

Airway smooth muscle remodeling is a dynamic process in severe long-standing asthma

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Background: The origin of the excess airway smooth muscle in asthma and when in the course of the disease it is acquired are uncertain.

Objectives: We examined the relative sensitivities of 2 markers of proliferation, proliferating cell nuclear antigen (PCNA) and Ki 67, in airway smooth muscle *in vivo* and *in vitro*. We then studied whether muscle remodeling is a dynamic process in asthma by quantifying proliferation rate and area. Finally we examined heparin-binding epidermal growth factor as a biomarker of remodeling.

Methods: We obtained bronchoscopic biopsies from subjects with moderate or severe asthma and healthy controls (n = 9/group). For *in vitro* studies, airway smooth muscle cells were cultured from tracheas of transplant donors. The proliferation rate was quantified from PCNA and Ki 67, co-localized to smooth muscle-specific α -actin cells *in vivo*. Muscle area was assessed morphometrically. We examined the expression of heparin-binding epidermal growth factor on tissues by *in situ* hybridization and by immunohistochemistry and in cells in culture by RT-PCR.

Results: Proliferating cell nuclear antigen and Ki 67 were highly correlated, but PCNA was a significantly more sensitive marker both *in vivo* and *in vitro*. Muscle area was 3.4-fold greater and the fraction of PCNA⁺ nuclei in muscle was 5-fold greater in severe asthma than in healthy subjects. Heparin-binding epidermal growth factor was upregulated in proliferating muscle cells in culture and in airway smooth muscle in severe asthmatic tissues.

Conclusion: Proliferating cell nuclear antigen is a highly sensitive marker of proliferation and heparin-binding epidermal growth factor is a potential biomarker during active remodeling of ASM in severe asthma. (J Allergy Clin Immunol 2010;125:1037-45.)

Key words: HB-EGF, PCNA, Ki 67, *in situ* hybridization, biomarker

Abbreviations used

ASM:	Airway smooth muscle
α -SMA:	α -smooth muscle actin
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
HB-EGF:	Heparin-binding epidermal growth factor
ISH:	<i>In situ</i> hybridization
LTD ₄ :	Leukotriene D ₄
PCNA:	Proliferating cell nuclear antigen

Airway remodeling, in particular change in smooth muscle mass, is postulated to account for many of the features of asthma.¹⁻⁴ The increase in airway smooth muscle (ASM) has been generally assumed to reflect growth by hyperplasia based on the finding of an increase in smooth muscle nuclei *per se* with increase in muscle.^{5,6} However, a recent study failed to find an excess of proliferating ASM cells, and alternative hypotheses to account for growth have been proposed, such as apposition of proliferating myofibroblasts in the subepithelial compartment to existing smooth muscle bundles.⁴ Epithelial-mesenchymal transition has been proposed as the mechanism by which these myofibroblasts are produced.⁷ A further possibility is that an excess of ASM is innate as observed in animal models of hyperresponsiveness⁸ or acquired early in life.⁹ Studies of airway remodeling in animal models support the concept of *in situ* proliferation of ASM cells in response to allergen challenge.¹⁰⁻¹² Proliferation of smooth muscle is observed in animals throughout the airway, including the trachea in allergen exposed rats,¹³ and in airways and pulmonary vessels in mice.¹²

The detection of proliferating cells may be accomplished by several techniques. Proliferating cell associated nuclear antigen (PCNA) and Ki 67 have been commonly used as markers of proliferation. In animal models of airway remodeling, PCNA has been demonstrated to detect ASM hyperplasia, and an associated change in muscle mass has also been documented in these studies.¹²⁻¹⁵ Ki 67 has been reported in the detection of human myocyte proliferation in culture.¹⁶ PCNA has a reported half-life of 20 hours, whereas Ki 67 has a half-life of 2 hours. The former may therefore be a more suitable marker for low-frequency events such as may be the case for proliferating ASM *in vivo*. In normal tissues, there is a correlation between the 2 markers,¹⁷ whereas in highly proliferative conditions like tumor, there is no correlation between the 2 markers,¹⁸ raising questions about the specificity of the technique under these circumstances. We wished to investigate the relative sensitivities of PCNA and Ki 67 and hypothesized that PCNA is a more sensitive marker of proliferation than Ki 67. We also reasoned that if active remodeling

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of ASM is a feature of asthma, it should be most easily discerned in severe asthma.

