

Original article

Augmented epithelial endothelin-1 expression in refractory asthma

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Background: Airway remodeling in patients with severe steroid-refractory asthma might result from a reduced ability of steroid therapy to limit the transcription of remodeling factors by the bronchial epithelium.

Objective: We sought to compare the levels of transcripts encoding remodeling factors in bronchial epithelium of healthy volunteers and of asthmatic patients with either steroid-sensitive or steroid-refractory disease and to correlate these levels with hallmarks of airway remodeling.

Methods: By means of real-time quantitative PCR, we assessed the levels of 14 transcripts encoding remodeling factors, matrix metalloproteinases, and extracellular matrix proteins in laser-capture microdissected bronchial epithelium of healthy volunteers, patients with mild steroid-untreated asthma, and patients with steroid-sensitive and steroid-refractory asthma ($n = 8-10$ in each group). Histologic features of airway remodeling and endothelin-1 (EDN1) immunolocalization were determined by using frozen specimens.

Results: Patients with steroid-refractory asthma had greater levels of EDN1 transcripts (4.1-fold increase, $P = .026$) and protein ($P = .0009$) in their bronchial epithelium compared with patients with steroid-sensitive asthma. EDN1 mRNA levels and protein expression in asthmatic patients were negatively correlated with prebronchodilator and postbronchodilator FEV₁ value ($r^2 \geq 0.193$, $P \leq .03$), and they were positively related to airway smooth muscle areas ($r^2 = 0.253$, $P = .01$ and $r^2 = 0.281$, $P = .005$ for EDN1 mRNA and protein expression, respectively).

Conclusion: Increased EDN1 synthesis by the bronchial epithelium characterizes severe refractory asthma and correlates with airway remodeling and airflow obstruction.

Clinical implications: Targeting EDN1 might represent a novel therapeutic strategy for severe steroid-refractory asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Endothelin-1, severe asthma, laser microdissection fibrosis, airway smooth muscle

Refractory asthma is a heterogeneous condition with different patterns of severity and diverse reasons for loss of control. Clinically, patients with refractory asthma can present with a variety of separate or overlapping conditions, including frequent exacerbations, irreversible airflow obstruction, and reduced sensitivity or resistance to corticosteroids.¹ The mechanisms behind the inability of corticosteroids to reverse airflow obstruction in refractory asthma are unclear. Persistent architectural alterations, defined as airway remodeling, might increase the thickness of the bronchial wall, thus contributing to irreversible airway narrowing in these patients.^{2,3}

Airway remodeling is characterized by subepithelial fibrosis, with thickening of the subepithelial basement membrane (SBM), fibroblast and myofibroblast accumulation, greater expression of fibrogenic growth factors, and augmented extracellular matrix (ECM) protein deposition in the subepithelial areas of the proximal airways.² Other features of airway remodeling include an increase in airway smooth muscle (ASM) mass caused by hypertrophy and hyperplasia, goblet cell hyperplasia, and new blood vessel formation.² Notably, steroid-based therapy poorly affects the fibrotic response and the increase in ASM mass in patients with severe asthma,⁴⁻⁷ suggesting that certain molecular pathways involved in the onset of these processes are less suppressible with corticosteroids.

Studies with bronchial biopsy samples and with isolated cells suggested that an aberrant repair of the airway epithelium to injury is a major event that precipitates airway remodeling in asthma.⁸ Thus abnormally repaired epithelium becomes a prominent source of ECM components, fibrogenic cytokines, chemokines, and growth factors that sustain activation and proliferation of fibroblasts and ASM cells and promote the differentiation of fibroblasts into myofibroblasts.⁸ Although this hypothesis has been widely discussed in the literature, only a limited number of studies addressed this issue in patients with refractory asthma.⁹⁻¹³ In addition, none of these reports has used a quantitative approach targeting specifically epithelial cell transcript expression.

In the present study we hypothesized that the epithelial expression of certain transcripts encoding remodeling factors would be relatively resistant to suppression by steroid therapy in patients with refractory asthma. We

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Abbreviations used

ASM: Airway smooth muscle
 CCL2: Chemokine (C-C motif) ligand 2
 CXCL8: Chemokine (C-X-C motif) ligand 8
 ECM: Extracellular matrix
 EDN1: Endothelin-1
 MCP: Monocyte chemotactic protein
 MMP: Matrix metalloproteinase
 SBM: Subepithelial basement membrane

applied the technique of laser-capture microdissection to collect bronchial epithelial cells from endobronchial biopsy samples of healthy control individuals, patients with mild steroid-untreated asthma, and patients with severe steroid-sensitive and steroid-refractory asthma. We assessed quantitatively the levels of the transcripts encoding fibrogenic cytokines, chemokines, and growth factors, namely IL-8/chemokine (C-X-C motif) ligand 8 (CXCL8),¹⁴ monocyte chemotactic protein (MCP) 1/chemokine (C-C motif) ligand 2 (CCL2),¹⁵ TGF- α , TGF- β 1 and TGF- β 2,¹⁶ the fibrotic and ASM-proliferating peptide endothelin-1 (EDN1),^{2,17} and some ECM proteins and their degrading matrix metalloproteinases (MMPs).¹⁸ The immunolocalization of selected encoded proteins was determined in frozen tissue sections. The expression of the epithelial transcripts encoding factors involved in airway remodeling was correlated with the degree of airflow obstruction and with hallmarks of airway remodeling, including the thickness of the SBM, the accumulation of fibroblasts/myofibroblasts in the bronchial submucosa, and the increase in ASM area.

METHODS**Subjects**

Ten healthy volunteers, 10 patients with mild steroid-untreated asthma, and 18 patients with severe steroid-treated asthma fulfilling National Institute of Health criteria¹⁹ were recruited (see Table E1 in the Online Repository at www.jacionline.org). All subjects had never smoked. A flow-volume curve was performed in all subjects, and FEV₁ was assessed before and after the inhalation of 400 μ g of salbutamol. All subjects provided written informed consent, and the protocol was approved by the Hôtel Dieu hospital Ethics Committee (CP 02819). Patients with severe steroid-treated asthma were further classified as steroid sensitive ($n = 10$) or steroid refractory ($n = 8$) on the basis of the reversibility of their airflow obstruction (prebronchodilator and postbronchodilator FEV₁ of $\geq 80\%$ and $\leq 60\%$ of predicted value, respectively) on 12 to 18 months of short- and long-lasting β_2 -agonist and high-dose inhaled steroid therapy (mean daily dose of inhaled beclomethasone equivalents, 3200 and 3600 μ g in patients with steroid-sensitive and steroid-refractory asthma, respectively) and at least 1 course of oral prednisone (see Table E1 and the Methods section of the Online Repository at www.jacionline.org).¹

Fiberoptic bronchoscopy and laser-capture microdissection

Bronchial biopsy specimens were obtained by means of fiberoptic bronchoscopy,^{7,20} and bronchial epithelial cells were collected by

means of laser-capture microdissection (PALM Microlaser Technologies, Bernried, Germany) on toluidine blue-stained frozen tissue sections (see Fig E1 in this article's Online Repository at www.jacionline.org). Total RNA was extracted, and RNA polyA was amplified and reverse transcribed with Moloney Murine Leukemia Virus enzyme (Invitrogen, Cergy-Pontoise, France). The expression of 17 transcripts, including 3 housekeeping genes, was evaluated by using quantitative real-time PCR with the Mx 3000P apparatus (Stratagene Europe, Amsterdam, The Netherlands). Only genes with cycle thresholds of less than 35 were included in the analysis. The expression of each transcript was normalized to the geometric mean of the levels of transcripts encoding the 3 selected most stable housekeeping genes: ubiquitin C, succinate dehydrogenase, and ribosomal protein 13a. Selection of housekeeping genes, calculation, and normalization were performed with geNorm software.²¹ Primers were designed with Primer Express 2 Software (Applied Biosystems, Foster City, Calif) and were synthesized with Genosys (Sigma, Lyon, France) (see Table E2 in this article's Online Repository at www.jacionline.org).

