation, and this effect continued throughout the first 24 h of incubation (data not shown). Incubation for 24 h with TNFα stimulated CINC/gro secretion in a dose dependent manner (Table 1). At the 500 ng/ml, TNF-α caused 20-fold secretion of CINC/gro compared to vehicle group. An effective dose of TNF-α on CINC/gro secretion is rather high as compared with that of previous reports. It may be related to the difference of cell sensitivity to TNF-α, since it has been reported that sensitivities to TNF-α are quite different between tumor cells and normal cells (24).

Discussion

In the presnt study, we examined the possibility of detection of CINC/gro immunoreactivity in the anterior pituitary gland. To the best of our knowledge, this is the first report to demonstrate that CINC/gro immunoreactivity was detected in the anterior pituitary gland by immunohistochemistry and

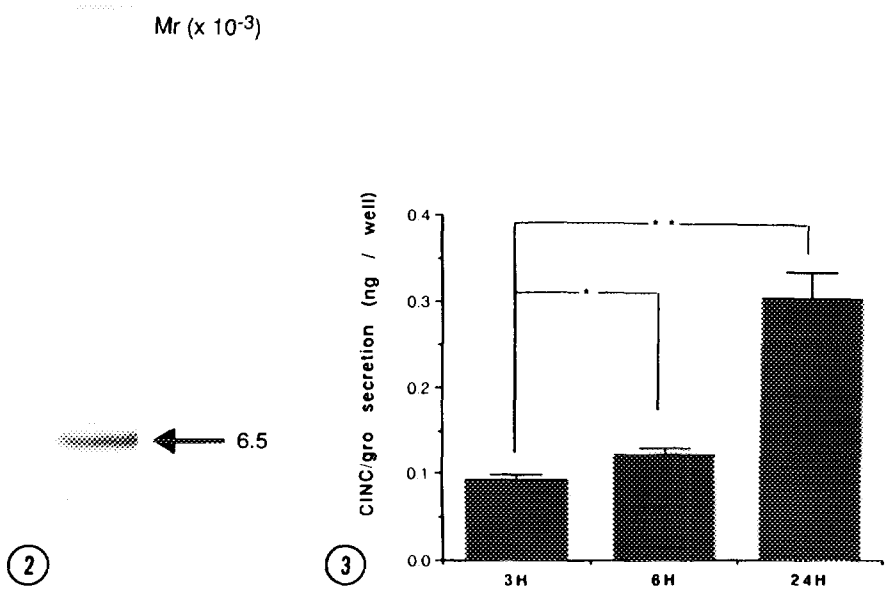

Figure 2. Detection of CINC/gro in normal anterior pituitary cells by immunoblotting. Lysates of anterior pituitary cells were subjected to SDS-PAGE followed by immunoblotting with anti-CINC/gro antiserum as described in Materials and Methods.

Figure 3. CINC/gro secretion by normal anterior pituitary cells. The cells were incubated with serum-free growth medium for the designated incubation times. The conditioned media were collected and later assayed for CINC/gro by ELISA. The values are the mean ± SEM of four independent experiments. *P<0.05 vs 3h-conditioned medium. **P<0.01 vs 3h-conditioned medium.

immunoblotting and that significant immunoreactive CINC/gro was also detected in the conditioned medium of normal anterior pituitary cells by a recently-developed enzyme-linked immunosorbent assay.

CINC/gro immunoreactivity was already detected in 3-h conditioned medium of anterior pituitary cells, and it increased in a time-dependent manner. The concentration of CINC/gro in the medium after 24 h of incubation was rather low, compared to the minimum effective concentration of CINC/gro on anterior pituitary hormone secretion (submitted for publication). However, the local concentration of CINC/gro *in vivo* may be greater than that in this culture system and incubation of anterior pituitary cells with 500 ng/ml TNFα for 24 h caused a 20-fold increase in the basal CINC/gro secretion, which almost corresponds to the minimum effective concentration of CINC/gro on anterior pituitary hormone secretion (submitted for publication).

It is of interest that we could not detect CINC/gro immunoreactivity in GH3-cell-conditioned medium but did detect CINC/gro-like immunoreactivity in a folliculo-stellate(FS)-like cell line (TtT/GF) which was recently established by

Table 1 Effect of TNF-α on CINC/gro secretion

TNF-α Concentration (ng/ml)	Secreted Concentration CINC/gro (ng/ml)
Vehicle	0.30 ± 0.02
1	1.16 ± 0.16 [a]
10	2.24 ± 0.22 [a]
100	3.80 ± 0.28 [a]
500	5.86 ± 0.26 [a]

Effect of TNFα on CINC/gro secretion in normal posterior pituitary cells. The cells were incubated with serum-free growth medium containing various concentration of TNFα (1-500 ng/ml) for 24 h. The conditioned media were collected and assayed for CINC/gro by ELISA. The values are the mean ± SEM of four independent experiments.
a : $p<0.01$ vs vehicle group.

Inoue et al. (25) (data not shown). The source of CINC/gro has not yet been determined, but we speculate that FS cells are one of the candidates for CINC/gro-producing cells in the pituitary. Histochemical studies are under way to clarify the nature of the CINC/gro-producing cells.

In conclusion, the present findings indicate that CINC/gro, a novel cytokine, is produced in pituitary gland and also suggests the possibility that CINC/gro may play some role as a modulator of anterior pituitary function, especially in the cross-talk mechanism between the immune and neuroendocrine systems.

Acknowledgments

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