

Increased activation of fibrocytes in patients with chronic obstructive asthma through an epidermal growth factor receptor-dependent pathway

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Background: Fibrocytes are circulating progenitor cells that are increased in asthmatic patients with chronic obstructive asthma (COA) and rapid decrease in lung function. Fibrocytes from patients with COA have a greater capacity for proliferation and differentiation.

Objective: We investigated whether epidermal growth factor receptor (EGFR) activation mediated the proliferation of fibrocytes in patients with COA and whether oxidative stress was involved in this activation.

Methods: Circulating fibrocytes from nonadherent non-T-cell mononuclear cell fractions from healthy subjects, asthmatic patients with normal pulmonary function, and patients with COA were determined by using flow cytometric coexpression of collagen I, CD45, and CD34 or EGFR or a disintegrin and metalloprotease domain 17 and placed in culture.

Results: Expression of EGFR was increased in fibrocytes from patients with COA compared with that seen in patients with NPF. AG1478 and gefitinib, inhibitors of EGFR tyrosine kinase, reduced fibrocyte proliferation and myofibroblast transformation. Increased expression of EGFR and fibrocyte proliferation and transformation were induced by hydrogen peroxide, and these effects were inhibited by N-acetylcysteine.

Conclusions: Enhanced fibrocyte proliferation and transformation found in patients with COA might be mediated through an oxidant-sensitive EGFR-dependent pathway. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Fibrocytes, epidermal growth factor receptor, a disintegrin and metalloprotease domain 17, asthma, oxidative stress

Abbreviations used

ADAM: A disintegrin and metalloprotease domain
 α -SMA: α -Smooth muscle actin
 COA: Chronic obstructive asthma
 EGF: Epidermal growth factor
 EGFR: Epidermal growth factor receptor
 FITC: Fluorescein isothiocyanate
 HB-EGF: Heparin-binding EGF-like growth factor
 H₂O₂: Hydrogen peroxide
 IMDM: Iscove modified Dulbecco medium
 NAC: N-acetylcysteine
 NANT: Nonadherent non-T
 NPF: Normal pulmonary function
 PE: Phycoerythrin
 PerCp: Peridinin chlorophyll-protein complex
 siRNA: Small interfering RNA
 TAPI-1: TNF protease inhibitor 1

Asthma is characterized by chronic persistent airway inflammation and structural remodeling changes that could lead to persistent airflow obstruction associated with chronic obstructive asthma (COA).^{1,2} Airway wall remodeling is characterized by subepithelial fibrosis with extracellular matrix deposition of collagen, tenascin, fibronectin, and proteoglycans and an increase in airway smooth muscle mass.³⁻⁵ We have shown that there are increased numbers of the circulating progenitor fibrocyte expressing CD34, CD45, and collagen I in patients with COA compared with numbers seen in asthmatic patients with normal pulmonary function (NPF).⁶ Fibrocytes can act as precursors of bronchial myofibroblasts after allergen challenge⁷ and could localize within the proximity of the thickened subbasement membrane and the airway smooth muscle bundle in patients with asthma.^{8,9}

Fibrocytes from asthmatic patients with COA also demonstrated a higher proliferative potential and increased differentiability into α -smooth muscle actin (α -SMA)-positive myofibroblasts.⁶ The mechanisms underlying fibrocyte proliferation and transformation are unclear. Epidermal growth factor receptor (EGFR) signaling might regulate a variety of physiologic responses, including proliferation, differentiation, motility, and survival.^{10,11} EGFR expression is increased in the epithelium of asthmatic patients, and activation of EGFR contributes to airway epithelial repair.^{12,13} EGFR ligands might bind to EGFRs in the extracellular domain, leading to ligand-dependent EGFR tyrosine phosphorylation. Alternatively, EGFR tyrosine phosphorylation can occur through ligand-independent EGFR phosphorylation,

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such as in response to oxidative stress.¹⁴ Reactive oxygen species within cells can act as second messengers in intracellular signaling cascades to transactivate EGFRs.^{15,16} Oxidative stress is increased in asthmatic patients depending on asthma severity.¹⁷ All members of the epidermal growth factor (EGF) family are type I transmembrane proteins and are expressed on cell surfaces that can be cleaved by cell-surface proteases,¹⁸ such as a disintegrin and metalloproteases (ADAMs). ADAM17 has been linked to increased EGFR-mediated cell proliferation and transformation^{19,20} and might be a regulator of EGFR activity.²¹ Reactive oxygen species might also directly induce the transcription of the EGFR ligand heparin-binding EGF-like growth factor (HB-EGF).^{22,23}

We hypothesized that activation of the EGFR pathway in fibrocytes from patients with COA contributes to their higher proliferative capacity. Because oxidative stress can lead to EGFR transactivation,^{24,25} we examined the role of oxidative stress relevant to the EGFR pathway in the proliferation and transformation of fibrocytes from patients with COA. In addition, we examined the expression and role of ADAM17.

METHODS

Patients

Thirty-two asthmatic patients with either NPF or with persistent COA were recruited from the outpatient clinics of Chang Gung Memorial Hospital (Table I). Patients with COA had a postbronchodilator FEV₁ of 60% of predicted value or less and had a rapid decrease in FEV₁ of 20% or greater over a 5-year follow-up ($32.8\% \pm 2.5\%$, $n = 16$). Asthmatic patients with NPF had prebronchodilator and postbronchodilator FEV₁ of greater than 80% of predicted value, and the decreasing rate of FEV₁ over 5 years was $-0.2\% \pm 1.8\%$ ($n = 16$). This study was approved by the Chang Gung Hospital Ethics Committee, and all subjects provided informed consent.

Isolation of circulating fibrocytes

PBMCs were separated from 100 mL of blood by using Ficoll-Hypaque density gradient centrifugation, as described previously.^{6,26} The nonadherent mononuclear cell fraction was separated by means of adherence and centrifugation and resuspended in Iscove modified Dulbecco medium (IMDM) and then mixed with 100 μ L of sheep red blood cells and depleted of E-rosette-forming cells by means of a second Ficoll-Hypaque centrifugation. T cells were further depleted by using magnetic beads coated with anti-CD3 mAb such that T cells were less than 1% of nonadherent non-T (NANT) cells.

