



Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils

Maho Suzukawa¹, Rikiya Koketsu¹, Motoyasu Iikura², Susumu Nakae³, Kenji Matsumoto³, Hiroyuki Nagase⁴, Hirohisa Saito³, Kouji Matsushima⁵, Ken Ohta⁴, Kazuhiko Yamamoto¹ and Masao Yamaguchi¹

Eosinophils are important effector cells in allergic diseases, but the mechanisms regulating their biological functions remain obscure. Interleukin-33 (IL-33) is a recently identified cytokine of the IL-1 family, and it reportedly accelerates the production of Th2-associated cytokines and promotes tissue inflammation. However, the action of IL-33 on effector cells such as eosinophils has remained unclear. In this study, we investigated the effects of IL-33 on eosinophil activation, assessed in terms of the cells' adhesiveness, expression of CD11b and apoptosis. Adhesiveness was quantified by measuring eosinophil peroxidase content of adherent eosinophils, and expression of CD11b was measured by flow cytometry. Apoptosis was determined by flow cytometry based on the ability of cells to bind annexin V. Real-time PCR analysis showed that eosinophils expressed mRNA for ST2, a putative receptor for IL-33. IL-33 at 1–100 ng/ml enhanced the adhesiveness and CD11b expression of eosinophils even more potently than IL-5. IL-33 maintained the viability of eosinophils. Treatment with neutralizing antibodies to ST2 eliminated the effects of IL-33 on eosinophil CD11b expression and cell survival. However, IL-33 did not elicit degranulation or leukotriene C4 synthesis in eosinophils. These findings indicate that IL-33 potently induces eosinophil adhesion and CD11b expression and enhances eosinophil survival. The IL-33-ST2 pathway might be an important regulator of eosinophil biology in the pathogenesis of Th2-biased allergic diseases.

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Blood and local tissue eosinophilia is an outstanding feature of allergic diseases and other disorders such as helminthic parasitic infections and various neoplasms.¹ In the allergic inflammation observed locally in bronchial asthma and atopic dermatitis, eosinophils constitute the major line of effector cells. They possess the capacity to generate and release a wide array of preformed mediators such as major basic protein and eosinophil peroxidase (EPO), as well as newly synthesized mediators including leukotriene (LT) C4 and platelet-activating factor.^{2,3} These mediators can cause tissue destruction, modify the smooth muscle tone and vascular permeability and also attract and activate other inflammatory cells. Eosinophils are thus thought to be an important source of proinflammatory mediators. However, only little has been known about which factors are primarily

responsible for the accumulation and activation of eosinophils *in vivo*.

ST2, also called DER4, Fit-1 or T1, is a member of the IL-1 receptor family originally identified as a serum-inducible secreted protein in murine fibroblasts.⁴ ST2 cDNAs have been also cloned from humans⁵ and rats.⁶ This receptor is expressed in both soluble and membrane-bound forms as a result of differential splicing,⁷ although the expression pattern of ST2 protein differs between humans and mice. ST2 is stably expressed on mouse Th2 cells, but not on mouse Th1 cells.⁸ For this reason, this molecule is considered to be a stable cell marker on murine Th2 effector cells. On the other hand, ST2 is inducible in human Th2 cells, and human Th2 cells express ST2 on their cell surface and secrete ST2 following activation.⁹ Several lines of evidence suggest that

¹Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; ²Department of Respiratory Medicine, National Disaster Medical Center of Japan, Tokyo, Japan; ³Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan;

⁴Department of Respiratory Medicine, University of Teikyo School of Medicine, Tokyo, Japan and ⁵Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Correspondence: Dr M Suzukawa, MD, PhD, Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.

E-mail: fueta-ky@um.in.ac.jp

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the ST2 expressed on Th2 cells⁹ and mast cells¹⁰ is linked to important Th2 effector functions.⁸ Exogenous administration of soluble ST2 has been demonstrated to effectively neutralize the putative ligand, resulting in alleviation of inflammation by abrogating Th2 cytokine production and induction of the eosinophilic inflammatory response.¹¹ Moreover, mice deficient in ST2 did not develop a Th2 response to *Schistosoma* egg antigen.¹² In addition, although the ligand for ST2 had not been known for years, elevated levels of the soluble form of ST2 were reported to be present in the circulation of patients with various inflammatory diseases.¹³ These results have collectively suggested that ST2 may be an important receptor mediating various inflammatory reactions.

Schmitz *et al*¹⁴ recently identified a new cytokine, interleukin-33 (IL-33), which mediates its biological effects through ST2 and accelerates production of Th2-associated cytokines by *in vitro* polarized Th2 cells. Furthermore, *in vivo* experiments have revealed that exogenous administration of IL-33 markedly increased expression of IgE, IgA, IL-4, IL-5 and IL-13 in the serum and led to obvious pathological changes including eosinophilic and mononuclear infiltration of arterial walls, lungs and intestinal tissues, increased mucus production and epithelial cell hyperplasia and hypertrophy. A very recent study showed that IL-33 induces IL-8 secretion and autocrine production of IL-13 in human cultured mast cells.¹⁵

Although these novel findings seem to imply that IL-33 potentiates the effector functions of Th2 cells and mast cells, there have been no report showing whether IL-33 acts directly on allergic inflammatory granulocytes such as eosinophils, or whether these inflammatory cells possess functional ST2. Therefore, we for the first time conducted a series of analyses designed to detect IL-33-induced eosinophil activation *in vitro*. In this report, we demonstrate that ST2 is expressed on eosinophils, and that IL-33 affects the viability, increases the adhesiveness and upregulates CD11b expression of human eosinophils.

MATERIALS AND METHODS

Reagents

The following reagents were purchased as indicated: human recombinant IL-33 (Adipogen Inc., Seoul, South Korea); human recombinant IL-18 (MBL, Nagoya, Japan); human IL-1 β (Wako, Osaka, Japan); human IL-5 (Peprotech, London, UK); human recombinant VCAM-1 and ICAM-1 (R&D, Minneapolis, MN, USA); Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden); PBS and RPMI 1640 medium (GIBCO, Grand Island, NY, USA); FCS and fibronectin (0.1% solution) (Sigma, St Louis, MO, USA).

The following antibodies were purchased as indicated: mouse anti-human ST2 neutralizing mAb (IgG1, clone 97203), mouse anti-IL-4 neutralizing mAb (IgG2b, clone 34019.111), mouse anti-IL-5 neutralizing mAb (IgG1, clone 14611) and mouse anti-GM-CSF neutralizing mAb (IgG1, clone 3209) (R&D); mouse anti-CD18 neutralizing mAb (IgG1, clone

L130) (BD Pharmingen, San Diego, CA, USA); control mouse IgG1 (MOPC21) and mouse IgG2a (UPC10) (Sigma); mouse anti-ST2 mAb (IgG1, clone HB12) (MBL); FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA); mouse anti-CD29 neutralizing mAb (IgG1, clone 4B4), FITC-conjugated mouse anti-CD16 mAb (IgG1, clone 3G8), PE-conjugated mouse anti-CD11b mAb (IgG1, clone Bear 1) and PE-conjugated mouse IgG1 (clone 679.1Mc7) (Coulter Immunotech, Marseille, France).

Isolation of Eosinophils, Neutrophils and Culture of Mast Cells

Leukocytes were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases.

Eosinophils were purified by density gradient centrifugation. In some experiments, eosinophils were further purified by negative selection using anti-CD16-bound beads (Miltenyi BioTech, Belgisch-Gladbach, Germany) as previously described.¹⁶ After this negative selection, the eosinophil purity was > 99%.

Human neutrophils were separated by density gradient centrifugation followed by positive selection using anti-CD14-bound micromagnetic beads (Miltenyi BioTech).¹⁶ The purity of neutrophils was approximately 96–99% after the selection.

Human cord blood-derived mast cells were obtained by culturing cord blood CD34-positive cells in the presence of stem cell factor (100 ng/ml) and IL-6 (50 ng/ml) for more than 10 weeks.¹⁷ Purity of mast cells assessed using Toluidine blue stain was > 99%.

Real-Time Quantitative PCR Analysis

Real-time quantitative PCR analysis was performed as previously described.¹⁶ In brief, total RNA was extracted from MACS-separated eosinophils, neutrophils and mast cells from separate donors using RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time PCR was performed using the 7500 Real Time PCR System (PE Applied Biosystems, Foster City, CA, USA). The primers and the probes for ST2 were designed by PE Applied Biosystems. A standard curve was constructed with serial dilutions of specific PCR products, which were obtained by amplifying peripheral leukocyte cDNA as previously described.¹⁸

ST2 Protein Expression

Highly purified eosinophils were used for flow cytometric analysis of ST2 expression. Eosinophils were incubated for 30 min at 4°C with 10 μ g/ml of either anti-ST2 mAb or control antibody and then stained with PE-conjugated goat anti-mouse IgG at 10 μ g/ml for 60 min at 4°C. For intracellular staining, the cells were fixed with PBS containing 4% paraformaldehyde at 4°C for 30 min followed by permeabilization in PBS containing 0.1% Tween 20 at 4°C for 30 min. The cells were then stained and analyzed using an Experimental

Physics and Industrial Control System, XL System II (Coulter, Miami, FL, USA).

Survival Assay

Highly purified eosinophils were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Differential analysis of apoptotic and live cells was performed using a MEBCYTO apoptosis kit (MBL) and flow cytometry as previously described.¹⁹ Early apoptotic cells were quantitatively determined by their ability to bind annexin V and exclude propidium iodide (PI). Cells stained with PI were considered to be necrotic cells. Cells without binding to annexin V or PI were judged to be alive.

Adhesion Assay

A 96-well culture plate (IWAKI, Tokyo, Japan) was coated with 100 µl of BSA (20 mg/ml), fibronectin (20 µg/ml), ICAM-1 (100 ng/ml) or VCAM-1 (100 ng/ml) dissolved in PBS overnight at 4°C. The coated wells were washed twice with blocking buffer (2% BSA in PBS) and incubated with 100 µl of this buffer for 1 h at 37°C. The wells were washed twice with RPMI 1640 medium containing 0.3% HSA before adding eosinophils.

Approximately 3×10^4 highly purified eosinophils were added to each well containing the stimulating reagent dissolved in RPMI 1640 medium including 0.3% HSA and incubated at 37°C in 5% CO₂ for 45 min. In some experiments, either anti-CD18 mAb, anti-CD29 mAb or control antibody at 10 µg/ml was added to each well. After incubation, the wells were gently washed twice with RPMI 1640 medium to remove nonadherent cells. Eosinophil adhesion was monitored by quantification of the EPO activity released from the adherent eosinophils as previously reported.^{16,20} In brief, 200 µl of 50 mM Tris-HCl, pH 8.0, containing 0.1% (v/v) Triton X-100, 0.1 mM O-phenylenediamine dihydrochloride (Sigma) and 50 mM hydrogen peroxide was added to each well. The plate was left at room temperature until reaching the desired extinction, and the reaction was terminated with 50 µl of 1 M sulfuric acid solution. The optimal density was read at 490/570 nm using an ELISA plate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). Data were analyzed with the Microplate Manager III program (Bio-Rad Laboratories), and the numbers of the adherent cells were calculated from a calibration curve established with varying known numbers of eosinophils. The adherent cells were expressed as a percentage of the total eosinophils added to each well.

