University of Tokushima

Pharmaceutical Sciences

Investigating the Suppressive Effect of Ampelopsis Glandulosa (Wild Grape) On Transcriptional Up-Regulation of Allergic Sensitive Genes

By

Rezwanul Islam

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Acknowledgement

Acknowledgement

The success and final outcome of this project were contributed through a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

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Sincerely,

Rezwanul Islam

Department of Molecular Pharmacology

Faculty of Pharmaceutical Sciences, Tokushima University Graduate School

Tokushima, Japan.

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Abstract

Abstract

Allergic rhinitis is an inflammatory disease caused by the activation of mucosal mast cells located in the epithelium of nasal cavity and is characterized by sneezing, itching, congestion, and rhinorrhea. The expression levels of allergic disease-sensitive genes are correlated with the severity of allergic symptoms in rhinitis patients. So, suppression of these gene expressions could be good therapeutics. PKCδ signaling-dependent H1R gene and NFAT signaling-dependent IL-9 gene are reported to be the sensitive genes for pollinosis. Here, we investigated the effect of wild grape on the signaling pathways for PKCδ-mediated H1R and NFAT-mediated IL-9 gene expressions. We found wild grape extract suppressed PMA-induced up regulation of H1R gene expression in HeLa cells. Treatment with wild grape extract also suppressed TDI-induced up-regulation of H1R gene expression in the nasal mucosa and alleviated nasal symptoms in TDI-sensitized allergic model rats. We have reported that NFAT signaling-dependent IL-9 gene is the additional sensitive gene for pollinosis and Awa-tea suppressed its gene expression in both *in vitro* and *in vivo* studies. In our present study we found combination treatment of wild grape extract with Awa-tea markedly alleviated nasal symptoms. Furthermore, these data suggested that combination of wild grape with Awa tea will have distinct clinical and therapeutic advantages over existing antihistamines. In addition, as wild grape also suppressed PMA-induced IL-33 gene up-regulation in Swiss3T3 cells and the expression level of IL-33 mRNA was correlated with the number of blood eosinophil in patients with pollinosis, wild grape could also be effective against eosinophilic inflammation.

Chapter 1

Effects of Wild grape on allergic rhinitis and its standardization

1.1. Introduction

Traditional medicine plays an important role in the health care sectors in many developing nations. The aim of the recent investigation was to scientifically record herbal medicines used by traditional healers to treat and manage allergic diseases. About 80% of developing countries relay on folk medicines for their primary health care supports [1]. Traditional medicine, particularly herbal medicines, is a key element in the public health sectors. Herbal medicine, often referred to as botanical medicine or herbalism, includes the use of plants or plants parts, to treat injuries or diseases [2]. Nowadays, folk medicine shows some of the key issues and major points in the history of medical sciences. Scientific research and clinical trials have assisted to organize the field of medicine and herbal medicine is showing a bright future for better therapies.

Proper utilization of herbal medicines and associated medicinal plants has been recorded by many researchers [3–5] although there are still many native cultures and colonies possess a huge volume of indigenous knowledge about folk medicines treating against various diseases, which are yet to be recorded scientifically. Documentation of those herbal medicines prevent the traditional culture heritage from being vanish for health care system of both present and future generations [6]. Through further pharmacological, biochemical and phytochemical scientific studies on folk medicine can lead to discover novel bioactive agents to treat various ailments. Instead of common interest natural products, specifically medicinal plants evaluate as an essential source of novel drugs, new drug leads,

and new chemical entities in molecular re-modeling, combinatorial and synthetic chemical approaches by several pharmaceutical industries. [7,8].

In addition, herbal medicine has been widely used all over the world to treat allergy and allergic complications. Allergic rhinitis (AR) is a symptomatic IgE-mediated inflammation of nasal membranes that is induced after allergen exposure. AR mainly characterized by rhinorrhea, sneezing, nasal itching, and nasal obstruction; which are often convoyed by postnasal drip, nasal mucosal swelling, cough, eye itching, and fatigue from nasal discomfort [9]. About 15% to 20% of world population is overblown by AR. In Westernmost nations ubiquity of AR tends to be higher than in other countries [10]. To treat AR, alleviation of nasal symptoms is highly focused.

Chinese herbal medicine (CHM) has a good fame to treat AR. CHM has been used against AR for centuries; the herbs resulting alleviation in AR symptom through their immune modulation and anti-allergic or anti-inflammatory effects [11]. Indeed, the activities of CHM have evaluated through several clinical studies, such as Yu-ping-feng San (YS), Cure-allergic-rhinitis syrup (CS), fermented red ginseng [12, 13]. That suggested that CHM therapy is useful for the treatment of nasal symptoms in AR patients [11].

A positive correlation between histamine H_1 receptor (H1R) mRNA expression and severity of allergic symptoms has been reported in the patient with pollinosis and toluene-2,4-diisocyanate (TDI)-sensitized allergy model rats [14, 15]. We previously reported that histamine stimulation increased H1R at both mRNA and

protein level via activation of the H1R in HeLa cells expressing H1R endogenously [16]. Stimulation with Phorbol 12-myristate 13-acetate (PMA) also up-regulated H1R gene expression in HeLa cells [16, 17]. The suppression of H1R gene up-regulation by several medicinal plants such as Kujin (the dried root of *Sophorae flavescensis*) [18], *Albizia lebeack* [19], *Teproshia perpurea* [20], has been reported.

In this present study, *Ampelopsis glandulosa*, commonly known as Wild Grape (WG) or Porcelain berry is one of the medicinal plants of interest to treat AR. WG is a species of plant native to Japan, China, India, Nepal, Myanmar, Vietnam, and the Philippines. It was reported that *A. brevipedunculata*, synonymous to *A. glandulosa* has anti-inflammatory, anti-hepatotoxic, and anti-osteoclastogenesis activity [21-24].

Another plant is *Morus alba*, known as white mulberry. Mulberry leaves have been used in traditional Chinese medicine for several decades and its use was first recorded in around 500 A.D. Mulberry leaves extract reported to possess several biological activities such as anti-diabetic, imunonutrition and anticancer activities, hepatoprotective activity, neuroprotective activity, anxiolytic and antidepressant activities [25].

We aimed to investigate the effects WG extract and Mulberry extract on AR through the suppression of H1R gene expression. The comparison of anti-allergic effects of these two extract has also been investigated and found that WG exhibited better suppressive activity of PMA-induced H1R gene up-regulation than that of

mulberry leaves extract in HeLa cells, which triggered to go with WG extract for further study.

1.2 Methods and Materials

1.2.1. Standardization

For standardization of WG extract, gallic acid (1 mg/ml in water) was used as a standardization marker. Freeze-dried WG extract (1 mg) was dissolved in 1 ml of water and applied on a HPLC system (Hitachi High-Tech Science, Tokyo) equipped with Cosmosil 5C18-MS-II column (4.6 ID x 250mm; Nacalai Tesque, Kyoto) at room temperature under constant flow rate of 1 ml/min using the solvent system of 20% acetonitrile in 0.05% trifluoroacetic acid. The HPLC chemical fingerprint was recorded by UV-VIS L-2420 detector (Hitachi High-Tech Science) at 254 nm. Standardization analyzes revealed that hot water extract of WG contains 12.2% of gallic acid (Fig 1.1).

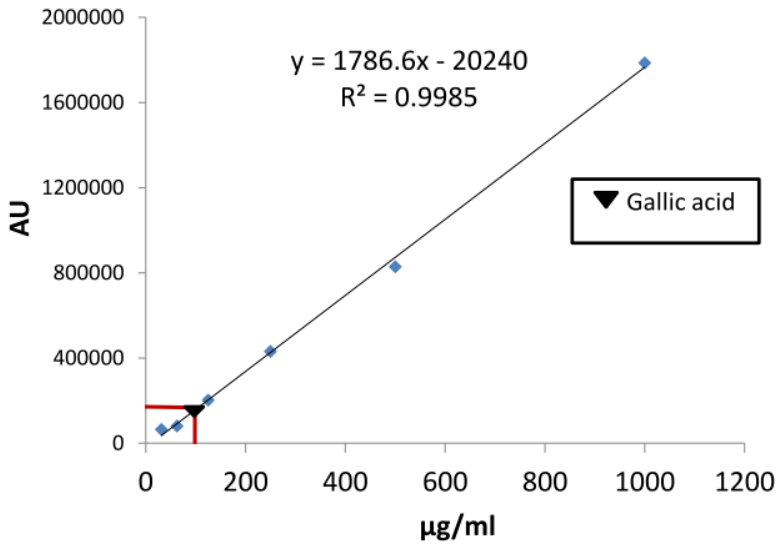

Fig. 1.1. Standardization of wild grape (WG) extract. According to methods and materials WG extract was prepared. One miligram of freeze-dried WG extract was dissolved in 1 ml of water. Gallic acid (1 mg/ml water) was used as a standardization marker. WG extract (1mg/ml) or gallic acid (500 µg/ml~15.625 µg/ml) was applied on a HPLC (Hitachi) equipped with Cosmosil 5C18 – MS-II 4.6 ID x 250mm at room temperature under constant flow rate 1ml/min and solvent system used 20% ACN in 0.05% TFA. The HPLC data recorded at UV-VIS L-2420 detector at 254 nm.

1.2.2. Cell culture

HeLa cells were cultured in MEM-α medium containing 8% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco) at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

1.2.3. Real-time quantitative RT-PCR

Nasal mucosa of rats was separated from the nasal septum, collected in RNAlater (Applied Biosystems, Foster City, CA, USA) 4 h after provocation with TDI, and stored at -80°C until used. Nasal mucosa was homogenized using a Polytron

(Model PT-K; Kinematica, AG, Littau/Luzern, Switzerland). Total RNA was isolated using RNAiso Plus reagent (Takara Bio Inc., Kyoto) according to the manufacturer's instructions. HeLa cells were serum-starved and the concentration of FBS in the culture medium for Swiss3T3 cells was reduced to 0.5% before subject to the mRNA determination. HeLa cells were cultured to 70% confluency in six-well dishes. The cells were treated with WG and mulberry for 24h before treatment with 100 nM PMA. After the stimulation with PMA for 3h, the cells were harvested and total RNA was prepared. The RNA samples (5 μg) were reverse transcribed to cDNA using PrimerScript RT Reagent Kit (Takara Bio Inc.). TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The nucleotide sequences of the primers and probes for H1R are listed in Table 2. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To standardize the starting material, endogenous control human GAPDH and rodent GAPDH control reagents (Applied Biosystems) were used, and data were expressed as a ratio of GAPDH mRNA.