A number of features of airway remodeling have been related to the epidermal growth factor (EGF) receptor (EGFR) and, in particular, one of its ligands, heparin binding-EGF (HB-EGF).¹⁹⁻²¹ Upregulation of both the EGFR and HB-EGF have been described in human asthmatic airway tissues.²⁰⁻²² HB-EGF is released from human airway epithelial cells by pressure, and HB-EGF is also mitogenic for smooth muscle.²³⁻²⁵ Therefore, if ASM remodeling is still an active phenomenon, then growth factors such as HB-EGF that potentially contribute to remodeling might also be expected to be increased in expression and to relate to the severity of asthma because of the relationship of an increase in ASM mass and disease severity.^{3,4,26}

To answer these questions, we examined the correlation between PCNA and Ki 67 immunoreactivity in cultured ASM stimulated to proliferate to varying degrees by fetal bovine serum (FBS) and HB-EGF. Having established the greater sensitivity of PCNA for the detection of proliferating ASM cells, we then studied bronchoscopic biopsies of human subjects with moderate or severe asthma for evidence of smooth muscle proliferation. We verified that HB-EGF was upregulated in proliferating ASM cells in culture and then examined its expression as a biomarker of ASM remodeling on asthmatic tissues.

METHODS

Study subjects

Subjects with moderate and severe asthma (American Thoracic Society criteria)²⁷ and healthy controls (n = 9/group) were recruited, and bronchoscopic biopsies were performed following American Thoracic Society guidelines. The study was approved by an institutional ethics committee, and written informed consent was obtained.

Fiberoptic bronchoscopy and tissue processing

Bronchoscopy was performed under light sedation, and endobronchial biopsy specimens were obtained from the right lung at various segmental and subsegmental carinae. Tissues were fixed and embedded in paraffin in random orientation before cutting. For *in situ* hybridization (ISH), biopsy specimens were fixed in 4% paraformaldehyde for 4 hours. For immunohistochemical stains, specimens were fixed in 10% buffered formalin. See the section on fiberoptic bronchoscopy in the Online Repository at www.jacionline.org for additional details.

Co-localization of proliferating cells to ASM in airway tissue

Proliferation of ASM cells was detected by double-immunostaining for PCNA or Ki 67 and α -smooth muscle actin (α -SMA).¹⁴ See the section on co-localization of proliferating cells to ASM in the Online Repository at www.jacionline.org for additional details.

Immunostaining for PCNA or Ki 67 using cytopins of human ASM cells

Primary cultures of human ASM cells were prepared from lung transplant specimens.²⁸ See the section on human ASM culture in the Online Repository at www.jacionline.org for further details. Cells were incubated with 10% FBS, 0.1% FBS, or 0.1% FBS treated with 10 ng/mL recombinant HB-EGF (R&D Systems, Minneapolis, Minn) for 4 or 24 hours. Cell cytopins were prepared and immunostained for PCNA or Ki 67. See the section on immunostaining for PCNA and Ki 67 in the Online Repository at www.jacionline.org for additional details.

Quantification of proliferating ASM cell

The numbers of PCNA or Ki 67 positive and negative ASM cells were counted in ASM bundles or cytopins of human ASM cells in 4 randomly chosen fields, and a proliferation index was calculated as a percentage of positive ASM cells out of total number of ASM nuclei.

