

Increased TGF- β 2 in severe asthma with eosinophilia

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Background: Airway eosinophilia and thickened subepithelial basement membrane have previously been reported to increase with increases in TGF- β expression. However, little is known regarding the expression of specific TGF- β isoforms (TGF- β 1, TGF- β 2, and TGF- β 3) in asthma, despite recent evidence suggesting that isoforms may have differing biologic activities. **Objective:** This study examined airway tissue expression of the 3 TGF- β isoforms and several downstream pathway elements in 48 patients with severe asthma with or without persistent eosinophilia, 14 patients with mild asthma, and 21 normal subjects.

Methods: Immunohistochemistry/immunofluorescence, quantitative real-time PCR and enzyme immunoassay were used to evaluate the 3 TGF- β isoforms, their receptors, collagen I deposition, connective tissue growth factor expression, and tissue inhibitor of metalloproteinases 1 levels.

Results: Of the isoforms, only TGF- β 2 was different among the groups and increased in severe asthma (overall $P < .0001$). The increase was due to severe asthma tissue eosinophils which, unlike eosinophils in other groups, expressed high amounts of TGF- β 2. Subjects with severe asthma also had the thickest subbasement membrane and highest tissue inhibitor of metalloproteinases 1 levels. In contrast, TGF- β receptor 1 and connective tissue growth factor were both consistently downregulated in asthma, regardless of severity.

Conclusion: TGF- β 2, expressed mainly by eosinophils, is the predominant isoform expressed in severe asthma, and is associated with increased profibrotic responses. Decreased expression of TGF- β receptor 1 and connective tissue growth factor in all asthma severity groups suggests a degree of activation of the TGF- β pathway in airway tissue of all asthmatic compared with normal airways. (*J Allergy Clin Immunol* 2005;115:110-7.)

Key words: TGF- β , eosinophils, connective tissue growth factor

Tissue eosinophilia and a thickened subepithelial basement membrane (SBM) are hallmark pathologic features of asthma.^{1,2} Recent studies suggest that severe asthma with persistent eosinophilic inflammation is

Abbreviations used

BAL:	Bronchoalveolar lavage
CTGF:	Connective tissue growth factor
SBM:	Subbasement membrane
TGF- β R:	TGF- β receptor
TIMP-1:	Tissue inhibitor of matrix metalloproteinases 1

associated with a thicker SBM, even in the presence of high-dose oral corticosteroids.^{3,4} Although the precise mechanisms leading to SBM thickening are not clear, both increased deposition of matrix (through growth factor-induced collagen synthesis) and inhibition of tissue degradation (through imbalances in proteases and protease inhibitors) are likely to play important roles.^{5,6} TGF- β family members, with their ability to induce collagens, inhibit proteases, and induce inhibitors of proteases, are likely candidates to contribute to these remodeling changes.⁷ TGF- β 1, the most intensely studied family member, has been reported to be increased in association with eosinophils and fibrosis in asthmatic airways in mild to moderate asthma.⁸ In contrast, TGF- β 2, although reported to be produced by airway epithelial cells, has not been evaluated in submucosal tissue.^{9,10}

Collagen I and tissue inhibitor of matrix metalloproteinases 1 (TIMP-1), both of which are increased in asthma, are strongly induced by TGF- β .¹¹⁻¹³ Although the relation to disease severity in the lungs has not been confirmed, TIMP-1 has been reported to be increased in serum of patients with steroid-dependent asthma.^{14,15} Connective tissue growth factor (CTGF) is also induced in fibroblasts by TGF- β , but its expression in asthma has been studied to only a limited degree.¹⁶

Almost all cell types can respond to TGF- β family members. However, limited studies have suggested that the different isoforms may have differential functions, depending on cell type, maturity, and environment.¹⁷⁻²⁰ These differences suggest that understanding the pattern of different isoforms in diseased states is of importance, but no studies have yet been reported in asthma.²¹⁻²³

Because we previously reported increases in pan-TGF- β in severe eosinophilic asthma, we wished to understand better which isoform was increased and whether there was evidence for downstream activation of pathway elements that may be involved in tissue remodeling.³ Therefore, airway expression of the 3 TGF- β isoforms, their receptors, and 3 downstream markers of TGF- β activation—TIMP-1, SBM collagen deposition, and CTGF—were measured in biopsy tissue from subjects with severe

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TABLE I. Subject characteristics

	Sex, M/F	Age, y*	FEV ₁ † (% predicted)	Eosinophils, #/mm ² *	Oral steroid dose, mg/d*
Severe asthma					
Eosinophil ⁺ , n = 24	8/16	39 (28-53)	51% ± 4%	86 (43-118)	32 (20-50)
Eosinophil ⁻ , n = 24	15/9	33 (23-42)	55% ± 4%	0 (0-5)	30 (10-40)
Mild asthma, n = 14	6/8	35 (28-47)	86% ± 2%	16 (1-37)	—
Normal subjects, n = 21	12/9	27 (23-33)	—	2 (0-11)	—
Overall P value	.18	.03	<.0001	<.0001	.40

*Median (interquartile range).

