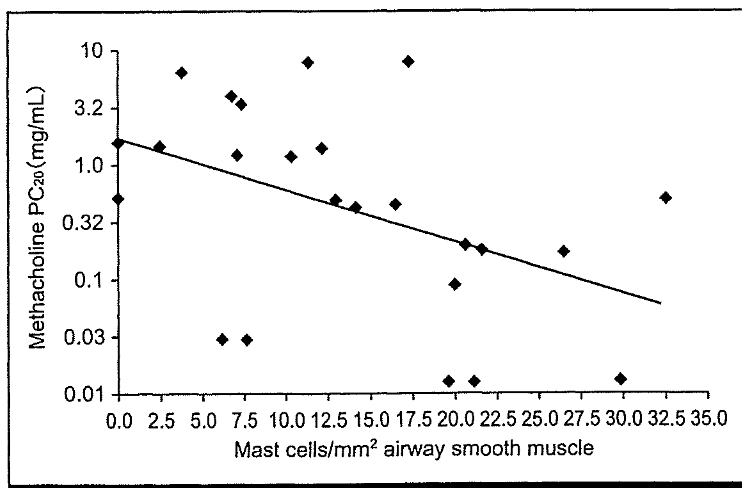

図7　気管支平滑筋細胞に発現するmast cellとメサコリン気道過敏性との関係

難治性喘息患者では平滑筋細胞に発現するmast cellの数/mm^{2}とMch PC_{20}は負の相関を示す.　（文献29より）

過敏性亢進と正の相関を示した（図7）と報告している[29]。この結果から平滑筋細胞の増生のメカニズムにmast cellが関与することが示唆される。

線維芽細胞増生，コラーゲン沈着には喘息重症度と関連がある，関連がないという双方の報告があり，例えばChuらはコラーゲン沈着，基底膜肥厚，好酸球浸潤，TGF-β陽性細胞数は軽症喘息と重症喘息では差がみられないと報告している[30]。一方でBenayounらはintermittent asthma, mild-to-moderate persistent asthma, severe persistent asthmaの3群で粘膜生検を施行し，好酸球数，好中球数，上皮剥離，基底膜肥厚，コラーゲン沈着には差を認めなかったが，粘液腺増生，平滑筋細胞増生，線維芽細胞増生は重症度に相関すると報告している[31]。気道上皮下の線維化については罹病期間，重症度，治療内容によってさまざまな研究結果があり，一定した見解は得られておらず，特に難治性喘息の特徴といえる所見も明確にはされていないと考えられる。

基底膜肥厚は喘息以外の呼吸器疾患でも肥厚することは知られており，罹病期間と基底膜肥厚の程度には相関を認めないという報告[32]が比較的多い。一般喘息では喘息重症度に比例して基底膜が肥厚するという報告や[33]，重症喘息で好酸球浸潤を伴うタイプにおいてのみ，基底膜の肥厚が認められたという報告がある[5]。

粘液線に関しては，中等症から重症喘息患者では気道分泌が亢進しており，また上皮傷害に基づく粘液線毛輸送不全も加わると気道内腔に粘液が滞留し気道抵抗が増大する。喘息死の剖検例を検討した報告では，著明な杯細胞の増生が認められ，中枢，末梢気道とともに気道粘膜に占める杯細胞の面積は健常者の約20～30倍に達するといわれている[33]。

VEGF (vascular endothelial growth factor)
PDGF (platelet-derived growth factor)
FGF (fibroblast growth factor)

血管のリモデリングに関してはKuwanoらの致死的な喘息発作患者では血管の拡張や増加が著しいという報告がある[34]。血管新生に関与する因子としてはvascular endothelial growth factor (VEGF), angiogenin, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF)などが挙げられる。Hoshinoらは喘息患者の気道粘膜ではVEGFの受容体であるflt-1, flk-1のmRNAレベルでの発現が亢進しており，さらにその陽性細胞数と血管占拠率が相関すると報告している[35]。

おわりに

難治性喘息のメカニズムというテーマにそって気道炎症，気道過敏性，気道リモデリングの観点から述べた。難治性喘息は喘息発症時にその因子が規定されているのか，環境，罹病期間，治療内容などにより難治性となりうるのか，まだまだ解明されていない点が多い。近年では気道炎症という面で制御性T細胞の解明が急速に進んでおり，難治性喘息の病態，ステロイド依存性のメカニズムなどの解明の糸口となることが期待される。

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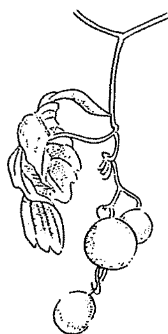

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原　著

小児気管支喘息治療における吸入ステロイド薬中止後の臨床経過の前方視的研究

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【背景・目的】小児気管支喘息治療における吸入ステロイド薬 inhaled corticosteroids (ICS) 治療のステップダウン，中止に関する臨床指標を検討する目的で前方視的観察研究を実施した．

【対象】ICS 100μg/日以下単独で3カ月以上完全コントロールされた患者を対象とした112名の小児．

【方法】メサコリン吸入負荷試験 methacholine challenge test (MCT) を実施し，provocative concentration causing a 20% fall in FEV1 (PC_{20}) が 0.5mg/mL 以上の患者に，中止後7カ月，16カ月，24カ月で評価を行った．

【結果】24カ月まで観察した63名のうち81%は，間欠型以下の症状で推移し，肺機能は有意な変化はなかった．MCT の PC_{20} は，全く発作の無かった群が低下傾向，それ以外では，有意に低下した．ICS 使用期間，血清総 IgE 抗体値で差があった．

【結論】完全コントロールで，MCT の PC_{20} が 0.5mg/mL 以上に改善していれば，中止しても80%以上は安定して経過することがわかった．

Key words: child asthma —— inhaled corticosteroids —— prospective study —— step down

緒　言

吸入ステロイド薬 inhaled corticosteroids (ICS) は現在の気管支喘息治療ガイドラインでの第1選択薬剤であり気道炎症を鎮静化し臨床症状を改善する効果があるが，吸入ステロイド薬を開始して十分な臨床症状のコントロールができた場合に，どのように治療をステップダウンし中止するかという課題がある[1)-3)]．これまで明確な指標がなく行われているが，本研究は，実際に臨床的に行われている方法で治療をステップダウンし，ICS を中止した場合にどのような経過になるかを，ICS 中

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Abbreviations: **BHR** "bronchial hyperresponsiveness", **FEV1%** "percentage of forced expiratory volume in one second", **GINA** "Global Initiative for Asthma", **ICS** "inhaled corticosteroids", **JPGL2008** "Japanese Pediatric Guideline for the Treatment and Management of Asthma 2008", **MCT** "methacholine challenge test", **PC_{20}** "provocative concentration causing a 20% fall in FEV1", **SNPs** "single nucleotide polymorphisms", **%V25** "percentage to predicted maximal expiratory flow in 25% vital capacity", **%V50** "percentage to predicted maximal expiratory flow in 50% vital capacity"

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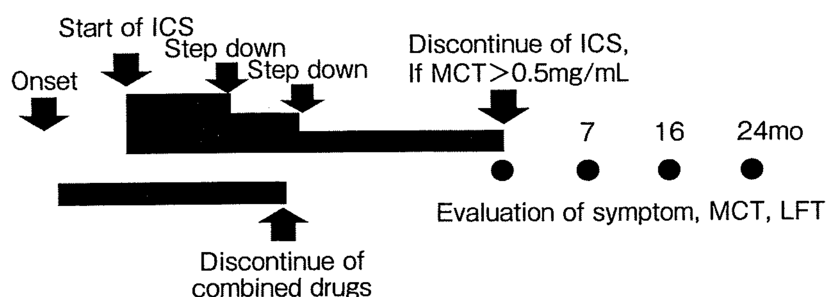

Fig. 1. Observation schedule.
All patients were treated with adequate inhaled corticosteroids (ICS) and step down to 100μg/day and stopped. After stopping ICS, clinical symptom and bronchial hyperreactivity are evaluated on the 7th, 16th and 24th month.
ICS: inhaled corticosteroids, MCT: methacholine challenge test, LFT: lung function test.