Table-1.1. Nucleotide sequences of primers and probes were used in this study.

Primer/probe name	Sequence
Human H1R mRNA	
Sense primer	5'-CAGAGGATCAGATGTTAGGTGATAGC-3'

Anti-sense primer	5'-AGCGGAGCCTCTTCCAAGTAA-3'
Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA

1.2.4. Statistical Analysis

The results are shown as the mean ± S.E.M. Statistical analyses were performed with analysis of variance with Dunnett's test and the Spearman's rank correlation method using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Values of $p < 0.05$ were considered statistically significant.

1.3 Results

1.3.1 Effects of WG and Mulberry extract on PMA induced H1R gene up-regulation

Stimulation of HeLa cells with PMA induced significant and transient increase in H1RmRNA with a maximum 3 h after PMA or histamine stimulation [26]. We investigated the effect of WG and Mulberry extract on PMA-induced up-regulation of H1R mRNA expression in HeLa cells. Pretreatment with WG suppressed PMA- and histamine-induced up-regulation of H1R mRNA in a dose-dependent manner but not Mulberry extract (Fig. 1.2A and 1.2B).

(A) (B)

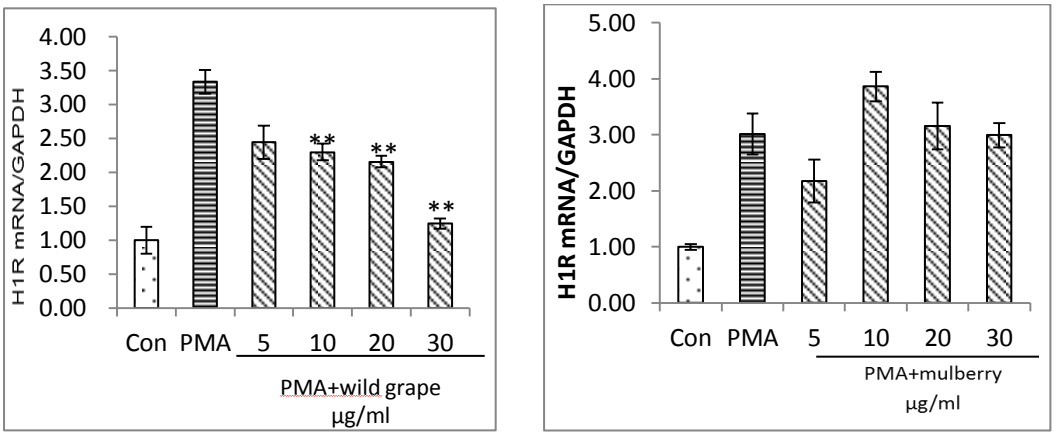

Fig. 1.2. Effect of WG and mulberry on PMA-induced H1R gene expression in HeLa cells.

HeLa cells were serum-starved for 24 h at 37 °C before treatment with 100 nM PMA for 3 h. After treated with WG (A) and mulberry (B) extract HeLa cells were incubated for 24 h before PMA. H1R mRNA was determined by quantitative RT-PCR. Data are expressed as means ± S.E.M. (n=3). $^{\#\#}p<0.01$ vs. control; *$p<0.05$, **$p<0.01$ vs. PMA

1.4. Discussion

Allergic disorders have gotten worldwide attention. The use of herbal medicines and innovative utilization of plants against AR have been passed down through generations. Histamine signaling has been identified as one of the major factors for allergic pathogenesis. Researchers have searched for synthetic as well as natural resources that have inhibitory effects on histamine signaling. In this study, we demonstrated that WG and mulberry extracts suppressed H1R gene expression.

In the past few years there has been increasing interest in the determination of suitable dietary sources of antioxidant phenolic compounds. Grapes are in greatest quantities around the world, whether processed or in their natural form, and they

also have one of the highest phenolic compound contents [26]. Gallic acid (3,4,5-trihydroxybenzoic acid) is one of the chemical components of WG. Gallic acid, a polyphenyl natural products, is known to have anti-oxidant, anti-inflammatory, anti-microbial, and radical scavenging activities. It has been reported that gallic acid modulates the inflammatory allergic reaction through the inhibition of histamine release and production of pro-inflammatory cytokine in mast cells [27].

Therefore, WG crude extract has been standardized by gallic acid standard using HPLC. Standardization analyzes revealed that hot water extract of WG contains 12.2% of gallic acid (Fig. 1.1). Thus, we hypothesized that gallic acid could be an active compound to suppress H1R gene up-regulation.

In this present study, 4 doses of both extracts (5, 10, 20, 30 µg/ml) were used to treat PMA induced HeLa cells. WG suppressed H1R gene up-regulation significantly and dose dependently. On the other hand, mulberry leaves extracts did not suppress H1R gene up regulation using the similar doses indicating that WG exhibited better suppressive activity of PMA induced H1R gene up-regulation than that of mulberry leaves extract in HeLa cells (Fig. 1.2). These data suggest WG samples could be a good medicine for allergic diseases. Besides that, WG derived active substances may contribute to develop new generation antihistamines that will have distinct clinical and therapeutic advantages over existing antihistamines.

Chapter 2

Wild grapes improved nasal symptoms in TDI rats

2.1. Introduction

Pollinosis is a seasonal allergic rhinitis caused by hypersensitivity to tree or grass pollens and affects approximately 40% of the Japanese population [28]. Histamine is one of the major chemical mediators of the allergic reaction, especially of the early-phase reaction and its action is mainly mediated through the activation of histamine H_1 receptor (H1R). Previously, we demonstrated that H1R gene expression is correlated with the severity of nasal acute symptoms such as sneezing and watery rhinorrhea in toluene-2,4-diisocyanate (TDI)-sensitized rats and patients with pollinosis [29,30]. We also showed that PKCδ signaling was involved in H1R gene expression and suppression of the H1R gene up-regulation alleviated these nasal symptoms in TDI-sensitized rats [31-34].

Th2 cytokines are also considered to be important for development of allergy. It was reported that interleukin (IL)-4, IL-5, and IL-13 are involved in the initiation and maintenance of allergic reaction [35-37]. We also reported that antihistamines suppressed Th2 cytokine gene up-regulations in TDI-sensitized rats and the expression level of H1R mRNA was correlated with those of IL-4 and IL-5 in patients with pollinosis [30,38]. In addition, accumulating evidence suggests the existence and important role of the histamine–cytokine network in allergic inflammation [39, 40].

Wild grape (*Ampelopsis glandulosa*, WG), common name porcelain berry, is a species of plant native to Japan, China, India, Nepal, Myanmar, Vietnam, and the Philippines. It was reported that *A. brevipedunculata*,

synonymous to *A. glandulosa* has anti-inflammatory, anti-hepatotoxic, and anti-osteoclastogenesis activity [21-24]. However, effect of WG on the PKCδ and NFAT signaling remains unknown.

In the present study, we investigated the effect of WG on PKCδ-mediated H1R gene expression responsible for the pathogenesis of acute symptoms of allergic rhinitis in TDI-sensitized rats. We also investigated the effect of WG on the gene expression of histamine signaling related Th2 cytokines, IL-4 and IL-5. This assay system may contribute to develop new generation antihistamines that will have distinct clinical and therapeutic advantages over existing antihistamines.

2.2. Materials and Methods

2.2.1. Animals

Six-week-old male Brown Norway rats weighing 180-220 g (Japan SLC, Hamamatsu) were used for the present study. Rats were allowed free access to water and food and kept in a room at 25 ± 2°C and 55 ± 10% humidity with a 12-h light/ dark cycle. The animals were divided into 7 groups comprising of the control, sensitized with TDI (Wako Pure Chemical, Osaka), and test groups, with 5 rats in each group. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of Tokushima University.

2.2.2. Sensitization and provocation with TDI and administration of wild grape

TDI sensitization in rats was performed using the protocol described at Kitamura [38]. In short, 10 µl 10% TDI solution in ethyl acetate (Wako Pure Chemical) was applied bilaterally to the nasal vestibule once a day for 5 consecutive days. This strategy was then repeated after a 2-day interval. Nine days after the second sensitization, 10 µl of 10% TDI solution was again applied to the nasal vestibule to provoke nasal allergic-like symptoms. Control rats were treated with ethyl acetate only according to the same schedule (Fig. 2.1). WG powder was supplied from Nab co., ltd. (Kamikatsu, Tokushima). WG powder (50 g) was boiled for 90 min, in 1liter of water, and then extracts were centrifuged and filtered. Finally, freeze-dried extracts were kept at -30°C until use. WG extract (50 mg/kg and 100 mg/kg) was dissolved in water at the concentration of 10 mg/ml on the day of the experiments and administered orally 1 h before the TDI sensitization for 3 weeks (Fig. 2.1). The number of sneezes and the extent of watery rhinorrhea considered as the indicator of nasal allergic-symptoms and were determined using the protocol of Abe et al [6]. After TDI provocation, the number of sneezes and watery rhinorrhea severity were examined for 10 min. Scaling from 0 to 3 was used as the basis to estimate the level of watery rhinorrhea described in the Table 2.1.

