

Expression and Localization of Purinergic P2Y₁₂ Receptor in Human Nasal Mucosa

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ABSTRACT

Background: Extracellular nucleotides such as ATP and UTP are released from essentially all cells, and they interact with cell surface P2 receptors to produce a broad range of physiological responses. P2Y₁₂ receptor is the major platelet receptor that mediates ADP-induced aggregation, P2Y₁₂ receptor inhibitors such as clopidogrel and prasugrel inhibit platelet aggregation, and thus, they are used in the treatment and prevention of coronary artery disease. Recently, studies have focused on the P2Y₁₂ receptor as a receptor for leukotriene E₄ (LTE₄), because this receptor is required for LTE₄-mediated pulmonary inflammation. To establish the presence of P2Y₁₂ receptor in human nasal mucosa, we investigated the expression and the localization of the P2Y₁₂ receptor in human nasal mucosa.

Methods: Human turbinates were obtained by turbinectomy from 12 patients with nasal obstruction refractory to medical therapy. The expression of P2Y₁₂ receptor was evaluated by RT-PCR, western blotting, and immunohistochemical analysis.

Results: RT-PCR analysis of total RNA extracted from human nasal turbinate, primary cultured human nasal epithelial cells and nasal vascular endothelial cells demonstrated the expression of P2Y₁₂ receptor mRNA. A band of approximately 55 kDa was detected in human turbinates by western blot analysis using anti-P2Y₁₂ receptor antibody. We could not find any differences between P2Y₁₂ receptor levels in allergic and non-allergic nasal mucosa. An immunohistochemical study revealed that epithelial cells, submucosal glands and vascular endothelial cells showed intense immunoreactivity for the P2Y₁₂ receptor.

Conclusions: The results may have important clinical implications for understanding the role of P2Y₁₂ receptor in upper airway diseases such as allergic rhinitis and non-allergic rhinitis.

KEY WORDS

allergic rhinitis, immunohistochemistry, P2Y₁₂ receptor, purinergic receptor, western blot

INTRODUCTION

Extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) regulate physiological responses through a multi-gene family of P2 receptors. P2 receptors are divided into 2 major classes: P2X receptors, which are nonselective cation channels and P2Y receptors, which are members of the G protein-coupled receptor (GPCR) superfamily.¹ P2Y₁₂ receptor is the major platelet receptor that mediates ADP-induced aggregation,² and P2Y₁₂ inhibitors such as clopidogrel and prasugrel are used in the treatment and prevention of coronary artery diseases.³ Re-

cently, the P2Y₁₂ receptor has been studied as the receptor for leukotriene E₄,⁴ because it was shown that not only ADP but also leukotriene E₄ (LTE₄) activates P2Y₁₂ receptor at nanomolar concentrations.⁵ Administration of exogenous LTE₄ into human airways causes airway obstruction and mucosal eosinophilia in patients with asthma,⁶⁻⁸ and purinergic P2Y₁₂ receptor is required for LTE₄-mediated pulmonary inflammation.^{9,10} Therefore, to understand the role of LTE₄ in allergic airway diseases, information about the localization of P2Y₁₂ receptor is very important. However, only a limited number of studies have been performed in this field. In the present study, western

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Table 1 Demographic characteristics of allergic and non-allergic patients

	Allergic rhinitis N = 6	Non-allergic patients N = 6
Sex (male/female)	2/4	3/3
Age (years)	31 (19-58)	39 (28-55)
Specific IgE to house dust mite (d1) (kU/L)	2.7 (1.0-13)	<0.35
Total IgE (kU/L)	210 (10-387)	110 (10-185)
Blood eosinophils (cells/ μ L)	370 (70-690)	135 (55-240)
Current nasal symptoms (number of patients)		
Nasal obstruction	6 (all patients)	6 (all patients)
Sneezing	4	0
Rhinorrhea	3	2

Data expressed as median values and range (in parentheses).

blotting, immunohistochemistry and RT-PCR analysis for P2Y₁₂ receptor were performed to confirm the expression of P2Y₁₂ receptor mRNA and protein in human allergic and non-allergic nasal mucosa.

