Expression of ADAMs and Their Inhibitors in Sputum from Patients with Asthma

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ADAMs (a disintegrin and metalloprotease) constitute a family of cell surface proteins containing disintegrin and metalloprotease domains which associate features of adhesion molecules and proteases. ADAMTSs (a disintegrin and metalloprotease with thrombospondin motifs) bear thrombospondin type I motifs in C-terminal extremity, and most of them are secreted proteins. Because genetic studies have shown that ADAM-33 gene polymorphisms are associated with asthma, we designed this study to assess mRNA expression profile of several ADAM and ADAMTS proteases in sputum from patients with asthma and to investigate the relationship between expression of these proteases and asthma-associated inflammation and airway obstruction. mRNA expression profile of selected ADAM and ADAMTS proteinases (ADAM-8, -9, -10, -12, -15, -17, and -33; ADAMTS-1, -2, -15, -16, -17, -18, and -19), their physiological inhibitors TIMP-1 and TIMP-3, and RECK, a membrane-anchored MMP activity regulator, was obtained by RT-PCR analysis performed on cells collected by sputum induction from 21 patients with mild to moderate asthma and 17 healthy individuals. mRNA levels of ADAM-8, ADAM-9, ADAM-12, TIMP-1, and TIMP-3 were significantly increased, whereas mRNA levels coding for ADAMTS-1, ADAMTS-15, and RECK were significantly decreased in patients with asthma compared with control patients. ADAM-8 expression was negatively correlated with the forced expiratory volume at the first second (FEV_1) ($r = –0.57$, $P < 0.01$), whereas ADAMTS-1 and RECK expressions were positively correlated to FEV_1 ($r = 0.45$, $P < 0.05$, and $r = 0.55$, $P = 0.01$, respectively). We conclude that expression of ADAMs and ADAMTSs and their inhibitors is modulated in airways from patients with asthma and that these molecules may play a role in the pathogenesis of asthma.

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INTRODUCTION

Asthma is a complex inflammatory disease of the conducting airways, leading to progressive lung function impairment linked to some morphological changes of airways (1,2). Histological studies have described that patients with asthma display airway wall abnormalities mainly consisting of an increase in muscle mass, mucous gland hypertrophy, inflammatory cells infiltration (eosinophils, lymphocytes, mast cells, neutrophils, etc.), and extracellular matrix changes. These structural changes in bronchial tree have clinical repercussions, being responsible for at least a part of airway hyperresponsiveness and increased rate of decrease in forced expiratory volume at the first second (FEV_1) reported to occur during the life of such patients (3).

ADAM (a disintegrin and metalloprotease) and ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) proteases represent a class of membrane-anchored or secreted proteases, respectively. To date, more than 30 members have been described in the ADAM family and 19 in the ADAMTS family (4). ADAMs and ADAMTSs share a conserved domain structure: an N-terminal signal sequence, a prodomain, a metalloprotease domain with a conserved consensus sequence (HEXGHXXGXXH), a disintegrin domain, a cysteine-rich region usually containing an epidermal growth factor (EGF) repeat, and a transmembrane domain, followed by a cytoplasmic tail. ADAMTSs display a variable number of copies of thrombospondin 1–like repeats. Some ADAM and ADAMTS proteinases are physiologically inhibited by tissue inhibitors of metalloproteases (TIMPs-1 and -3) (5,6). In addition, a membrane-anchored glycoprotein, RECK (reversion inducing cysteine-rich protein with Kazal motifs), known as a matrix metalloprotease inhibitor, has been reported to regulate the function of several ADAM and ADAMTS proteases (7). ADAMs and ADAMTSs are implicated in physiological processes such as cell fusion, cytokine and growth factor shedding, cell migration, and in some complex cascades of events such as muscle development, fertilization, and immune response (8,9). ADAM and ADAMTS family members are also involved in pathological processes such as cancer and inflammation (10).

Accumulating evidence suggests that matrix metalloproteinases (MMPs), proteases closely related to ADAMs, are implicated in the pathogenesis of asthma. High amounts of MMP-2 and -9 are found in the lung and bronchial tree from patients with asthma (11,12), and

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Table 1. Patient characteristics.

	Patients with asthma	Control subjects
n	21	17
Mean age, years	34 (20-67)	29 (23-40)
Sex, M/F	16/5	7/10
FEV_1, % predicted	88 (36-123)	92 (82-114)
FEV_1, mL	3354 (1164-4920)	3670 (2430-4130)
$PC_{20}M$, mg/mL	0.80 (0.3-6.2)	ND
Atopy, *n*	18/21	0/17
Therapy	21 NT	17 NT

Values are expressed as mean (range); $PC_{20}M$ is expressed as geometric mean (range). NT indicates not treated (short-acting β_2 agonists on demand); ND, not done; $PC_{20}M$, methacholine concentration inducing a 20% fall in FEV_1.

MMP-9 was reported to be the predominant MMP in asthma (13). The recent generation of MMP knockout mice has provided interesting tools to delineate the implication of MMPs in asthma (14,15). In a recent genomic study performed on 460 families, a locus linked to asthma and airway hyperresponsiveness was identified on chromosome 20. Subsequent analysis of polymorphisms in 23 genes in the p13 region of chromosome 20 led to the identification of ADAM-33 as a susceptibility gene in the pathogenesis of asthma (16). Using a microarray detection system for gene expression in a murine model of asthma, King et al. (17) demonstrated recently that ADAM-8 is overexpressed in experimental asthmatic lungs. The putative contribution of other ADAMs and ADAMTSs is not known.