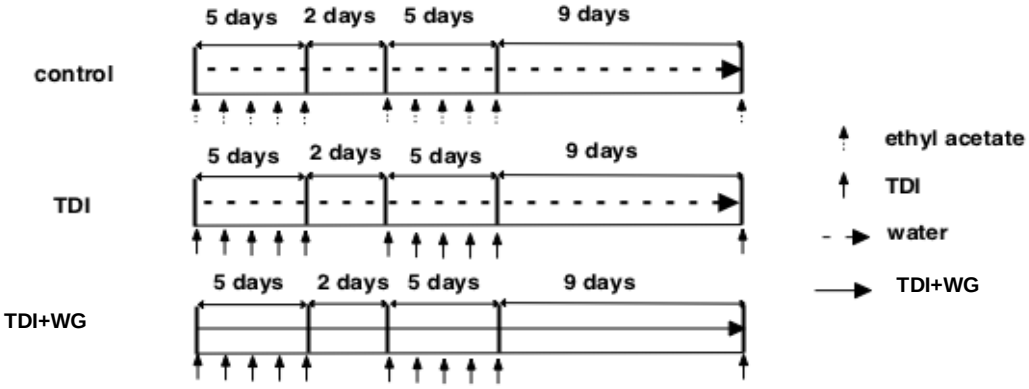

Fig 2.1. Rats were sensitized by intranasal application of 10 µl of 10% TDI in ethyl acetate for 2 weeks. After a 1-week interval, provocation was done with 10 µl of 10% TDI. The control group was sensitized with ethyl acetate only. The drug-treated group was orally treated with WG (25 and 50 mg/kg/day) and/or Awa-tea (40 mg/kg/day) once a day for 3 weeks. Drugs were administrated 1 h before rats were sensitized with TDI.

Table 2.1. Grading criteria of the severity of TDI-induced nasal symptoms in rats.

	Score			
Nasal response	0	1	2	3
Watery rhinorrhea	(-)	At the nostril	Between 1and 3	Drops of discharges from nose
Swelling and redness	(-)	Slightly swollen	Between 1and 3	Strong swelling with redness

2.2.3. Cell culture

HeLa cells were cultured in MEM-α medium containing 8% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco) at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

2.2.4. Real-time quantitative RT-PCR

Nasal mucosa of rats was separated from the nasal septum, collected in RNAlater (Applied Biosystems, Foster City, CA, USA) 4 h after provocation with TDI, and stored at -80°C until used. Nasal mucosa was homogenized using a Polytron (Model PT-K; Kinematica, AG, Littau/Luzern, Switzerland). Total RNA was isolated using RNAiso Plus reagent (Takara Bio Inc., Kyoto) according to the manufacturer's instructions. HeLa cells were serum-starved and the concentration of FBS in the culture medium for Swiss3T3 cells was reduced to 0.5% before subject to the mRNA determination. HeLa cells were cultured to 70% confluence in six-well dishes. The cells were treated with WG for 24h before treatment with 100 nM PMA. After the stimulation with PMA for 3h, the cells were harvested and total RNA was prepared. The RNA samples (5 μg) were reverse transcribed to cDNA using PrimerScript RT Reagent Kit (Takara Bio Inc.). TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The nucleotide sequences of the primers and probes for H1R are listed in Table 3. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To standardize the starting material, endogenous control

human GAPDH and rodent GAPDH control reagents (Applied Biosystems) were used, and data were expressed as a ratio of GAPDH mRNA.

Table 2.2. Nucleotide sequences of primers and probes used in this study.

Primer/probe name	Sequence
Human H1R mRNA	
Sense primer	5'-CAGAGGATCAGATGTTAGGTGATAGC-3'
Anti-sense primer	5'-AGCGGAGCCTCTTCCAAGTAA-3'
Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA
Rat H1R mRNA	
Sense primer	5'-TATGTGTCCGGGCTGCACT-3'
Anti-sense primer	5'-CGCCATGATAAAACCCAACTG-3'
Probe	FAM-CCGAGAGCGGAAGGCAGCCA-TAMRA
Rat IL-4 mRNA	
Sense primer	5'-CAGGGTGCTTCGCAAATTTTAC-3'
Anti-sense primer	5'-CACCGAGAACCCCAGACTTG-3'
Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA

2.2.5. Immunoblot Analysis

For the immunoblot analysis, 10 μg of each protein sample was separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was briefly rinsed in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and then incubated for 1 h at room temperature in TBS-T containing 5% skim milk (Difco) or 3% BSA (for detecting phosphoproteins; Sigma, St. Louis, MO, USA). The membrane was then incubated with a primary antibody [PKCδ(C-20) (sc-937, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phospho-PKCδ (Tyr^{311}) and β-actin (#2055S and #4697, Cell Signaling Technology Japan, Tokyo)] overnight at 4 °C. Proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Billerica, MA, USA).

2.2.6. Statistical Analysis

The results are shown as the mean ± S.E.M. Statistical analyses were performed with analysis of variance with Dunnett's test and the Spearman's rank correlation method using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Values of $p< 0.05$ were considered statistically significant.

2.3. Results

2.3.1. Effect of WG on PMA- and histamine-induced up-regulation of H1R mRNA in HeLa cells.

Stimulation of HeLa cells with PMA or histamine induced significant and transient increase in H1RmRNA with a maximum 3 h after PMA or histamine stimulation [39]. We investigated the effect of WG extract on PMA- or histamine-induced up-regulation of H1R mRNA expression in HeLa cells. Pretreatment with WG extract suppressed PMA- and histamine-induced up-regulation of H1R mRNA in a dose-dependent manner (Fig. 2.2A and 2.2B).

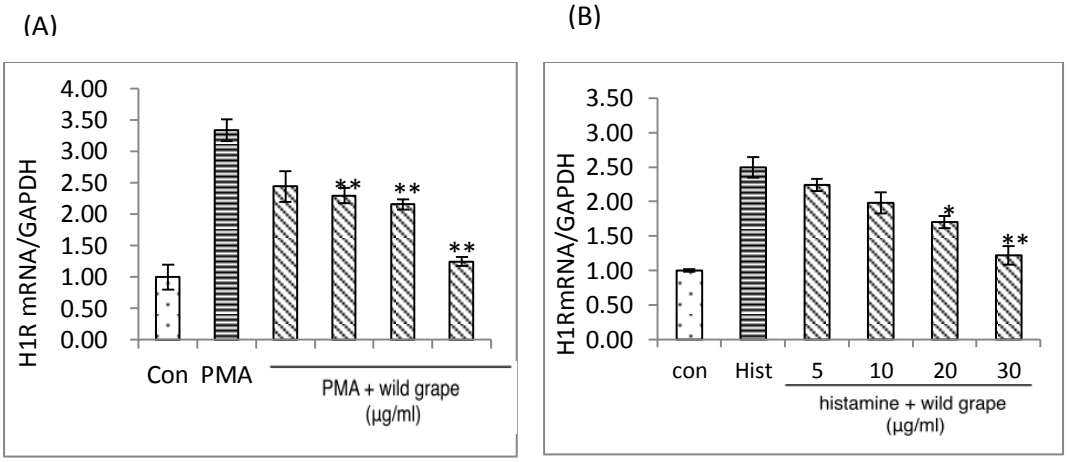

Fig. 2.2. Effect of WG extract on PMA- and histamine-induced up-regulation of H1R gene expression.

HeLa cells were serum-starved for 24 h at 37 °C before treatment with 100 nM PMA for 3 h (A) or 100 μM histamine for 3 h (B). WG extract were incubated for 24 h before PMA and histamine stimulation. H1R mRNA was determined by quantitative RT-PCR. Data are expressed as means ± S.E.M. (n=3). $^{\#\#}p<0.01$ vs. control; $^{*}p<0.05$, $^{**}p<0.01$ vs. PMA or histamine.

2.3.2. Effect of WG on TDI-induced nasal symptoms in TDI-sensitized rats.

Application of TDI caused nasal symptoms characterized by sneezing and watery rhinorrhea in TDI-sensitized rats. Pre-treatment with WG extratct (50 and 100 mg/kg/day) for 3 weeks significantly reduced TDI-induced sneezing and nasal core (Fig. 2.3A and 2.3B). Control rats showed no nasal symptoms after ethyl acetate provocation. In the previous study, we have demonstrated that suppression of PKCδ signaling alleviated TDI-induced nasal symptoms in TDI-sensitized rats [33]. Our in-vitro experiments data suggest that WG suppresses PKCδ signaling. Thus, we investigated the effect of WG extract on TDI-induced nasal symptoms in TDI-sensitized rats. Treatment with WG extract (50 mg/kg/day and 100 mg/kg/day) reduced TDI-induced sneezing and the nasal score (Fig. 2.3A and 2.3B).

(A) (B)

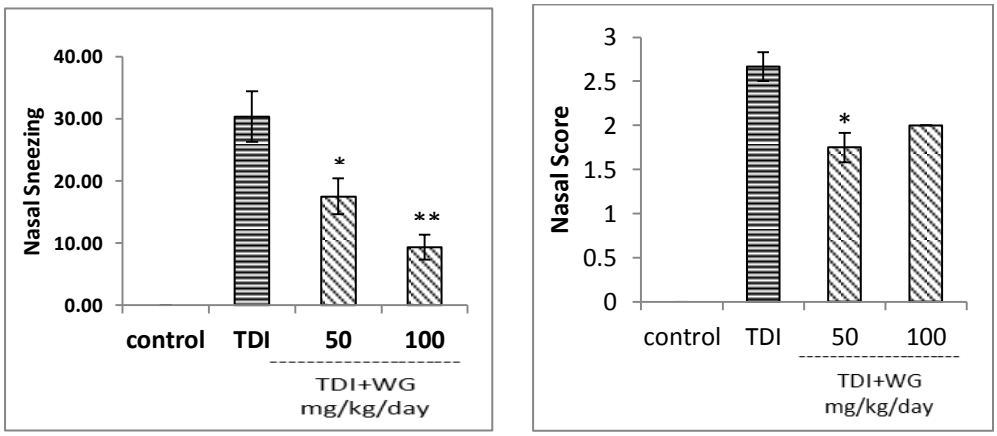

Fig. 2.3. Effect of WG extract on TDI-induced nasal symptoms in TDI-sensitized rats.

