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ORIGINAL ARTICLE

The role of heme oxygenase-1 in systemic-onset juvenile idiopathic arthritis

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Abstract We have determined the serum levels of heme oxygenase-1 (HO-1) in 56 patients with systemic-onset juvenile idiopathic arthritis (s-JIA) and compared these with serum HO-1 levels in healthy controls and patients with other pediatric rheumatic diseases. Serum HO-1 levels were measured by the sandwich enzyme-linked immunosorbent assay. The mean serum HO-1 level in s-JIA patients during the active phase was 123.6 ± 13.83 ng/ml, which was significantly higher than that in patients with polyarticular juvenile idiopathic arthritis (p-JIA), Kawasaki disease, systemic lupus erythematosus or mixed connective tissue disease ($P < 0.0005$). The serum levels of HO-1, cytokines and cytokine receptors in patients with s-JIA were also assessed at both the active and inactive phases. The serum HO-1 level in patients with s-JIA in the active phase was found to be significantly greater than that in patients with the disease in the inactive phase ($P < 0.0001$). An assessment of the relationships between serum HO-1 levels and other laboratory parameters or cytokines in patients with s-JIA did not reveal any strong correlations. These results suggest that the serum level of HO-1 may be a useful marker for the differential diagnosis of s-JIA. Further study will be necessary to elucidate the mechanism of HO-1 production and to clarify the role of HO-1 in the disease process.

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Introduction

Heme oxygenase-1 (HO-1), also known as heat shock protein 32, is the rate-limiting enzyme in the degradation of heme to biliverdin, free iron and carbon monoxide (CO) [1]. This protein has been shown to exert an important protective effect against cellular stress following inflammation, preventing the deleterious effects of heme as well as mediating anti-inflammatory and anti-apoptotic function via its products [2–4]. Both bilirubin and ferritin act as antioxidants [5], whereas CO suppresses apoptosis and the synthesis of inflammatory mediators, such as proinflammatory cytokines, nitric oxide and prostaglandins [6–8]. Thus, heme degradation products and the metabolic derivatives generated by HO-1 suppress toxic events in cells.

Increased expression of HO-1 is associated with anti-inflammatory events and can benefit the host in different situations of stress or injury. Therapies involving HO-1 chemical inducers or gene transfer, as shown in animal models, have had favorable effects on lung damage or pneumonia, ischemia/reperfusion-induced injury to the heart and liver and allogeneic heart transplantation [9–13], among others. Interleukin (IL)-10 or 15-deoxy-$\Delta^{12,14}$-PGJ_2, both known inducers of HO-1, alleviate adjuvant-induced arthritis [14] and lipopolysaccharide (LPS)-induced septic shock [15] in mice.

Systemic-onset juvenile idiopathic arthritis (s-JIA), a typical chronic inflammatory disease in childhood, manifests with inflammatory symptoms and signs, such as spiking fever, skin rash, arthritis, pericarditis, and

hepatosplenomegaly [16]. On occasion, patients progress into macrophage activation syndrome, which is associated with serious morbidity and can be fatal [17, 18]. This syndrome is characterized by a pro-inflammatory cytokinemia responsible for the development of pathological events [19, 20].

High serum levels of HO-1 in adult-onset Still's disease (ASD) and hemophagocytic syndrome (HPS) have been reported in adult patients. Both diseases can be attributed to pro-inflammatory cytokinemia, and the measurement of serum HO-1 levels may be useful for differentiating inflammatory diseases and in monitoring disease activity [21].

Here, we have determined the serum levels of HO-1 in 56 patients with s-JIA and compared these to with serum HO-1 levels in healthy controls and patients with other pediatric rheumatic diseases. Sera from patients suffering macrophage activation syndrome (MAS) and HPS were also examined. We found that HO-1 is highly expressed in children with active s-JIA as well as in those with other hyper-cytokinemic disease states, MAS and HPS.

Patients and methods

Patients

A total of 56 children with s-JIA were enrolled. Active disease was defined by elevated levels of C-reactive protein (CRP; ≥1.5 mg/dl) and inadequate response to corticosteroids at 0.2 mg/kg prednisolone equivalent or more for more than 3 months.

Patients with other rheumatic diseases, including polyarticular juvenile idiopathic arthritis (p-JIA) ($n = 15$), systemic lupus erythematosus (SLE) ($n = 13$), mixed connective tissue disease (MCTD) ($n = 25$), Kawasaki disease (KD) ($n = 17$), Takayasu aortitis ($n = 6$), macrophage activation syndrome (MAS), which was diagnosed by reference to clinical and laboratory findings based on the preliminary diagnostic guideline by Ravelli et al. [22] ($n = 7$), and HPS, which was defined by the hemophagocytic lymphohistiocytosis 2004 protocol [23] ($n = 6$). All blood samples from these rheumatic diseases were collected at the active phase. Sera from 15 normal children (ten boys and five girls) were used as healthy controls. The study was approved by the Human Ethics Review Board of this institution, and informed consent was obtained from each patient's parents before enrollment.

Methods

A total of 204 blood samples were collected and immediately centrifuged at 3000 g for 10 min; sera were aspirated and stored in aliquots at −80°C until use.

Serum HO-1 levels were measured by a human HO-1 enzyme-linked immunosorbent assay (ELISA) kit (Stressgen, Victoria, Canada) according to the manufacturer's instructions. This assay is basically a quantitative sandwich immunoassay, and concentrations of factors in the samples are quantified against absorbance readings from a standard curve.

Concentrations of serum IL-2, IL-4, IL-6, IL-10, tumor-necrosis factor (TNF)-α and interferon (IFN)-γ were determined by a cytometric bead array system (CBA) (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Concentrations of soluble IL-6 receptor (sIL-6R) and soluble TNF receptor-1 (sTNFR-1) were determined by ELISA using a Quantikine human sIL-6R kit (R&D Systems, Minneapolis, MN) and a Quantikine human sTNFR-1 kit (R&D Systems), respectively, according to the manufacturer's instructions. These two assays are also sandwich immunoassays, as previously described.

Statistical analysis

Data were expressed as mean ± standard error (SE). Differences were assessed by Student's t test and paired t test. For comparison between multiple groups, statistical significance was tested by ANOVA with the Tukey's HSD test or Dunnet's procedure for post hoc comparisons. Pearson's correlation test was performed to assess the relationship between each experimental data set. The accepted level of significance was set at $P < 0.05$. Sensitivity and specificity were calculated from the receiver operating characteristic (ROC) curve. These statistical analyses were performed on a Macintosh computer using JMP ver. 6.0.3 (SAS Japan, Tokyo, Japan).

Results

The biological characteristics (gender, age) and laboratory findings (white blood cell counts, C-reactive protein), erythrocyte sedimentation rate and measured serum heme oxygenase-1 level of each group of children are shown in Table 1.

