Suplatast Tosilate Inhibits Histamine Signaling by Direct and Indirect Down-Regulation of Histamine H₁ Receptor Gene Expression through Suppression of Histidine Decarboxylase and IL-4 Gene Transcriptions

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Allergic rhinitis (AR) is one of the most common allergic diseases in the world, affecting 5–22% of the general population, and its prevalence is increasing (1). It can affect people of both sexes and all ages and social and ethnic groups, and it has an impact on their quality of life and health. It is frequently associated with other chronic airway diseases: an estimated 60–80% of all patients with allergic rhinitis have concomitant asthma or bronchial hyperreactivity (2). Although drugs are available for its treatment, none is completely effective. Because of the high incidence of AR, its impairment of the quality of life, and the presence of comorbidities such as asthma, sinusitis, and otitis media, there is need for improved treatment options for this disorder. AR is an inflammatory disease of the upper airway that is accompanied by sneezing, itching, congestion, rhinorrhea, and loss of the sense of smell. It is considered that these symptoms are caused by IgE-mediated activation of mucosal mast cells located on the epithelium of the nasal cavity (3). Activation of mast cells results in the release of histamine and many other mediators. Histamine has been shown to elicit virtually all of the early phase responses, primarily through binding to the histamine H1 receptor (H1R) (4, 5). Iriyoshi et al. found that patients with AR had increased expression of H1R mRNA in nasal scrapings (6). Dinh et al. also reported that expression of H1R mRNA alters in patients with perennial AR (7).

Toluene-2,4-diisocyanate (TDI), a highly reactive industrial chemical, is one of the leading causes of occupation-related asthma in industrialized countries (8). We demonstrated previously that repeated intranasal application of TDI induced release of histamine from mast cells via neurogenic inflammation and led to the development of nasal hypersensitivity in guinea pigs and rats (9–11). Up-regulation of H1R at both the mRNA and protein levels was also observed in the nasal mucosa of TDI-sensitized nasal allergic model rats (12, 13). As histidine decarboxylase (HDC) is the only enzyme that synthesizes histamine from histidine in mammals, control of HDC activity is another important target for regulation of histamine signaling. It was reported that HDC mRNA, HDC activity, and the histamine content were significantly increased in the nasal mucosa of TDI-sensitized rats (13). These findings suggest that H1R and HDC genes are allergic disease-sensitive genes and the levels of H1R and HDC expression affect the severity of
allergic symptoms, and also that compounds that suppress histamine signaling are promising as anti-allergic drugs.

Th2 cytokines including IL-4, IL-5, IL-9, and IL-13 are also suggested to play important roles in the pathogenesis of allergic inflammation (14). Among them, IL-4 plays a central role in allergic inflammation: it is associated with the development of T lymphocytes, acts as a growth factor for Th2 cells, stimulates IgE synthesis, and is involved in mast cell activation (15). Previous reports that the expression of IL-4 and IL-5 mRNAs was up-regulated in nasal mucosa suggest the involvement of these cytokines in nasal hyperresponsiveness in TDI-sensitized rats (16, 17).

Increasing experimental evidence suggests the existence and importance of the histamine-cytokine network in allergic inflammation, in which histamine influences the expression and action of several cytokines and some cytokines modulate the production and release of histamine (18–20). Pretreatment with IL-4 primes the release of histamine, prostaglandins, leukotrienes, and cytokines in response to FceRI (21, 22). Histamine, on the other hand, modulates the releases of IL-4 and IFN-γ from T cells (23) and induces the release of IL-5 (24).

Suplatast tosilate (IPD-1151T) is an immunomodulator that suppresses eosinophil infiltration, IgE production, and allergic inflammation by suppressing the production of IL-4 and IL-5 (25). The efficacy of systemic administration of suplatast in nasal allergy has been reported (26). These reports indicated that suplatast could be useful for treatment and prevention of AR. In search of drugs that suppress histamine signaling, we found that suplatast almost completely blocked TDI-induced up-regulation of H1R mRNA in TDI-sensitized rats, while d-chlorpheniramine partially suppressed this up-regulation (27). This suggests the existence of more than one pathway for up-regulation of H1R gene expression in TDI-sensitized rats, one of which is sensitive to H1 antihistamines and the possibility that suplatast suppresses not only the H1 antihistamines-sensitive pathway but also an unknown pathway that is insensitive to H1 antihistamines. However, the effect of suplatast on histamine signaling has not yet been elucidated. In the present study, we evaluated the possible inhibitory effect of suplatast on TDI-induced nasal symptoms and the up-regulation of H1R, HDC, and IL-4 mRNAs in TDI-sensitized nasal allergy model rats. Additionally, we investigated a possible relationship between increases in the expression of IL-4 and H1R in nasal mucosa of TDI-sensitized rats.