METHODS

TISSUE PREPARATION

Human inferior turbinates were obtained after turbinectomy from 12 patients with nasal obstruction that was refractory to medical therapy. Informed consent was obtained from all patients, and this study was approved by the ethics committee of Sapporo Medical University (approval number: 24-14). All patients were nonsmokers, of which 6 had perennial allergy to mites, as diagnosed through questionnaires and the CAP test (Pharmacia, Uppsala, Sweden). The remaining 6 patients, who had concomitant septoplasty because of symptomatic nasal septal deviation, with a negative CAP test for major inhaled allergens such as mites, birch, grass, mugwort, and Japanese cedar, were included in the non-allergic rhinitis group as control. All medications, including antibiotics, were prohibited for at least 3 weeks prior to the study. The demographic and clinical characteristics of the patients are summarized in Table 1. The nasal mucosal specimens were (1) immediately frozen in liquid nitrogen and stored at -70°C for RNA and protein extraction for RT-PCR and western blotting, respectively, (2) placed into cold transfer medium (RPMI 1640 medium) for epithelial cell and vascular endothelial cell culture, and (3) fixed in 10% formalin for immunohistochemistry.

HUMAN NASAL VASCULAR AND EPITHELIAL CELL CULTURE

Vascular Endothelial Cell Culture

Human nasal vascular endothelial cells (HNVECs) were isolated from nasal inferior turbinates according

to a previously described protocol with minor modifications.¹¹ The nasal specimen was cut into 2-mm² sections and enzymatically digested using 0.2% collagenase type IV solution (Sigma, St. Louis, MO, USA) for 5 min at 37°C, washed with MCBF 131 medium (Sigma) containing 5% FCS and 2 ng/mL vascular endothelial growth factor (Invitrogen, Carlsbad, CA, USA), and placed in collagen type-I-coated 6-well culture plates (Sumitomo Bakelite, Osaka, Japan). After 24 h, the medium and the tissue pieces were discarded, and the culture plate was washed twice to remove floating cells. Fresh MCBF 131 medium (Sigma) containing 5% FCS and 2 ng/mL vascular endothelial growth factor was added, and the cells were cultured in a 5% carbon dioxide humidified atmosphere at 37°C. The culture medium was changed at day 1 and every 2 days thereafter. Monolayer cell confluence was achieved after 7-10 days of culture. Morphologic observations using a phase contrast microscope showed that HNVECs consisted primarily of vascular endothelial cells. More than 95% of the HNVECs showed positive reactions for anti-human CD31 antibody (Dako, Denmark). HNECs grown to 80% confluency were used for RT-PCR analysis and western blotting.

Epithelial Cell Culture

Human nasal epithelial cells (HNECs) were isolated from human nasal mucosa specimens according to a previously described protocol.¹² Nasal specimens were rinsed 2-3 times with Ham's F-12 medium (Sigma) supplemented with antibiotics and incubated in 0.1% protease type XIV in Ham's F-12 medium for 16 h at 4°C. After incubation 10% fetal bovine serum was added to neutralize protease activity and epithelial cells were detached by gentle agitation. Cell suspensions were filtered through a 60-mesh cell dissociation sieve (Sigma) and centrifuged at 500×g for 10 min at room temperature. The cell pellet was then resuspended in hormonally defined Ham's F-12 culture medium (Ham's HD). Cell suspensions were plated onto collagen type-I-coated 6-well culture plates (Sumitomo Bakelite) in Ham's HD medium and cultured in a 5% carbon dioxide humidified atmosphere at 37°C. The culture medium was changed at day 1 and every 2 days thereafter. Monolayer cell confluence was achieved after 6-8 days of culture. Morphologic observations using a phase contrast microscope showed that the HNECs consisted primarily of epithelial cells. More than 95% of the HNECs showed positive reactions for anti-human cytokeratin antibody (Dako). HNECs grown to 80% confluency were used for RT-PCR analysis.