The rats were sensitized and provoked as described in the Materials and Methods. The numbers of sneezes (A) and the nasal score (B) were measured over the 10-min period immediately after TDI

provocation. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}$p<0.01 vs. control; *<p0.05, **p < 0.01 vs. TDI; $^{\$}$p < 0.05, $^{\$\$}$p < 0.01 vs. Awa-tea; ‡p < 0.05 vs. wild grape at the corresponding concentration.

2.3.3. Effect of wild grape on TDI-induced up-regulations of H1R in the nasal mucosa of TDI-sensitized rats.

Sensitization with TDI increased H1R mRNA expression in the nasal mucosa of TDI-sensitized rats [29, 40]. Our previous data found that H1R mRNA expression reached a maximum after 4 h of TDI provocation. Pretreatment with WG extract (50 and 100 mg/kg/day) reduced H1R gene expression in the nasal mucosa of the TDI sensitized rats (Fig. 2.4).

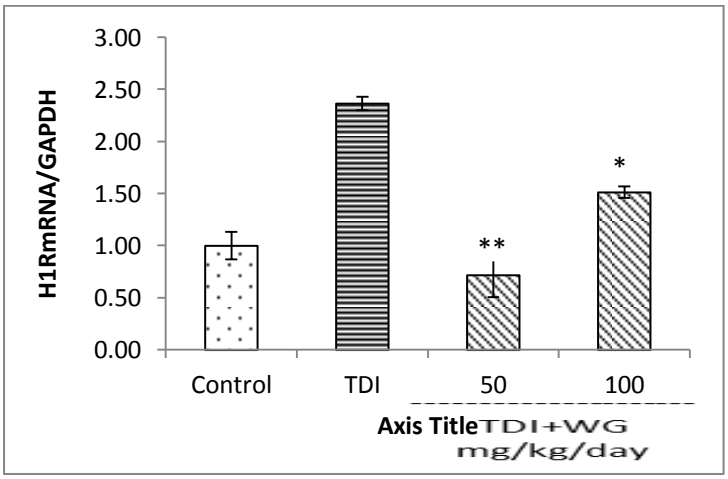

Fig. 2.4. Effect of WG extract on TDI-induced up-regulation of H1R gene expression in the nasal mucosa of TDI-sensitized rats.

The rats were sensitized and provoked as described in the Materials and Methods. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of H1R were determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n =

4). ##p<0.01 vs. control; *<p0.05, **p < 0.01 vs. TDI; ‡p < 0.05 vs. WG at the corresponding concentration.

2.3.4. Effect of wild grape on TDI-induced up-regulations of Th2 cytokine gene expressions in the nasal mucosa of TDI-sensitized rats.

Repeated sensitization with TDI also up-regulated gene expressions of Th2 cytokines such as IL-4 [29]. Treatment with WG (50 and 100 mg/kg/day) significantly suppressed TDI-induced up-regulation of IL-4 (Fig. 2.5).

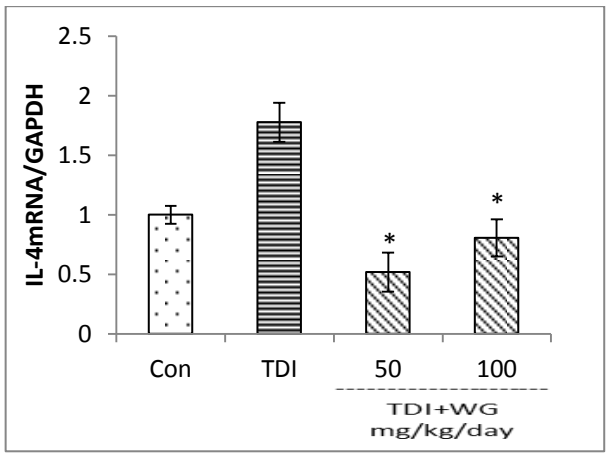

Fig. 2.5. Effect of wild grape on TDI-induced up-regulations of Th2 cytokine gene expressions in the nasal mucosa of TDI-sensitized rats.

The rats were sensitized and provoked as described in the Materials and Methods. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of IL-4 was determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). ##p<0.01 vs. control; *<p0.05, **p < 0.01 vs. TDI.

2.3.5. Effect of wild grape on PMA-induced phosphorylation of PKCδ at Tyr^{311} in HeLa cells.

Phosphorylation and dephosphorylation of PKCs regulate their activities, stabilities, and functions, and PKCδ is reportedly activated by phosphorylation at Tyr^{311} [41]. Previously, we showed that stimulation with histamine or PMA accelerate to increased phosphorylation of PKCδ at Tyr^{311} in HeLa cells [31]. We investigated the effect of WG on PMA-induced phosphorylation of PKCδ at Tyr^{311}. Pretreatment with WG extract suppressed PMA-induced phosphorylation of PKCδ at Tyr^{311} (Fig. 2.6).

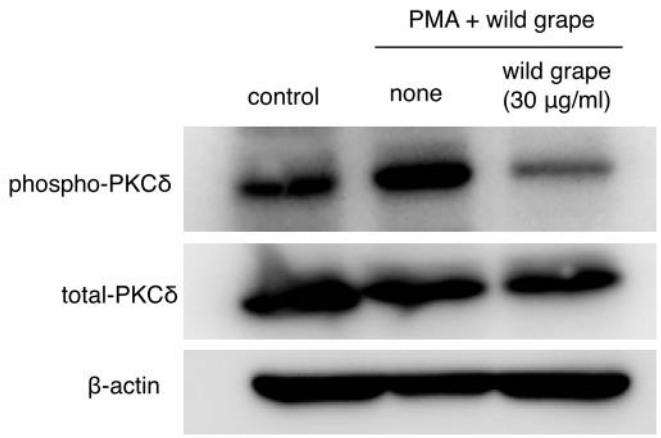

Fig. 2.6. Effect of wild grape on PMA-induced phosphorylation of PKCδ at Tyr^{311} in HeLa cells.

HeLa cells were serum-starved for 24 h and then treated with WG extract (30 μg/ml each) for 24 h before stimulation with 100 nM PMA for 10 min. Total cell lysates were prepared, and phosphorylation of PKCδ at Tyr^{311} was determined using immunoblot analysis.

2.4. Discussion

In the present study, we revealed that hot water extract of WG suppressed H1R in HeLa cells. In addition, treatment with WG extract alleviated TDI-induced acute nasal symptoms and suppressed TDI-induced H1R, IL-4 gene up-regulations in the nasal mucosa of TDI-sensitized rats (Fig. 2.4 and 2.5). However, WG extract (50 mg/kg/day) supressed both H1R and IL-4 gene expressions in TDI model rats but WG extract (100 mg/kg/day) showed a little up regulation. The small up-regulation by WG (100 mg/kg/day) may be occurred due to use of higher concentration of WG. However, histamine plays an important role for the pathogenesis of acute allergic symptoms [28]. WG suppressed histamine signalling through the inhibition of histamine-induced up-regulation of H1R gene expression. We reported that PKCδ signalling was involved in expression of H1R gene. As phosphorylation of Tyr^{311} was vital for the activation of PKCδ, western blot analysis suggested that inhibition of phosphorylation of PKCδ is the underlying mechanism of suppressive effect of WG on the up-regulation of H1R gene expression. (Fig. 2.6)

WG extract also suppressed TDI-induced up-regulation of IL-4 gene (Fig. 2.5), which expression levels were shown to be correlated with that of H1R gene [29]. The histamine-cytokine network plays important role in allergic inflammation, in which histamine influences the expression and action of several cytokines and some cytokines modulate the production and release of histamine [42-44]. Pretreatment with IL-4 triggers the release of histamine in response to FceRI [35, 45]. On the other hand, histamine affects the production of IL-4 [46].

We also showed that suppression of PKCδ signalling alleviated the acute nasal symptoms in TDI-sensitized rats [33]. Based on our investigation, treatment of WG improved TDI-induced nasal symptoms in TDI-sensitized rats, suggesting the effectiveness of these plants on the acute symptoms.

Chapter 3

Nasal allergic symptoms markedly alleviated by combined treatment Wild grape and Awa tea

3.1. Introduction

Nasal hypersensitivity is a representative incurable disease, so the development of therapeutics for high alleviation is needed. The symptoms are mostly sneezing, a runny nose and watery eyes. Histamine performed a crucial contribution in allergic inflammation. The biological effects of histamine are exhibited through G protein-coupled receptors, referred to as histamine H_1 receptor (H1R), histamine H_2 receptor (H2R), histamine H_3 receptor (H3R), and histamine H_4 receptor (H4R) [47].

Our results indicate that the PKCδ/ERK/PARP-1 signaling pathway is involved in histamine or PMA-induced up-regulation of HIR gene expression in HeLa cells [31].

A positive correlation between H1R mRNA expression and severity of allergic symptoms has been reported in the patient of pollinosis and toluene-2,4-diisocyanate (TDI)-sensitized allergic model rats [29, 30]. Even though, the compounds that suppress the up-regulation of H1R gene expression alleviate allergy symptoms [32,33].

We previously reported that histamine stimulation increased H1R at both mRNA and protein level via activation of the H1R in HeLa cells expressing H1R endogenously [39]. Stimulation with Phorbol 12-myristate 13-acetate (PMA) also up-regulated H1R gene expression in HeLa cells [31, 39]. Histamine and PMA-induced up-regulation of H1R gene expression was suppressed by rottlerin, a PKCδ selective inhibitor, indicating that the up-regulation of H1R gene expression

is PKCδ dependent [31]. Further studies showed that both histamine and PMA-induced up-regulation of H1R gene expression involved common downstream signaling mediators of PKCδ.

