Airway smooth muscle cells (ASMCs) have been reported to express Toll-like receptors (TLRs) and take part in the pathogenesis of asthma exacerbation. Though TLRs were found to activate epidermal growth factor receptor (EGFR) in airway epithelial cells, little is known about the association of TLR ligands with EGFR signaling pathways in ASMCs. Using primary cultured ASMCs from Brown Norway rats, TLR4, eotaxin, and RANTES mRNA were examined by real-time quantitative RT-PCR after stimulation with the TLR4 ligand, lipopolysaccharides (LPS). The concentration of RANTES protein in culture supernatant was measured by ELISA. The effect of EGFR signaling inhibitors on RANTES expression was examined as well. Phosphorylation of EGFR after stimulation was examined by Western Blotting. Rat ASMCs expressed TLR4 and eotaxin, and LPS upregulated RANTES production. The EGFR tyrosine kinase inhibitor AG1478, the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, and the matrix metalloproteinase (MMP) inhibitor GM6001 inhibited RANTES expression induced by LPS. LPS phosphorylated EGFR. TLR4 activation can induce RANTES expression via EGFR transactivation and PI3K/Akt pathway in rat ASMCs. MMP-induced EGFR proligand cleavage and ligand binding to EGFR seem to be involved in this pathway. These findings may be critical in the pathogenesis of asthma exacerbation by airway infection.

Key words: RANTES, airway smooth muscle, Toll-like receptor, asthma, rats

Introduction

The innate immune system is the first line of host defense against microorganisms. When microorganisms invade the body, phagocytes such as macrophages, neutrophils, and dendritic cells engulf and kill them. At the same time, these cells recognize conserved motifs on a group of pathogens as "pathogen-associated molecular patterns (PAMPs)". Toll-like receptors (TLRs) play an essential role in the innate recognition of PAMPs and in the triggering of adaptive immunity. Eleven TLRs have been found so far in the human genomes: TLR-1-TLR-11.

The risk of development of allergic disease is thought to be modified by infections through the activation of TLRs by PAMPs. Several TLRs or TLR ligands have been associated with asthma. Elevated levels of lipopolysaccharide (LPS), a variant of the TLR4 ligand, have been negatively correlated with the incidence of asthma in several studies. On the other hand, low-level inhaled LPS signaling through TLR4 has been found to be necessary for the induction of Th2 responses in inhaled antigens in a mouse model of allergic sensitization.

Airway smooth muscle cells (ASMCs) produce various pro-inflammatory cytokines and chemokines that may perpetuate airway inflammation and the development of airway remodeling. Human ASMCs have been shown to express TLRs. The expressions of TLR2, TLR3, and TLR4 in human ASMCs are regulated by cytokines and TLR ligands, and the activation of these receptors mediates chemokine release.
activating intracellular TLR3 in bronchial smooth muscle cells. Respiratory RNA virus infections stimulate the production of eotaxin-1/CCL11 and enhance eosinophilic inflammation of the airways in the Th2-dominant microenvironment. These findings suggest that ASMCs participate in the pathogenesis of exacerbation of inflammation during infection in asthmatic patients.

Infections of respiratory epithelial cells with rhinovirus, respiratory syncytial virus (RSV), and influenza virus are associated with the production of chemokines such as eotaxin-1/CCL11 and RANTES (regulated on activation, normal T cell expressed and secreted)/CCL5. The ASMCs are another major source of chemokines, including eotaxin and RANTES. RANTES, IL-8, and eotaxin are important chemokines for activation of eosinophils, critical effector cells in the pathogenesis of asthma. RANTES is a potent chemoattractant for eosinophils as well as for other cell types observed in allergic inflammation, including monocytes and memory T lymphocytes. Eotaxin is a highly selective chemoattractant for eosinophils. Production by airway smooth muscle cells of RANTES, IL-8, eotaxin implies a role for these structural cells to participate directly in the inflammatory process through recruitment and activation of eosinophils and neutrophils in the airways. Pretreatment of cytokine-treated airway smooth muscle cells with dexamethasone resulted in reduced expression of the RANTES gene and inhibition of its protein product.

Airway epithelial cells express high levels of several TLRs, and several TLR ligands enhance the expression of chemokines, cytokines, and host defense molecules by activating epithelial cells. Koff et al. showed that multiple TLRs in airway epithelial cells produce innate immune responses by activating epidermal growth factor receptor (EGFR) via an epithelial cell signaling cascade. Reactive oxygen species (ROS) were generated by stimulation with TLR1/2, 3, 5, and 6/2. ROS activate the latent form of tumor necrosis factor-α-converting enzyme (TACE), removing the prodomain and exposing the active domain to cleave transforming growth factor (TGF)-α proligand. TGF-α was released, then bound to and activated EGFR, initiating signaling for IL-8 and vascular endothelial growth factor (VEGF) production.