RT-PCR Analysis

Total RNA from nasal mucosa was extracted using TRIzol reagent (Invitrogen). Some of the RNA samples (1 μ g/ μ L) were treated with DNase I (10 U/ μ L)

for 30 min to avoid DNA contamination following the second RNA extraction. RT-PCR was carried out using a commercial One Step RNA PCR Kit (Takara Biomedicals, Tokyo, Japan). RNA (50 ng) was reverse-transcribed into cDNA by incubating with 5 U AMV Reverse Transcriptase XL at 50°C for 30 min. After denaturation at 94°C for 2 min, cDNA was amplified by 40 cycles of PCR (94°C for 30 s, 60°C for 30 s, and 72°C for 1 min) with 5 U of AMV-Optimized Taq DNA polymerase to amplify the genes of the P2Y₁₂ receptor. For P2Y₁₂ receptor, the forward primer: 5'-CTAAGATTCTCTCTGTTGTCATCTG-3' and the reverse primer: 5'-ACAGAGTGCTCTCTTTCACATAG-3' were used to generate a 432-bp fragment.¹³ PCR products were analyzed by agarose electrophoresis in a 1.5% gel and visualized by ethidium bromide staining.

Western Blot Analysis

For the western blot of P2Y₁₂ receptor, rabbit anti-human P2Y₁₂ receptor polyclonal antibody (product number P4871, Sigma) was used at 4 µg/mL.

Nasal mucosa and primary cultured nasal cells were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with a protease inhibitor cocktail (Sigma). The protein extracts were purified using PAGEprep Advance Kit (Thermo Fisher Scientific), and the protein concentration in the homogenates was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Protein extracts (20 µg protein each) were separated on a 4-12% sodium dodecyl sulphate (SDS)-polyacrylamide gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen). Membranes were then blocked and probed with rabbit anti-human P2Y₁₂ receptor polyclonal antibody (4 µg/mL; Sigma) or rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as an internal control. The bands were visualized using a WesternBreeze chromogenic western blot immunodetection kit (Invitrogen). The ratio of the expression of P2Y₁₂ receptor protein to GAPDH protein was evaluated by densitometry.

Immunohistochemistry

For immunohistochemistry of the P2Y₁₂ receptor, rabbit anti-human P2Y₁₂ receptor polyclonal antibody (product number P4871, Sigma) was used at 20 µg/mL. Deparaffinized sections were initially incubated with 3% H₂O₂ in methanol for 30 min to quench the endogenous peroxidase activity. After microwave treatment (10 min at 500 W in citrate buffer), the sections were incubated in the blocking solution (10% normal goat serum in PBS) for 30 min before incubation with the primary antibody. Then, the sections were incubated with anti-P2Y₁₂ receptor polyclonal

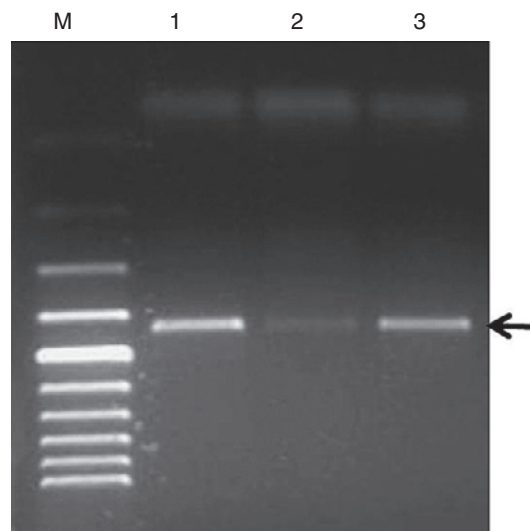


Fig. 1 Detection of P2Y₁₂ receptor mRNA by RT-PCR after 40 cycles of amplification from primary cultured human nasal epithelial cells, primary cultured human vascular endothelial cells, and inferior turbinate. RNA was extracted, and RT-PCR was performed using P2Y₁₂ receptor primers, generating a 432-bp fragment. Lane M: 100 bp ladder. Lane 1: PCR products from total RNA of primary cultured human nasal epithelial cells. Lane 2: PCR products from total RNA of primary cultured human nasal vascular endothelial cells. Lane 3: PCR products from total RNA of human inferior turbinate.