Immunohistochemistry and morphometry

Five-micrometer frozen biopsy sections were incubated with mouse mAbs directed against EDN1, α -actin, or propyl-4-hydroxylase (fibroblasts/myofibroblasts).⁷ An anti-mouse antibody conjugated to biotin was then added, followed by the avidin-alkaline phosphatase complex from the Vectastain kit (Vector, Burlingame, Calif), Fast Red staining, and light nuclear Mayer's hematoxylin counterstaining.⁸ Epithelial cells expressing EDN1 (in percentage of total epithelial cells) and propyl-4-hydroxylase (fibroblasts/myofibroblasts)-positive cells per millimeter of SBM were enumerated.⁷ Computer-assisted image analysis was used to assess morphologically intact epithelium (in percentages), SBM thickness (in micrometers) and ASM area (in percentage of total biopsy area) on Mayer's hematoxylin- and α -actin-stained tissue sections, respectively.^{7,22}

Statistical analysis

Data were analyzed statistically by using the StatView SE+Graphics program for Macintosh (Abacus Concepts, Berkeley, Calif). Results are expressed as medians (interquartile ranges). A nonparametric Kruskal-Wallis analysis of variance with the Benjamini and Hochberg correction for multiple testing of the 14 transcripts was performed. When the results of this test were significant, the Mann-Whitney *U* test, followed by the Benjamini and Hochberg correction, was used to determine significance across the groups. An overall *P* value of .05 or less was considered significant.

Additional information on the methods used is provided in the Online Repository at www.jacionline.org.

RESULTS**Transcript levels in microdissected bronchial epithelium**

Using quantitative real-time PCR, we found detectable amounts of the transcripts encoding all the factors tested (Table I; Table E3 in the Online Repository at www.jacionline.org). The highest expression was found for tenascin C, TGF- α , MMP-2, and fibronectin 1 (values of cycle threshold ranging from 22.6 to 25.6, Table I). Exemplary original quantitative real-time PCR amplification plots are shown in Fig E2 of the Online Repository at www.jacionline.org.

By using the correction for multiple testing, only the differences in levels of the transcripts encoding IL-8/CXCL8 and EDN1 across the 4 study groups reached

TABLE I. Levels of transcripts in laser-capture microdissected bronchial epithelium from control subjects and patients with steroid-untreated and steroid-treated asthma

Transcript	Basal Ct*	Asthmatic patients				P value‡
		Control subjects	Steroid untreated	Steroid sensitive	Steroid refractory	
		Relative intensity†	Fold change over control subjects (mean ratio)			
MCP-1/CCL2	26.4 (25.5-28.4)§	0.2 (0.1-0.8)	12.60	1.30¶	1.80¶	.011
IL-8/CXCL8	29.9 (26.2-30.7)	0.2 (0.1-1.8)	4.65	2.40	7.26#	.001
MMP-2	25.3 (24.7-26.7)	0.4 (0.3-0.6)	4.12	2.34	2.38	.329
EDN1	27.7 (27.4-30.3)	0.3 (0.1-0.4)	2.59	2.07	8.80 ¶#	.002
FN1	25.6 (25.5-26.8)	1.4 (1.1-1.8)	2.24	0.47	1.28	.525
TNC	22.6 (21.3-26.9)	0.4 (0.1-0.8)	1.91	1.54	1.80	.369
COL1A1	28.8 (28.2-30.0)	4.4 (2.9-6.4)	1.68	1.17	1.42	.660
TGF-α	23.9 (23.2-26.6)	0.9 (0.4-2.2)	1.49	1.34	1.15	.878
TGF-β2	30.3 (29.8-30.9)	1.7 (1.5-2.1)	1.02	0.63	0.80	.112
MMP-9	28.2 (28.1-32.4)	11.4 (6.8-17.1)	0.79	0.13	0.01	.040
COL3A1	26.5 (26.2-30.0)	8.3 (5.8-9.2)	0.59	1.06	0.99	.873
TGF-β1	28.9 (28.1-34.5)	1.2 (0.2-2.6)	0.44	0.28	0.18	.344
MMP-1	26.6 (26.8-33.7)	2.6 (0.1-4.4)	0.18	0.03	0.01	.259
COL4A1	26.7 (25.5-28.3)	0.5 (0.3-1.1)	0.19	1.98	2.49	.286

Ct, Cycle threshold; FN1, fibronectin 1; TNC, tenascin C; COL, collagen.

*Basal cycle threshold corresponds to the cycle threshold values measured in laser-capture microdissected bronchial epithelium from control subjects.

†Data represent the ratio of mRNA encoding each transcript over the geometric average of mRNA expression of the housekeeping genes ubiquitin C, succinate dehydrogenase, and ribosomal protein 13a.

‡Significance between the relative intensities of each transcript was assessed by using the nonparametric Kruskal-Wallis test with the Benjamini and Hochberg correction for multiple testing. Only P values in boldface are significant.

||Significant compared with control subjects.

¶Significant compared with patients with steroid-untreated asthma.

#Significant compared with patients with steroid-sensitive asthma (Mann-Whitney U test and Benjamini and Hochberg correction if global test result is significant).

§Data are presented as medians (interquartile ranges).

statistical significance (Table I; Table E3). Changes in the epithelial expression of MCP-1/CCL2 and MMP-9 mRNA was not significant by using the correction for multiple comparisons, despite the overall significance with use of the Kruskal-Wallis test ($P = .011$ and $P = .04$, respectively; Table I; Table E3).

When compared with control subjects, patients with mild steroid-untreated asthma had significantly higher epithelial amounts of the transcripts encoding EDN1 (2.6-fold increase, $P = .038$) and a 4.7-fold increase in IL-8/CXCL8 mRNA expression, although this latter difference failed to reach significance ($P = .227$). The levels of IL-8/CXCL8 and EDN1 mRNA were similar in patients with steroid-sensitive asthma compared with those in their counterparts with mild steroid-untreated asthma (Table I). In contrast, patients with steroid-refractory asthma had greater epithelial expression of EDN1 mRNA compared with that seen in control subjects, patients with mild steroid-untreated asthma, and patients with steroid-sensitive asthma (8.8-, 3.2-, and 4.1-fold increase and $P = .001$, $P = .024$, and $P = .026$, respectively; Table I). This was accompanied by higher levels of IL-8/CXCL8 transcripts in relation to those seen in patients with steroid-sensitive asthma ($P = .021$, Table I).

Assessment of protein expression by means of immunohistochemistry

As the most relevant and novel finding related to changes in the epithelial levels of the transcripts encoding

EDN1, the immunolocalization of the corresponding encoded protein was assessed in frozen biopsy sections collected from the same control and asthmatic subjects used for microdissection studies (Fig 1).

Normal bronchial epithelium failed to show significant immunoreactivity for EDN1 (Fig 1, A), and scarce positive cells were found in patients with mild steroid-untreated asthma (Fig 1, B). Patients with steroid-sensitive asthma exhibit an irregular pattern of expression, with the majority of EDN1 expression being confined on the luminal surface of the epithelium (Fig 1, C). Some weak and discontinuous EDN1 immunostaining was also seen in basal cells, irrespective of the group of asthmatic patients analyzed (ie, patients with mild steroid-untreated or steroid-sensitive asthma; Fig 1, B and C). In patients with steroid-refractory asthma EDN1 immunostaining was visibly increased and occurred uniformly throughout the epithelial layer (Fig 1, D). Quantification analysis showed a significantly greater proportion of epithelial cells staining for EDN1 in the severe asthma groups compared with that in the control group (1.9- and 2.6-fold increase and $P = .0002$ and $P = .0004$, respectively; Fig 1, E). EDN1 epithelial immunostaining was significantly increased in patients with steroid-refractory asthma in relation to that seen in patients with mild steroid-untreated asthma (1.7-fold increase and $P = .002$; Fig 1, E) and patients with steroid-sensitive asthma (1.4-fold increase and $P = .0009$; Fig 1, E).

