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Development of an *In Vitro* Test System Measuring Transcriptional Downregulatory Activities on IL-13

Choi, Jeong June[1], Bo-Kyung Park[1], Sunyoung Park[1], Chi-Young Yun[2], Dong Hee Kim[1,2], Jin Sook Kim[3], Eun Sook Hwang[4], and Mirim Jin[1,2]*

[1]*Laboratory of Pathology, College of Oriental Medicine, Daejeon University, Daejeon 300-716, Korea*
[2]*Traditional Medicine Bio-Research Center, Daejeon University, Daejeon 300-716, Korea*
[3]*Korea Institute of Oriental Medicine, Daejeon 305-764, Korea*
[4]*College of Pharmacy, Division of Life and Pharmaceutical Science, Center for Cell Signaling and Drug Discovery Research, Ewha Womans University, Seoul 120-750, Korea*

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Interleukin-13 (IL-13) has been proposed as a therapeutic target for bronchial asthma as it plays crucial roles in the pathogenesis of the disease. We developed an *in vitro* test system measuring transcriptional downregulatory activities on IL-13 as a primary screening method to select drug candidates from natural products. The promoter region of IL-13 (–2,048 to +1) was cloned into the upstream of a luciferase gene in the plasmid pGL4.14 containing the hygromycin resistance gene as a selection marker, generating pGL4.14-IL-13. The EL-4 thymoma and RBL-2H3 mast cells transiently expressing this plasmid highly produced the luciferase activities by responding to PI (PMA and ionomycin) stimulation up to 8-fold and 13-fold compared with the control, respectively, whereas cyclosporin A, a well-known antiasthmatic agent, significantly downregulated the activities. The BF1 clone of RBL-2H3 cells constitutively expressing pGL4.14-IL-13 was established by selecting surviving cells under a constant lethal dose of hygromycin treatment. The feasibility of this system was evaluated by measuring the downregulatory activities of 354 natural products on the IL-13 promoter using the BF1 clone. An extract from *Morus bombycis* (named TBRC 156) significantly inhibited PI-induced luciferase activities and IL-13 mRNA expression, but not the protein expression. Fisetin (named TBRC 353) inhibited not only PI-induced luciferase activities and mRNA expression, but also the IL-13 protein secretion, whereas myricetin (named TBRC 354) could not suppress the IL-13 expression at all. Our data indicated that this *in vitro* test system is able to discriminate the effects on IL-13 expression, and furthermore, that it might be suitable as a simple and time-saving primary screening system to select antiasthmatic agents by measuring transcriptional activities of the IL-13 promoter.

Keywords: IL-13, pGL4.14-IL-13 expressing stable cell lines, luciferase assay, natural therapeutic candidates for allergic disease

*Corresponding author
Phone: +82-42-280-2679; Fax: +82-42-280-2624;
E-mail: mirimj@dju.ac.kr

The expression of Th2-type cytokines such as IL-4, IL-5, and IL-13 is strongly associated with allergic disease. Specifically, IL-13, which is produced by Th2 cells as well as mast cells and basophils, has been suggested as a central mediator of bronchial asthma [25]. Acute administration of recombinant IL-13 was sufficient to induce asthmatic features, such as airway hyperresponsiveness, to a variety of antigenic stimuli, eosinophilic inflammation, and mucus overproduction in the murine asthma model [5, 26]. Moreover, the asthmatic phenotype caused by IL-13 *via* actions on epithelial and smooth muscle cells was more prominent than IL-4 [24, 29]. Furthermore, IL-13 was predominantly expressed, compared with IL-4, in the lesions of bronchial asthma [22] and was greatly upregulated by allergen challenge in bronchial tissue, bronchoalveolar lavage fluids, and sputum derived from asthma patients [9]. Therefore, IL-13 has been proposed as a molecular therapeutic target to develop new medicines for asthma treatment [10, 11, 19].

