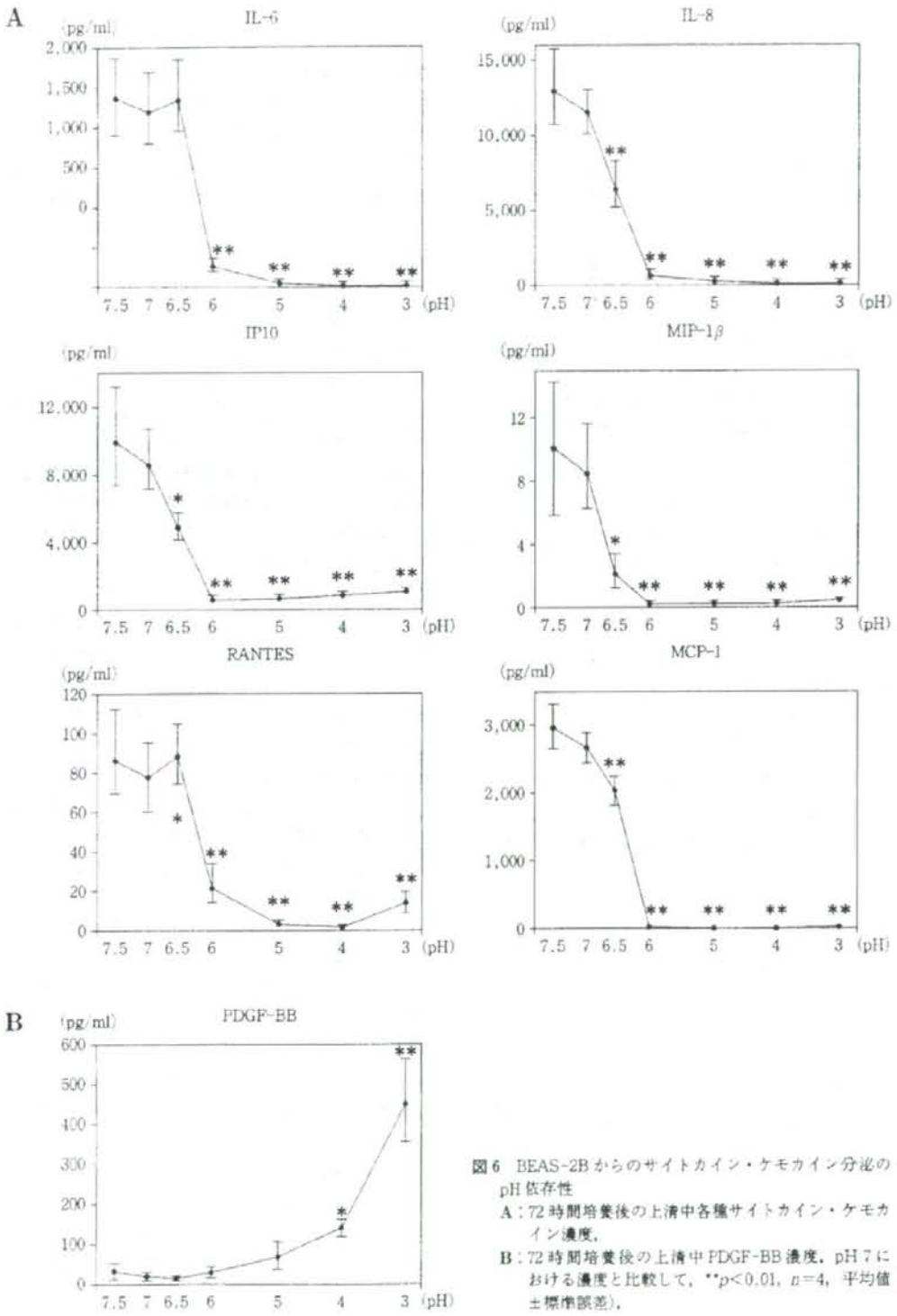

図6 BEAS-2Bからのサイトカイン・ケモカイン分泌のpH依存性

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．pH 7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に，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。

治療的観点からは，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，H_2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[10]。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[11]。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[11,12]，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らは酸逆流症状の問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を低下させうる自覚症状であり[13]，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[14,15]，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TXA_2の関与が示唆されている[16]。また，慢性咳嗽においては，PGE_2[16]，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う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-1β，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-b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，QEST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。

謝辞：本稿を終えるにあたり，帝京大学医学部内科学講座主任教授寺本民生先生ならびに直接ご指導，ご校閲頂きました長瀬洋之講師，中野純一講師，大田健教授に深く感謝の意を表します。また，本研究にご協力頂いた諸先生方および実験助手の田村知世さん，増田倫子さんに深く感謝致します。

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(論文受付日：2007年9月7日
論文受理日：2007年11月21日)

The relationship between bronchial asthma and gastroesophageal reflux disease -The effect of proton pump inhibitor on QUEST questionnaire-positive asthmatics-

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Background : The interaction between gatroesophageal reflux disease (GERD) and asthma symptoms has been widely suggested, but the diagnosis of GERD is relatively complicated for daily practice. Although QUEST is a simple questionnaire for diagnosing GERD, the utility of QUEST in GERD-complicated asthma patients has not been well established. We prospectively investigated the effect of proton pump inhibitor (PPI) on QUEST-positive asthmatics and verified the utility of QUEST in this study. In addition, as an acid aspiration has been assumed to be a factor for asthma exacerbation, we also investigated the effect of acid exposure on bronchial epithelial cells in *in vitro* studies.

Methods : Eighty-eight asthma patients were asked to fill QUEST for the analysis of the ratio of QUEST positive patients. Among those patients, 19 QUEST-positive stable asthmatics were enrolled into study and received 20 mg of omeprazole once daily for 4 weeks. We assessed the changes in QUEST score, pulmonary function, asthma symptoms, and QOL after treatment by omeprazole. We also cultured bronchial epithelial cell line BEAS-2B in low pH medium and analyzed the survival and cytokine and chemokine generation from BEAS-2B.

Results : Forty-eight of 88 asthma patients (54.5 %) were judged as QUEST positive and the positive correlation between QUEST score and the asthma severity was observed. Omeprazole significantly improved QUEST score, cough symptoms and these factors lead to the significant improvement in QOL. In *in vitro* studies, the significant cell death of BEAS-2B was induced and the levels of PDGF-BB in culture supernatant was elevated after exposure to low pH medium for 4 hour.

Conclusions : The utility of QUEST in diagnosing and treating the GERD-complicated asthmatics was confirmed. The acid exposure induced bronchial epithelial cell death and the secretion of PDGF-BB and these effects might potentially contribute to asthma exacerbation by GERD.

Key words : Asthma, gastroesophageal reflex disease, QUEST, cough, bronchial epithelial cells

Double-Stranded RNA and TGF-α Promote MUC5AC Induction in Respiratory Cells

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Viral infection is a major trigger for exacerbation of asthma and induces overproduction of mucins. We investigated whether dsRNA could amplify the induction of mucin by TGF-α in human bronchial epithelial cells, as well as the molecular mechanisms regulating MUC5AC expression. Human pulmonary mucoepidermoid carcinoma (NCI-H292) cells and normal human bronchial epithelial cells were exposed to polyinosinic-cytidyric acid (poly(I:C)) and TGF-α. Then, MUC5AC protein production, mRNA expression, and promoter activity were evaluated. Cells were pretreated with a selective inhibitor of ERK, and phosphorylation of ERK was examined by Western blotting. Furthermore, the expression of MAPK phosphatase 3 (MKP3) mRNA was evaluated and the effect of MKP3 overexpression was assessed. Poly(I:C) synergistically increased MUC5AC induction by TGF-α in both NCI-H292 and normal human bronchial epithelial cells. This increase was dependent on MUC5AC gene transcription. A MEK1/2 inhibitor (U0126) significantly inhibited MUC5AC production. Phosphorylation of ERK was enhanced by poly(I:C). TGF-α stimulation up-regulated MKP3 mRNA expression, while costimulation with poly(I:C) inhibited this up-regulation dose-dependently. Enhanced expression of MUC5AC mRNA by poly(I:C) in wild-type cells was completely suppressed in cells transfected with the MKP3 expression vector. dsRNA can synergistically amplify the induction of MUC5AC mucin by TGF-α. This synergistic effect on MUC5AC production may be due to enhanced activation of ERK through inhibition of MKP3 by poly(I:C). *The Journal of Immunology*, 2009, 182: 293–300.

In chronic airway diseases such as asthma, goblet-cell hyperplasia is an important feature (1). Excessive secretion of mucus by hyperplastic goblet cells causes airway plugging and contributes to morbidity and mortality in asthma patients (2, 3). To date, 19 different mucin genes have been identified. Among these, MUC5AC mucin is a major component of the mucus produced by airway epithelial cells (4), and its production is regulated by epidermal growth factor receptor (EGFR) signaling pathway (5, 6). EGFR and its ligands are not only expressed in patients with malignant lung tumors, but also in those with airway inflammatory diseases such as asthma (7). TGF-α is one of the ligands of EGFR, and it is known to play a critical role in phosphorylation of EGFR that leads to MUC5AC production in the airways (5).

Viral infection is a common cause of the exacerbation of asthma. Among the many viruses that infect the airways, human rhinovirus, respiratory syncytial virus, influenza virus, and parainfluenza virus are particularly common pathogens that induce the hypersecretion of mucus and exacerbation of asthma (8–10). These are RNA viruses that synthesize dsRNA during replication in infected cells. TLR3 recognizes dsRNA and was the first antiviral TLR identified (11). Because dsRNA is a universal viral molecule, TLR3 has been assumed to have a central role in the host response to infection by viruses (11). Previous studies have shown that stimulation with a synthetic analog of viral dsRNA (poly inosinic-cytidyric acid, poly(I:C))[2] is mediated by a pathway involving TLR3 that induces airway inflammation due to various cytokines and chemokines such as IL-8, IL-6, and RANTES (12). Despite the importance of excessive mucin production due to viral infection in triggering the exacerbation of asthma, the mechanisms causing such overproduction remain unknown.

We hypothesized that viral infection might synergistically amplify respiratory mucin gene expression and protein production induced by growth factors that are involved in the pathogenesis of asthma. Here, we demonstrate that a synthetic analog of viral dsRNA (poly(I:C)) synergistically increases the induction of respiratory mucin MUC5AC by TGF-α in human airway epithelial cells, both at the level of mRNA expression and protein production. This action depends on the activation of ERK, and the ERK pathway is enhanced through inhibition of MAPK phosphatase 3 (MKP3) by poly(I:C).