The level of HO-1 in the serum of 56 patients with s-JIA in the active phase was markedly elevated (123.6 ± 13.83 ng/ml) compared to that found in the normal controls (6.0 ± 0.6 ng/ml) or patients with p-JIA (7.9 ± 1.3 ng/ml), SLE (10.7 ± 2.6 ng/ml), MCTD (7.2 ± 1.5 ng/ml), KD (14.3 ± 2.9 ng/ml) ($P < 0.0005$ by ANOVA with Dunnet's procedure for post hoc comparisons). The level of HO-1 in the serum of children with Takayasu aortitis (92.0 ±38.1 ng/ml), MAS (196.7 ± 75.6 ng/ml) and HPS

Table 1 Profile of patients

Rheumatic condition	*n*	Sex (M/F)	Age (year)	WBC (/μl)	CRP (mg/dl)	ESR (mm/h)	HO-1 (ng/ml)
s-JIA	56	32/24	8.6 ± 0.6	18,600 ± 1,500	9.7 ± 1.2	52.1 ± 5.1	123.6 ± 13.83
p-JIA	15	6/9	11.4 ± 1.4	8,940 ± 1,020	6.9 ± 3.1	68.5 ± 15.0	7.9 ± 1.3*
SLE	13	3/10	12.9 ± 0.8	3,920 ± 900	0.05 ± 0.03	25.6 ± 8.1	10.7 ± 2.6*
MCTD	25	6/19	15.4 ± 1.6	5,510 ± 560	0.7 ± 0.2	42.0 ± 4.9	7.2 ± 1.5*
KD	17	9/8	4.8 ± 1.3	11,200 ± 1,590	14.5 ± 3.5	118.0 ± 6.3	14.3 ± 2.9*
Aortitis	6	0/6	14.9 ± 0.9	12,700 ± 2,170	7.1 ± 2.2	47.8 ± 10.9	92.0 ± 38.1
MAS	7	1/6	4.3 ± 1.4	14,900 ± 4,210	8.4 ± 2.9	49.8 ± 14.2	196.7 ± 75.6
HPS	6	4/2	6.5 ± 1.9	4,180 ± 700	1.0 ± 0.7	31.6 ± 14.8	142.3 ± 89.9
Controls	15	10/5	7.3 ± 1.0	–	–	–	6.0 ± 0.6*

Data are shown as the mean ± SE

WBC white blood cell, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *HO-1* Heme oxygenase-1, *p-JIA* polyarticular juvenile idiopathic arthritis, *s-JIA* systemic-onset JIA, *SLE* systemic lupus erythematosus, *MCTD* mixed connective tissue disease, *MAS* macrophage activation syndrome, *HPS* hemophagocytic syndrome, *KD* Kawasaki disease

* s-JIA vs. others, $P < 0.0005$ as determined by ANOVA with Dunnet's procedure for post hoc comparisons

(142.3 ± 89.9 ng/ml) were markedly elevated, but not significantly different from that in children with s-JIA.

The threshold HO-1 that differentiates patients at active phase with s-JIA from those with p-JIA was calculated to be 13.4 ng/ml, using the receiver operating characteristic curve (ROC) analysis; its sensitivity for diagnosis was 90.0%, and its specificity was 94.1% (Fig. 1). Similarly, we found a high sensitivity and specificity in the differential diagnosis of s-JIA relative to KD, SLE, MCTD using HO-1 serum levels (data not shown).

We next assessed levels of HO-1, cytokines (IL-2, 4, 6, 10, TNF-α, IFN-γ) and cytokine receptors (sIL-6R, sTNF-R1) in the serum of 44 (M:F = 21:23) patients with s-JIA at both the active and inactive phases. The clinical characteristics and laboratory findings [white blood cells (WBC), neutrophil counts, CRP, ESR, serum amyloid A (SAA), ferritin, fibrin degradation products (FDP-E) and urine beta-2 microglobulin (U-β2MG) and measured serum HO-1 level of these patients are shown in Table 2.

The serum HO-1 of s-JIA at the active phase was found to be significantly greater (98.5 ± 13.4 ng/ml) than that at the inactive phase (31.5 ± 4.3 ng/ml), indicating a marked reduction after successful therapy ($P < 0.0001$). The HO-1 level of inactive s-JIA patients was still higher than that of normal controls. By ROC analysis, The threshold HO-1 level was used as a laboratory marker for s-JIA disease activity in the ROC analysis and estimated as 41.5 ng/ml; its sensitivity for diagnosis was 62.7% and its specificity 77.2%. However, this degree of sensitivity and specificity is not sufficient to estimate disease activity. For a specificity of 95.0%, the threshold HO-1 level and the sensitivity would have to have been 101.8 ng/ml and 44.2%, respectively.

In terms of serum cytokines and their receptors, the levels of IL-4, IL-10 and sTNF-R1 were significantly increased at the active phase ($P = 0.01$, $P = 0.0005$ and $P = 0.0007$, respectively); in contrast, there were no significant differences between the active and inactive phase for the other cytokines. The IL-6 serum levels were particularly high in both the active (296.1 ± 54.3 pg/ml) and inactive phases (203.2 ± 50.8 pg/ml) of disease (no significant difference between these values). The concentration of sIL-6R was significantly higher at the inactive phase (423.0 ± 71.9 ng/ml) than that at the active phase (44.1 ± 6.8 ng/ml) ($P = 0.0003$).

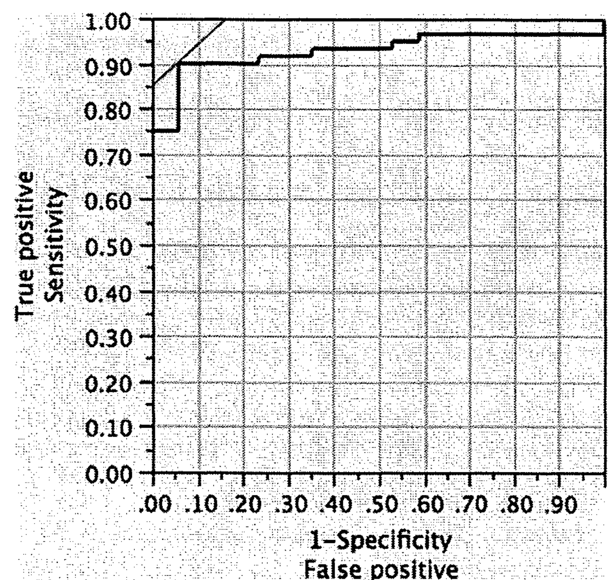

Fig. 1 Receiver operating characteristic curve of serum heme oxygenase-1 (HO-1) level used to differentiate patients with systemic-onset idiopathic arthritis (s-JIA) from patients with polyarticular JIA (p-JIA). The threshold serum HO-1 level was calculated to be 13.4 ng/ml (sensitivity 90.0%, specificity 94.1%)

Table 2 Profile of patients with systemic-onset idiopathic arthritis at active/inactive states