Materials and Methods

**Materials**

Suplatast tosilate was a gift from Taiho Pharmaceutical. [Pyridinyl 5-3H]mepyramine (specific activity, 20 Ci/mmol) was obtained from PerkinElmer. TaqMan assay reagents of rat and human GAPDH were from PerkinElmer. TRIzol reagent was purchased from Invitrogen. A BCA protein assay kit was from Sigma-Aldrich. Recombinant rat IL-4 (rIL-4) was from Wako Pure Chemical. All other chemicals were of analytical grade.

**Animals**

Six-week-old male Brown Norway rats (200–250 g; Japan SLC) were used. They were allowed free access to water and food and were kept in a room at 25 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle. They were divided into three groups of four rats each: a control group, a group sensitized with TDI (Wako Pure Chemical), and a test group. All procedures involving the animals were conducted in accordance with the Guidelines for Animal Experiments approved by the Ethical Committee for Animal Studies of the School of Medicine of the University of Tokushima.

**TDI sensitization and provocation and administration of suplatast**

Rats were sensitized with TDI by the method described by Kitamura et al. (12) with slight modifications. 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. 1). Suplatast (100 mg/kg) was dissolved in sterile water 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. 1). This dose seems to be high. However, Taiho Pharmaceutical, the manufacturer of suplatast, used 30–300 mg/kg for the preclinical animal studies in their reports and reported that 100 and 300 mg/kg, but not 30 mg/kg, of suplatast significantly prevented the expression of airway hyperresponsiveness. A similar dose of suplatast was also used in many papers for animal studies (28–32), in which some of them found that suplatast was effective when it was given at 100 mg/kg, and the effect of suplatast was not clear when it was given at lower dose. From these reports, we decided to use 100 mg/kg of suplatast to see a clear suppressive effect by suplatast on TDI-induced nasal symptoms and up-regulation of H1R, HDC, and IL-4 mRNAs. Our preliminary study to investigate the effect of repeated pretreatment with suplatast on TDI-induced nasal allergic-like symptoms and up-regulation of H1R, HDC, and IL-4 mRNAs revealed that repeated pretreatment with suplatast for 3 days or 1 wk suppressed TDI-induced nasal symptoms and up-regulation of these mRNAs more effectively than did its administration once before TDI provocation, and that single treatment with suplatast showed no significant effect (data not shown). From these findings and the fact that long-term use of suplatast is preferred clinically, we administered suplatast for 2 wk in this study.

**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 Abe et al. (9). After TDI provocation, animals were placed in a plastic 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.
Real-time quantitative RT-PCR

At the indicated time after provocation, rats were sacrificed and nasal mucosa was removed from the nasal septum, collected in RNA later (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 TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were reverse transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen). TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The nucleotide sequences of the primers and probes used in this study are listed in Table II.

The PCR products were verified by sequencing using a Genetic analysis system (Beckman CEQ 8000; Beckman Coulter). To measure the differences in starting material, GAPDH primers and probe reagents (Applied Biosystems) were used. For evaluating the effect of IL-4 on H1R expression in the nasal mucosa of normal (i.e., non-TDI-treated) rats, 10 µl of rrIL-4 (0–1.0 µg/rat) was administered into the bilateral nasal cavities of rats once or once a day for 3 days or 1 wk. Nasal mucosa was collected 4 h after the administration of histamine or IL-4 expression. H1R mRNA was determined. For quantification of the effect of histamine on IL-4 expression, 10 µl of histamine (0–3 mg/rat) in PBS was instilled into the nasal cavity bilaterally. Rats were sacrificed and the nasal mucosa was removed from nasal septum 4 h after rrIL-4 instillation, and H1R mRNA was quantified. For investigation of the effect of histamine on IL-4 expression, 10 µl of histamine (0–3 mg/rat) was administered into the bilateral nasal cavities of rats once or once a day for 3 days or 1 wk. Nasal mucosa was collected 4 h after the final administration of histamine, after which IL-4 mRNA was determined.