CD11b Expression

CD11b expression experiments were performed as previously described.²¹ Briefly, following stimulation, purified cells were incubated with 10 µg/ml of either PE-conjugated anti-CD11b mAb or PE-conjugated control mouse IgG1 and then stained with FITC-conjugated anti-CD16 antibody at 10 µg/ml.

Stained cells were analyzed by flow cytometry. Cells that stained negative for CD16 were identified as eosinophils. The median values of fluorescence intensity of eosinophils were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as previously described.²¹ Surface expression levels were semi-quantified using the following formula: $\Delta\text{MESF} = (\text{MESF of cells stained with anti-CD11b mAb}) - (\text{MESF of cells stained with control IgG})$.

Enzyme Immunoassay for LTC₄ and Eosinophil-Derived Neurotoxin

For enzyme immunoassay (EIA), 5×10^5 cells per ml of highly purified eosinophils were cultured with stimulating reagents in RPMI medium containing 0.3% HSA for 18 h at 37°C, and the supernatant was collected after centrifugation. Cell lysates were obtained by addition of 0.5% nonidet P-40 (Sigma) to the cell pellets. Samples were stored at -80°C until assay.

Immunoreactive LTC₄ was measured using an EIA kit for LTC₄ (Cayman Chemicals, Ann Arbor, MI, USA; detection range: 10–1000 pg/ml) by following the manufacturer's instructions.

The concentration of eosinophil-derived neurotoxin (EDN) was measured using an EDN ELISA kit (MBL) by following the manufacturer's instructions. The detection limit was 0.62 ng/ml.

Statistics

All data are expressed as the mean \pm s.e.m. Differences between values were analyzed by the one-way ANOVA test. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

RESULTS

Eosinophils Express ST2

First, eosinophil expression of mRNA for ST2 was quantified by real-time PCR in comparison with neutrophils and mast cells. As shown in Figure 1a, ST2 mRNA expression was observed in eosinophils as well as neutrophils and mast cells. Consistent with previous reports,¹⁰ mast cells expressed high levels of mRNA for ST2. The expression level of ST2 by eosinophils was lower than that by mast cells (about 10-fold lower) but much higher than that by neutrophils (about 10-fold higher). Certain cytokines have been previously reported to regulate their respective receptors;²² thus, we tested the effect of IL-33 on ST2 mRNA expression. However, the ST2 mRNA expression level by eosinophils did not change as a result of 4 h incubation with IL-33 at 100 ng/ml (Figure 1b). By flow cytometric analysis, we found that hardly any ST2 was expressed on the surface of eosinophils (data not shown), but intracellular staining of eosinophils showed a low but detectable level of ST2 (Figure 1c).

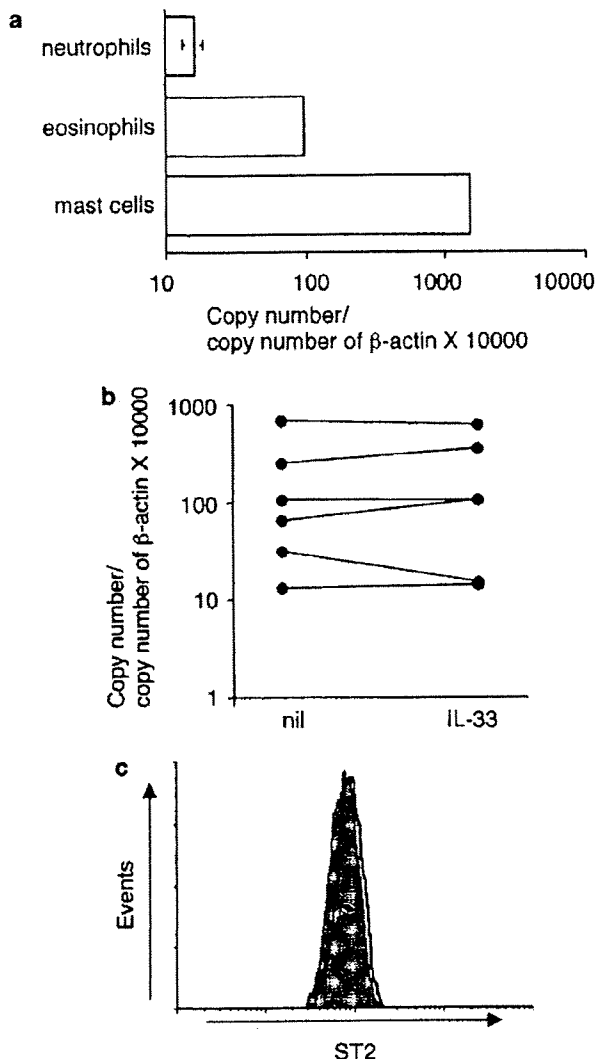


Figure 1 Real-time quantitative PCR analysis and flow cytometric detection for ST2, a receptor for IL-33. (a) Quantitative PCR was performed using cDNAs from highly purified eosinophils ($n = 10$), neutrophils ($n = 9$) and mast cells ($n = 4$). The data are calculated as follows: copy number of ST2 gene/copy number of β -actin gene \times 10 000. (b) Highly purified eosinophils ($n = 6$) were incubated with and without IL-33 at 100 ng/ml for 4 h before extraction of the RNA. The expression levels for the same donor are connected with a solid line in the graph. (c) Fixed eosinophils were analyzed for ST2 protein expression by flow cytometry. The cells stained with the control antibody are indicated with a thin line, and the cells stained with anti-ST2 antibody are indicated with a thick line. The data are representative of three separate experiments using cells from different donors and showing similar results.

IL-33 Upregulates Adhesiveness of Eosinophils

As *in vivo* administration of exogenous IL-33 in murine models resulted in local accumulation of eosinophils, we performed a migration assay on eosinophils with IL-33. However, IL-33 added to the lower chambers failed to attract

eosinophils. In addition, IL-33 added to the upper chambers did not show enhancement of eosinophil migration toward eotaxin (data not shown). Therefore, next, eosinophil adhesion was quantified by measuring EPO released from lysed adherent cells after 45-min incubation. As shown in Figure 2a, eosinophil adhesion to albumin-, fibronectin-, ICAM-1- and VCAM-1-coated wells was significantly upregulated by IL-33. This effect was apparent at a concentration of 1 ng/ml and increased up to 100 ng/ml. Notably, the effect of IL-33 at 100 ng/ml on eosinophil adhesion was significantly greater than that of IL-5 at 300 pM ($P < 0.01$), the most potent known eosinophil-activating cytokine. Two other cytokines of the IL-1 family, ie, IL-1 β and IL-18 at 100 ng/ml, did not show any effect on eosinophil adhesion. As shown in Figure 2b, adhesion of eosinophils to albumin-, fibronectin- and ICAM-1-coated wells in the presence of IL-33 was almost completely blocked by anti-CD18 neutralizing antibody, indicating that mainly β_2 integrin on IL-33-treated eosinophils is involved in the adhesion process to albumin, fibronectin and ICAM-1. On the other hand, adhesion to VCAM-1-coated wells was strongly diminished by the combination of anti-CD18 plus anti-CD29 antibodies, suggesting that eosinophil β_1 integrin is also involved in adhesion to VCAM-1.

IL-33 Augments CD11b Expression on Eosinophils

Eosinophils have been reported to express β_2 (CD11a, CD11b and CD18) integrins on their surface, and the levels of CD11b on eosinophils are enhanced by eosinophil-activating cytokines such as IL-5. In this study, expression of CD11b on eosinophils was analyzed by flow cytometry. As shown in Figure 3, IL-33 at 1–100 ng/ml significantly upregulated the expression of CD11b on eosinophils dose-dependently, and the effect of IL-33 at 100 ng/ml was stronger than that of IL-5 at 300 pM ($P < 0.001$). On the other hand, two other IL-1 family cytokines, IL-1 β and IL-18, did not show any effect on eosinophil CD11b expression.

To elucidate the role of ST2, neutralizing antibody for ST2 was added together with IL-33. When eosinophils were pretreated with anti-ST2 neutralizing antibody, the effect of IL-33 on CD11b expression was diminished, as shown in Figure 4a and b, indicating that IL-33 affected eosinophils by binding to and signaling through ST2. When IL-33 at 1 or 10 ng/ml was used to stimulate eosinophils, the effect declined significantly upon addition of anti-ST2 antibody. These results suggest that eosinophil CD11b expression is regulated by IL-33 and its receptor, ST2.

IL-33 Enhances Survival of Eosinophils

Next, we used highly purified eosinophils and analyzed the effect of IL-33 on their viability. As shown in Figure 5a and b, IL-33 at 10–100 ng/ml significantly enhanced the survival of eosinophils dose-dependently although the effect was weaker than that of IL-5 at 300 pM. IL-33 at 100 ng/ml increased the number of live eosinophils by approximately