Patient profile/parameters	Active	Inactive	P
Age (years)	8.9 ± 0.7	11.1 ± 0.8	0.059
WBC (/μl)	21,138 ± 2,071	7,202 ± 475	<0.0001*
Neutrophil (rate)	0.80 ± 0.02	0.64 ± 0.02	0.0002*
CRP (mg/dl)	10.6 ± 1.4	0.05 ± 0.03	<0.0001*
SAA (ug/ml)	819.7 ± 123.3	9.3 ± 4.8	<0.0001*
ESR (mm/h)	57.2 ± 6.2	3.4 ± 0.6	<0.0001*
Ferritin (ng/ml)	733.2 ± 295.9	22.2 ± 6.0	0.021*
FDP-E (ug/ml)	307.1 ± 36.5	68.2 ± 12.5	<0.0001*
U-β2MG (ug/ml)	435.4 ± 136.2	115.1 ± 13.1	0.027*
HO-1 (ng/ml)	98.5 ± 13.4	31.5 ± 4.3	<0.0001*

The data are shown as the mean ± SE

SAA Serum amyloid A, *FDP* fibrin degradation products, *U-B2MG* urine beta-2 microglobulin

* $P < 0.05$ as determined by paired t-test

We next examined the relationships between serum HO-1 levels and other laboratory parameters or cytokines in patients with s-JIA. As shown in Fig. 2a–d, moderate ($r > 0.40$), but not strong ($r > 0.7$) correlations were found between HO-1 and several laboratory parameters related to inflammation, such as SAA ($r = 0.579$, $P < 0.0001$), ESR ($r = 0.484$, $P < 0.0001$) and ferritin ($r = 0.434$, $P < 0.0001$). We could not find a strong correlation between serum HO-1 and IL-6 ($r = 0.297$, $P = 0.0082$), although this cytokine plays an important role in disease activity. Other parameters also lacked significant associations with serum HO-1 (data not shown). In a previous study on ASD and HPS [21], a close correlation between ferritin and serum HO-1 was reported ($r = 0.649$, $P = 0.0048$). Our data are thus not in agreement with that study.

Discussion

To the best of our knowledge, this study is the first to report on the levels of HO-1 in the serum of patients with pediatric rheumatic diseases. We found that the serum HO-1 level was significantly higher in patients with s-JIA than in those with p-JIA, KD, SLE or MCTD. On the basis of the serum HO-1 level, s-JIA could be differentiated from these diseases with a sensitivity and specificity >90%, suggesting that HO-1 level may be useful tool for the differential diagnosis of pediatric rheumatic diseases. We also found that the serum HO-1 level in two patients with primary Sjogren's syndrome was as low as that in the healthy controls (5.29 ± 3.48 ng/ml, $n = 2$) (data are not shown due to there being only two patients). However, the serum HO-1 levels in patients with HPS or MAS, although elevated, were not significantly different from that in patients with s-JIA. Such elevated levels were also measured in patients with Takayasu disease. Because of great inter-individual variability in these small numbers of patients, a definitive conclusion regarding the value of HO-1 level as a diagnostic factor will require further study in larger numbers of samples.

In contrast to SLE and MCTD, the main feature of which is immune complex-induced inflammation—i.e. tissue inflammation, tissue destruction and fibrosis induced by production of autoantibodies and immune complexes—s-JIA, MAS, HPS and Takayasu aortitis syndrome are systemic inflammatory diseases which progress to abnormal activation of vascular endothelial cells, apoptosis of cells and organ failure accompanied by excessive production of inflammatory cytokines [24]. Some studies have reported that both anti-inflammatory cytokines (IL-10 and IL-13) and inflammatory cytokines (IL-1 and TNF-α) induce HO-1 in endothelial cells and macrophages [25, 26]. In contrast, other studies have found that TNF-α suppresses the expression of HO-1 in human peripheral mononuclear cells [27]. From the pathological point of view, because the serum HO-1 increased only in diseases where cytokines were produced in excess, it could be hypothesized that there is some relationship between increased serum levels of cytokines and HO-1 production. However, no strong correlations have been found between serum HO-1 levels and those of either inflammatory cytokines, such as IL-6, IFN-γ and TNF-α, or anti-inflammatory cytokines, including IL-10. Furthermore, in our study, we found that the serum HO-1 level was only slightly higher in patients with KD than in healthy controls, although it has been widely accepted that the levels of TNF-α and other inflammatory cytokines are elevated in the acute phase of this disease [28]. These results suggest that the elevation of serum HO-1 and the nature and quantity of the cytokines elevated in serum, or the duration of the elevation, mutually affect one another. However, further study is necessary to elucidate associations of these factors.

We found that the serum concentrations of most of the cytokines and cytokine receptors measured in patients with s-JIA decreased with remission of the disease and that only the concentration of IL-6R remained significantly elevated in the inactive phase. Because most of the children with s-JIA enrolled in this study were treated with tocilizumab, a humanized antihuman IL-6 receptor antibody, the sIL-6R may have been elevated due to a negative feedback mechanism. Alternatively, it is possible that we measured membrane-bound IL-6R released into the serum by some undetermined mechanism.

Of the parameters measured in patients with s-JIA, we found correlations between serum HO-1 level and concentrations of SAA, FDP-E, ESR and U-β2MG.

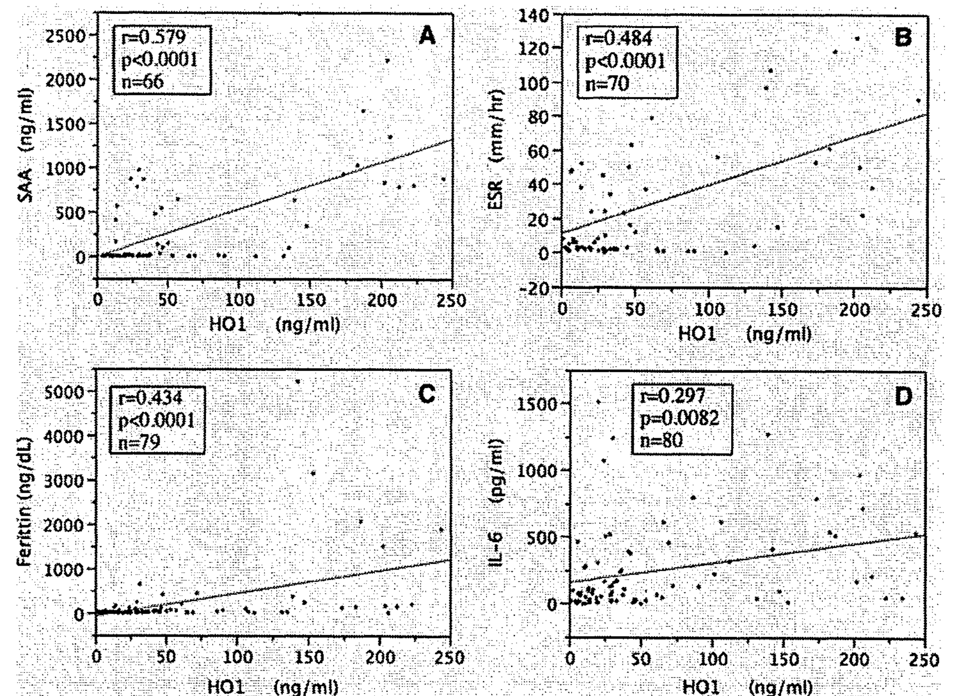

Fig. 2 Correlations between serum HO-1 and laboratory parameters, analyzed by Pearson's correlation test. *ESR* Erythrocyte sedimentation rate, *SAA* serum amyloid A, *IL* interleukin

Correlations between serum HO-1 and SAA, ESR or FDP-E suggest an association between HO-1 level and systemic inflammation, especially inflammation of the vascular endothelium due to hypercytokinemia. Several studies have raised the possibility that HO-1 acts as a cytoprotective factor in epithelial cells in the proximal tubule [29, 30]. It has also been reported that human HO-1 deficiency is characterized by interstitial lesions in the renal tubule [31, 32]. These results are concordant with the correlation between serum HO-1 level and the amount of U-β2MG in patients with s-JIA found in our study. Gene transcription of ferritin and HO-1 are both regulated by Nrf2 (nuclear factor, erythroid-derived 2, like2) [33, 34]. In contrast to the results of previous studies in adult patients, we failed to find any correlation between serum levels of ferritin and HO-1 in children with s-JIA, suggesting the possibility that the production of these factors is regulated by other mechanisms that are not mediated by Nrf2.