$[^3]H$Mepyramine binding assay

Nasal mucosa was homogenized in a Polytron in 10 vol (w/v) of ice-cold 50 mM Na$_2$/K phosphate buffer. The homogenate was centrifuged at 10,000 g for 30 min at 4°C, and the pellet was resuspended in 10 vol of ice-cold 50 mM Na$_2$/K phosphate buffer as a membrane sample for $[^3]H$Mepyramine binding assay. Membranes containing 100 µg of protein were incubated with 1 nM $[^3]H$Mepyramine in the absence and presence of 10 µM triprolidine for 60 min at 25°C in a final volume of 500 µl. Reactions were terminated by rapid vacuum filtration through Whatman GF/B filters presoaked in 1% polyethyleneimine, and the radioactivity trapped on the filters was counted in a liquid scintillation counter. Specific binding was defined as the radioactivity bound after subtraction of nonspecific binding as defined with 10 µM triprolidine.

HeLa cell culture and treatment with drugs

HeLa cells were cultured at 37°C under a humidified 5–95% CO$_2$-air atmosphere in MEM-α medium containing 8% FCS (Sigma-Aldrich) supplemented with 100 IU/ml penicillin (Sigma-Aldrich) and 50 µg/ml streptomycin (Sigma-Aldrich). HeLa cells were cultured to 70% confluence in 35-mm dishes and were serum starved for 24 h at 37°C before treatment with 10 µM histamine or 5 ng/ml rrIL-4. Cells were pretreated with 10 µM d-chlorpheniramine for 1 h or 100 µM suplatast for 24 h before histamine or IL-4 treatment. Then, they were stimulated with histamine or IL-4 for 3 h and H1R mRNA was determined.

Determination of histamine content in nasal lavage fluid

Nasal lavage was performed with the slightly modified method of Durland et al. (33). Rats were anesthetized with diethyl ether. Polyethylene tubing connected to a regulated vacuum was positioned in the left nostril that was kept under slightly reduced pressure. Washing with 1.5 ml of PBS prewarmed to 37°C was performed from the right to left nostril. The same procedure was repeated from the left to right nostril. Nasal exudates were then transferred in a new tube and 10 µ1 of perchloric acid (60%) was added. After mixing, nasal exudates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatant (200 µl) was transferred to another tube and kept at −20°C. The histamine content of the lavage fluid was determined in an HPLC system as described below.

Measurements of HDC activity and histamine content of nasal mucosa

Nasal mucosa was homogenized with 10 vol of ice-cold Histamine buffer consisting of 0.1 M potassium phosphate buffer (pH 6.8), 0.2 mM DTT, 0.01 mM pyridoxal-5-phosphate, 1% polyethylene glycol (average molecular weight, 300 kDa), and 100 µg/ml PMSF. The homogenate was centrifuged at 10,000 × g for 15 min at 4°C and the supernatant (designated as supernatant A) was collected. Half of supernatant A was dialyzed three times against an adequate volume of Histamine buffer for 6 h at 4°C (designated as supernatant B). The histamine content in supernatant A was determined with an HPLC system with cation exchanger (Tosoh) and the automated α-phthalaldehyfluorometric detection system (Hitachi) by the method described previously (13). HDC activity was determined by incubating supernatant B for 4 h at 37°C with 0.25 mM L-histidine. HDC activities were calculated based on the formation of histamine after subtraction of the blank value.

Determination of protein concentration

Protein concentration was determined with a BCA protein assay kit (Sigma-Aldrich) using BSA as a standard.

Statistical analysis

Results are shown as mean ± SEM. Statistical analysis was performed by one-way ANOVA with Dunnett’s test using GraphPad Prism software (GraphPad Software). Values of $p<0.05$ are considered to be statistically significant.

Results

Effect of suplatast on TDI-induced nasal allergic-like symptoms

Application of TDI causes nasal allergic-like symptoms characterized by sneezing and watery rhinorrhea in TDI-sensitized rats. Pretreatment with suplatast for 2 wk significantly reduced TDI-induced sneezing and rubbing (Fig. 2, a and b) but had no noticeable effect on the nasal score (Fig. 2c). Control rats showed no nasal allergic-like symptom after TDI provocation.