止の時点から前方視的に観察研究を実施し，臨床経過に影響を与える要因の推測をすることを目的に実施した．

対象と方法

対象患者は，平均年齢3.9±2.7歳で小児気管支喘息を発症し，アレルギー専門外来でアレルギー専門医により治療を継続してきた6〜26歳，平均年齢12.9±9.6歳の計112名（男性73名，女性39名）である．一部の成人年齢の患者は，小児喘息として発症し治療を継続しているため採用した．ICSを中止するにあたり次の条件を満たした患者を対象とした．治療開始早期からICSを導入し，小児気管支喘息治療管理ガイドラインの発表後からはガイドラインに沿った治療を行い，環境指導，日常生活指導を適切に行っていること．継続的にICSによる治療を行い，参加時点でICSとしてプロピオン酸フルチカゾン単独で100μg/日以下相当で治療を行い3カ月以上喘息発作がないこと，1秒率(FEV1%)が70%以上あること，今後の治療，検査に同意することを条件とした．対象患者の重症度は喘息を発症してからの経過中最も重症であったときの重症度で評価すると，軽症持続型が56名，中等症持続型が50名，重症持続型が6名であった[4]．

対象患者は，24時間以上ICSを中止してからメサコリン吸入負荷試験methacholine challenge test (MCT)を実施した．MCTでFEV1が20%以上低下したメサコリン吸入薬負荷濃度provocative concentration causing a 20% fall in FEV1 (PC20)が0.5mg/mL以上の場合に患者および保護者にICSを中止する事を説明し同意がとれた場合にICSを中止した．PC20が0.5mg/mL未満の場合は，気道過敏性が十分改善していないという判断でICSによる治療を継続することを勧めた．

ICS中止後は主要評価項目として平均7カ月，16カ月，24カ月時点でのMCTによるPC20と肺機能検査およびその間の臨床症状を測定した．付加的評価項目として，末梢血好酸球数，血清総IgE抗体，ダニ特異IgE抗体測定を行った（**Fig. 1**）．

MCTは，米国胸部疾患学会の方法（ATS法）に従い，メサコリン0.031mg/mL，0.063mg/mL，0.125mg/mL，0.25mg/mL，0.5mg/mL，1mg/mL，2mg/mL，4mg/mL，8mg/mL，16mg/mLの溶液を，LCネブライザー（PARI，松吉医科機器，東京）を使用して圧搾空気5L/min.で2分間吸入し，吸入後に2回スパイロメーター，AS-300（ミナト医科機器，東京）で測定した．PC20の算出は，FEV1が，前値に比べて20%以上低下した場合あるいは明かな喘鳴，呼吸困難がある場合を陽性として判定し，PC20をATS法で採用されている次の方法で計算し，気道過敏性の閾値とした[5]．

PC20＝antilog ［log C1＋(log C2－logC1)(20－R1)/(R2－R1)］

C1＝second-to-last methacholine concentration (concentration preceding C2).

Table 1 Evaluation of clinical course after stopping of inhaled corticosteroid.

Group	Clinical course description
A	No symptom at all
B	Accidental attack less than 3 times/year
C	Intermittent (attack less than 1 time/month)
D	Intermittent + EIA
E	Mild persistent (attack less than 1 time/week)
F	Dropped out
G	Continued ICS, PC_{20} more than 0.5mg/mL
H	Continued ICS, PC_{20} less than 0.5mg/mL

C2 = final concentration of methacholine (concentration resulting in a 20% or greater fall in FEV1)

R1 = percent fall in FEV1 after C1

R2 = percent fall in FEV1 after C2

ICS 中止後の臨床症状の評価は，観察期間中適時行いICS 中止後24カ月を最終評価とした．経過観察中の評価基準は，全く喘息発作，喘鳴がなかった群(A)，通常の生活では，発作，喘鳴がなかったが，アレルゲンとの予期せぬ接触等により発作を年2回以下まで起こした群(B)，年に数回から月に1回未満の喘鳴，小発作を起こす間欠型の群(C)，間欠型で運動誘発喘息を起こす群(D)，月に1回以上小発作が起こるようになった軽症持続型以上の群(E)に分類した．観察開始後の経過を分類するために，ICSを一旦中止したその後に患者の何らかの理由により再開または経過観察ができなくなった群(F)，気道過敏性PC20が0.5 mg/mL以上であったが患者側の何らかの理由でICSを中止しなかった群(G)，気道過敏性が0.5 mg/mL未満でICSを中止しなかった群(H)に分類した(**Table 1**)．経過観察中の明かな増悪は，軽症持続型(E群)以上になった場合として，その診断をした時点からICSによる治療を再開した．

統計解析

臨床経過によるグループ分類と臨床経過，喘息歴，気道過敏性検査等の統計は，t-検定を用いた．

結　果

臨床症状の経過

24カ月間の臨床経過の間で，入院を要する重篤な喘息発作を起こした者は無く，増悪した患者の重症度は軽症持続型までであった．ICS中止24カ月後の臨床症状の評価は，全く喘息発作および喘鳴がなかった群(A)が32例，通常の生活では，発作，喘鳴がなかったが，アレルゲンとの予期せぬ接触等により発作を年2回以下まで起こした群(B)が17例，間欠型の群(C)が2例，間欠型で日常で運動誘発喘息を起こす群(D)が3例，月に1回以上小発作が起こるようになった軽症持続型以上の群(E)が9例，脱落群(F)が11例，気道過敏性PC20が0.5mg/mL以上であったがICSを中止しなかった群(G)が11例，気道過敏性が0.5 mg/mL未満でICSを中止しなかった群(H)が27例であった(**Table 2**)．

予期せぬ発作を起こしたB群の発作の原因は，日常生活では全く発作，喘鳴を経験しなかったが，気管支炎罹患時に喘鳴，小発作を経験した症例，動物に対するアレルギーがあり，飼育している家に行ったときに喘鳴を認めた症例があった．脱落群(F)は，症状が軽症持続型に至らないが，患者の判断あるいは，医師との相談でICSを再開した症例，気道過敏性試験で前回より過敏性が亢進したために患者の判断でICSを開始した症例が含まれる．気道過敏性PC20が0.5mg/mL以上であったがICSを中止しなかった群(G)の理由は，中止時期が季節的あるいは，学校等の事情で中止に至らなかった症例が含まれる．