The expression of IL-13 is regulated at the transcriptional levels. It has been known that production of IL-13 is controlled by cooperation with several transcription factors including the NF-AT families, the GATA proteins, and AP-1. NF-ATc1 is the major transcription factor involved in regulating IL-13 transcription by cooperation with GATA proteins [14, 17, 21]. GATA-2 protein is able to induce excess AP-1 binding to the IL-13 promoter as well as increase IL-13 production in mast cells [16]. The human

and the mouse IL-13 gene promoters have evolutionary-conserved association of a consensus GATA-binding site and two GATA motifs [27]. In T cells, GATA-3, which requires activation of both STAT6 and NF-κB [4, 27], is known to facilitate the full transcriptional activity of the IL-13 gene promoter by the combination of these three motifs [15, 28]. GATA-3 binds a consensus GATA-binding site with histone acetyl transferase, CBP/p300, and RNA polymerase II, together leading to Th2 cell-specific histone acetylation followed by expression of the IL-13 gene [27].

Using the luciferase assay, we developed an *in vitro* test system that can easily detect the transcriptional downregulatory activities on the IL-13 promoter and we also investigated its feasibility as a drug screening system.

Materials and Methods

Materials and Cell Culture

pGL4.14 plasmid was purchased from Promega (Madison, WI, U.S.A.). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma (St. Louis, MO, U.S.A.). Cyclosporin A was obtained from Calbiochem (La Jolla, CA, U.S.A.). Lipofectamin 2000 was purchased from Invitrogen (Carlsbad, CA, U.S.A.) and luciferase substrate solution was purchased from Promega (Madison, WI, U.S.A.). EL-4 thymoma was purchased from the Korea Cell Bank (Seoul, Korea) and maintained in DMEM (Gibco-BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, U.S.A.) and 100 μg/ml of penicillin-streptomycin. The RBL-2H3 rat mast cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and cultured in MEM (Gibco-BRL, Grand Island, NY, U.S.A.) supplemented with 15% fetal bovine serum and 100 μg/ml penicillin-streptomycin and maintained at 37°C in a 5% CO_2 humidified air atmosphere.

TBRC Natural Product Library

The TBRC plant library was prepared as previously reported [3]. Briefly, the dried and powdered plant materials were extracted three times with 80% ethanol (EtOH) for 2 days at room temperature. The combined extracts were concentrated under reduced pressure. The residues (80% EtOH extracts) were diluted with distilled water and then partitioned three times successively with n-hexane, ethyl acetate, and butanol to afford the n-hexane, EtOAc, BuOH, and water-soluble fractions, respectively. Fisetin and myricetin were purchase from Sigma (St. Louis, MO, U.S.A.).

IL-13 Promoter Construction and Generation of Stable Cell Lines

The DNA fragment of the murine IL-13 promoter region extending from −2,048 to +1 was obtained by digesting the pIL-13-luc plasmid [13], generously gifted by Dr. E. S. Hwang (Ehwa Womans University, Seoul, Korea), with KpnI and XhoI, and cloned into pGL4.14 at the upstream of the luciferase gene, generating pGL4.14-IL-13.

Transient Transfection

EL-4 thymoma (1×10^6 cells/ml) or RBL-2H3 (3×10^5 cells/ml) cells were transfected with the plasmid in the presence of Lipofectamine 2000 according to the manufacturer's recommendation. Briefly, the mixture of DNA (0.4 μg) and Lipofectamine 2000 (1 μl) was added to each well of a 48-well cell culture plate. Following 12 h incubation, the cells were treated with test materials for 1 h and stimulated with 50 ng/ml of PMA and 0.5 μM of ionomycin for 16 h, and then cells were harvested to measure the luciferase activity.