EGFR and ADAM17 expression in circulating fibrocytes

Fibrocytes were confirmed by means of flow cytometry with both phycoerythrin (PE)-conjugated anti-CD34 and anti-CD45-peridinin chlorophyll-protein complex (PerCp) polyclonal antibodies (Becton Dickinson, San Jose, Calif) and fluorescein isothiocyanate (FITC)-conjugated anti-collagen I antibodies (Chemicon International, Temecula, Calif).^{6,7} Freshly isolated peripheral blood NANT cells (1×10^5 cells/mL) or NANT cells cultured for 24 hours were fixed with 4% paraformaldehyde and then washed twice with PBS (pH 7.0). The cell pellet was resuspended and immersed in 150 μ L of FACS permeabilizing solution (Becton Dickinson) and then incubated with mouse anti-human collagen I antibodies (Chemicon International), followed by anti-mouse FITC. Cell pellets were incubated with anti-CD34-PE and anti-CD45-PerCp antibodies. These CD45⁺ and collagen I⁺ cells are fibrocytes, as previously reported.⁷ To identify the expression of EGFR and ADAM17 on circulating fibrocytes, the NANT cells were fixed and incubated with mouse anti-human collagen I antibodies (Chemicon International), followed by anti-mouse PE. Then the cell pellet was incubated with anti-EGFR-FITC (Santa Cruz Biotechnology, Santa Cruz, Calif) and anti-CD45-PerCp

antibodies or anti-ADAM17-PE (R&D Systems, Minneapolis, Minn) and anti-CD45-PerCp antibodies. The cell suspension was examined with a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson, Mountain View, Calif). Compensation settings were established with CalBrite beads (Becton Dickinson Instrument Systems).

Immunofluorescence labeling

NANT cells were incubated with anti-phosphorylated EGFR rabbit antibody (Santa Cruz Biotechnology; 1:100) and then with anti-rabbit-cy2 antibodies (Chemicon; 1:250). Next, the cells were incubated with anti-CD34-PE (Becton Dickinson) and anti-CD45-PerCp (Becton Dickinson) and examined with a confocal laser scanning microscope (Leica TCS 4D, Wetzlar, Germany).

Culture of fibrocytes

NANT cells (5×10^5) were incubated with 1 mL of IMDM, 1% BSA, stem cell factor (50 ng/mL), 30% FCS, and AG1478 (Calbiochem; 0, 1, and 10 μ M/L) for 14 days to study the role of EGFR. We examined the effect of hydrogen peroxide (H₂O₂; 0.1 and 0.3 mmol/L) on the expression of EGFR on fibrocytes in the presence or absence of N-acetylcysteine (NAC; 10 mmol/L) for 24 hours. NANT cells (5×10^5) from asthmatic patients with NPF were treated with H₂O₂ (0.1 and 0.3 mmol/L) in the presence or absence of NAC (10 mmol/L) or gefitinib (1 μ M/L) for 14 days to determine whether the effect of oxidative stress on EGFR expression affected the proliferation and transformation of fibrocytes. NANT cells of asthmatic patients with COA were cultured for 14 days in the presence or absence of NAC (10 mmol/L) or of TNF protease inhibitor 1 (TAPI-1; Santa Cruz Biotechnology), an inhibitor of ADAM17 (1, 5, and 25 μ M/L). The number of fibrocytes after culture was determined by means of triple staining with anti-CD34, anti-CD45, and anti-collagen I antibodies and myofibroblasts by using anti-human α -SMA antibodies and analyzed by means of flow cytometry.

Role of ADAM17 in fibrocytes from patients with COA

We used small interfering RNA (siRNA) to knock down the expression of ADAM17. siRNAs (100 nmol/L) targeting ADAM17 (siADAM17) were transfected into NANT cells of patients with COA by using Lipofectamine reagents. Scrambled siRNAs were used as a negative control. The sequences of ADAM17 siRNA were target plus smart pool and were as follows: GAAGAACACGUGUAAAUUA, GCACAAAGAAUUAUGGUAA, UAU GGGAAACUCUUGGAUUA, and GGAAAUUGUCAUGUAUCC. After 48 hours, the cultured cells were assessed for the expression of ADAM17 in CD45⁺/collagen I⁺ fibrocytes by means of flow cytometry. Cells were also incubated in IMDM, 1% BSA, stem cell factor, and 30% FCS for 14 days.

Data analysis

Data are reported as means $\pm$ SEMs. Nonparametric statistical analyses were used with Kruskal-Wallis analysis, and subsequent analysis was performed with the Mann-Whitney *U* test. The null hypothesis was rejected at a *P* value of less than .05.

RESULTS

Increased expression of EGFR in circulating fibrocytes of patients with COA

We defined fibrocytes on the basis of concomitant expression of CD34⁺, CD45⁺, and collagen I⁺ cells in NANT cell fractions.⁶ The percentage of circulating fibrocytes was significantly increased in patients with COA compared with that seen in asthmatic patients with NPF and healthy subjects (Table I). We gated CD45⁺ fibrocytes and then examined for dual expression of collagen I and EGFR (Fig 1, A). We identified a population of CD45⁺/collagen I⁺/EGFR⁺ fibrocytes with the highest expression of EGFR in