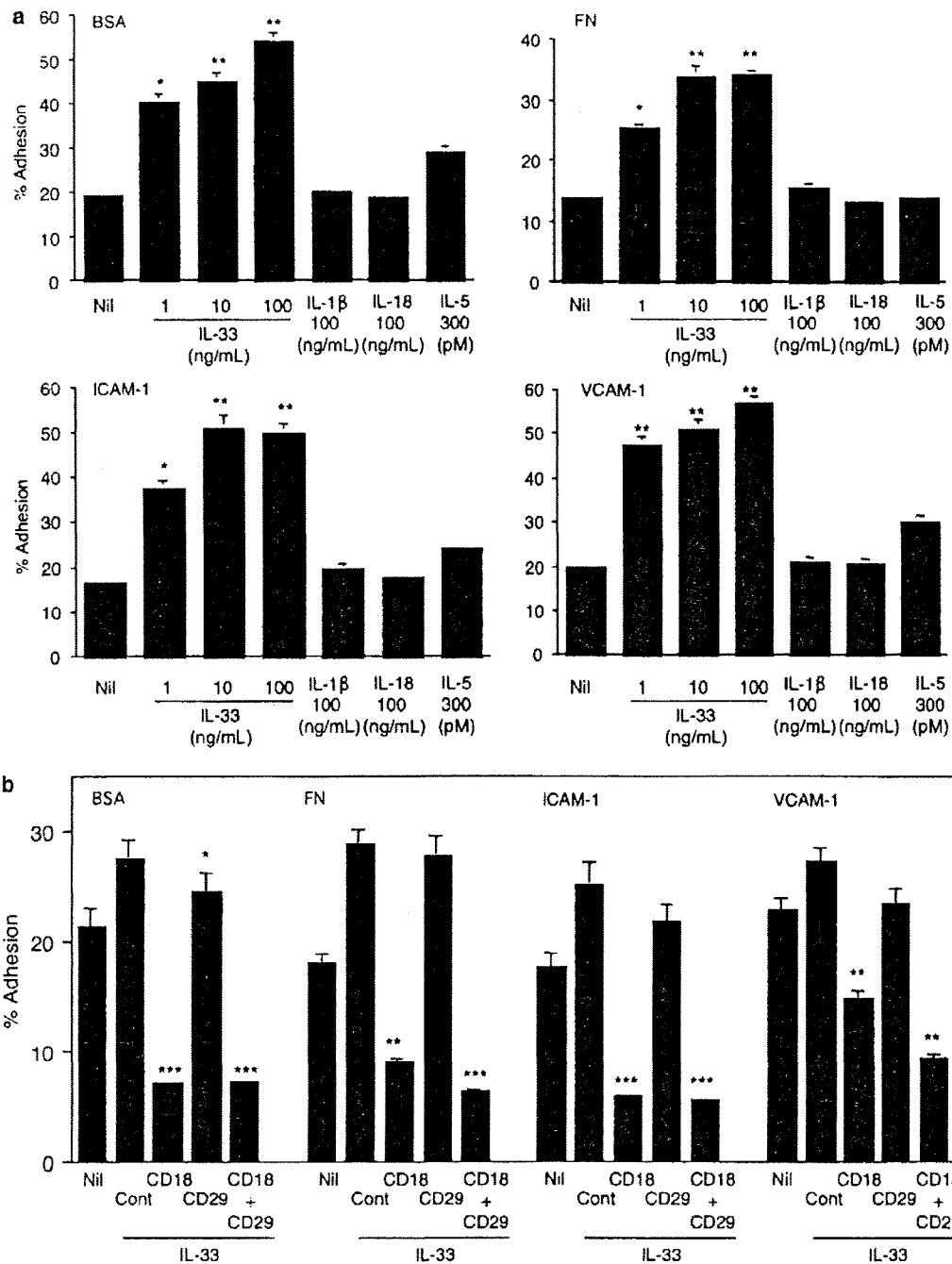


Figure 2 IL-33 enhances adhesion of eosinophils. (a) Human eosinophils were used to analyze adhesion to BSA-, fibronectin-, ICAM-1- or VCAM-1-coated culture plates. The cells were incubated with the indicated concentrations of IL-33 or IL-1 β at 100 ng/ml, IL-18 at 100 ng/ml or IL-5 at 300 pM for 45 min. The number of adherent cells is expressed as a percentage of the total number of cells placed in each well. Bars represent the s.e.m. ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs medium alone (nil). (b) Human eosinophils were preincubated with either neutralizing antibody or control antibody at 10 μ g/ml plus IL-33 at 100 ng/ml, for 45 min. The number of adherent cells is expressed as the percentage of the total number of cells placed in each well. Bars represent the s.e.m. ($n = 4-5$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs control antibody.

20% after 24 h. Apoptotic cells, ie, positive for annexin V staining and negative for PI staining, were significantly decreased by addition of IL-33 (Figure 5c). IL-1 β and IL-18,

at 100 ng/ml, did not enhance the survival of eosinophils, indicating that this effect is specific for IL-33 among these cytokines.

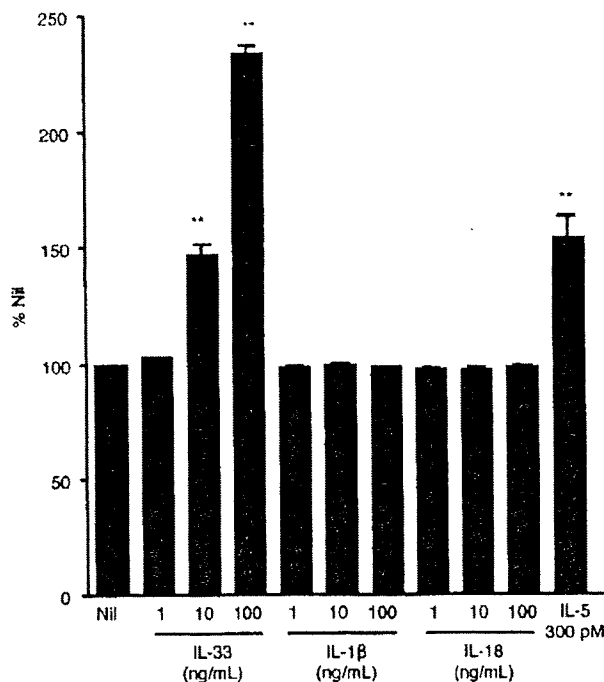


Figure 3 The effect of IL-33 on eosinophil CD11b expression. Human eosinophils were incubated with the indicated reagent for 30 min, and then the cell-surface expression of CD11b was analyzed by flow cytometry. The data are expressed as the percentage of the calculated MESF values of eosinophils cultured without stimulus (% nil). Bars represent the s.e.m. ($n = 3$). $^{*}P < 0.01$ vs nil.

To elucidate the role of ST2 in eosinophil survival, neutralizing antibody for ST2 was added together with IL-33. As a result, anti-ST2 antibody at 20 $\mu\text{g/ml}$ significantly down-modulated the effect of IL-33, as shown by a decreased number of live cells and an increased number of apoptotic cells (Figure 6a and b), indicating that IL-33 enhances eosinophil survival by signaling through the ST2 receptor.

It has been reported that eosinophils can produce IL-5 upon stimulation. We used neutralizing antibody for IL-5 to test the possibility that IL-33 enhancement of eosinophil survival was mediated by IL-5. However, anti-IL-5 antibody did not affect the number of viable eosinophils cultured with IL-33 (data not shown). Similar tests using anti-IL-4 and anti-GM-CSF neutralizing antibody gave the same result. These results indicate that the effect of IL-33 on eosinophil survival is not mediated by autocrine activation involving IL-5, IL-4 or GM-CSF.

Analysis of Degranulation and Lipid Mediator Synthesis

We conducted experiments to see whether IL-33 induces degranulation and lipid mediator synthesis in human eosinophils. Eosinophil degranulation was analyzed by measuring EDN, but IL-33 was negative for this activity (data not shown). LTC₄ synthesis was analyzed by ELISA, but

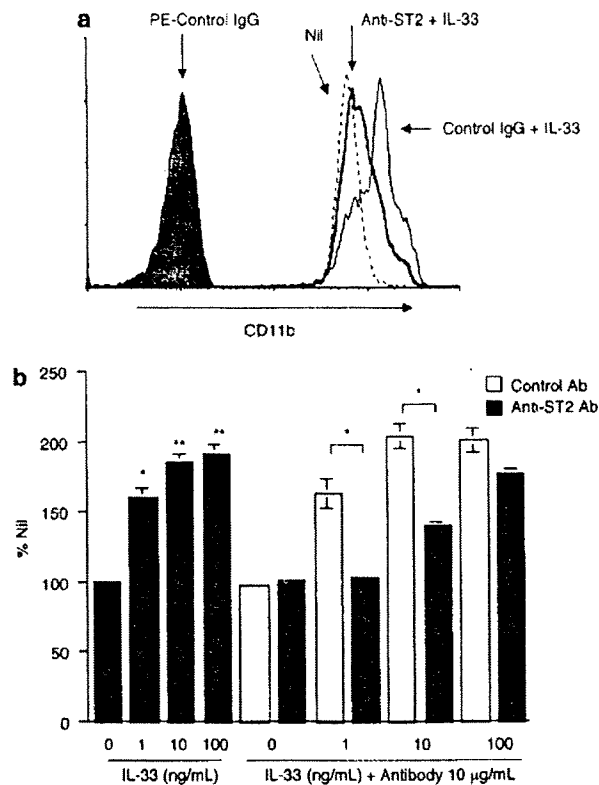


Figure 4 The effect of anti-ST2 neutralizing antibody on CD11b expression by eosinophils. (a) Human eosinophils were preincubated with anti-ST2 antibody at 10 $\mu\text{g/ml}$ for 60 min and then with IL-33 at 10 ng/ml for 30 min. The surface CD11b expression level was assessed by flow cytometry. Eosinophils stained with PE-control mouse IgG1 are shown as a shaded area. Data are representative of four separate experiments showing similar results. (b) Human eosinophils were preincubated with or without anti-ST2 antibody or control antibody at 10 $\mu\text{g/ml}$ for 60 min and then with IL-33 at the indicated concentrations for 30 min. The data are expressed as the percentage of the calculated MESF values of eosinophils cultured without antibodies or stimulus (% nil). Bars represent the s.e.m. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs nil.

no apparent release of LTC₄ was induced by IL-33 (data not shown).

DISCUSSION

In the present study, we assessed the potential role of IL-33 in regulation of eosinophil functions. We demonstrated that IL-33 is a potent activator of human eosinophils, enhancing their surface CD11b expression and adhesion and prolonging their life span. Surprisingly, the maximal enhancing effects of IL-33 on adhesion and CD11b expression were comparable to, or even greater than, the effects of IL-5, a potent eosinophil-activating cytokine. This is the first report showing direct effects of IL-33 on the biological functions of eosinophils. Importantly, the effects of IL-33 on eosinophils were limited to cell adhesion and survival, in clear contrast to

