ophores in dispersed zona glomerulosa cells[2,3] In vivo, AM prevents increased plasma aldosterone levels induced through the infusion of angiotensin II, a sodium-deficient diet or bilateral nephrectomy[21,22] These findings suggest that AM may have a role in inhibiting aldosterone secretion from zona glomerulosa cells. In addition, intravenous infusion of AM reduced aldosterone levels in humans[10,11,16] Thus, it is possible that AM directly inhibits aldosterone secretion in heart failure. Several lines of evidence show that AM has antioxidative effects[2,3] A recent study has demonstrated that angiotensin II-induced reactive oxygen species production through the activation of NADPH oxidase was significantly attenuated by AM in a concentration-dependent manner[23] Increased oxidative stress plays a major role in the pathogenesis of heart failure[24,25] Thus, the inhibitory effects of AM on aldosterone secretion and production of reactive oxidative species may be useful in the treatment for heart failure.

AM+hANP therapy appeared to increase UV, $U_{Na}V$, $U_{cAMP}V$ and $U_{cGMP}V$, whereas these variables did not change significantly after switching to hANP monotherapy. This suggests that the observed renal effects of AM+hANP therapy may be mainly due to a hANP effect. Many studies have demonstrated that AM has renal vasodilatory, natriuretic and diuretic actions[2] We also reported previously that intravenous infusion of AM ($0.05\,\mu g \cdot kg^{-1} \cdot min^{-1}$) increases GFR, UV and $U_{Na}V$ in rat and human heart failure[10,11,16] The possible reasons why an obvious renal effect from AM was not observed may be due to: (1) the low dose of AM ($0.02\,\mu g \cdot kg^{-1} \cdot min^{-1}$) used; and (2) the severe intensity of heart failure in the present study.

Thus, the present study indicated that the combination of AM and hANP would be good for: (1) potential strong preload and afterload reduction; (2) HR stability through mutual suppression of an AM-induced-HR-increase with hANP and a hANP-induced-HR-decrease with AM; and (3) neurohumoral changes.

We have the following limitations of the present study: (1) the study had a small number of cases and did not have enough subjects to detect a statistical difference in all time-points between baseline values and AM+hANP therapy with regard to hemodynamic parameters; (2) a fixed dose of AM was used regardless of the severity of heart failure, thus the response to the AM+hANP therapy might be blunted in severe ADHF patients; (3) subjects with different heart failure etiologies were included in the present study; and (4) patients with relatively different severities of heart failure were included. Despite these heterogeneities, AM+hANP therapy could show some beneficial hemodynamic and hormonal effects in ADHF.

In summary, we evaluated the effect of AM+hANP therapy in a small pilot study of patients with ADHF. The administration of AM+hANP was associated with a reduction in SVR, PAR, PCWP, mPA, MAP, aldosterone, BNP and d-ROM, and with an increase in CI, UV, $U_{Na}V$, $U_{cAMP}V$ and $U_{cGMP}V$. These data are preliminary and require confirmation in a larger clinical trial.

Acknowledgments

This work was supported in part by Scientific Research Grants-in-Aid 14570692, 18590787, and 20590837 from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan; Research Grant for Cardiovascular Diseases 17A-1 from the Ministry of Health, Labour and Welfare of Japan; and the Seki Minato Prize. We thank Ms. Keiko Ishikawa, Ms. Masako Minato, Ms. Kyoko Tabei and Ms. Machiko Sakata for technical assistance. We also thank Toshiyasu Iwase and Chiaki Koshikawa for preparing the adrenomedullin injection.

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Endocrine Journal 2009, 56 (9), 1119-1128

NOTE

Ghrelin Increases Hunger and Food Intake in Patients with Restricting-type Anorexia Nervosa: A Pilot Study

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Abstract. Ghrelin increases hunger sensation and food intake in various patients with appetite loss. Anorexia nervosa (AN) begins with psychological stress-induced anorexia and some patients cannot increase their food intake partly because of malnutrition-induced gastrointestinal dysfunction. The effects of ghrelin on appetite, food intake and nutritional parameters in anorexia nervosa (AN) patients were examined. Five female restricting-type AN patients (age: 14-35 y; body mass index: 10.2-14.6 kg/m^2) had persistently complained of gastrointestinal symptoms and failed to increase body weight. They were hospitalized for 26 days (6 days' pre-treatment, 14 days' ghrelin-treatment, and 6 days' post-treatment) and received an intravenous infusion of 3 μg/kg ghrelin twice a day. Ghrelin infusion improved epigastric discomfort or constipation in 4 patients, whose hunger scores evaluated by visual analogue scale questionnaires also increased significantly after ghrelin infusion. Daily energy intake during ghrelin infusion increased by 12-36 % compared with the pre-treatment period. Serum levels of total protein and triglyceride as nutritional parameters significantly increased after ghrelin treatment. There were no serious adverse effects including psychological symptoms. We found that ghrelin decreases gastrointestinal symptoms and increases hunger sensation and daily energy intake without serious adverse events in AN patients. Although the present study had major limitations of the lack of a randomized, placebo-controlled group, non-blindness of the investigators and the small number of patients recruited, it would contribute to further investigations for therapeutic potential of ghrelin in AN patients.

Key words: Ghrelin, Anorexia nervosa, Hunger, Food intake

(*Endocrine Journal* 56: 1119-1128, 2009)

GHRELIN is mainly secreted by the stomach during starvation and it exerts a potent stimulatory effect on food intake and growth hormone (GH) secretion [1-3]. Endogenous ghrelin and its receptors are involved in the regulation of food intake, adiposity, and GH secretion [4]. Intravenous infusion of ghrelin is reported to increase food intake and body weight in healthy subjects [5-7] and to stimulate appetite and food intake in patients with congestive heart failure [8], chronic obstructive pulmonary disease [9], cancer [10], and functional dyspepsia [11].

Anorexia nervosa (AN) usually begins with psychological stress-induced anorexia and is characterized by fear of weight gain, starvation-induced abnormal behaviors, and a variety of biochemical and endocrinological abnormalities due to malnutrition. Chronic malnutrition induces both functional and organic changes in the gastrointestinal tract [12-14]. Most AN patients complain of chronic or recurrent upper abdominal discomfort and fullness, and chronic constipation. Laboratory examinations of the stomach reveal atrophy of the mucosa, alteration of peristalsis, and de-

Received Jun. 9, 2009; Accepted Aug. 28, 2009 as K09E-168
Released online in J-STAGE as advance publication Sep. 16, 2009
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Table 1. Clinical profile of AN patients in the present study

Case No	1	2	3	4	5
Age on entry (yrs)	27	31	25	35	14
Height (cm)	161	157	156	154	150
Weight before illness (kg) (BMI kg/m^2)	48 (18.5)	48 (19.5)	44.2 (18.2)	50 (21.1)	43 (19.1)
Age of onset (yrs)	16	24	17	20	13
Duration of illness (yrs)	12	6	8	15	1
The minimal weight (kg) (BMI kg/m^2)	29 (11.2)	30 (12.2)	32 (13.1)	23 (9.70)	27.4 (12.2)
Weight on entry (kg) (BMI kg/m^2)	37.9 (14.6)	32.5 (13.2)	35.0 (14.4)	24.2(10.2)	28.2 (12.5)
The increment of daily energy intake (%)	12	36	16	33	14
Weight on the end of study (kg) (BMI kg/m^2)	36.4 (14.0)	31.5 (12.8)	35.7 (14.7)	26.6 (11.2)	28.4 (12.6)
Weight on 6 months after discharge (kg) (BMI kg/m^2)	43 (16.6)	38.5 (15.6)	38.2 (15.7)	28 8 (12.1)	34.5 (15.3)

layed emptying time [15]. Even after becoming fully motivated to gain body weight, AN patients often cannot increase their food intake because of malnutrition-induced gastrointestinal dysfunction, and this delays recovery. Currently prescribed appetite-stimulating drugs such as metoclopramide, cyproheptadine, and sulpiride are not always effective, and any increase in appetite may be minor. Therefore, there is a pressing need for effective appetite-stimulating therapies for AN patients.

To develop a possibly new medical treatment for AN, we investigated the effects of ghrelin on appetite, energy intake, and nutritional parameters in restricting-type AN patients without binge eating/purging as a pilot study.

Subjects and Methods

Subjects

Subjects in the present study comprised 5 Japanese female amenorrheic AN patients aged 26 ± 8 yr (mean ±SD) (range, 14-35 yr) and mean body mass index (BMI) of 13.0 ±1.8 kg/m^2 (range, 10.2-14.6 kg/m^2) (Table 1). Patients met the Diagnostic and Statistical Manual IV (DSM IV) criteria for AN [16], in addition to those of the Survey Committee for Eating Disorders of the Japanese Ministry of Health, Labor and Welfare [17]. All patients had restricting AN, and had never reported binge eating, vomiting or laxative/diuretic abuse. All subjects were tested to be negative for *Helicobacter (H) pylori*. None of the patients had started medication prior to the trial. Four patients except for case 5 had complained of such as epigastric discomfort, abdominal fullness or pain after eating and constipation for several years and had been treated with intensive pshychotherapy as well as supervision of dieticians. All patients had been admitted to undertake hyperalimentation therapy but then lost weight again. They had been motivated to gain weight, but could not increase their food intake, in part because of gastrointestinal discomfort. The study protocol was approved by the institutional review board of Tokyo Women's Medical University. All patients provided written informed consent to participate in this study.