TABLE I. Clinical characteristics of subjects and fibrocyte numbers

	Healthy subjects (n = 14)	Asthmatic patients with NPF (n = 16)	Patients with COA* (n = 16)	P value
Age (y)	45.1 ± 3.9	49.7 ± 3.3	56.1 ± 3.1	.098†
Sex (male/female)	7/7	6/10	6/10	.731‡
FVC (L)	3.2 ± 0.2	3.1 ± 0.1	1.8 ± 0.1	<.0001†
FVC (% predicted)	97.4 ± 4.2	91.4 ± 3.6	52.1 ± 3.9	<.0001†
FEV ₁ (L)	2.7 ± 0.2	2.5 ± 0.1	1.2 ± 0.1	<.0001†
FEV ₁ (% predicted)	94.1 ± 4.5	88.5 ± 2.9	44.2 ± 3.1	<.0001†
FEV ₁ /FVC ratio (%)	85.6 ± 1.2	81.6 ± 1.2	63.1 ± 3.0	<.0001†
FEV ₁ reversibility (%)	4.1 ± 0.7	18.5 ± 2.0	19.7 ± 1.8	
Decrease in FEV ₁ over 5 y (mL)§	ND	−0.7 ± 8.8	111.8 ± 14.8#	
PC ₂₀ (mg/mL)	>25	5.5 ± 0.7	1.5 ± 0.5#	
Atopic status (no.)	0	9	12	
Serum IgE (IU/mL)		386.0 ± 118.8	509.3 ± 147.2	
Fibrocytes in NANT cells (%)	3.0 ± 0.6	3.3 ± 0.4	23.4 ± 2.0	<.0001†
Medication over previous year				
Fluticasone alone (no.)	0	9	0	
Fluticasone with salmeterol (no.)	0	7	16	
Daily oral corticosteroids¶ (n)	0	0	5	

Data are shown as means ± SEMs.

FVC, Forced vital capacity; ND, not done.

*Three were exsmokers, each with a 10-year pack history, and had not smoked for 15 years.

†P values are analyzed among 3 different groups by using the Kruskal-Wallis test.

‡Analyzed by using the χ^2 test.§Measured as the FEV₁ at entry (year 1) minus the FEV₁ at year 5.

||Measured 5 years ago.

¶Daily dose of prednisolone was 5 to 10 mg/d.

#P < .001 compared with asthmatic patients with NPF.

fibrocytes from patients with COA compared with those in asthmatic patients with NPF and healthy subjects (Fig 1, B). Mean fluorescence intensity of EGFR expression on the fibrocytes was also increased in patients with COA compared with that seen in asthmatic patients with NPF or healthy subjects (Fig 1, B). Confocal image analysis also revealed an increased number of cells expressing phosphorylated EGFR in fibrocytes from patients with COA compared with those seen in asthmatic patients with NPF (Fig 1, C), indicating increased numbers of activated EGFR⁺ fibrocytes. In addition, there was increased expression of the EGFR family (human epidermal receptors 1 [EGFR], 2, and 3) in fibrocytes from patients with COA but not in fibrocytes from patients with NPF (Fig 1, D). There was a significant correlation between the number of circulating EGFR⁺ fibrocytes in the peripheral blood of patients with COA and the slope of the yearly decrease in FEV₁ ($r = -0.697$, $n = 16$, $P < .01$).

Inhibition of EGFR blocks proliferation of fibrocytes from patients with COA

After 14 days, spindle-shaped myofibroblasts, as confirmed by means of positive staining with α -SMA antibody, were observed, but this differentiation was blocked by the EGFR tyrosine kinase inhibitor AG1478 (Fig 2, A). AG1478 dose-dependently reduced the numbers of myofibroblasts obtained from asthmatic patients with COA. AG1478 had no effect on myofibroblasts grown from NANT cells of healthy subjects or asthmatic patients with NPF (Fig 2, B).

Another EGFR tyrosine kinase inhibitor, gefitinib (1 μ mol/L), also blocked the proliferation of fibrocytes and myofibroblast transformation from patients with COA (Fig 2, C).

Role of ADAM17 underlying EGFR activation in fibrocyte proliferation from patients with COA

We analyzed the dual expression of collagen I and ADAM17 on gated CD45⁺ cells using flow cytometry (Fig 3, A). There was a greater percentage of ADAM17⁺ fibrocytes and greater intensity of ADAM17 expression in NANT cells in patients with COA (Fig 3, B). We then examined whether the upregulated ADAM17 in patients with COA could serve as ectodomain shedding and whether potential ligands for EGFR activation mediated the enhanced proliferative capacity of fibrocytes. Incubation with TAPI-1 (1–25 μ mol/L), a broad-spectrum inhibitor of matrix metalloproteinases and ADAM proteins, resulted in an inhibition of fibrocyte proliferation and α -SMA⁺ myofibroblast transformation in patients with COA but not in patients with NPF, while maintaining the percentage of α -SMA⁺ myofibroblasts (Fig 3, C). The inhibitory effect of TAPI-1 was similar to that elicited by using the EGFR inhibitor gefitinib, suggesting that metalloproteinase activity is required for proliferative capacity of fibrocytes.

We used siRNA to knock down the number of fibrocytes expressing ADAM17 by 41.5% ± 4.1% in patients with COA, which resulted in a dramatic decrease of the highly proliferative capacity of fibrocytes with a concomitant reduction in myofibroblasts (Fig 4).

Role of oxidative stress underlying EGFR activation in fibrocytes from patients with COA

We examined the effect of the antioxidant NAC (10 mmol/L) on NANT cells of asthmatic patients with COA. The expression of EGFR and ADAM17 was significantly downregulated after a