Effect of suplatast on TDI-induced increases in H1R mRNA expression and H1R protein in nasal mucosa

Repeated application of TDI increased H1R mRNA expression in the nasal mucosa of TDI-sensitized rats. This expression was maximal after 4 h of TDI provocation (12). Pretreatment with suplatast

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Table I. Criteria for grading the severity of TDI-induced nasal allergic-like symptoms

<table>
<thead>
<tr>
<th>Nasal Response</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watery rhinorrhea</td>
<td>0</td>
<td>(−)</td>
</tr>
<tr>
<td>Swelling and redness</td>
<td>1</td>
<td>(−)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Between 1 and 3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Between 1 and 3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Primer/Probe Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat H1R mRNA</td>
<td>5'TTAAGCCTTCGCCGCTCCACT-3'</td>
</tr>
<tr>
<td>Antisense primer</td>
<td>5'CCCCAG AttAACAACCAACTG-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>FAM-CACCAGGCGGACGACCA-TAMRA</td>
</tr>
<tr>
<td>Human H1R mRNA</td>
<td>5'CAAAGAATCAATAGGTTGGTGAGAC-3'</td>
</tr>
<tr>
<td>Antisense primer</td>
<td>5'AGGGCCGGCCGGTGTCCCTTGGGAA-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>TAMRA-CAGGGTGCTTCGCAAATTTTAC</td>
</tr>
<tr>
<td>Rat HDC mRNA</td>
<td>5'GCGCGAAGAGGAACAAATTTTAC-3'</td>
</tr>
<tr>
<td>Sense primer</td>
<td>5'AAGAGCCGAGCGCCTGTGCCTGGA-3'</td>
</tr>
<tr>
<td>Antisense primer</td>
<td>5'AGGAACATGGACCCCCATCGTGGT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>TAMRA-FAM</td>
</tr>
<tr>
<td>Rat IL-4 mRNA</td>
<td>5'CACGCGGTCTCGGAAAAATTTTAC-3'</td>
</tr>
<tr>
<td>Sense primer</td>
<td>5'CCAACGAGACCCGACGCT-3'</td>
</tr>
<tr>
<td>Antisense primer</td>
<td>5'CACCGAGACCCGACGCT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>TAMRA-FAM</td>
</tr>
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significantly reduced H1R expression in the nasal mucosa of the TDI-sensitized rats (Fig. 3a). As suplatast inhibited H1R mRNA expression in nasal mucosa of TDI-sensitized rats, its effect on the expression of histamine receptor protein was also investigated. \[^{1}H\]mepyramine binding activity was significantly increased 24 h after TDI provocation in TDI-sensitized rats (Fig. 3b). Administration of suplatast significantly inhibited \[^{1}H\]mepyramine binding activity in nasal mucosa, indicating a decrease in the number of H1R in the nasal mucosa of TDI-sensitized rats.

**Effect of suplatast on TDI-induced increases in HDC mRNA expression and HDC activity**

Increase in the histamine content of the nasal mucosa of TDI-sensitized rats may result from increase in HDC activity. In TDI-sensitized rats, HDC mRNA and HDC activity in the nasal mucosa were significantly increased after TDI provocation and reached a maximum 4 and 9 h, respectively, after TDI provocation (13). Thus, we collected nasal mucosa 9 h after TDI provocation and investigated the effect of suplatast on HDC activity. As shown in Fig. 4a, pretreatment with suplatast markedly suppressed HDC activity in the nasal mucosa of TDI-sensitized rats. As increase in HDC activity observed in the nasal mucosa of TDI-sensitized rats may be due to up-regulation of HDC mRNA, and the suppressive effect of suplatast on HDC activity may be due to the inhibition of HDC mRNA expression. To explore this possibility, we evaluated the effect of suplatast on HDC mRNA expression in TDI-sensitized rats. Repeated application of TDI increased the expression of HDC mRNA in the nasal mucosa, and suplatast significantly suppressed TDI-induced up-regulation of HDC mRNA (Fig. 4b).

**Effect of suplatast on TDI-induced increase in the histamine content of nasal lavage fluid and nasal mucosa**

Nasal symptoms after TDI provocation were accompanied by a significant histamine release in the lavage fluid. We therefore investigated the effect of suplatast on histamine release in nasal lavage fluid. Suplatast markedly inhibited histamine release in the nasal lavage fluid (Fig. 4c). The histamine content in the nasal mucosa is also reported to increase significantly after TDI provocation with a maximum 9 h after TDI provocation (13). Thus, we next examined the effect of suplatast on the histamine content in the nasal mucosa 9 h after TDI provocation. Pretreatment with suplatast tended to decrease the nasal histamine content, but this difference was not statistically significant (Fig. 4d).

**Effect of suplatast on TDI-induced up-regulation of IL-4 mRNA**

IL-4 plays a crucial role in the pathogenesis of allergic disorders. Expression of IL-4 mRNA increased significantly in the nasal mucosa of TDI-sensitized rats, reaching a peak 4 h after TDI provocation. As suplatast is known as a Th2 cytokine inhibitor, we examined the effect of suplatast on the up-regulation of IL-4 mRNA after TDI provocation. Suplatast almost completely inhibited the up-regulation of IL-4 mRNA expression after TDI provocation (Fig. 5).