Generation of Stable Cell Line

To generate stable cell lines constantly expressing pGL4.14-IL-13, RBL-2H3 (3×10^5 cells/ml) cells were transfected with pGL4.14-IL13 plasmid in the same method described above, and cultured in the presence of hygromycin at the concentration of 400 μg/ml over 4 weeks. The surviving cells were obtained as single cells by 0.1% trypsin treatment, and continuously subcultured from a 96-well to 6-well scale. Each clone was stored at −80°C until use.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT) Assay

RBL-2H3 cells were placed 7×10^5 cells per well in 96-well plates. Cells were grown in the presence of various extracts at the concentration of 10 or 100 μg/ml at 37°C in a 5% CO_2 incubator. After 48 h of incubation with various extracts, viable cells were stained with 100 μl of MTT (5 mg/ml) for 4 h at 37°C. The medium was removed, and then formazan crystals were dissolved by the addition of 200 μl of dimethyl sulfoxide. Absorbance was measured at 540 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Luciferase Assay

To measure the luciferase activities, stable cell lines or cells transfected with the plasmid were treated with PMA (50 ng/ml) and ionomycin (500 nM) for 16 h in the absence or presence of testing materials. Cells were washed with PBS three times and extracted with 100 μl of lysis reagent (Promega, Madison, WI, U.S.A.) at 4°C for 20 min, and centrifuged at 12,000 rpm, 4°C, for 1 min. The supernatants were mixed with 100 μl of luciferase substrate solution, and the emitted light was measured using an Lmax II luminometer (Molecular Devices, Sunnyvale, CA, U.S.A.). Normalized luciferase activities in each study were expressed as mean±SEM for three independent experiments, each performed in duplicate.

Real-Time PCR for IL-13

RBL-2H3 (3×10^5) cells were treated with ionomycin 500 nM and PMA (50, ng/ml), and incubated in the absence or presence of testing materials for 16 h. Total RNAs were isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) as per the manufacturer's recommendations. The RNA sample was used for cDNA synthesis by using M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.). Real-time PCR was conducted on a Rotor-Gene 6000 (Corbett Research, Australia) using the iQ SYBR green supermix (Bio-Rad, Hercules, CA, U.S.A.) with rat IL-13-specific primers under the following conditions; initial denaturation for 3 min at 95°C, denaturation for 10 sec at 95°C, annealing for 20 sec at 60°C, and extension for 20 sec at 72°C, by 40 cycles. β-Actin was used as an internal control to normalize the IL-13 mRNA levels. Rat IL-13 forward; 5'-CAGTTGCAATGCCATCCACA-3', rat IL-13 reverse; 5'-AGCCACATCCGAGGCCTTT-3', rat β-actin forward; 5'-AAGAGAATTTCTTTGGTCCC-3', rat β-actin reverse; 5'-TAACTTGAGCCAGGATGTCT-3'.

ELISA for IL-13

RBL-2H3 (3×10^5) cells were treated with ionomycin 500 nM and PMA (50, ng/ml), and incubated in the absence or presence of

testing materials for 16 h. The levels of IL-13 in the culture supernatant were determined using a commercially available ELISA set (BioSource, Camarillo, CA, U.S.A.) according to the manufacturer's instructions. The detection limit was 15.6 pg/ml.

RESULTS

Establishment of an *In Vitro* Test System Measuring Downregulatory Effects on IL-13