Materials and Methods

Cell culture and stimulation

A human pulmonary mucoepidermoid carcinoma cell line (NCI-H292) was maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere with 5% CO_2. NCI-H292 cells were seeded into 12-well plates for the ELISA and luciferase assay, and into 6-cm dishes for Western blotting and mRNA analysis. Cells were grown until 70% confluence was reached,

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Received for publication July 10, 2008. Accepted for publication October 23, 2008.

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[2] Abbreviations used in this paper: poly(I:C), polyinosinic-cytidyric acid; AB-PAS, Alcian blue/periodic acid-Schiff; C_T, threshold cycle; EGFR, epidermal growth factor receptor; MKP, MAPK phosphatase; NHBE, normal human bronchial epithelial; RT, room temperature.

 0022-1767/08/$2.00

Table 1. *Primers used for quantitative real-time PCR analysis of gene expression*

Target mRNA	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
MUC5AC	TCA CAG CCG GGT ACG CGT TGG CAC AAG TGG	TGC TAT TAT GCC CTG TGT AGC CAG GAC TGC
β-actin	GTG GGG CGC CCC AGG CAC CA	CTC CTT AAT GTC ACG CAC GAT TTC
MKP3	CAC CGA CAC AGT GGT GCT CT	CTG AAG CCA CCT TCC AGG TAG
EGFR	TGC GTC TCT TGC CGG AAT	GGC TCA CCC TCC AGA AGG TT

and they were maintained overnight in serum-free medium before stimulation. Cells were exposed to poly(I:C) (Sigma-Aldrich) at 25 μg/ml or TGF-α (R&D Systems) at 4 ng/ml, or to a combination of both agents.

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza. NHBE cells were seeded at density of $1.3 \times 10^5/cm^2$ into 12-well plates containing bronchial epithelial growth medium (Lonza) supplemented with defined growth factors and retinoic acid from the SingleQuot kit (Lonza), and were incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were exposed to poly(I:C) (25 μg/ml) or TGF-α (4 ng/ml), or a combination of both agents, for 24 h.

Analysis of mucin

NCI-H292 cells were stained with Alcian blue and periodic acid-Schiff stains (AB-PAS). MUC5AC protein was measured as described previously (5). In brief, supernatants were collected at 24 h after stimulation and cell lysates were prepared with PBS, and 50 μl of each sample was incubated with bicarbonate-carbonate buffer (50 μl) at 40°C in a 96-well plate (Nunc) overnight. Plates were washed three times with PBS and blocked with 2% BSA for 1 h at 37°C. Plates were again washed three times with PBS and then incubated with 50 μl of mouse monoclonal anti-MUC5AC Ab (1/100) (Lab Vision/NeoMarkers), which was diluted with PBS containing 0.05% Tween 20 and dispensed into each well. After 1 h, the plates were washed three times with PBS, and 100 μl of HRP-sheep anti-mouse IgG conjugate (1/10,000) (Amersham Biosciences) was added to each well. After 1 h, the plates were washed three times with PBS. Color was developed with 3,3',5,5'-tetramethylbenzidine peroxidase solution (Kirkegaard & Perry Laboratories) and the reaction was stopped with 1 M H_2SO_4. The data were expressed as a fold induction on the same experimental day due to various mucin production with cell passage in NCI-H292 cells.

Real-time quantitative PCR analysis

Expression of MUC5AC, MKP3, and EGFR mRNA by NCI-H292 cells was determined by reverse transcription (RT), followed by the real-time quantitative PCR. Total RNA was extracted from lysates of differentiated NCI-H292 cells using Isogen (Nippon Gene) at 12 h after stimulation. RT was performed with 1 μg of total RNA and oligo(dT) primers in a 25-μl reaction mixture according to the manufacturer's protocol (Applied Biosystems). The sequences of the specific primer sets that were used for PCR are listed in Table 1 (13, 14).

Real-time PCR was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems) using SYBR Green (Applied Biosystems) as a dsDNA-specific binding dye. For MUC5AC and β-actin, initial denaturation was done at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The threshold cycle (C_T) was recorded for each sample to reflect the level of mRNA expression. A validation experiment confirmed linear dependence of the C_T value on the concentrations of MUC5AC and β-actin and the consistency of ΔC_T (mean C_T for MUC5AC − mean C_T for β-actin) in a given sample at different RNA concentrations. ΔC_T was therefore used as an indicator of relative mRNA expression. To determine the effects of different stimuli on MUC5AC gene expression compared with unstimulated cells, $\Delta\Delta C_T$ was calculated ($\Delta\Delta C_T$ = ΔC_T for stimulated cells − ΔC_T for unstimulated cells). MUC5AC mRNA expression was indexed to β-actin mRNA expression by using the formula $1/(2\Delta C_T) \times 100\%$. $2\Delta\Delta C_T$ was calculated to demonstrate the fold change of MUC5AC gene expression in stimulated cells compared with unstimulated cells.

Expression of MKP3 and EGFR mRNA by NCI-H292 cells was determined in the same manner.

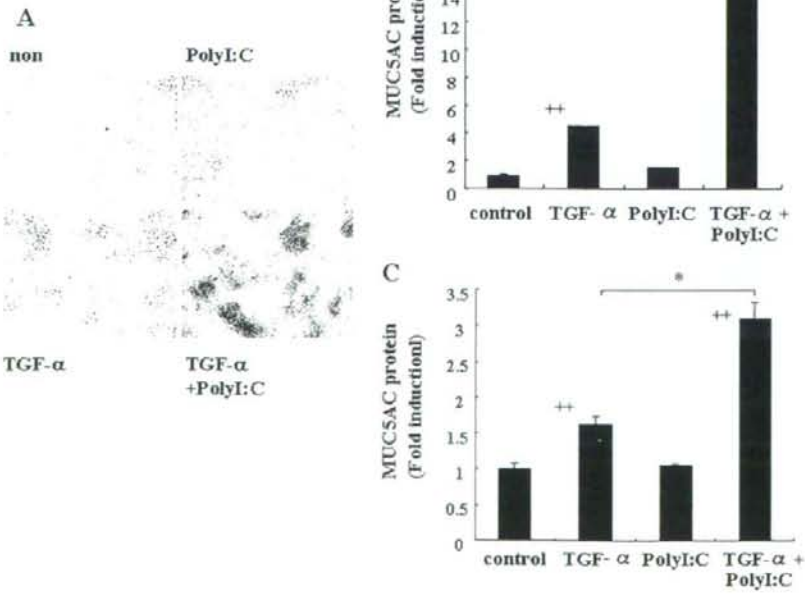

FIGURE 1. Synergistic effect of poly(I:C) and TGF-α on MUC5AC production. *A*, AB-PAS staining of NCI-H292 cells for identification of mucus glycoconjugates. Incubation with poly(I:C) (25 μg/ml) and TGF-α (4 ng/ml) for 24 h increased positive staining. Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced MUC5AC mucin production in NCI-H292 cell supernatant ($n = 9$) (*B*) and cell lysate ($n = 9$) (*C*). Cells were incubated with poly(I:C) and TGF-α for 24 h. Data are shown as means ± SD. ++, $p < 0.01$ compared with non-stimulated control cells. *, $p < 0.05$ and **, $p < 0.01$.

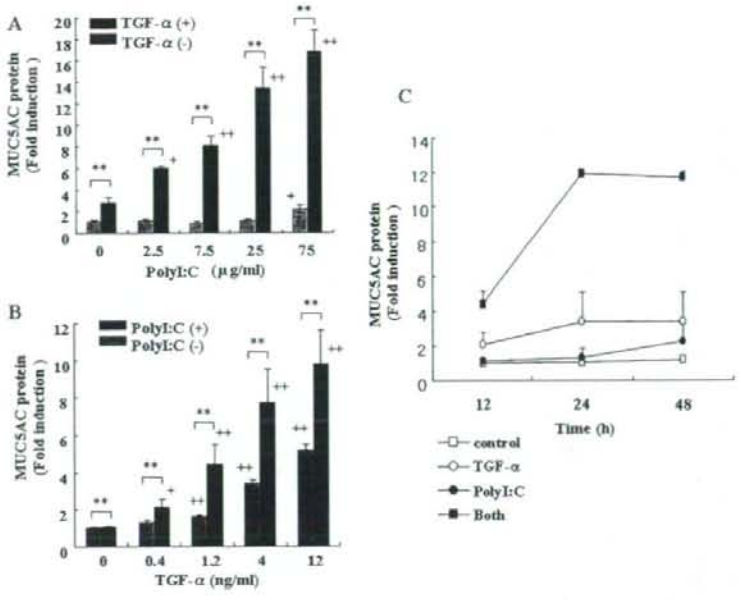

FIGURE 2. Dose responsiveness and time course of MUC5AC protein production. *A*, Effect of the poly(I:C) concentration on TGF-α (4 ng/ml)-induced MUC5AC mucin production in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF-α for 24 h. *B*, Effect of the TGF-α concentration on poly(I:C) (25 μg/ml)-induced MUC5AC mucin production in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF-α for 24 h. *C*, Effect of poly(I:C) (25 μg/ml) on the time course of induction of MUC5AC mucin production by TGF-α (4 ng/ml) in NCI-H292 cells ($n = 6$). Data are shown as means ± SD. +, $p < 0.05$ and + +, $p < 0.01$ compared with nonstimulated control cells. **, $p < 0.01$.

Reporter assay for the MUC5AC promoter

To investigate the regions of the MUC5AC promoter that were activated by poly(I:C) and TGF-α, the full-length human MUC5AC promoter was cloned into pGL3basic (a promoterless luciferase vector). This was then serially truncated using a combination of restriction enzyme digestion and PCR amplification to successively isolate regions of the promoter containing a large variety of potential transcription factor-binding sites (−1330 to −63).

NCI-H292 (0.8×10^5) cells were seeded into 12-well plates and grown overnight in complete medium. At 60% confluence, cells were rinsed with 1 ml of serum-free medium and incubated for 1 h. Then the cells were transfected using 1.3 μl of FuGENE 6 (Roche Applied Science) in 50 μl of RPMI 1640 medium per well plus 4 μl of MUC5AC promoter-luciferase plasmid DNA. At 1 h after transfection, cells were stimulated with poly(I:C) (25 μg/ml) and then incubated for 12 h before stimulation with TGF-α (4 ng/ml). Cell lysates were prepared, and reporter gene activity was determined by using a luciferase assay kit (Promega). The total protein concentration of samples was measured by spectrophotometry (NanoDrop from Thermo Scientific) to adjust for variations in harvesting of cells.

