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International Immunopharmacology 4 (2004) 1633–1644

International Immunopharmacology

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Glycyrrhizin and related compounds down-regulate production of inflammatory chemokines IL-8 and eotaxin 1 in a human lung fibroblast cell line

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Received 15 June 2004; received in revised form 1 July 2004; accepted 21 July 2004

Abstract

Glycyrrhizin (GL) is known to have various immunomodulating activities and has long been used clinically as an anti-allergic and anti-hepatitis agent. While the potency of GL against lung inflammatory diseases has been expected, the effect of GL on the lung has been poorly understood. Lung fibroblasts are known as a potent producer of inflammatory chemokines, IL-8 and eotaxin 1, by which neutrophils and eosinophils are strongly attracted during inflammation. Therefore, we studied the effects of GL on the production of these chemokines using a human fetal lung fibroblast cell line, HFL-1, stimulated with TNF-α and IL-4. Moreover, we examined the structure–activity relationships of GL to explore more beneficial compounds. 18α,β-GL inhibited IL-8 dose-dependently and inhibited eotaxin 1 slightly. 18α,β-Glycyrrhetic acid (GA) did not inhibit IL-8 but inhibited eotaxin 1. The effect of 18α,β-glycyrrhetic acid monoglucuronide (MGA) resembled that of 18α,β-GL but was weaker. Both 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-11-deoxo-olean-12-en-30-oic acid (11-deoxo-GL) and 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-oic acid (hetero-GL) exhibited inhibitory activity with significant cytotoxicity. 3β-[(2-*O*-β-D-Glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-olean-9,12-dien-30-oic acid (homo-GL) did not have cytotoxicity but its activity was mild like that of 18α,β-GL. 3β-[(2-*O*-β-D-Glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-ol (hetero-30-OH-GL) and 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-olean-9,12-dien-30-ol (homo-30-OH-GL) showed potent inhib-

Abbreviations: GL, glycyrrhizin; GA, glycyrrhetic acid; MGA, glycyrrhetic acid monoglucuronide; 11-deoxo-GL, 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-11-deoxo-olean-12-en-30-oic acid; Homo-GL, 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-olean-9,12-dien-30-oic acid; Hetero-GL, 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-oic acid; Homo-30-OH-GL, 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-olean-9,12-dien-30-ol;Hetero-30-OH-GL, 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-ol; SNMC, Stronger-Neo Minophagen C.

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doi:10.1016/j.intimp.2004.07.023

itory effects, at concentrations lower than 18α,β-GL with no significant cytotoxicity. These results suggest that GL-related compounds are effective in reducing chemokine production and that GL-modified compounds including hetero-30-OH-GL and homo-30-OH-GL appear most beneficial in view of their inhibitory capacity with less cytotoxicity.

Keywords: Glycyrrhizin derivatives; IL-8; Eotaxin 1; Human lung fibroblast

1. Introduction

Pulmonary fibroblasts have now been recognized as an active participant during inflammatory processes in the lung, playing a specialized role in the recruitment and regulation of immune cells that infiltrate the interstitial space. Through their regulatory mechanisms, lung fibroblasts generate many kinds of chemokines and cytokines, including IL-8, eotaxin 1, MCP-1, MIP-1α, RANTES, and GM-CSF, upon activation with inflammatory stimulants [1–3]. IL-8 (CXCL8) is one of the major chemokines with neutrophil chemotactic activity [4], and is produced by a variety of cells including lung fibroblasts activated with various stimulants [3–8]. On the other hand, eotaxin 1 (CCL11) was originally isolated as the predominant eosinophil chemoattractant in the lung lavage fluid of sensitized guinea pigs after allergic exposure and was cloned in 1996 by Kitaura et al. [9,10]. Eotaxin 1 can be produced by several types of cells, including lung or dermal fibroblasts [11–13], and lung or bronchial epithelial cells [13–15]. Production of eotaxin 1 is induced by IL-4 and suppressed by IFN-γ [11,13], and it is recognized that eotaxin 1 is responsible for the infiltration of eosinophils into allergic inflammatory sites [9–13].

Glycyrrhizin (GL), a triterpene glycoside extracted from licorice root (*Glycyrrhiza glabra*), consists of glycyrrhetic acid (GA) and two molecules of glucuronic acid at the C-3 position (see Fig. 1). For the past few decades, the GL preparation Stronger-Neo Minophagen C (SNMC) has extensively been used to treat chronic hepatitis in Japan [16,17], and the safety and efficacy of SNMC have been established. SNMC is now under clinical trial in Europe in patients with chronic hepatitis C [18,19]. So far, GL has been demonstrated to have a variety of activities, such as the production of several cytokines, namely IFN-γ [20], IL-2 [21], IL-12 [22], and IL-10 [23], and the augmentation of NK cell/NKT cell activity [24]. Also, GL administration induces anti-inflammatory, cytoprotective effects in vivo and anti-viral effects in vitro [25]. Some other pharmacological effects of GL and related compounds have been demonstrated [26–28].

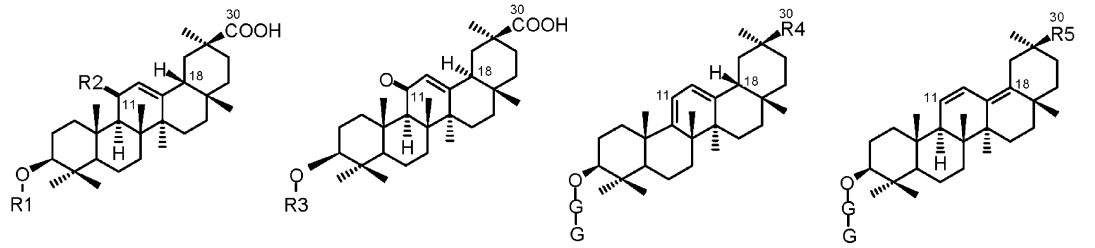
In early 2003, severe acute respiratory syndrome (SARS) raged in several countries and the event shook the world. Surprisingly, it was promptly reported that GL was effective against SARS-associated coronavirus [29]. A subsequent paper reported that an elevation of plasma inflammatory cytokines and chemokines was observed in SARS patients [30]. Taking these reports into consideration, we presumed that GL may act against the production of chemokines in the lung. However, the effect of GL on chemokine production by lung constituent cells remains to be identified.