antibody overnight at 4°C, washed, and incubated for 30 min with Alexa Fluor 488-labelled goat anti-rabbit IgG (diluted 1 : 100; Molecular Probes, OR, USA). Sections were mounted with SlowFade antifade kits (Molecular Probes) and examined under an Olympus BX51 microscope with a DP70 CCD camera (Olympus Optical, Tokyo, Japan). All images were processed with DP Controller and DP Manager software (Olympus Optical) for image analysis. Negative controls were obtained by replacing primary antibodies with rabbit immunoglobulin fraction (Dako).

Statistical Analysis

The protein levels of P2Y₁₂ receptor and GAPDH evaluated by western blot analysis were determined in duplicate, and data are presented as mean ± standard deviation. The non-parametric Mann-Whitney U test was used to determine the differences between perennial allergic rhinitis and non-allergic rhinitis. Values with $P \leq 0.05$ were considered statistically significant.

RESULTS

RT-PCR ANALYSIS

The results of RT-PCR for 40 cycles using total RNA extracted from human nasal mucosa (Fig. 1, lane 1),

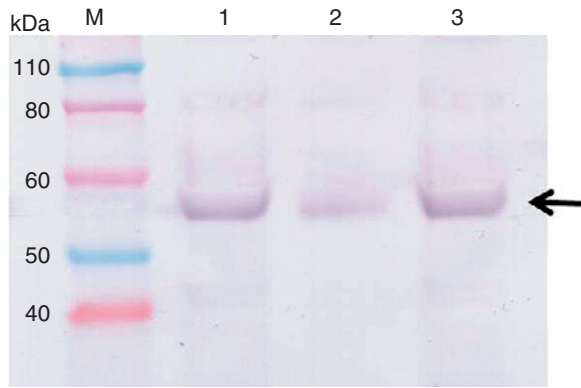


Fig. 2 Representative western blots for the P2Y₁₂ receptor protein in human nasal mucosa (lane 1: primary cultured human nasal epithelial cells, lane 2: primary cultured human nasal vascular endothelial cells, lane 3: human inferior turbinate). The antibody recognized a single protein of 55 kDa, referred to as the P2Y₁₂ receptor.

primary cultured human nasal vascular endothelial cells (Fig. 1, lane 2), and primary cultured human nasal epithelial cells (Fig. 1, lane 3), revealed the expression of P2Y₁₂ receptor mRNA. These results suggested the presence of P2Y₁₂ receptor mRNA in human nasal epithelial cells and vascular endothelial cells.

WESTERN BLOTTING

In order to determine the protein size of P2Y₁₂ receptor in human nasal mucosa, P2Y₁₂ receptor expression was confirmed by western blot analysis. As shown in Figure 2, the expression of a single band for P2Y₁₂ receptor protein in human nasal mucosa (lane 1: primary cultured human nasal epithelial cells, lane 2: primary cultured human nasal vascular endothelial cells, lane 3: human inferior turbinate) could be demonstrated.

Densitometric quantification of the bands was performed to evaluate the expression levels of P2Y₁₂ receptor (the ratio of P2Y₁₂ receptor/GAPDH) in allergic and non-allergic nasal mucosa (Fig. 3). The difference in P2Y₁₂ receptor expression between allergic and non-allergic nasal mucosa was not significant (allergic rhinitis: 0.52 ± 0.11 , non-allergic patients: 0.43 ± 0.12 , $P = 0.26$; Fig. 3B).

IMMUNOHISTOCHEMISTRY

The distribution of P2Y₁₂ receptor in the nasal mucosa was examined by immunohistochemical analysis. As shown in Figure 4A, significant immunoreactivity for the P2Y₁₂ receptor was detected in epithelial cells, vascular endothelial cells, and submucosal glands. The specificity of the staining was also confirmed by the absence of labeling with normal rabbit immunoglobulin (Fig. 4B).