Methods

Study design

Due to ethical reasons, randomized controlled or blind methods were not applied for the present study. Subjects were hospitalized for 26 days (day -6 to day 20) in Tokyo Women's Medical University Hospital (Figure 1). Food intake and subjective hunger sensation were measured for 24 days (day -5 to day 19). The pre-treatment period was defined as the 5 days before ghrelin injection (day -5 to day -1). Subjects received an intravenous infusion of ghrelin (3 μg/kg body weight) for 5 min twice a day (before breakfast and dinner) for 14 days (day 1 to day 14) [11]. After ghrelin infusion, subjects were monitored for the clinical efficacy and safety of ghrelin for 5 days (day 15 to day 19) as a post-treatment period. Since ghrelin at doses of 1 and 5 μg/kg tended to increase appetite dose-dependently and repeated administration of ghrelin at a dose of 3μg/kg increase food intake without severe adverse effects [6, 11], we chose 3μg/kg of ghrelin in the present study.

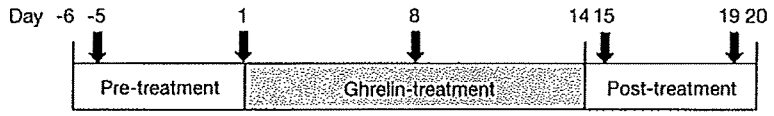

Fig. 1. The timeline of the present study
Subjects were hospitalized for 26 days (day −6 to day 20) and subjective hunger sensation was measured for 24 days (day −5 to day 19). The pre-treatment period was defined as the 5 days before ghrelin injection (day −5 to day −1). Subjects received an intravenous infusion of ghrelin for 14 days (day 1 to day 14). After ghrelin infusion, subjects were monitored for the clinical efficacy and safety of ghrelin for 5 days (day 15 to day 19) as a post-treatment period. Blood and urine samples for biochemical and endocrinological parameters were taken in the morning after overnight fasting and psychological assessment was done on day −5, day 1, day 8, day 15, and day 19, respectively.

Ghrelin used in the present study

Human ghrelin was prepared as previously described [11]. Acylated peptide was dissolved in 3.75 % D-mannitol to yield a final concentration of 180 μg/mL. The solutions were filtered and stored at -20°C in sterile vials. Examination by the Japan Food Research Laboratories (Tokyo, Japan) did not find any traces of endotoxin in the ghrelin solutions. A pyrogen test based on the Pharmacopoeia of Japan was also negative.

Assessment of food intake and attitudes toward food

The primary endpoint of this study was energy intake. Patients were initially served with an amount of food equivalent to their meals at home before hospitalization plus an additional 200 Kcal. Each dish was weighed before and after eating. Energy intake was calculated by dieticians as total energy, carbohydrate, fat, and protein intakes. When subjects ate all of the food served and wanted more, they were allowed to eat self-prepared foods yielding approximately 200 Kcal such as fruit or other snacks. Their attitudes toward food were evaluated by a questionnaire incorporating visual analogue scales (VAS) rating hunger, satiety, prospective consumption, fullness, desire for some meat or fish, desire of something salty, desire of something sweet and desire of something fatty. During pre- and post-treatment, AN patients answered VAS questionnaire at before and after every meal. During ghrelin treatment, they did at 15 min before ghrelin infusion and breakfast or dinner, 15 min after ghrelin infusion before breakfast or dinner, and after those meals. It is demonstrated that food intake correlates with perceptions of hunger and fullness as assessed by VAS in healthy volunteers [18].

Measurement of biochemical and endocrinological parameters

Blood and urine samples for biochemical and endocrinological parameters were taken in the morning after overnight fasting longer than 10 h on day -5, day 1, day 8, day 15, and day 19. Blood samples for ghrelin assay were collected in tubes with 1 mg/mL EDTA-2Na and 500 U/mL aprotinin. They were immediately centrifuged at 4°C, and plasma samples were then acidified with 1 normal HCl and stored at -80°C until assay.

Immunoradiometric assays were utilized to measure levels of plasma GH (Eiken Chemical Co. , Tokyo, Japan) and serum IGF-I (Daiichi Pharmaceutical Co., Tokyo, Japan). Plasma insulin measurements were performed using an ELISA kit (Eiken Chemical Co., Tokyo, Japan). Plasma levels of intact and desoctanoyl ghrelin were measured using Active Ghrelin and Desacyl-Ghrelin ELISA kits (Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively.

Psychological assessment

Depression and anxiety levels were evaluated using the Japanese versions of the self-rating depression scale (SDS) [19] and state-trait anxiety inventory (STAI) [20] on day -5, day 1, day 8, day 15 and day 19, respectively. Eating behaviors, weight, and body image concerns were also assessed by eating disorder inventory (EDI) [21] on the same time as described.

Statistics

Data are expressed as mean ± SE. Two-way analysis of variance (ANOVA) was used for energy and nutrient intakes and for biochemical and endocrinologic data. Appetite scores were analyzed by a Wilcoxon

signed rank test comparing the changes in VAS. Statistical analyses were performed using the computer statistical package SPSS (version 13.0.; SPSS Inc., Chicago, IL). Levels of significance were determined at $p<0.05$.

Results

Gastrointestinal symptoms and hunger sensation

After ghrelin injection, all patients except for case 2 reported that they had sensations of stomach activity or that their upper abdominal fullness disappeared. Borborygmi were frequently audible just after each ghrelin infusion in all patients. During ghrelin treatment, no patients reported constipation. As case 5 complained of loose stools, the dose of ghrelin was reduced to 1.5 μg/body weight from day 7 to day 14 and this improved her symptoms.

Hunger sensation evaluated by VAS was higher just after ghrelin infusion than that before ghrelin infusion in all patients except for case 2 (Figure 2). The stimulatory effects of ghrelin on hunger sensation disappeared after eating and did not last until next meal. Only in case 1, hunger scores before breakfast or dinner during ghrelin treatment were lower than those during both the pre- and post-treatment periods.

Food intake and body weight

The mean daily intakes of energy, carbohydrate, fat and protein are presented in Figure 3. The daily energy intake of the 5 patients during the pre-treatment period ranged from 825 to 1426 Kcal. During ghrelin infusion, all patients except for case 5 showed a statistically significant increase in daily energy intake. The mean increase in daily energy intake during ghrelin infusion was 20 ± 4 % when compared with the pre-treatment period. The mean food intake during ghrelin treatment in case 2, who did not report an increase in hunger sensation after ghrelin injection, significantly increased compared to that of pre-treatment. Analysis of nutrients revealed significant increases in daily intakes of carbohydrate (in 3 patients; cases 2, 3, and 4), fat (in 1 patient; case 4) and protein (in all patients). During the post-treatment period, daily energy, carbohydrate and protein intakes remained higher than those in the pre-treatment period in 3 patients (cases 2, 3, and 4). The daily fat intake during post-treatment period also remained higher than that in the pre-treatment period in 4 patients (cases 2, 3, 4, and 5). The increments of body weight in 5 patients were ranged from -1.5 to 2.4 kg during the ghrelin study (Table 1). Case 4 increased water and fat components evaluated by dual X-ray absorptiometry (data not shown).

Biochemical and endocrinological changes

Complete blood count did not change significantly during this study. Serum total protein and triglyceride levels significantly increased after ghrelin treatment (Table 2). Other nutritional markers including serum levels of transferrin and glucose showed a tendency to increase during and after ghrelin treatment, but this did not reach statistical significance. With the exception of case 4, in whom elevated transaminase levels due to malnutrition were improved by ghrelin treatment, liver function was stable over the study period.

Mean plasma levels of insulin and leptin did not increase significantly during ghrelin treatment. Although the elevated plasma level of GH decreased and the suppressed serum level of IGF-I improved during the study in case 4, other patients did not show a significant change in those parameters. Mean plasma levels of PRL and ACTH measured in the morning before ghrelin injection did not change significantly during ghrelin treatment.

We previously reported mean levels of plasma active and desacyl ghrelin in healthy young women as 29.9 ± 3.1 and 94.1 ± 7.5 pmol/L, respectively [22]. In the present study, the plasma levels of active ghrelin in AN patients ranged from 13 to 73 pmol/L (mean, 42) before ghrelin treatment and then did not show a significant change. Plasma levels of desacyl ghrelin in AN patients ranged from 80 to 731 pmol/L (mean, 280) before treatment, and then showed a tendency to decrease during ghrelin treatment.

Adverse effects

No serious adverse events occurred in all cases during ghrelin treatment. We did not detect any changes in vital signs or biochemical and endocrinologic data after ghrelin treatment. The only exceptions were loose stools in case 5 and an occasional warm sensation in the trunk or mild sweating in 2 subjects. No patients developed somnolence during ghrelin treat-