24カ月まで経過観察を行い，脱落群(F)を除いた評価では，24カ月間間欠型以下で経過したの

Table 2 Clinical course and group detail

Group		Whole	A	B	C	D	E	F	G	H
Number	case	112	32	17	2	3	9	11	11	27
Gender	M : F	75 : 37	23 : 9	12 : 5	2 : 0	1 : 2	7 : 2	7 : 4	6 : 5	17 : 10
Age	year	12.9 ± 9.6	13.3 ± 3.1	10.9 ± 3.7	11.3 ± 1.2	11.6 ± 4.3	10.9 ± 4.0	14.1 ± 5.6	20.2 ± 28.3	11.3 ± 3.7
Onset	year	3.9 ± 2.7	4.2 ± 2.8	3.4 ± 2.7	1.2 ± 0.3	7.7 ± 5.7	3.6 ± 2.0	4.7 ± 2.9	3.5 ± 2.7	3.6 ± 2.4
Severity	Mild	56	18	6	1	1	6	6	5	13
	Moderate	50	13	11	1	2	3	2	5	13
	Severe	6	1	0	0	0	0	3	1	1
Days using ICS	days	1262 ± 942	1507 ± 885*	1229 ± 671*	1416 ± 758*	734 ± 227*	797 ± 432*	1136 ± 774	962 ± 599	1359 ± 1388
Days w/o attack	days	633 ± 467	697 ± 603	598 ± 361	1201 ± 368	690 ± 158	582 ± 192	520 ± 434	520 ± 284	633 ± 494
Total IgE	IU/mL	1021 ± 1705	486 ± 551**	956 ± 1539	1798 ± 1670	1352 ± 1061	781 ± 820	502 ± 774	1205 ± 2165	1856 ± 2670**
HDM IgE	IU/mL	63.7 ± 36.7	51.6 ± 36.0	56.1 ± 39.9	55.3 ± 8.1	74.1 ± 44.9	69.3 ± 33.9	54.5 ± 38.1	75.2 ± 37.0	80.3 ± 32.0
Eosinophil	%	6.7 ± 4.4	4.8 ± 2.5#	6.0 ± 5.1	10.7 ± 5.4	6.2 ± 8.4	10.4 ± 5.6#	5.6 ± 5.6	6.2 ± 2.5	8.5 ± 4.0#

All patients have been classified into 8 groups according to their symptom after stopping of inhaled corticosteroid.
Group description refers Table 1.
Average ± SD. ICS: inhaled corticosteroids, HDM: house dust mite,
Days w/o attack: Number of attack free days from latest attack until stopping of inhaled corticosteroid.
*: Days using ICS: group A + B + C versus D + E: $p = 0.001$
**: Total IgE antibody: group A versus H: $p = 0.016$
#: Eosinophil: group A versus E + H: $p < 0.001$

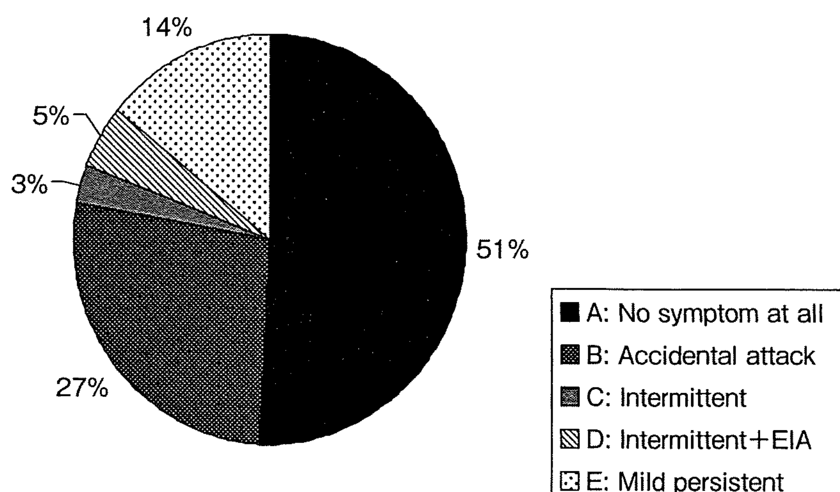

Fig. 2. Clinical course at 24 months after stopping of inhaled corticosteroid.
Group description refers Table 1.
Sixty-three out of 112 patients have been evaluated at 24 months after stopping of inhaled corticosteroids. 81% remains as no-symptom or symptom less than 1 time/month.

は，A～C群の81%であった（**Fig. 2**）．

各群の喘息歴の相違

はじめに，気道過敏性を測定し0.5mg/mL以上の患者でICSを中止し，その後24カ月までの臨床症状の評価でA～H群に分類した．ICSを中止するまでの臨床経過，喘息歴を比較しその後の臨床症状の違いに影響する要因の有無について検討した（**Table 2**）．

性別は，小児のこの年齢層では，一般的に男児が多く，対象者も同じ傾向であった．ICSの中止を検討した今回の経過観察開始時点の年齢，発症年齢は，各群とも有意差はなかった．経過中の最大重症度の比較では，各群に差はなかった．ICSを開始してから，今回中止するまでの使用期間は，中止後の発作のないあるいは少ないA, B, C群では1200日以上であり，悪化したD, E群では平均800日以下であり有意差（$p=0.001$）があった．今回ICSを中止するまでの無発作期間は633日で各群での差はなかった．

各群の検査値の相違

喘息歴と同様に，各群の血液検査を比較した．血清総IgE抗体値は，中止後全く発作の無いA群が486±551IU/mL，気道過敏性が0.5mg/mL以下であったH群が1856±2670IU/mLでありA群の平均は3分の1以下であった（p=0.016）．ダニ特異的IgE抗体値は，グループ間で差はなくいずれも高値であった．末梢血好酸球数は，全く発作のないA群が他の群に比較して低い傾向にあり，E+H群との比較では有意($p<0.001$)に低かった．

各群の肺機能検査の相違

A～Hの全ての群において，ICSを中止あるいは中止しようとした時点での，FEV1%は，平均85%以上あり，標準偏差のばらつきも10%以内で少なかった．また，24カ月まで経過を追った群でもほとんど変化がなかった．%V50は，平均は80%を上回っているが，ばらつきが20%前後あり低い症例もあったが，各群間の差はなかった．%V25は，平均は70%を上回っているが，ばらつきが20%前後あり低い症例があり，A，B群に比べ，気道過敏性が亢進しているH群では低い傾向があった．%V50, %V25ともに24カ月の経過中有意な変化はなかった（**Table 3**）．

各群の気道過敏性の相違

ICSを中止あるいは中止しようとした時点での112名全ての患者のPC_{20}の値を過去の最大重症

Table 3 Change of lung function after stopping of inhaled corticosteroid.