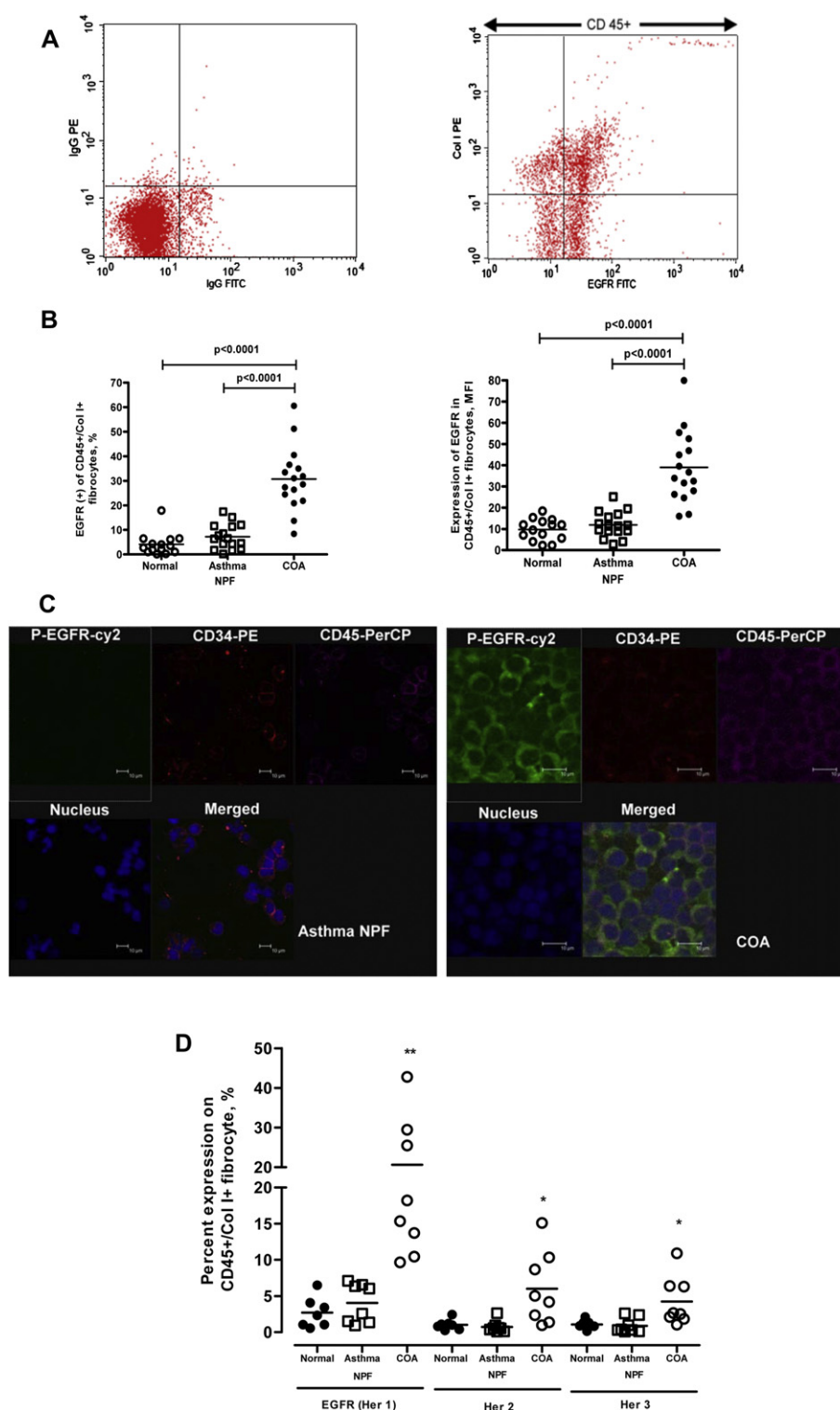


FIG 1. **A**, Flow cytometric analysis of NANT cells immunostained for collagen I, CD45, and EGFR. **B**, Percentages of CD45⁺/collagen I⁺/EGFR⁺ fibrocytes and EGFR expression in CD45⁺/collagen I⁺ as mean fluorescence intensity. Horizontal lines are mean values. **C**, Confocal image analysis of phosphorylated EGFR in CD34⁺ and CD45⁺ fibrocytes. **D**, Individual percentage of fibrocytes expressing human epidermal receptors (Her) 1, 2, and 3. * $P < .05$ and ** $P < .01$ compared with healthy subjects.

24-hour incubation with NAC (Fig 5, A and B). NAC decreased fibrocyte and myofibroblast numbers by half after 14 days of culture (Fig 5, C and D).

H₂O₂ (0.3 mmol/L) increased the expression of EGFR by approximately 2-fold and that of ADAM17 by 2.3-fold on fibrocytes isolated from asthmatic patients with NPF after 24 hours (Fig 6, A