In this study, we investigated the inhibitory effect of GL and related compounds on chemokine production by human lung fibroblast (HFL-1), which is known as a good producer for chemokines [12,31]. We focused on IL-8 and eotaxin 1, since both chemokines recruit polymorphonuclear cells such as neutrophils and eosinophils during lung inflammation. Also, we investigated the structure–activity relationship of GL derivatives.

2. Materials and methods

2.1. Materials

All GL-related compounds used here except for 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-ol (hetero-30-OH-GL) and 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-olean-9,12-dien-30-ol (homo-30-OH-GL) have already



	Compound	R1	R2	Compound	R3
Group 1	18β-GL	G-G	O	18α-GL	G-G
Group 2	18β-GA	H	O	18α-GA	H
Group 3	18β-MGA	G	O	18α-MGA	G
Group 4	11-deoxo-GL	G-G	H, H		

	Compound	R4	Compound	R5
Group 4	Homo-GL	COOH	Hetero-GL	COOH
Group 5	Homo-30-OH-GL	CH_2OH	Hetero-30-OH-GL	CH_2OH

GL : glycyrrhizin GA : glycyrrhetic acid MGA : glycyrrhetic acid monoglucuronide

G-G : 3-O-β-D-glucuronopyranosyl-(1→2)-β-D-glucuropyranoside

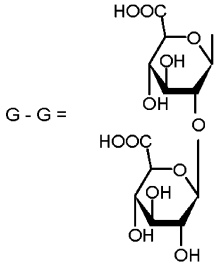


Fig. 1. Structures of glycyrrhizin and its derivatives.

been reported elsewhere [26,27,32]. Since the latter two compounds have not been reported elsewhere, herein, we briefly describe their chemical preparation.

(1) Hetero-30-OH-GL; 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13,(18)-dien-30-ol
hetero-GL [32] was treated with 10% hydrogen chloride in methanol at room temperature to give a dimethyl ester, which was then acetylated to afford a dimethyl ester pentaacetate (hetero-GL-2Me-5Ac) in 52% yield. Hetero-GL-2Me-5Ac was treated with ethyl chloroformate and triethylamine followed by sodium borohydride to afford hetero-30-OH-GL-2Me-5Ac in 88% yield. Hetero-30-OH-GL-2Me-5Ac was treated with sodium methoxide at room temperature to give hetero-30-OH-GL in 60% yield. MS (ESI) (−) *m/z* 791 [M-H]. ^{1}H-NMR (δ) ppm (CD_3OD): 2.39 (d, 1H,H-9, *J*=14.6), 3.16 (dd, 1H, H-3, *J*=4.3, 11.6), 3.75 (d, 1H, H-5′ , *J*=9.8), 3.79 (d, 1H, H-5″, *J*=9.2), 4.52 (d, 1H, H-1′ , *J*=7.3), 4.63 (d, 1H, H-1″, *J*=8.5), 5.56 (d, 1H, H-12, *J*=10.4), 6.38 (m, 1H, H-11). ^{13}C-NMR (δ) ppm (CD_3OD): 91.1 (C-3), 105.3 (C-1′), 106.2 (C-1″), 126.7 (C-11), 126.7 (C-12), 135.4 (C-18), 138.2 (C-13) 172.0, 172.4 (COOH).

(2) Homo-30-OH-GL; 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-olean-9,12-dien-30-ol
11-OH-GL [32] was treated with 10% hydrogen chloride in methanol at room temperature to give a mixture of homo dimethyl ester, which were then acetylated to afford a dimethyl ester pentaacetate (homo-GL-2Me-5Ac) in 75% yield. Homo-GL-2Me-5Ac was treated with ethyl chloroformate and triethylamine followed by sodium borohydride to afford homo-30-OH-GL-2Me-5Ac in 63% yield. Homo-30-OH-GL-2Me-5Ac was treated with sodium methoxide at room temperature to give homo-30-OH-GL in 57% yield. MS (ESI) (−) *m/z* 791 [M-H]. ^{1}H-NMR (δ) ppm (CD_3OD): 3.13 (dd, 1H, H-3, *J*=4.3, 11.6), 3.75 (d, 1H, H-5′ , *J*=9.8), 3.79 (d, 1H, H-5″,

J=9.2), 4.52 (d, 1H, H-1′ , *J*=7.3), 4.63 (d, 1H, H-1″, *J*=8.5), 5.53 (d, 1H, H-11, *J*=6.1), 5.57–5.61 (m, 1H, H-12). ^{13}C-NMR (δ) ppm (CD_3OD): 66.5 (C-30), 90.9 (C-3), 105.3 (C-1′), 106.2 (C-1″), 117.0 (C-11), 122.4 (C-12), 147.5 (C-13), 155.8 (C-9), 172.0, 172.4 (COOH).

