Combined Stimulation of Nasal Polyp Fibroblasts With Poly IC, Interleukin 4, and Tumor Necrosis Factor \(\alpha\) Potently Induces Production of Thymus- and Activation-Regulated Chemokine

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**Objective:** To examine the effects of cytokines and poly IC on the expression of thymus- and activation-regulated chemokine (TARC), a potent chemoattractant for helper T-cell type 2 (TH2) cells, in nasal polyp fibroblasts.

**Design:** Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis.

**Setting:** Academic research.

**Participants:** Primary fibroblast lines were established from human nasal polyp biopsy tissue specimens (n = 5) removed at polypectomy.

**Main Outcome Measures:** The expression of TARC messenger RNA (mRNA) was evaluated by real-time RT-PCR. The amount of TARC in the supernatants was measured by enzyme-linked immunosorbent assay.

**Results:** Combined stimulation with interleukin 4 (IL-4) and tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) or with poly IC and IL-4 induced TARC production. Combined exposure of cells to poly IC, IL-4, and TNF-\(\alpha\) resulted in substantial amounts of TARC release into the culture medium. Quantitative RT-PCR analysis revealed that simultaneous stimulation with those 3 compounds induced a tremendous increase in the amount of TARC mRNA in the nasal polyp fibroblasts.

**Conclusion:** Nasal polyp fibroblasts contribute to TH2 cell infiltration and RNA virus–induced exacerbation of TH2-type airway inflammatory conditions such as allergic chronic sinusitis.

**Arch Otolaryngol Head Neck Surg.** 2008;134(6):630-635

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VIRAL INFECTIONS ARE known to cause airway inflammatory diseases such as acute rhinitis and bronchitis and to exacerbate asthma. Among the many kinds of viruses that infect the airways, the common pathogens that exacerbate asthma include rhino-virus, respiratory syncytial virus, and influenza virus. Nasal polyposis and asthma are expressions of airway inflammatory disease. While one affects the upper airways and the other the lower airways, nasal polyposis and asthma have similar pathologic features, and they often coexist in the same patient. RNA virus infections also exacerbate nasal polyposis. RNA viruses synthesize double-stranded RNA (dsRNA) when replicating in infected cells. Double-stranded RNA stimulates production of type 1 interferons in dendritic cells and fibroblasts and has an important role as a trigger of host defenses against viral infections. In the upper airway, not only leukocytes but also connective tissue cells such as fibroblasts have been reported to secrete chemokines on stimulation with exogenous components such as poly IC, a synthetic analogue of dsRNA.

Nasal polyps may be found in patients with chronic sinusitis. Accordingly, nasal polyps and the paranasal sinus mucosa demonstrate similar histologic pictures in patients with chronic sinusitis. Moreover, chronic sinusitis is often associated with allergy. Histologic studies showed that tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) is expressed and that the densities of helper T-cell type 2 (TH2) cells and eosinophils and the levels of TH2 cytokines (interleukin [IL] 4 and IL-13) are increased in the sinus mucosa and in nasal polyps from patients with sinusitis who have allergy. However, the mechanisms underlying the recruitment of TH2 cells into the sinus mucosa and nasal polyps have yet to be clarified. Airway fibroblasts are major structural components of the mucosa and a rich source of cytokines and chemokines. Therefore, fibroblasts are thought to be involved in the local recruitment of inflammatory cells.
Thymus- and activation-regulated chemokine (TARC) is a member of the CC chemokine family and is a potent and selective chemoattractant for T_{H}2 cells, which express the corresponding chemokine receptor (CCR4) on their surfaces. Therefore, local production of TARC has important roles in the induction and maintenance of T_{H}2 cell infiltration into the sinus mucosa and nasal polyps. In 2003, a synergistic effect of IL-4/IL-13 and TNF-α on the expression of TARC in corneal and skin fibroblasts was reported. In this study, we investigated whether nasal polyp fibroblasts produce TARC on stimulation with poly IC, IL-4, and TNF-α.

**METHODS**

**REAGENTS**

Interleukin 4 and TNF-α were purchased from Genzyme Co (Cambridge, Massachusetts). Toll-like receptor 3 ligand (synthetic analogue of dsRNA [poly IC]) was purchased from InvivoGen Co (San Diego, California).