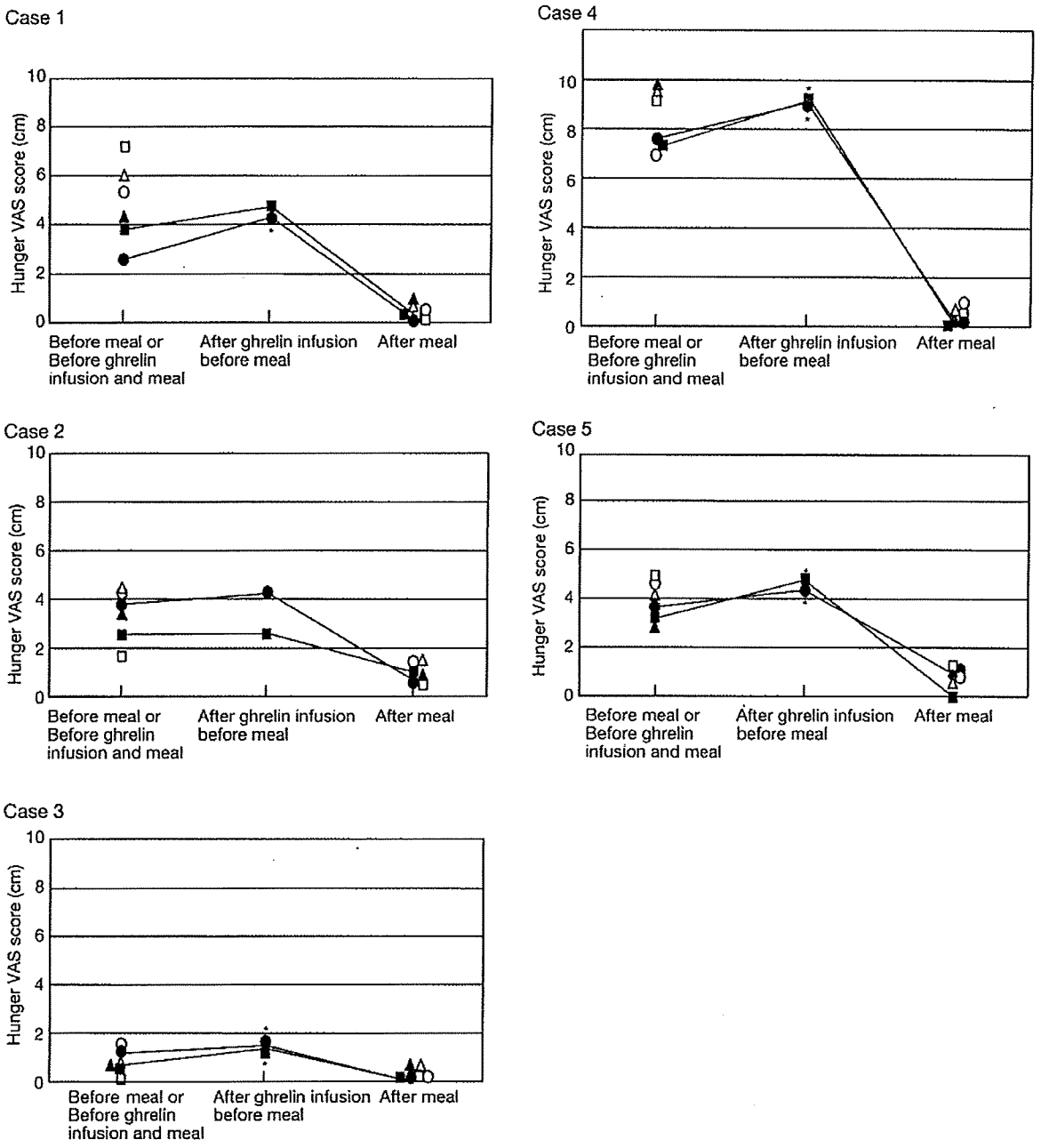

Fig. 2. Changes in hunger evaluated by VAS in AN patients

During pre- and post-treatment, AN patients answered VAS questionnaire at before and after every meal. During ghrelin treatment, they did at 15 min before ghrelin infusion, 15 min after ghrelin infusion before breakfast or dinner, and after those meals. Open circles (○), triangles (△) and squares (□) represent the mean of VAS hunger scores for breakfast, lunch, and dinner during pre and post-treatment periods, respectively. Closed circles (●), triangles (▲) and squares (■) represent the mean of VAS hunger scores for breakfast, lunch, and dinner during ghrelin treatment, respectively.

Data are expressed as mean. * $p<0.05$ *vs.* before ghrelin infusion

The mean of hunger scores before breakfast or dinner evaluated by VAS significantly increased after ghrelin infusion in all cases except for case 2.

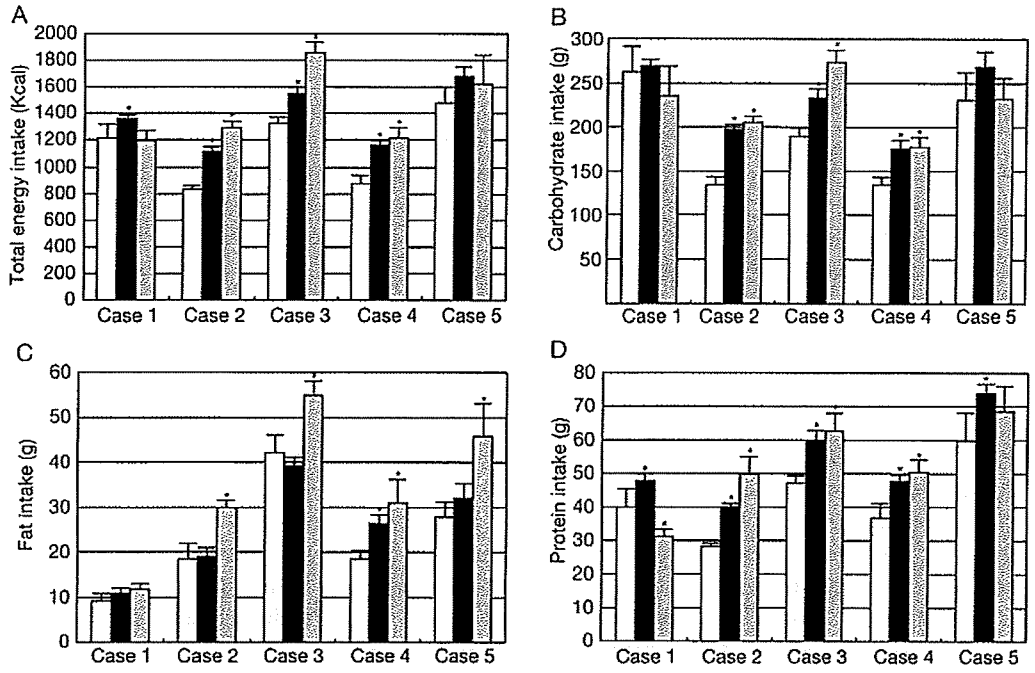

Fig. 3 Changes in the mean of total energy (panel A), carbohydrate (panel B), fat (panel C), and protein (panel D) intakes of AN patients.

Open (□), closed (■) and grey (▒) bars represent the mean of intake during pre-treatment, ghrelin treatment, and post-treatment periods, respectively. Data are expressed as mean ± SE. * $p<0.05$ *vs.* pre-treatment period.

Across the 5 patients, mean increase in daily energy intake during ghrelin infusion was 12-36%. Energy intake in the post-treatment period remained higher than that in the pre-treatment period in 3 patients.

Table 2. Changes in biochemical and endocrinological data in AN patients during the present study

	Day 1	Day 8	Day 15	Day 19
White blood cell (/μL)	3200±230	2820±331	2660±388	3240±614
Hemoglobin (g/dL)	12.7±0.8	13.0±1.2	12.7±1.1	13.0±0.9
Platelet (x10^4/μL)	15.8±2.4	16.0±2.4	15.6±1.9	16.4±1.8
Total protein (g/dL)	6.5±0.4	6.9±0.1	6.8±0.4	7.1±0.3*
Transferrin (mg/dL)	179±22	195±21	196±15	208±10
Retinol binding protein (mg/dL)	3.0±0.3	3.0±0.4	3.1±0.3	3.1±0.2
Blood sugar (mg/dL)	75±5	79±2	81±2	81±2
AST(U/L)	86±58	32±7	27±2	31±3
ALT(U/L)	164±139	60±35	37±11	38±10
Cholinesterase (U/L)	220±29	220±30	214±28	216±25
Triglyceride (mg/dL)	47±10	80±15*	72±9*	83±10*
Total cholesterol (mg/dL)	179±23	187±23	170±24	182±18
Immunoreactive insulin (U/mL)	2.00±0.29	1.57±0.46	2.21±0.46	2.39±0.27
Leptin (ng/mL)	1.4±0.3	1.2±0.2	1.3±0.1	1.3±0.1
GH (ng/mL)	16.6±14.6	11.2±10.3	8.7±7.0	3.6±1.9
IGF-I (ng/mL)	115±37	116±28	128±32	123±35
PRL (ng/mL)	10.8±2.0	8.2±1.3	9.9±1.5	9.3±1.5
ACTH (pg/mL)	28.3±7.4	19.1±4.3	22.9±2.6	25.1±3.5
Active ghrelin (pmol/L)	42±19	45±12	54±15	46±9
Desacyl ghrelin (pmol/L)	280±115	198±26	206±37	198±37

Data are expressed as mean ± SE. *$p<0.05$ compared to day 1.

ment. In terms of psychological tests, SDS and STAI showed no significant change during the study, and EDI did not show any increased fear of weight gain in these patients (data not shown).

Clinical course after discharge

All patients gained weight after discharge, as shown in Table 1. In case 3, menstruation resumed 6 months after discharge.

Discussion

The present study showed that ghrelin infusion (3 μg/kg twice a day) can decrease gastrointestinal symptoms and enhance hunger sensation and daily energy intake without serious adverse events in restricting-type AN patients. The major limitations of the present study relate to the lack of a randomized, placebo-controlled group and non-blindness of the investigators and the small number of patients recruited. A non-treated group is not possible due to ethical reasons. Although non-ghrelin infused subjects who receive intense counseling and supervision of dietitian might be considered as a control group, all subjects in the present study had already received those treatments as well as total parenteral nutrition during the previous admission but failed to increase body weight due to gastrointestinal symptoms. Since the daily energy intake of post-treatment period was still higher than that of pre-treatment period, we could not exclude a placebo effect of ghrelin. However, we insist that 4 patients who failed in gaining body weight for long periods but they could increase their food intake during and after ghrelin infusion. It is speculated as the patients told us that ghrelin triggered an improvement in gastrointestinal function, which ameliorated the fear of gastrointestinal discomfort after eating in these patients.

Ghrelin seems to improve gastrointestinal motility in AN patients in the present study. It is notable that borborygmi occurred immediately after ghrelin infusion and that abdominal fullness or constipation disappeared in all patients. Ghrelin plays a role in the regulation of gastrointestinal motility and acid secretion in rats [23-25] and increases the gastric emptying rate in normal-weight humans [26]. Although we did not investigate gastric emptying rate in AN patients after ghrelin injection, ghrelin improved epigastric discomfort. This was probably mediated partly through increased gastric peristalsis as shown in other diseases with gastrointestinal dysfunction [27-30].