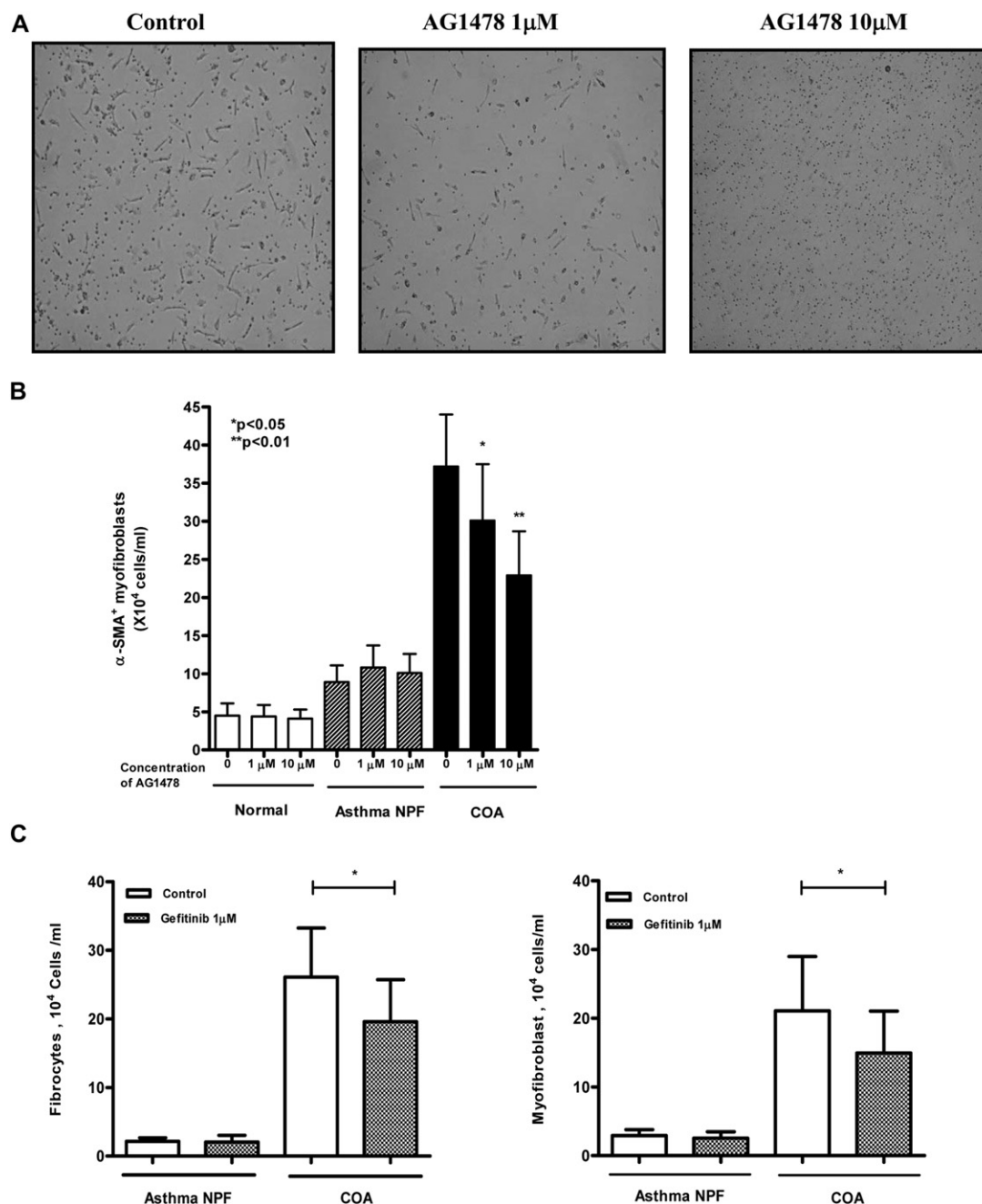


FIG 2. A, Spindle-shaped myofibroblasts from patients with COA receiving AG1478 ($\times 200$ magnification). B, Effect of the EGFR tyrosine kinase inhibitor AG1478 on the α -SMA⁺ myofibroblasts from healthy subjects ($n = 9$) or asthmatic patients with NPF ($n = 12$) or COA ($n = 12$). * $P < .05$ and ** $P < .01$ compared without AG1478. C, Effect of gefitinib (1 μ mol/L) on fibrocytes and myofibroblasts from asthmatic patients with NPF ($n = 6$) and COA ($n = 8$). * $P < .05$.

and B). NAC (10 mmol/L) did not have any effect on baseline expression of EGFR or ADAM17 but abrogated the effect of H₂O₂ (Fig 6, A and B). Next, NANT cells of asthmatic patients with NPF were treated with H₂O₂ (0.1 and 0.3 mmol/L) for 14 days. H₂O₂ increased fibrocyte proliferation and transformation into myofibroblasts (Fig 6, C and D). NAC did not affect baseline fibrocyte proliferation or myofibroblast transformation but almost completely inhibited the effect of H₂O₂ (Fig 6, C and D). The increased fibrocyte proliferation or myofibroblast transformation

induced by H₂O₂ (0.3 mmol/L) was also partly inhibited by gefitinib (Fig 6, C and D).

DISCUSSION

Major findings

We demonstrate that the EGFR pathway of activation underlies the increased proliferation and differentiation of fibrocytes from patients with COA, as demonstrated by inhibition of EGFR