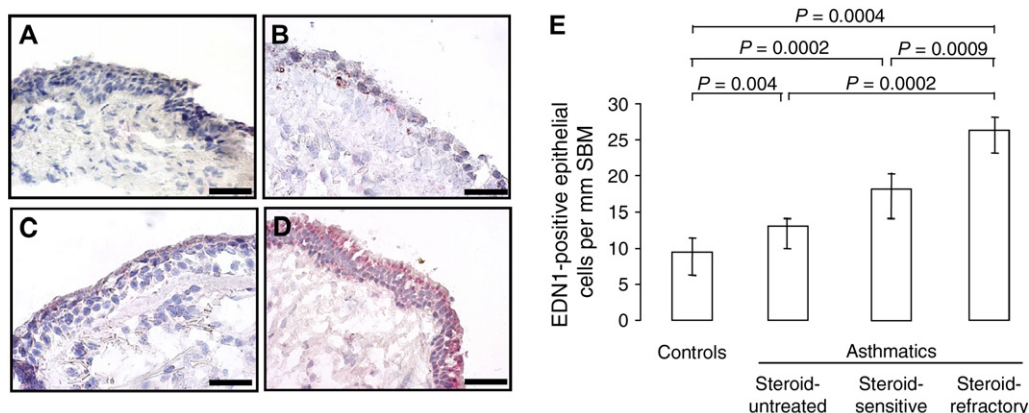


FIG 1. Representative immunostaining of EDN1 in bronchial biopsy specimens from control subjects (A), a patient with mild steroid-untreated asthma (B), and patients with severe steroid-sensitive (C) and steroid-refractory (D) asthma is shown. Scale bars = 250 μ m. E, proportion of EDN1-positive epithelial cells per millimeter of SBM in bronchial biopsy specimens from control subjects and the 3 groups of asthmatic patients. Bars represent median values, and error bars denote interquartile ranges.

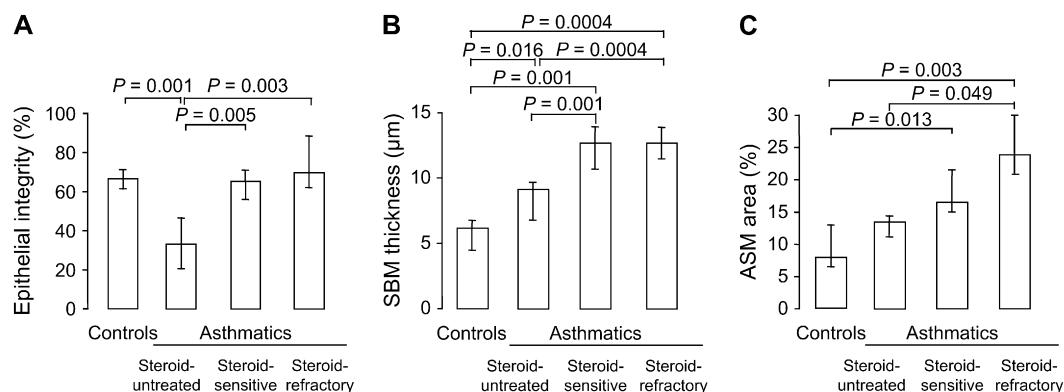


FIG 2. Epithelial integrity (A), SBM thickness (B), and ASM areas (C) in bronchial biopsy specimens from control subjects and asthmatic patients are shown. Bars represent median values, and error bars denote interquartile ranges.

Epithelial integrity and features of airway remodeling

A significant decrease in the proportion of the epithelial layer with an intact structure was seen in patients with mild steroid-untreated asthma compared with that seen in control subjects (Fig 2, A). Patients with severe steroid-sensitive and steroid-refractory asthma showed an apparent epithelial restitution, and the extent of morphologically intact epithelium in these latter groups was comparable with that measured in control subjects (Fig 2, A). SBM was thicker in the 3 groups of asthmatic patients than in the control subjects. Both patients with steroid-sensitive and steroid-refractory asthma had greater SBM thickness in relation to patients with mild steroid-untreated asthma (Fig 2, B). Scant mucosal fibroblasts/myofibroblasts (propyl-4-hydroxylase-positive cells) were enumerated in control individuals (Figs 1, E, and 3, C). These numbers were similar in patients with mild steroid-untreated asthma (Fig 3, B and E), and they were

significantly augmented in patients with severe asthma with either steroid-sensitive or steroid-refractory disease (Fig 3, C, D, and E). ASM area was larger in patients with steroid-sensitive and steroid-refractory asthma in relation to that seen in control subjects (Fig 2, C). However, patients with steroid-refractory asthma had the highest ASM areas, with values statistically significant compared with those of patients with mild steroid-untreated asthma ($P = .049$) but not those of patients with severe steroid-sensitive asthma ($P = 0.112$; Fig 2, C).

Correlation analyses

By means of univariate regression analysis (the Spearman rank-order method), we found that the individual epithelial levels of the transcripts encoding IL-8/CXCL8 and EDN1 correlated negatively with prebronchodilator and postbronchodilator FEV₁, whereas a positive correlation was found for MCP-1/CCL2 mRNA (Table II). In addition, EDN1 and IL-8/CXCL8 transcripts

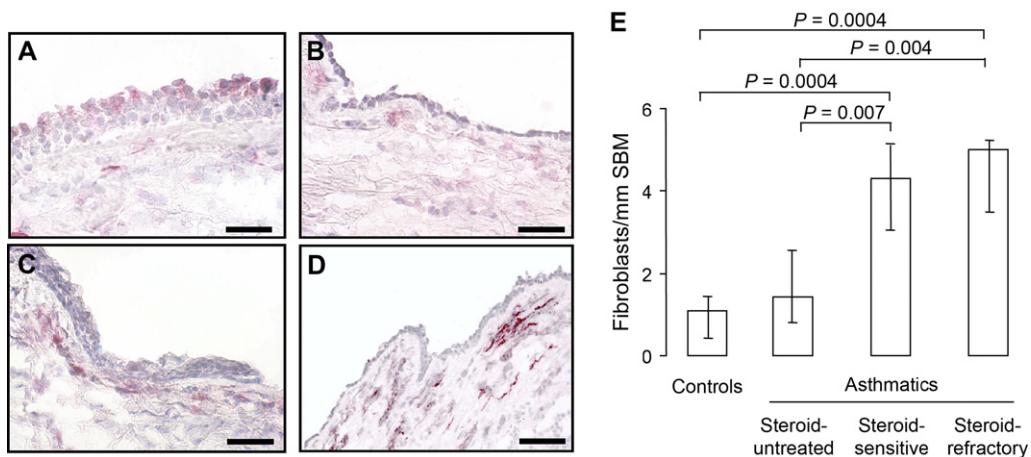


FIG 3. Representative immunostaining of fibroblasts and myofibroblasts (propyl-4-hydroxylase–positive cells) in bronchial biopsy specimens from a control subject (**A**), a patient with mild steroid-untreated asthma (**B**), and patients with severe steroid-sensitive (**C**) and refractory (**D**) asthma is shown. Scale bars = 250 μ m. **E**, Numbers of fibroblasts/myofibroblasts per millimeter of SBM in bronchial biopsy specimens from control subjects and the 3 groups of asthmatic patients. Bars represent median values, and error bars denote interquartile ranges.