Following salt formation compounds were dissolved in 5% EtOH containing serum free medium (10 mg/ml), and diluted with 1% fetal bovine serum (FBS) containing medium: 18α-GL (disodium salt), 18β-GL (ammonium salt), 18α,β-glycyrrhetic acid monoglucuronide (18α-MGA; potassium salt), 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-11-deoxo-olean-12-en-30-oic acid (11-deoxo-GL; ammonium salt), 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-18β-olean-9,12-dien-30-oic acid (homo-GL; diammonium salt), 3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-oic acid (hetero-GL; disodium salt), hetero-30-OH-GL (disodium salt), homo-30-OH-GL (diammonium salt). 18α-GA, 18β-GA, and 18β-MGA were dissolved in DMSO (10mg/ml), and diluted with 1% FBS containing medium. Final concentration of EtOH or DMSO was less than 0.1% except for high concentration of 18α-GL, 18β-GL, and 18β-MGA. For the latter three compounds, same concentration of EtOH or DMSO was used as comparable control.

2.2. Reagents

Human recombinant TNF-α was kindly provided by Dainippon Pharmaceutical (Suitashi, Osaka, Japan). Human recombinant IL-4 and rabbit polyclonal anti-human eotaxin 1 antibody were purchased from Peprotech EC (London, UK). Horseradish peroxidase-conjugated swine anti-rabbit antibody was obtained from Dako (Denmark).

2.3. Cell cultures

Human fetal lung fibroblasts (HFL-1, from normal embryonic lung, diploid, originally ATCC CCl-153) were purchased from Riken Cell Bank (Saitama, Japan). The cells were cultured to confluence in Ham's F-12 (GIBCO, Grand Island, NY) supplemented with 15% heat-inactivated FBS, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin in humidified 5% CO_2/95% air at 37 °C in 10-mm-diameter collagen-coated culture dishes (IWAKI, Chiba, Japan).

2.4. Experimental procedure

The cells were seeded at a density of 5×10^4 cells per well in 24-well collagen-coated plates and incubated overnight for adherance. After removing the culture media, various concentrations of each compound diluted with 1% FBS containing medium were added and then the cells were stimulated with TNF-α or with TNF-α plus IL-4 and incubated at 37 °C. Since some published reports have indicated that co-stimulation with TNF-α and IL-4 causes a synergistic upregulation of eotaxin 1 production and mRNA expression [11,13,15], we adopted this stimulating condition for eotaxin 1 measurements. In the time course experiments, the cells were incubated with TNF-α or TNF-α plus IL-4 for 0–48 h. In most experiments, the cells were incubated with 10 ng/ml of TNF-α for 24 h for IL-8, or with 10 ng/ml of TNF-α plus 20 U/ml of IL-4 for 48 h for eotaxin 1. At the indicated time, the supernanants were harvested and stored at −20 °C until the ELISA and lactate dehydrogenase (LDH) assay. In all the experiments, control cells were incubated with the compound vehicle for the same period of time as the experimental cells.

2.5. Measurement of chemokine production and cytotoxicity assay

The concentration of IL-8 or eotaxin 1 in the culture medium was measured by the ELISA method as described elsewhere [33,34]. As coating Ab, monoclonal anti-IL-8 Ab (4 μg/ml) or monoclonal anti-eotaxin 1 Ab (1.87 μg/ml) and as 2nd Ab, rabbit polyclonal anti-IL-8 Ab (2 μg/ml) or anti-eotaxin 1 Ab (0.5 μg/ml) were used. All the samples were assayed at least in duplicate. These systems did not cross-react with other members of chemokine families. Average concentrations of IL-8 and eotaxin 1 detected in the samples treated with stimulants alone were 25 and 12 ng/ml, respectively.

The cytotoxicity of the test compounds was evaluated by LDH assay. The release of LDH in the supernanants was determined as described with the LDH-Cytotoxic Test Kit (Wako, Osaka, Japan). Since LDH assay well correlated with the MTT assay, we have shown the sole data on LDH assay in this study.

2.6. RNA isolation and PCR amplification

IL-8 mRNA and eotaxin 1 mRNA were analyzed by RT-PCR. HFL-1 cells were incubated with each compound and total RNA was extracted from adherant cells using Rneasy Mini Kit (Qiagen, Hilden, Germany). The RNA was reverse transcribed according to the manufacturer's protocol (Takara Bio, Shiga, Japan) using oligo dT primer and 1 μg of total RNA for first-strand cDNA synthesis. PCR was performed at an annealing temperature of 57 °C and with 23 amplification cycles for IL-8 with 26 amplification cycles for eotaxin 1, and with 25 amplification cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as a housekeeping gene. The PCR products were applied to 2% agarose gel and electrophoresed in Tris borate/EDTA. Human IL-8 was amplified with primers 5′-GAGCCAGGAAGAAACCACCGGA-3′ (sense) and 5′-GCATCTGGCAACCCTACAACAGACC-3′ (antisense); human-eotaxin 1 was amplified with primers 5′-CCCAACCACCTGCTGCTTTAACCTG-3′ (sense) and 5′-AAAAATGGTGATTATTTATGGC-3′ (antisense); human-GAPDH was amplified with primers 5′-GTCAGTGGTGGACCTGACCT-3′ (sense) and 5′-TGAGGAGGGGAGATTCAGTG-3′ (antisense). For the RT-PCR, two doses were set up between which was the 50% inhibitory concentration (IC_{50}) value calculated from each ELISA data, and mRNA levels of IL-8 and eotaxin 1 of the cells treated with selected concentrations of each compound were compared.

2.7. Statistical analysis

Results were expressed as mean values±S.D. of three experiments. Statistical analysis was performed using Student's *t*-test. A *p* value less than 0.05 was considered significant.