ADAMs and ADAMTSs are thus in many aspects similar to MMPs that have been extensively studied in asthmatic pathology, and some members of the ADAM and ADAMTS family have been reported to be asthma-associated genes. In this context, the aim of the present work was to assess the expression of ADAM and ADAMTS proteases in human asthmatic pathology. We used RT-PCR performed on sputum cells to unveil some differences in locally expressed genes that could have been missed when performing micro-arrays. Studied proteases were selected among ADAMs and ADAMTSs on the basis of a documented ectodomain sheddase activity and a reported role in inflammation or in lung development.

The expression profile of ADAM and ADAMTS proteinases was established by semiquantitative RT-PCR analysis on RNA extracted from cells from induced sputum of patients with asthma and healthy control subjects.

MATERIALS AND METHODS

Patients

Twenty-one patients with asthma and 17 healthy control subjects were included in this study (Table 1). Patients were classified as having mild or moderate asthma following the GINA Guidelines (http://www.ginasthma.com/). Asthma diagnosis was established by symptoms of wheezing, cough, and/or breathlessness. Twenty-three men and 15 women with a mean age of 32 years (range, 20 to 67 years) were included in this study. Patients with asthma and healthy individuals were nonsmokers, did not experience any exacerbation in the last 3 months, and were not treated by drugs other than short-acting β_2 agonists on demand. Some subjects had asthma requiring controller therapy, and the sputum was obtained at their initial evaluation before anti-inflammatory therapy. All subjects underwent a prick test with common aeroallergens and a PC_{20} methacholine ($PC_{20}M$) measurement if their FEV_1 was > 70%. All subjects gave written informed consent, and the protocol of the study was approved by the local ethics committee from CHU, Liège.

Sputum Induction and Processing

As previously reported (13,18), subjects inhaled saline solution aerosols (NaCl 4.5%) or isotonic saline solution (NaCl 0.9%), depending on baseline FEV_1 values, for a fixed period of 15 or 20 min after premedication with 400 μg inhaled salbutamol. An ultrasonic nebulizer (Devilbiss 2000) with an output of 1.5 mL/min was used to perform aerosols. Measurement of FEV_1 was performed before the procedure and after every 5-min period. The inhalation of saline solution was discontinued if the decrease in FEV_1 was < 20% from the basal state. The patients were asked to rinse their mouths with tap water every 5 min. Samples were collected in a plastic container and kept at 4 °C. Samples were diluted twice with PBS containing 10 mM dithiothreitol (Calbiochem, San Diego, CA, USA) and centrifuged at 400*g* for 10 min at 4 °C to separate cellular and fluid phases. Cell counts were estimated on samples centrifuged (Cytospin) and stained with Diff Quick coloration (Dade, Brussels, Belgium). Cell viability was measured using the trypan blue exclusion method. Supernatants were stored at –80 °C until analysis.

Semiquantitative RT-PCR

Expression levels of ADAM and ADAMTS mRNAs were determined by semiquantitative RT-PCR using the GeneAmp thermostable RNA RT-PCR kit (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed on 10 ng total RNA at 70 °C during 15 min followed by 2 min of incubation at 95 °C for denaturation of RNA-DNA heteroduplexes. Total RNA was obtained from the sputum cell pellets using the cesium chloride ultracentrifugation method (19). The design of oligonucleotide primers specific for the different targets was based on sequences available in GenBank (http://www4.ncbi.nlm.nih.gov/PubMe