Real-time quantitative PCR and ELISA for HB-EGF in human ASM cells

Human smooth muscle (SM) cells were cultured in 10% FBS or 0.1% FBS for 3 or 6 hours. RNA was extracted from ASM cells, and then reverse transcription and real-time PCR for HB-EGF were performed. Relative mRNA expression was calculated by using the $\Delta\Delta$ cycle threshold method.²⁹ Individual data were normalized for the housekeeping gene, S9. See the section on reverse transcription and quantitative PCR in the Online Repository at www.jacionline.org for additional details.

A similar experiment was performed for the assessment of HB-EGF protein released into the medium after stimulation with 0.1% or 10% FBS. Medium was collected at 6 hours after stimulation, and a commercial sandwich ELISA assay (R&D Systems) was used following the manufacturer's instructions. Wells were spiked with authentic HB-EGF to verify recovery and to exclude significant loss of protein through adsorption.

Quantification of ASM mass in airway tissue

ASM mass was evaluated by measuring the area of α -SMA immunoreactivity using computer-assisted image analysis (Image Master 5; Photon Technology International, Media Cybernetics, Birmingham, NJ) and was normalized to the total area of the biopsy.

In situ hybridization and immunohistochemical staining for HB-EGF

Radiolabeled complementary RNA probes coding for human HB-EGF were prepared from cDNA,³⁰ and ISH was performed as previously described.³¹ See the section on *in situ* hybridization in the Online Repository at www.jacionline.org for additional details.

Immunostaining was performed as described.¹⁵ See the section on immunostaining in the Online Repository at www.jacionline.org for additional details.

Quantification of HB-EGF mRNA and protein in airway tissues

Hybridization between the radiolabeled complementary RNA probes (S-35) and mRNA encoding HB-EGF was identified from the development of the photographic emulsion overlaying the tissue sections. The density of silver grains and intensity of immunoreactivity for HB-EGF were both quantified for intact epithelium and smooth muscle with computer-assisted image analysis (Image Master 5). All measurements were performed by an observer who was unaware of the subject group.

Data analysis

Limited availability of tissue samples precluded the obtaining of complete data sets for all outcomes so that study groups were less than 9 for some outcomes. Data were presented as mean $\pm$ 1 SE. One-way ANOVA followed by the Fisher least significant difference test was performed for the comparison of multiple means. Correlation was explored with linear regression. Significance was accepted at the 5% confidence level.

RESULTS

Clinical characteristics of the subjects with asthma

Clinical characteristics of the patients with asthma and control subjects are shown in Table E1 in the Online Repository at