**Effect of IL-4 or histamine on nasal mucosal H1R or IL-4 mRNA expression in TDI-untreated normal rats**

As both histamine and IL-4 mRNA increased in nasal mucosa of TDI-sensitized rats, it is tempting to speculate that there is some relationship between histamine and IL-4 signaling pathways. This hypothesis is supported by recent findings that prophylactic treatment with H1 antihistamines suppressed the TDI-induced up-regulation of H1R and IL-4 mRNAs in TDI-sensitized rats (34). To explore this possibility, we applied rrIL-4 or histamine directly to...
the nasal cavity of rats and investigated the effect of IL-4 or histamine on the level of H1R or IL-4 mRNA expression. Preliminary studies showed that intranasal application of rIL-4 or histamine to normal rats increased the levels of H1R and IL-4 mRNA expression and these expressions were highest 4 h after IL-4 or histamine administration (data not shown). As shown in Fig. 6, administration of IL-4 to the nasal cavity of rats increased H1R mRNA expression dose-dependently. Intranasal application of histamine for 1 wk up-regulated the expression of IL-4 mRNA in a dose-dependent manner (Fig. 7a). However, single administration with histamine did not significantly increase IL-4 mRNA level (Fig. 7b).

**Effect of suplatast on histamine- or IL-4-induced up-regulation of H1R mRNA in HeLa cells**

Stimulation of HeLa cells with histamine induced significant and transient increase in H1R mRNA with a maximum 9 h after histamine stimulation (35). This histamine-induced H1R up-regulation was mediated by H1R (35). Pretreatment with 10 μM d-chlorpheniramine (used as a positive control) for 1 h completely blocked histamine-induced up-regulation of H1R mRNA (Fig. 8a). As suplatast inhibited H1R mRNA up-regulation in the nasal mucosa of TDI-sensitized rats, we investigated the effect of suplatast on histamine-induced up-regulation of H1R mRNA in HeLa cells. Pretreatment with suplatast failed to inhibit histamine-induced up-regulation of H1R mRNA in HeLa cells (Fig. 8a). Recently, we found that IL-4 also induced up-regulation of H1R mRNA in HeLa cells (S. Horio, K. Fujimoto, H. Mizuguchi, and H. Fukui, submitted for publication). Thus, we investigated whether suplatast inhibits IL-4-induced H1R mRNA up-regulation. As shown in Fig. 8b, suplatast suppressed IL-4-induced H1R mRNA elevation in a dose-dependent manner. In both cases, suplatast itself has no effect on up-regulation of H1R expression.

**FIGURE 4.** Effect of suplatast on TDI induced increases of HDC activity (a), HDC mRNA (b), and histamine content in the nasal lavage fluid (c) and in the nasal mucosa (d) of TDI-sensitized rats. Rats were sensitized and provoked as described in Materials and Methods. a, Rats were sacrificed 9 h after provocation and HDC activity in the nasal mucosa was measured by HPLC. b, Rats were sacrificed 4 h after provocation and HDC mRNA was determined by quantitative RT-PCR. c, Nasal lavage was performed on rats anesthetized with diethyl ether and nasal discharge was collected from both vestibules 10 min after provocation. Histamine content was measured by HPLC. d, Rats were sacrificed 9 h after TDI provocation and the histamine content of the nasal mucosa was measured by HPLC. Data are presented as means ± SEM (n = 4). *, p < 0.01 vs control; #, p < 0.01 vs TDI.

**FIGURE 5.** Effect of suplatast on TDI induced up-regulation of IL-4 mRNA in rat nasal mucosa of TDI-sensitized rats. Rats were sensitized and provoked as described in Materials and Methods. They were sacrificed 4 h after TDI provocation and IL-4 mRNA was determined by quantitative RT-PCR. Data are expressed as mean ± SEM (n = 4). *, p < 0.01 vs control. #, p < 0.01 vs TDI.