Western blot analysis

Cells (3.0×10^5) were washed with PBS and lysed in 300 μl of lysis buffer (0.5% Nonidet P-40, 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 3 mM *p*-amidinophenylmethanesulfonyl fluoride (Sigma-Aldrich), 5 mg/ml aprotinin (Sigma-Aldrich), 2 mM sodium orthovanadate (Sigma-Aldrich), 5 mM EDTA). Whole-cell extracts were subjected to electrophoresis on 7.5–12% Tris-glycine gel (XV Pantera gel; DRC) and then transferred to Sequi-Blot polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T (pH 7.5)) for 30 min at room temperature (RT) and

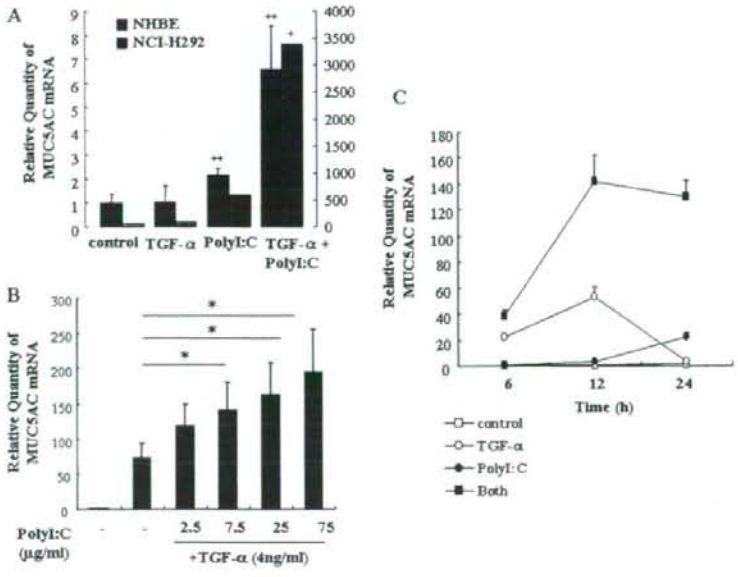

FIGURE 3. Dose responsiveness and time course of MUC5AC mRNA expression. *A*, Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced MUC5AC gene expression in NCI-H292 cells and NHBE cells. Cells were incubated with poly(I:C) and TGF-α for 24 h. Data are presented as the fold induction over the level in control NHBE cells. The right side of the y-axis is for NCI-H292 cells and the left side is for NHBE cells ($n = 6$). *B*, Effect of the poly(I:C) concentration on TGF-α (4 ng/ml)-induced MUC5AC mRNA expression in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF-α for 12 h. *C*, Effect of poly(I:C) (25 μg/ml) on the time course of induction of MUC5AC mRNA expression by TGF-α (4 ng/ml) in NCI-H292 cells ($n = 4$). Data are shown as means ± SD. +, $p < 0.05$ and + +, $p < 0.01$ compared with nonstimulated control cells. *, $p < 0.05$.

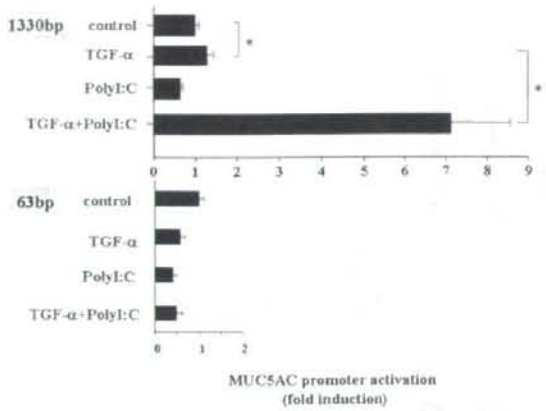

FIGURE 4. Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced *trans*-activation of the full-length (−1330) and the short-length (−63) MUC5AC promoter in NCI-H292 cells ($n = 6$). Data are shown as means ± SD. *, $p < 0.05$.

probed with primary anti-human phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) Ab and p44/42 MAPK Ab (Cell Signaling Technology) for 1 h at RT. The membranes were then washed with TBS-T and incubated with secondary donkey anti-rabbit Ig Ab conjugated to HRP (Amersham Biosciences) for 1 h at RT. Finally, Ab-Ag complexes were detected using an ECL chemiluminescent detection system according to the manufacturer's instructions (ECL plus Western blot detection system; Amersham Biosciences).

Cloning of MKP3 expression vector and transfection into NCI-H292 cells

A DNA fragment of the coding sequence of MKP3 was amplified by PCR using cDNA from poly(I:C)-treated NCI-H292 cells. The purified PCR product was digested with *Bam*HI and *Sal*I and cloned into the pAcGFP1-C1 vector (Clontech Laboratories). The plasmid was analyzed by digestion with restriction enzymes and DNA sequencing. Plasmids for transfection were purified with HiSpeed Plasmid Maxi kit (Qiagen). H292 cells were seeded into 6-well plates and grown to 50% confluence. Cells were transfected with 4 μg of expression vector with 10 μl of Lipofectamine 2000 (Promega) and grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). After 24 h, the medium was changed to RPMI 1640 supplemented with 10% FBS without antibiotics. Then, the cells were exposed to poly(I:C) (25 μg/ml), TGF-α (4 ng/ml), or a combination of both agents. After 12 h, the expression of MUC5AC and MKP3 mRNA was evaluated.

Other reagents

U0126 (a MEK1/2 inhibitor) was purchased from Sigma-Aldrich and monoclonal anti-human CXCL8/IL-8 Ab was purchased from R&D Systems. U0126 was dissolved in DMSO, while the monoclonal anti-human CXCL8/IL-8 Ab was dissolved in PBS. In all studies, the concentration of DMSO was 0.02–0.06%. U0126 (20 μM) (15, 16) and the anti-IL-8 Ab (2 μg/ml) were preincubated with cells for 1 h before adding poly(I:C) and TGF-α.

Phosphoprotein assay

Cells (3.0×10^5/ml) were seeded into 6-cm dishes and were treated with poly(I:C) for 1 h and then with TGF-α for 15 min. Protein lysates were prepared by using a cell lysis kit (Bio-Rad), and phosphorylated EGFR was detected with an EGFR (Tyr) assay kit (Bio-Rad) and a phosphoprotein testing reagent kit (Bio-Rad) according to the manufacturer's protocol. Briefly, 50 μl of cell lysate (adjusted to a protein concentration of 200–400 μg/ml) was plated into a 96-well filter plate coated with EGFR Ab-coupled beads and incubated overnight on a platform shaker at 300 rpm at RT. Total protein was measured with a Bio-Rad DC protein assay kit.

Statistical analysis

All data are expressed as the means ± SD. Results were analyzed by using the paired Student's *t* test or ANOVA as appropriate. Analyses were done with SPSS II software (SPSS Japan), and *p* values of <0.05 were considered significant.

Results

Poly(I:C) synergistically enhances MUC5AC protein production induced by TGF-α

First, we examined the ability of TGF-α and poly(I:C) to induce mucous glycoconjugate production assessed by AB-PAS staining in NCI-H292 cells (Fig. 1*A*). Twenty-four hours of incubation with TGF-α (4 ng/ml) increased PAS-positive staining, while poly(I:C) (25 μg/ml) alone did not affect staining. However, poly(I:C) enhanced the stimulatory effect of TGF-α on mucous glycoconjugate production (Fig. 1*A*). To quantify the MUC5AC mucin production, an ELISA was performed. TGF-α alone caused a 5-fold increase in MUC5AC mucin protein in cell supernatant (Fig. 1*B*) and a 1.5-fold increase in cell lysate (Fig. 1*C*) from NCI-H292 cells 24 h after stimulation. Poly(I:C) alone caused little increase in MUC5AC mucin protein; however, poly(I:C) strongly potentiated the effect of TGF-α. Thereafter, we evaluated MUC5AC mucin protein only in cell supernatant, because it was more prominent than cell lysate.

Next, we determined effects of dose responses of poly(I:C) (2.5–75 μg/ml) and TGF-α (0.4–12 ng/ml) on MUC5AC mucin production (Fig. 2, *A* and *B*). Although poly(I:C) alone did not

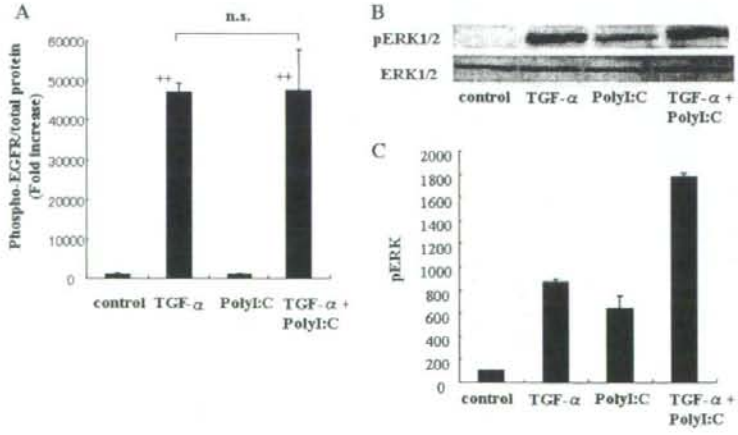

FIGURE 5. *A*, Relative phosphorylation of EGFR by poly(I:C) and TGF-α in NCI-H292 cells. Proteins extracted from samples collected were tested for the presence of phosphorylated EGFR by a Bio-Plex phosphoprotein assay kit using the Bio-Rad Luminex machine. The values plotted show the ratios of phosphorylated EGFR to total EGFR expressed as fold increase over control ($n = 6$). *B*, Phosphorylation of ERK1/2 by poly(I:C) (25 μg/ml) and TGF-α (4 ng/ml) in NCI-H292 cells assessed by performing Western blot analysis. *C*, Phospho-ERK was expressed as the fold increase in relative intensity ($n = 3$). Data are shown as means ± SD. ++, $p < 0.01$ compared with nonstimulated control cells.

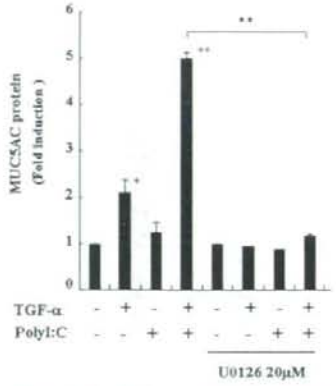

FIGURE 6. Effect of U0126 on poly(I:C)- and TGF-α-induced MUC5AC mucin production in NCI-H292 cells. Cells were preincubated with U0126 (a specific inhibitor of MEK1/2) at 20 μM (15, 16) for 1 h before adding poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml), and cells were analyzed 24 h after stimulation ($n = 6$). Data are shown as means ± SD. +, $p < 0.05$ and ++, $p < 0.01$ compared with nonstimulated control cells. **, $p < 0.01$.

significantly induce MUC5AC mucin production in every dose, costimulation with TGF-α caused an increase in MUC5AC mucin production with regard to poly(I:C) in a dose-dependent manner (Fig. 2*A*). TGF-α alone induced a dose-dependent increase in MUC5AC mucin production, and poly(I:C) enhanced the effect of TGF-α (Fig. 2*B*). Subsequent studies were focused on the time course of MUC5AC mucin production. Costimulation with poly(I:C) (25 μg/ml) and TGF-α (4 ng/ml) caused a small increase in MUC5AC mucin production 12 h after stimulation, with maximal levels of MUC5AC at 24 h (Fig. 2*C*). These results may imply that poly(I:C) synergistically up-regulates MUC5AC mucin production induced by TGF-α.