The existence of autoinflammatory syndromes has recently been recognized as the disease paradigm "fever of unknown origin". This group of syndromes includes chronic inflammatory neurological cutaneous articular (CINCA) syndrome, hyper-immunoglobulin D syndrome, familial Mediterranean fever and TNF receptor-associated periodic syndrome. Here, we measured HO-1 in a patient with CINCA syndrome and found it to be as high as that in patients with s-JIA (121.5 ng/ml, $n = 1$). Enhanced release of cytokines in patients with CINCA syndrome and the effective treatment of a CINCA syndrome patient with a cytokine inhibitor have been reported [35, 36]. Thus, it is possible that the increased serum level of HO-1 in a patient with CINCA syndrome reflects the existence of systemic inflammation due to hypercytokinemia in this disease. In actual clinical practice, infectious diseases are the underlying cause of the great majority of fever of unknown origin, with malignant tumors and drug allergies occasionally found to be the cause. In our study, serum HO-1 levels in a patient with abscesses induced by methicillin-resistant *Staphylococcus aureus* (7.8 ng/ml) and a patient with drug allergy (10.0 ng/ml) were as low as those in healthy controls (data not shown). Further study of serum HO-1 levels in larger numbers of patients with infectious diseases or malignant tumors are therefore required.

The origin of circulating HO-1 has not been definitely determined. It has been suggested that monocytes are a major source of HO-1 in the peripheral blood and that among organ-localized macrophages, alveolar macrophages, hepatic Kupffer cells and splenic macrophages constitutively produce HO-1. A study of blood HO-1 levels in adult patients with HPS and ASD found that the expression of HO-1 mRNA in peripheral blood mononuclear cells in most of these patients was not different from healthy controls, although increased expression was observed in some patients [21]. It has also been demonstrated that a $CD16^{high}$ $CCR2^{-}$ subpopulation of monocytes constitutively expressed HO-1 mRNA, whereas the $CD16^{low}CCR2^{-}$ subpopulation produced HO-1 only in response to tissue damage and/or inflammation [37]. Since

there is a possibility that macrophages activated in the reticuloendothelial system and inflammatory sites produce HO-1, both clinical and pathological studies are required to clarify this point in the future.

Conclusion

This study demonstrated that the serum level of HO-1 may be a useful marker for the differential diagnosis of s-JIA. Further study will be necessary to elucidate the mechanism of HO-1 production in other pediatric rheumatic diseases, autoinflammatory syndromes, infectious diseases and malignant tumors and to clarify the role of HO-1 in the disease process.

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

Hiromi Tadaki,*† Hirokazu Arakawa,[1]* Takahisa Mizuno,* Tomoko Suzuki,* Kiyoshi Takeyama,‡ Hiroyuki Mochizuki,* Kenichi Tokuyama,§ Shumpei Yokota,† and Akihiro Morikawa*

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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[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.

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Table I. *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

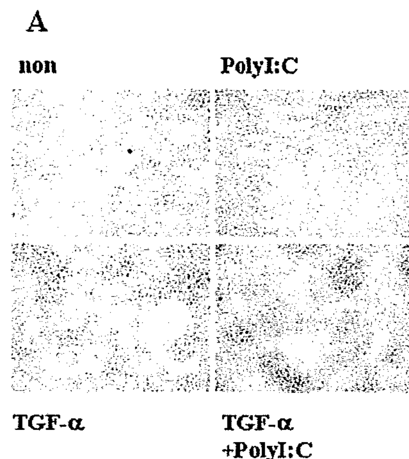
NCI-H292 cells were stained with Alcian blue and periodic acid-Schiff stains (AB-PAS). MUC5AC protein was measured as described previously (5). In brief, supernatants were collected at 24 h after stimulation and cell lysates were prepared with PBS, and 50 μl of each sample was incubated with bicarbonate-carbonate 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 I (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 ($\Delta\Delta C_T = \Delta C_T$ 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.



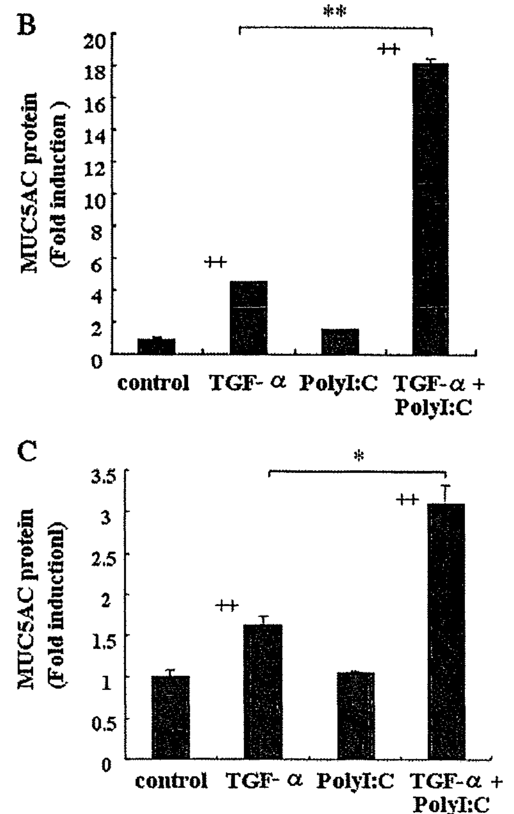


FIGURE 1. Synergistic effect of poly(I:C) and TGF-α on MUC5AC production. *A*, AB-PAS staining of NCI-H292 cells for identification of mucus glycoconjugates. Incubation with poly(I:C) (25 μg/ml) and TGF-α (4 ng/ml) for 24 h increased positive staining. Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced 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 **, $p < 0.01$.