**FIGURE 6.** Effect of IL-4 on H1R mRNA expression in the nasal mucosa of normal TDI-untreated rats. Rats were treated with recombinant rat IL-4 (0–1.0 μg/rat) intranasally and sacrificed 4 h later. H1R mRNA was determined by quantitative RT-PCR. Data are expressed as means ± SEM (n = 4). *, p < 0.01 vs control.
Discussion

In the present study, we used TDI-sensitized rat as a model of AR. Intranasal application of TDI caused neuropeptide-mediated release of histamine from mast cells in the nasal mucosa and led to the development of nasal allergic-like symptoms such as sneezing and watery rhinorrhea in TDI-sensitized guinea pigs and rats (9–12, 36, 37). Although AR, defined as an IgE-mediated disease, could be different from TDI-induced, non-IgE-mediated rhinitis, nasal allergic-like symptoms induced by TDI are similar to those observed in AR patients (38, 39). Additionally, TDI-sensitized rats also display many of the characteristic features of AR in humans, including infiltration of eosinophils and mast cells (40), increase in the level of cytokines (8, 41–43), elevation of H1R mRNA and protein level (12), increase in the HDC mRNA level, HDC activity, and histamine content (13), even though histamine release was triggered by neuropeptide, but not IgE, in TDI-sensitized animals. Furthermore, the expression of IL-4 and IL-5 mRNAs was also up-regulated in the nasal mucosa of TDI-sensitized rats after provocation with TDI (16, 17). Therefore, we consider that TDI-sensitized rats can be a model of AR.

Nasal allergic-like symptoms including sneezing and nasal rubbing were studied for 10 min just after TDI provocation to evaluate the effect of suplatast on early phase symptoms that were caused mainly by the release of preformed mediators during the sensitization process. Pretreatment with suplatast significantly reduced nasal sneezing and itching (Fig. 2, a and b) but could not prevent nasal swelling and rhinorrhea as evident by its effects on the nasal score (Fig. 2c). Histamine is reported to cause sneezing and nasal rubbing by its binding to H1R on the sensory nerve endings (44). On the contrary, other mediators such as leukotrienes and prostaglandins play major roles in swelling, rhinorrhea, and congestion (45). This could be the reason why suplatast had no noticeable effect on the nasal score.

Expression of H1R mRNA is reported to increase in epithelial, endothelial, and neural cells on the nasal mucosa in the patients with occupational rhinitis (46, 47). H1R binding activity in the nasal mucosa is also reported to increase during development of nasal allergy (48). We have reported that expression of H1R is up-regulated at both the mRNA and protein level in the nasal mucosa of TDI-sensitized rats (12, 13). Therefore, it is likely that increase in nasal allergic-like symptoms in TDI-sensitized rats is due to increase in H1R in the nasal mucosa. Suplatast significantly inhibited the H1R mRNA expression (Fig. 3a). Administration of suplatast also significantly inhibited TDI-induced increase in the level of H1R protein on the nasal mucosa of TDI-sensitized rats (Fig. 3b). These data suggest that suplatast inhibits nasal hypersensitivity by suppression of TDI-induced H1R up-regulation.

**FIGURE 7.** Effect of histamine on IL-4 mRNA expression in the nasal mucosa of normal TDI-untreated rats: dose-response (a) and time-course (b) studies. In a, 10 μl of histamine (0–3 mg/rat) was administered bilaterally in the nasal cavity of rats for 1 wk. In b, 3 mg per rat of histamine was instilled into the nasal cavity bilaterally once (represented as single) or once a day for 1 wk (represented as 1 wk). Nasal mucosa was collected 4 h after the final administration of histamine, and IL-4 mRNA was determined. Data are expressed as means ± SEM (n = 4). *, p < 0.05 vs control; **, p < 0.01 vs control.

**FIGURE 8.** Effect of suplatast on histamine-induced (a) or IL-4-induced (b) up-regulation of H1R mRNA in HeLa cells. HeLa cells were starved in serum-free medium for 24 h at 37°C before treatment with 10 μM histamine (a) or 5 ng/ml IL-4 (b). They were pretreated with 10 μM d-chlorpheniramine (used as a positive control) for 1 h or suplatast (100 μM in (a) and 0–200 μM in (b)) for 24 h before histamine or IL-4 treatment. H1R mRNA was determined by quantitative RT-PCR 3 h after histamine treatment or 4 h after IL-4 treatment. Data are represented as means ± SEM of four independent assays in triplicate. **, p < 0.01 vs control; ##, p < 0.01 vs histamine; ††, p < 0.01 vs IL-4.
Synthesis of histamine by HDC plays another important regulatory step in histamine signaling. HDC mRNA is increased in nasal mucosa of patients with AR (49). The finding that expression of HDC mRNA, HDC activity, and the histamine content of the nasal mucosa were significantly increased after TDI provocation indicates the importance of HDC in allergic responses (13). Pretreatment with suplatast significantly suppressed HDC activity and HDC mRNA expression in the nasal mucosa (Fig. 4, a and b). The histamine content of the nasal mucosa was also decreased, although not markedly (Fig. 4d). In suplatast-treated rats, ~30% of the HDC activity still remained (Fig. 4a), and the histamine in the nasal mucosa might be derived from this remaining activity. Since histamine is an important chemical mediator in AR, inhibition of its synthesis by decreasing HDC activity and gene expression may contribute to the beneficial effect of suplatast in AR. Symptoms of AR were caused by the activation of mucosal mast cells of the nostril and were accompanied by a significant histamine release in the lavage fluid. Suplatast significantly inhibited histamine release in the nasal lavage fluid (Fig. 4c). It was reported that suplatast suppressed the Ag-induced degranulation of mesentric mast cells and histamine release from peritoneal exudate cells of rats (50). Therefore, the decreased histamine content that resulted from suplatast pretreatment in the nasal lavage fluid may be due to suppression of histamine release from mast cells. Our data suggest suplatast inhibits the histamine signaling by suppression of both synthesis and release of histamine.