3. Results

3.1. Glycyrrhizin and its derivatives

To identify the inhibitory effect of glycyrrhizin and some derivatives on chemokine production in human lung fibroblasts, we focused on two main chemokines, IL-8 and eotaxin 1. Glycyrrhizin-related compounds are tentatively classified into five groups with these isomer conformations or relative structures (Fig. 1). We investigated the effects of these compounds on IL-8 and eotaxin 1 production and mRNA expression. Since no significant IL-8 and eotaxin 1 production was induced by these compounds alone (data not shown), the effects on TNF-α or TNF-α plus IL-4-induced chemokine production were evaluated.

3.2. Effects of 18α-GL and 18β-GL on IL-8 and eotaxin 1 production, mRNA expression, and cytotoxicity

Effects of 18α-GL and 18β-GL belonging to group 1 compounds, on chemokine production, mRNA expression and cytotoxicity are shown in Fig. 2. Both compounds exhibited similar inhibitory effects on IL-8 production in a gradual dose-dependent curve. Significant inhibition was observed at more than 300 μg/ml ($P<0.01$) and IL-8 production was reduced to 25% at 1000 μg/ml ($P<0.001$). The cytotoxicity of 18α-GL was slightly stronger than that of 18β-GL (Fig. 2A) with a CC_{30} of 330 μg/ml for 18α-GL vs. >1000 μg/ml for 18β-GL (Table 1). 18α-GL did not indicate significant effect on IL-8 mRNA expression, 18β-GL inhibited IL-8 mRNA expression moderately at 1000 μg/ml (Fig. 2B). On the other hand, the effects of both compounds on eotaxin 1 production and mRNA expression were rather different. 18α-GL had more of an inhibitory effect on eotaxin 1 production at a lower concentration (IC_{50}=25 μg/ml) than 18β-GL. The cytotoxicity of both compounds was mitigated in the presence of IL-4 (Fig. 2C) compared to TNF-α alone (Fig. 2A). The actions of both compounds on eotaxin 1 mRNA expression basically paralleled the ELISA data, i.e., 18α-GL inhibited the mRNA expression slightly at 10 and 100 μg/ml but 18β-GL did not inhibit it at the same doses (Fig. 2D).

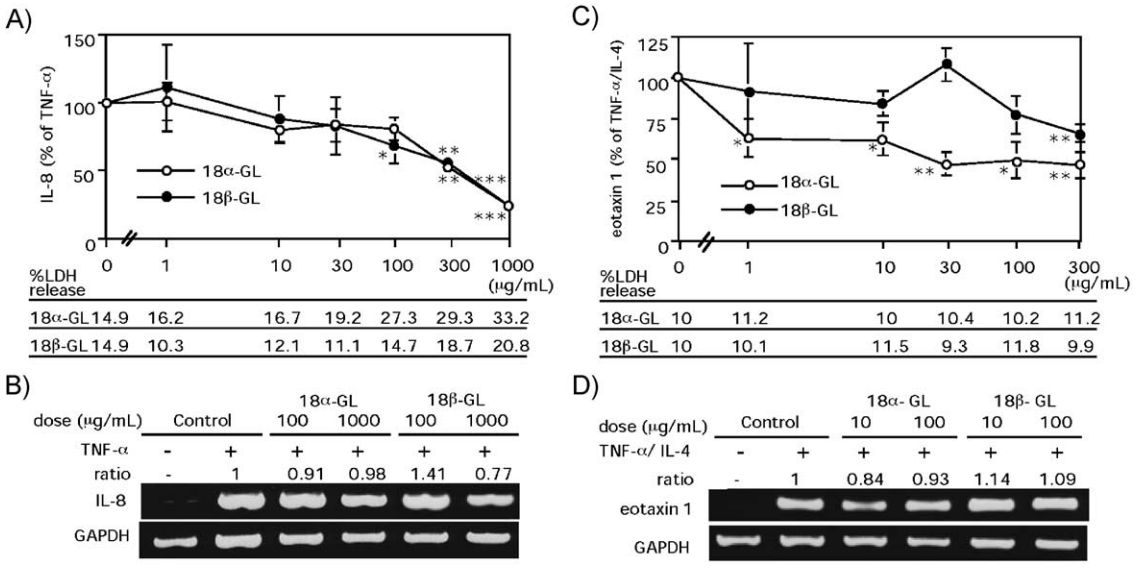


Fig. 2. Effect of 18α-GL and 18β-GL on IL-8 and eotaxin 1 production, LDH leakage, and mRNA expression. (A) Measurement of IL-8 secretion and LDH leakage. (B) RT-PCR analysis of IL-8 mRNA expression. (C) Measurement of eotaxin 1 secretion and LDH leakage. (D) RT-PCR analysis of eotaxin 1 mRNA expression. HFL-1 cells were seeded at densities of 5×10^4 cells per well in 24-well collagen-coated plates and incubated overnight for adherence. After removing the culture media, various concentrations of 18α-GL (○) or 18β-GL (●) diluted with 1% FBS-containing medium were added and then the cells were stimulated with TNF-α (10 ng/ml) for 24 h for IL-8 or TNF-α plus IL-4 (20 U/ml) for 48h for eotaxin 1 at 37 °C. Supernanants were collected and IL-8 or eotaxin 1 accumulation was measured by ELISA as described in the Materials and methods. Each point represents the mean±S.D. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the effect of TNF-α or TNF-α plus IL-4. The LDH release from cells over the course of the experiments was used as a measure of cytotoxicity. LDH activity in the supernanants was determined using a LDH-Cytotoxic Test Kit according to the manufacturer's protocol. The mean % LDH release of three independent experiments was shown as cytotoxicity index. Each S.D. was less than 5%. Total RNA (1 μg) extracted from 18α-GL, 18β-GL, and TNF-α-treated cells for IL-8 or TNF-α plus IL-4-treated cells for eotaxin 1 was used for RT-PCR with specific human IL-8 or eotaxin 1 primers and GAPDH primes as an internal control. Ratios for chemokine mRNA expression relative to GAPDH expression were calculated densitometrically (above RT-PCR photos).