Ghrelin infusion increased hunger scores evaluated by VAS questionnaires of AN patients in the present study. Although AN patients often report not to feel hunger or satiety sensation, hunger scores was higher just after ghrelin infusion than that before ghrelin infusion in 4 patients. Since the sensation of hunger is usually correlated with gastric emptying in humans [31], enhanced hunger sensation in AN patients may be caused in part by ghrelin-induced gastric motility. However, the stimulatory effects of ghrelin on hunger score did not last until the next meal. We considered that the short-term effect of ghrelin on hunger sensation is related to its rapid degradation. The plasma concentration of ghrelin reaches the peak at 15 min after injection and rapidly decreases [6]. Hunger scores before breakfast or dinner during ghrelin treatment were lower than those during both the pre- and post-treatment periods in case 1. It is likely that abdominal fullness induced by the increased amount of food eaten in the foregoing meal during ghrelin treatment probably disturbed the hunger sensation on the next meal.

In previous reports, continuous or repeated ghrelin infusion increased hunger sensation and food intake in healthy volunteers and various patients with appetite loss. Ghrelin infusion at a dose of 5 pmol/kg/min for 270 min increased food intake by 28 % in healthy young Caucasian volunteers [5] and by 31 % in middle-aged and elderly cancer patients [10]. Ghrelin infusion (2 μg/kg twice a day) for 3 weeks increased food intake and body weight by 0.8 kg in elderly patients with congestive heart failure [9], and by 1 kg in elderly patients with chronic obstructive pulmonary disease [8]. Moreover, in patients with functional dyspepsia, ghrelin infusion (3 μg/kg twice a day) for 2 weeks increased hunger sensation and food intake by 29 % without significant weight gain [11]. Since 1 kg weight gain requires 7000-8000 Kcal, the increase in energy intake achieved for 14 days in this study was not enough to lead to any considerable weight gain. Although case 4 gained 2.4 kg and showed remarkable improvement in nutritional parameters and malnutrition-related liver dysfunction, we believe that water retention during the refeeding period contributed to this weight gain [32]. A decrease in body weight of 2 patients (cases 1 and 2) during ghrelin study might be attributable to a decrease in malnutrition-induced fluid

retention or improvement in bowel movements.

There were two reports about the effects of ghrelin on appetite in AN patients. In one study, 5 pmol/kg/min ghrelin infusion for 300 min had little effect on appetite in severely emaciated as well as weight-recovered AN patients [33]. However, appetite was evaluated by VAS alone because the severely emaciated AN patients refused to eat in the study. Since it is well known that recognition of hunger and satiety in AN patients is generally impaired, appetite cannot be always analyzed correctly by VAS alone. Although 1 μg/kg ghrelin bolus infusion made AN patients feel hunger sensation in another study, their food intakes were not evaluated [34]. We therefore believe that studies aiming to investigate ghrelin as an appetite-stimulating substance should recruit only AN patients who are fully motivated to gain weight by psycho-educational therapy.

Adverse effects such as abdominal discomfort, diarrhea, transient flushing, truncal perspiration, and somnolence have been reported after ghrelin injection [6]. Two patients in the present study occasionally reported a warm sensation in the trunk and mild sweating. Since case 5 experienced mild abdominal pain and several episodes of loose stools per day, we reduced the dose of ghrelin to 1.5 μg/kg, which improved these symptoms. No other serious physical or biochemical deteriorations occurred. Moreover, malnutrition-related liver dysfunction and endocrinologic abnormalities in case 4 were improved after ghrelin treatment. Interestingly, ghrelin infusion increased somnolence in the study [33], however, none of the present 5 subjects reported increased sleepiness. We did not observe increased fear concerning weight gain, abnormal behavior, or unstable mental status owing to an increase in appetite during ghrelin treatment, and psychological tests did not demonstrate any significant change in mental state. The present patients who motivated to gain body weight felt happy to be able to eat after ghrelin infusion, and they were pleased to be free from uncomfortable gastrointestinal symptoms after this ghrelin study. It is notable that all patients gained weight after discharge.

In conclusion, we found that ghrelin decreases gastrointestinal symptoms and increases hunger sensation and daily energy intake without serious adverse events in AN patients. A double-blinded, randomized, and placebo-controlled study is indispensable for developing ghrelin as an effective appetite-stimulating therapy for AN patients. The present study would contribute to investigations for therapeutic potential of ghrelin in AN patients.

Acknowledgments

We would like to thank Dr. Chiori Shibasaki for the analysis of nutritional intake, and SRL (Tachikawa, Tokyo, Japan) for technical assistance with the ELISA assay for ghrelin. This study was supported by a fund from the Ministry of Health, Labor and Welfare of Japan. There is no conflict of interest that would prejudice the impartiality of the research.

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International Immunology, Vol. 21, No. 4, pp. 467–476
doi:10.1093/intimm/dxp014

CXCL13 production by an established lymph node stromal cell line via lymphotoxin-beta receptor engagement involves the cooperation of multiple signaling pathways

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Keywords: chemokine, NF-κB, protein kinase C, secondary lymphoid organ

Abstract

Non-hematopoietic mesenchymal stromal cells in secondary lymphoid organs play pivotal roles in tissue organization and immune responses by exhibiting specialized features such as the production of lymphoid homeostatic chemokines. However, the maturational process of stromal cells mediated by lymphotoxin-beta receptor (LTβR) signaling, a key for stromal maturation, remains unclear. Taking advantage of a stromal cell line established from mouse lymph node, which can produce a homeostatic chemokine, CXC chemokine ligand (CXCL) 13, by the engagement of LTβR but not by tumor necrosis factor (TNF) receptor (TNFR), we analyzed the details of intracellular signaling events during the maturational process. The activation of both canonical and non-canonical nuclear factor-κB (NF-κB) pathways was essential for CXCL13 induction; however, an excessive amount of non-canonical RelB–p52 complex was still insufficient for CXCL13 gene expression. Under RelB–p52-over-expressed conditions, TNFα could induce a markedly high amount of CXCL13 production, indicating that the downstream of TNFR contains an additional key component of signaling. We also found that protein kinase C activity plays a critical role in this process in addition to the NF-κB pathways. Taken together, it is suggested that the maturation of lymphoid stromal cells mediated by LTβR is accomplished by the cooperation of multiple signaling cascades.

Introduction

Secondary lymphoid organs (SLOs) are important anatomical locations in which lymphocytes are accumulated for the induction of efficient adoptive immune responses (1). Several distinct types of non-hematopoietic mesenchymal cell lineages designated as stromal cells support the tissue architecture of the SLO (2–5). Recent findings have compelled researchers to consider that stromal cells play pivotal roles in immune responses by producing various factors, including cytokines, chemokines and adhesion molecules, which regulate the motility and homeostasis of immune cells (6–8). In addition, stromal cells seem indispensable for the organogenesis and tissue remodeling of SLOs (9). However, the molecular and cell biological nature of these cells remain poorly understood.

Many studies using knockout mice have indicated that signals directing the activation of transcription factor nuclear factor-κBs (NF-κBs) mediated by lymphotoxins (LTs) and tumor necrosis factor (TNF) α are essential for the organogenesis and construction of tissue architecture of SLOs (9–11). The NF-κB family consists of five members, RelA, RelB, cRel, NF-κB1 (p50) and NF-κB2 (p52), which function as heterodimers composed of combinations of these members (11). TNF receptor (TNFR) transmits a signal that leads to the phosphorylation and degradation of inhibitor of NF-κB (IκB), which in turn triggers the translocation of RelA–p50 complex from the cytoplasm to nucleus (canonical pathway) and initiates a broad spectrum of inflammatory gene expression (11, 12). On the other hand, lymphotoxin-beta receptor (LTβR), a specific receptor for LTα1β2, triggers the phosphorylation and processing of p100 (NF-κB2 gene product) followed by the generation of p52 via NF-κB-inducing kinase (NIK) and IκB-kinase α and finally activates RelB–p52

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Received 12 November 2008, *accepted* 27 January 2009

Transmitting editor: T. Watanabe

Advance Access publication 27 February 2009

complex (non-canonical pathway) in addition to the canonical pathway (11, 13). The non-canonical pathway is known to regulate a restricted set of genes, including CXC chemokine ligand (CXCL) 13, which is a crucial chemokine for the organogenesis of SLOs (13–15). It is widely believed that lymphoid tissue inducer cells or lymphocytes, which produce LT and/or TNFα, transmit a signal to stromal cells expressing LTβR and TNFR followed by triggering the NF-κB-dependent expression of responsible genes required for SLO formation (9); however, this scenario is still hypothetical because it is assembled from evidence based on the phenotypical analysis of various knockout mice and huge numbers of biochemical examinations. Virtually no comprehensive verification of the cascades has been demonstrated in the strictly defined context of stromal cells as a sequential event from receptor engagement to gene expression and the production of functional protein. It is also unclear whether target genes of the non-canonical pathway such as CXCL13 could be induced simply by p100 processing and subsequent generation of the RelB–p52 complex or by the consequence of a cooperative effect with additional signaling components.

We previously established stromal cell lines from the mouse lymph node and showed that they preserved several features of stromal cells in SLOs (16, 17). One of these cell lines, BLS12, had a unique property to secrete CXCL13 efficiently upon LTβR engagement and closely resembled a recently identified stromal subset common to SLOs or organizer stromal cells in the anlagen (18); therefore, this cell line provides an opportunity to analyze the detailed activity inside lymphoid stromal cells. In this study, we took advantage of BLS12 cells to examine the LTβR-induced intracellular cascade toward the expression of CXCL13 and showed that protein kinase C (PKC) activity, as well as the activation of NF-κB pathways, play a critical role in this process. Our findings suggest that the maturation of lymphoid stromal cells mediated by LTβR is accomplished by the cooperation of multiple signaling pathways.