Group			A	B	C	D	E	F	G	H
Number	month	112	32	17	2	3	9	11	11	27
FEV1%	0	87.1 ± 5.44	87.4 ± 4.33	86.6 ± 5.10	93.6 ± 8.06	89.4 ± 6.43	89.2 ± 3.99	88.9 ± 5.02	85.6 ± 7.47	85.7 ± 5.45
	7	87.0 ± 5.88 (59)	87.0 ± 5.48 (29)	86.5 ± 6.61 (16)	88.8 ± 9.90 (2)	86.6 ± 7.60 (3)	84.8 ± 5.12 (5)	91.4 ± 5.18 (4)		
	16	86.8 ± 5.32 (47)	87.2 ± 5.98 (26)	87.0 ± 4.67 (14)	83.0 (1)	84.8 ± 8.49 (2)	85.7 ± 3.14 (3)	85.8 (1)		
	24	88.2 ± 5.46 (38)	89.1 ± 6.24 (21)	87.4 ± 3.92 (14)	85.2 (1)	86.7 ± 9.33 (2)				
%V50	0	89.6 ± 19.9	91.6 ± 16.2	90.5 ± 20.6	109.5 ± 4.95	105.3 ± 28.9	100.1 ± 18.5	86.7 ± 14.2	82.4 ± 20.9	84.0 ± 23.3
	7	88.4 ± 19.3	92.0 ± 19.4	86.0 ± 17.1	80.5 ± 20.5	89.7 ± 35.6	84.0 ± 21.7	77.0 ± 13.2		
	16	89.5 ± 19.1	93.0 ± 22.5	86.4 ± 12.9	71.0	73.5 ± 14.8	90.7 ± 13.4	86.0		
	24	90.8 ± 20.4	96.8 ± 19.0	86.1 ± 19.5	69.0	71.0 ± 29.7				
%V25	0	79.1 ± 23.2	81.0 ± 20.3	79.4 ± 27.4	114.5 ± 21.9	95.7 ± 34.1	89.0 ± 17.0	73.4 ± 20.1	74.5 ± 23.7	72.9 ± 23.4
	7	78.8 ± 25.8	83.7 ± 24.0	73.9 ± 24.3	78.5 ± 36.1	89.7 ± 54.8	67.5 ± 25.3	64.3 ± 12.1		
	16	79.8 ± 23.6	83.4 ± 26.9	77.1 ± 19.3	55.0	64.5 ± 12.0	81.7 ± 21.1	75.0		
	24	84.2 ± 25.0	91.1 ± 25.4	79.0 ± 21.3	59.0	60.5 (1)				

Lung function was measured by spirometer. Group description refers Table 1.
Average ± SD. ( ): patients number who were measured.

Table 4 Change of bronchial hyperreactivity in methacholine after stopping of inhaled corticosteroid.

Group	A	B	C	D	E	F	G	H
N	32	17	2	3	9	11	11	27
0 Mo	7.69 ± 6.66 *	5.78 ± 6.32 *	2.08 ± 0.10 *	5.66 ± 4.59 *	3.69 ± 3.46 *	6.36 ± 5.94	0.83 ± 0.47	0.23 ± 0.10
7 Mo	5.01 ± 5.78 (29)	3.39 ± 5.17 (16)	8.13 ± 11.1 (2)	0.21 ± 0.07 (3)	0.37 ± 0.36 (5)	7.48 ± 8.23 (4)		
16 Mo	7.70 ± 6.22 (29)	3.83 ± 6.32 (15)	6.06 (1)	4.05 ± 4.27 (3)	0.48 ± 0.39 (3)			
24 Mo	5.49 ± 5.78 (21)	4.97 ± 6.27 (14)	5.72 (1)	1.23 ± 1.34 (2)				

(mg/mL)

Group description refers Table 1.
0 month: group A + B versus C + D + E: $p < 0.01$
( ): patients number who were measured.

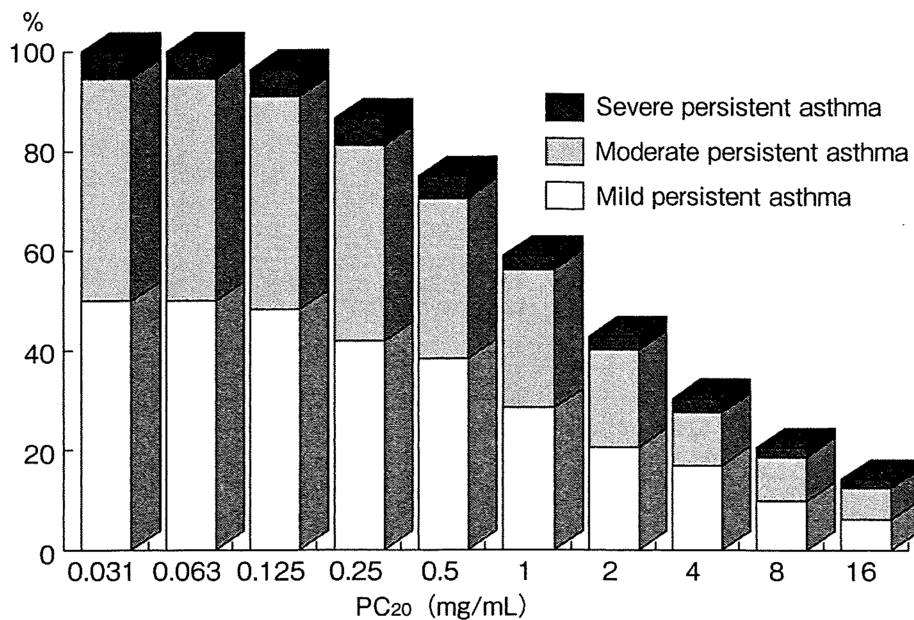

Fig. 3. Cumulative rate of patients with bronchial hyper-reactivity of every stages of MCT.

Each column shows rate of patient who did not react to that concentration of methacholine. Black, grey and white columns show the rate of patients who have had severe, moderate and mild persistent asthma respectively.

度と併せて比較したが重症度とは相関無かった．MCH での PC20 は，全員が 0.063mg/mL 以上であり，それ以上の濃度では線形的に患者が分布していた（**Fig. 3**）．今回の ICS 中止基準として採用した PC20 が 0.5mg/mL 以上の患者は 85 名であった．

ICS を中止あるいは中止しようとした時点での PC20 は，A 群が平均 7.69mg/mL，B 群が平均 5.78 mg/mL でその後に全く発作の無い A 群で気道過敏性が低い傾向があった．A+B 群と C+D+E 群での比較では有意にその後の発作の少ない A+B 群で気道過敏性が低かった（**Table 4**）．

ICS 中止後の A 群の経過は，7 カ月で一時的に有意に亢進し，その後 16 カ月で改善し，24 カ月では中止に比べ亢進していたことがわかった（**Table 4, Fig. 4**）．B 群および C 群も同様に，中止後 7 カ月で一時的に亢進し，16 カ月，24 カ月で改善するも中止時に比べて有意に亢進していた（**Table 4, Fig. 5**）．

考　案

吸入ステロイド薬が多くの喘息患者において症状をコントロールし，気道炎症を鎮静化することがわかり，世界中の喘息治療ガイドラインで第 1 選択薬剤として採用されている．吸入ステロイド薬による治療を開始して，症状のコントロールができているかどうかは，臨床症状による評価，肺機能検査，気道過敏性検査，細胞診，呼気ガス分析などさまざまな方法が提案され治療ガイドラインにも採用されているが，吸入ステロイド薬の減量，中止に関する明確な指標がない．

ガイドラインにおける治療ステップのステップダウン，中止に関して，Global Initiative for Asthma（GINA）では，最低量のコントローラーでコントロールされ一年間症状の再発がない場合に中止できる可能性があるというエビデンス D の記述がある[6]．小児気管支喘息治療・管理ガイドライン 2008（JPGL2008）では，ステップダウンを試みるのは，日常生活に支障がないこと，β2