The induction of acute IL-13 expression is largely dependent on its upstream promoter region at the transcriptional level in activated Th2 cells and mast cells. In an effort to establish an *in vitro* test system measuring downregulatory effects on IL-13 promoter activities, we used a luciferase reporter plasmid, pGL4.14. The promoter region (from −2,048 to +1) of murine IL-13, which contains three GATA-binding sites, an AP-1-binding site, and an NF-AT-binding site (Fig. 1B), was cloned upstream of the luciferase gene in this plasmid, generating pGL4.14-IL-13. To examine whether this plasmid works properly or not, we transiently transfected murine EL-4 thymoma with this plasmid and treated it with PMA and ionomycin (PI), which are known as potent activators for IL-13. The treatment with PI highly increased the luciferase activities in a dose-dependent manner. The maximum activation was shown at the concentrations of 100 ng/ml PMA and 1 μM ionomycin by approximately 9-fold compared with that of PBS-treated control (Fig. 2A). Cyclosporin A, a well-known antiasthmatic drug, was tested to validate this system. Treatment with cyclosporin A significantly reduced the PI-induced luciferase activities in a dose-dependent manner without affecting cell viability (Fig. 2B). Furthermore, we tested whether this reporter plasmid is compatible in RBL-2H3 cells, which is a mast cell line capable of producing Th2 cytokines such as IL-13. In transiently transfected RBL-2H3 cells, PMA (25 ng/ml) and ionomycin (250 nM) considerably induced luciferase activities by maximally 13-fold, which is comparable to the results of EL-4 thymoma (Fig. 3A). The PI-induced luciferase activity was also inhibited by cyclosporin A in a dose-dependent manner as shown in Fig. 3B. As these results indicated that our *in vitro* test system responded appropriately to external stimuli, we generated RBL-2H3 stable cells that are continuously expressed in this reporter system. Cells transiently transfected with this reporter plasmid were cultured in the presence of hygromycin at the concentration of 400 μg/ml over 4 weeks to select viable clones. Each attached cell of RBL-2H3 cells was cultured from a 96-well to a 6-well scale, and the surviving clones were tested for the responsiveness of their luciferase activities to PI. Among the selected 6 clones only one clone (named BF1) responded greatly to PI with 9-fold

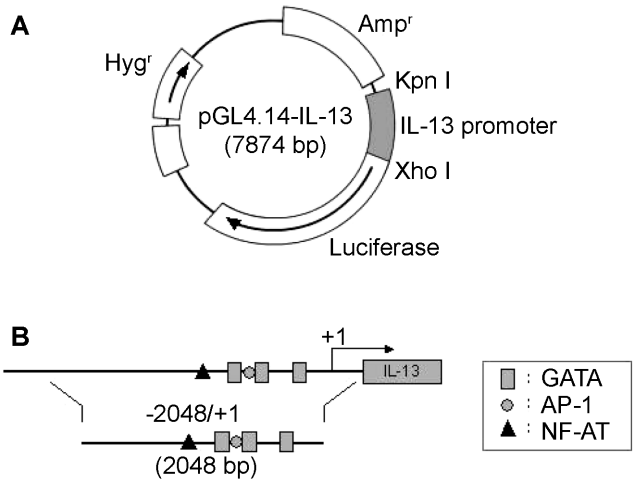

Fig. 1. A. Schematic diagram of pGL4.14-IL-13. The pGL4.14-IL-13 was generated by inserting the mouse IL-13 promoter into the pGL4.14 plasmid. **B**. Overview of the regulatory regions in the IL-13 promoter.

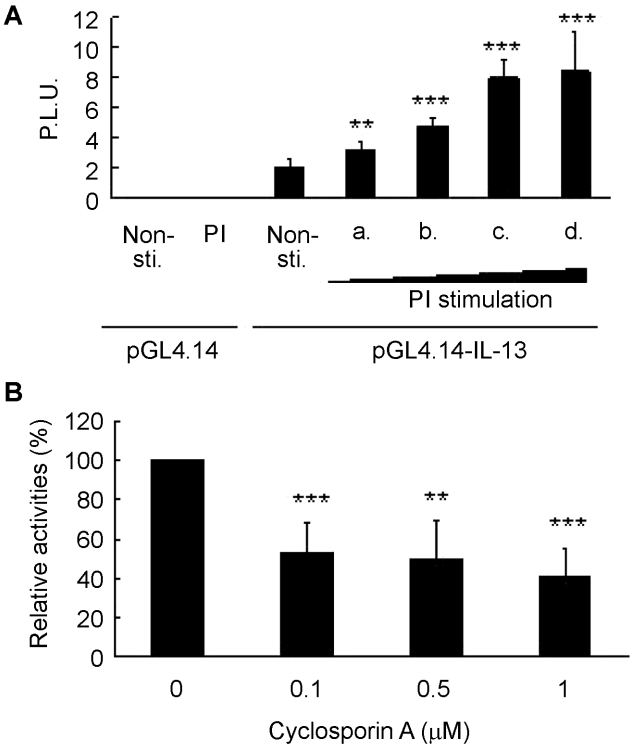

Fig. 2. Regulation of IL-13 promoter activities in EL-4 cells transiently transfected with pGL4.14-IL-13.