d/). Primers were designed to anneal to distinct exons, and the specificity of the selected sequences was verified with the NCBI BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/). Selected primers were obtained from Eurogentec (Seraing, Belgium): 5′-GTGAATCACG TGGACAAGCTAT-3′ (sense) and 5′-TTCTTGCTGTGGTCCTGGTTCA-3′ (antisense) for ADAM-8; 5′-AGAAG AGCTGTCTTGCCACAGA-3′ (sense) and 5′-TTTTCCCGCCACTGCACGAAGT-3′ (antisense) for ADAM-9; 5′-TTTGG ATCCCCACATGATTCTG-3′ (sense) and 5′-GGTTGGCCAGATTCAACAAA AC-3′ (antisense) for ADAM-10; 5′-GGAATTGTCATGGACCATTCAG-3′ (sense) and 5′-TTCCTGCTGCAACTG CTGAACA-3′ (antisense) for ADAM-12; 5′-AACATGGACCACTCCACCAGCA-3′ (sense) and 5′-TTCGAAGAGGCAGCT GCCCATT-3′ (antisense) for ADAM-15; 5′-TACAAAGGAAGCTGACCTGGTT-3′ (sense) and 5′-TTCATCCACCCTCGA GTTCCCA-3′ (antisense) for ADAM-17; 5′-ATGAGTGGCCTGATCACCCTCA-3′ (sense) and 5′-TGGTTCAAGTTTCGG TGCCGAG-3′ (antisense) for ADAM-33; 5′-CAGCCCAAGGTTGTAGATGGTA-3′ (sense) and 5′-TTCACTTCGATGTTG GTGGCTC-3′ (antisense) for ADAMTS-1; 5′-GAACCATGAGGACGGCTTCTCCT-3′ (sense) and 5′-GGCTGCAGCGGGACC AGTGGAA-3′ (antisense) for ADAMTS-2; 5′-GCCTGGCAGAAGAAGCTGAAC-3′ (sense) and 5′-GCTGTCCAGGAAGTC GGTGAT-3′ (antisense) for ADAMTS-15; 5′-TGCCAGTGGCAGTCTGGATTG-3′ (sense) and 5′-GCAAGGCAGATAGCT TGAGCG-3′ (antisense) for ADAMTS-16; 5′-GCTTCTGTCACTGGCAGAACG-3′ (sense) and 5′-GCGTACTGTGTGCTG GCTTCT-3′ (antisense) for ADAMTS-17; 5′GGCAAGAGACATGATCATGCC-3′ (sense) and 5′-ATACTGTCCTGCTTG CTTGGG-3′ (antisense) for ADAMTS-18; 5′-GTGCAGGTCAATCTTCGTGTG-3′ (sense) and 5′-AGCACACGATGGGTG GTCATT-3′ (antisense) for ADAMTS-19; 5′-CATCCTGTTGTTGCTGTGGCTGAT-3′ (sense) and 5′-GTCATCTTGATCTCA TAACGCTGG-3′ (antisense) for TIMP-1; 5′-CTTCTGCAACTCCGACATCGTGAT-3′ (sense) and 5′-CAGCAGGTACTGGTA CTTGTTGAC-3′ (antisense) for TIMP-3; 5′-CCTTCACAGGTCTGCCCTGTAA-3′ (sense) and 5′-TCGTCAGGCAGACTT GTGGTTT-3′ (antisense) for RECK; and 5′-GTTCACCCACTAATAGGGAA CGTGA-3′ (sense) and 5′-GATTCTGACT TAGAGGCGTTCAGT-3′ (antisense) for 28S. PCR products obtained with each pair of primers were digested with appropriate restriction enzymes to verify the specificity of amplification. PCR amplification conditions were optimized so that PCR products did not reach any saturation levels. Amplification started at 94 °C for 15 s, 68 °C for 20 s, and 72 °C for 10 s and was terminated by 2 min at 72 °C. Products were then resolved on polyacrylamide gels (10%) and stained with Gel Star (Biowhittaker, Rockland, MD, USA). With Quantity One software (Biorad, Hercules, CA, USA), we analyzed the intensity of each band. To normalize mRNA levels, the value of the band corresponding to each mRNA level was divided by the intensity of the corresponding 28S rRNA band used as an internal standard.

Western Blot Analysis

Western blots were performed on sputum supernatants that were loaded on gels respective to their protein contents (20 μg protein/lane) to normalize the method. Samples were migrated on a 12% polyacrylamide gel and transferred on nitrocellulose PDVF membrane (Perkin Elmer Life Sciences, CA, USA). Blockade with 10% milk/PBS-Tween was carried out during 2 h. The primary antibody [anti-ADAMTS-1 antibody diluted 1/300 (Sigma), anti-TIMP-1 antibody diluted 1/500 (Santa Cruz), and anti-TIMP-3 antibody diluted 1/500 (Chemicon)] was applied on membranes overnight. Proteins were detected by chemiluminescence after incubation with the secondary antibodies diluted 1/1000 [TIMP-3: rabbit anti-mouse, ADAMTS-1: rabbit anti-goat-IgG, and TIMP-1: swine anti-rabbit (all Dako, Denmark)].

Detection of sCD23 in Sputum Supernatants

ELISA measurement of the soluble form of CD23 (sCD23) was performed in the sputum fluid phase of patients with asthma and control subjects using a commercial ELISA (Bender MedSystems). Microwell strips were coated with a murine monoclonal antibody against human sCD23. Samples were incubated and wells were washed with the wash buffer provided. A biotin-conjugated monoclonal anti-sCD23 was added and incubated for 2 h. Streptavidin/HRP was added for 1 h. A colorimetric reaction did occur and was stopped by adding the stop solution provided. Absorbance was measured at 450 nm.

Immunostaining

Slides obtained by cytocentrifugation as described above were fixed in 4% paraformaldehyde and treated with PBS containing 0.5% Triton X-100. Slides were incubated during a 1-h period in a goat anti–ADAM-8 antibody (Santa Cruz Biotechnology) at a dilution of 1/500 and, after rinsing, in a rabbit anti-goat antibody (Dako, Denmark) coupled with streptavidin/biotin.

Statistical Analysis

Results of cell counts are expressed as median (range). mRNA levels are reported as mean ± SEM. Differences between level of ADAM and ADAMTS proteinase expression in patients with asthma and in healthy individuals were evaluated by the Mann-Whitney test. Correlations were sought by calculating Spearman's coefficient of correlation. Values of $P < 5\%$ were considered statistically significant.

RESULTS

Characteristics of Patients

Patients with asthma and healthy individuals had similar mean ages. The patients with asthma were classified as having mild to moderate asthma according to GINA guidelines and all subjects were selected among nonsmokers.

Table 2. Total and differential cell counts in sputum of asthmatic and control patients.

	Patients with asthma	Control subjects
Sputum weight, g	4.35 (1.43-8.92)	3 (0.75-6.74)
Viability, %	92 (77-100)	92 (71-100)
Squamous cells, %	10 (0-33)	5 (0-13)
Total nonsquamous cell, $\times 10^3$/g	1150 (260-25390)	1457 (150-2180)
Macrophages, %	41.6 (4-83)[a]	59.8 (25-76.4)
Macrophages, $\times 10^3$/g	550 (85-5388)	761 (334-1513)
Eosinophils, %	5.2 (0-67)[a]	0.2 (0-7.2)
Eosinophils, $\times 10^3$/g	59 (0-3555)[a]	2.1 (0-94)
Neutrophils, %	23.2 (1.4-80.6)	17 (5-55)
Neutrophils, $\times 10^3$/g	343 (7-20464)	187 (42-1150)
Lymphocytes, %	1.8 (0-5.2)[a]	4.4 (0-13)
Lymphocytes, $\times 10^3$/g	16 (0-196)	45 (0-170)
Epithelial cells, %	8 (1.4-48.4)	6.8 (1.8-33.2)
Epithelial cells (10^3/g)	145 (25-465)	131 (32-375)

Data are expressed as median (range). [a]$P < 0.05$ vs. control subjects.