TABLE II. Univariate correlation analyses in asthmatic patients (n = 28, Spearman rank-order method)

Marker	Prebronchodilator FEV ₁		Postbronchodilator FEV ₁		SBM thickness		Fibroblasts/ myofibroblasts		ASM area	
	r ²	P value	r ²	P value	r ²	P value	r ²	P value	r ²	P value
IL-8/CXCL8 mRNA	0.280	.005	0.232	.01	0.043	NS	0.001	NS	0.058	NS
EDN1 mRNA	0.193	.03	0.225	.003	0.016	NS	0.069	NS	0.253	.01
EDN1 epithelial staining	0.484	<.001	0.399	<.001	0.419	<.001	0.204	.02	0.281	.005

NS, Not significant.

correlated positively between each other ($r^2 = 0.323$, $P = .005$), and EDN1 mRNA was positively related to ASM areas (Table II).

None of these transcripts correlated with the extent of epithelial integrity. In addition, the proportion of epithelial cells expressing EDN1 negatively correlated with pre-bronchodilator and postbronchodilator FEV₁ values ($P = .0004$ and $P = .022$, respectively; Table II) and was positively related to ASM areas ($P = .002$, Table II) and to EDN1 mRNA ($r^2 = 0.195$, $P = .02$).

The main significant correlations are shown in Figs E3 and E4 in the Online Repository at www.jacionline.org.

DISCUSSION

Using laser-capture microdissection coupled to quantitative real-time PCR, we demonstrated that the levels of IL-8/CXCL8 and EDN1 mRNA were significantly augmented in the bronchial epithelium from patients with refractory asthma. These subjects' FEV₁ values failed to improve to greater than 70% of predicted value and less than 15% from baseline value after 14 days of treatment with 0.75 mg/kg/d oral prednisone, and they maintain severe airflow obstruction despite long-term treatment with high doses of

inhaled steroids. A group of patients with sensitive asthma (ie, asthmatic patients normalizing their lung function on a similar treatment regimen) was examined in parallel to establish whether the observed transcriptional modulations were due to asthma severity or to steroid use. Finally, a group of patients with mild steroid-untreated asthma was included to determine the levels of the expression of the different transcripts in the absence of a potential confounding effect linked to steroid therapy. The expression of EDN1 mRNA was correlated with epithelial integrity and with hallmarks of airway remodeling that have been previously associated with asthma severity, such as SBM thickness, mucosal fibroblast numbers, and ASM area.^{7,10,23}

When compared with healthy control individuals, patients with mild asthma had significantly higher epithelial levels of the transcripts encoding EDN1 and a trend toward a greater expression IL-8/CXCL8 mRNA. This was accompanied by a significant SBM thickness, thus corroborating previous observations showing that the production of these molecules, together with features of airway remodeling, are present in patients with mild asthma with normal respiratory function.²⁴⁻²⁶ In our hands the levels of TGF- α , TGF- β 1, TGF- β 2, and MMP-9 mRNA were not significantly modulated in patients with mild asthma, suggesting that the bronchial epithelium

might not represent the main source of these factors in the airways. Supporting this hypothesis, previous studies have shown that TGF- β 1, TGF- β 2, and MMP-9 originate from mucosal eosinophils^{23,27} and that neutrophils contribute substantially to MMP-9 production in the airways.⁴

An increase in the epithelial levels of IL-8/CXCL8 was observed in patients with steroid-refractory asthma compared with that seen in patients with steroid-sensitive asthma, a finding that extends previous observations showing a greater immunoreactivity for this chemokine in the bronchial epithelium and higher levels in induced sputum of patients with severe asthma.^{12,28} IL-8/CXCL8 overexpression is believed to contribute to neutrophilic inflammation but also to airway remodeling by promoting ASM cell contraction and migration.¹⁴

The most salient finding of the current report relates to epithelial EDN1 transcript, the increased levels of which distinguished patients with steroid-refractory asthma from patients with steroid-sensitive asthma. Immunohistochemical analysis corroborated the data obtained with quantitative real-time PCR because they illustrate a striking disease-related increase in the proportion of epithelial cells expressing EDN1, with the highest immunostaining observed in patients with refractory asthma.

EDN1 is a potent bronchoconstrictor 21-amino-acid peptide that plays an important role in the pathogenesis of airway remodeling through the activation of several functions in structural cells.¹⁷ These functions include the synthesis of ECM proteins from lung fibroblasts and bronchial epithelial cells,²⁹⁻³¹ the differentiation of fibroblasts into myofibroblasts,³² and the proliferation of ASM cells, fibroblasts, and myofibroblasts.³³⁻³⁵ Studies with bronchial biopsy samples demonstrated that EDN1 is weakly expressed by the normal airway epithelium and that this expression is upregulated in mild and moderate asthma.^{36,37} Conflicting observations, however, have been reported concerning the effect of corticosteroids on EDN1 expression. Hence although immunoreactive EDN1 was downregulated *in vivo* by steroid therapy,³⁷ *in vitro* studies with human bronchial epithelial cells demonstrated failure by dexamethasone to prevent EDN1 synthesis in response to TNF- α , most likely because of the absence of any identifiable glucocorticoid-responsive element in the EDN1 promoter region.³⁸ It was speculated that *in vivo* inhibition of epithelial EDN1 expression observed in patients with steroid-treated asthma resulted from an indirect suppressive effect on the synthesis of TNF- α ,³⁸ a cytokine the levels of which continue to be increased in the airways of patients with severe asthma despite steroid therapy.³⁹ Although the expression of TNF- α has not been examined in this study, these observations suggest that sustained local generation of this cytokine might contribute to the epithelial EDN1 overexpression presently reported.

Finally, we ascertained whether the epithelial levels of transcripts encoding IL-8/CXCL8 and EDN1 varied in relation with asthma severity and with the extent of epithelial integrity and airway remodeling. Although the assessment of airway epithelium integrity by means of

bronchial biopsy is confounded by an artifactual cell desquamation caused by tissue sampling,⁴⁰ we found a loss of epithelial integrity in patients with mild steroid-untreated asthma, whereas in patients with severe asthma with either sensitive or refractory disease, the airway epithelium appeared fully restored, probably as a result of high-dose long-term steroid therapy.^{7,41}

The assessment of airway remodeling demonstrated the existence of larger ASM areas, greater SBM thickness, and higher mucosal fibroblast/myofibroblast numbers in patients with severe steroid-refractory and steroid-sensitive asthma compared with that seen in patients with mild asthma and control subjects. These results substantiate in part our previous observations performed in distinct subjects and showing that the main bronchial wall changes seen in patients with severe asthma consist of fibroblast/myofibroblast accumulation beneath the SBM and in increased ASM mass.⁷

Univariate regression analyses demonstrated that epithelial IL-8/CXCL8 and EDN1 transcripts, as well as the proportion of EDN1-positive epithelial cells, negatively correlated with prebronchodilator and postbronchodilator FEV₁ values. However, only EDN1 transcript and protein showed positive correlation with ASM areas. These correlations argue for an important role played by EDN1 in the pathogenesis of the remodeling response in the airways, particularly of ASM hypertrophy and hyperplasia, and in the respiratory function abnormalities seen in patients with refractory asthma. Finally, neither the levels of the EDN1 and IL-8/CXCL8 transcripts nor the extent of EDN1 immunostaining correlated with the proportion of morphologically intact epithelium, suggesting that the observed differences in the expression of these molecules between patients with severe steroid-sensitive and patients with steroid-refractory asthma reflect functional abnormalities of the bronchial epithelium rather than a disparity of its integrity.