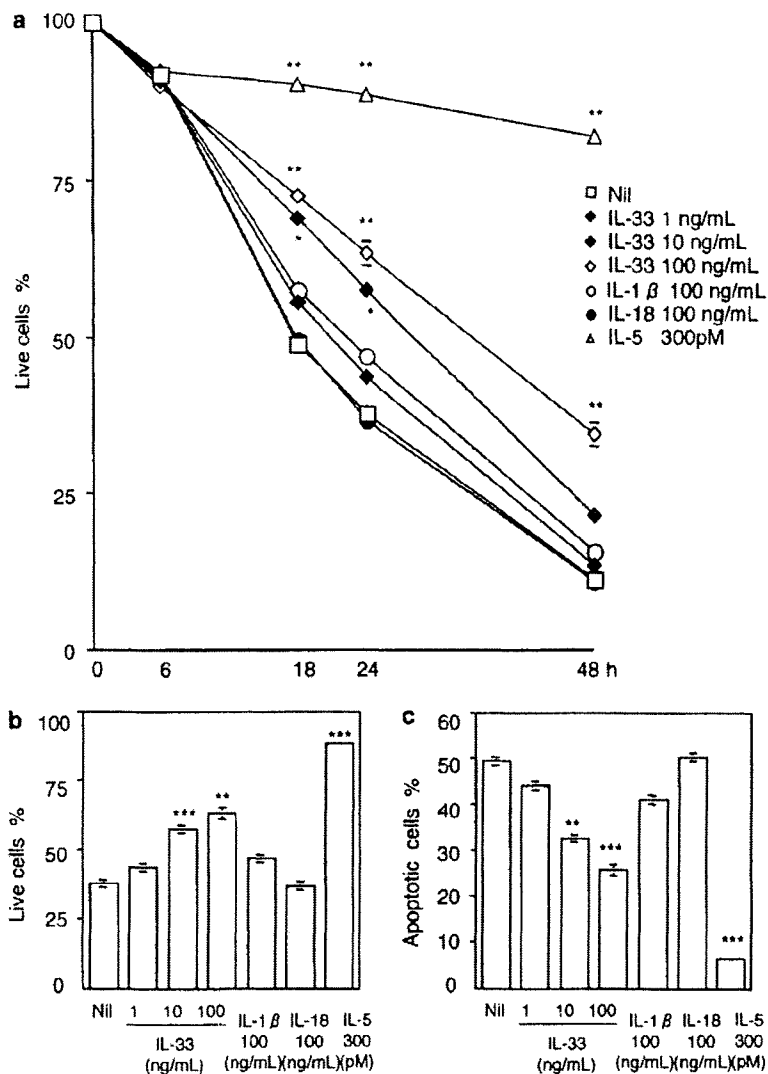


Figure 5 IL-33 enhances survival of eosinophils. (a) Time course of survival of human eosinophils. Highly purified eosinophils were cultured in medium alone or in the presence of IL-33 (1, 10 and 100 ng/ml), IL-1 β (100 ng/ml), IL-18 (100 ng/ml) or IL-5 (300 pM) for the indicated times. The cells were analyzed by double staining with annexin V and PI. Live cells were negative for both annexin V and PI. Data are expressed as percentages of total cell numbers. (b, c) Viable (b) and apoptotic eosinophils (c) after 24-h incubation. Early apoptotic cells were defined as annexin V-positive and PI-negative. Bars represent the s.e.m. (n = 4). *P < 0.05, **P < 0.01 and ***P < 0.001 vs medium alone.

IL-5, which also affects mediator synthesis and release by eosinophils.^{23,24}

IL-33 is a recently identified cytokine belonging to the IL-1 family. It has increasingly been thought that IL-33 may be involved in the pathogenesis of Th2-polarized inflammation. Schmitz *et al*¹⁴ demonstrated that IL-33 induces Th2-polarized cells to produce Th2 cytokines such as IL-5 and IL-13. In addition, *in vivo* exposure to IL-33 causes histological changes in the lungs and gastrointestinal tract, including eosinophilic and mononuclear cell infiltration, increased mucus production, and epithelial cell hyperplasia and hypertrophy.¹⁴ Although the principal source of IL-33 in

Th2-related allergic diseases has yet to be determined, this cytokine can reportedly be produced by many cell types including epithelial cells and smooth muscle cells.²⁵ Thus, IL-33 secreted by various cell types may act collectively to induce allergic inflammation through Th2 cell differentiation and a direct effect on Th2 effector cells, including eosinophils.

Among the IL-1 family cytokines we tested, IL-33 was the only one that activated eosinophil functions. In previous studies, IL-18, another IL-1 family cytokine, has been demonstrated to induce IL-8 production by eosinophils and to enhance antigen-induced eosinophil recruitment in mouse airways.^{26,27} IL-1 β has also been reported to enhance

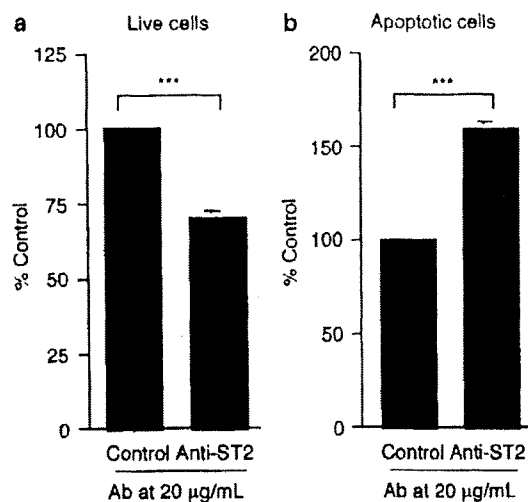


Figure 6 Effect of anti-ST2 antibody on IL-33-induced eosinophil survival. Highly purified eosinophils were cultured with IL-33 at 10 ng/ml and anti-ST2 neutralizing antibody at 20 µg/ml for 24 h. Live (a) and apoptotic (b) cells were analyzed by flow cytometry. Data are expressed as percentages of corresponding cells cultured with control antibody. Bars represent the s.e.m. ($n = 5$). *** $P < 0.001$ vs control.

mediator release from IgE-stimulated eosinophils and adhesiveness of eosinophils to endothelium.²⁸ Thus, the IL-1 family cytokines are considered to be important proinflammatory cytokines in allergic inflammation. The IL-1 family member most closely related to IL-33 is reported to be IL-18.¹⁴ In clear contrast to IL-33, the main cellular source of IL-1 β and IL-18 seems to be hematopoietic cells. Moreover, IL-1 β and IL-18 are not considered to be selective Th2-related cytokines, as they can also promote Th1-associated responses. On the basis of our present study, IL-33 seems to have different roles from IL-1 β and IL-18. Thus, IL-33 promotes Th2-associated responses, at least partly through direct activation of eosinophils, and this action seems unique among IL-1 family cytokines.

An IL-1 receptor family member, ST2, has been shown to function as an important effector molecule of Th2 responses in a number of experimental settings,^{6,11} and it is an active receptor for IL-33. It has been demonstrated that expression of ST2 on Th2 cells is induced by proinflammatory substances, including TNF, IL-1 α , IL-1 β , IL-6, IL-5 and PMA, and importantly, crosslinking of ST2 provided a costimulatory signal for Th2 cells and directly induced Th2 cell proliferation and type 2 cytokine production.^{29,30} Thus, ST2 might be important in the pathogenesis of diseases of the Th2 phenotype.

In the present study, real-time PCR analysis of eosinophils revealed that eosinophils express mRNA for ST2, although the expression level of mRNA was lower than that of mast cells which have already been reported to express ST2 on their cell surface.¹⁰ Although surface expression of ST2 on eosinophils was hardly detectable, ST2 was shown to be

present in the cells by intracellular flow cytometric analysis. At present, we do not know whether presumably faint levels of surface ST2 would suffice for triggering cell activation signals, or whether intracellular ST2 in eosinophils is also involved in cell activation by IL-33. However, based on the successful blocking of IL-33's effects by IL-33-neutralizing antibodies, we can reasonably say that ST2 protein expressed by eosinophils has functional relevance. In our present study, a high-affinity ST2 ligand, IL-33, induced a strong eosinophil adhesion response with efficacy higher than that of IL-5. The IL-1 β , TNF- α , IFN- γ , IL-5 and IL-4 inflammatory cytokines upregulate the expression of adhesion molecules on the endothelium and eosinophils and increase eosinophil binding to endothelial cells.³¹ On the basis of the findings of this study, IL-33, like other proinflammatory cytokines, also upregulated expression of CD11b, a component of Mac-1, on eosinophils. Therefore, the enhancement of eosinophil adhesiveness by IL-33 is at least partially due to this enhanced expression of CD11b. Neutralization experiments confirmed that β 1 and β 2 integrins are critically involved in the adhesion process of IL-33-treated eosinophils. Also, IL-33 prolonged the life span of eosinophils, although the effect was weaker than that of IL-5. We have shown that the survival-enhancing effect of IL-33 is not due to autocrine production of IL-4, IL-5 or GM-CSF, since neutralization of those cytokines did not affect IL-33-induced prolongation of eosinophil survival. On the other hand, at relatively high concentrations (10–20 µg/ml) anti-ST2 antibody was fairly efficient at inhibiting IL-33-induced upregulation of CD11b expression and survival of eosinophils, but those abrogating effects were not complete. We speculate that, in eosinophils, a small amount of IL-33 that can bind to cell-surface ST2 even in the presence of anti-ST2 antibody may act efficiently to modulate cell functions. Another possibility is that, in eosinophils, intracellular ST2 may not be completely blocked by anti-ST2 antibody. In any case, our results indicate that ST2 is an important receptor through which IL-33 mediates most, if not all, of various aspects of eosinophil activation.

Eosinophils are considered to be the most prominent cells at sites of allergic inflammation. Tissue eosinophils are believed to contribute to exacerbation of inflammation by an autocrine or paracrine mechanism. Therefore, marked reduction of tissue-infiltrated eosinophils is considered to be a promising therapeutic target for allergic diseases. On the basis of the findings of our present study, neutralization of ST2 abolished the effect of IL-33 on eosinophil activation indicating that IL-33 affected eosinophil function through ST2 although ST2 protein levels were low in eosinophils. In addition, in the clinical setting, elevated ST2 protein levels were reported in the sera of patients with asthma exacerbation, and the severity of asthma exacerbation correlated with the levels of serum ST2.¹³ Furthermore, it has been demonstrated in a murine asthma model that administration of recombinant ST2 fusion protein attenuated eosinophilic inflammation of the airway and suppressed IL-4 and IL-5

production.¹¹ Thus, the IL-33–ST2 pathway may be actively involved in the pathogenesis of eosinophil-related allergic diseases such as asthma and eosinophilic gastroenteritis. In this context, several studies have shown that inhibition of the ST2-related signal pathway leads to abrogation of allergic diseases.^{8,11,12}

In summary, we have explored for the first time the receptor expression and functions of IL-33 in human eosinophils, and we found that this cytokine potently activates eosinophils. In combination with previous reports by others, our results strongly suggest that the IL-33–ST2 pathway may be critically involved in the pathogenesis of allergic diseases. The effects of IL-33 on other inflammatory cells also need to be explored in detail; these information will help us understand the detailed mechanisms underlying clinical allergic diseases.

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DISCLOSURE/DUALITY OF INTEREST

We have no duality of interest to declare.