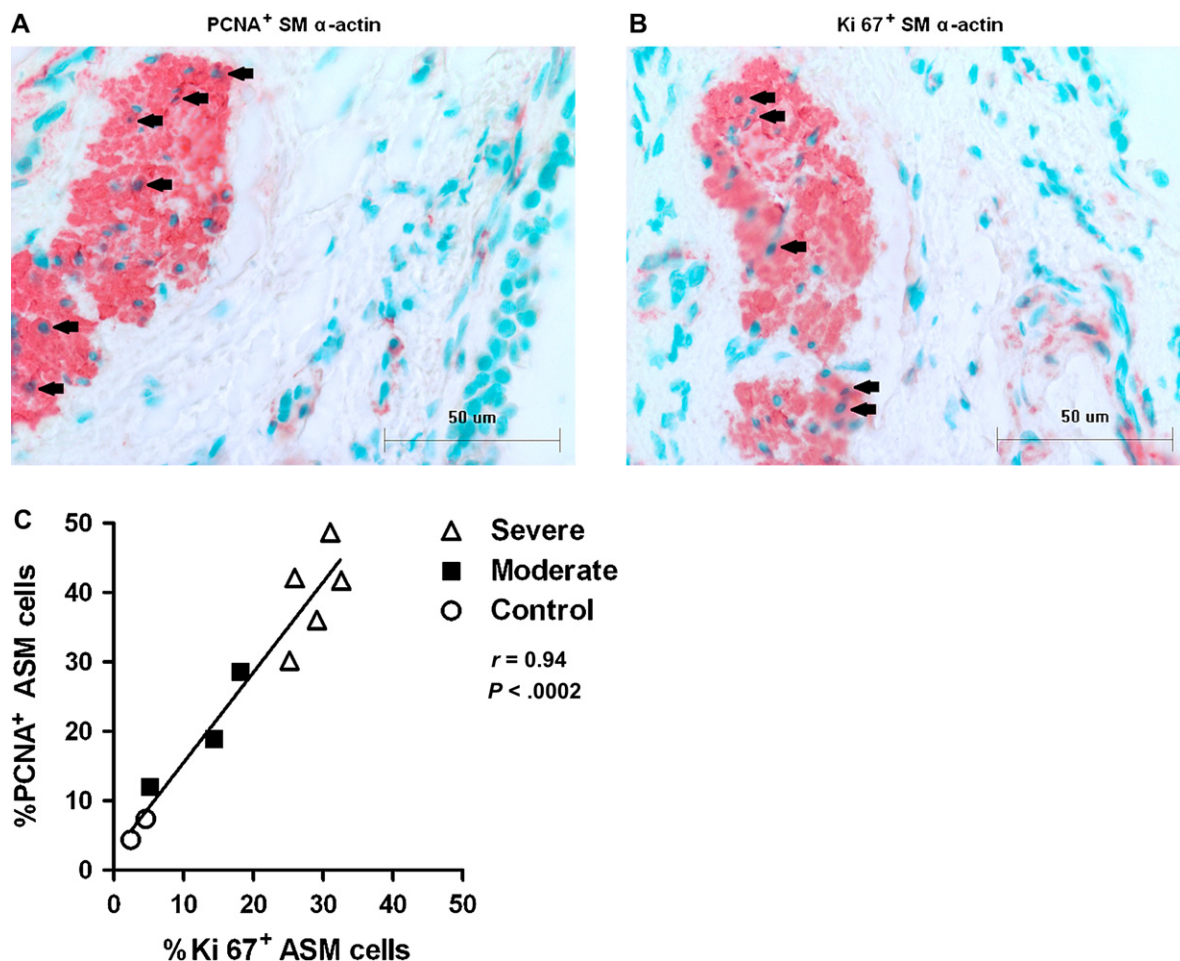


FIG 1. Correlation between PCNA and Ki 67 in airway tissues. PCNA⁺ or Ki 67⁺ nuclei were immunolocalized to α-SMA⁺ cells by using consecutive sections of bronchial biopsies. An illustrative example is shown (A and B). PCNA and Ki 67 proliferation indices were correlated (C). Data are mean cell percentages ± SE (n = 4 in severe, 3 in moderate, and 2 in control subjects). Scale bar = 50 μm.

www.jacionline.org. The subjects studied did not differ in age or in sex distribution. There was a significant difference in FEV₁ among the groups. The mean duration of asthma in the patients with severe asthma was 23.8 years and 23.4 years in the patients with moderate asthma.

Correlation between PCNA and Ki 67 *in vivo* and *in vitro*

To determine whether PCNA⁺ and Ki 67⁺ ASM cells were correlated in airway tissues, we immunolocalized PCNA⁺ or Ki 67⁺ nuclei to α-SMA⁺ cells by using serial sections of bronchial biopsies. An illustrative example is shown in Fig 1, A and B. To obtain a range of signals, biopsies from 4 severe, 3 moderate, and 2 control subjects were examined by double immunostaining. The percentage of PCNA⁺ nuclei in ASM compared with total nuclei in ASM was highly significantly correlated with the percentage of Ki 67⁺ nuclei in ASM ($r = 0.94$; $P < .0002$; Fig 1, C). The regression equation had a slope of 1.30 ± 0.12 , reflecting the greater sensitivity of PCNA over Ki 67. The slope was significantly greater than 1 ($P = .038$).

