Suppressive Activity of Lycoricidinol (Narciclasine) against Cytotoxicity of Neutrophil-Derived Calprotectin, and Its Suppressive Effect on Rat Adjuvant Arthritis Model

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Received February 16, 1999; accepted April 15, 1999

Calprotectin is a calcium- and zinc-binding protein complex that is abundant in cytosol of neutrophils. The concentration of calprotectin in extracellular fluids is greatly increased under various inflammatory conditions *in vivo*. We recently demonstrated that calprotectin inhibited cell growth and induced apoptosis of various cell types including tumor cells and normal fibroblasts; therefore, extracellular calprotectin might cause tissue destruction in severe inflammatory diseases. We previously found that an alkaloid, lycorine inhibits induction of apoptosis by calprotectin. In this paper, we examined the inhibitory activities of other Amaryllidaceae alkaloids, namely, lycoricidinol, hippeastrine and ungerine against the cytotoxicity of calprotectin. Lycoricidinol (narciclasine) inhibited calprotectin-induced cytotoxicity at more than 10-fold lower concentration ($IC_{50}=0.001-0.01 \mu\text{g/ml}$) than lycorine, while the effects of the latter two alkaloids were very weak. Therefore, we next checked the prophylactic effect of lycorine and lycoricidinol on the adjuvant arthritis model in rats. Lycoricidinol, but not lycorine, significantly suppressed the degree of swelling of adjuvant-treated as well as untreated feet, suggesting that lycoricidinol might be a candidate as a the drug having marked suppressive activity for inflammation which might be influenced by calprotectin.

Key words lycoricidinol; lycorine; calprotectin; neutrophil; apoptosis; inflammation

Neutrophils are known to be inflammatory cells which accumulate in the first phase of a local inflammatory site. In addition to the roles of phagocytic and killing functions of microorganisms, it has recently been recognized that neutrophils modulate inflammatory and immunological reactions through production of a variety of protein factors, including proteolytic enzymes and cytokines.^{1,2)}

We recently reported that neutrophils contain a factor having novel cytostatic and cytotoxic activity against various tumor and normal cell types,^{3,4)} and identified that factor as calprotectin which is abundant in cytosol of neutrophils.⁴⁾ Calprotectin is a calcium- and zinc-binding protein complex composed of 8 and 14 kD subunits.⁵⁾ The subunits have also been termed migration inhibitory factor-related protein (MRP)-8 and MRP-14,⁶⁾ respectively, and the terms S100A8 and S100A9 were recently proposed because the subunits belong to the S-100 protein family.⁷⁾ We observed that calprotectin inhibits growth of a variety of normal cells including lymphocytes,³⁾ macrophages⁴⁾ and fibroblasts,⁸⁾ as well as many tumor cell lines.^{4,9)} In addition to the negative effect on cell growth, it induces apoptotic cell death of all tumor cell lines investigated⁹⁾ and of normal fibroblasts.⁸⁾

Calprotectin is increased in extracellular fluids under many inflammatory conditions. For example, synovial fluids of rheumatoid arthritis sometimes contain more than $100 \mu\text{g/ml}$ of calprotectin.^{10,11)} Also, an extremely high concentration (1–20 mg/ml) was noted in the supernatant fluid of a subcutaneous abscess.¹²⁾

Although the exact role of calprotectin *in vivo* is still unclear, it may be possible that it exerts a regulatory role in inflammatory processes through its growth-inhibitory and apoptosis-inducing activities to cells that migrate into inflam-

matory sites. On the other hand, the factor may cause tissue destruction in a condition where a very high concentration of calprotectin exists in local body fluid for a prolonged period. Although these hypotheses should be examined by *in vivo* experiments, it is important to develop drugs which regulate the cytotoxicity of calprotectin to elucidate its *in vivo* role, as well as to control inflammatory reactions. We recently searched for substances having inhibitory activity against the cell death-inducing activity of calprotectin among plant materials which have been traditionally used as Chinese medicines to counteract inflammation.¹³⁾ We reported that the extract of *Crinum asiaticum* var *siacum* shows strong inhibitory activity against calprotectin-induced apoptosis. Our purification studies revealed that an Amaryllidaceae alkaloid, lycorine is the main substance responsible for the inhibition: lycorine inhibited the apoptosis induced by calprotectin and also restored DNA synthesis which was suppressed by calprotectin.¹³⁾ It reportedly has inhibitory activity against protein synthesis.^{14–16)} However, at low concentrations which inhibit calprotectin cytotoxicity, it causes attenuation of calprotectin-augmented protein synthesis without severe damage against basal protein synthesis: the inhibitory action of lycorine is considered to be mediated by suppressing calprotectin-augmented protein synthesis.¹³⁾