3.3. Effects of 18α-GA, 18β-GA, aglycone form of α,β-GL, and 18α-MGA, 18β-MGA

Next, we examined 18α-GA and 18β-GA (group 2), which are aglycones of 18α-GL and 18β-GL. The compounds did not inhibit but rather increased IL-8 production at less than 10 μg/ml (Fig. 3A). The results of RT-PCR basically corresponded with the results of ELISA but not at 10 μg/ml (Fig. 3B,D). Concentrations of more than 30 μg/ml were cytotoxic (Fig. 3A,C) and we did not test higher doses. Of note is that these compounds affected eotaxin 1 production at two phases; enhancing at low doses (1–3 μg/ml) with 1.5–2-fold increase of eotaxin 1 production and inhibiting at 10 μg/ml. The inhibitory effect of 18α-GA was stronger than that of 18β-GA (Fig. 3C).

In addition, effects of 18α-MGA and 18β-MGA (group 3), monoglucuronide forms of 18α,β-GL were tested. As shown in Fig. 4A,B, no marked inhibition of IL-8 and eotaxin 1 production was exhibited by these monoglucuronide forms.

3.4. Effects of 11-deoxo-GL, homo-GL and hetero-GL

Five compounds, classified as groups 4 and 5, form a partly common structure in which the carbonyl group at the C-11 position has been reduced.

Inhibitory effects of 11-deoxo-GL, homo-GL, and hetero-GL (group 4) are shown in Fig. 5. 11-Deoxo-GL and hetero-GL drastically decreased IL-8 production at around 100 μg/ml. While approximately 40% cytotoxicity was observed at 100 μg/ml of both

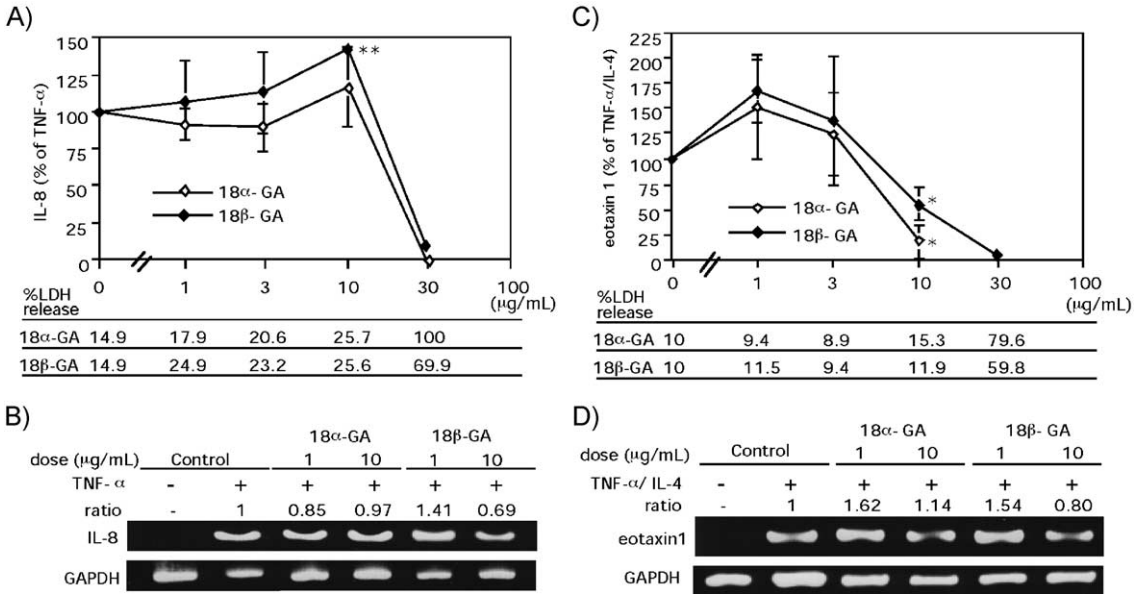


Fig. 3. Effect of 18α-GA and 18β-GA on IL-8 and eotaxin 1 production, LDH leakage, and mRNA expression. (A) Measurement of IL-8 secretion and LDH leakage. (B) RT-PCR analysis of IL-8 mRNA expression. (C) Measurement of eotaxin 1 secretion and LDH leakage. (D) RT-PCR analysis of eotaxin 1 mRNA expression. 18α-GA (◇), 18β-GA (◆). Each point represents the mean±S.D. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the effect of TNF-α or TNF-α plus IL 4. The mean % LDH release of three independent experiments was shown as cytotoxicity index. Each S.D. was less than 5%. Ratios for chemokine mRNA expression relative to GAPDH expression were calculated densitometrically (above RT-PCR photos).

compounds (Fig. 5A), morphological change of the cells was detected at this concentration. Namely, the fibrous shape of the cells changed to a round shape (data not shown). The action of homo-GL was similar to that of 18α,β-GL. Homo-GL moderately inhibited IL-8 production like 18α,β-GL as shown in Fig. 2A. In contrast to 11-deoxo-GL and hetero-GL, homo-GL did not show notable cytotoxicity even at high concentrations (Fig. 5A). The results of RT-PCR for these three compounds corresponded with the ELISA data (Fig. 5B). On the other hand, these three compounds decreased eotaxin 1 production at less than 100 μg/ml in a dose-dependent manner (Fig. 5C). Homo-GL was rather weaker than the other two compounds, and its inhibitory effect on eotaxin 1 mRNA expression was not so evident (Fig. 5D).