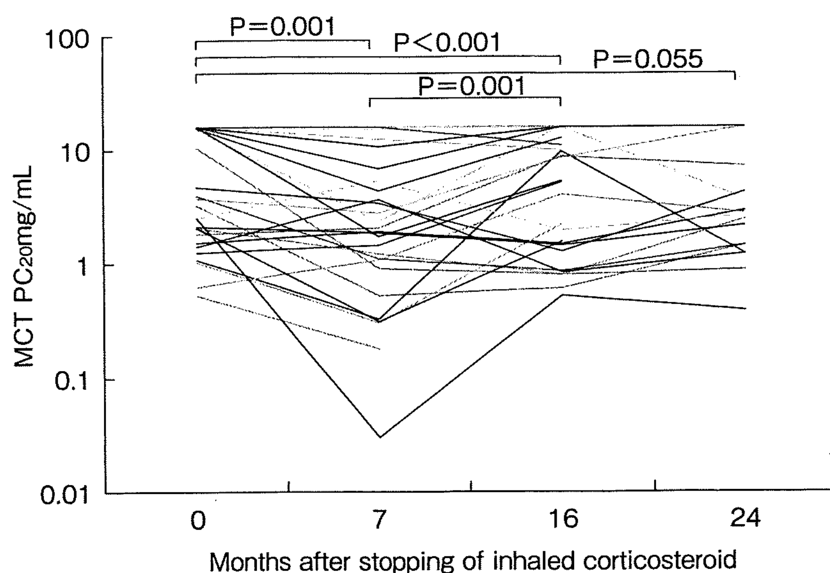

Fig. 4. Change of bronchial hyperreactivity in no symptom group (A) after stopping of inhaled corticosteroid.

Bronchial hyperreactivity was measured by methacholine challenge test (MCT) followed by the method of American Thoracic Society at 0, 7, 16 and 24 months after stopping inhaled corticosteroids.

PC_{20}: provocative concentration causing a 20% fall in FEV1

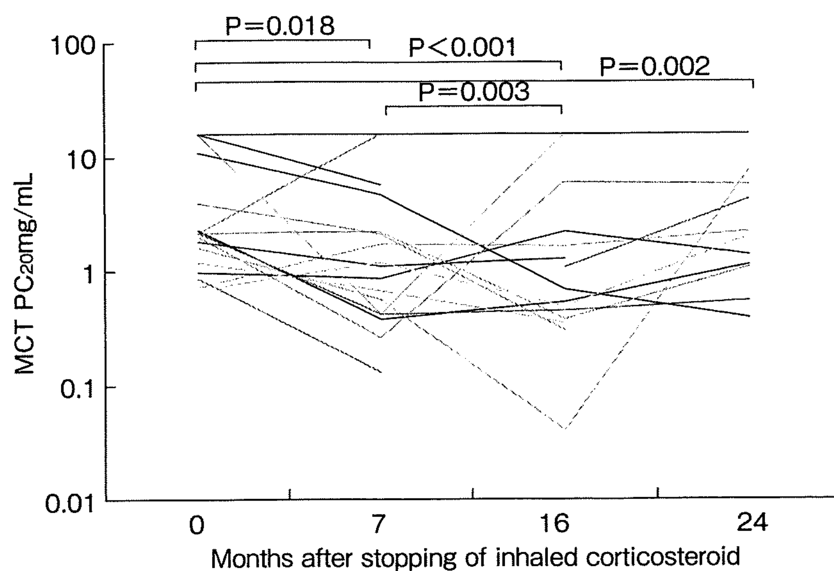

Fig. 5. Change of bronchial hyperreactivity in group B and C after stopping of inhaled corticosteroid.

Bronchial hyperresopnsiveness was measured by methacholine challenge test (MCT) followed by the method of American Thoracic Society at 0, 7, 16 and 24 months after stopping inhaled corticosteroids.

PC_{20}: provocative concentration causing a 20% fall in FEV1

刺激薬の必要性がないコントロールされた状態が3カ月以上続いた場合とし，長期管理薬の中止時期に関しては，一定の基準はないとしながら，最少量の薬物で間欠型以下に見かけ上の重症度がコントロールされ，肺機能も良好であれば中止可能であるとしている[1].

この観察研究では，喘息治療を専門とする医師により治療が導入され，ガイドライン出版後は，ガイドラインに沿った治療を行い，症状がコントロールされ，一定の基準で吸入ステロイド薬を中止した場合に，喘息寛解状態を維持できることを期待して臨床症状を主要観察項目として前方視的に実施し，寛解維持に関連する要因を見いだすことを目的とした．

対象とした患者は，旧国立小児病院，国立成育医療センターアレルギー科の専門外来で経過観察をしていた患者である．喘息発症時期に関しては，乳幼児期の喘息診断であることから，3回以上の喘鳴発作あるいは明かな喘息発作を基準としても，患者申告によるものであり正確には判断できていないが，平均3.9歳であった．発症からICSによる治療開始期間に関しては，要因として検討したかった項目であるが前述の理由で不正確になるため採用しなかった．治療内容は，現在のJPGL 2008に近似した内容で開始され，最も早くICSを導入した患者は1991年から開始していた．ガイドライン出版後は，これに準拠した治療を行い，必要に応じてピークフローモニタリング，喘息教室での患者教育を徹底して実施した．コントローラーのステップダウンは，3カ月以上の無発作を確認しながら，抗アレルギー薬，ロイコトリエン受容体拮抗薬，ICSを担当医の判断で減量し，最終的にICS最小量である100μg/日まで減量した．従って，本研究の対象者は，標準的な治療で十分なコントロールができ，比較的順調にステップダウンできた症例であり，難治例である最重症持続型患者，重症持続型患者や患者教育が不十分あるいは社会的に治療が困難な症例は含まれていない．

吸入ステロイド薬中止にあたっては，3カ月以上の経過観察をしながらステップダウンを行い，最終的にICS100μg/日で3カ月以上症状がないことを第1条件とした．これは，今まで報告が無いことから専門家の経験に基づくものであるが，現在のJPGL2008でも専門家の意見として採用されている．本研究では，対象者に肺機能検査，メサコリン吸入負荷試験を実施して，FEV1%が80%以上，PC_{20}が0.5mg/mL以上改善していることを第2条件とした．気道過敏性試験は，専門施設でしか実施できないのが現状であるが気道過敏性の改善は，JPGL2008においても治療目標に挙げられている項目であり観察項目として採用することとし，本試験のICS中止にあたっては，倫理上明らかに気道過敏性の高い症例については，ICS治療の継続が望ましいという判断をした（H群）．気道過敏性亢進という判断は，米国胸部疾患学会の基準では，PC_{20}が1.0mg/mL以下を中等度過敏性があるとしているが，中止後の経過観察を十分に行えるため0.5mg/mL以上を中止基準として採用した．このため，ICS中止にあたり，患者への説明を行いICS中止を保留することを患者が選択できるようにした（G群）．

ICS中止後の臨床症状の観察は，1～3カ月ごとの外来受診時に行い，MCTは学校スケジュール等との調整を行いながら実施して平均7カ月後，16カ月後，24カ月後となった．

ICS中止後24カ月間の臨床症状からA～E群に分類した．症状の程度は，A群は，基本的なアレルギー疾患に対する生活環境指導を守っていれば，全く問題なく日常生活を送れる状態であり，B群は，同様にしていても，アレルゲン暴露をうけたり，気管支炎により発作を併発してしまった例である．C群は，JPGL2008の重症度分類の間欠型に相当するものであり，E群は，軽症持続型に相当するようにした．途中の脱落例を除くと，ICS中止後81%の患者は，間欠型以下で経過することができている．