The group of Amaryllidaceae alkaloids contain many biologically active compounds,¹⁷⁾ some of them show cytotoxic and antiviral activities.^{18,19)} Lycoricidinol, which was reported to have regulating activity for plant cell growth²⁰⁾ has more potent inhibitory capacity for protein synthesis of mammalian cells than lycorine.¹⁴⁾ In this paper, to search for more effective compounds to inhibit the cytotoxic activity of calprotectin, we studied the inhibitory activities of three

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more Amaryllidaceae alkaloids; lycoricidinol and two commercially available alkaloids, hippeastrine and ungerine. We found that lycoricidinol inhibited calprotectin cytotoxicity with more than 10-fold lower IC_{50} than lycorine. We then examined whether lycorine and lycoricidinol suppress the rat adjuvant-induced arthritis model, because calprotectin has been suggested to be involved in the pathological processes of rheumatic arthritis.^{10,11} The results suggested that lycoricidinol exerts a prophylactic effect on adjuvant arthritis, and it is possible that it is a candidate for an anti-inflammatory medicine having a new mechanism of action.

MATERIALS AND METHODS

Alkaloids Lycorine hydrochloride, hippeastrine hydrobromide and ungerine nitrate were purchased from Latoxan (Rosans, France). Lycoricidinol (also termed narciclasine) was purified from bulbs of *Steinbergia lutea*,²¹ which were purchased from a nursery in Heiwaen, Nara, Japan. The bulbs were cultivated and a voucher of the plant is on file in our laboratory. The plant material (fresh weight, 3.1 kg) was extracted with hot MeOH, and the MeOH extract was concentrated under reduced pressure. The viscous concentrate was suspended in H₂O (1 l) and extracted with *n*-BuOH (1 l × 2). Column chromatography of the *n*-BuOH-soluble phase on silica gel and elution with a stepwise gradient mixture of CHCl₃-MeOH (9:1; 4:1; 2:1), and finally with MeOH alone, gave eight fractions (I–VIII). Fraction V was further fractionated on a silica gel column eluting with CHCl₃-MeOH (9:1) to afford lycoricidinol (150 mg) as an amorphous solid. Copies of the original spectral data (UV, IR, ¹H-NMR and ¹³C-NMR spectra) of lycoricidinol are obtainable from the authors.

Animals Male Wistar rats and male C3H/He mice were purchased from Japan SLC, Inc. (Shizuoka). Male Wistar-Lewis rats were obtained from Charles River Japan Inc. (Kanagawa).

Cell Lines MM46, a transplantable ascites tumor from a spontaneous mammary carcinoma in a C3H/He mouse, was passaged weekly in the peritoneal cavity of these mice.

Preparation of Rat Calprotectin Sample Partially purified calprotectin was obtained as previously described¹³ and was used throughout in this work. Briefly, 20 mg of heat-killed *Enterococcus faecalis* was injected into the peritoneal cavity of Wistar rat. Twenty-four hours later, PECs were collected with 0.15 mol/l phosphate-buffered saline (PBS), washed and suspended in PBS. The suspension was sonicated for 30 s in an ice bath 5 times with a Branson sonifier (Model 185), and a soluble fraction was obtained by centrifugation at 12000 *g* for 10 min at 4 °C. After the fraction was precipitated with 70% (NH₄)₂SO₄, the precipitate was applied to Sephadex G-100 column (2 × 90 cm) and eluted with PBS. The cytotoxic activity to MM46 cells of each fraction was checked and the active fractions were pooled and stored at -30 °C until use.