3.5. Effects of hetero-30-OH-GL and homo-30-OH-GL

Finally, we examined the effects of hetero-30-OH-GL and homo-30-OH-GL, group 5 compounds. These compounds are reduced forms of hetero-GL and homo-GL, modified at the C-30 carboxyl group. Both hetero-30-OH-GL and homo-30-OH-GL were more effective in inhibiting IL-8 and eotaxin 1 production than 18α,β-GL, hetero-GL, and homo-GL. Both compounds inhibited chemokine release at lower concentrations, and no apparent cytotoxicity was observed (Fig. 6A,C). Both IL-8 and eotaxin 1 mRNA expression were inhibited dose-dependently and the results corresponded with the ELISA data (Fig. 6B,D).

3.6. Inhibitory concentration (IC_{50}) against IL-8 and eotaxin 1 production and cytotoxic concentration (CC_{30}) of glycyrrhizin and related compounds

The inhibitory concentration and cytotoxic concentration of glycyrrhizin and related compounds were calculated and are shown in Table 1. Taking the concentration ranges of IC_{50} and CC_{30} into consideration, we assumed that hetero-30-OH-GL and homo-30-OH-GL were most valuable for the inhib-

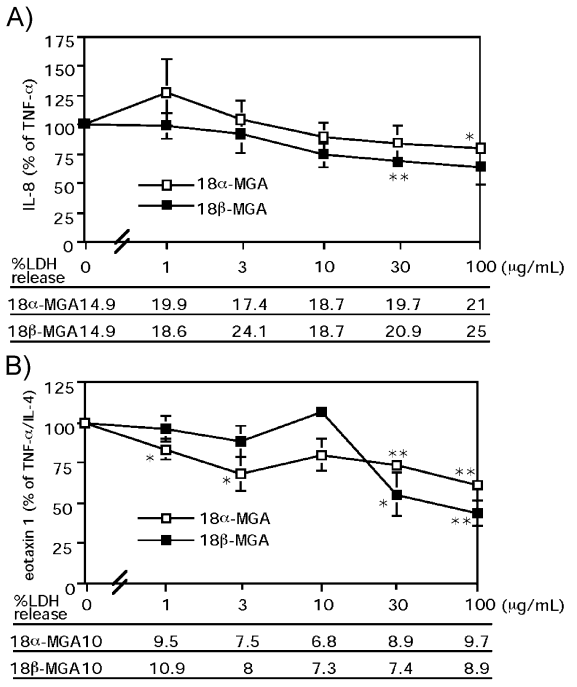


Fig. 4. Effect of 18α-MGA and 18β-MGA on IL-8 and eotaxin 1 production and LDH leakage. (A) Measurement of IL-8 secretion and LDH leakage. (B) Measurement of eotaxin 1 secretion and LDH leakage. 18α-MGA (□), 18β-MGA (■). Each point represents the mean±S.D. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the effect of TNF-α or TNF-α plus IL-4. The mean % LDH release of three independent experiments was shown as cytotoxicity index. Each S.D. was less than 5%.

ition of both IL-8 and eotaxin 1 production by HFL-1 cells, in view of their safety and efficacy.

4. Discussion

Several reports indicated that GL has immunomodulatory capacity through the regulation of Th1 and Th2 type cytokines [20–23,35]. In addition, Sasaki et al. [36] reported that GL induces the generation of CC-chemokines, MIP-1β and RANTES by peripheral blood mononuclear cells of HIV-infected patients. In this study, we focused on the modulatory effects of GL and its derivatives on the IL-8 and eotaxin 1 production by human lung fibroblasts. Since glucuronic acid and the C11, C18, and C30 positions of GL and its derivatives seem to be important as activity modulating positions [26–28], we modified these parts.

First, we removed glucuronic acid or changed the configuration of the hydrogen atom of GL at C-18. In view of the structure–activity relationships among GLs, GAs, and MGAs, glucuronic acid had different effects on IL-8 and eotaxin 1 generation. 18α,β-GL inhibited IL-8 production in a gradual dose dependent manner, and 18α-GL showed inhibitory effect on eotaxin 1 production. However, mRNA level was not well correlated with the amount of chemokines. Therefore, 18α,β-GL may regulate the chemokines production also by the posttranscriptional level like at the protein secretion or degradation. Further experiments are needed to explore in more detail. When one molecule of glucuronic acid was removed from GLs, MGAs did not show significant differences in IL-8 and eotaxin 1 production, but cytotoxicity increased with a CC_{30} of more than 100 μg/ml compared with more than 1000 μg/ml by 18α,β-GL. Moreover, when two molecules of glucuronic acid were removed from GLs, GAs indicated rather different actions on IL-8 and eotaxin 1 production. That is, while GAs did not show any inhibitory action against IL-8 production up to 10 μg/ml, GAs showed inhibitory effect of eotaxin 1 production at less than 10 μg/ml, with increasing cytotoxicity at more than 30 μg/ml. The results of both IL-8 and eotaxin 1 mRNA expression at 10 μg/ml were not necessarily corresponded with the result of ELISA. The reasons for this remain unclear. This concentration seems to be close to the cytotoxic concentration, so the effects of GAs on the protein level or mRNA level might be unstable. Alternatively, the timing at which eotaxin 1 production starts is later than that of IL-8, so this kinetic difference may reflect the different effects of GAs. As for the stereospecificity of the C18 position, there were no great differences between 18α- and 18β-compounds except for eotaxin 1 production; 18α-GL has a much stronger effect on eotaxin 1 production than 18β-GL.