AR is also characterized by the increase in Th2 cytokine productions such as IL-4 and IL-5. It is known that IL-4 involves allergic responses at several levels of regulation. It is associated with development of Th2 cells, stimulation of IgE synthesis, and activation of mast cells (51). Although suplatast is known as a Th2 cytokine inhibitor, the effect of suplatast on IL-4 gene expression has not yet been elucidated. Our data clearly show that suplatast suppressed IL-4 (Fig. 5) and IL-5 (data not shown) expression at the transcriptional level. We have not checked the effect of suplatast on the eosinophil infiltration corresponding with increases in IL-4 and IL-5 expression in this study. However, Irifune has already reported the infiltration of eosinophils and mast cells after TDI provocation in TDI-sensitized guinea pigs (40). We observed similar eosinophil infiltration after TDI provocation in TDI-sensitized rats (unpublished observation). As it is well established that IL-4 and IL-5 play important roles to recruit eosinophils and that suplatast inhibits IL-4 and IL-5 formations, we expect that eosinophil infiltration should be suppressed in suplatast-treated group. However, we have no data to support this.

It is well established that interactive cytokines network in allergic inflammation. Accumulating evidence also suggests the importance of the histamine-cytokine network, in which histamine influences the expression and actions of several cytokines, and some cytokines modulate the production and release of histamine as well (18, 19, 52). We have shown that H1R mRNA up-regulation was partially suppressed by H1-antihistamines such as d-chlorpheniramine and olopatadine in TDI-sensitized rats (27). This TDI-induced H1R mRNA up-regulation was completely inhibited by suplatast (Fig. 3a). These findings suggest the existence of multiple, H1R-mediated and non-H1R-mediated pathways for H1R mRNA up-regulation. Recently, we reported that prophylactic treatment with H1-antihistamines suppressed TDI-induced up-regulation of both H1R and IL-4 mRNAs in TDI-sensitized rats (34). Direct administration of IL-4 into the nasal cavity of non-TDI-treated normal rats up-regulated the expression of H1R mRNA (Fig. 6). Up-regulation of H1R mRNA expression by IL-4 was also reported in HUVECs and rheumatoid synovial fibroblasts (53, 54). On the other hand, intranasal application of histamine for 1 wk caused an increase in IL-4 mRNA elevation in normal rats (Fig. 7a). However, single administration with histamine did not significantly increase IL-4 mRNA level (Fig. 7b). We think that this duration of treatment with histamine is necessary for IL-4-synthesizing cells to migrate to the nasal mucosa in response to histamine, although we have not identified which cells are responsible for IL-4 synthesis. These findings suggest the existence of cross-talk between histamine and IL-4, in which histamine up-regulates IL-4 gene expression through H1R, and IL-4, in turn, up-regulates the expression of H1R mRNA and increases in the expression of H1R. In our previous study, we showed that stimulation of HeLa cells with histamine causes up-regulation of H1R mRNA (35). As suppression of this histamine induced up-regulation of H1R by d-Chlorpheniramine, this histamine-induced H1R up-regulation in HeLa cells is mediated by H1R (35). Additionally, IL-4 also induced up-regulation of H1R mRNA in HeLa cells (Fig. 5b and S. Hori, K. Fujimoto, H. Mizuguchi, and H. Fukui; submitted for publication). Thus, it is likely that IL-4 is one of the candidates responsible for H1R mRNA up-regulation in TDI-sensitized rats.