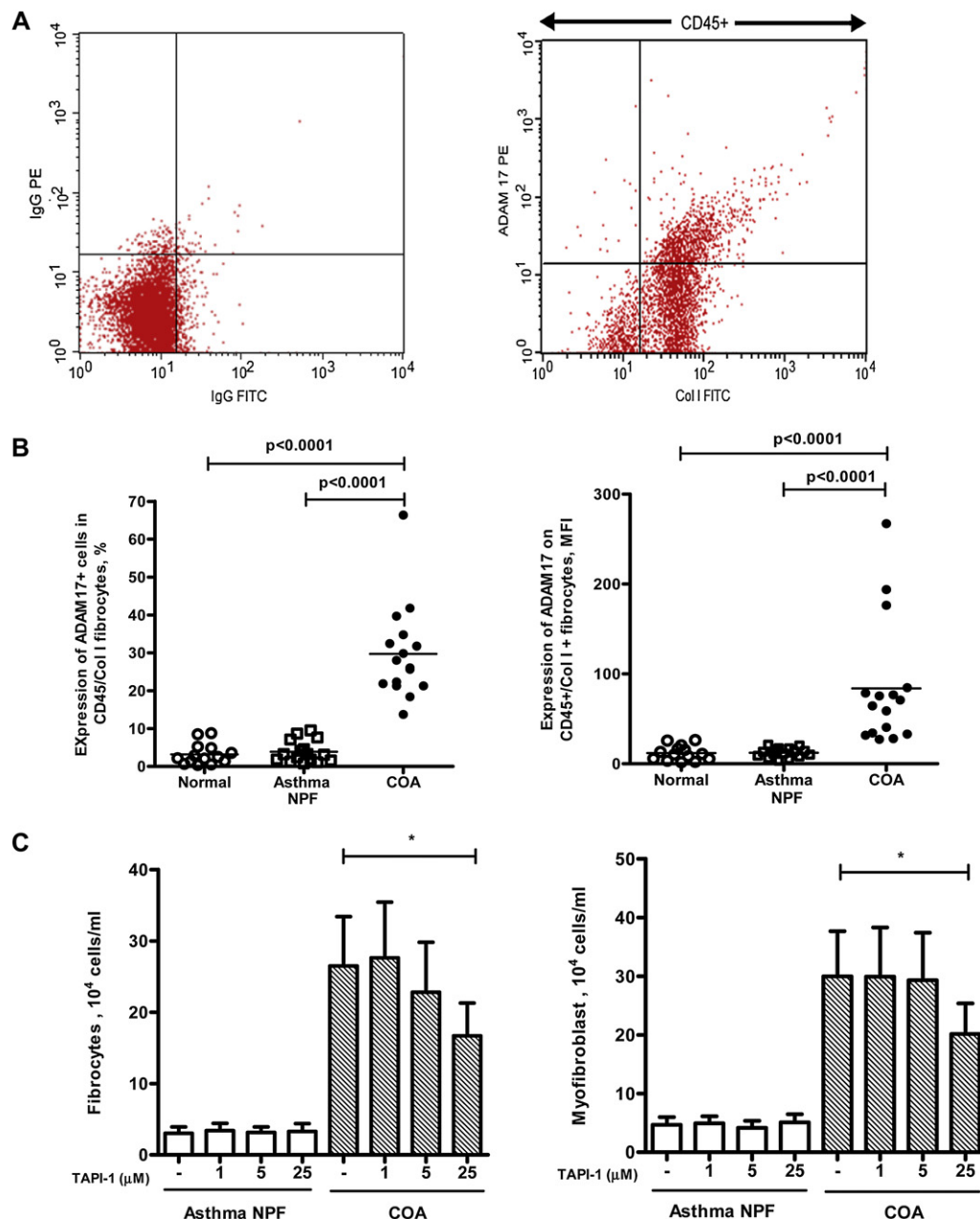


FIG 3. A, Example of flow cytometric analysis of ADAM17 and collagen I expression on CD45⁺ cells. B, CD45⁺/collagen I⁺/ADAM 17⁺ fibrocytes (left) and ADAM17 activity as mean fluorescence intensity (right) in healthy subjects (n = 14) or asthmatic patients with NPF (n = 16) or COA (n = 16). C, Fibrocytes and myofibroblasts from patients with NPF (n = 8) and COA (n = 9) incubated with TAPI-1. Data are presented as means ± SEMs. *P < .05.

activity. EGFR expression was upregulated in these fibrocytes but not in those from healthy subjects or asthmatic patients with NPF. Our data reveal a mechanism involving the expression and activity of EGFR, underlying the important phenotypic differences of increased proliferative and transformative responses to oxidative stress of fibrocytes from asthmatic patients with COA compared with those with NPF. In addition, we provide evidence for an involvement of ADAM17 in the EGFR-mediated increased fibrocyte proliferation and transformation, probably through increased cleavage of the EGFR ligands.

Role of EGFR in fibrocyte proliferation and transformation

EGFR signaling in asthmatic epithelial cells regulates epithelial cell proliferation, differentiation, migration, and adhesion and could be important in bronchial epithelial repair and airway wall remodeling.^{12,27,28} A similar role for EGFR signaling could be attributed to fibrocytes. We found an increased expression of the EGFR family on fibrocytes from patients with COA, with the greatest expression for EGFR/human epidermal receptor 1. Expression of EGFR has been reported to be increased in airways

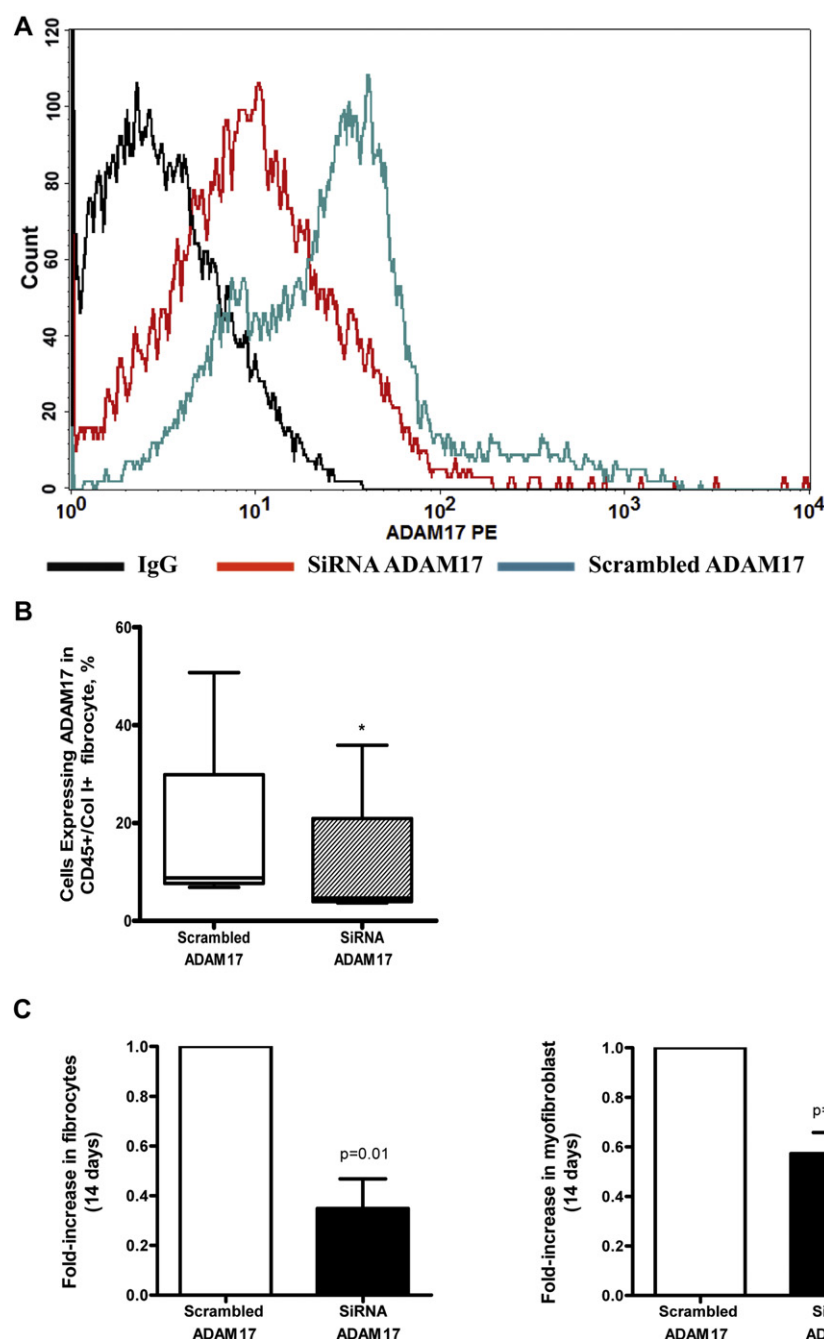


FIG 4. A, Flow cytometric histogram of ADAM17 expression after transfection with ADAM17 (red), scrambled siRNA (blue), or IgG (black). B, Percentage of ADAM17⁺ fibrocytes after ADAM17 or scrambled siRNA knock down ($n = 5$). Maximum to minimum, 25% to 75% percentiles as boxes, and medians are shown. * $P < .05$. C, Transfection with ADAM17 siRNA decreased fibrocyte proliferation (left) and myofibroblast transformation (right).