Phase III trials are ongoing on the effects of selective EDN1 receptor antagonists in idiopathic pulmonary fibrosis,⁴² a severe and irreversible lung disease characterized by an abnormal repair of the alveolar epithelium and by the accumulation of fibroblasts and ECM components in the peripheral airways.⁴³ The current report proposes the new concept that limiting EDN1 production might also represent a novel and valuable therapeutic tool for attenuating airway remodeling and respiratory function deterioration in patients with severe steroid-refractory asthma.

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METHODS

Subjects

Twenty-eight asthmatic subjects who never smoked, mainly atopic subjects fulfilling the criteria of the “Guidelines for the diagnosis and management of asthma” of the National Heart, Lung, and Blood Institute/World Health Organization,^{E1} were recruited (Table E1). Not all asthmatic patients experienced exacerbations within the 2 months preceding the bronchoscopy. A flow-volume curve was performed in all subjects on at least 3 occasions before bronchoscopy, and each measurement was separated by a 6-month interval. FEV₁ and forced vital capacity were assessed before and after the inhalation of 400 µg of salbutamol. Each subject had documented reversibility of FEV₁ (15% from baseline value) after inhalation of 400 µg of salbutamol at least once within the 18 months before bronchoscopy. A group of 10 healthy volunteers receiving no medication was also studied. All of them had never smoked, had normal pulmonary function, had no bronchodilator response, had no chronic illness, had no history of allergy or asthma, and had negative skin prick test responses. All subjects provided written informed consent, and the protocol was approved by the Hôtel Dieu hospital Ethics Committee (CP 02819).

The 28 asthmatic patients were distributed into 2 groups as follows: 10 patients with mild asthma and 18 patients with severe asthma. Patients with mild asthma had normal baseline lung function (prebronchodilator FEV₁ ≥80% of predicted values) and required only inhaled short-acting β₂-agonists, taken as needed, to achieve control. Patients with severe asthma were defined on the basis of the requirement of high-dose inhaled steroids (>1000 µg of fluticasone or equivalent per day) and a long-acting β₂-agonist as add-on therapy to achieve control.^{E1} These patients with severe asthma were further subdivided into those with steroid-sensitive asthma (n = 10) and those with steroid-refractory asthma (n = 8), according to the guidelines of the American Thoracic Society on refractory asthma.^{E2} The criteria used for defining refractory asthma was a failure of FEV₁ to improve to greater than 70% of predicted value and less than 15% from baseline value after 14 days of treatment with 0.75 mg/kg/d prednisone taken orally^{E2} and the persistence of airflow obstruction (ie, prebronchodilator FEV₁ <70% of predicted value). Patients with refractory asthma also met at least 2 of the following criteria: (1) daily use of a rescue inhaler, (2) 3 or more courses of oral steroids in the last year, (3) clinical deterioration on reduction of inhaled or oral steroid use, and (4) a history of a near-fatal asthma event.^{E3}

Twelve to 18 months before fibroscopy, patients with steroid-sensitive asthma had prebronchodilator median FEV₁ values of 60.0% of predicted value (interquartile range, 51.8% to 70.0%) and median postbronchodilator FEV₁ values of 72.5% of predicted value (interquartile range, 64.8% to 84.0%). Respiratory function in these subjects was improved after 12 to 18 months of treatment with short- and long-acting β₂-agonists and a mean daily dose of 3200 µg of inhaled beclomethasone equivalents. At the time of fibroscopy, these subjects had a median prebronchodilator FEV₁ value of 81.0% (interquartile range, 80.3% to 88.3%) and a median postbronchodilator FEV₁ value of 96.5% (interquartile range, 88.0% to 101.8%; Table E1).

Patients with steroid-refractory asthma had a median prebronchodilator FEV₁ value of 44.5% (interquartile range, 44.0% to 49.0%) and a median postbronchodilator FEV₁ value of 52.0% (interquartile range, 48.0% to 57.8%) that persisted during 12 to 18 months, despite treatment with a mean daily dose of 3600 µg of inhaled beclomethasone equivalents in association with short- and long-acting β₂-agonists on a regular basis or at least 1 course of oral steroids (0.75 mg/kg/d prednisone for 14 days).^{E3} Their median prebronchodilator FEV₁ value at the time of the fibroscopy was 42.0% (interquartile range, 38.5% to 49.5%) and the median postbronchodilator FEV₁ value was 51.5% (interquartile range, 45.0% to 61.0%; Table E1).

Fiberoptic bronchoscopy and biopsy collection

The bronchoscopy was performed by the same operator, according to the guidelines outlined by the American Thoracic Society,^{E4} as previously described.^{E5} The subjects were premedicated with 200 µg of inhaled salbutamol, and the nose and larynx were anesthetized with 2% and 0.5% xylocaine spray, respectively. Six biopsy specimens were then taken from the subcarinae of the right middle lobe (B4-B5) and the lower lobe (truncus basalis bifurcations: B7-B8, B8-B9, and B9-B10). A standard-type biopsy forceps and an OLYMPUS bronchoscope (model BF P20; Olympus Optical, Tokyo, Japan) were used. Four bronchial biopsy specimens were immediately put on cork disks, covered by Tissue-Tek compound (Sakura Finetek, Zoeterwoude, The Netherlands), and snap-frozen in liquid nitrogen. The frozen blocks were kept at –80°C until the assessment of immunohistochemistry and morphometry, as described below. The remaining 2 biopsy specimens were immediately put in RNA-DNA-free tubes and frozen at –80°C until use.

Laser-capture microdissection

Adjacent 7-µm tissue sections were cut in a cryostat (Leica CM3000; Leica, Rueil-Malmaison, France), immediately mounted on DNase-RNase-free glass slides (Palm Microlaser Technologies, Bernried, Germany), fixed in 70% ethanol for 30 seconds, rinsed, air-dried, rapid stained with toluidine blue, and dried in 100% ethanol. Intact areas of the bronchial epithelium were microdissected with a Laser Microdissector (PALM Microlaser Technologies, Bernried, Germany), placed immediately in PCR tubes containing 15 µL of RNeasy lysis buffer (Qiagen, Courtaboeuf, France), and frozen at –80°C until use. Injured bronchial epithelium (ie, a single layer of basal cells with no intact ciliated cells) was excluded.

Between 50 and 80 sections from each biopsy specimen were microdissected (median of 0.025 mm² of dissected epithelial area in each biopsy section), and the collected epithelium from a single biopsy specimen was pooled in the same tube. One biopsy specimen was used for each subject, and approximately 5000 epithelial cells, corresponding to 5 ng of total RNA, were obtained.

Exemplary photomicrographs showing selection and removal of bronchial epithelial areas by means of laser-capture microdissection are shown in Fig E1.

Epithelial cell RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was lysed and isolated with a RNeasy Micro kit (Ambion, Cambridgeshire, United Kingdom) according to the manufacturer's instructions, and RNA quality was assessed with RNA PicoChips (Agilent Technologies, Waldbronn, Germany). Forty-four biopsy specimens were microdissected, and 6 of them were discarded because RNA quantity and quality were insufficient for performing adequate quantitative real-time PCR analyses. RNA polyA was amplified with the MessageAmp aRNA kit (Ambion), and reverse transcription was performed in a final volume of 50 µL for 2 hours at 37°C by using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). The obtained cDNAs were diluted 10 times and amplified by using quantitative real-time PCR using SybrGreen JumpStart Taq Ready Mix detection kit (Sigma). In all assays cDNAs were amplified by using a standardized program (JumpStart Taq Polymerase activation step; 40 cycles of 30 seconds at 94°C and 1 minutes at 60°C and a dissociation step). All assays were performed in a volume of 20 µL, and primers were used at a final concentration of 0.33 µmol/L. Reactions were carried out with the PCR Mx 3000P apparatus (Stratagene Europe, Amsterdam, The Netherlands).