A. Cells were stimulated with varying concentrations of PMA and ionomycin or PBS for 16 h and the luciferase activities were measured by a luminometer. a, PMA (5 ng/ml) and ionomycin (50 nM); b, PMA (25 ng/ml) and ionomycin (250 nM); c, PMA (50 ng/ml) and ionomycin (500 nM); d, PMA (100 ng/ml) and ionomycin (1 μM). **B**. Cells were pretreated with cyclosporin A at the indicated concentrations or PBS for 1 h, and then stimulated with PMA (50 ng/ml) and ionomycin (500 nM) for 16 h. Relative activities were calculated by relative luciferase activities to those of the PBS-treated group. Data are presented as mean±SD of three experiments. $^{**}p<0.01$; $^{***}p<0.005$ *vs*. nonstimulated (**A**) or PBS-treated (**B**) group (Student's *t*-test). R.L.U., relative luciferase unit.

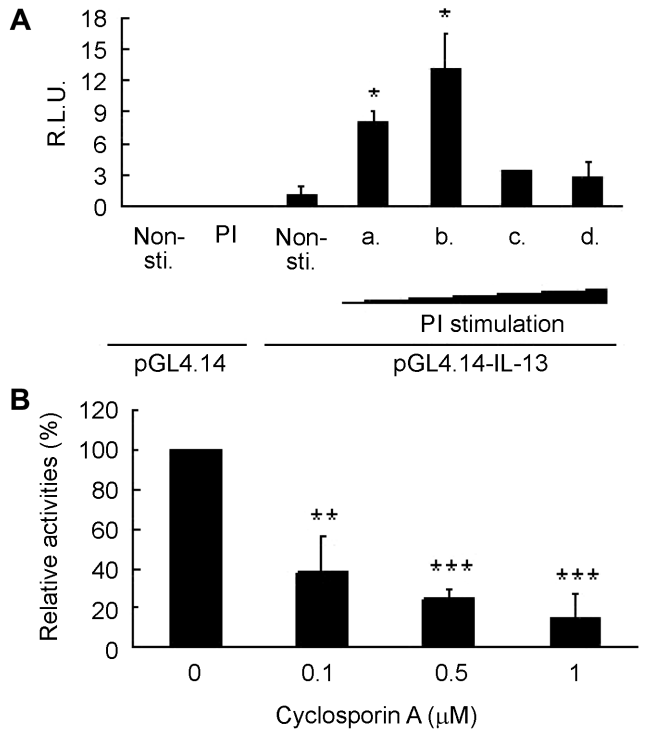

Fig. 3. Regulation of IL-13 promoter activities in RBL-2H3 cells transiently transfected with pGL4.14-IL-13.
A. Cells were stimulated with varying concentrations of PMA and ionomycin or PBS for 16 h and the luciferase activities were measured by a luminometer. a, PMA (5 ng/ml) and ionomycin (50 nM); b, PMA (25 ng/ml) and ionomycin (250 nM); c, PMA (50 ng/ml) and ionomycin (500 nM); d, PMA (100 ng/ml) and ionomycin (1 μM). **B**. Cells were pretreated with cyclosporin A at the indicated concentrations or PBS for 1 h, and then stimulated with PMA (25 ng/ml) and ionomycin (250 nM) for 16 h. Relative activities were calculated by relative luciferase activities to those of the PBS-treated group. Data are presented as mean±SD of three experiments. *p<0.05; **p<0.01; ***p<0.005 *vs.* nonstimulated (**A**) or PBS-treated (**B**) group (Student's *t*-test). R.L.U., relative luciferase unit.