Poly(I:C) synergistically enhances MUC5AC mRNA expression induced by TGF-α

To determine whether induction of MUC5AC mucin protein induced by poly(I:C) and TGF-α was a result of increased MUC5AC gene transcription, we investigated levels of MUC5AC mRNA, determined by real-time quantitative RT-PCR in NCI-H292 and NHBE cells. TGF-α (4 ng/ml) alone caused little increase in MUC5AC mRNA expression in NCI-H292 cells and NHBE cells upon 24 h of stimulation (Fig. 3*A*). Poly(I:C) (25 μg/ml) alone induced a small but significant increase in MUC5AC mRNA expression in both NCI-H292 cells and NHBE cells, and poly(I:C) strongly potentiated the effect of TGF-α (Fig. 3*A*). A clear dose response was observed at 12 h following stimulation with both poly(I:C) (2.5–75 μg/ml) (Fig. 3*B*) and TGF-α (0.4–12 ng/ml) (data not shown). Costimulation with poly(I:C) and TGF-α caused a small increase in MUC5AC mRNA expression 6 h after stimulation, which continued to a peak at 12 h after stimulation (Fig. 3*C*).

Poly(I:C) and TGF-α cause synergistic trans*-activation of the MUC5AC promoter*

We next investigated whether the MUC5AC promoter was activated by poly(I:C) and TGF-α. After 8 h, TGF-α alone induced a small but significant activation of the full-length MUC5AC promoter construct (−1330) (Fig. 4). Poly(I:C) did not activate the full-length MUC5AC promoter construct (−1330), but poly(I:C) strongly enhanced the activation induced by TGF-α, with 6-fold induction over that in unstimulated transfected control cells ($p < 0.05$) (Fig. 4). This was observed when poly(I:C) was added 12 h before TGF-α stimulation (Fig. 4), but not when the two agents were added at the same time (data not shown). There was no difference in the level of activation of the short-length MUC5AC promoter construct (−63) among TGF-α, poly(I:C), and both stimulations (Fig. 4). These results indicate that the −1330 to −63 region contains the elements regulating induction of the MUC5AC promoter by poly(I:C) and TGF-α.

Transactivation of the MUC5AC promoter by poly(I:C) and TGF-α is mediated via an ERK signaling pathway

Since it was shown that induction of MUC5AC-specific mucin protein by poly(I:C) and TGF-α was a result of increased MUC5AC gene transcription, we next investigated the upstream signaling leading to activation of the promoter.

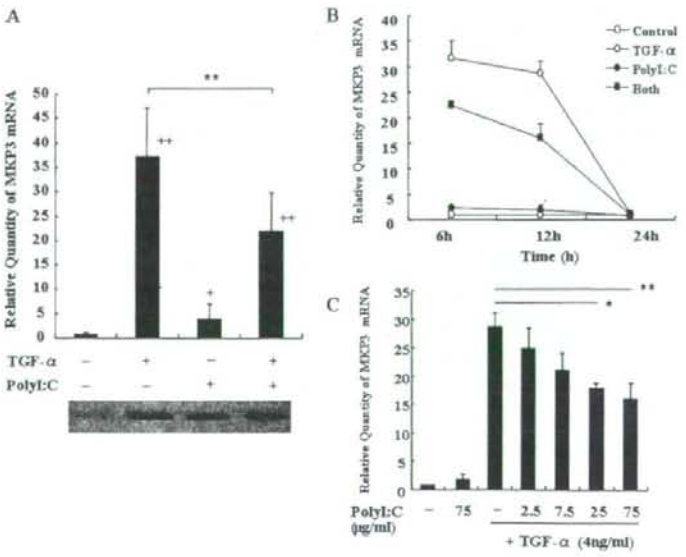

FIGURE 7. *A, Upper*, Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced MKP3 mRNA expression in NCI-H292 cells ($n = 8$). *Lower*, To verify MKP3 expression in NCI-H292 cells, expression of MKP3 after stimulation with poly(I:C) (25 mg/ml) and TGF-α (4 ng/ml) was examined by standard RT-PCR. In brief, 25 μl of reaction mixture consisted of 1 μl cDNA, 1 pmol MKP3 primer sets, and 12.5 μl AmpliTaq Gold PCR Master mix (Applied Biosystems). PCR was performed by an initial denaturation step at 95°C for 5 min followed by 30 cycles with a denaturation step at 95°C for 30 s, an annealing step at 60°C for 30 s, and an extension step at 72°C for 30 s. *B*, Time course of induction of MKP3 mRNA expression by poly(I:C) (25 μg/ml) and TGF-α (4 ng/ml) in NCI-H292 cells ($n = 4$). *C*, Effect of the poly(I:C) concentration on TGF-α (4 ng/ml)-induced expression of MKP3 mRNA in NCI-H292 cells ($n = 4$). Data are shown as means ± SD. +, $p < 0.05$ and ++, $p < 0.01$ compared with nonstimulated control cells. *, $p < 0.05$ and **, $p < 0.01$.

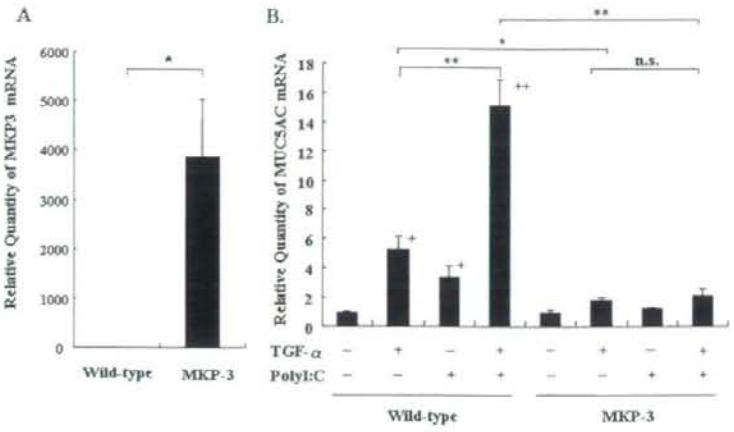

FIGURE 8. *A*, Effect of a MKP3 expression plasmid cloned into the pAcGFP1-C1 vector on MUC5AC mRNA expression in NCI-H292 cells. The level of MKP3 mRNA was enhanced significantly in cells transfected with the MKP3 expression plasmid. *B*, Enhanced expression of MUC5AC mRNA was noted in wild-type cells after 12 h of coincubation with TGF-α (4 ng/ml) and poly(I:C) (25 μg/ml), but it was completely abolished in cells transfected with the MKP3 expression vector ($n = 6$). Data are shown as means ± SD. +, $p < 0.05$ and ++, $p < 0.01$ compared with nonstimulated control cells. *, $p < 0.05$ and **, $p < 0.01$.

First, since TGF-α induces MUC5AC mucin production through the ligand-dependent *trans*-activation of EGFR in NCI-H292 cells (5), we examined the importance of EGFR activation for synergistic induction of MUC5AC mucin production by poly(I:C). We evaluated EGFR mRNA expression and phosphorylation of EGFR by RT-PCR and the Bio-Plex phosphoprotein assay, respectively. As a result, we found that poly(I:C) did not up-regulate EGFR mRNA expression upon 12 h of stimulation (data not shown) or increase the phosphorylation of EGFR (Fig. 5*A*).

Second, since previous studies have demonstrated that increased production of MUC5AC mucin protein after activation of the EGFR signaling pathway was exclusively MEK/ERK-dependent (17), we investigated the requirement of ERK. Western blot analysis revealed that poly(I:C) synergistically enhanced the phosphorylation of ERK by TGF-α stimulation (Fig. 5, *B* and *C*). This finding was compatible with the result of chemical inhibition by MEK1/2 inhibitor (U0126). U0126 inhibited the induction of MUC5AC protein production by poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml) compared with absence of the inhibitor at 24 h after stimulation (Fig. 6). These data suggest that *trans*-activation of the MUC5AC promoter by poly(I:C) and TGF-α is mediated via an ERK signaling pathway.

Poly(I:C) inhibits TGF-α-induced MKP3 mRNA expression

Having demonstrated that the ERK-dependent signaling was required in MUC5AC induction, still unclear is the mechanism interacting between TLR3-dependent signaling stimulated by poly(I:C) and EGFR-dependent signaling stimulated by TGF-α. Since MKP3 is known to be a member of the phosphatase family that inactivates ERK1/2, we examined the effect of poly(I:C) on MKP3 mRNA expression. A real-time quantitative RT-PCR showed that expression of MKP3 mRNA was up-regulated upon 12 h of stimulation with TGF-α, and MKP3 mRNA up-regulation by TGF-α was inhibited by stimulation with poly(I:C) (Fig. 7*A*). Stimulation with TGF-α led to a moderate increase in MKP3 mRNA expression at 6 h, followed by a decrease at 24 h (Fig. 7*B*). Costimulation with poly(I:C) dose-dependently inhibited this up-regulation, and inhibition was seen from 6 h after stimulation (Fig. 7, *B* and *C*).

Effect of the MKP3 expression vector

To further demonstrate the role of MKP3 on MUC5AC mucin induction, we investigated the effect of MKP3 expression vector. MKP3 mRNA levels were significantly enhanced in cells transfected with the MKP3 expression plasmid cloned into the pAcGFP1-C1 vector when compared with wild-type cells (Fig. 8*A*). Enhanced expression of MUC5AC mRNA was noted in wild-type cells 12 h after coincubation with TGF-α (4 ng/ml) and poly(I:C) (25 μg/ml), but was completely abolished in cells transfected with the MKP3 expression vector (Fig. 8*B*). These data suggest that the inhibition of MKP3 mRNA expression by poly(I:C) leads to synergistic MUC5AC mucin induction.