MTT Assay MTT assay was used to evaluate the target cell death.²² MM46 cells (1 × 10⁴ cells/well) were cultured in 96-well microtest plates (Corning, Corning, NY, U.S.A.) with 200 μ l medium (RPMI 1640, 100 U/ml penicillin, 60 μ g/ml kanamycin supplemented with 5% FCS). To the cultures were added test samples in addition to the calprotectin sam-

ple at the start of culture, and incubation followed at 37 °C in a humidified atmosphere of 5% CO₂ in air. After 20 or 24 h of culture, 25 μ l of 5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to each well and plates were incubated for an additional 3 or 4 h. After 150 μ l of the supernatants was discarded, 100 μ l of acid-isopropanol solution (0.04 N HCl in 2-propanol) was added to each well and the optical density at 588–630 nm was measured by an MTP-100 microplate reader (Corona Electric, Ibaraki).

Measurement of Net Protein Synthesis To examine net protein synthesis in MM46 cells, the cells (1 × 10⁴ cells/well) were pulsed with 925 KBq/ml L-[3,4,5-³H(N)]-leucine (³H-Leu; 4.4 TBq/mmol, Moravsek Biochemicals, Brea, CA, U.S.A.). After the indicated periods, cells were lysed with 0.5% SDS and precipitated with 5% trichloroacetic acid (TCA). The resulting TCA-insoluble substances were collected in glass filters with Labo mash LM-101 (Futaba Medical, Tokyo) using 5% TCA as washing solution. The isotope amounts were quantified in a liquid scintillation counter.

For the statistical analysis of these *in vitro* studies, the differences between each value of the test groups and their respective control (without alkaloid) were analyzed by Dunnett method using Stat-Flex software (Ver. 4, Artech, Osaka).

Rat Adjuvant-Induced Arthritis Model A suspension of liquid paraffin (0.05 ml) containing 5 mg heat-killed *Mycobacterium butyricum* (Lot. 0640-33-7, Difco Laboratories, Detroit, MI, U.S.A.) was injected intradermally into the left hindpaw of each male Wistar-Lewis rat (8 weeks of age).²³ The day of adjuvant injection was designated as day 0. Lycorine and lycoricidinol were injected intraperitoneally on day 0, 1, 2, 3, 5, 6, 7, 8, 9 and 10, respectively. As a positive control, hydrocortisone (20 mg/kg) was administered orally on each of the 10 d above. The development of arthritis was assessed by measuring the adjuvant-injected or uninjected paw volume with a plethysmometer. The data obtained from six rats per group were expressed by mean ± S.D. Dunnett method was used for statistical evaluation of difference between different groups of animals.

RESULTS

Comparison of Inhibitory Activities of 4 Amaryllidaceae Alkaloids against Cytotoxicity of Calprotectin

We first examined the inhibitory activities of lycorine and the three other Amaryllidaceae alkaloids, lycoricidinol, hippeastrine and ungerine (their structures are depicted in Fig. 1). As shown in Fig. 2, MM46 cells incubated with calprotectin sample underwent about an 80% decrease in MTT-reducing activity. This decrease was restored by 0.01 μ g/ml lycoricidinol, although the agent itself caused a small inhibitory effect at that concentration (Fig. 2b). The IC_{50} of lycoricidinol against calprotectin was between 0.001–0.01 μ g/ml, which is more than 10-fold lower than that of lycorine, which had an IC_{50} of about 0.1 μ g/ml (Fig. 2a). The results obtained from MTT assay were confirmed by morphological observation: apoptotic morphology and the subsequent necrotic shape of MM46 cells cultured with calprotectin disappeared in the presence of 0.01 μ g/ml lycoricidinol (data not shown). Although hippeastrine also inhibited calprotectin-induced decrease in MTT reduction, the effect was only marginal

(IC_{50} = 10–100 μ g/ml), while ungerine caused a slight inhibition at 100 μ g/ml (Fig. 2d).

Effects of the Amaryllidaceae Alkaloids on Net Protein Synthesis in Target Cells We previously reported that the apoptosis-inducing reaction of calprotectin requires target protein synthesis, and that calprotectin actually enhanced target protein synthesis in the induction phase of apoptotic cell death.²⁴ Although lycorine reportedly has an inhibitory activity for translational processes,^{14–16} 1 μ g/ml lycorine reduced calprotectin-induced protein synthesis in target cells to near the basal (no addition) levels: lycorine did not entirely inhibit protein synthesis at the concentrations inhibiting calprotectin cytotoxicity (Fig. 3a). The possibility was raised that the attenuation of up-regulated protein synthesis is re-

lated to the inhibition mechanism of lycorine. We therefore examined whether the effects of the other alkaloids on the target protein synthesis that is augmented by calprotectin.