†Mean ± SEM.

asthma with and without eosinophils, as well as subjects with mild asthma and control subjects. In addition, the source of the increased TGF- β 2⁺ cells in severe asthmatics was evaluated.

METHODS

Subjects

Severe asthma was defined by: (1) FEV₁ of $\leq 70\%$ predicted on more than 1 occasion over the previous year, (2) 10 mg/d prednisone or equivalent for at least 50% of the previous year while taking other standard asthma medications, (3) >2 urgent care visits for asthma in the previous year, and (4) ongoing symptoms.³ Patients with severe asthma were divided into those with persistent tissue eosinophilia (>22 eosinophils/mm² tissue, equivalent to ≥ 2 SDs from the mean value measured in normal controls) or not, as previously described.^{3,24} Mild asthma was defined as an FEV₁ $\geq 80\%$ predicted on β -agonists alone. Normal subjects had no history of any respiratory illness. There were no current smokers, but mild allergies and atopy was allowed. The study was approved by the National Jewish Institutional Review Board, and all subjects gave informed consent.

Bronchoscopy and tissue processing

Bronchoscopy with endobronchial biopsy and bronchoalveolar lavage (BAL) were performed as previously described.³ Unconcentrated BAL fluid samples were stored at -80°C . Biopsy tissues were fixed in acetone and embedded in glycol-methacrylate resin or fixed in 4% paraformaldehyde and embedded in paraffin.³

Immunostaining

Glycol-methacrylate-embedded tissue sections were immunostained by using primary antibodies detecting eosinophils (major basic protein, clone BMK-13; Accurate Chemical & Scientific Corp, Westbury, NY), collagen type I (Biodesign International, Kennebunkport, Me) and TGF- β isoforms TGF- β 1, TGF- β 2, and TGF- β 3 (all from Santa Cruz Biotechnology, Santa Cruz, Calif). Staining for TGF- β receptors (TGF- β Rs) TGF- β RI, TGF- β RII, TGF- β RIII (all from Santa Cruz Biotechnology) and CTGF (R&D Systems, Minneapolis, Minn) was performed on paraffin-embedded tissue sections that were deparaffinized, with antigens retrieved through heating in citrate buffer pH 5.5. Appropriate controls of immunostaining procedure by omitting primary antibodies were included.

To determine specific cell types expressing TGF- β 2 and TGF- β 1, dual immunofluorescent labeling techniques with appropriate fluorescein-conjugated (for cell markers) and rhodamine-conjugated (for TGF- β isoforms) secondary antibodies were applied (Dako, Carpinteria, Calif).

A Scion image analysis program (National Institutes of Health, Bethesda, Md) was used to obtain tissue morphometric data and determine cell counts (per square millimeter of submucosal tissue),

thickness of the SBM, and percentage of smooth muscle area stained for TGF- β Rs and CTGF (measured on tissue sections that were not counterstained).

Quantitative real-time PCR

Total cellular RNA was extracted from endobronchial tissue biopsies by using TRIzol reagent (Life Technologies, Rockville, Md). Reverse transcription followed by real-time PCR was performed for TGF- β 1 (predeveloped assay reagent catalog #4327054F and TGF- β 2, Assay On Demand, ID #Hs 00234344_m1; all from Applied Biosystems, Foster City, Calif). 18S ribosomal RNA served as the internal control. The threshold cycle was recorded and relative mRNA expression levels calculated by using the ΔCt method.

Enzyme immunoassay

Protein levels of TIMP-1 in unconcentrated BAL fluid were measured with commercial enzyme immunoassay kit (Oncogene, Boston, Mass). Undetectable levels of TIMP-1 in BAL fluid were reported as 0.1 pg/mL.