Anti-IL-8 Ab does not inhibit poly(I:C)- and TGF-α-induced MUC5AC mRNA expression

Poly(I:C) is known to increase the expression of mRNA for various chemokines and cytokines (18, 19). In our preliminary study, we measured the cytokine and chemokine levels in the supernatant after stimulation with costimulation of TGF-α and poly(I:C) by using a Bio-Plex cytokine assay. In that study, only the IL-8 level was synergistically high due to costimulation of TGF-α and poly(I:C). Therefore, we chose IL-8, and to investigate the role of IL-8, NCI-H292 cells were preincubated with anti-IL-8 Ab 1 h before stimulation with poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml). Anti-IL-8 Ab did not inhibit the increase in the expression of MUC5AC mRNA induced by 12 h of stimulation with poly(I:C) and TGF-α (Fig. 9).

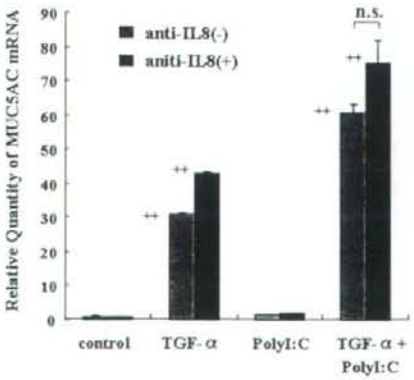

FIGURE 9. Effect of the anti-IL-8 Ab on poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml)-induced MUC5AC mRNA expression in NCI-H292 cells ($n = 4$). Data are shown as means ± SD. ++, $p < 0.01$ compared with nonstimulated control cells.

Discussion

In this study, we found that poly(I:C) synergistically increased the production of MUC5AC induced by TGF-α in both NCI-H292 and NHBE cells. This increase was dependent on activation of the MUC5AC promoter, and the upstream signaling pathway was ERK-dependent. The most interesting finding of this study was that expression of MKP3, which is one of the negative regulators of MAPK, was up-regulated by TGF-α and this up-regulation was inhibited by poly(I:C), indicating that MKP3 has a central role in the synergistic induction of MUC5AC production by poly(I:C) and TGF-α.

Mucin hypersecretion and goblet cell hyperplasia are characteristic features of airway inflammatory diseases such as asthma (1, 2). Since hypersecretory diseases are associated with abnormal epithelial cell growth and differentiation, and epithelial damage leads to repair and remodeling (19, 20), both inflammatory mediators and growth factors may be involved in stimulating mucin production from goblet cells. It has been postulated that activation of the EGFR pathway is a common denominator in the induction of MUC5AC mucin, a major component of mucus in the airways. Takeyama et al. have shown that stimulation of EGFR by its ligands, EGF and TGF-α, causes MUC5AC production by airway epithelial cells both in vitro and in vivo (5), and this effect is potentiated by TNF-α (5). In the present study, we found that using AB-PAS staining, ELISA, and RT-PCR, poly(I:C) synergistically amplified the induction of MUC5AC mucin induced by TGF-α at both the mRNA and protein levels in NCI-H292 cells.

In NHBE cells, MUC5AC mRNA expression was much lower than that in NCI-H292 cells, but poly(I:C) still synergistically amplified the expression of MUC5AC mRNA induced by TGF-α, indicating that synergic induction of MUC5AC by poly(I:C) and TGF-α may be generalizable to normal human epithelial cells. The lower expression of MUC5AC mRNA may be explained by not using an air-liquid interface in culturing NHBE cells. Indeed, studies done in air-liquid interface or monolayers would provide us important results. However, previous studies have demonstrated that both NCI-H292 and NHBE cells share key components of the signaling pathways upstream and downstream of EGFR responsible for mucin production (21), suggesting that NCI-H292 cells are a valid model of mucin production in normal cells. Therefore, our further studies investigating the mechanisms of the signaling pathway were done in NCI-H292 cells.

In the present study, we found that synergistic induction of MUC5AC mucin production by poly(I:C) and TGF-α was dependent on activation of the MUC5AC promoter within the proximal −1330/−63 region. Additionally, we investigated upstream signaling by using an inhibitor and Western blot analysis, and we found that the process was ERK-dependent. Our data are in agreement with findings reported by Hewson and coworkers, showing that increased production of MUC5AC mucin protein after activation of the EGFR signaling pathway was exclusively MEK/ERK-dependent (17). Furthermore, we found that poly(I:C) synergistically enhanced the phosphorylation of ERK induced by TGF-α. Therefore, we concluded that *trans*-activation of the MUC5AC promoter by poly(I:C) and TGF-α occurs exclusively via an ERK signaling pathway.

Receptor regulation has an important role in controlling the actions of several mediators. Yamamoto et al. demonstrated that IL-4-induced production of eotaxin-3 in airway epithelium was enhanced due to up-regulation of the IL-4 receptor by IFN-γ (22). In the present study, to determine whether the synergistic effect of poly(I:C) was due to up-regulation of the EGFR, we evaluated EGFR mRNA expression and EGFR phosphorylation. However, up-regulation of EGFR mRNA expression and the phosphorylation of this receptor by stimulation with poly(I:C) were not observed.

Since we had found that ERK was required for the synergistic effect of poly(I:C) on MUC5AC production induced by TGF-α, we proceeded to investigate this further by evaluating the role of MKP3, which is a member of the phosphatase family that inactivates ERK1/2. MKP3 is predominantly localized in the cytoplasm and has a highly specific role in the dephosphorylation and inactivation of ERK1/2 (23–26). MKP3 is an immediate early gene and is transcriptionally up-regulated after ERK2 activation (27, 28). Our present finding that MKP3 mRNA expression was 37-fold higher following stimulation with TGF-α is in agreement with previous reports that MKP3 is up-regulated after activation of the ERK2 pathway (27–29). Additionally, we found that this up-regulation was inhibited by stimulation with poly(I:C), and that overexpression of MKP3 completely abolished the increase in expression of MUC5AC mRNA. These data indicate that when NCI-H292 cells are stimulated by TGF-α alone, MUC5AC protein production remains under autoregulation to a certain extent by negative feedback via MKP3. However, when additional stimulation with poly(I:C) is added, MKP3 mRNA expression is partially down-regulated. This leads to synergistic activation of ERK, synergistic *trans*-activation of the MUC5AC promoter, and finally to synergistic production of MUC5AC protein.

Posttranscriptional events are also important in regulation of gene expression. A detailed examination of the time course of MUC5AC mRNA expression revealed that it was maximal at 12 h and decreased at 24 h after treatment with TGF-α alone. In contrast, costimulation with poly(I:C) and TGF-α caused a significant time-dependent increase in MUC5AC mRNA expression for up to 24 h. Furthermore, analysis of mRNA stability by real-time quantitative RT-PCR demonstrated that poly(I:C) did not alter the stability of MUC5AC mRNA (data not shown). Accordingly, the additional stimulation with poly(I:C) significantly increased and prolonged the induction of MUC5AC mRNA expression induced by TGF-α without affecting the rate of MUC5AC mRNA degradation.

Poly(I:C) is known to increase the expression of mRNA for various chemokines (IP-10, RANTES, LARC, MIP1α, IL-8, GRO-α, and ENA-78) and cytokines (IL-1β, GM-CSF, and IL-6), as well as the cell adhesion molecule ICAM-1 (18, 19). To determine whether IL-8 has an important role in the synergistic effect of poly(I:C) and TGF-α on MUC5AC production, we investigated the potential role of IL-8 by preincubation with an anti-IL-8 Ab in the cells. The anti-IL-8 Ab did not inhibit MUC5AC mRNA expression, indicating that IL-8 has no role in the process. This finding was consistent with a previous report showing that IL-8 alone had no effect on MUC5AC protein production in NCI-H292 cells (30).

Also, the role of IFN may be an important point particularly in the context of poly(I:C) and asthma. We have not done studies directly on IFN-α and IFN-β. However, to further investigate whether extracellular factors (such as chemokines and cytokines) released by poly(I:C) stimulation up-regulated TGF-α-induced MUC5AC production, we changed the culture medium at 12 h after poly(I:C) stimulation and then stimulated the cells with TGF-α. Although the extracellular factors had been removed, it did not alter the synergic expression of MUC5AC mRNA (data not shown), suggesting that extracellular factors including IFN-α and IFN-β released by poly(I:C) may not contribute to the enhanced MUC5AC expression.

In conclusion, poly(I:C) synergistically increases the production of MUC5AC induced by TGF-α in airway epithelial cells, due to inhibition of MKP3 expression. Studies completed with viruses,

especially rhinovirus and also inactivated viruses, would provide us with important perspectives. Further studies will be needed to analyze the interaction between viruses and TGF-α. Viral respiratory tract infections are the most common triggers for the exacerbation of asthma (31, 32), and mucin overproduction is one of the mechanisms involved. The present findings may help to explain the excessive production of mucus in asthmatic patients with viral infection. Mucus plugging of the airways is a feature of fatal asthma in both adults and children (33, 34). At present, there are no effective therapies to relieve the symptoms induced by hypersecretion of mucus due to viral infection in asthmatic patients. Our findings may provide a mechanism to explain mucin overproduction and a potential strategy for therapy.

Acknowledgments

We thank Tomoko Endo and Chinori Iijima for their excellent technical assistance.

Disclosures

The authors have no financial conflicts of interest.

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ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 4, 2008

DOI: 10.1089/ars.2007.1960

Forum News & Views

Does Leukotriene Affect Intracellular Glutathione Redox State in Cultured Human Airway Epithelial Cells?

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ABSTRACT

Leukotrienes (LTs) are one of the most important mediators in the pathophysiology of asthma. We measured the intracellular amounts of reduced glutathione (GSH) and oxidized glutathione (GSSG) in cultured human airway epithelial cells. LTC_4 affects the GSH/GSSG ratio by activating signals to increase interleukin-8 (IL-8) production. Pretreatment with a reducing agent, glutathione monochrome ester (GSH-OEt), and with a leukotriene receptor antagonist, montelukast, significantly suppressed LTC_4-induced time-dependent changes in the intracellular redox state, and also suppressed upregulation of IL-8 production by suppressing NF-κB activation. Our observations led to the hypothesis that LTC_4-induced oxidative stress is likely to contribute to amplification of airway inflammation. *Antioxid. Redox Signal.* 10, 821–828.

AIRWAY INFLAMMATION AND REDOX

ASTHMA IS A CHRONIC INFLAMMATORY AIRWAY DISEASE that affects children and adults of all ages (4). Although the pathogenesis of asthma remains incompletely defined, there is ample evidence that asthma is mediated by oxidative stress. The imbalance between reactive oxygen species (ROS) and antioxidants is termed oxidative stress. Most environmental factors, including oxidants, ultraviolet light, radioactivity, infections, and allergic responses to allergens, act as oxidative stress upon cells (6, 12). The cells obtained from the peripheral blood and lungs of patients with asthma generate increased amounts of ROS, such as superoxide radicals and hydrogen peroxide (H_2O_2), and the increase correlates with disease severity (16). Cell functions are activated and inactivated by the balance between intracellular oxidation and reduction (redox state), which in turn closely correspond to the surrounding environment. Epithelial cells are the first cells to encounter inhaled allergens, and asthma is a disorder involving the airway epithelium that is more vulnerable to environmental injury and responds to this by impaired healing, in addition to inflammation (9). As the first barrier of the airway, to maintain a steady state, airway epithelial cells possess mechanisms that eliminate oxygen radicals, tending to counteract intracellular shifts toward the oxidized state (20). Understanding how allergic respiratory diseases are exacerbated requires consideration of the effect of inflammation as an oxidative stress on airway epithelial cells, the initial site of injury from inflammatory cells, and/or pro-inflammatory mediators.