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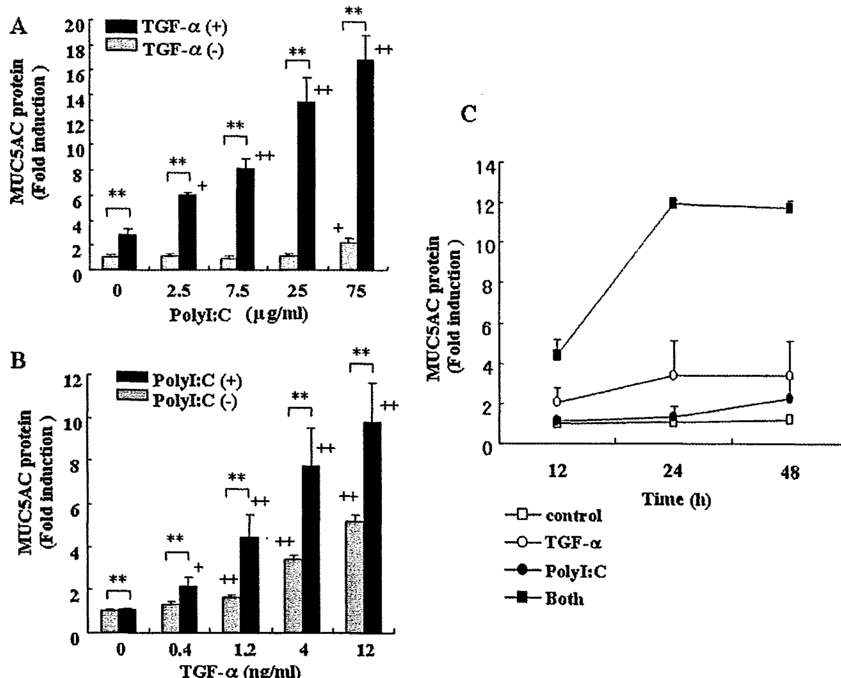

FIGURE 2. Dose responsiveness and time course of MUC5AC protein production. *A*, Effect of the poly(I:C) concentration on TGF-α (4 ng/ml)-induced MUC5AC mucin production in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF-α for 24 h. *B*, Effect of the TGF-α concentration on poly(I:C) (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

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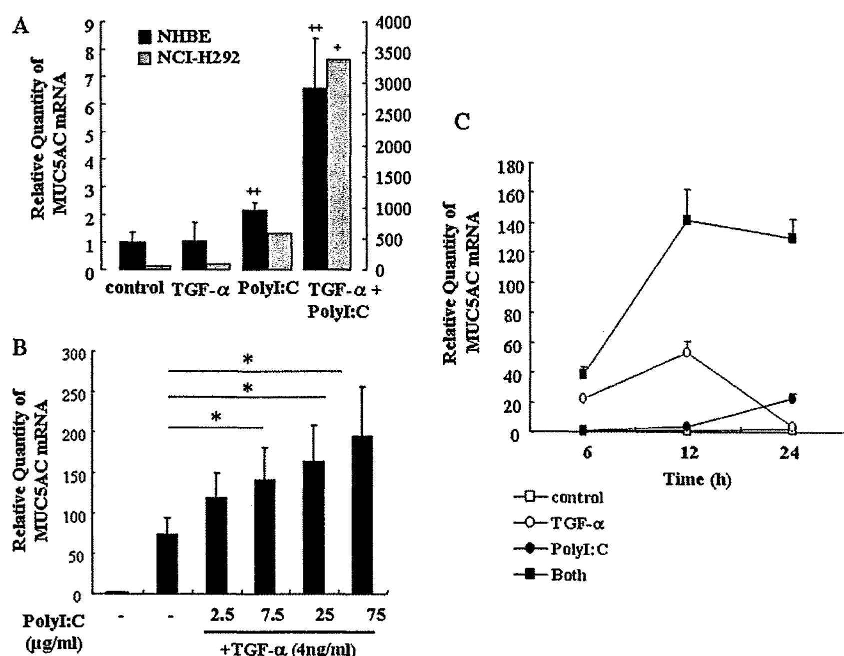

FIGURE 3. Dose responsiveness and time course of MUC5AC mRNA expression. *A*, Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced MUC5AC gene expression in NCI-H292 cells and NHBE cells. Cells were incubated with poly(I:C) and TGF-α for 24 h. Data are presented as the fold induction over the level 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. *, $p < 0.05$.

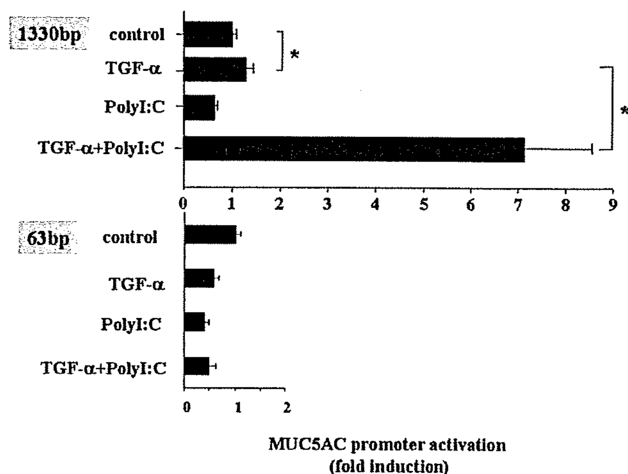

FIGURE 4. Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced *trans*-activation of the full-length (−1330) and the short-length (−63) MUC5AC promoter in NCI-H292 cells ($n = 6$). Data are shown as means ± SD. *, $p < 0.05$.

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

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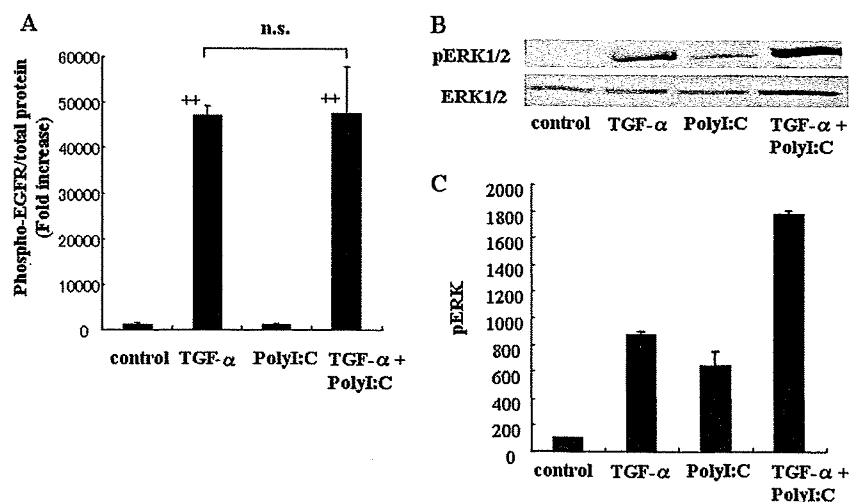

FIGURE 5. *A*, Relative phosphorylation of EGFR by poly(I:C) and TGF-α in NCI-H292 cells. Proteins extracted from samples collected were tested for the presence of phosphorylated EGFR by a Bio-Plex phosphoprotein assay kit using the Bio-Rad Luminex machine. The values plotted show the ratios of phosphorylated 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.