Statistical analysis

For the majority of parameters, nonparametric Kruskal-Wallis tests were used to determine differences among the groups. When there was a significant overall difference ($P \leq .05$), intergroup pairwise comparisons were performed by using the Dunn procedure. The log-transformed data for mRNA levels were normally distributed, and an ANOVA test was applied to compare the groups. Graphs in that instance present means and SEMs, which were reconverted back to their original scale. Spearman correlations (r_s) were used to measure the strength of association between variables. A value of $P \leq .05$ was considered significant. All testing was performed by using JMP or SAS (SAS Institute, Cary, NC).

RESULTS

Subject characteristics

Eighty-three subjects, including 24 eosinophil⁺ (eos⁺) and 24 eosinophil⁻ (eos⁻) subjects with severe asthma, were enrolled in this study (Table I). Control subjects were younger than subjects with asthma (overall $P = .03$). There was no difference in oral steroid doses between the 2 severe groups, and $\sim 25\%$ subjects in both groups were only on high-dose inhaled corticosteroids at the time of bronchoscopy.

Tissue expression of TGF- β 1, TGF- β 2, and TGF- β 3

TGF- β 1⁺ and TGF- β 3⁺ cell counts were similar in all groups (Fig 1, A and C). In contrast, TGF- β 2⁺ cells were

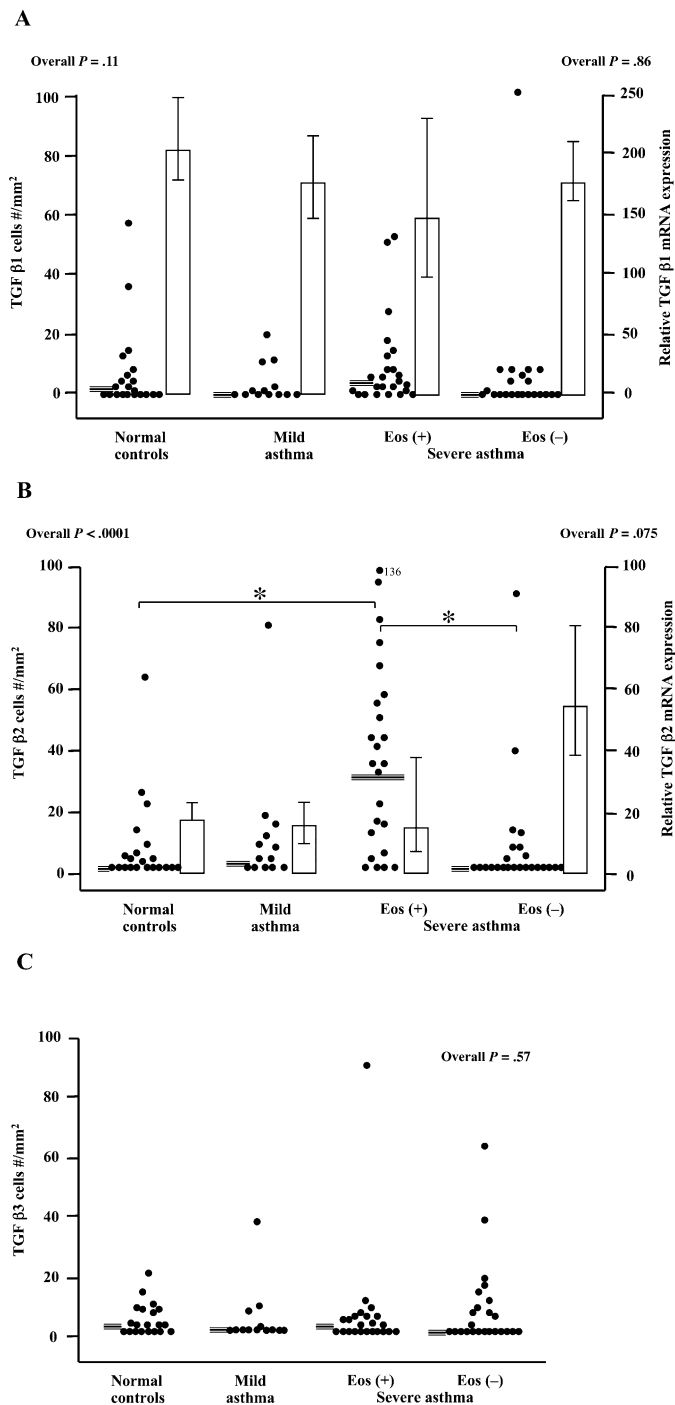


FIG 1. Numbers of positive cells (filled circles) and relative tissue mRNA expression (bars with SEM) for (A) TGF- $\beta 1$, (B) TGF- $\beta 2$, and (C) TGF- $\beta 3$ cells in airway submucosa of normal subjects, subjects with mild asthma, and subjects with severe asthma with and without persistent eosinophilia. * $P < .05$ for pairwise comparisons.