**CELL SOURCE AND CULTURE**

Primary fibroblast lines were established from human nasal polyp biopsy tissue specimens (n=5) removed at polypectomy and characterized as previously described. Only fibroblast lines between the fourth and seventh passages were used in this study. All nasal polyp specimens had been obtained from patients with allergic chronic sinusitis (5 men; mean [SEM] age, 44.4 [9.69] years). All patients were atopic, diagnosed on the basis of elevation of at least 1 specific IgE against 8 common aeroallergens (5 men; mean [SEM] age, 44.4 [9.69] years). All patients were atopic, diagnosed on the basis of elevation of at least 1 specific IgE against 8 common aeroallergens that were tested. All subjects gave written informed consent, and the study protocol was approved by the Ethics Committee of Nippon Medical School Hospital, Tokyo, Japan.

Before cytokine assay, fibroblasts (1×10^6 cells) were plated in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, New Jersey). Before real-time polymerase chain reaction, fibroblasts (2×10^6 cells) were plated in 60-mm culture dishes (Corning Inc, Corning, New York). The plated fibroblasts were allowed to grow to confluence in regular growth medium (Dulbecco modified Eagle medium) containing a combination of fetal bovine serum (10%), penicillin G sodium chloride (100 U/mL), streptomycin sulfate (100 µg/mL), and amphotericin B (2.5 µg/mL). The same culture medium was used when fibroblasts were exposed to various stimuli. At specified time points, supernatants were collected and centrifuged for cytokine protein assay, and cells were collected for RNA extraction.

**MEASUREMENT OF TARC RELEASE**

Nasal polyp fibroblasts were cultured with poly IC, IL-4, and TNF-α, alone and in combination. After 72 hours of culture, supernatants were collected, and TARC concentrations were determined. In dose-dependent experiments, cells were cultured with combinations consisting of a series of concentrations of poly IC, IL-4 (10 ng/µL), and TNF-α (10 ng/µL); a series of concentrations of IL-4, poly IC (50 µg/µL), and TNF-α (10 ng/µL); and a series of concentrations of TNF-α, poly IC (50 µg/µL), and IL-4 (10 ng/µL). In time-course experiments, cells were treated with poly IC (50 µg/µL), IL-4 (10 ng/µL), and TNF-α (10 ng/µL). Supernatants were assayed for TARC after 12, 24, 36, 48, 60, 72, 84, and 96 hours of culture using enzyme-linked immunosorbent assay (ELISA).

**ANALYSIS OF TARC MESSENGER RNA**

We investigated the effects of poly IC, IL-4, and TNF-α, alone and in combination, on the TARC messenger RNA (mRNA) in nasal polyp fibroblasts. The cells were cultured for 48 hours, after which the amount of TARC mRNA in cell lysates was assayed by quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR).

Total cellular RNA was extracted and purified (RNeasy mini kit; Qiagen GmbH, Hilden, Germany). The total RNA (2 µg) was reverse transcribed at 37°C for 60 minutes using random primers (Takara, Kyoto, Japan) and reverse transcriptase (Omniscript, Qiagen GmbH) according to the manufacturers’ protocols.

Quantitative real-time RT-PCR was performed (TaqMan assay and ABI Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, California). The primers and fluorogenic probes for TARC and β-actin were purchased from Applied Biosystems. The amplification conditions were 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Data analysis was performed using sequence detector system software (Applied Biosystems). Threshold cycles were used to calculate arbitrary mRNA concentrations by using the relative standard curve method. The standard curve was constructed using serial dilutions of complementary DNA containing the message for TARC. The level of TARC mRNA was normalized to the level of β-actin mRNA.

**CYTOKINE ASSAY**

The levels of TARC in culture supernatants were measured by ELISA using a commercially available kit (human TARC ELISA; R and D Systems, Minneapolis, Minnesota), which detects recombinant and natural TARC. The sensitivity of this assay system was greater than 7.0 pg/mL. These data are presented as picograms of TARC per 1×10^6 cells.

**STATISTICAL ANALYSIS**

Paired Wilcoxon test was used for statistical analysis. P < .05 was considered statistically significant.