Th2 cytokines are suggested to play important roles in the pathogenesis of allergic inflammation [48]. Previously reported that the expression of IL-4 and IL-5 mRNAs was upregulated in nasal mucosa of TDI-sensitized rats suggest the involvement of these cytokines in nasal hyperresponsiveness [43, 49]. Increasing experimental evidences suggest the existence and important role of the histamine–cytokine network in allergic inflammation [42, 43]. Although H1R mRNA up-regulation was completely suppressed by the antihistamines and anti-allergic compounds from natural sources but 40 % of symptoms remain unchanged. IL-9 elevated through NFAT signaling was thought to be second major allergic signaling pathway. We discovered that gene expression of H1R and IL-9 participates 90% of acute symptoms.

Histamine and cytokines are important pro-inflammatory mediators in nasal allergic inflammation. The blockage or inhibition of these two mediators may provide additional benefits compared to a single mediator inhibition [50]. Combination therapy seems to be a more effective strategy than monotherapy in the treatment of allergic rhinitis in patients with moderate to severe symptoms [51].

Natural products are believed to be less toxic compare to the synthetic chemical compounds. Awa-tea, a domestic fermented tea in Tokushima, Japan, was

attempted to elucidate, and revealed to possess suppressive effect of IL-9 gene expression through NFAT signaling.

Ampelopsis glandulosa, commonly known as Wild Grape (WG) or Porcelain berry is one of the medicinal plants of interest to treat allergic rhinitis. WG is a species of plant native to Japan, China, India, Nepal, Myanmar, Vietnam, and the Philippines. It was reported that *A. brevipedunculata*, synonymous to *A. glandulosa* has anti-inflammatory, anti-hepatotoxic, and anti-osteoclastogenesis activity [21-24]. In the previous chapters, we have demonstrated the inhibitory effect of WG extract samples on H1R mRNA gene expression in HeLa cells and alleviated TDI-sensitized nasal symptoms in the allergic model rats.

We aimed to make a combination therapy with WG extract and Awa tea in TDI-sensitized rats. Based on results above combination therapy using different drugs having suppressive effect on PKC and NFAT signaling is expected for high alleviation.

3.2. Methods and Materials-

3.2.1. Animals

Six-week-old male Brown Norway rats weighing 180-220 gm (Japan SLC, Hamamatsu) were used for the present study. Rats were allowed free access to water and food and kept in a room maintained at 25 ± 2°C and 55 ± 10% humidity with a 12-h light/ dark cycle. The animals were divided into 7 groups comprising

of the control, sensitized with TDI (Wako Pure Chemical, Osaka), and test groups, with 5 rats in each group. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of Tokushima University.

3.2.2 TDI sensitization and provocation and administration of Wild grape vine and Awa Tea

Rats were sensitized with TDI by the method described by [52]. Briefly, 10 µl of a 10% solution of TDI in ethyl acetate (Wako Pure Chemical) was applied bilaterally to the nasal vestibule once a day for 5 consecutive days. This sensitization procedure was then repeated after a 2-day interval. Nine days after the second sensitization, 10 µl of 10% TDI solution was again applied to the nasal vestibule to provoke nasal allergic-like symptoms. Control rats were treated with ethyl acetate only according to the same schedule (**Fig. 3.1**). WG (25 mg/kg and 50 mg/kg) and Awa tea (40 mg/kg) were dissolved in water as concentration 10 mg/ml on the day of the experiments and administered orally 1 h before the sensitization for 14 days starting 7 days after the first sensitization (**Fig. 3.1**). Repeated pretreatment with WG, Awa tea and combination of WG and Awa tea for 3 weeks suppressed TDI-induced nasal symptoms and up-regulation of allergic sensitive gene mRNAs.

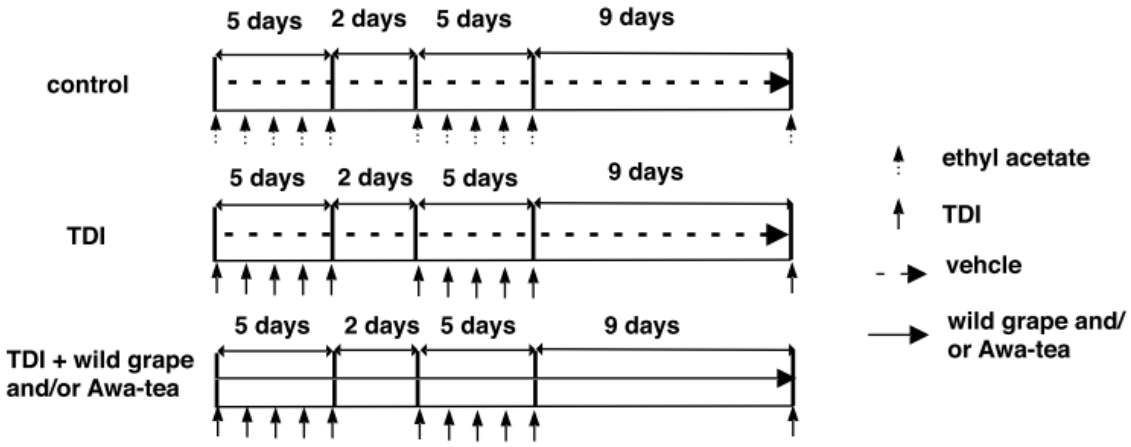

Fig. 3.1. Experimental protocol.

Rats were sensitized by intranasal application of 10 µl of 10% TDI in ethyl acetate for 2 weeks. After a 1-week interval, provocation was done with 10 µl of 10% TDI. The control group was sensitized with ethyl acetate only. The drug-treated group was orally treated with WG and/or Awa once a day for 3 weeks. Drugs were administrated 1 h before rats were sensitized with TDI.

3.2 3. Evaluation of nasal allergic-like symptom

Nasal allergic-like symptom was measured by means of the number of sneezes and the extent of watery rhinorrhea using the method of [53]. After TDI provocation, animals were placed in a cage (one animal per cage) and the number of sneezes and severity of watery rhinorrhea were examined for 10 min. The extent of watery rhinorrhea was measured according to the criteria given in the Table I on a scale ranging from 0 to 3.

Table 3.1. Criteria for grading the severity of TDI-induced nasal responses in rats.

	Score			
Nasal response	0	1	2	3
Watery rhinorrhea	(-)	At the nostril	Between 1and 3	Drops of discharges from nose
Swelling and redness	(-)	Slightly swollen	Between 1and 3	Strong swelling with redness

3.2.4. Real-time quantitative RT-PCR *in-vivo*

At the indicated time after provocation, rats were sacrificed and nasal mucosa was removed from the nasal septum, collected in RNAlater (Applied Biosystems), and stored at -80°C until assayed. Nasal mucosa was homogenized in a Polytron (Model PT-K; Kinematica). Total RNA was isolated using RNAiso Plus reagent (Takara Bio Inc., Kyoto, Japan) according to the manufacturer's instructions. RNA sample (5 μg) was reverse transcribed to cDNA using PrimerScript RT reagent Kit (Takara Bio Inc.). TaqMan primers and probe were designed using Primer Express software (Applied Biosystems, Foster City, CA,

USA). The nucleotide sequences of the primers and probes used in this study are listed in Table 3.2. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To standardize the starting material, GAPDH primers and probe reagents (Applied Biosystems) were used.

3.2.5. Cell culture

HeLa cells were cultured in MEM-α medium containing 8% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco) at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. RBL-2H3 cells were cultured in E-MEM containing 10% FBS, 100,000 U/mL penicillin, and 10 mg/mL streptomycin.

3.2.6. Real-time Quantitative RT-PCR *in-vitro*

HeLa cells were serum-starved and the concentration of FBS in the culture medium for Swiss3T3 cells was reduced to 0.5% before subject to the mRNA determination. HeLa cells and RBL-2H3 cells were cultured to 70% confluency in six-well dishes. The cells were treated with WG or Awa-tea for 24h before treatment with 100 nM of PMA (for HeLa cells and Swiss3T3 cells) or 1 μM of iomomycin (for RBL2H3 cells). After the stimulation with PMA for 3h or ionomycin for 2h, the cells were harvested and total RNA was prepared. The RNA samples (5 μg) were reverse transcribed to cDNA using PrimerScript RT Reagent Kit (Takara Bio Inc.). TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The nucleotide sequences of the primers and probes for H1R and IL-9 are listed in Table 3.2. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To

standardize the starting material, endogenous control human GAPDH and rodent GAPDH control reagents (Applied Biosystems) were used, and data were expressed as a ratio of GAPDH mRNA.

Table 3.2. Nucleotide sequences of primers and probes used in this study.

Primer/probe name	Sequence
Human H1R mRNA	
Sense primer	5'-CAGAGGATCAGATGTTAGGTGATAGC-3'
Anti-sense primer	5'-AGCGGAGCCTCTTCCAAGTAA-3'
Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA
Rat H1R mRNA	
Sense primer	5'-TATGTGTCCGGGCTGCACT-3'
Anti-sense primer	5'-CGCCATGATAAAACCCAACTG-3'
Probe	FAM-CCGAGAGCGGAAGGCAGCCA-TAMRA
Rat IL-4 mRNA	
Sense primer	5'-CAGGGTGCTTCGCAAATTTTAC-3'
Anti-sense primer	5'-CACCGAGAACCCCAGACTTG-3'

Probe FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA

Rat IL-5 mRNA

Sense primer 5'-CAGTGGTGAAAGAGACCTTGATACAG-3'

Anti-sense primer 5'-GAAGCCTCATCGTCTCATTGC-3'

Probe FAM-TGTCACTCACCGAGCTCTGTTGACG-TAMRA

Rat IL-9 mRNA

Sense primer 5'- CAGAGGATCAGATGTTAGGTGATAGC -3'

Anti-sense primer 5'- AGCGGAGCCTCTTCCAAGTAA -3'

Probe FAM- CTTCTCTCGAACGGACTCAGATACCACCT-TAMRA

3.2.7. Statistical Analysis

The results are shown as the mean ± S.E.M. Statistical analyses were performed with analysis of variance with Dunnett's test using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Values of $p < 0.05$ were considered statistically significant.