Glutathione, the most abundant nonprotein tripeptide containing a sulfhydryl group, plays a prominent role in antioxidant protection of the lung. In humans, glutathione is 100-fold more concentrated in the airway epithelial lining fluid than in plasma (29). Oxidants or oxidative stress have been reported to activate transcription factors, including NF-κB and phosphorylation of MAP kinase (19, 22). Inflammation-related changes in intracellular redox state in lung macrophages and monocytes have a potent effect on cytokine production (11, 28). Furthermore, the intracellular redox system is affected by oxidative stress induced by inflammatory cells during allergic reactions in the airway mucosa. However, changes of intracellular redox state and redox-related reactions in airway epithelial cells are largely uncharacterized.

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LEUKOTRIENE AND INFLAMMATION

Leukotrienes (LTs), one of the most important mediators of the pathophysiology of asthma, are known to induce bronchoconstriction, airway inflammation, edema, and mucus hypersecretion (18). There are two families of leukotrienes: LTB_4 and a second group (LTC_4, LTD_4, LTE_4), called cysteinyl-leukotrienes (Cys-LTs), bind to highly selective receptors to induce bronchoconstriction and inflammation associated with asthma (14). Analysis by enzyme immunoassay of exhaled breath condensate from patients with asthma has proved that asthma patients have higher levels of Cys-LTs and 8-isoprotane (a marker of oxidative stress) than normal subjects (1). In a B cell line (BL41-E95-A), oxidative stress (H_2O_2, diamide) enhanced activity of the cellular 5-lipoxygenase (5-LO) , which catalyzes the first two steps in leukotriene biosynthesis (30). Depletion of thiol antioxidants induced the production of Cys-LTs and selective phosphorylation of MAP kinase in lung fibroblasts (2). Previous studies have demonstrated an association between production of Cys-LTs and oxidative stress. In this study, we investigated whether Cys-LTs can induce oxidative stress in cultured human airway epithelial cells, and evaluated the direct effect of Cys-LTs as an oxidative stressor through measurement of reduced glutathione (GSH) and oxidative glutathione (GSSG). We also studied cytokine regulation induced by Cys-LTs with a leukotriene receptor antagonist, montelukast, which is widely used as an anti-asthmatic drug.

LEUKOTRIENE AND INTRACELLULAR REDOX STATE

LTC_4 exposure and intracellular redox state

To determine an informative concentration and duration of Cys-LTs exposure, we compared six various stimulated concentration of LTC_4: from 10^{-7} to 10^{-12} *M* at 2 and 6 h. LTC_4 exposure dose-dependently induced an oxidized state within Calu-3 cells at 2 h after stimulation (Fig. 1). We chose a final LTC_4 concentration of 10^{-8} *M* as suitable for subsequent study because it induced a reproducible oxidized state at 2 h and a rebound reaction at 6 h.

We measured the intracellular redox state in Calu-3 cells immediately and at 2, 4, 6, 12, 24, and 48 h after LTC_4 exposure. In the 10^{-8} *M* group after exposure for 2 h, the GSH/GSSG ratio decreased immediately to a mildly oxidized state, whereas the GSH/GSSG ratio fell below 100% compared with the control state. At 4 h after LTC_4 exposure, the ratio gradually declined to a severely oxidized state where the GSH/GSSG ratio fell to the minimal value, then the GSH/GSSG ratio increased dramatically to reach a maximum reduction at 6 h after LTC_4 exposure (Fig. 2A). Figure 2B shows the time course of the absolute values of GSH and GSSG. At 4 h after LTC_4 exposure, the GSH slightly decreased, and thereafter the GSH increased significantly to reach a maximum reduction at 6 h after LTC_4 exposure (Fig. 2B). The GSSG increased at 24 h after LTC_4 exposure. The control group, exposed to PBS as vehicle (10 μl/well), did not show any significant changes in the intracellular redox state at any time point (data not shown).

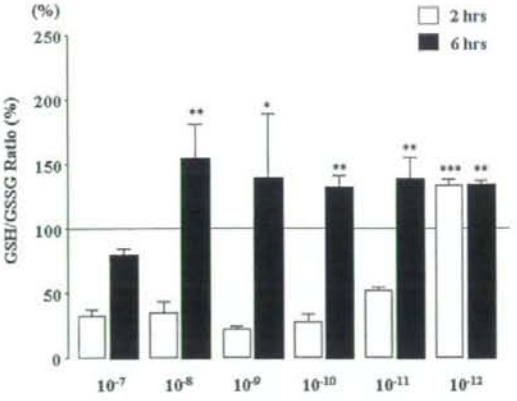

FIG. 1. Dose-dependent effect of LTC_4 on intracellular redox. Results following LTC_4 (10^{-7}–10^{-12} *M*) exposure for 2 h show the intracellular redox state declining to indicate an oxidized state, with the GSH/GSSG ratio showing <100% of control-state values ($n = 6$). At 6 h after exposure, the GSH/GSSG ratio increased. In 10^{-8} *M* concentration, LTC_4 induced a clear series of oxidation and reduction. $*p < 0.05$, $**p < 0.02$, $***p < 0.001$, compared with 10^{-7} *M* LTC_4.

LTC_4 exposure and pretreatment with glutathione modulators

Calu-3 cells were pretreated with the reducing reagent, glutathione monochrome ester (GSH-OEt), at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} *M* for 4 h, and then exposed to LTC_4 at a concentration of 10^{-8} *M*. In the LTC_4 group (exposed to LTC_4 without pretreatment), the GSH/GSSG ratio at 2 h after LTC_4 exposure showed a decreased of >25%, indicating that cells were in an oxidized state, peaking at 6 h after LCT_4 exposure (Figs. 3A and B). On the other hand, in the OEt+LTC_4 group (GSH-OEt pretreatment and exposed to LTC_4), the GSH/GSSG ratio exceeded 100%, and a reduced intracellular state was maintained for 6 h after LTC_4 exposure. LTC_4-induced intracellular oxidative state at 2 h was inhibited by pretreatment with GSH-OEt, thus resulting in the continuous reductive state in human epithelial cells.

LTC_4 exposure and pretreatment with Cys-LTs receptor antagonists

Calu-3 cells were pretreated with Cys-LTs receptor antagonist, montelukast, at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M* for 2 h before and during exposure to LTC_4 at a concentration of 10^{-8} *M*. In the LTC_4 group (exposed to LTC_4 without pretreatment), the GSH/GSSG ratio at 4 h after LTC_4 exposure decreased to nearly 50%, indicating that cells were in a severely oxidized state (Fig. 4), while addition of montelukast at concentrations of 10^{-6} and 10^{-7} *M* attenuated the significant decreases (Fig. 4). There was no significantly difference in the GSH/GSSG ratio between montelukast pretreatment (10^{-6}, 10^{-7} *M*) group and control group at 4 h after LTC_4 exposure.

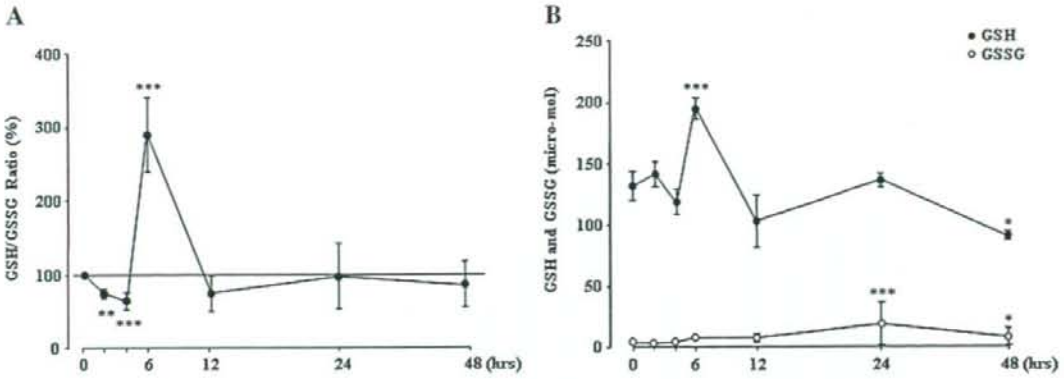

FIG. 2. (A) Change of GSH/GSSG ratio after LTC_4 exposure. Results following LTC_4 (10^{-8} *M*) exposure for 2 h show the intracellular redox state declining to indicate a mildly oxidized state, with the GSH/GSSG ratio showing <100% of control-state values ($n = 6$). At 4 h after exposure, the ratio gradually declined to severely oxidized state where the GSH/GSSG ratio fell to the minimal value. Then the GSH/GSSG ratio increased dramatically, and the maximum reduction was reached at 6 h after LTC_4 exposure. $^{**}p < 0.02$, $^{***}p < 0.001$, compared with 0 h. **(B)** Change of GSH and GSSG after LTC_4 exposure. At 4 h after LTC_4 exposure, the GSH slightly decreased, thereafter the GSH increased significantly to reach a maximum reduction at 6 h after LTC_4 exposure. The GSSG increased at 24 h after LTC_4 exposure ($n = 6$). $^{*}p < 0.05$, $^{***}p < 0.001$, compared with 0 h.