**RESULTS**

**IL-4, TNF-α, AND POLY IC INDUCTION OF TARC PRODUCTION BY NASAL POLYP FIBROBLASTS**

TARC was negligible in the supernatants of nonstimulated cells. No effect on TARC release was seen with poly IC, IL-4, and TNF-α. However, combined stimulation with IL-4 and TNF-α and with poly IC and IL-4 induced TARC release by the fibroblasts. Simultaneous exposure of the cells to poly IC, IL-4, and TNF-α resulted in substantial amounts of TARC release into the culture medium (Figure 1).

The release of TARC by the cells increased in a dose-dependent manner with each of the combinations (Figure 2). In time-course experiments, the TARC release by the fibroblasts started after 24 hours and increased sharply between 36 hours and 72 hours in response to simultaneous stimulation with poly IC, IL-4, and TNF-α (Figure 3).
EXPRESSION OF TARC GENE IN NASAL POLYP FIBROBLASTS

No significant effect on the amount of TARC gene mRNA was seen with either TNF-α or IL-4. In contrast, stimulation with poly IC, poly IC and TNF-α, poly IC and IL-4, or IL-4 and TNF-α induced significant amounts of TARC mRNA. Simultaneous exposure of cells to poly IC, IL-4, and TNF-α induced a tremendous increase in the abundance of TARC mRNA compared with that in nonstimulated cells (Figure 4).

COMMENT

In the present study, we demonstrated that combined stimulation of cultured nasal polyp fibroblasts with poly IC, IL-4, and TNF-α resulted in substantial amounts of TARC release into the culture medium in a time- and dose-dependent manner. Similarly, quantitative RT-PCR analysis revealed that the combined stimulation induced a tremendous increase in the amount of TARC mRNA in the fibroblasts. Given that chronic sinusitis is often associated with allergy and that the expression of TH2 cytokines (such as IL-4 together with a proinflammatory cytokine, TNF-α) is enhanced in the sinus mucosa and in nasal polyps from patients with sinusitis who have allergy, our results suggest that RNA virus–induced activation of fibroblasts in the upper airway may contribute to the pathogenesis of TH2-type inflammation by promoting the infiltration of TH2 cells, leading to eosinophilic inflammation.

The paranasal sinuses are the target of a substantial number of infectious agents, including viruses. Acute vi-
nal infections often predispose patients to the development of chronic sinusitis. Chronic sinusitis is characterized by edema, fibroblast hyperplasia, and persistent inflammation that can lead to narrowing of the sinus ostia with further obstruction of the sinus drainage, leading to secondary bacterial infection. Recent evidence suggests that viral infections may contribute to not only acute inflammatory reactions but also persistent inflammation in sinusitis. Continuous rhinovirus infection of turbinate epithelial cells in chronic sinusitis has been demonstrated. It was also reported that, in patients with allergic sinusitis, nasal polyps contain large numbers of IL-4- and TNF-α–positive cells. Although TGF-β cells are considered to be the primary source of these cytokines, mast cells and eosinophils also can produce these cytokines. In fact, all 3 cell types express these cytokines in nasal polyps.

Histologic findings demonstrated that sinus tissues from patients with allergic sinusitis were extensively infiltrated with eosinophils. Along with eosinophils, the density of TGF-β cells and the levels of TGF-β cytokines are also increased in the sinus mucosa and in nasal polyps from patients with allergic sinusitis. Considering our results, viral infections in a TGF-β cytokine– and TNF-α–rich milieu may tremendously promote TARC production by nasal polyp fibroblasts, resulting in recruitment of TGF-β cells into the upper airway.

Although the major cellular targets for respiratory RNA viruses are the upper and lower respiratory epithelial cells, rhinovirus was able to infect airway fibroblasts and induced chemokine expression. The viral infection might have damaged the infected epithelial cells, causing leakage of virus-derived dsRNA that stimulates the surrounding cells, including fibroblasts. The respiratory epithelial cells of patients with asthma release more viral RNA than the respiratory epithelial cells of healthy subjects. This is because the respiratory epithelial cells of patients with asthma have a reduced capacity for production of interferon beta, resulting in increased replication of the infecting virus. In addition, cellular necrosis is accompanied by membrane disruption. Leakage of rhinovirus-derived RNA into the blood during upper respiratory tract rhinovirus infections is more prominent in persons with asthma than in those without asthma. Besides viral RNA, cellular RNA leaked from, or associated with, necrotic cells is capable of activating toll-like receptor 3 with its secondary structures that contain double-stranded sequences.