of asthmatic patients^{12,27} and also in epithelium of asthmatic patients.²⁸ Fibrocytes from patients with COA have greater functional proliferative and transformative capacities.⁶ The number of EGFR⁺ fibrocytes and the intensity of EGFR expression were increased in these patients. Confocal imaging revealed that most of the EGFR expressed on fibrocytes was in an activated phosphorylated state. Inhibition of EGFR activity by the specific EGFR tyrosine kinase inhibitors AG1478 and gefitinib attenuated fibrocyte proliferation and myofibroblast transformation,

confirming the role of EGFR in the increased proliferative and transformative capacities of fibrocytes from patients with COA.

Upregulated EGFR expression in patients with COA might also increase the vulnerability of fibrocytes to proliferation and differentiation. The EGF family is comprised of members such as EGF, TGF- α , amphiregulin, HB-EGF, betacellulin, epiregulin, epigen, and neuregulin-1 to neuregulin-6.²⁸ All members of the EGF family are type I transmembrane proteins and are expressed on cell surfaces that can be cleaved by cell-surface proteases.¹⁸

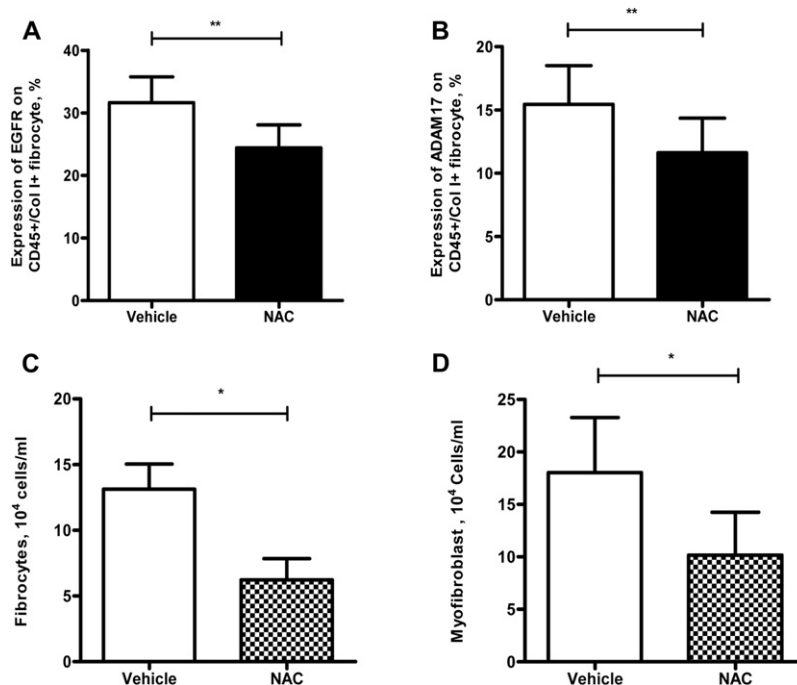


FIG 5. Effect of NAC on the expression of EGFR (A) and ADAM17 (B) on fibrocytes from patients with COA (n = 6). Effect of NAC on fibrocyte proliferation (C) and transformation into myofibroblasts (D) of patients with COA (n = 7) for 14 days. Data represent means $\pm$ SEMs. * $P < .05$ and ** $P < .01$.

This cleavage by ADAMs is a crucial step in the control of ligand availability and receptor activation. In this study the expression of ADAM17 was found to be upregulated in fibrocytes of patients with COA, and a metalloproteinase inhibitor, TAPI-1, reduced fibrocyte proliferation with a proportional decrease in myofibroblasts. In addition, transfection with siRNA to knock down ADAM17 also decreased the highly proliferative capacity of fibrocytes from COA with a concomitant reduction in myofibroblasts. Thus ectodomain shedding of ligands by ADAM17 might play an important role in the functional transactivation of upregulated EGFRs in those patients. Even without exogenous EGFR ligands, the upregulated ADAM17 might provide auto-crine ligand to transactivate EGFR, mediating fibrocyte proliferation and transformation.

Although the knock down of ADAM17 with ADAM17 siRNA was modest, the effect observed on fibrocyte proliferation was substantial. One explanation could be that there were off-target effects of the siRNA knock down, although this is less likely because we have compared with the effects of scrambled siRNA. Off-target effects could relate to inactivation of other pathways associated with EGFR activation, such as the nonreceptor protein tyrosine kinase or phosphoinositide 3-kinase/Akt signaling pathways that might also control fibrocyte proliferation.²⁹⁻³¹