Genes with cycle thresholds of greater than 35 were considered insufficiently expressed to perform an accurate analysis, and therefore they were excluded. The intensity of gene expression was calculated by using a cycle threshold of less than 35, and it was normalized to the geometric mean of the levels of transcripts encoding the 3 most stable housekeeping genes: ubiquitin C, succinate dehydrogenase A, and ribosomal protein L13a.^{E6,E7} These transcripts were highly expressed in laser-capture microdissected bronchial epithelium from control subjects (median cycle threshold, 15.4 [interquartile range, 15.0-16.3], 15.4 [interquartile range, 15.3-16.1], and 21.9 [interquartile range, 20.4-21.4] for ubiquitin C, succinate dehydrogenase A and ribosomal protein L13a, respectively). These values showed less than 8% variation across the 4 patient groups (data not shown).

The final results represent the mean of 1 to 3 independent quantitative real-time PCRs performed in duplicate. Primer sequences (Table E2) were designed with Primer Express software (Applied Biosystems) and were synthesized by using Genosys (Sigma). Their sequences were blasted against Basic Local Alignment Search Tool (BLAST) database (<http://www.ncbi.nlm.nih.gov/BLAST>). The quality of primers was verified by performing dissociation curves (1 minute at 95°C, 30 seconds at 60°C, 20 minutes to reach 95°C, and 1 minute at 95°C) in samples with cycle thresholds of 35 or less. When RNA from microdissected bronchial epithelium had a cycle threshold of greater than 35, RNA extracted from pooled bronchial biopsies was amplified by means of quantitative real-time PCR to ensure primer quality.

Exemplary original quantitative real-time PCR amplification plots are shown in Fig E2.

Immunohistochemistry and morphology

Serial 5- μ m sections of bronchial biopsy specimens collected from the same subjects were cut in a cryostat (Leica CM3000), collected on glass slides previously coated with β -methacryloxypolytrimethoxysilane (Sigma, St Quentin Fallavier, France), and fixed in acetone for 10 minutes at room temperature.

Immunohistochemistry was performed as described previously^{E5} by using monoclonal mouse anti-human antibodies directed against EDN1 (clone 1C4, 1:100 dilution; Chemicon, Abcys, Paris, France), α -actin (clone 1A4, 1:200 dilution, Sigma), or propyl-4-hydroxylase (fibroblasts/myofibroblasts; clone 5B5, 1:250 dilution; DakoCytomation, Trappes, France).^{E5} An anti-mouse antibody conjugated to biotin and the avidin-alkaline phosphatase complex from the Vectastain kit (Vector) were next used, followed by Fast Red staining (DakoCytomation) and light nuclear Mayer's hematoxylin counterstaining. The specificity of the immunostaining was assessed by using the corresponding isotype (ie, mouse IgG; DakoCytomation) instead of the primary antibody.

EDN1 immunostaining was quantified in intact areas of the bronchial epithelium, and results were expressed as positive cells per millimeter of SBM. Propyl-4-hydroxylase-positive cells were enumerated in the bronchial submucosa, excluding glands and ASM, in a zone of 60 μ m in depth, along the length of the SBM.^{E5}

The proportion of epithelium with apparent normal morphology was assessed by using morphometric analysis with computer-assisted image analysis (Microvision Instruments; Histolab, Evry, France). Biopsy sections were stained with Mayer's hematoxylin, and morphologically intact epithelium (in percentages) was defined as the total length of the SBM with intact epithelium divided by the total length of the SBM.^{E5,E8} SBM thickness (in micrometers) was assessed on tissue sections stained with Mayer's hematoxylin by using computer-assisted image analysis from the base of the bronchial epithelium to the outer limit of the reticular lamina of the basement membrane. Measurements (20-50) were made at regular intervals of 50 μ m along the length of the SBM (median value, 4150 μ m).^{E5}

The area of the bronchial submucosa occupied by the ASM was determined by using computer-assisted image analysis on α -actin-stained tissue sections, and final results are expressed as percentages of ASM area over the total biopsy area.^{E5} Similar biopsy areas were found in control subjects (median, 0.31 mm² [interquartile range, 0.23-0.43 mm²]), in patients with mild steroid-untreated asthma (median, 0.29 mm² [interquartile range, 0.20-0.33 mm²]), in patients with steroid-sensitive asthma (median, 0.28 mm² [interquartile range, 0.23-0.39 mm²]), and in patients with steroid-refractory asthma (median, 0.35 mm² [interquartile range, 0.24-0.39 mm²]).

All parameters were examined in 2 to 6 serial sections from the same biopsy specimen, at 3 different depths for each biopsy specimen, and in sections from 3 to 4 different biopsy specimens. Sections were randomly assigned and analyzed by an observer who was unaware about the clinical group assignment. The final figure was the mean of all the measurements obtained for each subject. For all parameters, the coefficients of variation between biopsy specimens from the same subject and between areas of the same biopsy specimen were of 5% to 10% and 6% to 9%, respectively. Immunohistochemical measurements and SBM thickness were made at a magnification of $\times 400$. Total biopsy and ASM areas were assessed at a magnification of $\times 60$.

Statistical analysis

Data were analyzed statistically by using the StatView SE+Graphics program for Macintosh (Abacus Concepts). Results are expressed as medians (interquartile ranges). The nonparametric Kruskal-Wallis ANOVA test was used to compare the 4 groups of subjects. To account for multiple tests because of the 14 transcripts, we used a Benjamini and Hochberg correction to assess the significance of the global Kruskal-Wallis test for each transcript. This correction is a common approach to control the increased false-discovery rate with a large number of tests.^{E9} A family-wise error rate of 0.05 was selected. The *P* values for each test were sorted in ascending order: $P_1 < P_2 < \dots < P_{14}$. The smallest *P* value, *P*₁, was compared with $(0.05 \times 1)/14 = 0.0036$, and if *P*₁ was less than .0036, *P*₂ was compared with $(0.05 \times 2)/14$, etc. If *P*₁₃ was lower than $(0.05 \times 13)/14$, then the highest *P* value, *P*₁₄, was compared with $(0.05 \times 14)/14$ and was considered significant if *P*₁₄ was less than .05. If the global test was significant with this correction for a given transcript, then 6 pairwise comparisons using the nonparametric Mann-Whitney *U* test for unpaired values were performed. Again, a Benjamini and Hochberg correction was used to assess the significance of the 6 comparisons across groups within the transcript. For the other statistical analyses, no correction for multiple testing was performed because they were mainly exploratory. Correlation coefficients (*r*²) were calculated by using the nonparametric Spearman rank-order method. Only asthmatic subjects were included in the correlation analysis. *P* values of .05 or less were considered significant.