Investigations of TARC expression and the effects of neutralizing antibodies to TARC in vivo have revealed an important role for this chemokine in TGF-β cell–mediated inflammation. In addition to immune cells such as monocytes and dendritic cells, tissue-resident cells such as epithelial cells and fibroblasts in the nasal mucosa, epithelial cells in the lung fibroblasts but not epithelial cells in the cornea, and epithelial cells and fibroblasts in the skin produce TARC in response to cytokines and bacterial products such as lipopolysaccharide. Our data reinforce the notion that nasal fibroblasts could be a main source of TARC, particularly in the case of viral infections of the upper airway.

The mechanisms underlying how TARC production is induced by nasal polyp fibroblasts remain obscure. In 2001, it was shown that poly IC or TNF-α leads to activation of nuclear factor κB (NF-κB), which regulates TARC expression in epithelial cells. Interleukin 4 induces TARC expression via STAT6 in human T cells. Matsukura et al demonstrated that eotaxin promoter contains overlapping consensus binding sites for 2 transcription factors, NF-κB and STAT6, and that stimulation with these 2 transcription factors results in further activation of the promoter. It is possible that NF-κB and STAT6 induced by poly IC and IL-4 and by IL-4 and TNF-α act positively at the overlapping binding sites on the TARC promoter. The synergistic TARC production seems to be induced mainly by postreceptor events after the binding of each stimulus to its receptor but may also involve induction of receptors. It has been demonstrated that stimulation with TNF-α induced a 2- to 3-fold increase in IL-4 receptor α-chain expression in endothelial cells and that poly IC up-regulated IL-4 receptor α-chain expression in epithelial cells. Therefore, the potent induction of TARC by simultaneous stimulation with poly IC, IL-4, and TNF-α may be, in part, heralded by receptor events. Further studies will be necessary to understand the mechanism by which TARC production occurs in our experimental system.

Asthma and allergic chronic sinusitis, as well as nasal allergy, are expressions of TGF-β-type airway inflammatory disease and share the same characteristic pathogenic features (ie, the presence of inflammatory cells such as activated eosinophils, mast cells, and TGF-β cells). These cells may be the main source of IL-4 and TNF-α. Respiratory viral infections are thought to be a cause for exacerbation of these diseases. Recently, Stephens et al reported that circulating TGF-β cells in the blood of allergic individuals entered the airways in response to viral infection and became activated to trigger allergies. Accordingly, it is speculative whether IL-4 and TNF-α, which are released from these cells, are capable of potently inducing TARC production by fibroblasts in the presence of RNA virus infections. TARC, which is released lo-
Thymus- and activation-regulated chemokine (TARC) messenger RNA (mRNA) expression in nasal polyp fibroblasts. Nasal polyp fibroblasts were incubated for 48 hours with poly IC (50 µg/mL), interleukin 4 (IL-4) (10 ng/mL), and tumor necrosis factor α (TNF-α) (10 ng/mL), alone and in combination. Total RNA was isolated from the nasal polyp fibroblasts. After complementary DNA synthesis, the analysis for TARC mRNA was performed. The ratio of TARC mRNA to β-actin mRNA was determined. Box plots represent median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; error bars, 10th and 90th percentiles. *P<.05 compared with control cultures. †P<.05 compared with cultures stimulated with IL-4.

In conclusion, we have shown that poly IC, IL-4, and TNF-α act positively on nasal polyp fibroblasts to augment TARC production. This finding supports the hypothesis that infection with an RNA virus such as rhinovirus together with an IL-4- and TNF-α-rich milieu induces fibroblasts to produce TARC explosively. We believe that this scenario may, in part, explain how upper airway inflammatory diseases such as allergic chronic sinusitis are exacerbated when an RNA virus infection occurs in the upper airway.

Submitted for Publication: September 19, 2007; final revision received November 7, 2007; accepted November 11, 2007.

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Drafting of the manuscript: Nonaka, Oghara, Fukumoto, and Sakamushi. Critical revision of the manuscript for important intellectual content: Nonaka, Pawankar, and Yagi.


Financial Disclosure: None reported.

Funding/Support: This work was supported by grant-in-aid for scientific research 17591804 from the Ministry of Health, Labor, and Welfare, Japan (Dr Nonaka).

Additional Contributions: Sachiko Saito, BS, provided technical assistance.

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