To clarify the role of LPS on asthma exacerbation by airway infection, we examined LPS-induced chemokine expression in ASMCs and also aimed to investigate signaling pathways which might involve TLR4 and EGFR.

Materials and Methods

Animal preparation and treatment

Nine- to 13-week-old male naïve BN rats (Sankyo Labo Service Corporation, Inc., Tokyo, Japan) were used for the experiments. All procedures with the animals were in compliance with policy for the ethical treatment of animals observed by the animal facility at Tokyo Medical and Dental University, and were approved by the Tokyo Medical and Dental University (Tokyo, Japan) Animal Care Committee. Tracheas were harvested from naïve BN rats and digested for 30 minutes with elastase (Sigma-Aldrich Japan K.K., Tokyo, Japan) and collagenase (Sigma-Aldrich Japan K.K., Tokyo, Japan) to isolate ASMCs. The cells were cultured in Dulbecco's Modified Eagle Medium - high glucose (Sigma-Aldrich Japan K.K., Tokyo, Japan) and Nutrient Mixture F-12 (GIBCO® Invitrogen, Life Technologies Japan Ltd., Osaka, Japan) (DMEM/F-12) medium with 10% sterile-filtered heat-inactivated fetal bovine serum (Moregate Biotech, Bulimba, Australia), 50 U of penicillin, 50 µg of streptomycin (Penicillin-Streptomycin, liquid, GIBCO® Invitrogen, Life Technologies Japan Ltd., Osaka, Japan), and 2.5 µg of amphotericin (Sigma-Aldrich Japan K.K., Tokyo, Japan). ASMCs were plated on 6-well plates after 3 to 6 passages and grown to confluence. Positive staining of the cells by anti-smooth muscle α-actin antibody was used to confirm the presence of airway smooth muscle cells. Confluent ASMCs on 6-well plates were starved in the absence of FBS for 24 hours, then stimulated with LPS from Escherichia coli O55:B5 (Sigma-Aldrich, St. Louis, MO, USA) for designated periods of time.

AG1478 (EGFR tyrosine kinase inhibitor) was purchased from Calbiochem (Darmstadt, Germany), LY294002 (selective phosphoinositide 3-kinase (PI3K) inhibitor) was purchased from Cayman Chemical (Ann Arbor, MI, USA), and GM6001 (broad-spectrum MMP inhibitor) was purchased from Biomol International, Inc. (Plymouth Meeting, PA, USA). For inhibitory experiments, ASMCs were pretreated with AG1478, LY294002, or GM6001 at 30 minutes before stimulation. Doses of these inhibitors we used were less than the levels of cytotoxicity for ASMCs.

Real-time quantitative PCR

Total RNA was extracted from ASMCs using RNeasy Mini Kit (QIAGEN, Maryland, USA) according to the manufacturer’s instructions. Complementary DNA was generated in a 20 µg reaction using 0.5 µg of total RNA.
oligo(dT)12-18 primers (Invitrogen, Life Technologies Japan Ltd., Osaka, Japan) and SuperScript™ II (Invitrogen, Life Technologies Japan Ltd., Osaka, Japan). Real-time quantitative PCR using specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TLR4, eotaxin, RANTES was performed with the Mini Opticon (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sequences and annealing temperature of PCR primers are shown in Table 1. The sequences of primers for TLR4, eotaxin, RANTES, and GAPDH have been previously published21-24. The PCR primers were generated by Sigma-Aldrich Japan KK. (Tokyo, Japan).

Melting curve analysis was used to assess the specificity of the amplification products, as well as primer-dimer formation. The mRNA encoding endogenous GAPDH was quantified as a reference gene and used to correct for variations in the cDNA content among samples. The comparative threshold cycle method was used to analyse the gene expression, and relative gene expression was normalized to GAPDH.

Real-time quantitative PCR was used to examine the mRNA expression of each gene at baseline and after treatment with LPS (1-10 µg/ml). Additional analyses were also performed to assess RANTES mRNA expression after pretreatment with inhibitors of the EGFR signaling pathway followed by stimulation with LPS.