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■ 原 著 ■

気管支喘息と胃食道逆流との関連についての 臨床的・実験的検討— QUEST 問診票に基づく 胃食道逆流診断と治療的介入の効果—

竹 澤 (矢野) 智 湖

帝京大学医学部内科学講座

背景・目的：気管支喘息では高率に胃食道逆流（以下 GERD）が合併し、GERD は喘息増悪因子として位置づけられている。簡便な GERD 診断法として、QUEST 問診表が開発されており、気管支喘息患者における GERD 合併を、QUEST 問診票を用いて診断・治療することの有用性を検討した。また、GERD による喘息増悪機序として、気道内への酸微量誤嚥の関与が想定されているため、酸曝露が気道上皮細胞株の生存や活性化におよぼす影響を *in vitro* で検討した。

対象と方法：気管支喘息患者 88 名における QUEST 陽性率を調査した。さらに、QUEST 陽性の気管支喘息患者 19 名に、オメプラゾール 20 mg を 4 週間投与し、QOL、喘息症状、呼吸機能の評価した。また、低 pH で気道上皮細胞株 BEAS-2B を培養し、生存とサイトカイン・ケモカイン産生に及ぼす影響を検討した。

結果：気管支喘息患者における QUEST 陽性率は 54.5 % であった。QUEST スコアは喘息重症度と正相関し、使用薬剤よりも喘息重症度と強く相関していた。オメプラゾール投与によって、QUEST スコアと咳嗽が有意に改善し、QOL も向上した。*In vitro* の検討では、酸曝露 4 時間後より気道上皮細胞死が惹起され、PDGF-BB 分泌が増強した。

結論：気管支喘息患者における GERD 合併を、QUEST 問診票を用いて診断・治療する診療モデルの妥当性が示唆された。また、酸曝露による気道上皮細胞死、PDGF-BB 分泌亢進が喘息増悪に関与している可能性が示唆された。

キーワード：気管支喘息，胃食道逆流，QUEST 問診票，咳嗽，気道上皮細胞

はじめに

胃食道逆流症（Gastroesophageal Reflux Disease, 以下 GERD）は胃食道逆流による身体的合併症や、逆流関連症状により健康な生活を障害しているものを指す¹⁾。身体的合併症とは、食道炎などの局所病変に加え、喘息、肺炎、嘔声、咽頭炎などの合併症を指し、健康な生活の障害とは、胸

焼けなどの自覚症状とそれに由来する Quality of Life（以下 QOL）の低下を指す¹⁾。そして、週に 2 回以上の症状があれば、QOL に悪影響を与えるとされる¹⁾。

気管支喘息に GERD が高頻度に合併することは 1960 年代より報告されている²⁾。疫学的には、喘息患者の 40 % にびらん性食道炎があることや、80 % 以上に異常逆流を認めることが報告されている³⁾。GERD が喘息病態を悪化させる機序としては酸の微量誤嚥や、逆流に伴う迷走神経反射によ

(指導：大田 健教授，長瀬洋之講師)
学位申請論文

る気道抵抗の増大などの関与が想定されており、気管支喘息増悪因子として、GERD合併の有無を検討することは、気管支喘息の治療管理の点からも重要である。

しかしながら、呼吸器外来における日常診療では、GERDの診断は必ずしも容易ではなく、増悪因子として検索されていないことも多い。GERDの診断は、胸やけ、呑酸などの定型的症状に関する詳細な問診を基本とするが、確定診断には24時間食道pHモニタリングが有用である。また、気管支喘息とGERDの関連を検討した報告においても、食道pHモニタリングや上部消化管内視鏡検査に基づくGERD診断が行われている。しかしながら、これらの検査は比較的侵襲が大きく、結果も迅速には得られにくい。

簡便なGERD診断法として、Questionnaire for the Diagnosis of Reflux Disease (以下QUEST)⁴⁾や、Frequency Scale for the Symptoms of GERD (FSSG)⁵⁾といった問診表が開発されている。QUEST問診票は1998年に開発され⁴⁾、胸焼けなどの典型的なGERD症状をスコア化し、高い特異度、感度を有している。消化器疾患領域ではGERD診断に広く用いられているが、気管支喘息患者において、GERDの典型的症状がいかにか修飾されるかについては不明であり、気管支喘息患者においてQUEST問診票の有用性を検証する必要がある。しかしながら、現時点ではQUEST問診票のみに基づいて気管支喘息患者におけるGERDの合併を診断し、GERD治療の気管支喘息病態への効果を検討した報告はない。

そこで、今回の研究目的は、1) 気管支喘息患者における、QUEST陽性率を調査すること、2) 気管支喘息患者におけるGERD合併を、QUEST問診票を用いて診断・治療することの有用性を検討し、日常診療に応用可能な診療体系を確立すること、とした。具体的には、QUEST陽性喘息患者にプロトンポンプ阻害薬 (proton pump inhibitor, 以下PPI) を投与し、QOL、喘息症状、呼吸機能を評価した。

さらに、GERDによる気管支喘息増悪の機序として、気道内への酸微量誤嚥の関与が想定されている。気道上皮細胞は、外界への最前線に位置し、種々のサイトカイン、ケモカインの産生を介して

気道炎症形成に関与することが明らかとなっているが、酸曝露による気道上皮細胞傷害や炎症性メディエーター産生へ及ぼす影響に関しては不明な点が多い。そこで、気道上皮細胞株を用いて、塩酸による低pHの及ぼす影響を*in vitro*で検討し、GERDによる気管支喘息増悪の一機序を検討することとした。

対象と方法

本研究計画は、帝京大学医学部倫理委員会の承諾を得た。研究に参加する際は、研究内容を文書で説明し、同意書への署名を得た。

1. 気管支喘息患者におけるQUEST陽性率の調査

帝京大学医学部附属病院の呼吸器・アレルギー外来に気管支喘息にて通院中の連続受診患者94名を対象にQUEST問診票を配布し、88名から有効回答を得た (平均年齢50.7±1.7歳、男性:41名、女性:47名)。QUEST問診票は7問からなり、逆流症状、誘因、発現・消失パターンに重み付けをしたスコアが配点されており、最高点は18点である。カットオフ値を4点以上とすると、感度70%、特異度は46%、カットオフ値を6点以上とすると、感度54%、特異度は60%と報告されている⁴⁾。気管支喘息の診断および重症度判定は、喘息予防・管理ガイドラインに基づいて行った⁶⁾。

2. QUEST陽性気管支喘息患者に対するプロトンポンプ阻害薬投与の効果

QUEST問診票の有効回答を得た88名のうち、下記のクライテリアを満たし、試験参加の同意を得た安定期気管支喘息患者19名を対象とした。

(1) QUESTスコア4点以上、(2) H₂阻害薬、PPIを内服していない、(3) 気管支喘息治療薬を4週間以上変更せずに使用している、(4) 4週間以上気管支喘息発作を認めない、(5) 経口ステロイド薬を内服していない。19名 (平均年齢57.0±2.9歳、男性:8名、女性:11名) の患者背景を表1に示す。喘息罹患年数は、17.2±2.5年、喘息重症度はステップ2:12名、ステップ3:7名であった。

PPI投与開始前の4週間を観察期間とし、観察期間終了後にオメプラゾール20mgの投与を1

表1 プロトンポンプ阻害薬投与対象例の患者背景

患者	年齢(歳)	性別	喘息病型	罹患年数(年)	喘息ステップ	QUESTスコア
1	44	F	ND	41	2	6
2	37	F	A	14	2	6
3	67	M	NA	12	2	8
4	75	M	NA	16	2	8
5	55	F	NA	4	3	8
6	56	F	A	20	3	8
7	57	F	NA	27	2	8
8	50	F	A	10	2	9
9	62	M	NA	22	3	11
10	68	M	A	14	2	11
11	60	F	ND	11	3	12
12	75	M	NA	15	3	15
13	36	M	A	26	2	6
14	65	F	A	12	2	10
15	72	F	NA	9	2	12
16	70	M	ND	7	3	6
17	47	F	A	21	2	9
18	46	F	A	43	3	6
19	41	M	ND	3	2	11

M：男性，F：女性，A：アトピー型，NA：非アトピー型，ND：Not determined

カ月行った。オメプラゾールは朝食後，1日1回の内服とした。観察期間，内服期間を通して，気管支喘息治療薬は変更せずに継続した。

評価項目はQUESTスコア，呼吸機能，QOL，朝および夕のピークフロー，症状点数とし，PPI内服前後で検討した。QOLは，疾患特異的なAsthma Quality of Life Questionnaire（以下AQLQ）日本版と疾患非特異的なMedical Outcomes Study 36-Item Short-Form Health Survey（以下SF-36）日本版を用いて評価した。ピークフローは携帯用ピークフローメーター（Mini-Wright，Clement Clarke，Harlow，UK）で測定した。症状点数は，日本アレルギー学会の方法⁷⁾，喘息日誌に記録された呼吸困難感，喘鳴，咳嗽の程度から0～9点の間でスコア化した。咳嗽（強い咳：1点，弱い咳：0.5点），痰量（多い：2点，少ない：1点，認めない：0点），痰の切れ（悪い：1点，良い：0点）もおおのスコア化し，観察期間と内服期間のそれぞれ最終の1週間のスコアを合計して算出した。QOLに関しては17名，呼吸機能検査，喘息日誌は13名が評価可能であった。

3. 酸曝露が気道上皮細胞株の生存およびサイトカイン産生に及ぼす影響

1) 気道上皮細胞培養

気道上皮細胞株は，正常人の気道上皮細胞をアデノウイルスとSV40のハイブリットにより腫瘍化した細胞株であるBEAS-2Bを用いた。まず，BEAS-2BをLHC-9培養液（GIBCO，Grand Island，USA）で培養し，Confluentに達するまで約1週間増殖させた。その後，trypsin-EDTAで剥離し， 2×10^4 個（in200 μ l/well）を96穴プレート（Iwaki，Tokyo）に分注した。培養液は1%ペニシリン・ストレプトマイシン（GIBCO）および5%ウシ胎児血清（Fetal Calf Serum，以下FCS，JRH bioscience，USA）を添加したDMEM/F-12（GIBCO）を用い，Confluentに達するまで，37℃下，CO₂インキュベーター中で3日間培養した。

2) 酸曝露が気道上皮細胞からの炎症性メディエーター産生に及ぼす影響

Confluentに達したBEAS-2B細胞の培養液を，pH 3，4，5，6，6.5，7，7.5に調整した培養液に置換してさらに培養後に上清を回収し，-80℃で保存した。培養液は1%ペニシリン・ストレプトマイシンおよび0.1%ウシ血清アルブミン

(Bovine Serum Albumin, 以下 BSA) を添加した DMEM/F-12 (DMEM/F-12-0.1% BSA) を 1N HCl を用いて pH を調整して用いた。培養上清の検討は、デュプリケートないし、トリプリケート由来のデータを平均して 1 パッセージのデータとした。

