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Effect of Glycyrrhetinic acid on Histamine Synthesis and Release

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The effect of glycyrrhetinic acid (18β-glycyrrhetinic acid, GA) on histamine synthesis and release was investigated in cocultured mast cells with Swiss 3T3 fibroblasts. GA has strong dose dependent inhibitory activity for histamine synthesis and release in cocultured mast cells. GA (50 μM) inhibited about 85% of histidine decarboxylase (HDC) activity. The appearance of cells staining positively with berberine sulfate was also decreased in the presence of GA. It indicates that transdifferentiation of cultured mast cells (CMCs) was also inhibited.

Key words : Histidine Decarboxylase, Swiss 3T3 fibroblast, Mast cell, Glycyrrhetinic Acid

INTRODUCTION

Glycyrrhetinic acid (GA) is an aglycone of glycyrrhizin, another major active component of licorice root (Kitagawa *et al.*, 1984), and is a representative medicine with a long history of use in Oriental pharmacology (Baba *et al.*, 1987; Doll *et al.*, 1962; Finney *et al.*, 1958; Kumagai *et al.*, 1964; Pompei *et al.*, 1979). GA has much stronger inhibitory activity than glycyrrhizin in 12-0-tetradecanoyl phorbol-13-acetate (TPA)-induced glucose transport, and has stronger binding activity to mineralocorticoid and glucocorticoid receptors (Armanini *et al.*, 1983). GA consists 18α - and 18β-stereoisomeric forms (Davidson *et al.*, 1986; Wang *et al.*, 1991) but 18β-GA is more effective than 18α-GA as an anti-mutagen, anti-tumor initiating agent (Wang *et al.*, 1991).

Previous studies have demonstrated that GA had a remarkable inhibitory effect on the antigen-stimulated histamine release in cultured mast cells (Imanishi *et al.*, 1989). The mechanism of the action of GA on mast cells has been suggested to be mediated by some effect on phospholipid metabolism, which is generally assumed to be involved in the induction of an antigen-stimulated cellular response in sensitized mast cells (Kennerly *et al.*, 1979; Ulmann *et al.*, 1975). Some anti-inflammatory steroids antagonize mouse skin tumor promotion *in vivo* (O'Brian *et al.*, 1988; O'Brian *et al.*, 1988; Weinstein *et al.*, 1988). That is, the antagonism of tumor promotion by GA may be consequence of either its interactions with steroid receptors.

In long-term coculture with fibroblasts, CMCs showed a phenotypic change to connective tissue-type mast cells (CTMCs) (Ebi *et al.*, 1992). In this report, to investigate the effects of GA on histamine synthesis, histamine release, and differentiation of CMCs, we used a coculture system with Swiss albino/3T3 fibroblasts.

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MATERIALS AND METHODS

Mice and cells

Mice were sacrificed by decapitation after ether anesthesia. Spleens were removed, and cell suspensions were prepared as described previously (Nakahata. *et al.*, 1982). The Swiss albino/3T3 fibroblast cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). This cell line was adapted to grow in α-minimum essential medium (α-MEM; Flow Laboratories, Irvine, UK) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Drug

GA was purchased from Sigma Chemical Company, St. Louis, MO.

Establishment of CMC

Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) was prepared as des-

cribed previously (Nakahata *et al.*, 1982). Spleen cells (2.0×10^6/mL) from 3-month-old WBB6F1-+/+ mice were incubated for 5 days in (α-MEM; containing a 1 : 300 dilution of PWM (GIBCO, Grand Island, NY), 10% FCS, and 10^{-4} mol/L 2-mercaptoethanol (Sigma Chemical Co, St Louis, MO). The conditioned medium was centrifuged, filtered through a 0.22 μm filter (Millipore Corp, Bedford, MA), and stored at -80°C. PWM-SCM contains at least IL-3, IL-4 (Hamaguchi *et al.*, 1987), and IL-9 (Hultner *et al.*, 1989; Moeller *et al.*, 1990) and is more effective than recombinant IL-3 for development and maintenance of CMCs. Culture flasks (Nunc, Roskilde, Denmark) containing 2×10^7 spleen cells and 5 mL α-MEM supplemented with 10^{-4} M 2-mercaptoethanol, 10% FCS, and 10% PWM-SCM were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Half of the medium was replaced every 7 days, and more than 95% of cells were CMCs 4 weeks after the initiation of the culture (Nakano *et al.*, 1985).