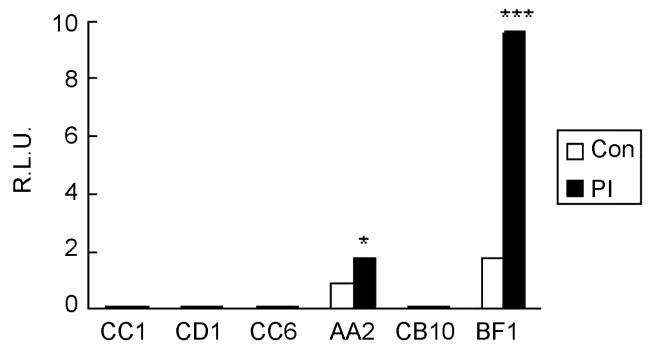

Fig. 4. Establishment of an RBL-2H3 stable cell line constantly expressing the luciferase reporter system.
The indicated six clones were obtained by selecting the survival cells under a lethal dose of hygromycin (400 ng/ml) for over 4 weeks after transient transfection with pGL-4.14-IL-13. The clones were stimulated with PMA (50 ng/ml) and ionomycin (500 nM) or PBS for 16 h, and the luciferase activities were measured by a luminometer. Data are presented as mean±SD of three experiments. *p<0.05; ***p<0.005 *vs.* PBS-treated group (Student's *t*-test). R.L.U., relative luciferase unit.

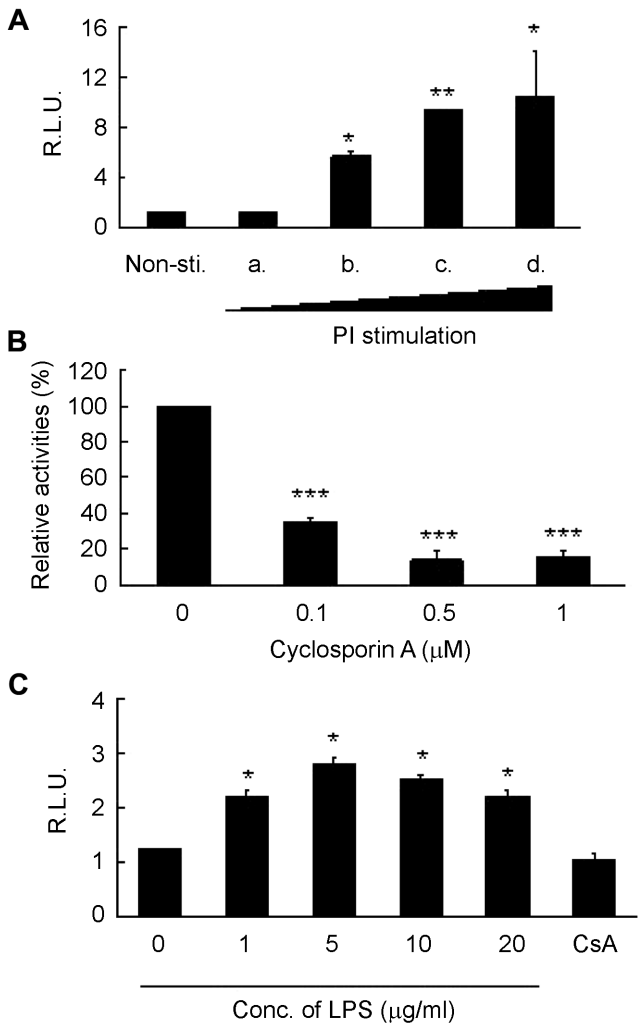

Fig. 5. Regulation of IL-13 promoter activity in BF1 clone.
A. Cells were stimulated with varying concentrations of PMA and ionomycin or PBS for 16 h, and the luciferase activities were measured by a luminometer. a, PMA (5 ng/ml) and ionomycin (50 nM); b, PMA (25 ng/ml) and ionomycin (250 nM); c, PMA (50 ng/ml) and ionomycin (500 nM); d, PMA (100 ng/ml) and ionomycin (1 μM). **B**. Cells were pretreated with indicated concentrations of cyclosporin A or PBS for 1 h, and then stimulated with PMA (50 ng/ml) and ionomycin (500 nM) for 16 h. Relative activities were calculated by relative luciferase activities to those of the PBS-treated group. **C**. Cells were stimulated with indicated concentrations of LPS or PBS for 16 h, and the luciferase activities were measured by a luminometer. Data are presented as mean±SD of three experiments. *p<0.05; **p<0.01; ***p<0.005 *vs.* nonstimulated (**A**) or PBS-treated (**B** and **C**) group (Student's *t*-test). R.L.U., relative luciferase unit.