The correlation between the 2 proliferation markers was also studied *in vitro* by using cytopspins of human ASM cells. An illustrative example is shown in Supplemental Fig E1, A and B. The

percentage of PCNA⁺ nuclei compared with total nuclei in the field also showed a highly significant correlation with Ki 67 ($r = 0.95$; $P < .0001$; Fig E1, C). The slope of the relationship was 1.34 ± 0.07 , and was also significantly greater than 1 ($P < .0001$), again indicating a greater sensitivity of PCNA over Ki 67.

HB-EGF and ASM cell proliferation

To examine the effect of HB-EGF on human ASM cells, cells were incubated with 0.1% FBS or 0.1% FBS treated with 10 ng/mL recombinant human HB-EGF for 4 or 24 hours. Cytopspin slides were prepared from cell suspensions, and immunostaining for PCNA or Ki 67 was performed as previously mentioned. The proliferation index was then calculated. HB-EGF significantly induced ASM cell proliferation at 24 hours compared with 0.1% FBS using either Ki 67 or PCNA immunostaining as a marker of proliferation ($P = .001$, $P = .00002$, respectively; Fig 2, A).

Effects of stimulation of ASM with FBS on HB-EGF mRNA expression and HB-EGF protein release

To investigate the association between HB-EGF mRNA expression and proliferation of human ASM cells, cells were cultured in 10% FBS or 0.1% FBS for 3 or 6 hours. Normalized

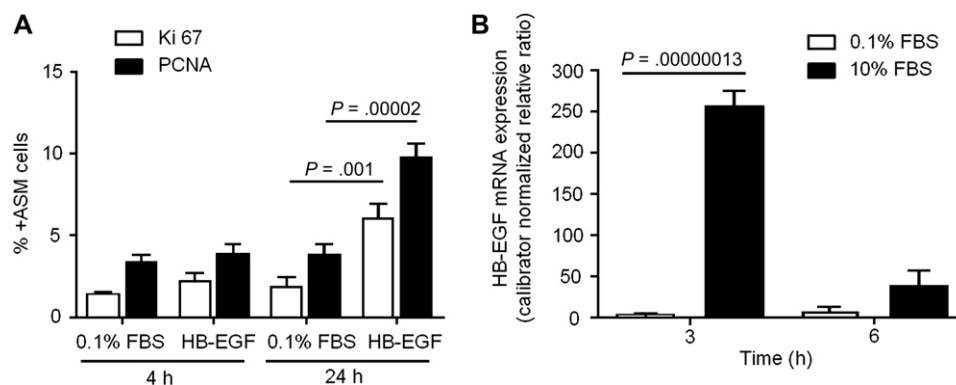


FIG 2. HB-EGF and ASM cell proliferation. Human ASM cells were incubated with 0.1% FBS or 0.1% FBS treated with 10 ng/mL recombinant human HB-EGF for 4 or 24 hours. PCNA or Ki 67 proliferation index was then calculated by immunostaining using cytopins of human ASM cells (**A**). Human ASM cells were cultured in 10% FBS or 0.1% FBS for 3 or 6 hours, and HB-EGF mRNA expression was examined (**B**). Bars show mean $\pm$ SE.

HB-EGF mRNA expression showed a significant increase in 10% FBS at 3 hours (257.6 ± 17.83) compared with 0.1% FBS (3.75 ± 1.36 ; $P < .0001$; Fig 2, B). A similar experiment was performed to assess the release of HB-EGF into the medium under the same conditions. HB-EGF was undetectable at 6 hours after stimulation by FBS at either concentration ($n = 3$). There was >80% recovery of authentic HB-EGF instilled into the culture wells.

Assessment of ASM mass

Airway smooth muscle bundles were identified on the basis of their immunoreactivity for α -SMA (illustrated in Fig 3, A and B). ASM mass was determined morphometrically and expressed as the ratio of the area of ASM to the total area of the biopsy specimen. Normalized area of ASM (ASM_{area}) showed a 3.4 fold greater amount in severe asthma (0.32 ± 0.09) compared with control subjects (0.10 ± 0.03 ; $P = .009$; Fig 3, C).