3.3 Results

3.3.1. Effect of wild grape and Awa-tea on ionomycin-induced IL-9 gene expression in RBL-2H3 cells.

Previously, we have shown that stimulation with ionomycin increased the level of IL-9 mRNA and NFAT signaling is involved in this transcription [14]. We investigated the effect of WG and Awa-tea on NFAT-mediated IL-9 gene expression in RBL-2H3 cells. Treatment with WG extract showed no inhibition for ionomycin-induced up-regulation of IL-9 gene expression (Fig. 3.2A). On the other hand, treatment with Awa-tea dose-dependently suppressed ionomycin-induced IL-9 gene up-regulation in RBL-2H3 cells (Fig. 3.2B), suggesting that Awa-tea suppressed NFAT signaling.

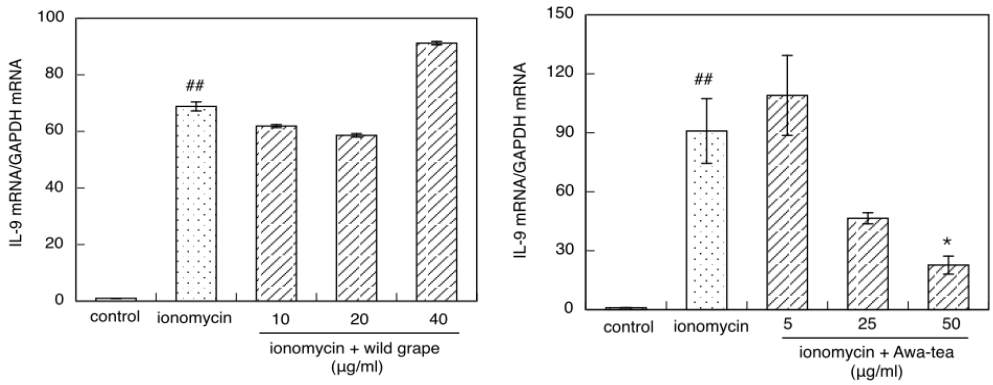

Fig. 3.2. Effect of WG extract and Awa-tea on ionomycin-induced up-regulation of IL-9 gene expression in RBL-2H3 cells.

RBL-2H3 cells were treated with 10-40 μg/ml of wild grape (A) or 5-50 μg/ml of Awa-tea for 24 h before ionomycin (1 μM) stimulation. After 2 h of stimulation with ionomycin, the RBL-2H3 cells were harvested and IL-9 mRNA was determined by real-time quantitative RT-PCR. The data are expressed as the mean ± S.E.M. (n=3). $^{\#\#}p<0.01$ vs. control; $^{*}p<0.05$, vs. ionomycin.

3.3.2 Effect of WG extract and/or Awa-tea on TDI-induced nasal allergy-like symptoms

Application of TDI for 3 weeks caused nasal symptoms in TDI sensitized rats **(Fig. 3.3)**. As previously our data shown Awa-tea alone significantly reduced TDI-induced sneezing and the nasal score. As WG extract inhibited H1R mRNA up-regulation in *in vitro* experiment, pretreatment with WG extract for 3 weeks also significantly reduced TDI-sensitized sneezing, rubbing, and nasal score **(Fig. 3.3A and 3.3B)**. Combined treatment with WG extract and Awa-tea markedly reduced TDI-induced sneezing and the nasal score **(Fig. 3.3)**. Control rats showed no nasal allergy-like symptoms after TDI provocation.

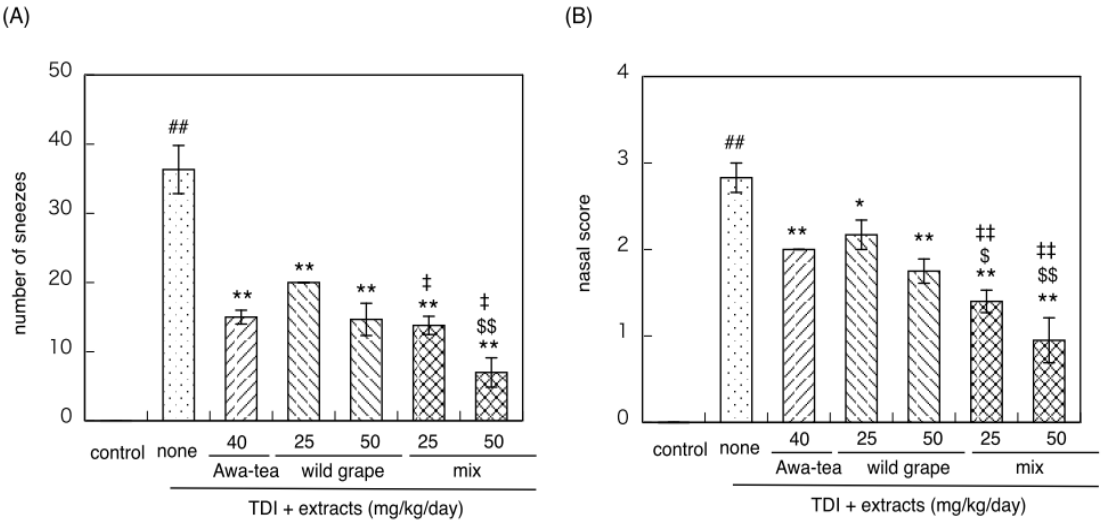

Fig. 3.3. Effect of WG extract and/or Awa-tea on TDI-induced nasal symptoms in TDI-sensitized rats.

The rats were sensitized and provoked as described in the Materials and Methods. The numbers of sneezes (A) and the nasal score (B) were measured over the 10-min period immediately after TDI

provocation. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}p<0.01$ vs. control; *<p0.05, **p < 0.01 vs. TDI; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. Awa-tea; $^{\ddagger}p < 0.05$ vs. wild grape at the corresponding concentration.

3.3.3 Effect of WG and/or Awa tea on TDI-induced increases in H1R mRNA expression in nasal mucosa

Sensitization with TDI increased H1R mRNA expression in the nasal mucosa of TDI-sensitized rats. Our previous data found that H1R mRNA expression reached a maximum after 4 h of TDI provocation [40]. Therefore, we investigated the effect of WG extract on TDI-induced H1R mRNA elevation using the nasal mucosa isolated 24 h, after TDI provocation. Pretreatment with WG extract significantly reduced H1R expression in the nasal mucosa of the TDI sensitized rats **(Fig. 3.4).**

(A) **(B)**

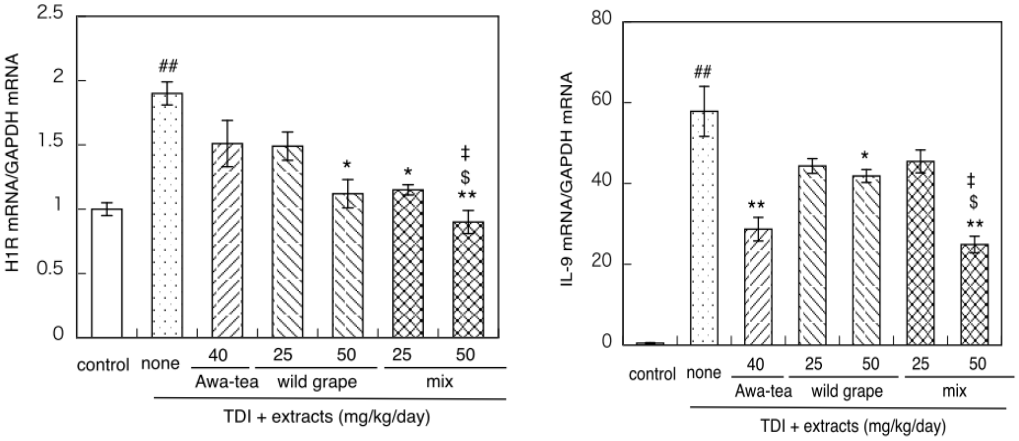

Fig. 3.4. The rats were sensitized and provoked as described in the Materials and Methods. The rats were sensitized and provoked as described in the Materials and Methods. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of H1R (A) and IL-9 (B) were determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}p<0.01$ vs. control; *<p0.05, **$p < 0.01$ vs. TDI; $^{\$}p < 0.05$ vs. Awa-tea; $^{\ddagger}p < 0.05$ vs. wild grape at the corresponding concentration.

3.3.4.Effect of wild grape and/or Awa-tea on TDI-induced up-regulations of Th2 cytokine gene expressions in the nasal mucosa of TDI-sensitized rats.

Repeated sensitization with TDI also up-regulated gene expressions of Th2 cytokines such as IL-4 and IL-5 [29, 33]. Treatment with WG (50 mg/kg/day) significantly suppressed TDI-induced up-regulation of IL-4 and IL-5 (Fig. 3.5A and 3.5B). Treatment with Awa-tea also showed significant suppression of up-regulation of these genes except IL-5 gene. Significant suppression of TDI-induced up-regulation of gene expression of these cytokines was also observed in combination of WG with Awa tea.

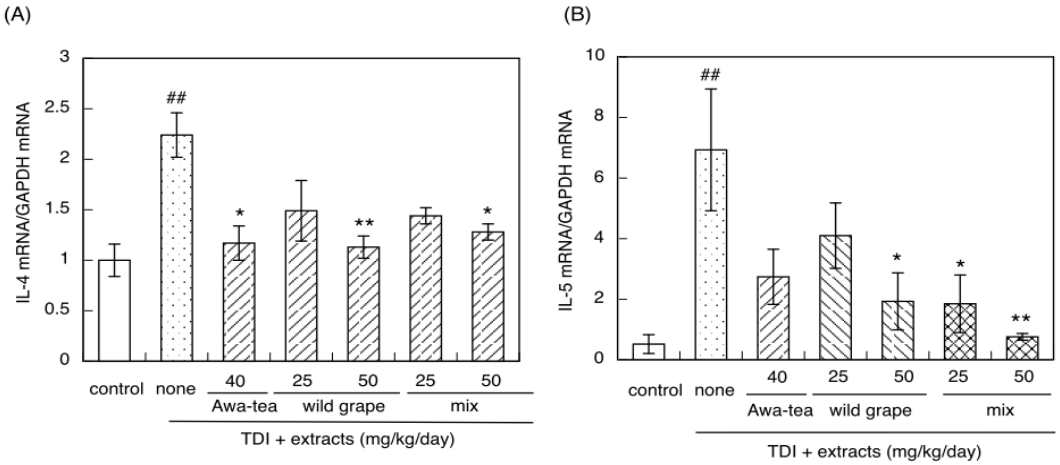

Fig. 3.5. Effect of WG and/or Awa tea on TDI-induced increases in H1R mRNA expression in nasal mucosa.