induction of luciferase activities compared with the control (Fig. 4). When we treated the BF1 clone with PI of varying concentrations, the luciferase activities were significantly increased in a dose-dependent manner (Fig. 5A). There was maximum increase (approximately 11-fold compared with control) at the concentrations of 50 ng/ml PMA and 500 nM ionomycin. The luciferase activity was almost completely inhibited by cyclosporin A over the concentration of 0.5 μM (Fig. 5B). Additionally, we tested whether this

cell line responds to LPS, which is known as an IL-13 activator [23]. As shown in Fig. 5C, LPS at the concentration of 5 μg/ml induced the luciferase activity by approximately 3-fold compared with the control, which is much less than that shown in PI treatment, and the activity was also suppressed by cyclosporin A. These data suggested that BF1 stable cell lines expressing pGL4.14-IL-13 might be a suitable *in vitro* test system for measuring IL-13 promoter activities by means of luciferase activities.

Exploring Possible Use of the Established Test System

To assess the feasibility of this test system, we screened 354 natural products prepared from plants for their effects on the PI-induced luciferase activities. They were tested for cytotoxicity in RBL-2H3 cells, and then BF1 clones were cultured in 96-well plates with various samples at concentrations that did not affect cell viability. We first examined whether the treatments with natural products inhibit the PI-induced luciferase activities. Then, the selected products having suppressive effects on luciferase activities were further examined as to whether they inhibit the PI-induced IL-13 mRNA expression, using quantitative real-time PCR. Finally, the levels of IL-13 in the supernatants of PI-treated RBL-2H3 cell cultures were determined by ELISA to confirm whether the suppression on PI-induced luciferase activities results in the inhibition of protein expression. As summarized in Table 1, we were able to categorize our data into three groups. An extract (named TRBC 156) prepared from *M. bombycis*, a representative of group 1, significantly suppressed the PI-induced luciferase activities at the concentration of 1–3 μg/ml in a dose-dependent manner, and decreased the mRNA expression, similar to the results from the luciferase assay. However, TBRC 156 could not significantly inhibit the IL-13 protein production. Next, fisetin (named TBRC 353), a representative of group 2, greatly suppressed the luciferase activities and the mRNA expression up to approximately 65% and 99%, respectively, at the concentration of 30 μM, without affecting cell viability. Consistent with the results from the luciferase assay and real-time PCR, fisetin greatly inhibited the level of IL-13 protein in the cell culture supernatant. In contrast, group 3, which is represented by myricetin (TBRC 354), could suppress neither PI-induced luciferase activities and mRNA expression nor IL-13 protein expression. These results indicate that this system has the potential to be used as a primary *in vitro* screening system by discriminating the inhibitory effects by natural products on IL-13 expression.

DISCUSSION

Since pivotal roles of IL-13 in the pathogenesis of bronchial asthma have been established, the therapeutic agent blocking action of IL-13 or its signal pathways are currently being developed as antiasthmatic drugs. Indeed, several biological agents including soluble IL-13 receptors [18] and anti-IL-13 antibody [2] are under preclinical studies and/or clinical trials for bronchial asthma; however, their safety level and high cost for patients are still of concern. On the contrary, development of novel therapeutics from natural sources has been impeded by a lack of scientific evidence in preclinical studies as well as clinical trials despite their proven safety by a long history of use and relatively low expense. Because it is impractical to test the therapeutic effects of a large number of natural sources by consumptive methods such as *in vivo* animal experiments, an efficient primary screening system is definitely needed. In this study, we established an *in vitro* test system measuring the

Table 1. Exploring possible use of the established test system.