24カ月後の臨床経過に影響しているICS中止以前の要因として，ICSの治療期間が長いこと血清総IgE抗体が少ないこと，末梢血好酸球数が低いことが中止後の経過が良いことに影響していた．しかし，再発を予測できるカットオフ値を求

めることはできなかった．また，肺機能検査ではFEV1は，全症例で十分な改善が得られていたが末梢気道の指標とされる%V50，%V25は一部の症例で低値であったが，その後の経過を予測する因子にはならなかった．気道過敏性を示すMCTでは，ICS中止時のPC_{20}が，A+B群でC+D+E群に比べて高値であり，予後判定のカットオフ値は見いだせないがMCTでの気道過敏性が改善している方がその後の経過の良いことがわかった．

ICS中止後の気道過敏性は，A～D群全体では，ICSを中止して7カ月後に一時的に気道過敏性が亢進し，16カ月後には再び改善し，24カ月後には中止時の値より低下した値であった（**Fig. 4，5**）．症状の安定していたA群の気道過敏性の変動は，途中有意差をもって変動しているが24カ月時点では，中止時と比較して有意な低下ではなかった．一方B，C群では24カ月で有意に低下している．この変動は個々の症例では，1～2濃度の変化であり，臨床的にはそれほど大きな変動とは考えにくい．16カ月，24カ月では比較的安定した動きをしめしていることから，7カ月の時点で一旦低下するのは，ICS中止時の気道過敏性検査を，ICS中止後24時間以上あけて実施しているが，検査の時点でまだICSの影響が残っていた可能性がある．また，逆にこうした安定した患者では，低容量のICSが気道過敏性を改善できる可能性を示唆する．

小児喘息において，喘息がコントロールされ治療をステップダウンし中止する報告としては，PEAK studyが報告されている[7]．PEAK studyは，ICS治療期間を2年間と限定しその後1年間の経過を観察し，2年間の投与期間では喘息は改善しないと結論している．本研究とは，研究デザインが全く異なること，年齢層の違い，薬物療法以外治療の違い，評価方法の違いがあるが本研究で示されたことは，小児喘息は，ICSを使用し，症状を完全にコントロールし平均約4年間以上ICS治療を行い，薬物療法以外の発作原因に対する環境指導，生活指導を適切に行いながらステップダウンしてくることで最終的にICSの最少使用量で維持できる症例があること，さらに，一定以上（MCTのPC_{20}が0.5mg/mL以上改善あるいは，それ以上）に気道過敏性が改善している場合には，ICSを中止しても80%以上の患者が間欠型以下で経過することができ，悪化した場合も中等症以上にはいたらないことがわかった．成人での報告では，Tsurikisawaらは，374名の日本人成人喘息の予後を後方視的に検討し，アセチルコリンによる気道過敏性が改善していても重症例では寛解しないことがあることを報告している[8]．

ICSの中止に関しては，本研究でもICS中止あるいは中止しようとした時点での気道過敏性が，ICS治療単独最低用量で3カ月以上症状がなくても，まだまだ過敏性を示す症例が多く存在することから，中止にあたってのさらなる検討が必要である．これまでICSの推奨最低使用量としてプロピオン酸フルチカゾン換算で100μg/日が一般的であるが，それ以下の使用での報告がある．最低使用量を継続することで，より安定した気道過敏性を維持し，quality of lifeが保たれるのであれば患者負担の少ない最低使用量の指針ができることが望まれる．

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PROSPECTIVE OBSERVATIONAL STUDY OF CLINICAL COURSE AFTER STOPPING INHALED CORTICOSTEROIDS IN CHILD ASTHMA

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Background: Prospective study was carried out to observe the clinical course and find out the clinical index to predict the outcome after stopping of inhaled corticosteroids (ICS).
Subjects: One hundred twelve asthmatic children (aged 12.9 ± 9.6 years old) who were totally controlled with 100μg/day of inhaled corticosteroids and have not experienced asthma symptom over 3 months, were enrolled.
Methods: Patients with over PC_{20} 0.5mg/mL of methacholine challenge test (MCT) were recommended to stop ICS therapy. Clinical course, lung function and bronchial hyperreactivity were followed at 7, 16 and 24 months after stopping of ICS.
Results: Eighty-one percent of 63 patients followed up to 24 months run on as symptom less than one time per month without remarkable change of lung function. PC_{20} in group of no asthma symptom during 24 months (group A) decreased weakly. That of other groups decreased remarkably. Days using ICS and plasma IgE antibody at stopping of ICS were different in clinical course at 24 months after stopping ICS.
Conclusion: Over 80% of totally controlled asthmatic children with MCT PC_{20} over 0.5mg/mL could stop ICS therapy and run on without relapse.

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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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[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.

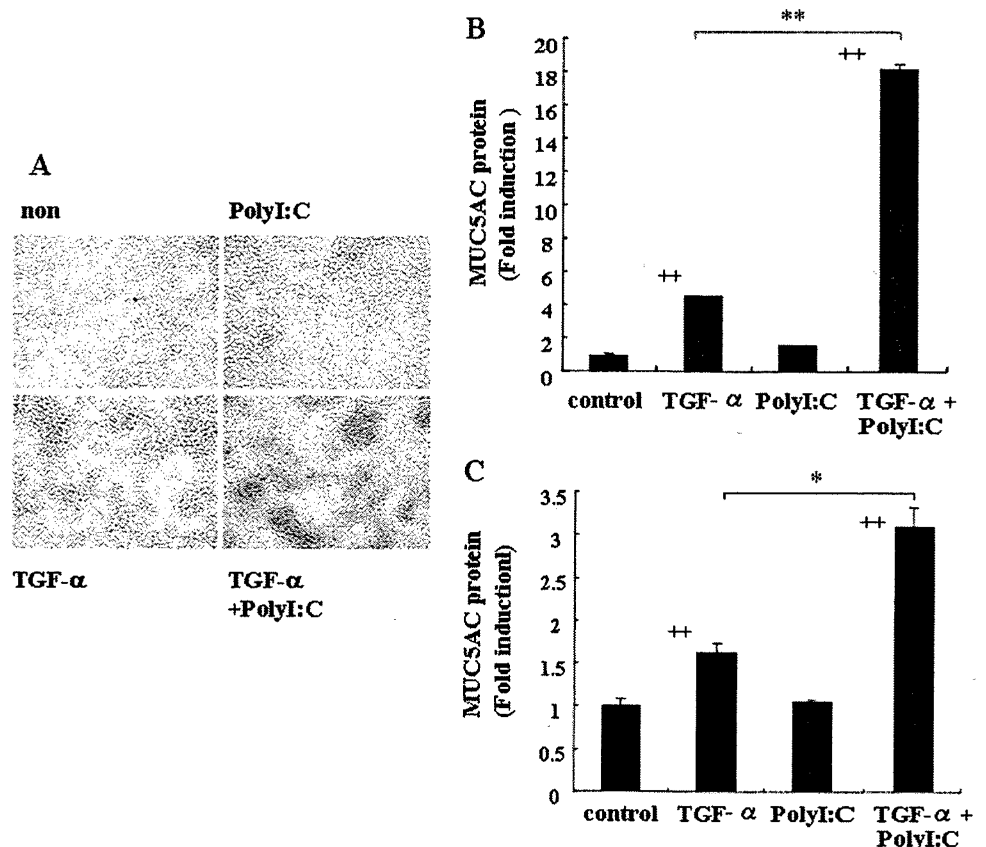

FIGURE 1. Synergistic effect of poly(I:C) and TGF-α on MUC5AC production. *A*, AB-PAS staining of NCI-H292 cells for identification of mucus glycoconjugates. Incubation with poly(I:C) (25 μg/ml) and TGF-α (4 ng/ml) for 24 h increased positive staining. Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced 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 nonstimulated control cells. *, $p < 0.05$ and **, $p < 0.01$.