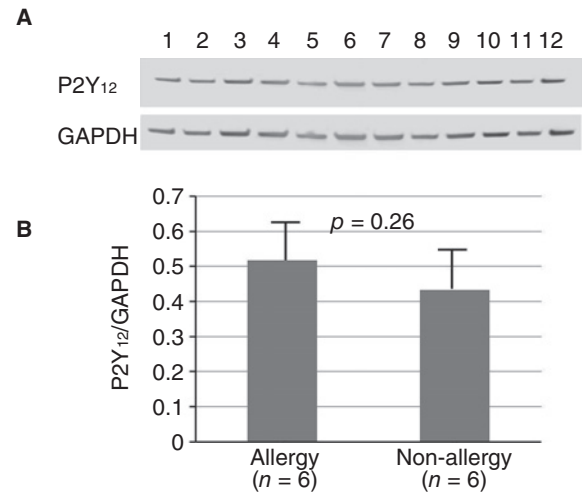


Fig. 3 **A:** Western blots showing P2Y₁₂ receptor protein level in allergic nasal mucosa (lanes 1-6) and non-allergic nasal mucosa (lanes 7-12). **B:** Densitometric analysis of the relative protein level of the P2Y₁₂ receptor is shown. The data are expressed as mean $\pm$ SD and normalized to GAPDH.

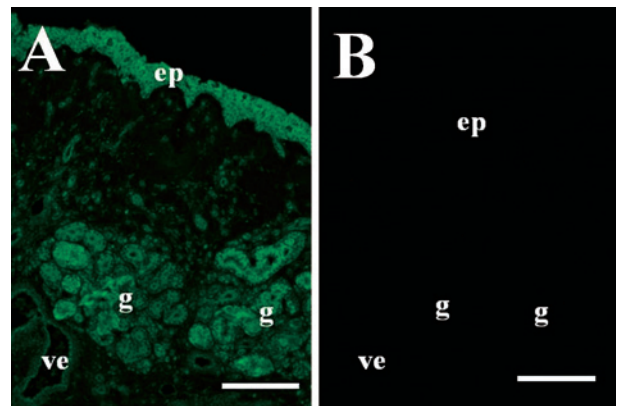


Fig. 4 Immunofluorescence staining for P2Y₁₂ receptor in human nasal mucosa. Inferior turbinates were stained with anti-human P2Y₁₂ receptor antibody (**A**) or normal rabbit immunoglobulin (**B**). P2Y₁₂ receptor immunoreactivity was observed mainly on vascular endothelial cells (ve), epithelium (ep) and submucosal glands (g). Scale bar = 200 μ m.

DISCUSSION

This is the first study to clarify the expression and the localization of the P2Y₁₂ receptor in human nasal mucosa. Although P2Y₁₂ receptor inhibitors such as clopidogrel and prasugrel have been used clinically, P2Y₁₂ receptor has mostly been studied in cardiovascular and cerebrovascular disease.³ In northern blots of human tissue extracts, Hollopeter *et al.* found P2Y₁₂ receptor mRNA in platelets and the brain only.¹⁴ Zhang *et al.* observed strong expression of

P2Y₁₂ receptor in the platelets and in all brain regions (plus spinal cord) tested, weak expression in lungs, pituitary, and adrenal gland, but no expression in any other tissues.¹⁵ The finding of western blot analysis showed that the molecular mass of the nasal P2Y₁₂ receptor is approximately 55 kDa, which is similar to that of P2Y₁₂ receptor found in human platelets and the internal mammary artery,¹⁶ although their expected size is 40 kDa. Such a difference could be because the nasal P2Y₁₂ receptor has post-transcriptional modifications, such as glycosylation and lipoylation.¹⁷ In addition, we could not detect any differences in the P2Y₁₂ receptor expression between allergic and non-allergic nasal mucosae obtained from the non-smoking patients. Our recent experiments using primary cultured nasal epithelial cells revealed no significant difference of P2Y₁₂ receptor level in between allergic and non-allergic epithelial cells (data not shown). To the best of our knowledge, only one study has previously identified an agent that either upregulates or downregulates P2Y₁₂ receptor expression. The study showed the effect of nicotine on P2Y₁₂ receptor expression in four cell lines.¹⁸ It is likely that long-term exposure to nicotine is necessary for P2Y₁₂ receptor up-regulation, because platelets from smokers have higher levels of P2Y₁₂ receptor than platelets from non-smokers.¹⁸ From these observations, it is possible that smoking accelerates LTE₄-induced airway inflammation via up-regulation of P2Y₁₂ receptor. Recently, it was shown that 5 single-nucleotide polymorphisms (SNPs) in the P2Y₁₂ gene are associated with lung function in a large family-based asthma cohort.¹⁹ Thus, the inhibitors of P2Y₁₂ receptor could lead to new therapeutic targets for asthma and allergic rhinitis.