The rats were sensitized and provoked as described in the Materials and Methods. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of IL-4 (A) and IL-5 (B) were determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}p<0.01$ vs. control; *<p0.05, **p < 0.01 vs. TDI.

3.4. Discussion

Combination therapy is a new strategy to manage allergic rhinitis and its complications. Combination therapy of histamine and leukotriene antagonists produces symptomatic improvement as well as improved quality of life [55]. Moreover, medicinal plants that suppressed H1R mRNA expression alleviated TDI-induced nasal symptoms [32-34, 54]. The positive correlation between the up-regulation of H1R gene mRNA and the allergic symptoms in the nasal mucosa of the patients with polinosis has been reported. [40, 56]. We also demonstrated that the PKCδ/ERK/PARP-1 signal pathway is involved in histamine and PMA-induced up regulation of H1R gene expression in HeLa cells [31]. Antihistamines alleviated the nasal symptoms in the TDI-sensitized allergic model rats but not completely, whereas antihistamines suppressed the H1R gene expression until basal level [57]. Data suggest that there is may be another signaling involved in the pathogenesis of allergic symptoms. Recently, we have demonstrated that NFAT signaling-mediated IL-9 gene is the additional allergic rhinitis-sensitive gene and suppression of both signaling markedly alleviated nasal symptoms in allergy model

rats [57]. WG samples significantly suppressed both PMA and histamine induced up-regulation of H1R gene expression **(Fig. 2.2)** in Hela cells, where as it didn't suppress IL-9 gene expression in RBL-2H3 cells On the other hand, extract of Awa-tea significantly suppressed the IL-9 gene expression (Fig. 3.2). That's why in this study we investigated the combination effect Awa-tea and WG to treat allergic symptoms in TDI-induced allergy model rats. Nasal allergic symptoms during the 10 min just after TDI provocation represents the early phase that is derived from the release of preformed histamine during the sensitization process [40]. WG extract (25 mg/kg/day, 50 mg/kg/day) and Awa-tea (40 mg/kg/day) alleviated nasal symptoms significantly. However, the combination therapy of WG with Awa-tea markedly alleviated the nasal symptoms compare to the mono therapies **(Fig. 3.3)**.

We previously reported that the H1R gene expression level is positively correlated to the severity of allergic symptoms in TDI sensitized allergy-model rats [14, 32, 39, 54]. Oral administration of WG extract dose dependently suppressed H1R gene expression and the higher dose (50mg/kg/day) showed significant inhibition of TDI-induced increase in the level of H1R protein on the nasal mucosa of TDI-sensitized rats (Fig. 3.4). Awa-tea also tended to decrease H1R mRNA but the suppression was not significant. Whereas, combination of WG and Awa-tea suppressed the H1R gene expression dose dependently and up to basal level **(Fig. 3.4)**.

The disruption of helper T cell type1/2 (Th1/Th2) results in the clinical expression of nasal allergy and asthma [58]. There is ample of evidence of concurrence of

cytokines and histamine release in which histamine influences the expression and actions of several cytokines [43, 48, 50]. We showed that antihistamines suppressed TDI-induced up regulation of gene expression of these Th2 cytokines [30]. We also exposed the correlation of H1R expression with the gene expression of IL-4 and IL-5 in TDI-sensitized rats [30, 38]. These findings suggest that gene expression of H1R and Th2 cytokines are correlated and that suppression of H1R gene expression leads to decreased Th2 cytokine production.

We showed that pretreatment with combination group of WG with Awa-tea (25 mg/kg/day and 50 mg/kg/day) significantly suppressed the TDI-induced up-regulation of IL-4 and IL-5 dose dependently compared to the single treatment groups (Fig. 3.5). Treatment with WG showed the suppression of TDI-induced IL-9 gene expression although it could not suppress ionomycin-stimulated IL-9 gene up-regulation in RBL-2H3 cells. WG extract also suppressed TDI-induced up-regulation of IL-4 and IL-5 genes, which expression levels were shown to be correlated with that of H1R gene [29]. The histamine-cytokine network plays important role in allergic inflammation, in which histamine influences the expression and action of several cytokines and some cytokines modulate the production and release of histamine [42-44]. Pretreatment with IL-4 primes the release of histamine in response to FceRI [35, 45]. On the other hand, histamine affects the production of IL-4 and IL-5 [46]. Furthermore, IL-9 promotes Th2-specific allergic responses and upregulates IL-4 and IL-5 [59, 60]. Accordingly, suppression of IL-9 expression affects the expression levels of these Th2 cytokines. IL-4 together with TGF-β was shown to enhance IL-9 production from

activated T cells [61]. Therefore, it is likely that suppressive effect of IL-9 gene up-regulation by wild grape was through the inhibition of histamine–cytokine network.

Our previous report demonstrated that NFAT-mediated IL-9 gene expression is additional signalling responsible for the pathogenesis of acute nasal symptoms [57]. We also showed that suppression of both PKCδ signaling and NFAT signaling markedly alleviated the acute nasal symptoms in TDI-sensitized rats [57]. These data suggest that WG extract and Awa-tea suppressed both *in vitro* and *in vivo* H1R mRNA and IL-9 gene up-regulation respectively. Oral administration of WG extract combination with Awa-tea markedly alleviated nasal allergic symptoms through inhibition of both PKCδ/ERK/PARP-1 and NFAT signaling by the suppression of TDI-induced H1R and IL-9 mRNA up-regulation in the nasal mucosa of TDI-sensitized rats. Thus, we can propose that combination of WG with Awa-tea could be a good therapeutics for allergic diseases.

3.5. Conclusion

Combination therapy is a new strategy to manage allergic rhinitis and its complications. Our data suggest that oral administration of WG extract combination with Awa-tea markedly alleviated nasal allergic symptoms through inhibition of both PKCδ/ERK/PARP-1 and NFAT signaling by the suppression of TDI-induced H1R and IL-9 mRNA up-regulation in the nasal mucosa of TDI-sensitized rats. Thus, we can propose that combination of WG with Awa-tea could be a good therapeutics for allergic diseases.

Chapter 4

Effect of Wild grape in preventing eosinophilia through inhibiting IL-33 gene

4.1. Introduction

Allergic diseases, including allergic asthma, allergic rhinitis (AR) and atopic dermatitis are characterized by an increased number of eosinophil granulocytes in the circulating blood [62]. Eosinophil, a multifunctional leukocyte plays a crucial role in Th_2 mediated allergic diseases [63]. The prevalence of seasonal AR, pollinosis, is increasing in the developed world. Patients with AR and allergic asthma demonstrate comparable local and systemic eosinophil inflammation. Eosinophils have been considered as major effector cells in the pathogenesis of asthma [64]. IL-33 has emerged as an important mediator in the immunopathogenesis of allergy and asthma. However, the role of IL-33 in eosinophil-mediated inflammation has not been fully explored. IL-33 exacerbated eosinophil-mediated airway inflammation by increasing the levels of eosinophils, macrophages, lymphocytes and IL-13 in the lungs. [65]

In several genome-wide related studies of allergic diseases, IL-33 has taken interests because of its strong interaction between both it and its receptor, ST2, in particular, we focus on accumulating evidence how IL-33 relates to eosinophils as well as how this may provide new concepts for the progression of allergy [66].

However, very little is known about the role of IL-33 for the development of AR. The important role of eosinophil for late phase reaction of allergic rhinitis and other eosinophilic inflammations such as asthma and eosinophilic sinusitis has been reported [28]. Release of leukotrines from eosinophils causes nasal mucosal swelling, observed in a late phase [67, 68]. Genome-wide association studies

uncovered IL-33 gene is susceptible for asthma onset [69, 70]. IL-33 is a cytokine belonging to the IL-1 superfamily and is known to induce helper T cells, mast cells, eosinophils, and basophils to produce Th2 cytokines [71]. Thus, IL-33 is considered to be crucial for the induction of Th2-dominant immune responses [72]. In AR patients the significant elevation of IL-33 levels in serum has been reported [73]. However, relationship between the level of IL-33 gene expression and eosinophils was not elucidated yet.

Antihistamines do not have any effect on allergic chronic inflammation whereas, natural products from medicinal plants, either as pure compounds or as standardized extracts have been demonstrated to have anti-allergic activity. Those substances are believed to be less toxic compare to the synthetic chemical compounds. Here we found that extract from wild grapes (WG) extract suppressed PMA-induced IL-33 gene up-regulation in Swiss3T3 cells and the expression level of IL-33 mRNA was correlated with the number of blood eosinophil in patients with pollinosis, suggesting that WG could also be effective to prevent eosinophilia.

4.2. Materials and Methods

4.2.1. Analysis of IL-33 mRNA expression in the nasal mucosa of patients with pollinosis

IL-33 mRNA was determined using the nasal mucosa samples of patients as described previously [38]. Patient information, preparations for scraping the nasal mucosa, evaluation of nasal symptoms, and other experimental conditions were previously reported [30]. Blood samples were collected and blood cell

analyses were conducted to count eosinophils. Nasal mucosa scrapings were obtained from patients by an otolaryngologist as previously described [30]. Total RNA was isolated and reverse-transcribed using the RNAqueous Micro Kit (Applied Biosystems) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), respectively. The nucleotide sequences of the primers and probes for human IL-33 are listed in (Table 4.1). Universal Probe Library #27 (Roche Diagnostics, Basel, Switzerland) were used for the IL-33 probes. The pumilio RNA binding family member 1 (PUM1) primer and probe kit (Hs 00206469-m1, Applied Biosystems) was used to generate a standard [30]. The data are expressed as the ratio of IL-33 mRNA to PUM1 mRNA as previously described. The ethics committee of Tokushima University Hospital and Yashima General Hospital approved this study; written informed consent was obtained from each patient before the study commenced.