Materials and methods

Cell culture

BLS12 cells were maintained in 10% FCS DMEM medium supplemented with antibiotics as described previously (16). BLS12 cells stably transfected with IκBαSR or NIK have also been described (16, 18). Cells were stimulated with 10 ng ml^{-1} murine TNFα (Peprotech, Rocky Hill, NJ, USA) and/or 0.5–0.75 μg ml^{-1} goat anti-mouse LTβR antibody (R&D Systems, Minneapolis, MN, USA) for several hours or days. For pharmacological inhibition of PKC activity, cells were incubated with medium containing various concentrations of bisindolylmaleimide-I (BIM-I), Gö6983, Gö6976 and Ro-32-0432 (Calbiochem/Merck, Darmstadt, Germany) for 1–24 h or myristoylated pseudosubstrates (PS) for PKCα/β (Calbiochem) or PKCζ/λ (Biosource/Invitrogen, Carlsbad, CA, USA) for 24 h.

Retrovirus-mediated gene transfer

BLS12 cells were transfected with genes using a pMX-puro or -hyg retrovirus vector system (16, 19). pMX-hyg vector was constructed by replacing the puromycin resistance gene with the hygromycin resistance gene cassette. pMX-puro-p100ΔGRR was provided by D.V. Novack (20). Complementary DNA (cDNA) encoding human p52 was excised from p100ΔGRR by *Eco*RI and *Xho*I digestion and sub-cloned into pCMV-3Tag vector (Stratagene, La Jolla, CA, USA) in frame with a C-terminal 3xMyc tag. Subsequently, p52-Myc cDNA fragment was ligated into pMX-puro. Mouse RelB cDNA provided by J. Stavnezer (21) was sub-cloned into pCMV-3Tag vector in frame with an N-terminal 3xFlag tag and Flag-RelB fragment was subsequently ligated into pMX-hyg. BLS12 cells infected with retroviruses were selected by 5–10 μg ml^{-1} puromycin or 130 μg ml^{-1} hygromycin.

ELISA

CXCL13 produced by BLS12 cells in culture supernatants was detected using a sandwich ELISA system (Duoset; R&D Systems) according to the manufacturer's recommendations.

Reverse transcription–PCR

Total RNA was extracted from BLS12 cells using TRIzol reagent (Invitrogen) and cDNA was synthesized using Superscript III reverse transcriptase and oligo(dT)12-18 primer (Invitrogen). Four- or 5-fold serial dilutions of cDNA were amplified by PCR with ExTaq DNA polymerase (Takara Bio, Otsu, Japan) and the following specific primer pairs: GAPDH, 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′; CXCL13, 5′-TTGAACTCCACCTCCAGGCA-3′ and 5′-CTTCAGGCAGCTCTTCTCTT-3′; CXCL10, 5′-AGACATCCCGAGCCAACCTT-3′ and 5′-GTTAAGGAGCCCTTTTAGAC-3′; CXCL12, 5′-AAACCAGTCAGCCTGAGCTAC-3′ and 5′-TTACTTGTTTAAAGCTTTCTC-3′; RelB, 5′-CGACAAGAAGTCCACCAACA-3′ and 5′-GGAAGCAGGGAAGAAATCAG-3′; NF-κB2, 5′-GCCTAGCCCAGAGATATGGA-3′ and 5′-GCAGGACACCCAGATTATTA-3′; NIK, 5′-ATGAAGGACAAGCAGACAGG-3′ and 5′-GCTTTCCCATCACCACTTCT-3′; LTβR, 5′-TACCAGATGTGAGATCCAGG-3′ and 5′-AGGATCCTTGGCCCTGTCAGA-3′.

Western blotting

Whole-cell lysates were prepared by lysing 1×10^6 cells in 200 μl radio immuno protein assay (RIPA) buffer; 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 1% Nonidet P-40, 50 mM NaF, 1% sodium deoxycholate, 0.5 mM Na_3VO_4, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin and 1 μg ml^{-1} pepstatin A. After insoluble materials were removed by centrifugation, lysates were boiled with the appropriate amount of SDS–PAGE loading buffer. Equal amounts of total cell lysates were separated by SDS–PAGE and transferred onto polyvinylidene fluoride membranes (Immobilin-P; Millipore, Billerica, MA, USA). The membranes were soaked in a blocking solution (1% skim milk and 0.05% Tween 20–PBS) for 1 h and then incubated with primary antibodies for 1 h. After washing with Tween 20–PBS, membranes were incubated with appropriate HRP-conjugated secondary antibodies (The Jackson Laboratory, Bar Harbor, ME, USA) for 1 h. Specific bands were visualized by an ECL-Plus (GE Healthcare, Little Chalfont, UK). NF-κB p52 (sc-298), RelB (sc-226), RelA (sc-109) and tubulin (sc-5286) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz,

CA, USA). Phospho-p44/42 MAP kinase (Thr202/Tyr204), p44/42 MAP kinase, IκBα and phospho-IκBα (Ser32) antibodies were from Cell Signaling Technology (Danvers, MA, USA). PKCα and PKCλ antibodies were from BD Biosciences (San Jose, CA, USA). Lamin-B1 antibody was from Zymed/Invitrogen.

Immunoprecipitation

Cells were lysed with RIPA buffer on a rotator for 1 h at 4°C. After insoluble materials were removed by centrifugation, the soluble supernatants were precleared with protein G Sepharose 4 Fast Flow (GE Healthcare). Samples were then immunoprecipitated with anti-Myc antibody (MBL, Nagoya, Japan) and 20 μl of protein G Sepharose beads. Protein G-bound protein complexes were washed intensively with RIPA buffer and boiled in loading buffer for SDS–PAGE. Immunoprecipitated proteins were detected by western blotting.

Results

LTβR-dependent CXCL13 production by BLS12 cells requires both canonical and non-canonical NF-κB pathways

Our previous study demonstrated that the treatment of BLS12 cells with an agonistic anti-LTβR antibody can trigger the production of CXCL13 in the culture supernatant (18) (Fig. 1A). The addition of TNFα markedly enhanced anti-LTβR antibody-dependent CXCL13 production, although TNFα alone had no effect. The production of CXCL13 protein well correlated with the mRNA expression in this system (Fig. 1B). In contrast, an inflammatory chemokine, CXCL10, a typical target gene of the canonical pathway, was induced by TNFα or anti-LTβR antibody, while CXCL12 was constitutively expressed in BLS12 cells.

We next examined the intracellular signaling components at several time points after BLS12 stimulation (Fig. 1C). As is well known, TNFα induced the rapid phosphorylation and degradation of IκBα (20 min after stimulation in our assay), and thereafter, the protein level recovered within an hour as a result of the up-regulation of IκBα gene expression (11), indicating transient activation of the canonical pathway after TNFR engagement. No remarkable enhancement of p100 processing coupled with the production of p52 was observed by TNFα. Instead, p100 and RelB were clearly augmented after 6 h of TNFα treatment. By contrast, LTβR engagement led to weak phosphorylation and slight reduction of IκBα at 20 min, while p100 processing and p52 generation, as well as a slight increase of RelB, were observed after 6 h. Strikingly, simultaneous stimulation with TNFα and anti-LTβR antibody resulted in the further accumulation of p100, p52 and RelB. Collectively, since the canonical pathway is known to regulate the expression of RelB and NF-κB2 (p100/p52) genes, strong activation of the canonical pathway by TNFα could enhance RelB and p100 expressions without the production of p52. In contrast, LTβR signaling induced intense activation of the non-canonical pathway but weak activation of the canonical pathway. Simultaneous stimulation of TNFR and LTβR exhibits a synergistic effect on the activation of both pathways.

To clarify the role of the canonical pathway, a phosphorylation-deficient mutant of IκBα (IκBαSR), which is known to block the

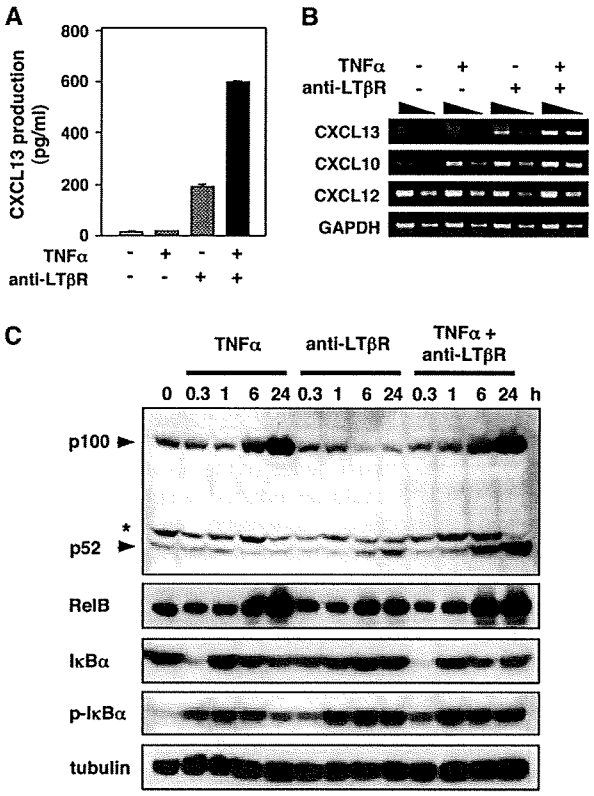

Fig. 1. LTβR transmits signal for CXCL13 production in BLS12 cells. (A and B) BLS12 cells express CXCL13 in response to LTβR engagement. BLS12 cells were stimulated with TNFα, anti-LTβR antibody or combinations of stimulants for 48 h. Secretion of CXCL13 in supernatants was measured by ELISA and shown as the mean ± SD (A). Transcripts for CXCL13, CXCL10 and CXCL12 were detected by reverse transcription–PCR analysis (B). The amounts of PCR products amplified from 5-fold serial dilutions of cDNAs were standardized relative to GAPDH. (C) Activation of NF-κB canonical and non-canonical pathways by LTβR engagement. Whole-cell lysates prepared from BLS12 cells with the indicated stimulations and time points were examined for p100/p52, RelB, IκBα, phospho-IκBα and tubulin by western blotting. Asterisk denotes a non-specific band.