TBRC No.	Conc. (μM)	Cell viability (%)	Percent of inhibition		
			Luciferase assay	Real-time PCR	ELISA
156	1	>95	67±1.4**	−19±7.4	2.3±14.5
(*M. bombycis*)	3	>100	78±0.8**	4.5±2.1	34.9±19.7
	10	>90	85±0.1**	31.8±3.8*	−2.6±19.5
353	1	>100	11.2±5.1*	27.4±10.8	8.5±9.4
(Fisetin)	3	>100	1.5±28.5	42.5±8.9*	14.1±14.9
	10	>100	22.3±12.3*	70.1±7.3**	69.5±10.5**
	30	>95	63.5±20.5*	99.5±17.7**	86.1±14.5**
354	1	>100	8.6±3.4	9.4±5.5	3.9±18.4
(Myricetin)	3	>100	14.3±6.5	−24.1±16.9	14.3±8.3
	10	>100	−2.4±2.7	−0.2±0.9	25.1±6.1
	30	>100	−8.1±3.0	−32.4±23.5	12.6±12.9

Cells were pretreated with test materials for 1 h at the indicated concentrations and then stimulated with PMA (50 ng/ml) and ionomycin (500 nM) for 16 h. MTT assay, luciferase assay, real-time PCR, and ELISA were performed as described in Materials and Methods. Percent of inhibition was calculated as follows:

Percentage of inhibition=(1−activity of test material-treated group/activity of PI-treated group)×100.

Data are presented as mean±SD of three experiments. *p<0.05; **p<0.01; ***p<0.005 *vs*. PI-treated group (student's *t*-test).

downregulatory effects of natural products on IL-13 promoter activity, and this system provided a successful primary screening method capable of differentiating the effects on IL-13 expression.

Fisetin and myricetin are naturally occurring flavonols. Fisetin has been known to strongly suppress IL-4 and IL-13 syntheses in allergens and anti-IgE antibody-stimulated human basophils as well as RBL-2H3 cells [6, 8, 12], and the intake of natural products containing fisetin [1] is negatively associated with asthma prevalence and severity [20], whereas myricetin has been reported even at 30 μM to have no inhibitory effects on Th2 cytokines [7]. By the means of simple luciferase assays, we could reproduce the previous results distinguishing between fisetin and myricetin in regard to the downregulatory effects on the IL-13 promoter. The downregulatory effects were continuously demonstrated at the levels of mRNA expression and protein production using quantitative real-time PCR and ELISA, respectively. Among the TBRC plant library containing approximately 300 plants extracts, TBRC 156 was detected as a downregulator of PI-activated IL-13 promoter, and it also inhibited the mRNA expression although there was a gap between luciferase activities and real-time PCR (85.01 *vs* 31.5) in the inhibition percentage (Table 1). However, TBRC 156 showed no inhibitory effects on the production of IL-13 protein. We assume that TBRC 156 might directly inhibit the enzyme activities of luciferase, resulting in overestimating the inhibitory effects on the IL-13 promoter. Another possibility is that it might support the posttranslational and/or posttranslational process for IL-13 protein production and secretion. Therefore, additional testing for the selected candidates to detect IL-13 production (such as ELISA in this study) should be followed to confirm the results from the luciferase assays.

Our test system seems to have some strength as a primary screening method. The experimental procedure is simple. The time spent from the sample treatment to measuring luciferase activity takes less than 24 h, and with relatively low expense as we usually performed the experiment in a 96-well plate using inexpensive reagents. Additionally, the procedure for luciferase assay is quite simple. With two steps including lysing cells and adding substrate, we could screen hundreds of extracts within 30 min, which enables us to perform massive screening with minimum labor. Using our *in vitro* test system, we are expecting to screen a variety of plant libraries as well as chemical libraries to select drug candidates for allergic diseases such as asthma.

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