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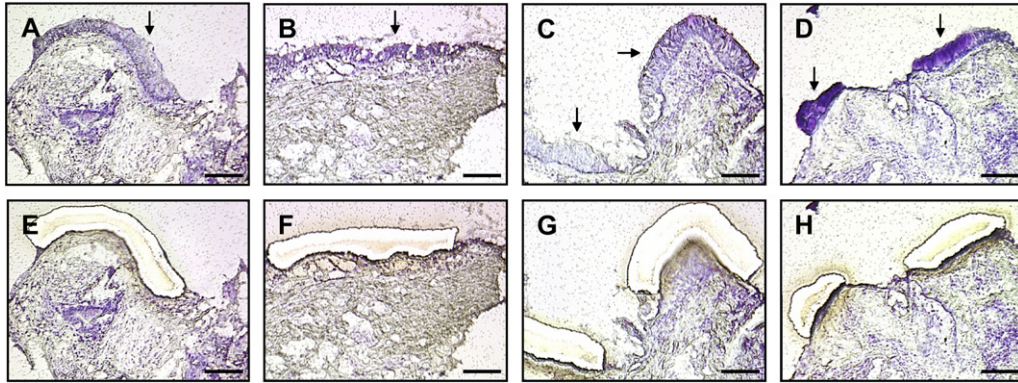


FIG E1. Laser-capture microdissection of bronchial epithelium from control subjects and asthmatic patients. Toluidine blue–stained frozen tissue sections from a control subject (**A** and **E**), a patient with mild asthma (**B** and **F**), a patient with severe steroid-sensitive asthma (**C** and **G**), and a patient with severe steroid-refractory asthma (**D** and **H**) are shown. Arrows in Fig E1, A through D, point to intact areas of bronchial epithelium, which were subsequently removed, as shown in Fig E1, E through H. Bars indicate 100 μ m.

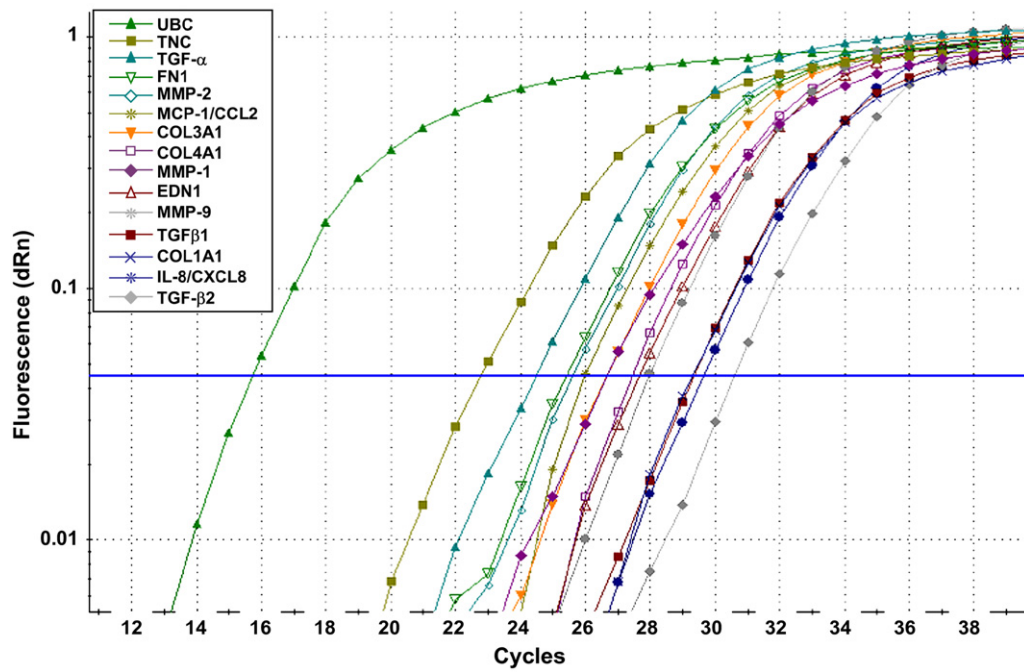


FIG E2. Exemplary real-time PCR amplification plots showing cycle threshold values for the 14 transcripts analyzed and for the housekeeping gene ubiquitin C (*UBC*) in laser-capture microdissected bronchial epithelium from a control healthy subject. *TNC*, Tenascin C; *FN1*, fibronectin 1; *COL*, collagen.

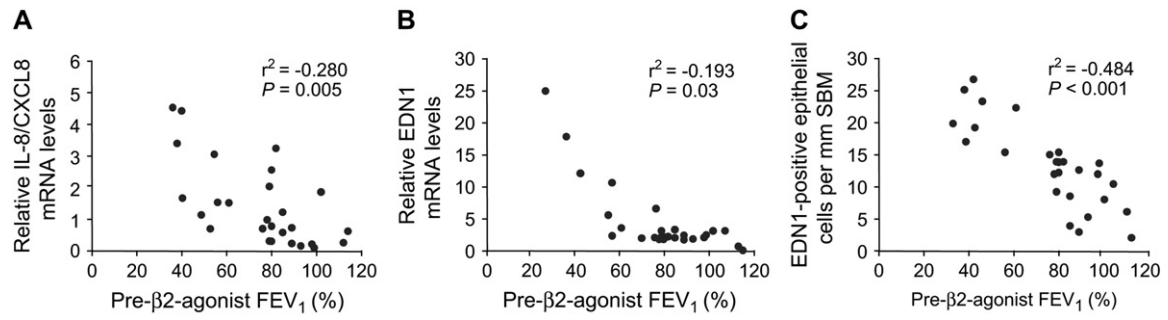


FIG E3. Correlation analyses (Spearman rank-order method) in asthmatic patients ($n = 28$). Prebronchodilator FEV₁ value (in percent predicted values) was correlated with the epithelial levels of the transcripts encoding IL-8/CXCL8 (**A**) and EDN1 (**B**) and with the number of EDN1-positive epithelial cells per square millimeter of SBM in bronchial biopsy specimens (**C**).

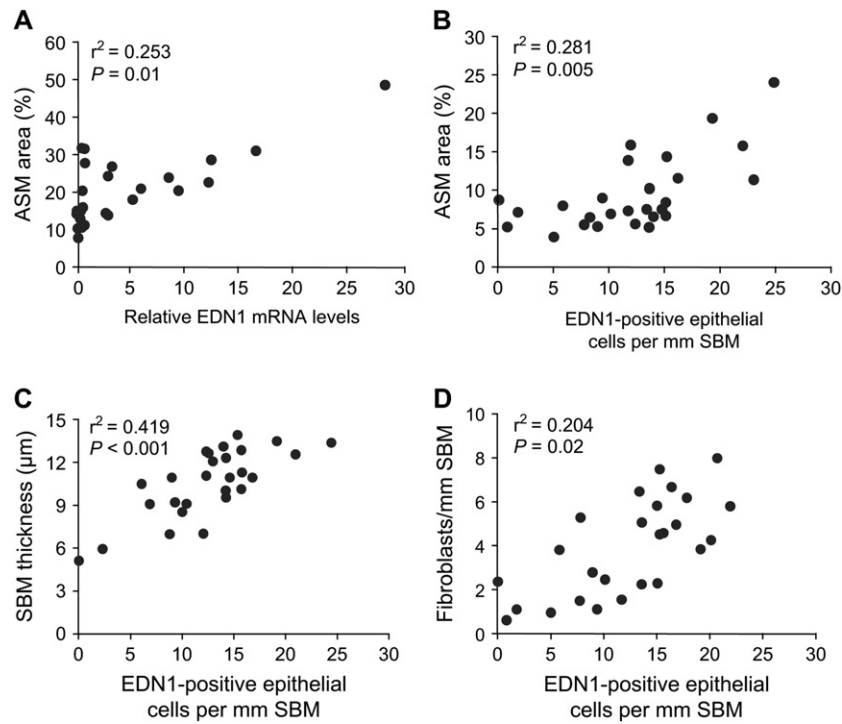


FIG E4. Correlation analyses between epithelial EDN1 mRNA (**A**) and protein (**B-D**) expression and hallmarks of airway remodeling in asthmatic patients (n = 28). The epithelial levels of the transcript encoding EDN1 was correlated with ASM area (Fig E4, A), and the number of EDN1-positive epithelial cells per square millimeter of SBM in bronchial biopsy specimens was related to ASM area (Fig E4, B), SBM thickness (Fig E4, C), and the number of fibroblasts beneath the SBM (Fig E4, D).