nuclear translocation of RelA–p50 but not RelB–p52 complex, was introduced into BLS12 cells by retrovirus vector. In transfected cells, stimulation-induced expressions of CXCL13 and CXCL10 were completely suppressed (Fig. 2A and B). Importantly, the basal expressions of both RelB and NF-κB2 under steady-state conditions were significantly reduced, whereas the expressions of LTβR and NIK were unchanged (Fig. 2B). Basal protein levels of RelB and p100 were also markedly diminished in this transfectant (Fig. 2C), indicating that the canonical pathway is essential for basal expression as well as stimulation-dependent up-regulation of RelB and NF-κB2. Therefore, as some reports have already suggested (12, 13, 15), regulating the production of the non-canonical pathway components is one of the important roles of the canonical pathway. We next introduced p100ΔGRR, a processing-resistant mutant of p100, into BLS12 cells to block the non-canonical pathway. In this transfectant, CXCL13 production was strongly inhibited, while CXCL10 was significantly

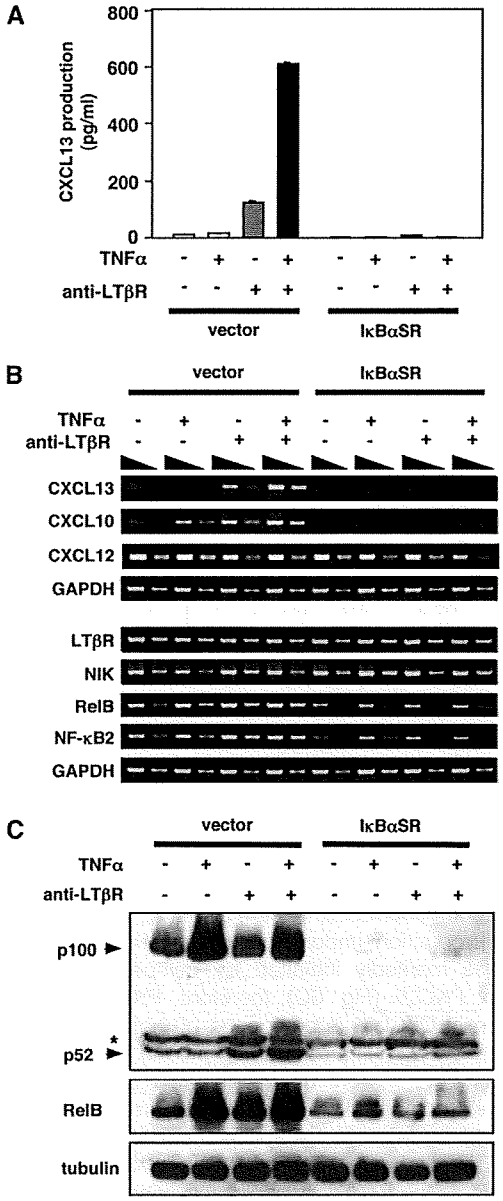

Fig. 2. LTβR-dependent CXCL13 production requires the activation of NF-κB canonical pathway. (A and B) IκBαSR inhibits LTβR-dependent CXCL13 production in BLS12 cells. BLS12 cells stably transfected with control vector or IκBαSR were stimulated for 48 h. CXCL13 production in supernatants was measured by ELISA (A), and transcripts for the indicated genes were detected by reverse transcription–PCR (B). (C) p100/p52, RelB and tubulin in cell lysates were examined by western blotting. Note that IκBαSR reduced RelB and p100/p52 proteins and the transcripts.

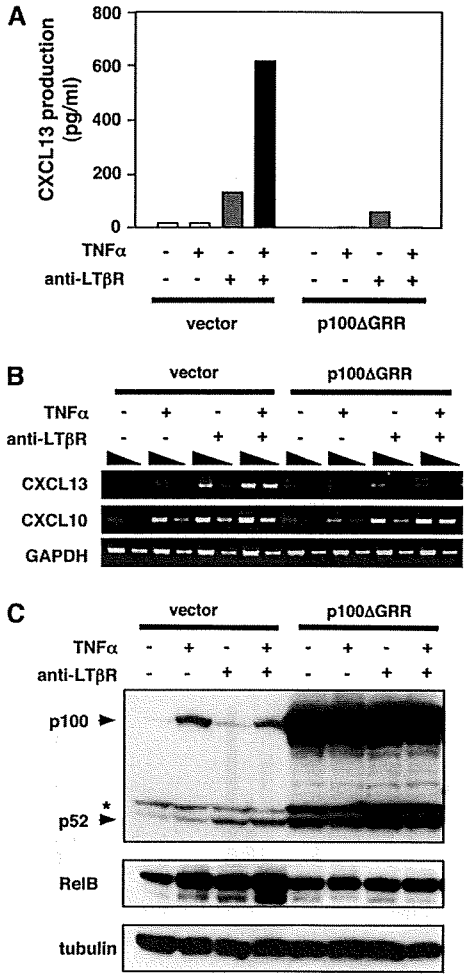

Fig. 3. LTβR-dependent CXCL13 production requires the activation of NF-κB non-canonical pathway. (A and B) p100ΔGRR inhibits LTβR-dependent CXCL13 production in BLS12 cells. BLS12 cells stably transfected with vector or p100ΔGRR were stimulated for 48 h. CXCL13 production in supernatants was measured by ELISA (A), and transcripts of the indicated genes were detected by reverse transcription–PCR (B). (C) p100/p52, RelB and tubulin in cell lysates were examined by western blotting.

induced especially in TNFα + anti-LTβR antibody treatment, demonstrating that CXCL13 but not CXCL10 expression is affected by p100ΔGRR (Fig. 3). The p100ΔGRR transfectant stimulated by TNFα or anti-LTβR antibody showed slightly lower expression of CXCL10 compared with the control cells. This might reflect potentially inhibitory effect of p100 to the canonical pathway under a certain condition (20, 22). Together, these results indicate that the activation of both canonical and non-canonical pathways play indispensable roles in CXCL13 induction.

Excessive RelB and p52 are still insufficient for CXCL13 induction and an additional signaling pathway seems to be required

In general, RelA, RelB and p50 proteins are present abundantly in the cytoplasm, whereas the amount of p52 protein is maintained in relatively low levels in a steady state and only raised by adequate stimuli that trigger the processing of the precursor protein p100 (13, 15, 23), which would be a rate-limiting step for the activation of the non-canonical pathway. To address whether p100 processing followed by the generation of p52 itself is sufficient for LTβR-dependent CXCL13 production, BLS12 cells were infected with

a retrovirus vector encoding p52, which is expected to bypass the p100 processing step; however, such a transfectant did not produce CXCL13 under unstimulated conditions (Fig. 4), suggesting that the generation and accumulation of p52 alone is insufficient for CXCL13 induction. It is worth noting that anti-LTβR antibody or TNFα + anti-LTβR antibody led to a markedly high level of CXCL13 secretion by this transfectant compared with control cells, indicating that there is a clear enhancing effect on CXCL13 gene expression by p52 over-expression. More interestingly, treatment of TNFα alone also induced a large amount CXCL13 production in p52-transfected BLS12 cells, similar to TNFα + anti-LTβR antibody treatment. Under these conditions, the levels of endogenous p100, p52 and RelB were comparable to the control (Fig. 4C). Over-expression of RelB alone did not show such a phenomenon, while cells transfected with both RelB and p52 displayed a pattern of CXCL13 production resembling to p52-transfected cells. Therefore, the induction of CXCL13 requires more than the accumulation of non-canonical RelB–p52 complex, suggesting that additional signaling pathways play a pivotal role in this process. Importantly, as the over-expression of both RelB and p52 without any stimulation was unable to induce CXCL13, the effect of TNFα on CXCL13 production at least in RelB–p52-transfected cells is unlikely due to the up-regulation of RelB and p100 by activation of the canonical pathway.

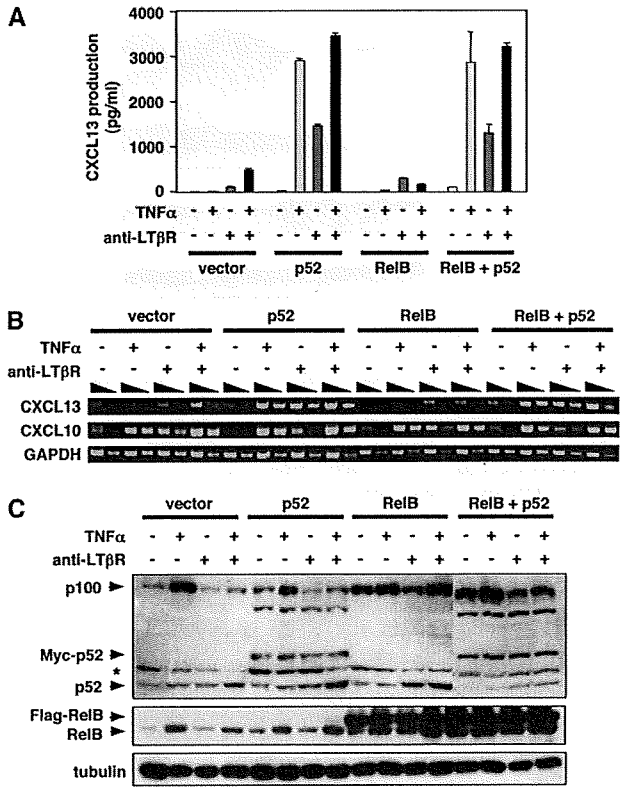

Fig. 4. Excess p52 and/or RelB are insufficient for CXCL13 production, while TNFα can induce CXCL13 under p52- or RelB–p52-over-expressing conditions. (A and B) BLS12 cells stably transfected with vectors, p52 and/or RelB were stimulated for 48 h. CXCL13 production in supernatants and transcripts of the indicated genes were examined by ELISA and reverse transcription–PCR, respectively. (C) p100/p52, RelB and tubulin in cell lysates were examined by western blotting.