The mean value FEV_1 of patients with asthma was not significantly different from that of control subjects (Table 1). We found that 12 of 21 patients with asthma did require high or moderate doses of inhaled steroids after their initial evaluation, during which sputum was obtained.

Cell Counts in Induced Sputum

Total cell numbers and cellular composition of induced sputum were compared between the groups (Table 2). As expected, eosinophil counts were higher in patients with asthma than in healthy individuals ($P < 0.05$). Moreover, in patients with asthma, the percentage of macrophages and lymphocytes was reduced compared with control subjects, although these differences were not significant for absolute cell counts.

Semiquantitative RT-PCR

mRNA expression levels of ADAM-8, -9, -10, -12, -15, -17, and -33, ADAMTS-1, -2, -15, -16, -17, -18, and -19, TIMP-1 and -3, and RECK were assessed by semiquantitative RT-PCR in asthma and control samples (Table 3). Levels of ADAM-8, ADAM-9, ADAM-12, TIMP-1, and TIMP-3 mRNAs were expressed in significantly higher amounts in patients with asthma than in healthy individuals (Figure 1). In sharp contrast, mRNA transcripts for ADAMTS-1, ADAMTS-15, and RECK were significantly decreased in sputum from patients with asthma compared with control subjects (Figure 2). ADAM-10, -15, and -17 mRNAs were expressed in both patients with asthma and control subjects, with no significant difference detected between the two groups. The other ADAM and ADAMTS proteinases (ADAM-33, ADAMTS-2, -16, -17, -18, -19) were not detectable in our samples.

Patients with asthma requiring therapy with high or moderate doses of anti-inflammatory agents had significantly higher RECK and ADAMTS-15 expression in sputum cells compared with those who did not require a high dosage of inhaled steroids (defined as a dose of budesonide of less than 400 μg/day or a dose of fluticasone of less than 250 μg/day) ($P < 0.05$). No differences were found regarding the other proteases or inhibitors assessed.

Western Blot Analysis

We assessed the proteins corresponding to ADAMTS-1, TIMP-1, and TIMP-3, which are secreted mediators, in sputum fluid phase. We found detectable amounts of these molecules in most of our samples. Corroborating the results obtained by RT-PCR measurements of mRNA levels, bands corresponding to ADAMTS-1 were significantly decreased in patients with asthma compared with control subjects, and bands corresponding to TIMP-1 were increased in fluid phase from patients with asthma. TIMP-3 protein measurement in sputum fluid phase was not different between the groups (Figure 3).

sCD23 Detection in Sputum Supernatants

CD23 is a substrate for ADAM-8, and we report here for the first time that levels of the cleaved form of CD23, soluble CD23 (sCD23), were significantly increased in sputum from patients with

Table 3. mRNA levels.

	Patients with asthma	Control subjects
ADAM-8	0.396 ± 0.042[a]	0.080 ± 0.007
ADAM-9	0.677 ± 0.018[a]	0.617 ± 0.013
ADAM-10	0.497 ± 0.016	0.547 ± 0.015
ADAM-12	0.145 ± 0.012[a]	0.090 ± 0.011
ADAM-15	0.684 ± 0.019	0.637 ± 0.022
ADAM-17	1.127 ± 0.024	1.113 ± 0.026
ADAMTS-1	0.184 ± 0.021[a]	0.273 ± 0.023
ADAMTS-15	0.188 ± 0.025[a]	0.522 ± 0.029
TIMP-1	0.522 ± 0.037[a]	0.270 ± 0.017
TIMP-3	0.425 ± 0.047[a]	0.150 ± 0.018
RECK	0.457 ± 0.030[a]	0.644 ± 0.035

mRNA levels were determined in asthmatic and control samples by semiquantitative RT-PCR and normalized to 28S rRNA. Data are expressed in arbitrary units as mean ± SEM. [a]$P < 0.05$ vs. control subjects.