TABLE E1. Characteristics of control and asthmatic subjects

Characteristic	Control subjects	Asthmatic patients			P value*
		Steroid untreated	Steroid sensitive	Steroid refractory	
Subjects examined (n)	10	10	10	8	—
Age (y)†	50.0 (42.5-52.8)	41.5 (37.5-52.5)	53.0 (37.5-56.5)	53.0 (36.0-71.0)	.449
Sex (male/female)	4/6	4/6	6/4	4/4	—
Atopy	0	10	9	7	—
Asthma duration (y)	NA	17.5 (7.3-23.5)	23.0 (18.5-39.3)	24.5 (13.5-33.8)	.357
FEV ₁ before β_2 -agonists (%)	101.0 (100.0-107.3)	98.5 (87.0-111.5)	81.0 (80.3-88.3)‡§	42.0 (38.5-49.5)‡§	.0001
FEV ₁ after β_2 -agonists (%)	107.0 (104.5-112.5)	99.5 (91.0-115.3)	96.5 (88.0-101.8)	51.5 (45.0-61.0)‡§	.0001
On short-acting β_2 -agonists	0	10	10	8	—
On long-acting β_2 -agonists	0	0	10	8	—
On inhaled steroids	0	0	10	8	—
Mean daily dose of BDP equivalents	0	0	3200 μ g	3600 μ g	—
On oral steroids	0	0	0	0	—

NA, Not applicable; BDP, beclomethasone dipropionate.

*Data were analyzed statistically by using the nonparametric Kruskal-Wallis test.

†Age, asthma duration, and percent predicted pre- β_2 -agonist and post- β_2 -agonist FEV₁ value are expressed as medians (interquartile ranges).

‡P < .05 compared with control subjects.

§P < .05 compared with patients with steroid-untreated asthma.

||P < .05 compared with patients with steroid-sensitive asthma (Mann-Whitney U test).

TABLE E2. Primer sequences used for quantitative real-time PCR

GeneBank identifier	Transcript	Forward sequence/reverse sequence
NM_000584	IL-8/CXCL8	GGCCAAGGCCAAGAGAA GGGTTGCCAGATTTAACAGAAAAA
NM_002982	MCP-1/CCL2	AAGCTGTGATCTTCAAGACCATTTGT GTTAGCTGCAGATTCTTGGGTTGT
NM_003236	TGF- α	GACAGCAGCCAACCCTGATC AAGCACAAATTCTCCTCCCTTACC
NM_000660	TGF- β 1	ACTCATTCAAGTCACCATAGCAACACT CGCCTGGCCTGAACACTACTTTT
NM_003238	TGF- β 2	CTGCTAATGTTATTGCCCTCCTACA CACCTAGATCCCTCTTGAATCA
NM_001845	COL1A1	CTTTCATTTCATCTCTCAAACCTTAGTTT CCCCGCATGGGTCTTCA
NM_000090	COL3A1	CGAAGAATCCATCCTTGCCATCTTCG ACGCGGTGAGGCTGGTATTC
NM_001845	COL4A1	CTAATCACAACTGAATGACTTGACTTCA AAATGGCCCGAATGTGCTTA
X02761	FN1	TGGACCAGAGATCTTGGATGTTT CGCCTAAAACCATGTTCTCAA
X56160	TNC	GGTCCACACCTGGGCATTT TTGCTGAATCAAACAACAAAACAGA
NM_004530	MMP-1	ACCCTCAGAGCCACCCTAA GCCCTCTTGAGACAGTCTCCATT
NM_004530	MMP-2	CTGCTGGCTGCCTTAGAACCT AACCATCACTATGTGGGCTGAGA
NM_004994	MMP-9	CGAACTTTGACAGCGACAAG TCAGGGCGAGGACCATAGA
NM_001955	EDN1	TGGGATCAGAGCAGGAGCAT AGATGAAAGAAGAGACCAAAGCAGTTAC
NM_004168	SDHA	TGTGTCCATGTCATAACTGTCTTCATA AAGAATGAAGCAAGGGACAAAGG
NM_012423	RPL13a	CCTGGAGGAGAAGAGGAAAGAGA TTGAGGACCTCTGTGTATTTGTCAA
NM_004168	Ubiquitin C	CACTTGGTCCTGCGCTTGA TTTTGGGAATGCAACAACCTT

COL, Collagen; *FN1*, fibronectin 1; *TNC*, tenascin C; *SDHA*, succinate dehydrogenase complex, subunit A; *RPL13a*, ribosomal protein 13a.

TABLE E3. Levels of transcripts in laser-capture microdissected bronchial epithelium from control subjects and patients with steroid-untreated and patients with steroid-treated asthma

Transcript	Control subjects	Asthmatic patients			P value†
	Relative intensity*	Steroid untreated	Steroid sensitive	Steroid refractory	
MCP-1/CCL2	0.20 (0.15-0.81)‡	2.52 (0.86-3.59)§	0.26 (0.02-0.64)	0.36 (0.27-0.43)	.011
IL-8/CXCL8	0.21 (0.12-1.79)	0.98 (0.59-1.99)	0.50 (0.25-0.93)	1.53 (1.03-3.66)¶	.001
MMP-2	0.37 (0.27-0.57)	1.52 (0.38-4.83)	0.77 (0.18-1.55)	0.88 (0.46-1.64)	.329
EDN1	0.25 (0.07-0.36)	0.65 (0.31-1.43)§	0.62 (0.48-0.88)§	2.20 (1.36-5.91)§	.002
FN1	1.39 (1.13-1.75)	3.12 (1.00-5.15)	0.65 (0.19-3.46)	1.78 (0.55-17.32)	.525
TNC	0.38 (0.04-0.78)	0.73 (0.15-1.56)	0.59 (0.28-0.84)	0.69 (0.37-2.02)	.369
COL1A1	4.42 (2.89-6.39)	7.41 (3.20-17.81)	5.19 (2.48-8.61)	6.28 (2.51-26.30)	.660
TGF-α	0.94 (0.36-2.25)	1.40 (0.86-1.85)	1.26 (0.95-1.58)	1.09 (0.69-1.62)	.878
TGF-β2	1.66 (1.48-2.09)	1.70 (1.60-4.62)	0.63 (0.46-1.12)	1.33 (0.29-1.75)	.112
MMP-9	11.41 (6.78-17.12)	9.03 (4.64-15.91)	1.52 (0.93-4.29/)	1.03 (0.30-1.65)	.040
COL3A1	8.27 (5.77-9.23)	4.91 (3.94-8.86)	8.75 (3.92-37.24)	8.15 (3.72-32.28)	.873
TGF-β1	1.22 (0.22-2.57)	0.54 (0.32-2.19)	0.34 (0.22-1.64)	0.22 (0.05-0.96)	.344
MMP-1	2.62 (0.02-4.44)	0.47 (0.09-2.03)	0.09 (0.02-1.87)	0.04 (0.01-0.89)	.259
COL4A1	0.51 (0.31-1.08)	0.10 (0.05-0.27)	1.50 (0.20-2.55)	1.27 (0.17-7.33)	.286

FN1, Fibronectin 1; TNC, tenascin C; COL, collagen.

*Data represent the ratio of mRNA encoding each transcript over the geometric average of mRNA expression of the housekeeping genes ubiquitin C, succinate dehydrogenase, and ribosomal protein 13a.

†Significance between the relative intensities of each transcript was assessed by using the nonparametric Kruskal-Wallis test with the Benjamini and Hochberg correction for multiple testing. Only P values in bold are significant.

§Significant compared with control subjects.

||Significant compared with patients with steroid-untreated asthma.

¶Significant compared with patients with steroid-sensitive asthma (Mann-Whitney U test and Benjamini and Hochberg correction if global test is significant).

‡Data are presented as medians (interquartile ranges).