### Western blot

BN rat ASMCs were grown to confluence, starved, and stimulated with LPS for designated periods of time, as described in the section on animal preparation and treatment. To prepare positive controls for ASMC phosphorylation, ASMCs were stimulated with 10 ng/ml of EGF (R&D systems, Inc., Minneapolis MN, USA) for 30 seconds. The cells were washed twice with ice-cold PBS, lysed in RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.25% Na-deoxycholate, 1% NP-40 ) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and sonicated for 10 seconds with Vibra-Cell™ (Sonics & Materials Inc., Newton, CT, USA). Lysates were centrifuged at 14,000 rpm for 10 minutes and supernatants were collected. Supernatant proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15 µg/lane-7.5% gel) and transferred to a PVDF membrane (Immobilon™, Millipore Corp, Billerica, MA, USA). Immunoblotting was performed with anti-phospho-EGFR (Tyr1068)(1H12) mouse monoclonal antibody and anti-EGFR (C74B9) rabbit monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4 ℃, and immune complex was detected by Amersham ECL plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, England) using Anti-mouse Ig, Horseradish Peroxidase linked F(ab’)2 fragment (from sheep) and ECL™ Anti-rabbit IgG, Horseradish Peroxidase linked F(ab’)2 fragment (from donkey) (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England (formerly Amersham

### Chemokine ELISA assay

Eotaxin and RANTES in culture supernatant were measured by Quantikine® Mouse CCL11/Eotaxin Immunoassay and Quantikine® Mouse RANTES Immunoassay (R&D systems, Inc., Minneapolis MN, USA) which were proven to have cross-reactivity with rats according to the manufacturer’s instructions.

### Table 1. PCR primer sequences, annealing temperatures, and the expected sizes of PCR products.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temperature (℃)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’-AATGCATCTGCACCACCAACTGC-3’</td>
<td>5’-GGAGGCGATGAGCCCATGAGT-3’</td>
<td>56</td>
<td>546</td>
</tr>
<tr>
<td>TLR4</td>
<td>5’-CCAGAGCCGGTGTTGATCT-3’</td>
<td>5’-TCAAGGGCTTTCATCAAC-3’</td>
<td>62</td>
<td>239</td>
</tr>
<tr>
<td>eotaxin</td>
<td>5’-AACTCTCATGCTGATTACCATA-3’</td>
<td>5’-TTTGGAGTTGGTGGTTGT-3’</td>
<td>62</td>
<td>202</td>
</tr>
<tr>
<td>RANTES</td>
<td>5’-CCATATGGCGGACACCA-3’</td>
<td>5’-CCCACCTTCTCTCTGGGTG-3’</td>
<td>69</td>
<td>168</td>
</tr>
</tbody>
</table>
Statistical analysis

Data are presented as median ± range or quantile deviation (QD). Multiple comparisons of mRNA levels and chemokine concentrations were performed by Kruskal-Wallis test as one-way ANOVA followed by Dunnett post hoc test using GraphPad Prism version 5 for Windows (MDF Co., Ltd., Tokyo, Japan). A P value of less than 0.05 was considered statistically significant.

Results

**TLR4 mRNA expression in rat ASMCs**

To determine whether rat ASMCs express TLR4 mRNA and whether the expression of this receptor was affected by its ligand, we performed real-time quantitative RT-PCR for TLR4 at baseline and after stimulation with LPS. TLR4 mRNA was detected on rat ASMCs under basal conditions. The expression level was not affected by the administration of its ligand (Fig. 1).

**RANTES and eotaxin mRNA expression induced by TLR4 ligand**

Having observed that BN rat ASMCs express TLR4, we performed real-time quantitative PCR for eotaxin and RANTES after stimulation with LPS to ascertain the expression of chemokine with TLR4 stimulation. Eotaxin mRNA was also detected on rat ASMCs under basal condition. No upregulation of eotaxin mRNA was observed in response to the administration of LPS (Fig. 2A). The expression of RANTES mRNA, meanwhile, was hardly detectable under the basal condition, but significantly upregulated by the administration of LPS (Fig. 2B).

**RANTES protein production induced by TLR4 ligand**

From the result of RANTES and eotaxin mRNA expression, we investigated the production of these proteins in culture supernatant. The RANTES protein concentration in culture supernatant was measured at time points after cell treatment with LPS. RANTES was not detected in culture supernatant under baseline condition. When the cells were stimulated with LPS, however, they produced RANTES protein (Fig 2C). No eotaxin was detected in the culture supernatant under any condition (data not shown). These results demonstrate that the activation of TLR4 by its ligand influences the RANTES expression.