Quantification of PCNA⁺ nuclei in ASM bundles in airway tissue

To determine whether ASM cells were undergoing hyperplastic growth, we immunolocalized PCNA⁺ nuclei to α -SMA⁺ cells. An illustrative example is shown in Fig 3, A and B. Biopsies from 9 severe, 6 moderate (insufficient biopsy material was available for 3 subjects), and 9 control subjects were used for double immunostaining. The percentage of PCNA⁺ nuclei in ASM compared with total nuclei in ASM was significantly greater in severe asthma (31.30 ± 4.71) compared with moderate asthma (11.90 ± 2.65 ; $P = .0008$) and control subjects (6.20 ± 0.84 ; $P = .00001$; Fig 3, D).

HB-EGF mRNA expression in bronchial biopsies by *in situ* hybridization

Biopsy tissues were available from 5 severe, 7 moderate, and 5 control subjects for *in situ* hybridization. HB-EGF mRNA expression was detected in subepithelial mucous glandular cells, inflammatory cells, epithelial cells, and smooth muscle bundles as illustrated in the dark field images generated from autoradiograms (Fig 4, B, E, H). The total HB-EGF mRNA expression in ASM (density $\times$ normalized ASM_{area}) was significantly increased in patients with severe asthma (8.20 ± 1.87) compared with patients

with moderate asthma (3.21 ± 0.70 ; $p = 0.008$) and control subjects (2.69 ± 0.87 ; $P = .007$; Fig 4, C). There was also a significant increase in the density of HB-EGF mRNA expression within ASM in severe asthma (37.28 ± 4.07) compared with control subjects (22.32 ± 2.57 ; $P = .02$; Fig 4, F). In contrast with ASM, epithelial HB-EGF mRNA expression was comparable among all groups (Fig 4, I). No significant hybridization signal was present with the antisense RNA probe in either epithelium or ASM (not shown).

Immunoreactivity for HB-EGF

We performed immunohistochemical stains to localize the site of the protein expression of HB-EGF in the airway tissues. Biopsies from 8 subjects in each group were available for study. HB-EGF immunoreactivity was detected in subepithelial mucous glandular cells, inflammatory cells, epithelial cells, and smooth muscle bundles as shown in bright field images (Fig 5, A, D, G) and corresponding immunofluorescence and 3-dimensional images (Fig 5, B, E, H; C, F, I).

Total HB-EGF protein expression (density $\times$ ASM_{area}) in ASM was significantly increased in patients with severe asthma (6.90 ± 2.42) compared with patients with moderate asthma (1.78 ± 0.51 ; $P = .02$) and control subjects (0.71 ± 0.31 ; $P = .006$; Fig 6, A). There was also a significant increase in the HB-EGF density of expression within ASM in severe asthma (19.62 ± 5.1) compared with control subjects (6.62 ± 1.61 ; $P = .01$; Fig 6, B). In contrast with ASM, epithelial HB-EGF protein immunoreactivity was comparable among all groups (Fig 6, C).

DISCUSSION

In the current study, we evaluated the relationship between 2 markers of proliferation, PCNA and Ki 67, in human ASM cells, both *in vitro* and *in situ*, in asthmatic and control airway biopsies. The 2 markers were highly correlated, but PCNA was a significantly more sensitive marker compared with Ki 67. We chose to include a group of patients with quite severe asthma in the study because airway remodeling is most marked in these subjects. The subjects with severe asthma in the current study had a 3.6-fold greater ASM mass than control subjects. There was also evidence that this remodeling was ongoing; the fraction of proliferating