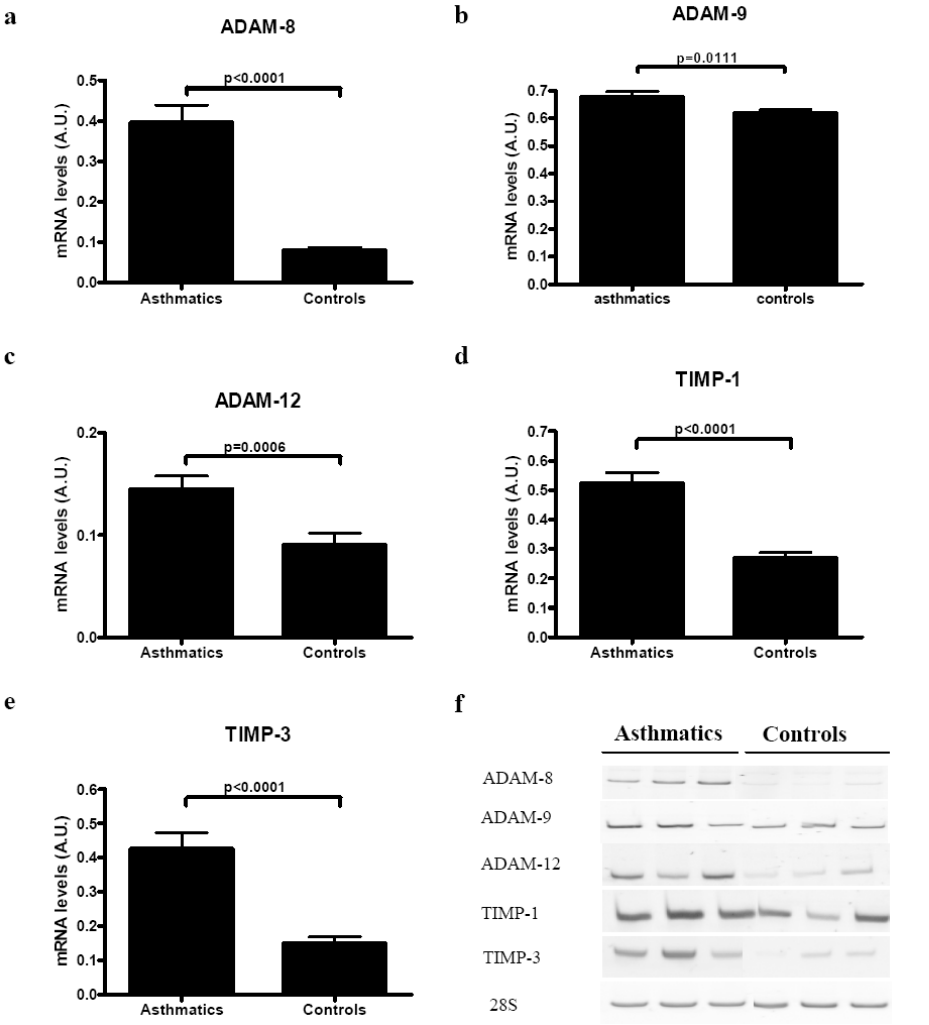

Figure 1. Statistical analysis of mRNA levels in sputum cells from patients with asthma measured by semiquantitative RT-PCR. Results are expressed in arbitrary units (AU) corresponding to density obtained for the band corresponding to measured mRNA further divided by the density of the band corresponding to 28S rRNA in the same sample (A-E). Representative examples of mRNA transcripts for different members of ADAM proteases and different inhibitors. Semiquantitative RT-PCR was performed on 10 ng total RNA from sputum cells (F).

asthma compared with control subjects ($P < 0.05$) (Figure 4).

Correlation of ADAM, ADAMTS, RECK, FEV_1, and Inflammation

In patients with asthma, ADAM-8 mRNA expression was negatively correlated with FEV_1 ($r = –0.57$, $P = 0.007$), indicating that a worsened asthma phenotype was associated with ADAM-8 overexpression (Figure 5A). In sharp contrast, amounts of ADAMTS-1 and RECK mRNA transcripts were both positively correlated with FEV_1 ($r = 0.45$ and $P = 0.04$, $r = 0.55$ and $P = 0.01$, respectively) (Figure 5B,C). In addition, ADAM-8 expression in sputum cells from patients with asthma was correlated with eosinophils ($r = 0.5$, $P = 0.02$) (Figure 5D) but also with neutrophils ($r = 0.56$, $P < 0.05$). ADAMTS-1 and RECK expression was inversely correlated with sputum neutrophils ($r = –0.57$, $P < 0.01$) and eosinophils ($r = –0.54$, $P < 0.05$), respectively. TIMP-3 expression was correlated with sputum eosinophils ($r = 0.69$, $P < 0.001$) (data not shown). Furthermore, ADAMTS-1 mRNA levels were significantly correlated with RECK mRNA levels in patients with asthma ($r = 0.6$; $P = 0.03$) (Figure 5E).

ADAM-8 Immunostaining

Because a positive correlation was found between ADAM-8 mRNA expression and eosinophil counts, anti–ADAM-8 immunohistochemistry was performed to document eventual ADAM-8 production by eosinophils. As illustrated in Figure 6, an intense staining of eosinophils was observed in sputum cells, confirming that eosinophils are able to produce ADAM-8.

DISCUSSION

We describe for the first time an increase in the expression of several members of the ADAM protease family in sputum cells from patients with asthma. In sharp contrast, we also demonstrate that the expression of other members of this family is downregulated in this disease. Indeed, we found levels of expression of ADAM-8, -9, and -12, along with physiological inhibitors of many ADAMs, TIMP-1 and -3, to be overexpressed in sputum cells from patients with asthma compared with matched healthy subjects. On the contrary, ADAMTS-1, -15, and RECK expression was decreased in sputum from patients with asthma.

The enhancement of ADAM-8 expression in sputum cells from patients with asthma is in line with a recent study performed on a mouse model of asthma (17). Indeed, increased ADAM-8 expression was evidenced by in situ hybridization in the peribronchial area of animals sensitized and exposed to experimental allergens. The study demonstrated also that IL-4 and IL-13 induced an overexpression of ADAM-8, suggesting that ADAM-8 could play a major role in the interplay between Th2 cytokines and lung inflammation (17). ADAM-8 appears as a multifunctional protease.