As shown in Fig. 3b, lycoricidinol gradually reduced the amount of 3 H-Leu incorporation into MM46 cells both in the absence and the presence of calprotectin. Lycoricidinol at 0.01 μ g/ml at which it exerted marked inhibition on the cytotoxicity significantly reduced the protein synthesis, although the effect seemed to be partial.

Hippeastrine alone, however, exerted no significant effect on target protein synthesis at concentrations up to 10 μ g/ml (Fig. 3c). Hippeastrine at 100 μ g/ml, at which it potently inhibited calprotectin cytotoxicity, largely reduced the enhanced 3 H-Leu incorporation by calprotectin. However, it is to be noted that even at 100 μ g/ml, hippeastrine by itself did not cause any reduction in protein synthesis. As to the ineffective alkaloid, ungerine, no significant effect was observed against target protein synthesis up to 100 μ g/ml (Fig. 3d). These data showed that suppression of calprotectin-augmented protein synthesis is correlated with the inhibitory actions on the cytotoxicity of calprotectin.

Effect of Lycorine and Lycoricidinol on Adjuvant-Induced Arthritis Model of Rats A high amount of calprotectin was reportedly present in the synovial fluid of many patients with rheumatoid arthritis.^{10,11} Since calprotectin has very broad target specificity in its growth-inhibitory and apoptosis-inducing activities,^{3,4,8,9} the factor may influence the inflammatory processes of this condition by controlling the growth or survival states of cells constituting the inflammatory tissue. Accordingly, we next studied the prophylactic effect of lycorine and lycoricidinol on the rat adjuvant arthritis model.

As shown in Fig. 4a, intraperitoneal administration of 1 and 5 mg/kg of lycorine did not suppress the adjuvant arthritis, whereas hydrocortisone showed significant prophylactic

Fig. 1. Structures of Lycorine, Lycoricidinol, Hippeastrine and Ungerine

Fig. 2. Effects of Lycorine, Lycoricidinol, Hippeastrine and Ungerine on Cytotoxicity of Calprotectin for MM46 Cells

MM46 cells were cultured with (closed circles) or without (open circles) calprotectin in the presence of indicated concentrations of lycorine (a), lycoricidinol (b), hippeastrine (c), or ungerine (d). The extent of cytotoxicity was assessed by MTT assay as described in Materials and Methods. The cells were pulsed with MTT during 20 to 24 h (a and b) or 24 to 27 h (c and d) of the culture. Bars in these figures represent mean $\pm$ S.D. of triplicate estimations. ** p < 0.01, vs. control (without alkaloids) values (Dunnett test).

Fig. 3. Effects of Lycorine, Lycoricidinol, Hippeastrine and Ungerine on Protein Synthesis of MM46 Cells

MM46 cells were cultured with (closed circles) or without (open circles) calprotectin in the presence of the indicated concentrations of lycorine (a), lycoricidinol (b), hippeastrine (c), or ungerine (d). The cells were pulsed with ³H-Leu during 3 to 6 h (a and b) or 4 to 7 h (c and d), and the net ³H-Leu incorporation was estimated as described in Materials and Methods. Bars in these figures represent mean ± S.D. of triplicate estimations. **p* < 0.05, ***p* < 0.01, vs. control (without alkaloids) values (Dunnet test).

effect on day 3 and day 9. On the other hand, injection of 1 mg/kg of lycoricidinol on consecutive days from 0 to 10 significantly suppressed the swelling of adjuvant-treated feet as well as untreated feet on day 14, suggesting that lycoricidinol shows a later effect than hydrocortisone. The inhibitory effects became obscure on day 21 (Fig. 4b). Because the body weights of the lycoricidinol-injected rats were not significantly different with control arthritis rats, the inhibitory effect seemed not to be due to nonspecific toxicity of this substance for the examined rats.