LEUKOTRIENE AND CYTOKINE/CHEMOKINE UPREGULATION

Cys-LTs -induced upregulation of cytokine production

The concentration of interleukin-8 (IL-8) in control cells unexposed to LTC_4 was 185.75 ± 23.92 ng/ml. After exposing Calu-3 cells to LTC_4, we measured the changes in production of IL-8. At 24 h after exposure, IL-8 values (282.49 ± 16.14 ng/ml) were significantly higher than in the control group ($p < 0.001$, Fig. 5). We also investigated the effects of the LTC_4-induced changes in IL-8 production following pretreatment with GSH-OEt (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} *M*) or montelukast (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M*). Pretreatment with GSH-OEt (10^{-3}, 10^{-4}, and 10^{-5} *M*) dose-dependently suppressed LTC_4-induced upregulation of epithelial cell-derived IL-8 production (Fig. 5). Also, pretreatment with 10^{-6} or 10^{-7} *M* montelukast

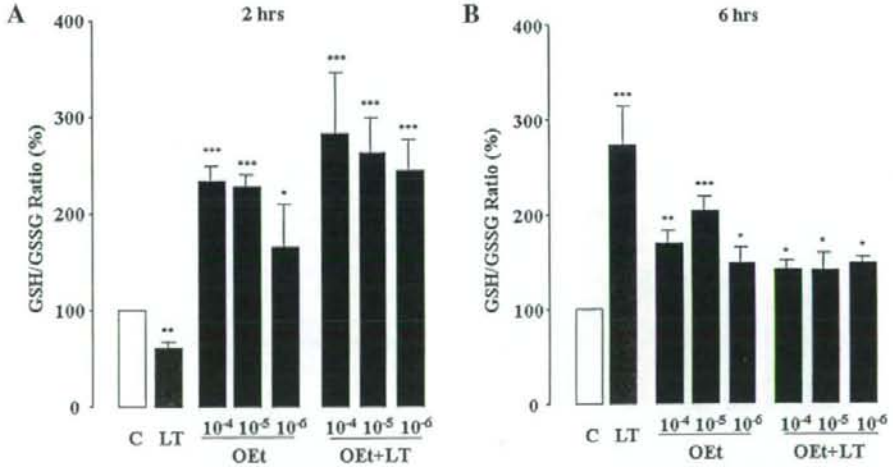

FIG. 3. (A) Effect of antioxidant on LTC_4-induced redox change at 2 h. GSH-OEt increased GSH/GSSG ratio after LTC_4 exposure in Calu-3 cells at 2 h ($n = 6$). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC_4 was 2 h. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with control. **(B)** Effect of antioxidant on LTC_4-induced redox change at 6 h. GSH-OEt suppressed after LTC_4 -induced increase of GSH/GSSG ratio in Calu-3 cells at 6 h ($n = 6$). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC_4 was 2 h. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control.

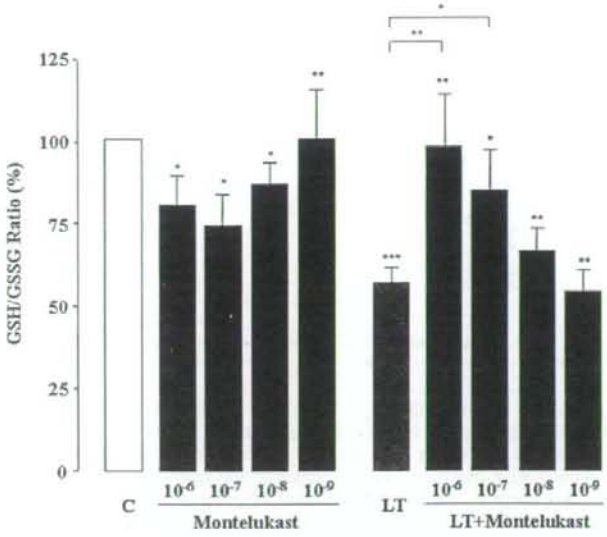

FIG. 4. Effect of LT antagonist on LTC_4-induced redox change. Montelukast had a protective effect on GSH/GSSG ratio after LTC_4 exposure in Calu-3 cells ($n = 6$). Pretreatment of montelukast was for 2 h, and incubation time of LTC_4 was 2 h. $^*p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$, compared with control.

significantly inhibited LTC_4-induced upregulation of epithelial cell-derived IL-8 production (Fig. 6).

Effect of Cys-LTs exposure upon NF-κB p65 binding activity

Compared with the control sample, the concentration of NF-κB p65 showed a significant increase after LTC_4 exposure ($p < 0.001$, Fig. 7). Pretreated with 10^{-4} or 10^{-5} *M*, GSH-OEt suppressed LTC_4-induced activation of NF-κB p65 in Calu-3 cells, and the inhibitory effect was dose dependent. This was also the case for montelukast at concentrations of 10^{-6} and 10^{-7} *M* (Fig. 7).

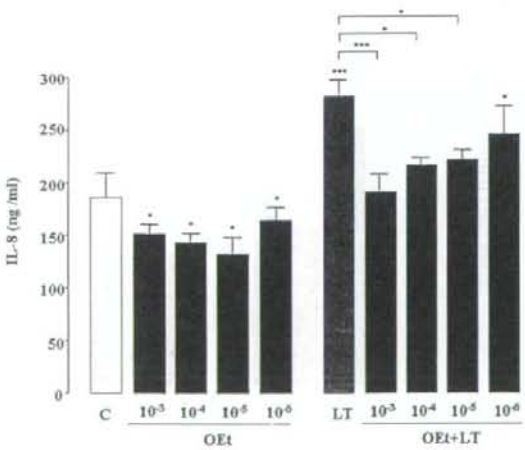

FIG. 5. Effect of LTC_4 and antioxidant on IL-8 production in Calu-3 cells. GSH-OEt suppressed LTC_4-induced IL-8 production in Calu-3 cells ($n = 6$). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC_4 was 2 h. $^*p < 0.05$, $^{***}p < 0.001$, compared with control.

CONCLUSION AND OPEN QUESTIONS

A vast body of evidence suggests that the intracellular redox state regulates various aspects of the cellular function (12), while that in various cell types of glutathione constitutes the first line of cellular defense against oxidative injury, acting as the major intracellular redox buffer (20). However, in the studies published so far, little has been reported regarding whether some important inflammatory molecules, such as Cys-LTs, can induce oxidative stress on the glutathione redox system in human airway epithelial cells or how changes in the intracellular redox state affect cytokine regulation. In this study, we examined whether Cys-LTs can induce oxidative stress in cultured human airway epithelial cells (Calu-3) and evaluated the direct effect of Cys-LTs as an oxidative stressor through measurement the intracellular redox state in Calu-3 cell. We monitored the intracellular glutathione redox balance, as indicated by the GSH/GSSG ratio. We also studied cytokine regulation induced by this oxidative stress.

One key finding of the present study is that the glutathione redox balance in airway epithelial cells was affected by LTC_4-induced oxidative stress. At 4 h after Calu-3 cells were exposed to 10^{-8} *M* LTC_4, the intracellular redox state gradually changed from a reduced to a severely oxidized state, followed by a prolonged reduced state. Airway epithelial cells have effective mechanisms to prevent a prolonged oxidized state, particularly the glutathione redox system, which can rapidly return cells from an oxidized to a reduced state (27). Although previous studies have demonstrated an association between the production of Cys-LTs and oxidative stress (2, 30), this is the first study of the effect of Cys-LTs on the intracellular redox state,

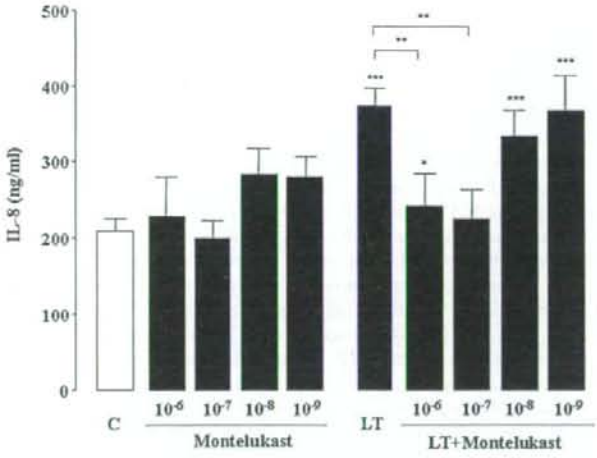

FIG. 6. Effect of LTC_4 and montelukast on IL-8 production in Calu-3 cells. Montelukast had a significant effect on LTC_4-induced IL-8 production in Calu-3 cells ($n = 6$). Pretreatment of monterukast was for 2 h, and incubation time of LTC_4 was 2 h. $^*p < 0.05$, $^{**}p < 0.02$; $^{***}p < 0.001$, compared with control.

and it proved that Cys-LTs can induce oxidative stress in cultured human airway epithelial cells. The decreased intracellular GSG/GSSG ratio in Calu-3 cells may be induced by the increased ROS formation after exposure to LTC_4. Electron spin resonance is the only method that can be used to measure free radicals directly, but the evanescent nature of many ROS makes them difficult to measure. Therefore, the most common technique is to quantify ROS indirectly by measuring the products damage caused by ROS, including the shift in the balance of reduced and oxidized glutathione (6). GSH is capable of reducing a wide variety of disulfides by transhydrogenation and acts as a major reductant of cellular protein disulfides (8).

This study clearly demonstrated that LTC_4 stimulated airway epithelial cells to produce IL-8. Cys-LTs are one of the most important mediators of the pathophysiology of asthma (18). They are produced mainly by eosinophils and mast cells. Studies have proved that Cys-LTs not only elicit bronchoconstriction as potent constrictors of smooth muscle, but also play an important role in airway remodeling. Recently, Perng *et al.* reported that LTC_4 induced transforming growth factor β_1 (TGF-β_1) in the airway epithelium through a P38 mitogen-activated protein (MAP) kinase activation mechanism (21). In our experiment, LTC_4 upregulated epithelial cell-derived IL-8 production. IL-8, a member of the α-chemokine family and one of the most abundant cytokines produced by airway epithelial cells, exhibits a variety of biological activities including neutrophils and T-lymphocyte chemotactic activity (3, 9). Recently, IL-8 has been implicated in the pathogenesis of the allergic inflammation of asthma, and neutrophils have been found to predominate over eosinophils as the major inflammatory cell type in bronchoalveolar lavage fluid and sputum samples of patients with acute exacerbated asthma (5).

Studies of inflammation-related changes in the intracellular redox state in pulmonary macrophages and monocytes show that these changes have a potent effect on cytokine production (11, 28). The IL-8 gene has been reported to be activated by

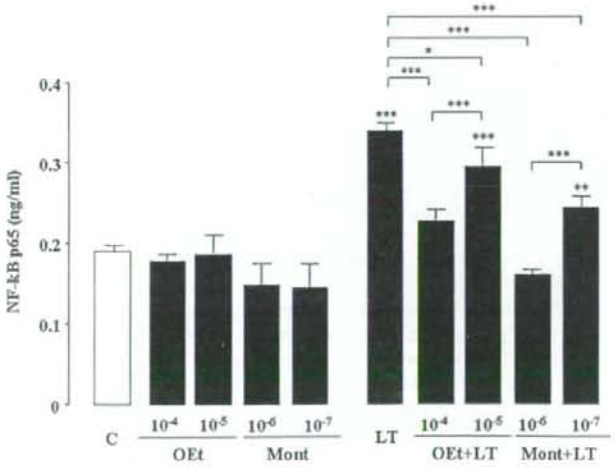

FIG. 7. Effect of LTC_4 on NF-kB p65 binding activity in Calu-3 cells. LTC_4 had an effect on NF-kB p65 binding activity in Calu-3 cells ($n = 5$). Pretreatment of GSH-OEt and montelukast was for 4 h and for 2 h. Incubation time of LTC_4 was 2 h. $^*p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$, compared with control.

oxidative stress (10, 17). In our previous study, oxidative stress affected the signal transfer system in airway epithelial cells, resulting in upregulation of cytokine and chemokine production, especially of IL-8 (27). It may be surmised that LTC_4 upregulated IL-8 production by means of oxidative stress in Calu-3 cells.