different between the groups (Fig 1, B; overall $P < .0001$). Eosinophil⁺ subjects with severe asthma had the highest number of TGF- $\beta 2$ ⁺ cells (32/mm² [10-53]), which was significantly higher than the TGF- $\beta 2$ ⁺ cell count in eos⁻ subjects with severe asthma and normal controls (0/mm² [0-6] and 2/mm² [0-7], respectively). Subjects with mild

asthma were intermediate and not significantly different from either severe asthma group or normal controls (4/mm² [0-12]). Although not analyzed in this study, TGF- $\beta 1$ and TGF- $\beta 2$ staining was also present in epithelial cells, and occasional TGF- $\beta 2$ staining was detected in smooth muscle.

Biopsy tissue samples from 13 normal, 12 mild, 10 eos⁻, and 5 eos⁺ subjects with severe asthma were analyzed for TGF- β 1 and TGF- β 2 mRNA expression by quantitative real-time PCR (Fig 1, A and B). TGF- β 1 mRNA levels, similar to cellular data, were not significantly different among groups ($P = .86$). TGF- β 2 mRNA tended to be higher in eos⁻ subjects with severe asthma (overall $P = .075$). There was no correlation between TGF- β 1/TGF- β 2 protein expression and their mRNA levels.

Identification of TGF- β 2⁺ cells

As noted, TGF- β 2⁺ cells were highest in eosinophil⁺ severe asthma. Their staining characteristics and distribution by immunocytochemistry suggested that virtually all positive cells were eosinophils. In addition, TGF- β 2⁺ cells in severe asthma strongly correlated with eosinophil cell counts ($r_s = 0.73$; $P < .0001$). Therefore, double immunofluorescence staining studies were performed to confirm that eosinophils were the primary source of TGF- β 2 in submucosal tissue. Indeed, tissue eosinophils in eosinophil⁺ severe asthma consistently colocalized with TGF- β 2⁺ cells, but that was not true for tissue eosinophils in mild asthma or occasional normal subjects with high eosinophil numbers (Fig 2, A-C).

TGF- β 1 could not be colocalized to tissue eosinophils in mild asthma or normal subjects either, and only a small fraction of eosinophils in severe asthma stained positive for TGF- β 1 (not shown). Along with those findings, there was no correlation between eosinophils and either TGF- β 1⁺ or TGF- β 2⁺ cells in mild asthma and normal subjects, whereas TGF- β 1 in severe asthma correlated with eosinophils ($r_s = 0.40$; $P = .005$).

TGF- β receptors and CTGF

These markers were evaluated in a subset of subjects that, besides acetone-fixed/glycol-methacrylate-embedded tissue, also had paraformaldehyde-fixed/paraffin-embedded tissue samples available. TGF- β RI, TGF- β RII, and CTGF expression were detectable in smooth muscle in all subject groups. TGF- β RIII was detectable only in low levels in the epithelium and was not further quantified.

Expression of TGF- β RI, TGF- β RII, and CTGF was measured in airway smooth muscle of 12 eos⁺ subjects with severe asthma, 11 eos⁻ subjects with severe asthma, 9 subjects with mild asthma, and 11 normal subjects. The mean ($\pm$ SEM) smooth muscle area analyzed in biopsy tissues was 0.11 ± 0.01 mm² for TGF- β RI and TGF- β RII, and 0.09 ± 0.01 mm² for CTGF. Figs 3 and 4 show a typical pattern of staining for markers quantified in Fig 5. Expression of TGF- β RI was apparently decreased in asthma regardless of severity compared with normal subjects, but comparison across the 4 subject groups did not reach statistical significance ($P = .08$). CTGF was also decreased in asthma, different across the 4 subject groups, and significantly higher in normal subjects than in eosinophil⁺ subjects with severe asthma (overall $P = .03$; respective groups were 7.4% [3.1-13] vs 0% [0-4.8]; $P = .02$). TGF- β RII was not significantly different among