Second, we examined the structure–activity relationship of 18β-GL-modified compounds in group 4. Comparing the effects of 11-deoxo-GL, homo-GL, and hetero-GL which were modified at C-11 and C-18, 11-deoxo-GL and hetero-GL indicated strong inhibition at 100 μg/ml but cytotoxicity arose simultaneously. Hetero-GL is a previously reported com-

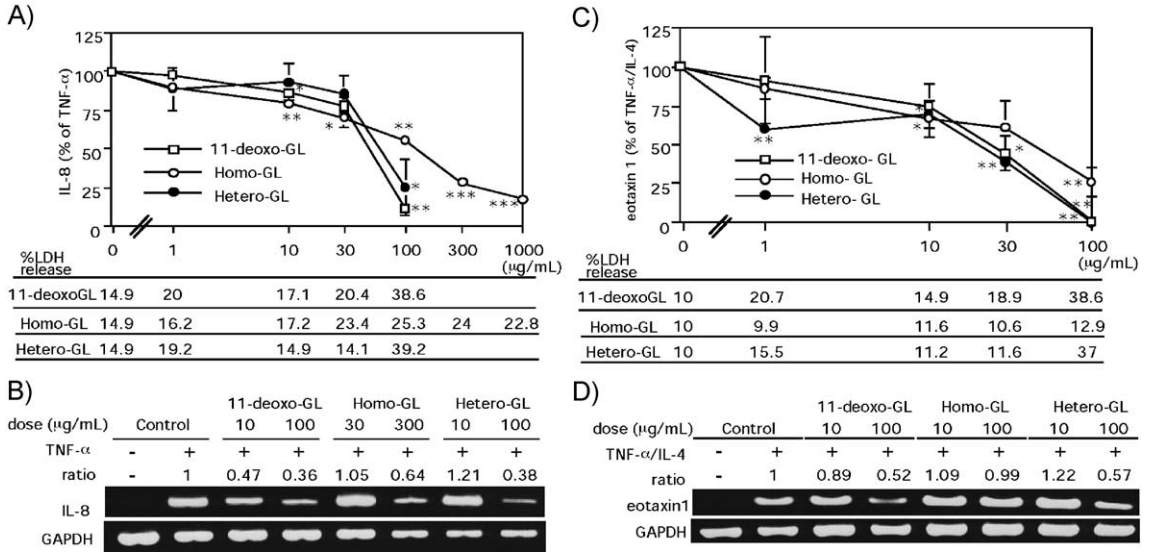


Fig. 5. Effect of 11-deoxo-GL, homo-GL, and hetero-GL on IL-8 and eotaxin 1 production, LDH leakage, and mRNA expression. (A) Measurement of IL-8 secretion and LDH leakage. (B) RT-PCR analysis of IL-8 mRNA expression. (C) Measurement of eotaxin 1 secretion and LDH leakage. (D) RT-PCR analysis of eotaxin 1 mRNA expression. 11-Deoxo-GL (□), homo-GL (○), hetero-GL (●). Each point represents the mean±S.D. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the effect of TNF-α or TNF-α plus IL-4. The mean % LDH release of three independent experiments was shown as cytotoxicity index. Each S.D. was less than 5%. Ratios for chemokine mRNA expression relative to GAPDH expression were calculated densitometrically (above RT-PCR photos).

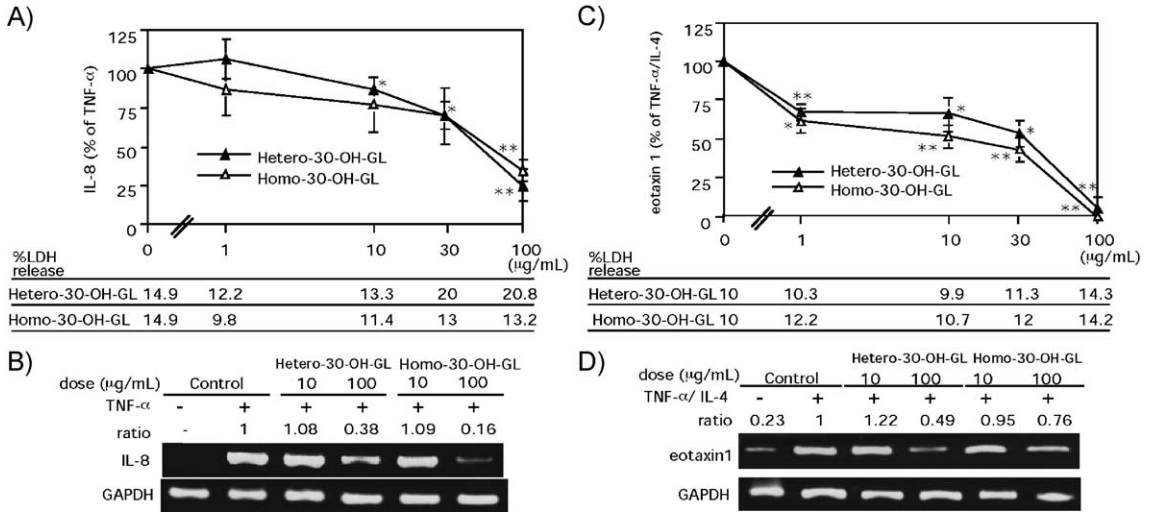


Fig. 6. Effect of hetero-30-OH-GL and homo-30-OH-GL on IL-8 and eotaxin 1 production, LDH leakage, and mRNA expression. (A) Measurement of IL-8 secretion and LDH leakage. (B) RT-PCR analysis of IL-8 mRNA expression. (C) Measurement of eotaxin 1 secretion and LDH leakage. (D) RT-PCR analysis of eotaxin 1 mRNA expression. Hetero-30-OH-GL (▲), homo-30-OH-GL (△). Each point represents the mean±S.D. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the effect of TNF-α or TNF-α plus IL-4. The mean % LDH release of three independent experiments was shown as cytotoxicity index. Each S.D. was less than 5%. Ratios for chemokine mRNA expression relative to GAPDH expression were calculated densitometrically (above RT-PCR photos).