Table 4.1. Nucleotide sequences of primers and probes used in this study.

Primer/probe name	Sequence
Human IL-33 mRNA	
Sense primer	5'- AGGCCTTCACTGAAAACAGG -3'
Anti-sense primer	5'- TACCAAAGGCAAAGCACTCC -3'

4.2.2. Cell culture

Swiss3T3 cells were cultured in DMEM medium containing 10% FBS, 100,000 U/mL penicillin, and 10 mg/mL streptomycin.

4.2.3. Real-time quantitative RT-PCR

The concentration of FBS in the culture medium for Swiss3T3 cells was reduced to 0.5% before subject to the mRNA determination. Swiss3T3 cells were cultured to 70% confluence in 6-well dishes. The cells were treated with WG extract for 24 h before treatment with 100 nM of PMA. After the stimulation with PMA for 3 h, the cells were harvested and total RNA was prepared. The RNA samples (5 μg) were reverse transcribed to cDNA using PrimerScript RT Reagent Kit (Takara Bio Inc.). TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The IL-33 primers and probe kit (Mm00505403_m1, Applied Biosystems) was used to determine mouse IL-33 mRNA. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To standardize the starting material, endogenous control human GAPDH and rodent GAPDH control reagents (Applied Biosystems) were used, and data were expressed as a ratio of GAPDH mRNA.

4.3. Result

4.3.1. IL-33 mRNA level was correlated with the number of blood eosinophil in patients with polinosis

Although it was reported that serum level of IL-33 proteins were significantly higher in patients with pollinosis than in controls [74], our previous data showed that no suppression of IL-33 mRNA in the nasal mucosa was observed, suggesting that IL-33 is not involved in the therapeutic effect of pre-seasonal treatment with antihistamine for patients with pollinosis [38]. As IL-33 gene was reported to be the gene related to asthma that was characterized by eosinophilic airway inflammation, we investigated the relationship between the IL-33 mRNA level and the number of blood eosinophils in patients with pollinosis. The IL-33 mRNA level was not correlated with that of H1R (Fig. 4.1A). On the other hand, the blood eosinophil number was well correlated with the expression level of IL-33 gene ($r = 0.74$, $p<0.01$ by the Spearman's rank correlation method; Fig. 4.1B).

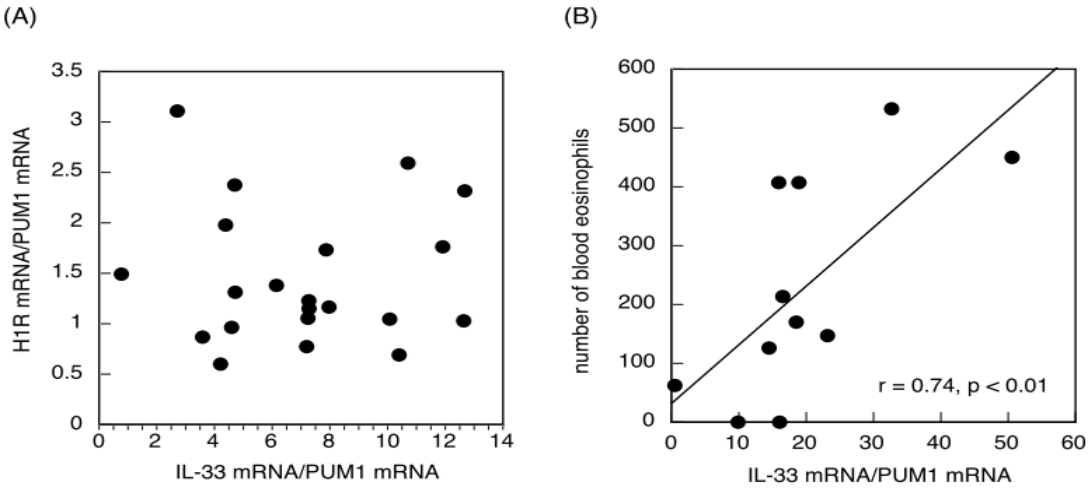

Fig.4.1. Relationship between the expression levels of IL-33 mRNA and that of H1R mRNA and the number of blood eosinophils

The scraping of the nasal mucosa from the patients with pollinosis were collected and total RNA was isolated using the RNAqueous Micro Kit as described in [30,38]. IL-33 and H1R mRNA was determined by quantitative real-time RT-PCR. Data for the expression of H1R mRNA were obtained from Mizuguchi et al., [30]. Correlation was analyzed by Spearman's rank correlation test.

4.3.2. Effect of WG extract on PMA-induced IL-33 gene expression in Swiss3T3 cells.

IL-33 gene is up-regulated by PMA stimulation in Swiss3T3 cells. Thus, we investigated the effect of WG extract on the PMA-induced up-regulation of IL-33 gene expression in Swiss3T3 cells. Treatment with WG extract dose dependently suppressed PMA-induced IL-33 gene up-regulation (Fig. 4.2).

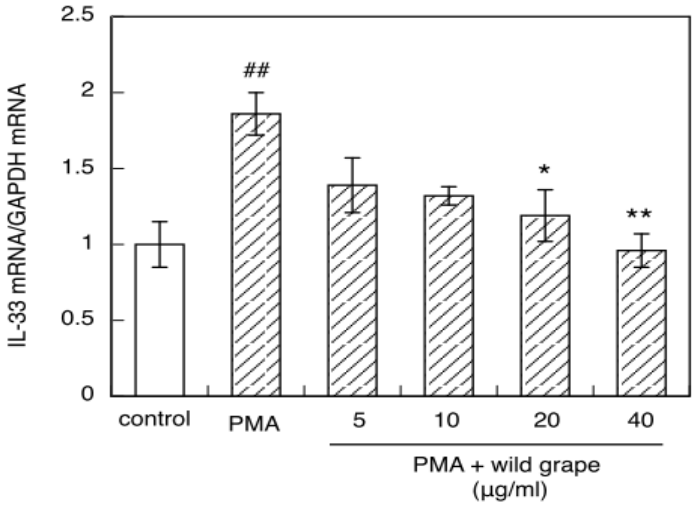

Fig. 4.2. Effect of WG extract on PMA-induced IL-33 gene expression in Swiss3T3 cells.

The concentration of FBS in the culture medium for Swiss3T3 cells were reduced to 0.5% before subject to the mRNA determination. WG extract (5-40 μg/ml) was incubated for 24 h before 100

nM of PMA stimulation. After 3-h stimulation with PMA, the cells were harvested and total RNA was prepared. IL-33 mRNA was determined by quantitative RT-PCR. Data are expressed as means ± S.E.M. (n=3). $^{\#\#}p<0.01$ vs. control; $*p<0.05$, $**p<0.01$ vs. PMA.

4.4. Discussion

Eosinophils are also generally regarded as the major effector cells in type 2 inflammatory diseases, including asthma, and allergy [75,76]. Eosinophil functions that can have a significant impact on allergic inflammation. It plays an important role in the pathogenesis of the chronic nasal symptoms [28]. IL-33 contributed to the manifestation of chronic allergic symptoms through an increase number of eosinophil. Chronic symptom of AR is mainly the late-phase responses consist of congestion, fatigue, malaise, and irritability at 6 to 24 hours after exposure to an allergen. The major pathologic change associated with late-phase responses is influx of inflammatory cells, such as eosinophils, into the nasal mucosa [75, 77, 78]. It has been reported that, Expression of IL-33 protein and its down regulation in inflamed nasal epithelial cells of patients with AR has been reported [79]. However, the role of IL-33 for the development of AR is not completely known. We showed here that the expression level of IL-33 mRNA was not correlated with that of H1R but correlated with the number of blood eosinophils in the patients with Japanese cedar pollinosis (Fig. 4.1). This suggests that suppression of up-regulation of IL-33 gene expression decreases the number of eosinophils, resulting in the improvement of symptoms caused by eosinophilic inflammation. Here, we examined the effect of WG on the IL-33 gene up-regulation that could be closely

related to improvement chronic symptoms of allergic rhinitis. WG suppressed PMA-induced IL-33 up-regulation in Swiss3T3 cells significantly in a dose dependent manner (Fig. 4.2). This suggests that WG could be effective to alleviate eosinophilic inflammation.

Conclusion

5. Conclusion

H1R plays an important role in many allergic responses including AR, a representative incurable disease, so the development of therapeutics for high alleviation is needed. Natural products from medicinal plants, either as pure compounds or as standardized extracts have been demonstrated to have anti-allergic activity both *in vitro* and *in vivo*. Those substances are believed to be less toxic compare to the synthetic chemical compounds. WG extract showed suppression on both *in vitro* and *in vivo* H1R mRNA up-regulation. It also suppressed IL-33 gene expression, suggesting that WG extract could be effective for both acute and chronic nasal symptoms and bio-active compounds from WG extract could be good therapeutics for allergic diseases and eosinophilia. Furthermore, Combination therapy is a new strategy to manage AR and its complications. Our data suggest that oral administration of WG extract combination with Awa-tea markedly alleviated nasal allergic symptoms through inhibition of both PKCδ/ERK/PARP-1 and NFAT signaling by the suppression of TDI-induced H1R and IL-9 mRNA up-regulation in the nasal mucosa of TDI-sensitized rats. Thus, we can propose that combination of WG extract with Awa-tea could be effective to treat allergic diseases.

Abbreviations

AR: Allergic Rhinitis

WG: Wild grapevive

H1R: Histamine H_1 receptor

PMA: Phorbol 12-Myristate 13-Acetate

PKCδ: Protein Kinase C-δ

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

IL: Interleukin

RT-PCR: Reverse transcription polymerase chain reaction

TDI: Toluene, 2-4-diisocyanate

Th2: Helper T cell type 2

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