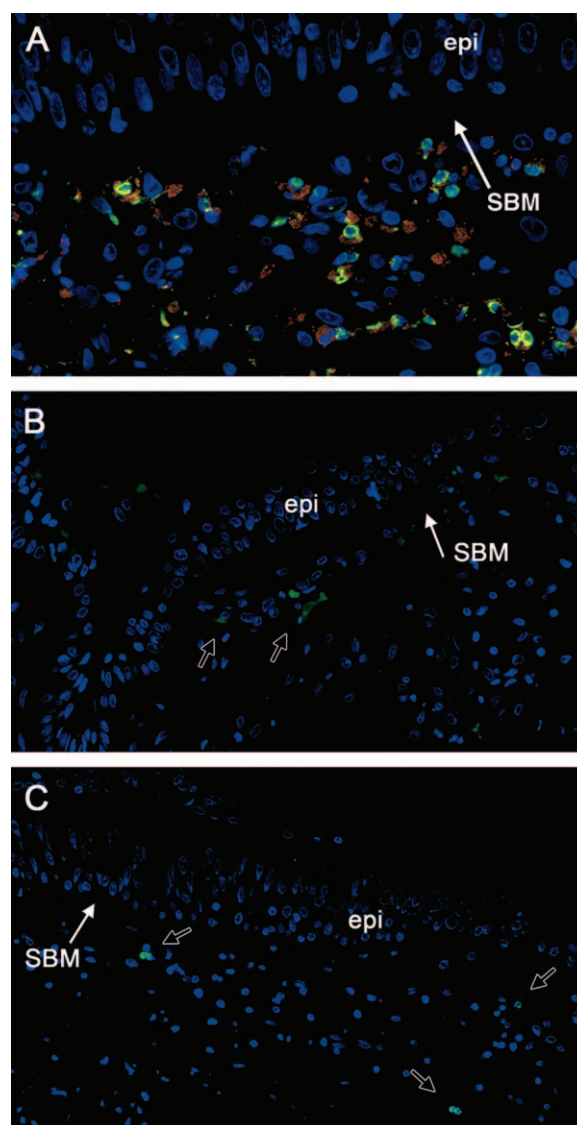


FIG 2. Double immunofluorescence of airway tissue colocalizes ~80% of TGF- β 2⁺ cells (red) with eosinophils (green) in an eosinophil⁺ subject with severe asthma (A). There are no TGF- β 2⁺ eosinophils (hollow arrows) in a subject with mild asthma (B) or a normal control (C). Filled arrows point at the epithelial layer (epi) and SBM. Original magnification 200 $\times$ for A, 100 $\times$ for B and C.

the groups ($P = .44$). Signal for TGF- β RII and CTGF was observed as a diffuse staining in epithelium as well.

TIMP-1 levels and SBM thickness

Eosinophil⁺ subjects with severe asthma had the highest levels of TIMP-1 in BAL fluid (overall $P = .007$), which was significantly higher than in all other groups (Fig 6, A). SBM thickness also differed among the groups (overall $P = .007$). However, although eos⁺ subjects with severe asthma had a thicker membrane ($8.56 \mu\text{m}$ [7-9.49]) than subjects with mild asthma ($5.78 \mu\text{m}$ [4.38-7.24]) or normal subjects ($6.12 \mu\text{m}$ [4-7.95]), there was no significant difference between eos⁺ or eos⁻ severe asthma

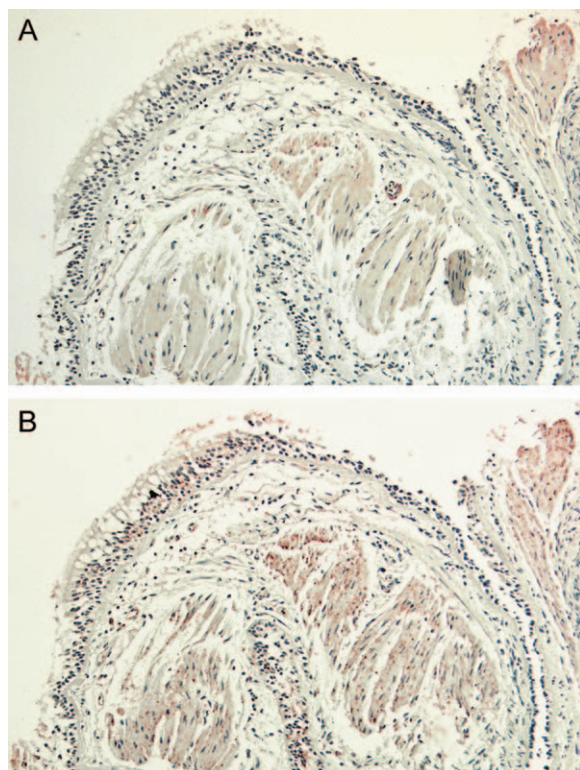


FIG 3. Representative photographs of (A) TGF- β RI and (B) TGF- β RII immunostaining in airway smooth muscle. Original magnification 100 $\times$.