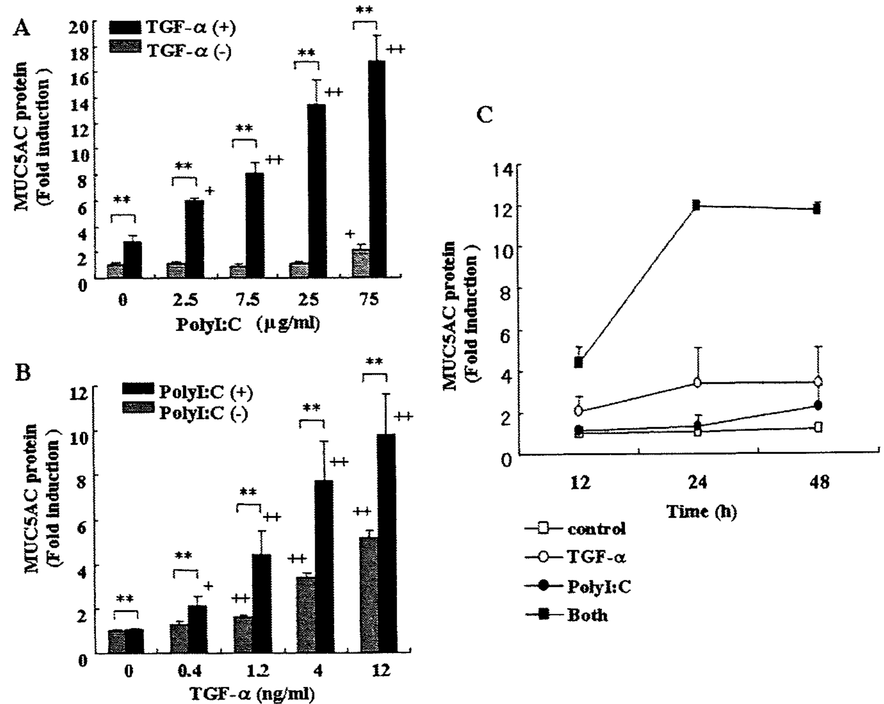

FIGURE 2. Dose responsiveness and time course of MUC5AC protein production. *A*, Effect of the poly(I:C) concentration on TGF-α (4 ng/ml)-induced MUC5AC mucin production in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF-α for 24 h. *B*, Effect of the TGF-α concentration on poly(I:C)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

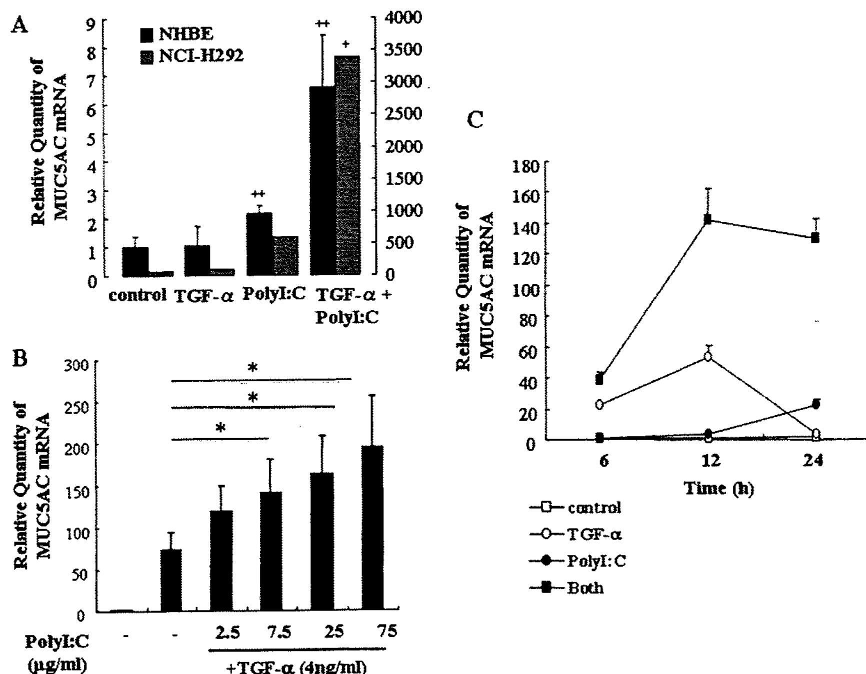

FIGURE 3. Dose responsiveness and time course of MUC5AC mRNA expression. *A*, Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced MUC5AC gene expression in NCI-H292 cells and NHBE cells. Cells were incubated with poly(I:C) and TGF-α for 24 h. Data are presented as the fold induction over the level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

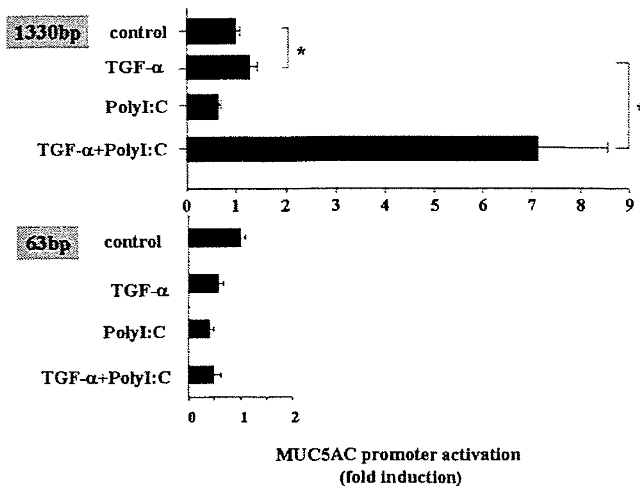

FIGURE 4. Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced *trans*-activation of the full-length (−1330) and the short-length (−63) MUC5AC promoter in NCI-H292 cells ($n = 6$). Data are shown as means ± SD. *, $p < 0.05$.