**Effects of EGFR tyrosine kinase inhibitor and PI3K inhibitor on RANTES expression**

To investigate the mechanism of RANTES upregulation by TLR4 ligand, we examined the effects of two inhibitors of the EGFR signaling pathway. Rat ASMCs were pretreated with the EGFR tyrosine kinase inhibitor AG1478 or the PI3K inhibitor LY294002 before stimulation by the TLR4 ligand. Both AG1478 and LY294002 inhibited RANTES mRNA and protein expression in culture supernatant induced by LPS (Fig. 3A, B).

**Effect of MMP inhibitor on RANTES expression**

Next, we tested the effect of broad spectrum MMP inhibitor. Rat ASMCs were pretreated with MMP inhibitor GM6001 before the stimulation with the TLR4 ligand. GM6001 inhibited RANTES mRNA and protein expression in culture supernatant after stimulation with LPS (Fig. 3C).

These results suggested that phosphorylation of the EGFR tyrosine residues and PI3K was involved in the RANTES expression induced by the TLR4 ligand. We also learned that MMP was involved in RANTES expression induced by the TLR4 ligand.

**Transactivation of EGFR in the RANTES expression induced by TLR4 ligand**

Finally, we performed Western blotting for phosphorylated-EGFR to determine whether the
transactivation of EGFR is involved in the RANTES expression induced by the TLR4 ligand. Stimulation with the EGFR ligand EGF induced phosphorylation of EGFR for use as a positive control. EGFR was also phosphorylated in cells stimulated with LPS (Fig. 4). This indicates EGFR transactivation with LPS.

Discussion

Our study has shown that rat ASMCs express TLR4, and that TLR4 ligand induces RANTES expression, presumably via EGFR transactivation and the PI3K/Akt pathway. The RANTES expression induced by the TLR4 ligand was inhibited by pretreatment with the EGFR tyrosine kinase inhibitor AG1478 and the PI3K inhibitor LY294002. Moreover, pretreatment with the MMP inhibitor GM6001 inhibited the RANTES expression induced by the TLR4 ligand. From these results, we assumed that this RANTES expression induced by TLR4 ligand was associated with the MMP-induced EGFR proligand cleavage and ligand binding to EGFR.

GM6001 is a potent broad-spectrum inhibitor of MMPs, TACE, and a disintegrin and metalloproteases (ADAMs). Strong inhibition of RANTES mRNA by pretreatment with GM6001 at 24hr may come from several inhibitory ways by GM6001 in addition to the inhibition of the cleavage of EGFR proligands. RANTES protein expression at 24 hours by LPS was not significantly inhibited by pretreatment with GM6001. Compared to the inhibition of RANTES mRNA by GM6001, it is possible that in 24 hours, RANTES protein expression induced by LPS-TLR4-NFκB pathway compensate for the suppression of RANTES by GM6001.

Epithelial cells express mRNA for TLR1-TLR10, and several known TLR ligands trigger the epithelial cell expression of chemokines, cytokines, and host defense molecules. Rat ASMCs expressed TLR4 in our experiment, but the ligand of the TLR4 brought about no increase in the level of receptor expression. In human ASMCs, LPS has no effect on TLR4 mRNA. Our result was consistent with that of human ASMCs. TLR signal triggers downstream kinase cascades which activate nuclear factor κB (NF-κB), mitogen-activated protein kinase, interferon-regulatory factor 3, and interferon-regulatory factor. This, in turn, leads to the production of proinflammatory cytokines, chemokines, type 1 interferons, hematopoietic factors, acute-phase proteins, and antimicrobial factors. In response to infectious stimuli, airway epithelial cells and airway smooth muscle cells are both reported to produce chemokines such as eotaxin and RANTES, and to contribute to airway inflammation. In our study, stimulation of rat ASMCs with the TLR4 ligand induced RANTES expression. Upon observing no upregulation of eotaxin mRNA expression by the TLR4 ligand, we focused on the RANTES expression induced by...
Figure 3: Effects of AG1478, LY294002, and GM6001 on RANTES mRNA and protein expression in ASMCs after the TLR4 ligand stimulation. Primary cultured rat ASMCs were starved for 24 hours, then pretreated with EGFR tyrosine kinase inhibitor AG1478 (A), PI3K inhibitor LY294002 (B), and MMP inhibitor GM6001 (C) at 30 minutes before stimulation with LPS for designated periods of time. The mRNA expression of RANTES at baseline and after treatment with LPS was examined by real-time quantitative PCR. The comparative threshold cycle method was used to analyse the gene expression, and relative gene expression was normalized to GAPDH. RANTES protein expression in culture supernatant was measured by ELISA. RANTES/GAPDH mRNA levels and RANTES protein levels pretreated with DMSO (A, C) or PBS (B) and stimulated with LPS are presented as 1.