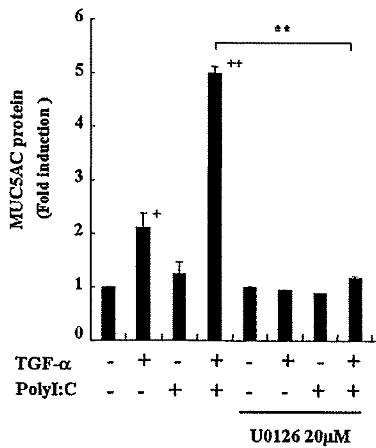

FIGURE 6. Effect of U0126 on poly(I:C)- and TGF-α-induced MUC5AC mucin production in NCI-H292 cells. Cells were preincubated with U0126 (a specific inhibitor of MEK1/2) at 20 μM (15, 16) for 1 h before adding poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml), and cells were analyzed 24 h after stimulation ($n = 6$). 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.

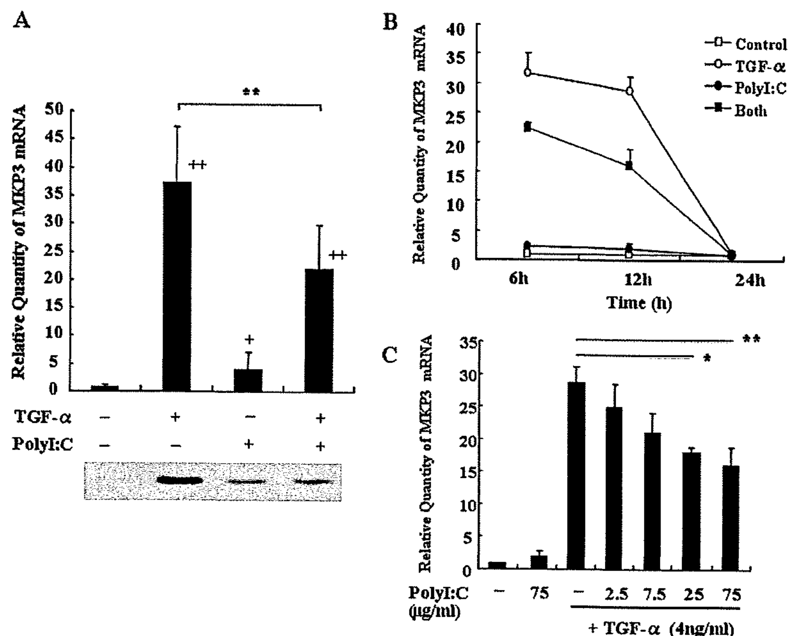

FIGURE 7. *A*, *Upper*, Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced MKP3 mRNA expression in NCI-H292 cells ($n = 8$). *Lower*, To verify MKP3 expression in NCI-H292 cells, expression of MKP3 after stimulation with poly(I:C) (25 mg/ml) and TGF-α (4 ng/ml) was examined by standard RT-PCR. 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 **, $p < 0.01$.

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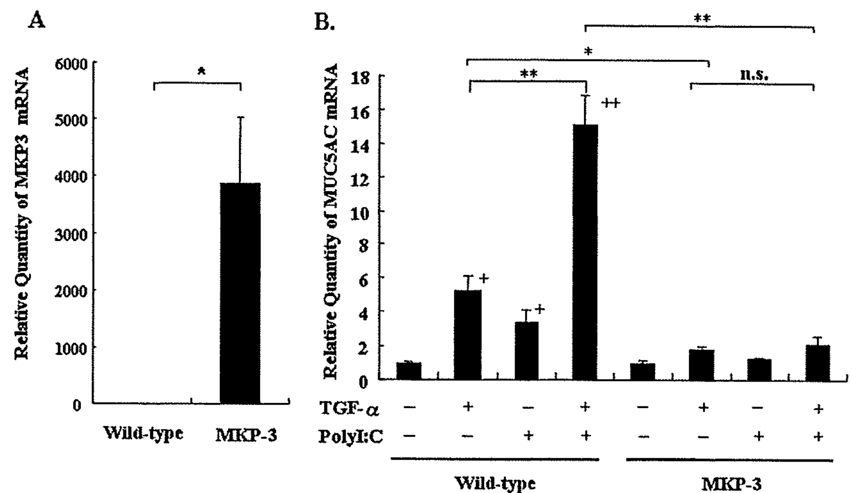

FIGURE 8. *A*, Effect of a MKP3 expression plasmid cloned into the pAcGFP1-C1 vector on MUC5AC mRNA expression in NCI-H292 cells. The level of MKP3 mRNA was enhanced significantly in cells transfected with the MKP3 expression plasmid. *B*, Enhanced expression of MUC5AC mRNA was noted in wild-type cells 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).

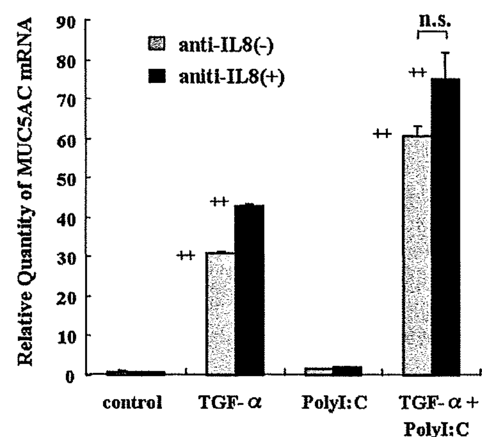

FIGURE 9. Effect of the anti-IL-8 Ab on poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml)-induced MUC5AC mRNA expression in NCI-H292 cells ($n = 4$). Data are shown as means ± SD. ++, $p < 0.01$ compared with nonstimulated control cells.

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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,

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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.

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Disclosures

The authors have no financial conflicts of interest.

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ORIGINAL ARTICLE

Improvement of reduced serum cartilage oligomeric matrix protein levels in systemic juvenile idiopathic arthritis patients treated with the anti-interleukin-6 receptor monoclonal antibody tocilizumab

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Abstract In this study, we determined serum cartilage oligomeric matrix protein (COMP) levels in systemic juvenile idiopathic arthritis (sJIA) patients during both the active and the remission phases to investigate how the growth cartilage turnover changed under tocilizumab treatment. Specimens were collected from 201 healthy children under 16 years of age with no growth impairment, and paired sera were collected from 11 sJIA patients treated with tocilizumab. Disease activity was assessed from white blood cell count, erythrocyte sedimentation rate, C-reactive protein, and ferritin, and the COMP concentration was determined by sandwich enzyme-linked immunosorbent assay. Serum COMP concentrations were found independent of age, and the mean value in healthy children was 17.74 ± 5.6 U/L. The mean serum COMP in sJIA patients during the active phase was 10.75 ± 3.9 U/L, lower than that of healthy children. The mean serum COMP in the remission phase (14.89 ± 3.9 U/L) was significantly higher than that in the active period ($P < 0.05$). These results suggested that in sJIA patients, a reduced serum COMP concentration is a useful marker of active disease and growth impairment, and that the growth cartilage turnover suppressed during the active phase is improved in the remission phase under tocilizumab treatment.