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

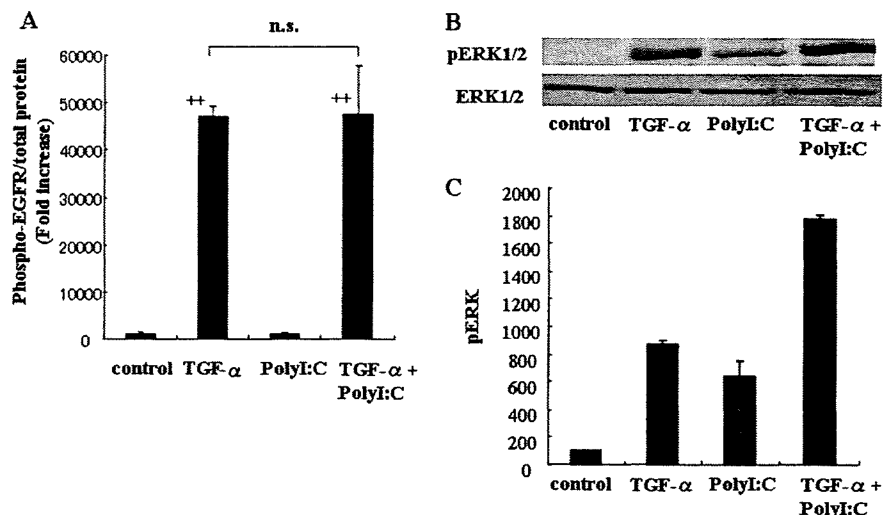

FIGURE 5. *A*, Relative phosphorylation of EGFR by poly(I:C) and TGF-α in NCI-H292 cells. Proteins extracted from samples collected were tested for the presence of phosphorylated EGFR by a Bio-Plex phosphoprotein assay kit using the Bio-Rad Luminex machine. The values plotted show the ratios of phosphorylated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

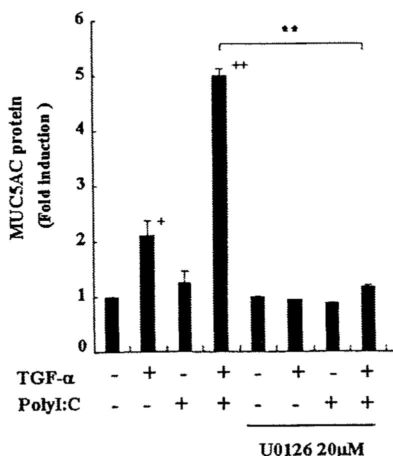

FIGURE 6. Effect of U0126 on poly(I:C)- and TGF-α-induced MUC5AC mucin production in NCI-H292 cells. Cells were preincubated with U0126 (a specific inhibitor of MEK1/2) at 20 μM (15, 16) for 1 h before adding poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml), and cells were analyzed 24 h after stimulation ($n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

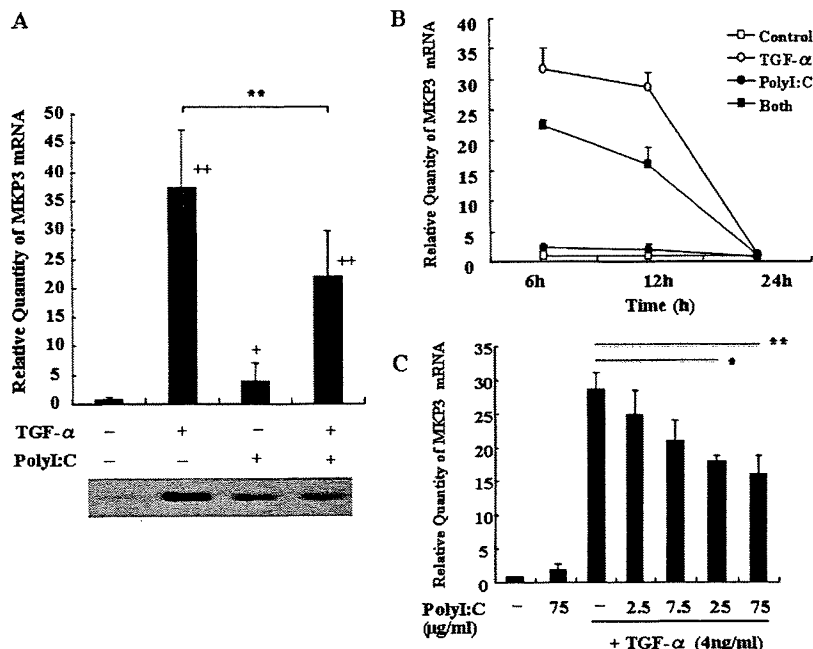

FIGURE 7. *A, Upper*, Effect of poly(I:C) (25 μg/ml) on TGF-α (4 ng/ml)-induced MKP3 mRNA expression in NCI-H292 cells ($n = 8$). *Lower*, To verify MKP3 expression in NCI-H292 cells, expression of MKP3 after stimulation with poly(I:C) (25 mg/ml) and TGF-α (4 ng/ml) was examined by standard RT-PCR. In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

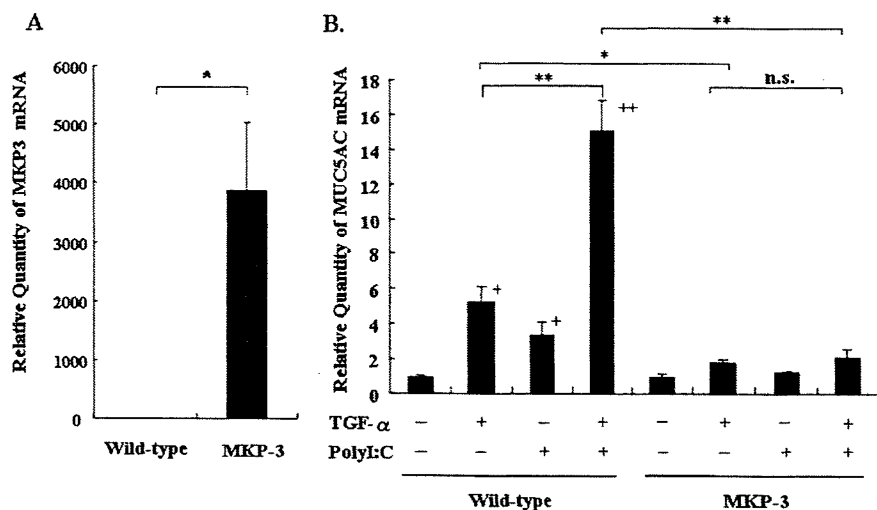

FIGURE 8. *A*, Effect of a MKP3 expression plasmid cloned into the pAcGFP1-C1 vector on MUC5AC mRNA expression in NCI-H292 cells. The level of MKP3 mRNA was enhanced significantly in cells transfected with the MKP3 expression plasmid. *B*, Enhanced expression of MUC5AC mRNA was noted in wild-type cells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

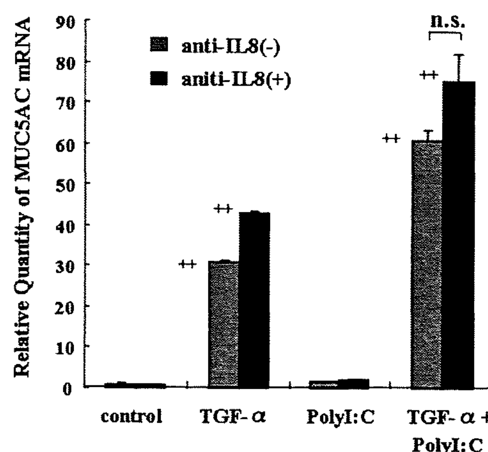

FIGURE 9. Effect of the anti-IL-8 Ab on poly(I:C) (75 μg/ml) and TGF-α (4 ng/ml)-induced MUC5AC mRNA expression in NCI-H292 cells ($n = 4$). Data are shown as means ± SD. ++, $p < 0.01$ compared with nonstimulated control cells.