(Fig 6, B). Both TGF- β 1⁺ and TGF- β 2⁺ cells in tissue correlated with TIMP-1 levels in BAL fluid (correlations with all subjects included $r_s = 0.23$; $P = .04$; and $r_s = 0.40$; $P = .0004$, respectively). Interestingly, there was no direct relationship between TGF- β 1⁺ or TGF- β 2⁺ cells and SBM thickness.

DISCUSSION

The current study, designed to evaluate specifically TGF- β isoforms and TGF- β -related pathways in asthma, is the first to identify TGF- β 2 as a prominent TGF- β isoform in severe asthma and its association with eosinophils. TGF- β 2 expression in eosinophils was specific to severe asthma, because TGF- β 2 could not be colocalized to tissue eosinophils in mild/nonsteroid-treated subjects with asthma or normal subjects. This study further reports evidence for activation of downstream TGF- β -related pathways, including high levels of the profibrotic factor TIMP-1 and a thicker SBM. Finally, we describe a decrease in CTGF and TGF- β RI expression in airway smooth muscle of patients with asthma regardless of severity or inflammatory pattern. Previous studies reported increased TGF- β 1 and pan-TGF- β expression in tissue inflammatory cells of patients with asthma, but no other isoforms were studied.^{8,25,26} In contrast, we report no difference in protein expression of TGF- β 1 (or TGF-

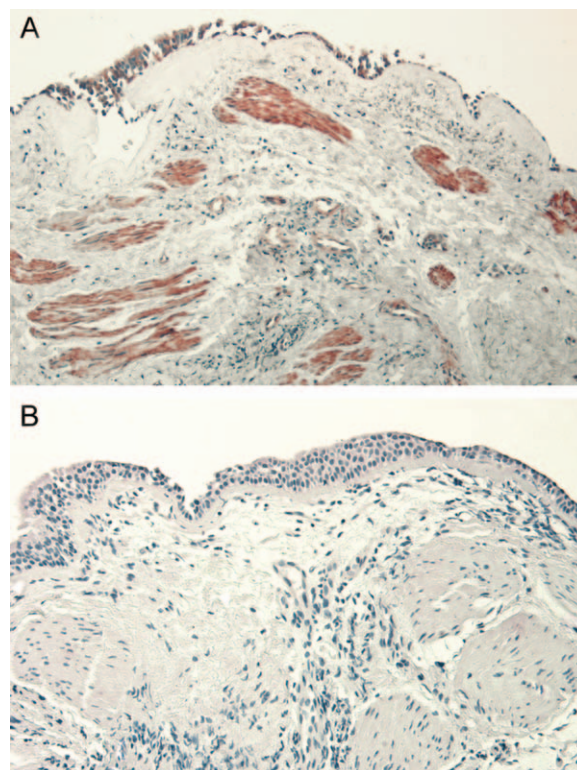


FIG 4. Immunostaining for CTGF in endobronchial biopsy tissue of a normal control (A) and a subject with severe asthma (B). Original magnification 100 $\times$.

β 3) between normal controls and patients with asthma of varying severity. The difference between these studies may be a result of the substantially larger numbers of subjects per group in the current study, differences in the subject population, and the different pathology methods used.⁸ Although Minshall et al⁸ reported colocalization of TGF- β 1 mRNA with eosinophils, colocalization with protein was not evaluated. In this study, TGF- β 1 protein was rarely colocalized to eosinophils, and then only in severe asthma. This low level of TGF- β 1 expression by tissue eosinophils is consistent with the observation that even after allergen challenge, no more than 50% of eosinophils in the skin of atopic subjects were positive for TGF- β 1, and that this percentage decreased over time despite persistent eosinophilia.²⁷ Interestingly, preliminary observations on eosinophils in nontissue compartments suggest that neither severe asthma or normal BAL eosinophils, nor normal peripheral blood eosinophils stain positive for any of the TGF- β isoforms. However, they do stain positive for pan-TGF- β , implying that expression of specific TGF- β isoforms in these eosinophils is likely to be low. Taken together, these data strongly suggest that various factors in the tissue environment including cytokines, chemokines, and extracellular matrix elements increase TGF- β expression by tissue eosinophils. Interestingly, hyaluronan fragments, commonly present at tissue injury sites, have been shown to increase TGF- β 1