Coculture with fibroblasts

Swiss albino/3T3 fibroblasts (1×10^4 cells) were suspended in 2 ml α-MEM supplemented with 10% FCS, and seeded in 35-mm culture dishes. The culture medium was aspirated and replaced with 2ml of fresh medium every 2 days. After 3 days, the fibroblasts formed a confluent monolayer is each dish containing 0.5 to 1.0×10^6 cells. The medium of the confluent cultures was aspirated, and 5×10^5 CMCs suspended in 2 ml α-MEM supplemented with 10% FCS and 50% WEHI-3-conditioned medium was added with or without GA. The medium in CMCs /fibroblasts coculture was changed every 2 days as follows. The medium was collected, and the supernatant obtained by centrifugation at 120 g for 5 minutes was used for sampling. Cells were resuspended in 1ml of fresh medium, and then returned to the the coculture. At various times after the initiation of coculture, the numbers of mast cells were estimated from the total hemocytometer count and the proportion of mast cells. The proportion of mast cells was determined by staining cytospin preperations (Shandom Southern, Elliott, IL) with alcian blue.

Assays of HDC activity and histamine content

For HDC assay, the CMCs were homogenized in 0. 5 ml of cold Solution A (0.1 M potassium phosphate buffer (pH 6.8), 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5-phosphate, 1% polyethylene glycol (average molecular weight 300, *W/W*), and 0.5 mg/ml each of leupeptin and chymostatin) in a Polytron homogenizer (Kinematica, Lucern, Switzerland) operated at the maximum setting for two 10-s periods in an ice bath. The homogenate was centrifuged at 10,000×g for 20 min, and the supernatant was dialyzed three times against 100 volumes of Solution A. HDC activity was assayed as described previously (Watanabe *et al.*, 1979). Briefly, the dialysate was incubated with 0.25 mM L-histidine for 15 h and histamine was separated from histidine on a short column of Amberlite CG-50, and measured fluorometrically by the o-phthalaldehyde method as described by Watanabe *et al.* (Watanabe *et al.*, 1979). For histamine analysis, CMCs were homogenized with 1.0 ml of 3% perchloric acid, and the homogenate was centrifuged as described above. The supernatants and cell-free culture media containing 3% perchloric acid were subjected to histamine analysis. Histamine was measured fluorometrically by the o-phthalaldehyde method in HPLC system as described by Yamatodani *et al.* (1982).

Alcian blue and berberine sulfate staining

After coculture, CMCs and fibroblasts were collected in Eppendorf microcentrifuge tubes containing 0.5 ml of phosphate-buffered saline (PBS). After washing with PBS, the samples were immediately spun in a cytocentrifuge at 600 rpm for 5 min, and the slides were stained with alcian blue or berberine sulfate. Specimens stained with berberine sulfate were examined using an epifluorescent microscope (Olympus, Tokyo, Japan). Enerback (Enerback, 1974) demonstrated that berberine sulfate specifically stains heparin-containing granules of CTMCs by cytofluorometry. We confirmed this by showing that the fluorescence disappeared after heparinase digestion (Nakano *et al.*, 1985).

Statistical analysis

Statistical analysis was performed using ANOVA, and a probability value of <0.05 was considered to be significant.

RESULTS

CMCs were cocultured with Swiss 3T3 fibroblasts in the presence or absence of GA. After two weeks of coculture, cells were collected by centrifugation, and histamine contents were assayed in both the culture medium and in the cells. As shown in Table I, when 50 μM GA was added to the coculture system, histamine content in the cultured medium was decreased to approximately 30%. Histamine release in the cells was also decreased to about 45% under the same conditions (Table I). Longer incubation with GA also showed a significant decrease in histamine content in the cells (Fig. 1) and in the supernatants (Fig. 2). These results indicated that not only the production but also the release of histamine were in-

Table I. Histamine Content of the Pellets and Supernatants after Coculture with Various Concentrations of GA

Addition	Histamine (μg/10^6 cells)	
	Pellets	Supernatants
None (GA)	1.36±0.01	5.40±0.01
50μM	0.97±0.05*	2.96±0.06**
400μM	0.89±0.02**	2.70±0.06**
1600μM	0.87±0.02*	2.17±0.04**

Values are the mean SE of triplicate samples. *,Significantly different from single culture, *; P<0.05, **; P<0.01.