Role of oxidative stress in fibrocytes

The excessive amount of oxidative stress described in patients with asthma,^{32,33} particularly in those with COA,³⁰ might play an important role in activating fibrocytes, numbers of which are increased in this condition. Our study demonstrates a contributory role for oxidative stress in enhanced fibrocyte proliferation and transformation in patients with COA, as shown by the attenuating effect of NAC. Activation of the EGFR pathway by H₂O₂ in

fibrocyte proliferation and transformation was implied by the inhibitory effect of the EGFR tyrosine kinase inhibitor gefitinib. There are previous reports indicating that H₂O₂ is required for myofibroblast differentiation³⁴ and for the transactivation of EGFR.³⁵ The upregulated expression of EGFR and ADAM17 in fibrocytes of patients with COA was related to intracellular oxidants, as shown by the inhibitory effects of NAC. H₂O₂ has been reported to induce tyrosine phosphorylation of EGFR through metalloproteinase-dependent HB-EGF release.^{36,37} Oxidative stress in fibrocytes of patients with COA might upregulate ADAM17 to release ligands for activation of the EGFR pathway. Thus the intrinsic difference in the proliferative and transformative capacity of fibrocytes between patients with COA and those with NPF might be attributed to intracellular oxidative stress, as demonstrated by the attenuating effect of the antioxidant NAC on baseline EGFR and ADAM17 expression, as well as on fibrocyte proliferation in patients with COA and on the enhancing effect of exogenous H₂O₂ on fibrocyte proliferation in asthmatic patients with NPF. However, oxidative stress can also induce metalloproteinase-independent EGFR phosphorylation.^{38,39} We did not directly measure the release of EGFR ligands because of the low number of fibrocytes.

The significance of our *in vitro* findings on fibrocytes from patients with asthma must be extrapolated with care to the *in vivo* situation, particularly in terms of the pathogenesis of chronic airflow obstruction in asthmatic patients. First, the extent of the involvement of the oxidative stress and ADAM17 pathway we have demonstrated *in vitro* is modest. Second, there are likely to be other mechanisms operative in a disease such as asthma with regard to the proliferation of fibrocytes and the development of chronic airflow obstruction. Other pathways that activate fibrocytes might include p38 mitogen-activated protein kinase,⁴⁰ TGF- β 1,⁴¹ and endothelin-1.⁷ Endothelin-1 and TGF- β 1 induce

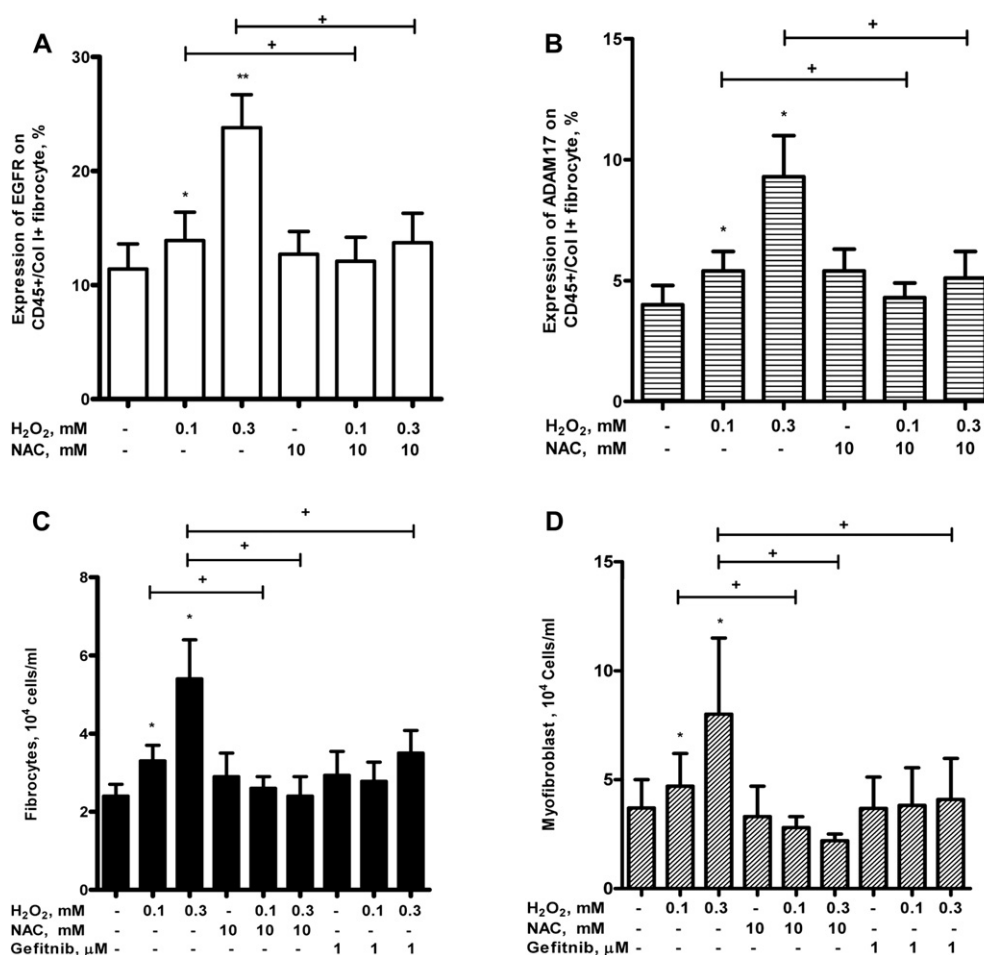


FIG 6. **A** and **B**, Effect of H₂O₂ on EGFR expression (Fig 6, **A**; n = 7) and ADAM17 (Fig 6, **B**; n = 7) on fibrocytes from asthmatic patients with NPF and of NAC on the expression of EGFR and ADAM17. **P* < .05 and ***P* < .01 compared with vehicle. +*P* < .05. **C** and **D**, Effects of H₂O₂, NAC, or gefitinib on fibrocyte proliferation (Fig 6, **C**) and transformation into myofibroblasts (Fig 6, **D**). **P* < .05 compared with vehicle. +*P* < .05.

the proliferation of circulating fibrocytes *in vitro* and promote their differentiation into mature myofibroblasts that produce fibronectin and collagen III.⁷

Conclusion

We have shown that an oxidative stress-sensitive upregulation of the EGFR pathway contributes to enhanced proliferative capacity and also to differentiation into myofibroblasts of fibrocytes from asthmatic patients with chronic airflow obstruction. This mechanism could contribute to chronic airflow obstruction and loss of lung function.

Clinical implications: Circulating fibrocytes from patients with COA have a greater capacity for proliferation and transformation. This effect is mediated through an EGFR-dependent pathway that most likely involves oxidant pathways.

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