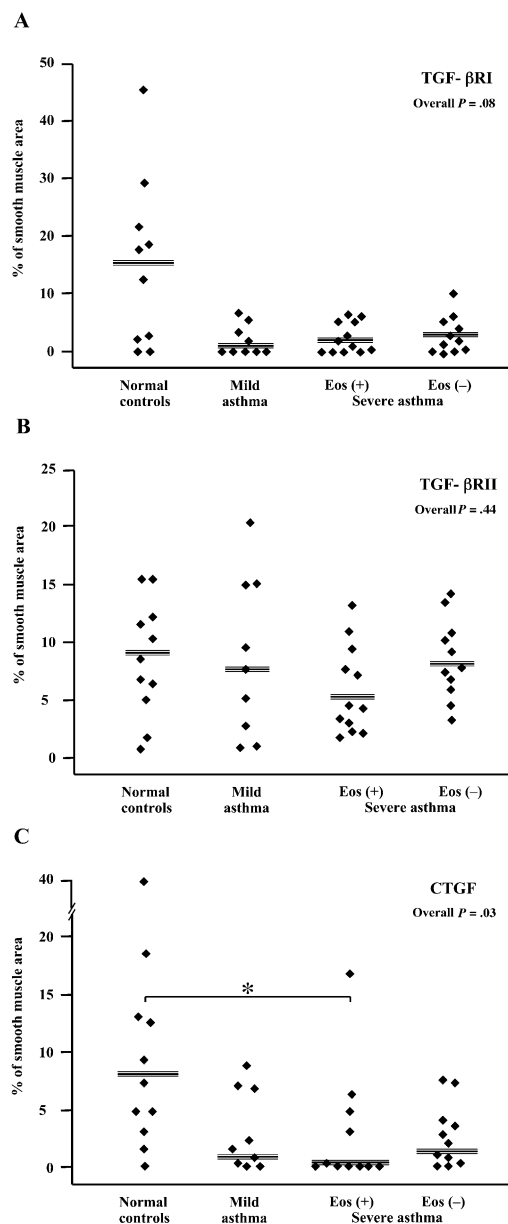


FIG 5. Percentages of smooth muscle area stained for (A) TGF-βRI, (B) TGF-βRII, and (C) CTGF in airways of normal subjects, subjects with mild asthma, and subjects with severe asthma with and without persistent eosinophilia. * $P < .05$ for pairwise comparison.

expression by eosinophils.²⁸ Whether similar mechanisms exist for TGF-β2 and TGF-β3 induction remains to be explored.

Interestingly, although eos⁻ subjects with severe asthma did not have eosinophils to express TGF-β2, TGF-β2 mRNA levels tended to be highest ($P = .075$) in that group. This increase in TGF-β2 mRNA, in the absence of eosinophils or other positive inflammatory cells, implies a significant contribution from structural cells such as smooth muscle cells or epithelial cells. Occasional smooth muscle staining for TGF-β2 as well as

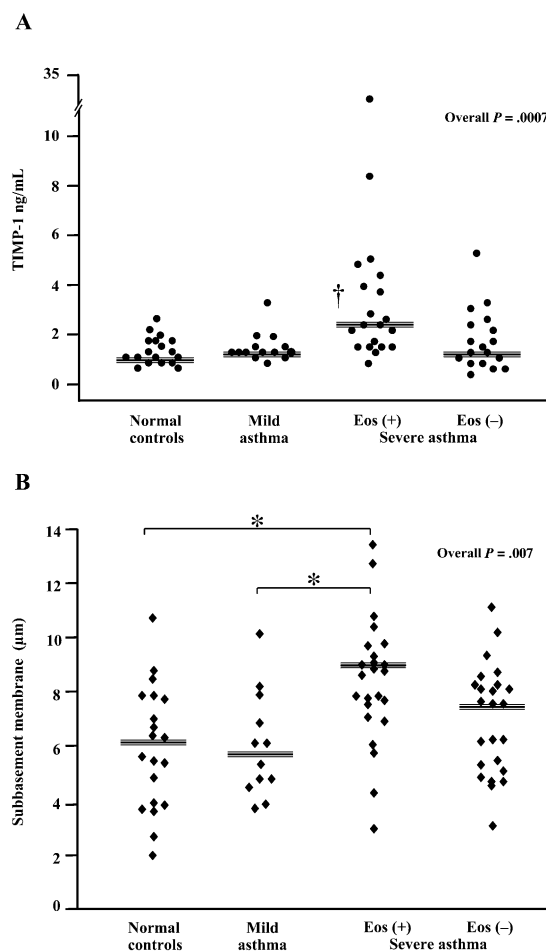


FIG 6. TIMP-1 levels in BAL fluid (A) and SBM thickness (B) from normal subjects, subjects with mild asthma, and subjects with severe asthma with and without persistent eosinophilia. * $P < .05$ for pairwise comparison; † $P < .05$ compared with all other groups.