Keywords Cartilage oligomeric matrix protein (COMP) · Systemic idiopathic juvenile arthritis (sJIA) · Interleukin-6 (IL-6) · Anti-IL-6 receptor monoclonal antibody · Growth impairment

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Introduction

Cartilage oligomeric matrix protein (COMP) is a secreted 550-kDa homopentameric glycoprotein that belongs to the thrombospondin family and is primarily found in the extracellular matrix of cartilage, ligament, and tendon [1–5]. The COMP protein is predominantly synthesized by chondrocytes and synovial cells [6]. In adults, the serum COMP level is considered to be a predictable factor of joint destruction in early osteoarthritis and early rheumatoid arthritis (RA) [7–10]. On the other hand, in children the serum COMP concentration is considered to be a reliable biomarker of growth cartilage turnover in the growth phase because the longitudinal growth is associated with an increased production of COMP mainly from chondrocytes in the growth plate [11].

Systemic juvenile idiopathic arthritis (sJIA) is a chronic inflammatory disorder involving growth retardation, characterized by markedly elevated circulating levels of interleukin-6 (IL-6) and severe multi-organ diseases. We have already reported that the anti-IL-6 receptor monoclonal antibody, tocilizumab is effective in children with sJIA [12–14]. Moreover, the long-term effects of tocilizumab were remarkable catch-up growth (15–18 cm/year) and increased bone mineralization [15, 16].

We examined the serum COMP levels in sJIA patients during the active disease and remission phases to investigate how the growth cartilage turnover changed under tocilizumab treatment. Additionally, we measured the

serum bone alkaline phosphatase (BAP) level as a bone constructing/remodeling marker.

Subjects and methods

Healthy controls

Specimens were collected from 201 healthy children (117 boys, 84 girls) under 16 years of age (mean age 9.34 years), who had no growth impairment and who served as healthy controls.

Patients

Eleven patients, diagnosed as having sJIA according to the International League of Associations for Rheumatology (ILAR) classification criteria (1997) [17], were enrolled in the present study. They included a group of 11 sJIA, Japanese children (four boys and seven girls), of 1–8 years. All of them were treated with tocilizumab because they could not tolerate (or showed inadequate response to) systemic corticosteroids and immunosuppressants. Consequently, their typical symptoms of inflammation and laboratory abnormalities were sharply improved.

Paired sera from the 11 sJIA patients were obtained in both the active and the remission phases under tocilizumab treatment. The period between the active disease and remission phases varied (2–55 months, average duration 23 months). Disease activity was assessed from white blood cell count (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and ferritin, and the remission phase was denoted by a fall of serum CRP (CRP; < 1.5 mg/dL). The changes in the value of the blood test used for assessing disease activity in the sJIA patients before and after the start of tocilizumab treatment are given in Fig. 1.

Quantification of serum COMP concentration

After collection, blood samples were centrifuged and the serum obtained was stored at −80°C. Serum COMP concentration was quantitatively determined using a human COMP assay kit, COMP® ELISA (MBL, Nagoya, Japan). The kit is a sandwich enzyme-linked immunosorbent assay (ELISA) utilizing two monoclonal antibodies directed against separate antigenic determinants on the human COMP molecule.

Serum BAP concentration and matrix metalloproteinase-3 (MMP-3) concentrations were measured at the same time using a commercial enzyme immunoassay (EIA) kit, Osteolinks-BAP (DS Pharma Biomedical, Osaka, Japan) and the MMP-3 test (The Binding Site, Birmingham, UK), respectively.

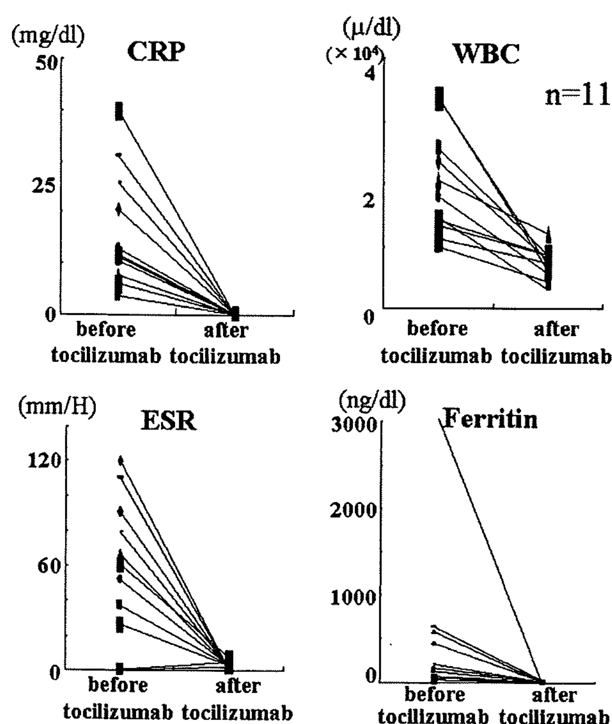

Fig. 1 Changes in the blood-test values used for assessing disease activity in the systemic juvenile idiopathic arthritis (sJIA) patients after the start of tocilizumab treatment. All values (*WBC*, *ESR*, *CRP* and *Ferritin*) decreased significantly after the start of tocilizumab treatment. *WBC* white blood cell count, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein

Statistical analysis

The Wilcoxon matched pairs test (two-tailed) was used to compare results of consecutive measurements in individual patients. Correlations were calculated using Spearman's correlation coefficient. P values < 0.05 were considered significant.

Results

Serum COMP concentration in the control group (Fig. 2)

The results showed that serum COMP concentrations were not related to age in 201 healthy children aged up to 16 years. The mean serum COMP in 201 healthy children was 17.74 ± 5.6 U/L.

Serum COMP concentrations during the active disease and remission phases in sJIA patients (Fig. 3)

In the sJIA patients, the mean serum COMP during the active phase was 10.75 ± 3.9 U/L, significantly lower than that of healthy children and the mean serum COMP in the

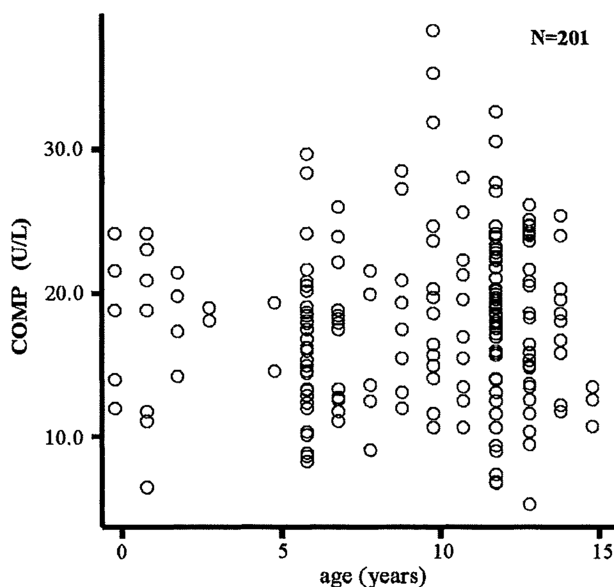

Fig. 2 Serum cartilage oligomeric matrix protein (*COMP*) concentration in the control group