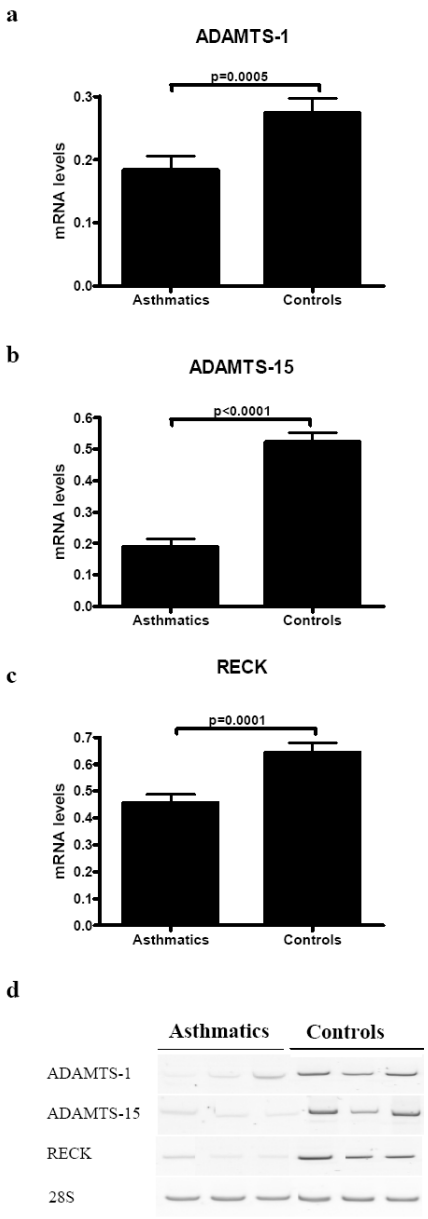

Figure 2. Quantitative analysis of decreased mRNA levels in cells from asthmatic sputum measured by semiquantitative RT-PCR. Results are expressed in arbitrary units (AU) and normalized with 28S values for each sample (A-C). Representative examples of semiquantitative RT-PCR for some ADAMTS proteinases and RECK realized on 10 ng total RNA from sputum cells (D).

Membrane–bound ADAM-8 cleaves the low-affinity IgE receptor (CD23), leading to the release of sCD23 (20). sCD23 could play a significant role in the control of inflammatory cascade leading to asthmatic phenotype, because sCD23 is capable of binding IgE and plays multiple non–IgE-related roles, regulating cell–cell interaction and cytokine production (21). sCD23 regulates at least the activation of monocytes and lymphocytes (21-23). sCD23 also appears to be implicated in regulatory loops leading to TNF-α, IL-1β, IL-8, and granulocyte-macrophage colony-stimulating factor production (23,24). Moreover, a very recent study demonstrated that segmental allergen challenges lead to an increase of sCD23 in bronchoalveolar lavage of patients with asthma, suggesting a role for a protease such as ADAM-8 in regulatory loops attempting to control allergen-induced inflammation (25).

In our study, we report for the first time increased levels of sCD23 in sputum supernatants from patients with asthma compared with control subjects; no correlation was found between sCD23 measurements in fluid phase and ADAM-8 expression in cell pellets. This lack of correlation is to our view not sufficient to rule out a putative relationship between ADAM-8 overexpression in sputum cells and CD23 shedding. The biological significance of such a lack of correlation between a protein and mRNA expression is potentially limited by possible posttranslational modifications of the molecules and, most importantly in the present study, by the fact that ADAM-8 expression is measured in a mixture of cells (sputum pellets) that does not allow identification of the relative contributions of different cell types to ADAM-8 expression and potentially to CD23 shedding (20). ADAM-8 mRNA expression was, however, correlated with eosinophil numbers in the sputum, and eosinophils were stained with an anti–ADAM-8 antibody, suggesting that those cells could be a major source of this protease. One can thus speculate that ADAM-8 could be a part of a complex regulatory system leading to an adaptive reaction following allergen exposure. Our data do not exclude that ADAM-8 could take part in the cascade

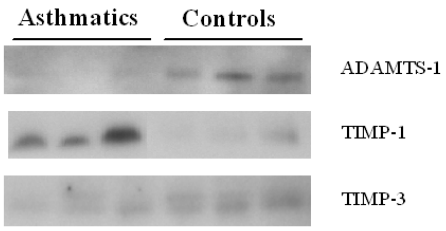

Figure 3. Representative examples of Western blots obtained for secreted proteins (ADAMTS-1, TIMP-1, and TIMP-3) in the sputum fluid phase of asthmatic and healthy patients.

of pro-inflammatory mediators in asthma.

ADAM-9 mRNA levels were increased in sputum cells from patients with asthma. ADAM-9 expression was previously reported to be increased in breast and prostate carcinomas. The potential role played by ADAM-9 in inflammation could relate to its ability to bind α_v-β_5 integrin, which is implicated in some inflammatory disorders (26). The potential role of ADAM-9 in asthma could also be ascribed to its potential capacity to activate TNF-α, one of the mediators most crucially implicated in asthma pathogenesis (27,28). Nevertheless, in the present study we did not raise any argument suggesting that ADAM-9 could be a prominent TNF-α activator over ADAM-17 (also

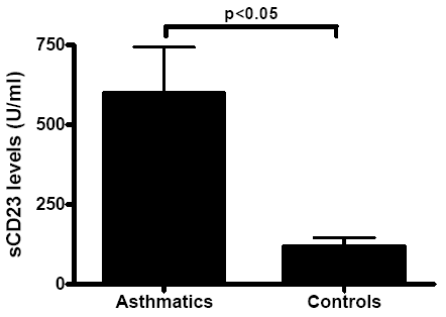

Figure 4. sCD23 measured by ELISA in the sputum fluid phase of patients with asthma and healthy control subjects (mean ± SEM).