epithelial cell staining for TGF-β2 was observed, consistent with previous reports.^{9,29,30} In contrast, the high TGF-β2 protein but lower mRNA levels in the eosinophil⁺ severe group are perhaps a result of the visual observation that TGF-β2 in eosinophils, similar to IL-13, may be stored in granules.³¹ Because mRNA levels of proteins stored in neutrophil granules are low, a similar relationship between TGF-β2 mRNA levels and protein may exist in eosinophils as well.³²

In addition to increased expression of TGF-β2 mRNA and/or protein in severe asthma, we observed downstream evidence for increased TGF-β-related activity, including high levels of the profibrotic factor TIMP-1 and a thicker SBM. TIMP-1, a profibrotic/matrix-sparing protein strongly upregulated by TGF-β1 and TGF-β2, was present in highest amounts in the severe asthma group with the highest eosinophil counts and TGF-β2 protein levels.³³ Even when measured in BAL fluid, TIMP-1 levels correlated strongly with both TGF-β1⁺ and TGF-β2⁺ cells. This suggests that TGF-β2 may increase

production of TIMP-1, by structural cells or even by the eosinophil itself.^{34,35} Interestingly, although the SBM thickness was numerically greatest in eos⁺ severe asthma, the differences in thickness between eos⁺ and eos⁻ subjects with severe asthma were relatively small. These small differences are likely reflective of the multiple regulatory elements contributing to SBM thickness and the long-term nature of this structure.

In contrast, one of the TGF- β Rs, TGF- β RI, was consistently downregulated in subjects with asthma, regardless of severity. The downregulation of this receptor in severe asthma can be explained by evidence for increased TGF- β 2 protein or mRNA expression. The mechanisms for the downregulation in mild asthma are less clear, because the expression of either TGF- β isoform in that group did not differ from that for normal subjects. However, the effects of TGF- β depend not only on the amount but also on the degree of activation. In the persistent inflammatory setting of mild asthma, it is conceivable that more TGF- β is active compared with normal tissue. Barbato et al³⁶ recently reported downregulation of TGF- β RII with no difference in TGF- β RI expression. However, these observations were made on submucosal inflammatory/nonstructural cells in childhood asthma and therefore are difficult to compare with our findings. Differential expression of TGF- β Rs was reported previously in experimental glomerulonephritis in rats, where expression of TGF- β RII changed little with the inflammatory and repair course.²³ Therefore, it could be that *in vivo* TGF- β RII is the more constitutive receptor and is less likely to be changed in the face of chronic stimulation. Nevertheless, several *in vitro* studies suggest that TGF- β RII rather than TGF- β RI may be an important regulatory component in TGF- β signaling.^{37,38}

To our knowledge, this is the first immunohistochemical report of CTGF protein in human airways. The results suggest higher expression of CTGF in smooth muscle from normal subjects than in subjects with asthma, which is an intriguing but also clearly a controversial finding. TGF- β is widely accepted as the most potent inducer of CTGF, and CTGF is believed to mediate many of the profibrotic/wound healing effects of TGF- β in tissue.³⁹⁻⁴¹ In addition, previous studies have suggested that smooth muscle cells isolated from subjects with asthma expressed higher amounts of CTGF mRNA than cells from normal subjects.¹⁶ However, the complex *in vivo* inflammatory environment associated with asthma likely involves similarly complex modulation of CTGF expression. For instance, activation of peroxisome proliferator-activated receptor γ , reportedly increased in smooth muscle from subjects with mild asthma, apparently interferes with TGF- β signaling *in vitro*, leading to a downregulation of CTGF *in vitro*.^{42,43} We suggest that the higher levels of CTGF present in the normal smooth muscle may be part of the ongoing breakdown and repair process required for normal airway function. However, further study is indicated.

In conclusion, we present the first report of prominent TGF- β 2 expression in severe asthma, with the eosinophil identified as a major cell source of the protein in patients

with persistent eosinophilic inflammation. The concurrent increase in TIMP-1, a factor strongly induced by TGF- β , as well as the significantly thickened SBM in both severe asthma groups support a high level of activation of this pathway. However, the decrease in TGF- β RI expression in all asthma groups suggests that activation of TGF- β pathway may be present in all severities of asthma. Finally, there is an indication that the normal CTGF response to TGF- β may be blunted in asthma. Further exploration of the intricate balances involved in TGF- β signaling in asthma are warranted.

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