Oxidants or oxidative stress have been reported to activate transcription factors, including NF-κB, and to promote phosphorylation of MAP kinase (19, 22). We also confirmed that oxidative stress influenced the NF-κB pathway. Like most inflammatory mediators, IL-8 expression can be induced by activation of specific transcription factors such as NF-κB, which then bind to DNA sites located in the promotor region of the relevant gene (17). NF-κB has been shown to be redox state responsive (19), with binding of NF-κB to IL-8 promotors being highly sensitive to changes in the intracellular redox state (29).

On the other hand, Cys-LTs are recognized by at least two receptor types: Cys-LT_1 and Cys-LT_2. The Cys-LT_1 receptor mRNA is found in the spleen, lung tissue, and smooth muscle cells (1), and Cys-LT_2 receptor mRNA is confirmed in human and rat airway epithelial cells (25). It remains unclear which of these is involved in the mechanism of LTC_4-induced upregulation of IL-8 production. Also, we cannot ascertain whether LTC_4 is able to directly induce NF-κB activation via the Cys-LTs receptor in human airway epithelial cells. A previous study reported that LTC_4 did not directly induce NF-κB activation in U-937 cells (human monocytic leukemia cell line) (15). Further investigations are necessary to clarify the relationship between the LTC_4-induced intracellular redox state and IL-8 related signal transfer in airway epithelial cells.

Our results also suggested that pretreatment with montelukast, a Cys-LT_1 receptor antagonist, suppressed LTC_4-induced oxidation and significantly reduced the upregulation of IL-8 production in cultured human airway epithelial cells. At present, montelukast is widely used in the treatment of asthma. The anti-asthmatic effect may be rendered not only by the antileukotriene activity, but also by other types of pharmacological activity. Simeonova *et al.* demonstrated that montelukast inhibited TNF-α-induced NF-κB activation in THP-1 cells (human monocytic leukemia cell line) (24). Pranlukast, another Cys-LT_1 receptor antagonist, has been reported to inhibit TNF-α-induced NF-κB activation in U-937 cells, which have Cys-LT_1 receptors on their membranes, and T cells (Jurkat) which do not have Cys-LT_1 receptors on their membranes (15). The underlying mechanism of the inhibitory effect on NF-κB activation is unclear. Taking a previous study into consideration (15, 24), antagonism of Cys-LT_1 receptor may be partially related to the inhibition of NF-κB activation, and it is likely that Cys-LT_1 receptor antagonists have another antagonism that inhibits NF-κB activation. Cys-LT_1 receptor antagonists compose a new class of drugs, currently being investigated, and further research will expand our knowledge of their anti-inflammatory potential.

Our results demonstrated that, in cultured human airway epithelial cells, pretreatment with a reducing agent significantly suppressed LTC_4-induced time-dependent changes in the intracellular redox state and LTC_4-induced upregulation of epithelial cell-derived IL-8 production by suppressing NF-κB activation, which in turn confirmed that the LTC_4-induced upregulation of IL-8 production was mainly a consequence of oxidative stress. This finding suggests that pretreatment with antioxidative agents could protect against LTC_4-induced oxidative stress. Accordingly, pretreatment with reducing agents may protect against ozone-induced upregulation of IL-8 production in cultured human airway epithelial cells (27), so antioxidative drugs may have a prophylactic effect against inflammation-induced exacerbation of respiratory symptoms. The modulation of IL-8 production, in relation to oxidative stress, has an important role in inflammatory cell recruitment and activation (24), and treatment with reducing agents might benefit patients with airway diseases associated with inflammation, such as asthma. The protective effects of reducing reagents on Cys-LTs exposure *in vivo* require further preclinical investigation.

In conclusion, LTC_4 can affect the intracellular glutathione redox state in human airway epithelial cells, thereby activating signals and thus causing them to increase the production of cytokine. These findings may provide a basis for understanding the interrelationships between oxidative stress and airway inflammation. Therapeutic interventions that either augment endogenous antioxidant defenses or result in a decreased exposure to environmental oxidative stress might therefore be beneficial as adjunctive therapies for asthma and allergic respiratory disorders.

ACKNOWLEDGMENTS

We thank Mitsuyoshi Utsugi, M.D., Kunio Dobashi, M.D., Masatomo Mori, M.D., Ms. Tomoko Endo, and Ms. Chinori Iijima (Gunma University, Maebashi, Japan) for general scientific and technical support.

ABBREVIATIONS

GSH, reduced glutathione; GSH-OEt, glutathione monochrome ester; GSSG, oxidized GSH; redox, reduction-oxidation' NF-κB, nuclear factor-κB.

APPENDIX

Culture of human airway epithelial cells

The Calu-3 cell line obtained frozen from the American Type Culture Collection (Rockville, MD), was grown in T75 tissue-culture flasks (Coster, CA), containing a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% fetal calf serum (FCS) at 37°C in an atmosphere including 5% CO_2 and 95% air. When the cells were 80–90% confluent, they were detached with 0.05% trypsin and 0.02% ethylenediamine tetraacetate (EDTA), and seeded into wells in six-well plastic tissue culture dishes at 10^5 cells/cm^2. They then were grown to confluence, which typically required 10–12 days (23).

LTC_4 stimulation

In experiments involving Cys-LTs stimulation, confluent Calu-3 cells were serum-starved for 24 h before the addition of LTC_4. Cells were stimulated with LTC_4 at various concentrations or exposed to ve-

hicle (phosphate buffer solution, PBS). Based on previous experiments of cultured airway epithelial cell, we finally exposed confluent Calu-3 cells to 10^{-8} *M* LTC_4.

Cell exposure to glutathione modulators or pretreated with Cys-LTs receptor antagonists

To increase the concentration of cellular GSH, some Calu-3 cells were either incubated with GSH-OEt at concentrations of 10^{-4}, 10^{-5}, or 10^{-6} *M* for 4 h before and during exposure to LTC_4 (7). Glutathione modulators were added in the presence of FCS-free medium to minimize the influence of FCS on cellular GSH. At the same time, FCS-free medium was added to the control samples. In selected experiments, Calu-3 cells were incubated with the Cys-LTs receptor antagonist, montelukast, at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} *M* for 2 h before and during exposure to LTC_4. Cell viability, determined by trypan blue dye exclusion, always exceeded 90%.

Measurements of intracellular GSH and GSSG concentration

Calu-3 cells were washed three times with cold wash buffer (0.1 *M* sodium phosphate and 5 m*M* EDTA; pH 7.5) and immediately thawed in 100 μl of lysis buffer (0.1% Triton-X, 0.1 *M* sodium phosphate, and 5 m*M* EDTA; pH 7.5) for 5 min. Lysates then were acidified with 15 μl of 0.1 *N* HCl, and protein was precipitated with 15 μl of 50% sulfosalicylic acid. After centrifugation, the supernatant was collected for GSH and GSSG assays. The total cellular glutathione concentration was assayed by a GSSG-reductase-DTNB recycling procedure by the method of Tietze (26), as modified by Buchmuller–Rouiller and coworkers (7). GSH was oxidized by DTNB and then reduced by β-NADPH in the presence of glutathione reductase.

Formation of 2-nitro-5-thiobenzoic acid was monitored by comparing absorbance at 405 nm with that of standard samples of GSH in lysis buffer. GSSG was assayed by Griffith's method (13). Briefly, standard solutions of GSSG or aliquots of samples were mixed with 2 μl of 2-vinylpyridine per 100 μl of sample volume. All solutions were adjusted to pH 7.5 with triethanolamine. After incubation for 60 min at room temperature, the assay was performed as described for total glutathione.

Quantitation of IL-8

Concentrations of IL-8 in culture supernatants were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems, Minneapolis, MN), in accordance with the manufacturer's instructions. Samples of each supernatant were collected 24 h after LTC_4 or PBS exposure. The assays could detect IL-8 concentrations exceeding 3 pg/ml.

Nuclear protein extraction and quantitation of NF-κB p65 binding activity

Nuclear protein extraction was carried out using a nuclear extract kit (Active Motif, Carlsbad, CA) as follows. Calu-3 Cells (10^5/cm^2) in six-well plastic tissue culture dishes were cultured for 10–12 days. After incubation with varied concentrations of GSH-OEt for a pretreatment time of 4 h or with montelukast for a pretreatment time of 2 h, the cells were exposed to LTC_4 or PBS, respectively. At 6 h following the LTC_4 exposure, nuclear proteins were extracted. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were harvested after washing twice with 2 ml of ice-cold PBS, centrifuging at 500 *g* for 5 min after each wash. Cells were resuspended in lysis buffer (10 m*M* HEPES at pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* dithiothreitol (DTT), and protease inhibitor cocktail). A syringe with a 27-gauge needle was used to disrupt the cells by 10 repetitions of drawing the cell suspension and then ejecting it. The disrupted cell suspension then was centrifuged at 11,000 *g* for 20 min; the supernatant was removed, and the nuclear pellet was resuspended in extraction buffer [20 m*M* HEPES at pH 7.9, 1.5 m*M* $MgCl_2$, 0.42 *M* NaCl, 0.2 m*M* EDTA, 25% (vol/vol) glycerol, 1 m*M* DTT, and protease inhibitor cocktail]. Nuclei were disrupted with a syringe using the method described above, and centrifuged for 5 min at 21,000 *g*. Protein concentration of the nuclear extract was measured by the Lowry method.

NF-κB p65 expression was measured using the TransA NF-κB p65 kit (Active Motif).

Reagents

GSH-OEt, LTC_4, IL-4, and TNF-α were purchased from Sigma Chemical. (St. Louis, MO). montelukast sodium was purchased from Merck Co. (Whitehouse Station, NJ). Nicotinamide adenine dinucleotide phosphate (β-NADPH), 5-5,-dithiobis-2-nitrobenzoic acid (DTNB), and glutathione reductase were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Statistical analysis

All values are expressed as mean ± SE. Nonparametric analysis of variance (Kruskal–Wallis method) was used to determine significant overall differences between groups. We used the Mann–Whitney *U* test to determine significant differences between individual groups. A value of $p < 0.05$ was considered to indicate significance.

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