LTβR-dependent CXCL13 expression involves PKC activity

To identify the signaling pathways participating in the LTβR-dependent induction of CXCL13, we treated BLS12 cells with various pharmacological inhibitors and found that PKC inhibitors, including BIM-I, Gö6983 (Fig. 5A and B), Gö6976 and Ro-32-0432 (data not shown), markedly inhibited the production of CXCL13. For further analysis, we selected Gö6983, which exhibited a clear dose-dependent inhibition of CXCL13 production and almost complete blockade was observed at 30 μM. Gö6983 showed no significant influence on the transcripts of RelB and NF-κB2, and the increase of RelB and p52 proteins due to stimulations was comparable to the control experiment (Fig. 5C and D), suggesting that RelA/p50 activity at least for the induction of RelB and NF-κB2 is unaffected by the inhibition of PKCs in BLS12 cells. However, it is worth noting that the treatment of anti-LTβR antibody or TNFα + anti-LTβR antibody in the presence of Gö6983 led to marked accumulation of p100 (described later).

We previously have demonstrated that the forced expression of NIK in BLS12 cells causes constitutive CXCL13 production (18). In NIK-over-expressing cells, PKC inhibitors suppressed CXCL13 production in a dose-dependent manner (Fig. 6A), suggesting that PKC functions downstream of NIK. Moreover, PKC inhibitors were able to suppress TNFα-dependent CXCL13 production by RelB–p52-over-expressing BLS12 cells (Fig. 6B), suggesting that PKC activity is also a signaling component downstream of TNFR. TNFα-dependent CXCL13 production under RelB–p52-over-expressing conditions was markedly inhibited by cell-permeable PS for PKCα/β or PKCζ/λ (Fig. 6C); therefore, PKC activity in the production of CXCL13 is composed of more than one subtype of PKC. We confirmed that the protein levels of PKCα/β as well as PKCζ/λ were unchanged in IκBαSR-expressing BLS12 cells compared with the control, irrespective of the presence or absence of TNFα stimulus (data not shown). In addition, TNFα-induced up-regulation of PKC activity, which is detected as the PKC-dependent activation of ERK MAP kinase (24, 25), was also unaffected in IκBαSR transfectant (data not shown), indicating that PKC pathways mediated by at least the above subtypes is fundamentally independent of the NF-κB canonical pathway.

PKC participates in CXCL13 expression other than p100 processing and nuclear translocation of RelB–p52 complex

Gö6983 treatment caused no significant alteration in the amounts of total, cytoplasmic or nuclear RelB and p52 after anti-LTβR antibody stimulation compared with the control experiment, except for the accumulation of p100 (Fig. 5D–F). In particular, in the presence of Gö6983, nuclear p100 was markedly increased at 24–48 h post-stimulation. Since some reports have shown that p100 can bind RelA–p50 as well as RelB–p52 complexes and suppress their transcription-inducing activity (20, 22), it raises the possibility that the

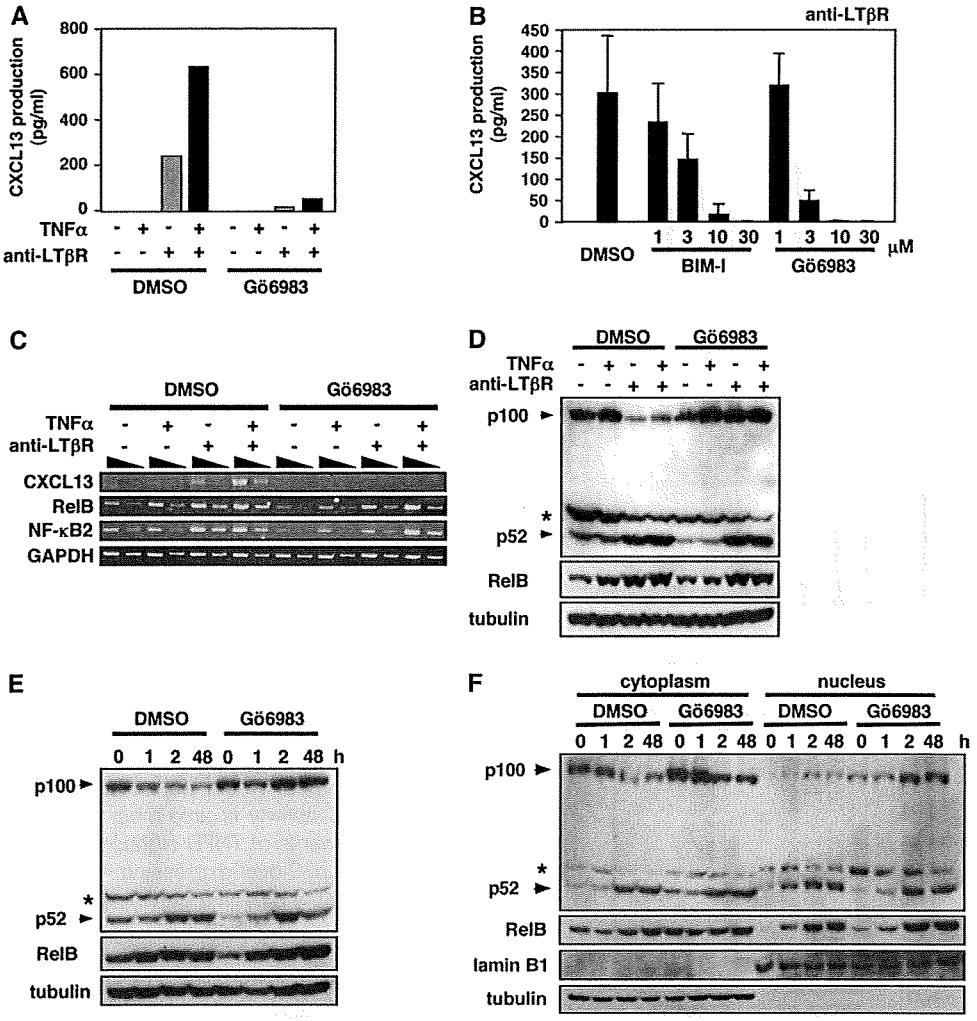

Fig. 5. LTβR-dependent CXCL13 production requires PKC activity. (A–C) PKC inhibitors suppress LTβR-dependent CXCL13 production. BLS12 cells were stimulated in the presence of dimethyl sulfoxide (DMSO) or Gö6983 (30 μM) for 48 h (A and C). Alternatively, cells were pre-treated with DMSO or various concentrations of BIM-I or Gö6983 for 1 h prior to stimulation for 48 h (B). CXCL13 production in supernatants and transcripts of the indicated genes were examined. (D) p100/p52, RelB and tubulin in cell lysates were examined by western blotting. (E and F) Gö6983 does not inhibit nuclear translocation of RelB and p52 but leads to the accumulation of p100. BLS12 cells were stimulated with anti-LTβR antibody in the presence of DMSO or Gö6983 (30 μM) for the indicated periods. Whole-cell extracts (E), cytoplasmic or nuclear extracts (F) were examined for p100/52, RelB, lamin-B1 and tubulin.

accumulation of nuclear p100 might inhibit CXCL13 expression via suppressing RelB–p52 activity. However, the following evidence excludes this possibility, at least in our experimental setting. First, although the protein level of p100 is markedly increased in BLS12 cells stimulated with TNFα and anti-LTβR antibody, strong CXCL13 production is readily observed. Second, over-expression of RelB and p52 in BLS12 cells clearly augmented p100 expression under steady-state conditions and TNFα further enhanced the accumulation of p100 (Figs 4C and 7). Even in such a situation, however, CXCL13 production induced by TNFα was extremely high and Gö6983 was able to inhibit it without further accumulation of p100 (Fig. 7A–C). In addition, the formation of RelB–p52 complex and the amount of nuclear p100 were constant, irrespective of Gö6983 treatment (Fig. 7D and E). Therefore, it is unlikely that the expression of CXCL13 is affected by nuclear p100 accumulation in this cell context. Taken together, PKC activity is required for CXCL13 expression other than p100 processing or the increase of RelB.