上清中サイトカイン・ケモカイン濃度は、Human Cytokine 27-Plex Panel Kit (BIO-RAD, Hercules, USA) を用い、蛍光マイクロビーズアレイシステムである Luminex[®] (Hitachi, Tokyo) で測定し、27 種類のターゲットの濃度を測定した。各ターゲットに対する特異的二次抗体には、ターゲット毎に異なる蛍光強度で標識された 27 種類のビーズが結合しており、ビオチン結合特異的二次抗体、ストレプトアビジン-PE でターゲットの濃度を検出した。すなわち、50 μ l の上清をビーズ結合一次抗体と 30 分反応させ、洗浄後二次抗体と 30 分反応、さらに洗浄後、ストレプトアビジン-PE と 10 分反応させ、Luminex[®] で測定した。プレート壁への抗体付着を防ぐために、サンプルは 0.5% 以上の蛋白を含有させた。スタンダードは 0.5% BSA を含有する DMEM/F-12 で溶解し、また培養上清には、2.5% BSA を含有する DMEM/F-12 で BSA を添加した。

測定に際しては、低 pH 下で抗体反応を行うと、抗体の結合性が低下する可能性を考え、抗体反応条件を検討した。スタンダード溶解液の pH を 3~7.5 に調整したところ、pH 4 以下で抗体反応させると、測定値が低値となったため、pH 7 に戻した状態で抗体と反応させた。さらに、低 pH 下で蛋白を放置した場合、分解される可能性を考え、各種ターゲットの蛋白を含有するスタンダードを pH 3~7.5 に調整した溶解液中で 48 時間孵置し、回収後に pH 7 に戻して抗体と反応させた。IL-4, IL-6, IL-7, IL-9, IL-13, IL-17, PDGF-BB, MCP-1, RANTES, Eotaxin, MIP-1 β , IL-8, IP-10 は、pH 3 に孵置後も pH 7 で孵置した際と同等の結果が得られ、低 pH 下でも分解されないことが示唆された。以上から、上記のターゲットについて、上清を pH 7 に調整後に抗体と反応させて Luminex[®] で測定した。

3) 酸曝露が気道上皮細胞生存に及ぼす影響

pH 3~7 の各 pH で培養後の BEAS-2B 細胞の

培養上清を回収後、PBS200 μ l で付着細胞を洗浄し、PBS5 μ l およびトリプシン-EDTA20 μ l を各 well に分注して BEAS-2B を剥離した。この後各 well を Pipes A (25 mM Pipes, 119 mM NaCl, 5 mM KCl, 0.03% ヒトアルブミンを含有) 200 μ l で 2 回洗浄して細胞を回収した。細胞生存の解析は、MEBCYTO apoptosis Kit (MBL, Nagoya) を用いて行った。死細胞には FITC 標識 annexin V が結合することを利用して生細胞の分画をフローサイトメトリー (FACS Caliber, Becton Dickinson, USA) を用いて定量した。

4. 統計

喘息重症度と QUEST スコアの相関解析には Spearman 順位相関係数の検定を行った。QUEST スコアを規定する背景因子の解析は、ステップワイズ重回帰分析を行った。PPI 治療前後の各種パラメーターの比較は Wilcoxon 符号付順位和検定を用いた。BEAS-2B を用いた検討における多群間の比較には、一元配置分散分析法 (one-factor ANOVA) を用い、有意差を認めた場合、Fisher's PLSD 検定で群間の比較を行った。結果は平均値 \pm 標準誤差で示した。いずれの検定においても $p < 0.05$ をもって有意と判定した。

結 果

1. 気管支喘息患者における QUEST 問診票陽性率

まず、気管支喘息患者に QUEST 問診票を配布し陽性率を調査した。88 例中 4 点以上は 48 例 (54.5%)、6 点以上は 36 例 (40.9%) であった。また、気管支喘息重症度と QUEST スコアとは強い正相関を示した (図 1A, $p < 0.01$)。QUEST 陽性率と気管支喘息重症度との相関も検討したが、QUEST スコアのカットオフを 4 点以上、6 点以上とした場合の双方で強い正相関を示した (図 1B, $p < 0.01$)。

QUEST スコアを規定する背景因子について検討する目的で、QUEST スコアを目的変数とし、年齢、性別、気管支喘息重症度、 β_2 刺激薬、テオフィリン薬、吸入ステロイド薬、経口ステロイド薬の各薬剤使用を説明変数としたステップワイズ重回帰分析を行った。予測式として、QUEST スコア = 2.22x 気管支喘息重症度ステップ - 0.971 (r^2

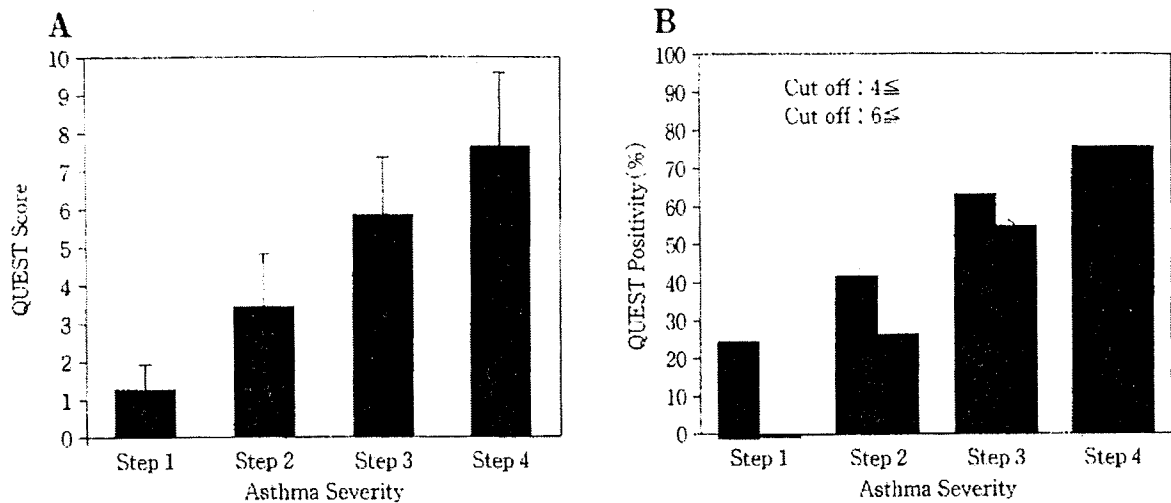


図1 気管支喘息重症度とQUEST score (A) およびQUEST陽性率 (B) との関連
 A: 気管支喘息重症度とQUEST scoreは有意な正相関を認めた ($p < 0.05$).
 B: 気管支喘息重症度とQUEST陽性率も, Cut off 4点以上 ($p < 0.05$), 6点以上 ($p < 0.05$) の双方で正相関を示した ($n = 88$, Step 1 ($n = 8$), Step 2 ($n = 37$), Step 3 ($n = 39$), Step 4 ($n = 4$)).

表2 PPI投与前後の呼吸機能検査, 喘息症状の変化 ($n = 13$)

		Baseline	Post-treatment	<i>p</i>
呼吸機能検査	FEV1.0 (L)	1.99±0.14	1.94±0.14	0.067
	% FEV1.0	87.9±4.7	84.8±5.1	0.106
	ピークフロー (朝) (l/min)	309.9±24.1	303.2±26.1	0.058
	ピークフロー (夕) (l/min)	313.9±21.1	314.3±23.8	0.913
喘息症状	痰量	0.7±0.2	0.5±0.1	0.236
	咳嗽	2.9±1.1	1.1±0.3	*0.036
	夜間点数	2.8±1.7	1.5±0.6	0.753
	症状点数	5.1±1.8	4.5±1.5	0.799

=0.120, $p = 0.0006$) が得られ, 使用薬剤よりも気管支喘息重症度がQUESTスコアに強く相関する因子として採用された。

2. QUEST陽性気管支喘息患者に対するプロトンポンプ阻害薬投与の効果

安定期気管支喘息患者において, PPI投与前後で各種パラメーターを比較した(表2)。QUESTスコアはPPI治療前後で有意に改善した(図2: $8.9 \pm 0.6 \rightarrow 6.2 \pm 1.0$, $p < 0.01$)。呼吸機能検査では, 1秒量, %1秒量(1秒量/予測1秒量 $\times 100$)、朝のピークフロー値は, 有意差には達せず, 夕のピークフロー値も改善を認めなかった。喘息症状に関しては, 夜間症状, 日内症状, 痰量のいずれも, 有意差には達しなかった。

一方, 咳嗽はPPI投与によって有意に改善した(図3: $2.9 \pm 1.1 \rightarrow 1.1 \pm 0.3$, $p < 0.05$)。PPI投

与前には13例中8例で咳嗽を認めたが, うち6例で投与後に改善した。

また, QOLもPPI投与によって有意に改善した。AQLQでは, 感情(図4A: $5.13 \pm 0.26 \rightarrow 5.50 \pm 0.23$, $p < 0.05$) および, 環境刺激(図4B: $5.16 \pm 0.31 \rightarrow 5.65 \pm 0.21$, $p < 0.05$)、SF-36では心の健康(mental health)が有意に改善した。(図4C: $71.8 \pm 3.9 \rightarrow 78.2 \pm 3.0$, $p < 0.05$)。

3. 酸曝露が気道上皮細胞株の生存および炎症性メディエーター産生に及ぼす影響

1) 酸曝露が気道上皮細胞生存に及ぼす影響

BEAS-2B細胞を種々のpHで経時的に培養し, 細胞生存を検討した結果を図5に示す。4時間後の早期より, pH依存性に生細胞比率の低下が認められ, pH5以下では, 有意に生細胞比率が減少していた。その後も時間経過に伴って, 生細

胞比率は減少し、24時間後には、pH5以下ではほとんどの細胞が細胞死に陥った。pH6においても24時間後には有意な細胞死を認め、以後経時的に細胞死は増加した。

2) 低pHが気道上皮細胞からの炎症性メディエーター産生に及ぼす影響

BEAS-2B細胞をpH7で72時間培養後の上清において測定可能であったサイトカイン・ケモカ

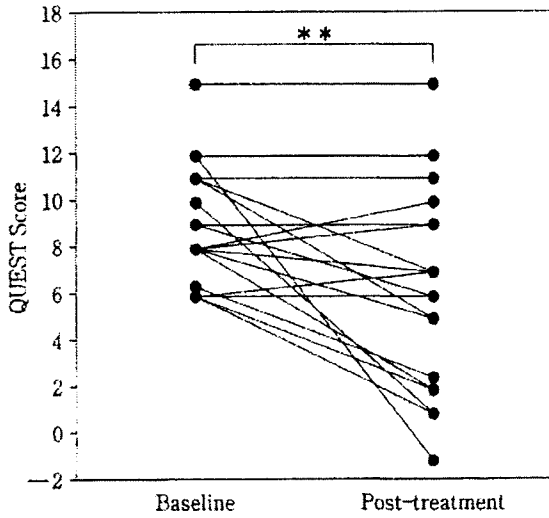


図2 PPI投与によるQUEST scoreの変化 (n=19, **p=0.006)