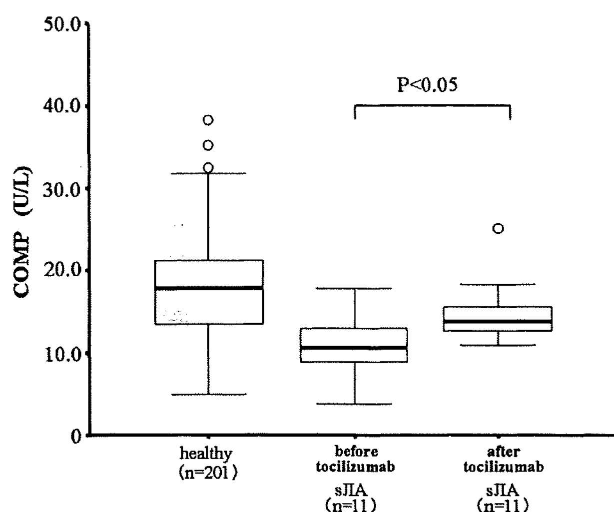

Fig. 3 Serum cartilage oligomeric matrix protein (*COMP*) concentrations during the active disease and remission phases in the systemic juvenile idiopathic arthritis (sJIA) patients. *Line* in the *box* represents the median, and the *upper* and *lower ends of the box* show the 25th and 75th percentiles of the population

remission phase during treatment with tocilizumab was (14.89 ± 3.9 U/L), significantly higher than that in the active phase ($P < 0.05$).

Serum BAP and MMP-3 concentrations in sJIA patients (Fig. 4a, b)

The serum MMP-3 concentrations were high during the active phase, but decreased in the remission phase during

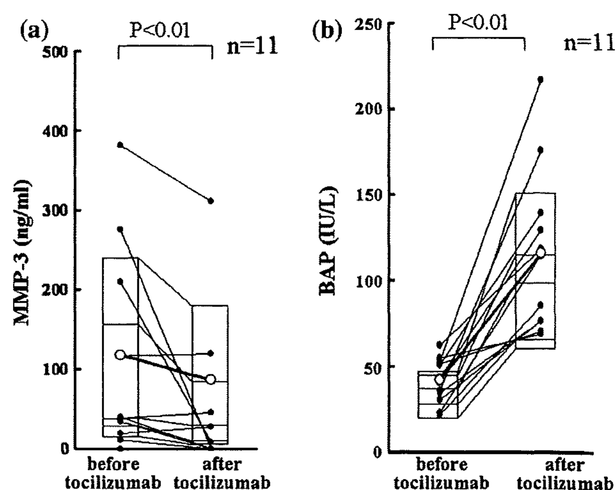

Fig. 4 **a** Serum matrix metalloproteinase-3 (*MMP-3*) concentrations during the active disease and remission phases in the systemic juvenile idiopathic arthritis (sJIA) patients. **b** Serum bone alkaline phosphatase (*BAP*) concentrations during the active disease and remission phases in sJIA patients

tocilizumab treatment ($P < 0.01$). On the other hand, the serum BAP concentrations increased significantly in the remission phase ($P < 0.01$).

Discussion

In the present study, we found that the serum COMP concentrations in sJIA patients during the active disease phase were lower than those of the healthy controls, but they significantly increased in the remission phase after the patients underwent tocilizumab treatment. These findings indicted that in sJIA patients, the growth cartilage turnover was suppressed during the active disease phase, but it was improved in the remission phase after tocilizumab treatment was commenced.

In adults, the serum COMP level has been considered to reflect the articular cartilage turnover because adults are past the growth phase, and growth cartilage is no longer present. It reflects the degree of destruction in the articular cartilage, and is used clinically as a marker of synovitis and as a predictive factor for joint destruction in cases of RA [10, 18]. However, in children, the serum COMP concentration has been considered to reflect the longitudinal growth, which results from chondrocyte proliferation and differentiation in the growth plate. Previous studies revealed that the highest level of COMP mRNA was detected in chondrocytes in the central region of the growth plate [19, 20], and that serum COMP concentration increased after the start of growth hormone treatment in short children [21].

Firstly, we measured the serum COMP concentrations in 201 healthy children between 0 and 16 years of age to

investigate the correlation between serum COMP levels and age. We expected to find age-associated differences in serum COMP concentration, but the children showed no correlation between age and serum COMP concentration. But the mean serum COMP concentration in 201 healthy subjects under 16 years of age was 17.74 ± 5.6 U/L, higher than the adult reference values published in the test kit documentation. Similarly, a previous study reported that serum COMP levels in subjects under 16 years of age were significantly higher than those in subjects aged 16 or older [11]. Accordingly, the serum COMP concentration in children apparently reflects the turnover of chondrocytes in the growth plate. In this study, there were large differences among individuals in the serum COMP levels, so it was impossible to establish a standard range of serum COMP concentrations in children in the growth phase.

Some previous studies have already revealed that the serum COMP concentrations in sJIA patients were significantly low [11, 19]. Children with early-onset and long-duration systemic diseases have abnormalities in growth. Once the systemic disease begins, the body height of the affected child is usually recorded as being steady during the course of the disease. The results of our study also revealed that serum COMP levels in sJIA patients during active disease phase were lower than those observed in healthy children. In addition, we found that the suppressed COMP levels significantly increased in the remission phase after tocilizumab treatment was commenced. At the same time, we evaluated the serum MMP-3 concentration, which is a clinically useful marker for predicting joint destruction and for disease activity in RA [22]. As a result, the serum MMP-3 concentrations in the sJIA patients were high during the active disease phase, but decreased after tocilizumab treatment was begun. In sJIA patients, the serum MMP-3 concentration reflects the disease activity of arthritis, as it does in RA, but the serum COMP level may reflect not arthritis so much as the longitudinal growth.

To our knowledge, this is the first report demonstrating the improvement of serum COMP levels in sJIA patients under treatment with tocilizumab. These results suggest that a reduced serum COMP level in sJIA patients may be a useful marker of active disease and growth retardation, but not of arthritis, and that the growth cartilage turnover suppressed during the active disease phase was improved in the remission phase during treatment with tocilizumab.

This study also evaluated the serum BAP concentration, which is one of the bone formation markers for osteoblastic activity. Bone alkaline phosphatase is released by osteoblasts during the mineralization process and is a well-characterized biochemical marker of bone formation [23, 24]. The serum BAP concentration also increased significantly in the remission phase in sJIA patients. These results indicated that in sJIA patients, both growth cartilage turnover and bone turnover may be improved as a result of the successful inhibition of inflammation under treatment with tocilizumab. In consequence, catch-up growth will occur.

Many factors—hormonal, nutritional, and other—as well as physical exercise, may affect the production of COMP protein, so that tocilizumab may cause changes in growth plate chondrocytes producing COMP protein, in collaboration with other factors. Further consideration is therefore needed.

In summary, our observations in the present study suggested that a reduced serum COMP concentration may be a useful marker of active disease and growth impairment in sJIA patients, and that marked increases of serum COMP and BAP are reliable biochemical indicators that retarded growth was improved by tocilizumab treatment. Tocilizumab blocks IL-6/IL-6 receptor-mediated inflammation, resulting in improvement of both growth plate cartilage turnover and bone turnover in sJIA patients.

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Conflicts of interest statement All authors have no conflicts of interest to be declared.

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