Discussion

When receiving external signals through TNFR or LTβR, which activates NF-κB, stromal cells in SLOs probably express specific features for constructing tissue structure and supporting immune reactions. Analysis of the details of the 'maturational process' in the context of strictly defined stromal cells has been limited because of the absence of a suitable experimental system. In this study, we showed that stromal cell line BLS12, which can proliferate efficiently *in vitro* and preserves

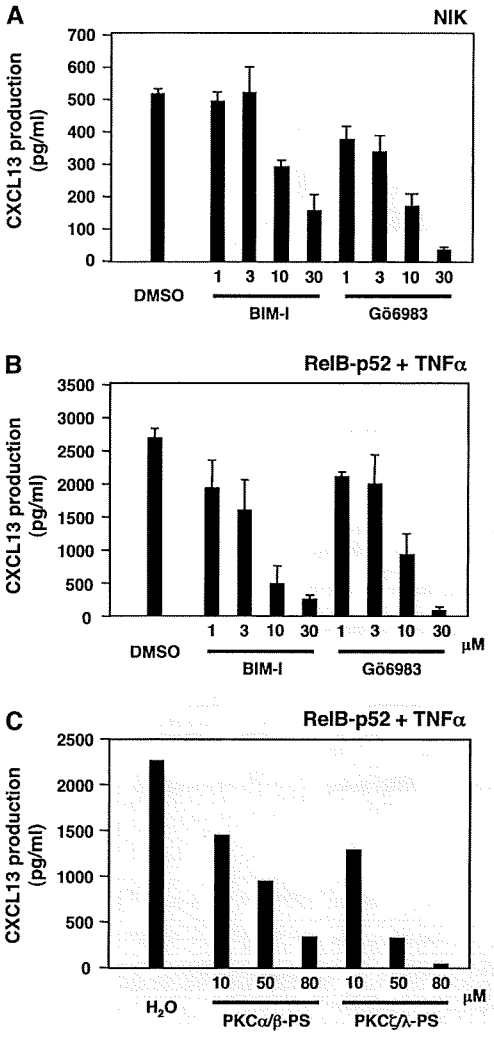

Fig. 6. PKC inhibitors suppress CXCL13 production in NIK- or RelB–p52-transfected BLS12 cells. (A) BLS12 cells stably transfected with NIK were treated with DMSO, BIM-I or Gö6983 for 48 h. CXCL13 production was examined by ELISA. (B) BLS12 cells stably transfected with vector or RelB–p52 were pre-treated with DMSO, BIM-I or Gö6983 for 1 h prior to TNFα stimulation for 24 h and CXCL13 production was examined. (C) Transfectant for RelB–p52 was stimulated with TNFα for 24 h in the presence of myristoylated PS for PKCα/β or ζ/λ, and CXCL13 production was examined.

several characteristics of SLO stromal cells, is very suitable for this purpose. Stimulation of BLS12 cells by LTβR engagement induced the activation of both canonical and non-canonical pathways and, as a consequence, CXCL13 secretion. This indicates that stimulated BLS12 cells exhibit the typical state of activated stromal cells in SLOs.

A number of studies have revealed that a broad spectrum of transmembrane receptors and intracellular mediators is able to activate the canonical RelA–p50 complex and induce the expression of diverse target genes (11, 12). In comparison, the activation of non-canonical RelB–p52 complex is triggered by a restricted set of receptors and only a limited number of target genes have been identified (13–15). Although the precise role of the non-canonical pathway in immune responses remains obscure, it is clear that this pathway is indispensable for the organogenesis of SLOs, especially for the expression of functional molecules in stromal cells (9–11).

By over-expressing dominant-negative mutants of IκBα or p100 in BLS12 cells, we confirmed that the induction of CXCL13 due to LTβR engagement requires both canonical and non-canonical pathways in this stromal cell context. In particular, the canonical pathway is crucial for steady-state expression as well as stimulation-induced up-regulation of RelB and NF-κB2; therefore, the non-canonical pathway necessarily cannot function without the canonical pathway. Several reports have supported this notion (12, 13, 15) and it would be a rational explanation of why RelA knockout mice (in combination with TNFR deficiency for rescue from perinatal lethality) lack all SLOs (26). The activation of RelB–p52 requires signal-dependent phosphorylation and processing of p100 to generate p52 (11, 13). To artificially bypass this step, we introduced p52 to BLS12 cells; however, over-expression of p52 or even of RelB–p52 could not induce CXCL13, indicating that the presence of excess non-canonical complex is insufficient to trigger this phenomenon. It is important that TNFα treatment led to marked CXCL13 production in p52- or RelB–p52-over-expressing BLS12 cells, suggesting that the downstream of TNFR contains an additional signaling component required for the expression of CXCL13 gene.

Searching for pharmacological inhibitors that can block LTβR-induced CXCL13 expression revealed that PKCs (at least two subtypes) would be an additional component for signaling. PKC activity is also most likely one of the downstream components of TNFR signaling that triggers CXCL13 expression in p52-over-expressing BLS12 cells. Since the activation of PKCs by TNFα has shown to be very rapid (27, 28), it seems unlikely that the initial activation of PKCs is regulated by RelA–p50 complex. We also confirmed that PKCα/β and PKCζ/λ proteins and a part of PKC activity induced by TNFα was unaffected in IκBαSR-transfected cells, suggesting that signaling mediated by PKCs is fundamentally distinct pathway from the NF-κB canonical pathway. On the other side, previous reports have suggested that PKCs are involved in NF-κB activation, especially on the canonical pathway, through different mechanisms (29–34). At least in BLS12, however, the inhibition of PKC activity after LTβR stimulation showed no gross alteration in the expression and nuclear localization of RelA, RelB and p52 compared with the control. It is worth noting that, in the presence of PKC inhibitor, remarkable accumulation of p100 after stimulation was observed. Although the reason for this phenomenon is still unclear, it raised the possibility that the increase of p100 might be a cause of CXCL13 suppression by PKC inhibitor. However, even though p100 accumulation is also observed in BLS12 treated with TNFα + anti-LTβR antibody or RelB–p52-over-expressing transfectant, a large amount of CXCL13 production is still readily induced by stimulation, indicating that the increased amount of endogenous p100, at least at a level owing to PKC inhibition, has no significant influence on CXCL13 expression. There are other possible mechanisms by which PKC could be involved in CXCL13 expression, e.g. by promoting the transcription-inducing activity of RelB–p52 complex via direct or indirect

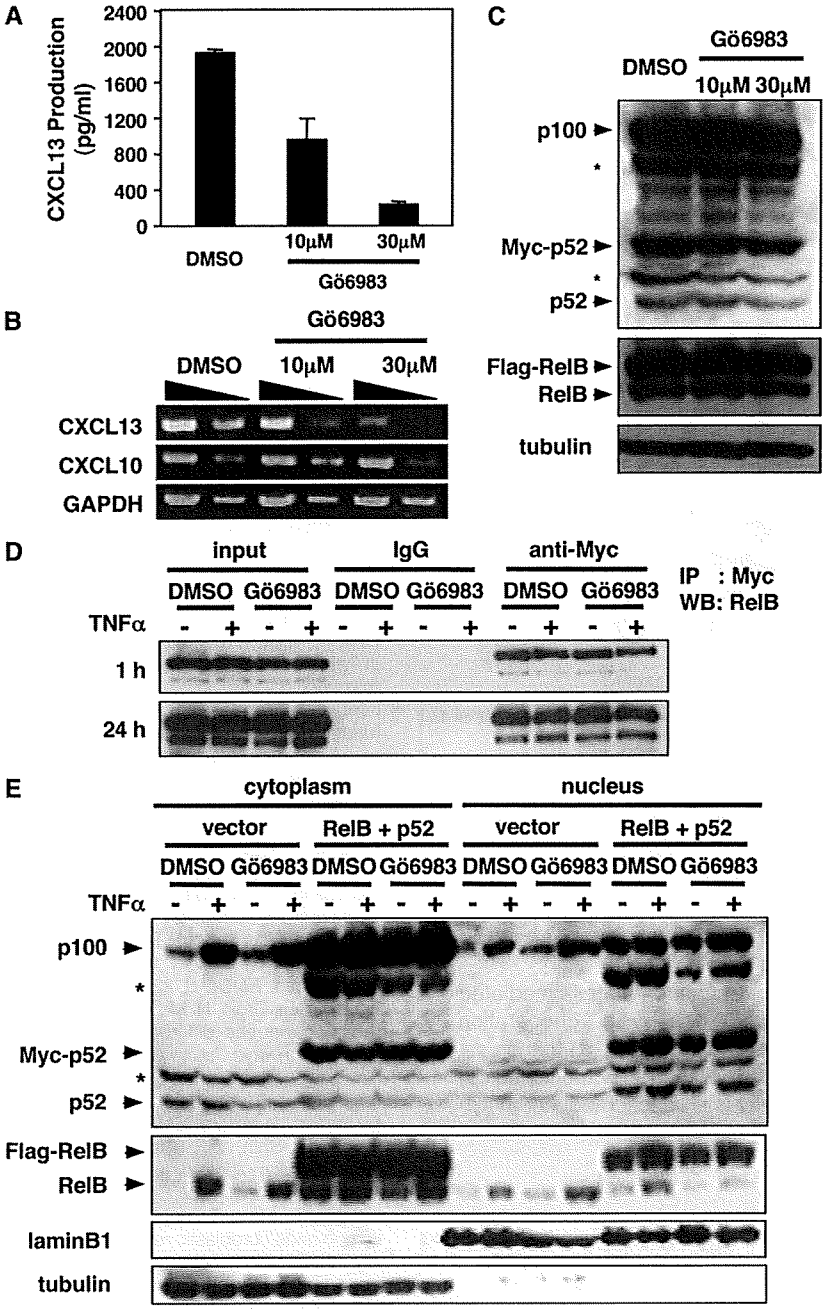

Fig. 7. Gö6983 inhibits TNFα-induced CXCL13 production by BLS12 cells over-expressing RelB and p52. (A and B) BLS12 cells stably transfected with RelB and p52 were stimulated with TNFα for 24 h in the presence of DMSO or Gö6983 (10 or 30 μM), and CXCL13 secretion (A) or transcripts (B) was examined. (C–E) Gö6983 does not influence the total amounts, complex formation and nuclear translocation of p52 and RelB. RelB–p52-transfected BLS12 cells were stimulated with TNFα in the presence of DMSO or Gö6983 (10 or 30 μM) for 24 h and examined for p100/p52, RelB and tubulin. (D) RelB–p52-over-expressing cells were stimulated with TNFα in the presence of DMSO or Gö6983 (30 μM) for 1 or 24 h. Cell lysates were immunoprecipitated with control mouse IgG or anti-Myc antibody and blotted with RelB antibody.

phosphorylation or the activation of other transcription factors or regulators. In addition, RelA–p50 complex might directly involve in CXCL13 gene transcription in cooperation with RelB–p52 complex. These are important issues to be addressed in the future. Besides PKC inhibitors, we also found pharmacological inhibitors that clearly inhibit LTβR-induced CXCL13 expression (H. Suto and T. Katakai, unpublished observation), suggesting that several different signaling components are involved in this process besides NF-κB pathways.