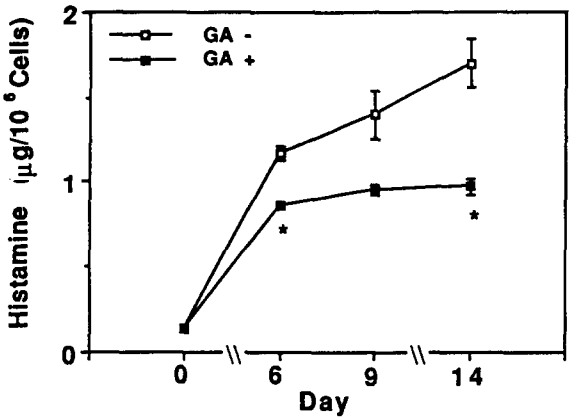

Fig. 1. Effects of GA on the histamine content in cells during coculture. The cells were harvested, washed twice with PBS, and then assayed for histamine content. Each value is the mean±SE of triplicate samples. *, Significantly different from GA (-) control, *; P<0.05

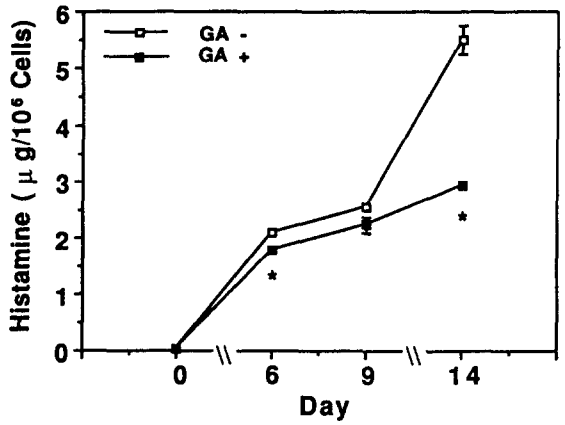

Fig. 2. Effects of GA on the histamine content in the supernatants during coculture. The supernatants were collected at various times after the initiation of coculture and then assayed for histamine content. Each value is the mean ±S.E. of triplicate samples. *, Significantly different from GA (-) control. *; P<0.05

hibited by GA. Next, we examined the HDC activity in the presence of GA. As shown in Fig. 3, an 80% decrease of HDC was observed when 50 μM GA was added to the medium. This result showed that the production of histamine was caused by GA via its inhibitory action against HDC. After two weeks of co-

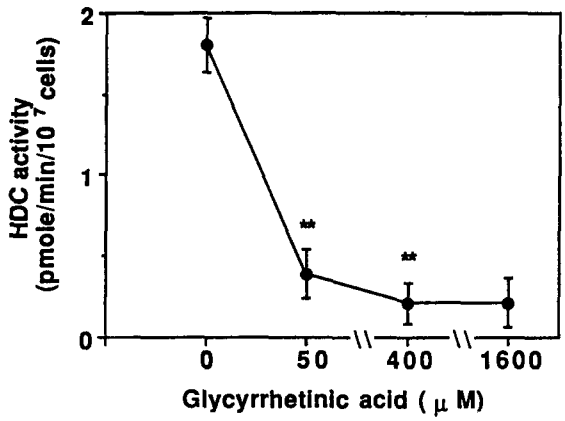

Fig. 3. Effects of GA on HDC activity after coculture in the presence of various concentrations of GA. The cells were harvested and then assayed for HDC activity (pmol/min/10^7 cells). Each value is the mean±S.E. of three determinations. **, Significantly different from coculture in the absence of GA. **; P<0.01

Table II. The Proportion of Mast Cells Stained Positively with Berberine Sulfate after Coculture with Various Concentrations of GA

Addition	Berberine sulfate-positive mast cells (%)
None (GA)	7.03
50 μM	1.78
400 μM	0.70
1600 μM	0.87

culture, mast cells were stained with berberine sulfate. The proportion of berberine sulfate-positive mast cells was 7.03% in the absence of GA. In contrast, only 1. 78% of cocultured mast cells stained positively with berberine sulfate when 50 μM GA was added to the medium (Table II), indicating that GA also effected a phenotypic change in mast cells.

DISCUSSION

This study provides further evidence that GA inhibits the synthesis and release of histamine by the supression of HDC activity in mouse mast cells cocultured with Swiss 3T3 fibroblasts. Although it is not clear whether the inhibitory effect of GA against HDC activity was at the transcriptional level or translational level, the results were consistent with those of previous reports (Imanishi *et al.*, 1989). Also, we demonstrated that GA can inhibit the differentiation of CMCs by berberine sulfate staining which is a marker of mast cells differentiation.

Stem cell factor (SCF) which is produced in fibroblasts and increases the histamine content in mast cells has been characterized (Tsai *et al.*, 1991). Although it is not still clear which pathway is inhibited by GA, the inhibitory activity of GA was clear and rational from the point of view of its use as an anti-allergy and anti-inflammatory drug in traditional Orien-

tal medicine. In this report, we confirmed that the inhibition of histamine production was due to the decrease of HDC activity by GA. We also showed that GA inhibits the differentiation of CMC to CTMC-like cells.

Further experiments to elucidate the mechanism of action of GA as a down-regulator of excess reactions in CMCs cocultured with Swiss 3T3 fibroblasts are currently in progress in our laboratory.

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