Our present immunohistochemical studies, western blotting, and RT-PCR analysis clearly show the expression of the P2Y₁₂ receptor on not only nasal vascular endothelial cells but also nasal epithelial cells at the mRNA and protein level. With respect to the expression of P2Y₁₂ receptor on nasal epithelial cells, a previous study identified P2Y₁₂ receptor mRNA in human primary cultured nasal epithelial cells by RT-PCR analysis.¹³ The extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) regulate various physiological responses via cell surface receptors namely, purinoreceptors, and they exert autocrine or paracrine effects on ion transport,²⁰ fluid transport,²¹ ciliary beat frequency,^{20,22} and mucin secretion.²³ Thus, the purinoreceptors in nasal epithelial cells may play an important role in nasal secretion under inflammatory conditions. Until recently, there were only a few reports on the expression of subtypes of P2 receptors in nasal epithelial cells.^{13,24} Further, it is still unclear whether the P2Y₁₂ receptor in nasal epithelial cells is functionally active. Recently, we found LTE₄ caused the secretion of MUC5AC mucin from lung epithelial

NCI-H292 cells via activation of P2Y₁₂ receptor (unpublished data), suggesting therapeutic potential of P2Y₁₂ receptor antagonists on allergic mucous secretion. In order to clarify the roles of P2Y₁₂ receptor in nasal epithelial cells, a study using cultured nasal mucosa-derived epithelial cells is now in progress at our laboratory.

In this study, we also found expression of the P2Y₁₂ receptor on vascular endothelial cells. P2Y₁₂ receptor was originally identified in platelets.¹⁴ Subsequent studies have demonstrated that the receptor is expressed in vascular endothelial cells.^{18,25} In endothelial cells, ATP generally causes an increase in [Ca²⁺] via the activation of P2Y receptors, which are coupled to phospholipase C and inositol 1,4,5-triphosphate-induced Ca²⁺ mobilization.¹ ATP increases vascular permeability,^{26,27} vasodilator production such as endothelial nitric oxide synthase (eNOS)²⁸ and expression of intercellular adhesion molecule-1 (ICAM-1)²⁹ in human vascular endothelial cells through a Ca²⁺-dependent pathway. These may be important physiological roles for P2Y receptors in nasal endothelial cells.

The present study showed the significant expression of P2Y₁₂ receptor expression in the nasal submucosal glands. With regard to the P2Y receptor subtypes in human submucosal glands, only the presence of P2Y₂ receptor and P2Y₄ receptor in a cell line (MM39) of human tracheal gland cells has been reported by using RT-PCR.³⁰ To date, no detailed study has reported the existence or the roles of the P2Y₁₂ receptor in airway submucosal glands. Activation of the P2Y receptors on nasal glands may cause mucus secretion, because P2Y-receptor agonist 2-methylthio-ATP evoked an increase in K⁺ and Cl⁻ conductance in avian nasal gland cells.³¹

In conclusion, this study provides important information about the localization of the P2Y₁₂ receptor on the nasal mucosa. The present findings should be of considerable interest for understanding the potential roles of P2Y₁₂ receptor in upper airway diseases such as allergic rhinitis and non-allergic rhinitis.

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