DISCUSSION

Among newly examined alkaloids, lycoricidinol had remarkable inhibitory activity against calprotectin-mediated induction of cytotoxicity. It had inhibitory activity stronger than lycorine: the respective IC₅₀ of lycorine and lycoricidinol was about 0.1 μg/ml and 0.001–0.01 μg/ml, so that the specific activity of lycoricidinol was more than 10-fold higher. The effect of hippeastrine was very low since its IC₅₀ was much higher than that of lycorine, while ungerine showed scarcely any inhibition.

In a previous study it was observed that calprotectin-induced apoptosis reaction requires target protein synthesis for its induction phase, and that calprotectin actually upregulates the net amount of protein synthesis of MM46 cells during the early period of the reaction.¹³⁾ Although lycorine reportedly has inhibition activity against protein synthesis,^{14–16)} we observed that it suppresses the upregulated synthesis to near the basal level at low concentrations showing inhibition against calprotectin.¹³⁾

To learn whether lycoricidinol and two other alkaloids, hippeastrine and ungerine suppress the calprotectin-augmented protein synthesis in a lycorine fashion, we examined their effects on net protein synthesis of MM46 cells. Like lycorine, 100 μg/ml of hippeastrine inhibited the augmented

A. Lycorine

B. Lycoricidinol

Fig. 4. Effects of Lycorine (A) and Lycoricidinol (B) on Induction of Rat Adjuvant Arthritis

Lycorine, lycoricidinol or hydrocortisone were administered to adjuvant-treated rats according to the schedule described in Materials and Methods. A. (□) Saline control, (▨) 5 mg/kg lycorine, (▩) 20 mg/kg lycorine, (■) 20 mg hydrocortisone. B. (□) Saline control, (▨) 0.2 mg/kg lycoricidinol, (▩) 1 mg/kg lycoricidinol, (■) 20 mg hydrocortisone.

protein synthesis of calprotectin-treated cells to the control level, whereas it did not affect the synthesis of untreated cells at that concentration. Accordingly, hippeastrine as well as lycorine may exert the effect solely on the calprotectin-upregulated protein synthesis *via* an unknown mechanism. The almost ineffective alkaloid, ungerine, showed scarcely any inhibitory activity against protein synthesis. It was reported that lycoricidinol has very strong capability to inhibit protein synthesis at much lower concentrations than lycorine.¹⁴⁾ Unlike lycorine or hippeastrine, 0.01 $\mu\text{g}/\text{ml}$ of lycoricidinol which is close to the halfway effective concentration for inhibition of calprotectin cytotoxicity, reduced the amounts of augmented protein synthesis of MM46 cells cultured with calprotectin to below the basal level. Lycoricidinol at that concentration also suppressed the protein synthesis of untreated cells. It is, therefore, suggested that the inhibition mode of lycoricidinol against calprotectin-upregulated protein synthesis is different from those of lycorine and hippeastrine. Nevertheless, it is still possible that the upregulated protein synthesis by calprotectin is related to the mechanism inducing cytotoxicity, and that these alkaloids inhibit calprotectin action through suppression of the augmented protein synthesis.

Body fluids in inflammatory tissues contain high amounts of calprotectin.⁵⁾ Titers of calprotectin in synovial fluids of rheumatoid arthritis patients sometimes reportedly reached 100 $\mu\text{g}/\text{ml}$, which is high enough to induce apoptosis *in vitro*.^{10,11)} Moreover, it was also reported that expression of calprotectin gene in the peritoneal exudate cells was correlated with genetic susceptibility of rats to the development of adjuvant arthritis.²⁵⁾ These findings, together with ours,^{8,9)} suggest that the factor influences the process of arthritis. We found that lycoricidinol, which is the most powerful inhibitor of cytotoxicity of calprotectin, exerted a prophylactic effect on the adjuvant-induced arthritis model, whereas lycorine did not, even when it was administered into rats at 5 times higher doses than lycoricidinol. Nevertheless, it remains to be determined whether calprotectin actually affects the pathological process of adjuvant arthritis. It is also probable that the prophylactic effect of lycoricidinol is mediated by other mechanisms; for instance, it is important to ascertain whether the alkaloid inhibits the production of cytokines from inflammatory cells. Although the exact mode of action remains to be

elucidated, this study suggests that the Amaryllidaceae alkaloid lycoricidinol may be candidate as a new drug having anti-inflammatory capacity.

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