These findings suggest the existence of a "vicious circuit" between histamine and IL-4, in which histamine up-regulates IL-4 gene expression through H1R, and IL-4, in turn, up-regulates the expression of H1R mRNA, and increases in the expression of H1R make cells more sensitive to histamine and exacerbate the allergic symptoms. As shown in Figs. 3a and 5, suplatast suppressed the TDI-induced mRNA elevations of both H1R and IL-4. Consequently, suplatast suppresses not only H1R-mediated but also IL-4-induced H1R gene expression and may shut down the histamine-IL-4 vicious circuit and ameliorate allergic symptoms in TDI-sensitized rats. The finding that stimulation of H1R with histamine caused up-regulation of histamine-induced H1R expression in HeLa cells suggests the existence of another vicious circuit between histamine and H1R, in which the histamine-induced up-regulation of H1R gene expression results in increase in the amount of H1R protein and this makes cells more sensitive to histamine and may exacerbate the allergic symptoms. Decrease in the histamine content of nasal lavage fluid by suplatast could shut down this second vicious circuit in the nasal mucosa. Suplatast failed to inhibit histamine-induced up-regulation of H1R mRNA in HeLa cells (Fig. 8a), while suplatast suppressed IL-4-induced H1R up-regulation in a dose-dependent manner (Fig. 8b). These data suggest that suplatast has no direct effect on the H1R-mediated pathway for H1R up-regulation but suppresses the IL-4-mediated pathway in HeLa cells. The inhibitory effect of suplatast on TDI-induced H1R-mediated H1R up-regulation in TDI-sensitized rats is due to decrease in the histamine content through suppression of HDC transcription.

Recently, Johnson et al. developed and characterized a murine model of TDI-induced rhinitis (55). They showed that inflammation induced by TDI is associated with a Th1/Th2 mixed immune response and showed that the lower airways showed no evidence of inflammation and that the inflammation and gene expression changes were isolated to the upper airways. In this respect, TDI-induced rhinitis is different from allergen-induced rhinitis, and our data may not be able to explain all the effect of suplatast observed clinically. Nevertheless, they are valuable to explain some recent clinical data. Shimizu et al. showed that suplatast inhibited Ag-induced mucus hypersecretion and eosinophil infiltration when it was given in the effector phase, that is, treatment for 3 days before OVA provocation in OVA-sensitized mice (32). They also showed there was no effect when suplatast is administered in induction phase. Our preliminary study showed that repeated pretreatment with suplatast for >3 days suppressed TDI-induced nasal symptoms and up-regulation of H1R, HDC, and IL-4 mRNAs in the...
nasal mucosa more effectively than did its single administration, which showed no significant effect (data not shown). These two findings indicate that long-term treatment with suplatast is effective in both TDI-induced and allergen-induced rhinitis. Nagakura et al. compared the efficacy and safety of omalizumab, anti-IgE Ab, with suplatast and concluded that omalizumab was more effective than suplatast tosilate against patients with moderate to severe Japanese cedar pollen-induced seasonal allergic rhinitis (56). In such patients, the serum-free IgE level is extremely high, and suppressive effect of omalizumab on the decrease in free IgE level should be much stronger than suplatast. Therefore, chemical mediators other than histamine, such as leukotrienes and prostaglandins, ameliorate allergic symptoms in suplatast-treated patients even though suplatast inhibits histamine signaling. It is well known that H1 antihistamines inhibit histamine signal by their binding to H1R. Recently, Yoshihara et al. investigated the effectiveness of early intervention with suplatast for prophylaxis of pediatric atopic asthma and compared the efficacy of suplatast with ketotifen, one of the H1 antihistamines, and found that suplatast is more effective for the primary prevention of wheezing and asthma in children than ketotifen (57). Their findings can be explained by our data. Suplatast inhibits both H1R-mediated and IL-4-induced H1R up-regulation. On the other hand, H1 antihistamines inhibit only H1R-mediated H1R up-regulation. Accordingly, suplatast is more effective than ketotifen.

In conclusion, we have shown that suplatast alleviates the nasal symptoms of AR in TDI-sensitized rats. Its mechanism would be related to its inhibitory effect on H1R-mediated and IL-4-mediated H1R gene up-regulations induced by TDI. Suppression of TDI-induced up-regulation of IL-4 mRNA could cause suppression of IL-4-mediated H1R gene up-regulation. Additionally, suppression of HDC gene expression and histamine release also could result in suppression of H1R-mediated H1R gene up-regulation. We have also shown that histamine is not only a mere chemical compound released in response to stimulations but also could be a key regulator that controls the histamine IL-4 vicious circuit that plays a crucial role in exacerbation of AR symptoms.

Disclosures

The authors have no financial conflicts of interest.

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