A. RANTES mRNA expression in ASMCs and RANTES concentrations in culture supernatant after AG1478 pretreatment (1, 10 μM) and LPS (10 μg/ml) stimulation
B. RANTES mRNA expression in ASMCs and RANTES concentrations in culture supernatant after LY294002 pretreatment (1, 10 μM) and LPS (10 μg/ml) stimulation
C. RANTES mRNA expression in ASMCs and RANTES concentrations in culture supernatant after GM6001 pretreatment (10, 20 μM) and LPS (10 μg/ml) stimulation

Bars represent median ± QD of 5 experiments as fold numbers of basal control.

Multiple comparisons of mRNA levels and chemokine concentrations were performed by Kruskal-Wallis test as one-way ANOVA followed by Dunnett post hoc test. *: p<0.05 compared with cells pretreated with DMSO (A, C) or PBS (B).

Figure 4: EGFR phosphorylation by the TLR4 ligand. Western blotting for phosphorylated-EGFR (P-EGFR) and EGFR was performed with rat ASMCs after 30-second treatment with LPS (10, 20 μg/ml). Stimulation with EGF used as a positive control caused EGFR phosphorylation. The results presented are representative of two experiments performed on different cell lines.
The EGFR receptor family has four members, EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. A family of ligands, the EGFR-related peptide growth factors, binds to the ErbB receptors and induces the formation of homo- and hetero-dimers, which in turn leads to the phosphorylation of specific tyrosine residues and the activation of intracellular signaling pathways. The EGFR signaling pathway elicits protective responses by the epithelium such as mucin production and secretion for mucociliary clearance of invading organisms, neutrophil recruitment for bacterial killing, and epithelial wound healing for repair of injured tissue. Human ASMCs express mRNA and protein of all members of the EGFR family, and EGFR expression is increased in the airway epithelium and smooth muscle of asthmatics. We hypothesized that other than the main pathway of LPS-TLR4-NF-κB, EGFR signaling might be involved in the RANTES expression induced by the TLR4 ligand. The EGFR tyrosine kinase inhibitor AG1478 and PI3K inhibitor LY294002 inhibited RANTES expression induced by LPS. The EGFR ligands EGF, TGF-α, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, and epiregulin activate the receptors directly by binding to EGFR. These ligands are formed as inactive transmembrane precursors which undergo ectodomain cleavage to release mature active growth factor. Several metalloproteases, including ADAM 10,-12,-15, and -17, have been reported to cleave EGFR proligands in response to G-protein-coupled receptor (GPCR) activation. Since the MMP inhibitor GM6001 inhibited RANTES upregulation by the TLR4 ligand, we can assume that RANTES expression induced by the TLR4 ligand is linked to metalloprotease induced EGFR proligand cleavage and ligand binding to EGFR. Our results indicate that TLR4 ligand LPS induces RANTES expression via TLR recognition, EGFR phosphorylation, PI3K activation, Akt phosphorylation and activation of the NF-κB pathway. Ligand-bound TLR4 in our experiments appears to stimulate EGFR via putative metalloproteinase-induced mechanisms.

There are important limitations of this study. First, we were not able to prove that the TLR4 ligand leads metalloproteinase-induced EGFR proligand cleavage. ELISA for rat EGFR ligands was not available. Second, we tested for the direct effect of some major EGFR ligands, EGF, HB-EGF and TGF-α without LPS on the induction of RANTES in the same series of experiments, but failed to observe its upregulation. Moreover, there is another possibility of "ligand-independent" EGFR phosphorylation. When epithelium cells are treated with GPCR activators, no soluble ligands are detected in the culture medium. Therefore, we assumed two possibilities. The first is that other minor ligands might
be involved in the pathway of RANTES induction by LPS. The second is that "ligand-independent" EGFR phosphorylation is also associated with EGFR transactivation in ASMCs. Third, among the various signaling pathways downstream of activated EGFR, we only investigated the PI3K/Akt pathway. Other signaling pathways such as the STAT pathway and RAS-MAPK pathway may also play roles in RANTES expression induced by the TLR4 ligand. The EGFR signaling pathway and its component step of ligand recognition by TLR4 merit further investigation in studies on asthma exacerbation by infection.

In conclusion, we found that in addition to the main pathway through NF-κB, TLR4 activation can induce RANTES expression through the EGFR axis in rat ASMCs. Bacterial infection stimulates TLR4, which then leads to EGFR activation and the production of chemokines such as RANTES. The TLR-ligand-induced chemokine signaling pathway through EGFR from the ASMCs might play a crucial role in exacerbations of bronchial asthma during airway infection.

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References


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