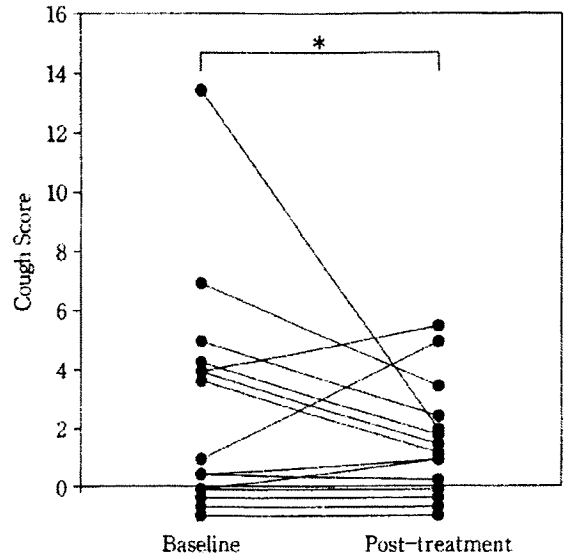


図3 PPI投与による咳嗽症状の変化 (n=13, *p=0.036)。

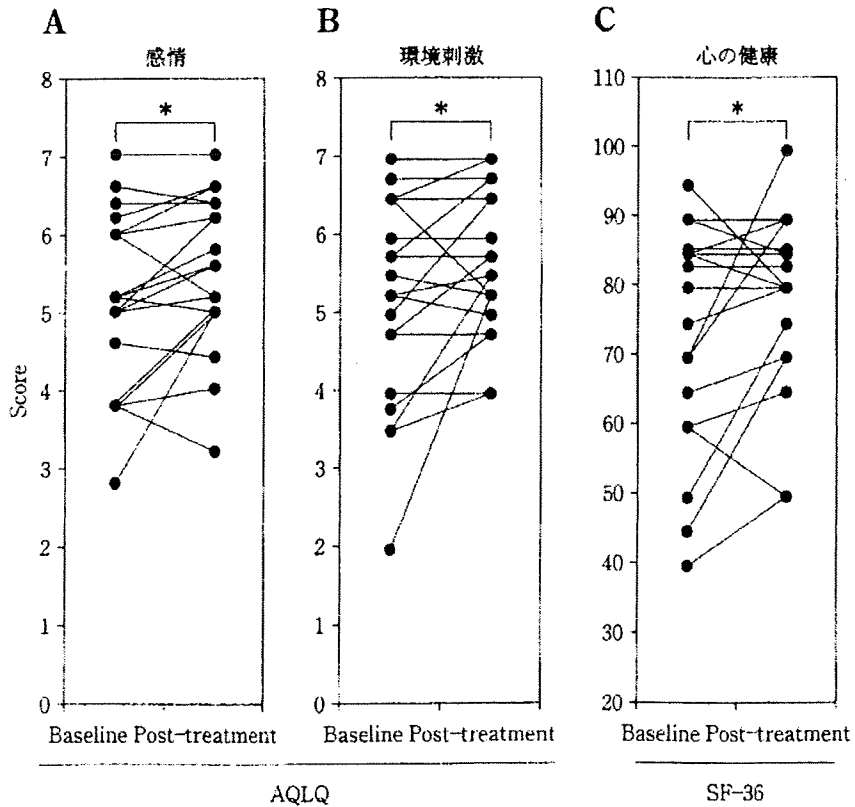


図4 PPI投与によるQOLの変化 (n=19, *p<0.036)。

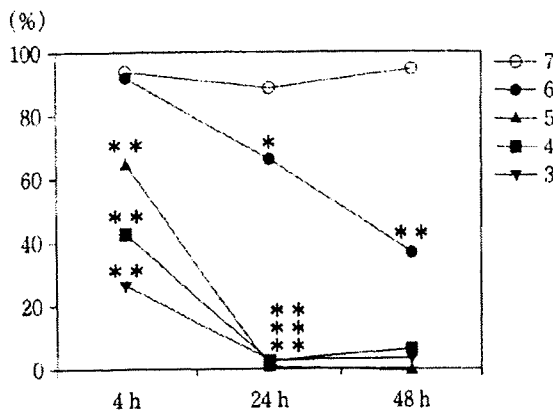


図5 BEAS-2B生存率の経時的变化
総細胞数を100%とし、Annexin V陰性の生細胞数の占める比率を示した
($n=6$, 平均値±標準誤差). 各培養時間におけるpH7での生存率と比較して, * $p<0.05$, ** $p<0.01$.

インはIL-6, MCP-1, RANTES, MIP-1 β , IL-8, IP10であった。これらのサイトカイン・ケモカインはpH依存性に分泌が減弱した。MCP-1, MIP-1 β , IL-8, IP10はpH6.5で、IL-6, RANTESはpH6で有意に産生が減弱していた(図6)。一方、PDGF-BBは、pH7で72時間培養後には、 17.7 ± 9.8 pg/mlと低値であったが、pH4より低いpHでの培養では、有意にPDGF-BB分泌が増強した(pH3: 454.5 ± 102.6 pg/ml, $p<0.01$)。時間経過も検討したが、pH3では、培養4時間後より有意にPDGF-BB分泌が増強していた(pH7: 0.2 ± 0.0 pg/ml vs pH3: 123.6 ± 14.8 pg/ml, $p<0.01$)。

以上から、酸曝露によって早期より気道上皮細胞死が惹起され、PDGF-BB産分泌が増強することが観察された。

考 察

本研究では、気管支喘息患者のQUEST陽性率は、カットオフ値を4点以上とした場合54.5%、6点以上とした場合40.9%であり、気管支喘息患者は高率にGERDを合併していることが示唆された。また、QUESTスコアは気管支喘息重症度と正相関すること、QUESTスコアは使用薬剤よりも気管支喘息重症度と強く相関することが示された。さらに、QUEST陽性患者にPPIを投与すると、QUESTスコアおよび咳嗽は有意に改善し、

QOLも向上したことから、気管支喘息患者におけるGERD合併を、QUEST問診票を用いて診断・治療する診療モデルの妥当性が示唆された。

GERDと気管支喘息の関連は1960年代から報告されており²⁾、喘息患者の40%にびらん性食道炎があることや、80%以上に異常逆流を認めることが報告されている³⁾。既報では、気管支喘息患者におけるGERDの診断は、比較的侵襲の高い上部消化管内視鏡やpHモニタリングで行われているものが多く、QUEST問診票を用いた報告は2報のみである。気管支喘息患者におけるQUEST陽性率は41~73%と報告されており⁴⁾、われわれの検討でもカットオフ値を4点以上とした場合54.5%と高率に陽性であった。QUEST陽性率が気管支喘息患者において、非喘息患者と比して有意に高いかどうかは、非喘息患者における調査が必要であるが、気管支喘息重症度とQUESTスコアが有意な正相関を示したことは、両病態の間に強い関連性があることを示唆している。

QUEST問診票は、胸焼けなどの典型的なGERD症状をスコア化しており、消化器疾患領域では広く用いられているが、気管支喘息患者において、GERDの典型的な症状がいかに修飾されるかについては不明であり、治療的に介入してQUEST問診票の有用性を検証した報告はない。今回の検討では、PPI投与後に逆流症状を示すQUESTスコアは19例中11例で、また全体としても有意に改善しており、気管支喘息患者においてもGERD診断にQUEST問診票を適用することの妥当性が示唆された。

また、気管支喘息重症度とGERD合併率の相関に関する詳細な報告はなかったが、われわれの検討では気管支喘息重症度とQUESTスコアは強い正相関を認め、両病態の関連が定量的に示唆された。さらに、気管支拡張薬である β_2 刺激薬やテオフィリンによる下部食道括約筋の弛緩が、GERDを増悪させる可能性も指摘されているが、興味深いことに、今回行った重回帰分析では、QUESTスコアを最も強く規定する因子は、使用薬剤ではなく気管支喘息重症度であった。Sontagらも、気管支喘息患者において、気管支拡張薬使用の有無はGERD合併率と関連しなかったことを報告している⁵⁾。これらのことは、薬剤による修飾のみで

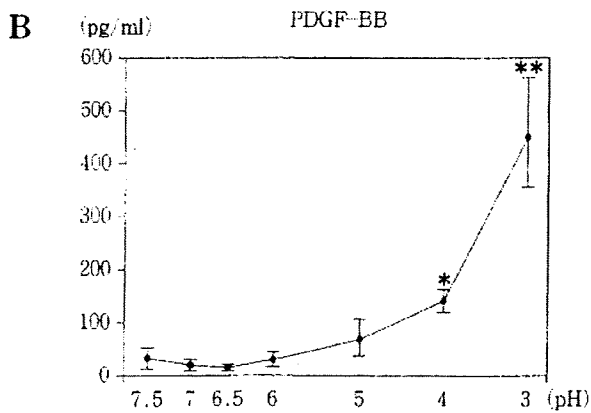
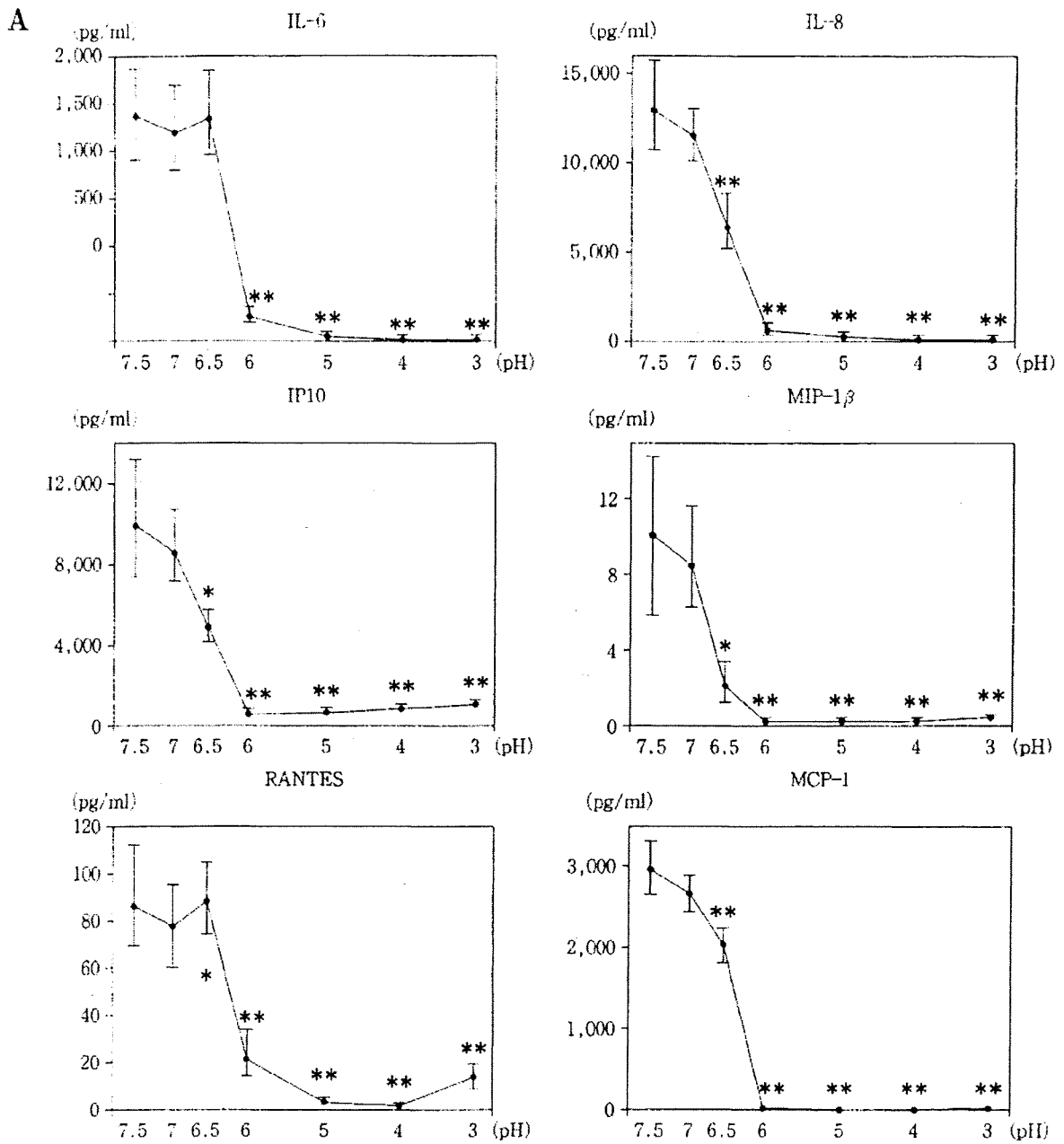