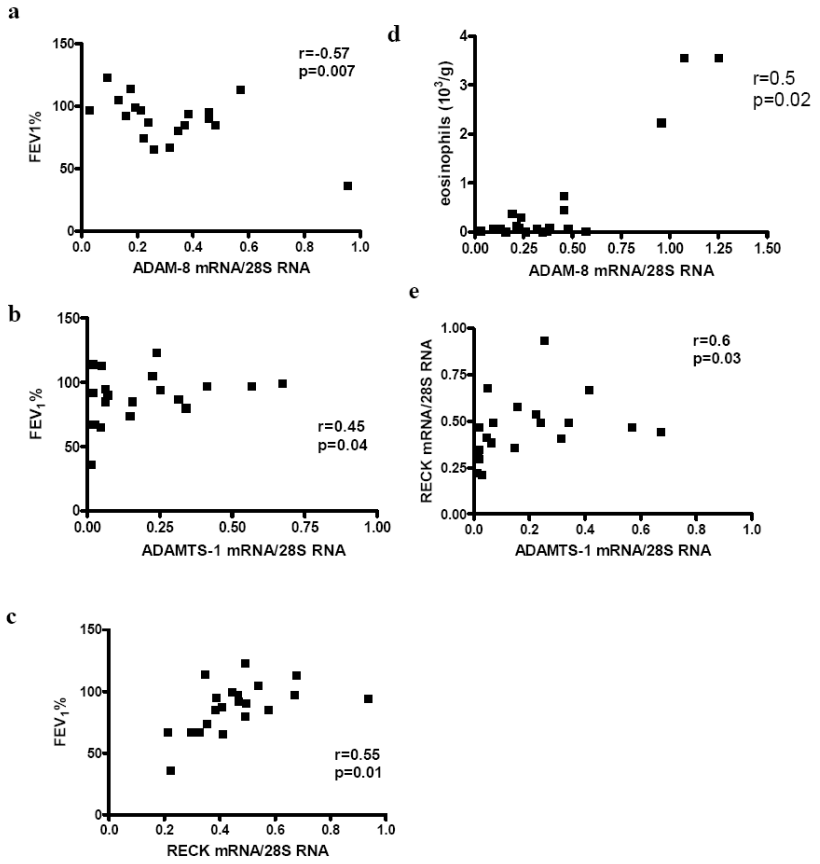

Figure 5. (A) Relationship between ADAM-8 mRNA levels and FEV_1 (%) in patients with asthma. (B) Relationship between the level of ADAMTS-1 mRNA and FEV_1 (%) in asthmatic population. (C) Correlation between RECK mRNA expression and FEV_1 (%) predicted. (D) Relationship between ADAM-8 expression and eosinophils (absolute cell counts). (E) Relationship between the ADAMTS-1 mRNA:28S RNA ratio and the RECK mRNA:28S RNA ratio.

related to TNF-α converting enzyme) or ADAM-10.

ADAM-12, which is a protease widely expressed by cancer cells (29), was overexpressed in cells issued from asthmatic sputum. This study is the first report of a potential implication of ADAM-12 in an inflammatory process in humans. The only other report about an increased expression of ADAM-12 in inflammation came from a study of a murine model of encephalomyelitis (30). ADAM-12 could contribute to the regulation of inflammation through modulation of cell–cell interactions by its ability to bind integrin α_9-β_1, which is able to bind specific extracellular matrix components such as tenascin or fibronectin (31).

Although ADAM-33 has recently been reported as an asthma-associated gene, we were not able to detect the expression of this protease in sputum. This finding is expected because ADAM-33 is expressed mainly in mesenchymal cells (fibroblasts, smooth muscle cells, etc.) but not in the inflammatory cells and bronchial epithelial cells found in sputum (16,32). Nevertheless, some authors have very recently reported that amounts of ADAM-33 protein in bronchoalveolar lavage from patients with asthma are correlated with lung function loss, suggesting that soluble ADAM-33 (which is not explored in the present study) could play a role in the modulation of airway caliber in asthma (33).

Of great interest is the significant decrease in ADAMTS-1 levels detected at the mRNA level in the sputum cells of patients with asthma and at the protein level in sputum supernatants from patients with asthma. ADAMTS-1 is a secreted protease activated by furin cleavage of its prodomain in the secretory pathway (34). ADAMTS-1 probably exerts many functions, because it was reported to inhibit angiogenesis by sequestration of VEGF, to be overexpressed in wounded skin, and to degrade cartilage components (6,35). Moreover, ADAMTS-1 could play a crucial role in inflammatory processes, because its production is greatly enhanced by some pro-inflammatory mediators such as LPS in vitro (10). ADAMTS-1 could play a potential role in the cascade of events leading to bronchial remodeling in asthma. Indeed, fibroblast growth factor (FGF)-2 was reported as a potentially important mediator in the pathogenesis of subepithelial fibrosis, because it was detected in higher amounts in the subepithelial area from humans with asthma (36). FGF-2 bioactivity appears to be controlled by ADAMTS-1, and high amounts of this protease inhibit FGF-2 by forming