Table 1
Inhibition of IL-8, eotaxin 1 production by glycyrrhizin derivatives and their cytotoxicity

Compounds	IL-8 TNF-α stimuli for 24 h		Eotaxin 1 TNF-α/IL-4 stimuli for 48 h	
	IC_{50} (μg/ml)	CC_{30} (μg/ml)	IC_{50} (μg/ml)	CC_{30} (μg/ml)
18α-GL	330	330	25	>1000
18β-GL	370	>1000	>300	>1000
18α-GA	(18)	11	7	13
18β-GA	(22)	11	12	15
18α-MGA	>100	>100	>100	>100
18β-MGA	>100	>100	50	>100
11-dcoxo-GL	50	75	25	78
Homo-GL	130	>1000	42	>1000
Hetero-GL	60	82	20	83
Hetero-30-OH-GL	52	>100	33	>100
Homo-30-OII-GL	60	>100	12.5	>100

Inhibitory concentration (IC_{50}) against IL-8 or eotaxin 1 production and cytotoxicity (CC_{30}) of each compound were calculated from the ELISA and LDH assay data.

pound that has inhibitory action against HIV and herpes simplex virus replication [26]. Therefore, the pharmacological potency of hetero-GL can be expected, but we found that the range of efficacy and toxicity is narrow. We demonstrated here that the effect of homo-GL on IL-8 and eotaxin 1 production was weaker than that of 11-deoxo-GL and hetero-GL but with less cytotoxicity. As the effect of homo-GL on eotaxin 1 production, mRNA level was not correlated with the protein level. Homo-GL also may modulate eotaxin 1 production by the porttranscriptional level as well as GLs. Moreover, as for hetero-30-OH-GL and homo-30-OH-GL (group 5), modified compounds of hetero-GL and homo-GL at the C-30 position, both compounds showed moderate inhibitory activity against IL-8 and eotaxin 1 expression. That is, the IC_{50} of hetero-30-OH-GL and homo-30-OH-GL against IL-8 and eotaxin 1 was lower than that of GLs and the cytotoxicity was less than that of hetero-GL. In terms of IC_{50}, homo-30-OH-GL (IC_{50} for IL-8 was 60 μg/ml and IC_{50} for eotaxin 1 was 12.5 μg/ml) was superior to homo-GL. These results suggest that the reduction of the carboxyl group at C-30 may augment the inhibitory activity without changing the cytotoxicity. Taken collectively, modification of GLs at the C-11 and C-18 positions may augment the activity of GLs.

GL has been used widely and effectively as a treatment for chronic hepatitis [16,19]. Furthermore, GL has been described as an anti-viral agent against many kinds of viruses, including herpes simplex virus type 1 [37], HIV [38], human cytomegalovirus (CMV) [39], influenza virus [40], SARS-associated coronavirus [29], etc. Murayama et al. [41] demonstrated that IL-8 enhanced human CMV replication in human lung fibroblasts. Also, an elevation of IL-8 was observed in SARS patients [30]. Thus, it seems that there is a close connection between viral infection and IL-8 production in the lung. In addition, IL-8 is known as a significant attractant for inflammatory cells in chronic obstructive pulmonary disease (COPD) [42]. Therefore, the results of our study that GL and related compounds reduced IL-8 generation in human lung fibroblasts explain the activity of these anti-inflammatory drugs against lung diseases. Eotaxin 1 is thought to be a potent eosinophil chemotactic factor, recruiting eosinophils into the airways after allergic stimulation. This notion is supported by some in vivo experiments [9,43]. Moreover, the correlation between eotaxin and eosinophil accumulation in bronchoalveolar lavage of asthmatic patients has been identified [44]. In this study, we demonstrated that GL derivatives had inhibitory capacity against eotaxin 1 production caused by TNF-α plus IL-4 stimulation in lung fibroblasts. These results would imply that GL derivatives could be useful as anti-allergic and anti-inflammatory agents in lung eosinophilic diseases like asthma. GL also has the ability to enhance Th1 cytokines, IFN-γ [20], IL-2 [21], and IL-12 [22] and to modulate Th2 cytokines, IL-10 enhancement [23], or IL-4 reduction in $CD4^+$ T cells [34]. Because the generation of eotaxin 1 is induced by IL-4 and is suppressed by IFN-γ [11,13], GL-related compounds may improve allergic and inflammatory lung diseases not only by inhibiting proinflammatory chemokine production but also by shifting the Th1 axis. Although GL needed high doses i.e., ~1 mg/ml to inhibit IL-8 and eotaxin 1 production, we demonstrated that other GL-related compounds, especially hetero-30-OH-GL and homo-30-OH-GL, are effective at much lower doses (less than 100 μg/ml). Thus, we assume that these compounds are more beneficial from the perspectives of their inhibitory concentration and cytotoxicity.

In summary, the present study showed the inhibitory effect of GL and related compounds on IL-8 and eotaxin 1 production by human lung fibroblasts. Notably, GL-modified compounds, hetero-30-OH-GL and homo-30-OH-GL, are presumed to be good candidates with their inhibitory activity against both IL-8 and eotaxin 1 production.

Acknowledgements

We are grateful to Mr. Motohumi Miura for technical assistance. We express great thanks to Dr. Hideo Inoue, Dr. Atsushi Karino, Dr. Takashi Naito, and Mr. Kazuhiro Hirabayashi for their scientific advice. We thank Mr. Tomohiko Shinozawa, Mr. Masataka Sugiura, and Mrs. Yukiko Nakayama for their support.

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