図6 BEAS-2Bからのサイトカイン・ケモカイン分泌のpH依存性
 A: 72時間培養後の上清中各種サイトカイン・ケモカイン濃度,
 B: 72時間培養後の上清中PDGF-BB濃度, pH7における濃度と比較して, ** $p < 0.01$, $n = 4$, 平均値±標準誤差).

はなく、気管支喘息病態とGERDの関連には他の生物学的メカニズムが関与していることを示唆している。気管支喘息重症度とQUESTスコアが正相関を示した因果関係については、まず、気管支喘息病態がGERDを惹起する背景として、1) 重症喘息では閉塞性換気障害が強く、胸腔内の圧較差が大きいため、GERDを高頻度に誘発する、2) 喘息の発症と経過に関する調査票⁶⁾を用いたわれわれの検討では、ステップ4患者21名のうち、76.2%で心身症の存在が示唆され、重症喘息では心理的ストレスが酸分泌亢進に働く、3) 重症喘息では、気管支拡張剤の多剤併用が潜在的なGERD合併のリスクとなる、ことなどが仮説として想定されるが、3)については、上述のように否定的な見解もある。一方、逆にGERDが気管支喘息病態を重症化させる機序としては、4) 今回 *in vitro* の検討で示唆されたように、気道内への酸微量誤嚥が、気道傷害を介して喘息病態を悪化させる、5) GERDによって迷走神経系がさらに賦活化され、気道過敏性を亢進させる、ことなどが想定されるが、5)については後述のように否定的な見解もある。気管支喘息病態とGERD病態の因果関係については双方の機序が考えられ、結論づけることは困難であるが、心身医学的側面からのアプローチはほとんどなされておらず、今後の検討課題と考えられる。

治療の観点からは、GERDを治療することで、気管支喘息病態がいかにか改善するかのランダム化比較試験が1980年代から行われるようになった。初期には、H₂阻害薬の効果が複数の試験で検討され、1990年代にはPPIを用いた検討も行われてきた。比較的少数を対象とした試験では、呼吸機能や喘息症状の改善を示す検討もみられたが、一貫した結果は得られていない。メタアナリシスでも、GERD治療によって、全体としては気管支喘息病態の有意な改善は認めないとしている¹⁹⁾。われわれの検討でもPPI投与後の1秒量やピークフロー値の有意な改善は認めなかった。

これらの報告は、評価項目として気管支喘息症状や呼吸機能を検討しているが、気管支喘息発作やQOLに及ぼす効果に関しては報告が少ない。近日、PPI長期投与が気管支喘息発作へ及ぼす影響についてはじめて報告され、GERD治療によ

って発作回数が減少することが報告された¹⁴⁾。また、気管支喘息患者において、GERD治療がQOLに及ぼす影響を検討した報告は2報のみであるが^{14,15)}、双方においてQOLの改善が報告されている。Levinらは24時間pHモニタリングによって、Littnerらは酸逆流症状の間診でエントリーを行っているが、われわれはQUEST問診票のみでエントリーを行い、やはりQOLの有意な改善を認めた。

今回のわれわれの検討では、PPI治療によって、気管支喘息患者の咳嗽症状が有意に抑制された。これまでのランダム化比較試験では、評価項目として、咳嗽の改善に注目して記載している報告はほとんどない。咳嗽はQOLを低下させる自覚症状であり¹⁹⁾、今回の検討で認められたQOLの改善には、QUESTスコアの減少で示された逆流症状の改善に加えて、咳嗽の改善も寄与している可能性が示唆された。

気管支喘息患者の咳嗽が、GERDによって増悪するメカニズムには不明な点が多いが、気管支喘息患者では咳感受性が亢進していること^{16,19)}、非喘息患者においても酸逆流があると咳感受性が亢進すること、そして気管支喘息患者に酸逆流が合併するとさらに咳感受性が亢進することが報告されている。

気管支喘息における咳感受性亢進の機序として、シクロオキシゲナーゼやTXA₂の関与が示唆されている¹⁹⁾。また、慢性咳嗽においては、PGE₂¹⁶⁾、神経ペプチドCGRP (calcitonin-gene-related-peptide)¹⁷⁾の関与が報告されている。気管支喘息は気道の慢性炎症を病理学的特徴としており、局所で増加したメディエーターが咳感受性の亢進に寄与している可能性がある。

また、非喘息患者で、酸逆流が咳嗽の原因となることを示した報告は多く^{18,19)}、食道遠位のpH低下が咳嗽と関連することが報告されている。この機序に関しては、酸刺激によって神経末端から分泌されるタキキニン様物質が、食道迷走神経を刺激し、求心性に情報伝達され、遠心性に気道症状が出現することが示唆されている²⁰⁾。また、気道系の感覚神経として、酸などの刺激物質を認識する化学受容体があるが、食道にも同様に化学受容体があり、気道および食道の化学受容体を持つ神

経末端は、解剖学的には孤束核の近傍に位置しており、食道と気道系の神経学的相互作用が示唆されている²¹⁾。

さらに、気管支喘息において、酸逆流が咳感受性をさらに亢進させる知見も報告されている。軽症気管支喘息患者の食道内に塩酸を投与した試験では、咳感受性は亢進するものの、呼吸機能は変化しないことが報告されている²²⁾。近日、喘息患者にPPI投与を行うと、咳感受性は改善するが、気道過敏性は改善しないことも報告された²³⁾。咳感受性と気道過敏性や呼吸機能にPPIが及ぼす影響の差異についてのメカニズムは不明であるが、これは、PPI投与を行った臨床試験で、気道過敏性の改善を認めた報告がないことと一致する知見であり、今後さらなるメカニズムの解明が必要である。

GERDが喘息病態を悪化させる機序のうち、酸の誤嚥が喘息病態をいかに悪化させるかの機序を明らかにする目的で、*in vitro*の実験を行った。気道上皮細胞は、外界への最前線に位置し、単なる物理的バリアではなく、種々の疾患においてサイトカイン、ケモカインなどの産生を介して気道炎症形成に関与することが明らかとなっており、酸の微量誤嚥に際しては、酸曝露の影響を直接うける細胞と考えられる。今回の検討では、酸曝露後4時間より有意な生細胞数の減少が認められた。喘息気道の病理学的特徴は、気道上皮の剥離、損傷とされており、既存の気道上皮傷害が、酸曝露によってさらに増悪することが、喘息病態悪化に寄与する可能性が示唆された。

さらに、酸曝露が気道上皮細胞からの炎症性メディエーター産生に及ぼす影響を検討した。種々の炎症細胞集積・活性化に関与するケモカインに関しては、CCケモカインであるMCP-1, RANTES, MIP-1 β , またCXCケモカインであるIL-8, IP-10のすべてで分泌が抑制された。一方、増殖因子であるPDGF-BBの産生は酸曝露によって亢進していた。PDGFは線維芽細胞の遊走・増殖因子であり²⁴⁾、PDGF-A鎖, B鎖からなるダイマーから形成され、PDGF-AA, PDGF-AB, PDGF-BBのアイソフォームが存在する。PDGF-BをもつPDGF-BBやPDGF-ABは、PDGF-AAより強い線維芽細胞増殖や遊走を惹起することが

示されている^{25,26)}。疾患との関連では、線維化が中心的な病態を形成する肺線維症において広く検討されてきており、マウス肺にPDGF-Bを強制発現すると肺線維化が亢進することが報告されている²⁷⁾。気管支喘息病態においても、気道の基底膜下の線維化が気道リモデリングを惹起し、非可逆的気道閉塞の原因となることが示唆されている。気道リモデリングに関与する分子としては、TGF- β の重要性が示唆されているが²⁸⁾、当研究室では喘息モデルマウスにおいて、PDGF-Bの阻害が、気道過敏性や気道壁肥厚を抑制することを示しており、PDGF-Bの重要性が示唆されている²⁹⁾。今回の検討では、気道上皮細胞におけるPDGF-BB蛋白分泌は酸曝露によって亢進しており、気道の酸曝露がPDGF-BBを介して気道リモデリングを惹起する機序が示唆された。

今回、QUEST問診票を用いた検討で、気管支喘息患者にGERDが高率に合併することが示唆され、特に重症度と有意に相関することから、気管支喘息増悪因子としてのGERDを検索することの重要性が示唆された。さらに、PPI投与によって、QUESTスコアおよび咳嗽は有意に改善し、QOLも向上したことから、気管支喘息患者におけるGERD合併を、QUEST問診票を用いて診断・治療する診療モデルの妥当性が示唆された。吸入ステロイド薬をはじめとする抗炎症療法の進歩により、気管支喘息の管理は大きく進歩したが、いまだに重症例は存在し、喘息死も年2,000人を超えている。背景因子として看過されがちなGERDを短時間で検索し、治療することが、気管支喘息症例の管理およびQOL向上につながりうるものと考えられた。

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