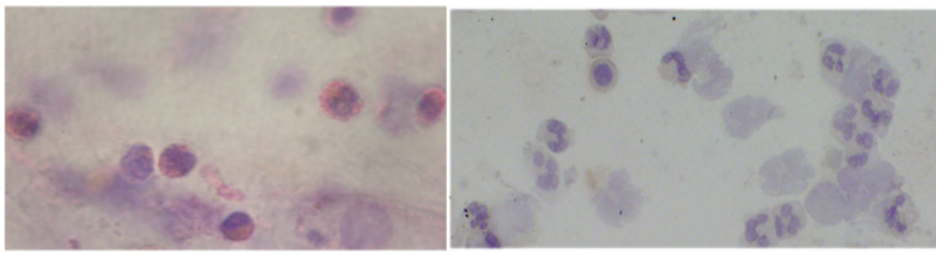

Figure 6. Immunostaining of sputum cells using an anti–ADAM-8 antibody. Left panel shows a representative example of a sputum cytospin incubated with the anti–ADAM-8. Right panel shows an analogous sample, incubated with a nonimmune control antibody.

complexes and subsequently inhibit its fibrogenic activity (35). In patients with asthma, one can hypothesize that low levels of ADAMTS-1 in bronchial walls would lead to unrepressed FGF-2 stimulation of fibroblast activity and production of extracellular matrix and, in the end, bronchial remodeling. A potential implication of ADAMTS-1 in the inhibition of bronchial remodeling by modulations of the extracellular matrix production is also suggested by the positive correlation found in the present study between ADAMTS-1 mRNA expression and FEV_1. This correlation reinforces the hypothesis of a protective effect of ADAMTS-1 against extracellular matrix changes in the bronchial walls.

We describe here that ADAMTS-15 mRNA expression was decreased in sputum cells from patients with asthma. ADAMTS-15 was first described as an aggrecanase displaying the ability to degrade collagen II (6). Interestingly, in line with our findings, levels of ADAMTS-15 mRNA were decreased in osteoarthritis compared with normal joints (37). Our results suggest a role for ADAMTS-15 in asthma-related inflammation, and one can thus speculate that a substrate other than collagen II could exist for ADAMTS-15.

We assessed the expression and production of TIMP-1 and -3 because those two proteins have been reported as strong inhibitors of many members of the ADAM and ADAMTS proteases family, whereas TIMP-2 and -4 did not display such an activity (38). TIMP-1 levels were increased in the sputum from patients with asthma at the mRNA and protein levels. This finding has already been described previously (12). The attention was drawn on TIMP-1 because of its role in MMP, and particularly MMP-9, inhibition. It appears now that the situation is much more complex and that this molecule could establish links between MMPs and ADAM-ADAMTS systems. Moreover, TIMP-1 can bind some pro- and activated forms of MMPs, being responsible for the fine tuning of MMP activity. To date, no such mechanisms have been described for ADAMs, but TIMP-1 and TIMP-3 could be responsible for ADAM-ADAMTS modulation. We describe here for the first time that TIMP-3 expression was increased in sputum cells from patients with asthma. This suggests that TIMP-3 could be implicated in the cascade of events leading to the asthmatic phenotype. TIMP-3 appears generally as an extracellular matrix–bound protein, and our results clearly indicate that it is produced by immune and inflammatory cells and that its production is not restricted to cells embedded in the extracellular matrix. We did not assess ADAM:TIMP or ADAMTS:TIMP ratios, suggested in some published papers as being indicative of the "net" MMP activity, because it appears now that those inhibitors are capable of restraining the activity of dozens of proteases. In this context, the biological and pathological significance of such a ratio between one TIMP and one ADAM or ADAMTS is questionable.

Another novelty of this work is the report that RECK, a membrane-bound MMP inhibitor, was found to be downregulated in the sputum cells from patients with asthma. The known roles of RECK are the inhibition of MMP-2, -9, and -14 (39,40) and a possible role in cancer development, because it was reported to inhibit both angiogenesis and metastasis progression (7,40). Our results suggest that RECK is expressed at the surface of cells contained in the bronchial lumen (mainly inflammatory cells) and could play a role in the control of inflammation. RECK levels were correlated with the expression of ADAMTS-1 and FEV_1 in our study. This suggests a protective role for that antiprotease against the impaired airway caliber.

In this study, we assessed by Western blot the amounts of secreted mediators (ADAMTS-1, TIMP-1, TIMP-3) in the sputum fluid phase, whereas membrane-bound mediators (ADAM-8, ADAM-9, ADAM-12, ADAM-15, ADAM-17, and RECK) have been explored exclusively by mRNA assessment.

As we designed this study as a proof-of-concept to demonstrate the interest of studying ADAM/ADAMTS in asthma, we used a technique that refers to the expression of mediators by a pool of different cells contained in the induced sputum (see Table 2). Some incomplete information is available from the literature on the respective production of cells present in the bronchial tree. For instance, ADAM-8 is reported to be expressed in polymorphonuclear cells, monocyte-macrophages, and stimulated epithelial cells (17,20); ADAM-12 is faintly expressed by normal bronchial epithelial cells (29); ADAM-17 is expressed by bronchial epithelial cells (41); RECK is present at the surface of macrophages (42); and TIMP-1 is produced by neutrophils, macrophages, epithelial cells, and lymphocytes (43-45).

Together, our results show that members of the ADAM and ADAMTS families are modulated in sputum from patients with asthma and, due to their varied potentialities, could play a role in the control of inflammation, airway remodeling, and bronchoconstriction. Our study implicates some ADAMs and ADAMTSs, modulation of which was not reported previously by other techniques. Further studies using animal models are required to confirm the implication and the precise role of those molecules in the pathogenesis of asthma and to validate putative new therapeutic targets.

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