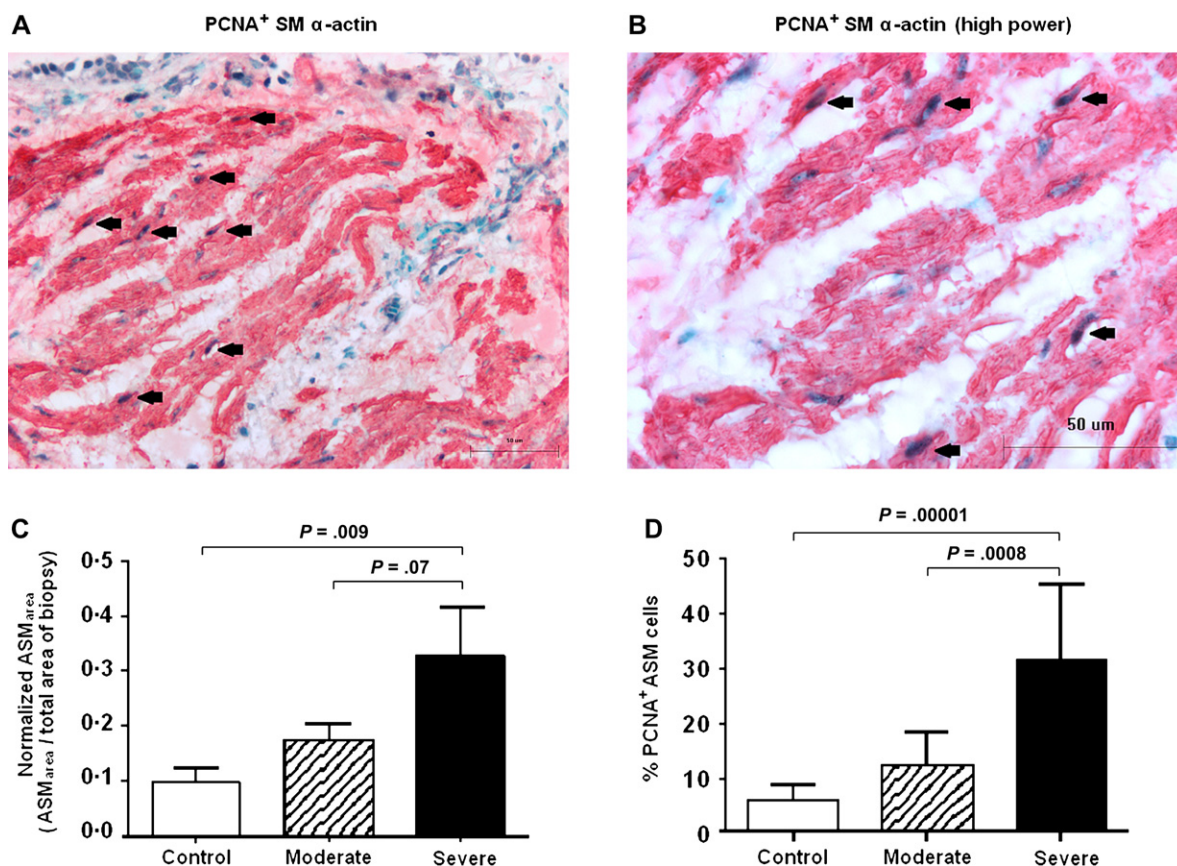


FIG 3. Measurement of ASM mass and quantification of proliferating ASM cells *in vivo*. ASM mass and hyperplasia were examined by co-localization of PCNA immunoreactivity with α-SMA. An illustrative example is shown (A and B). ASM mass was expressed as the area of ASM normalized to total area of the biopsy specimen (C), and PCNA proliferation index was calculated (D). Bars show mean ± SE (n = 6 in moderate, 9 in severe and control subjects). Scale bar = 50 μm.

ASM cells detected by PCNA positivity was 5-fold higher in the patients with severe asthma compared with control subjects and almost 3-fold that of subjects with moderate asthma. There was also an increase in expression of 1 of the EGFR ligands, HB-EGF, in ASM. This ligand was overexpressed in proliferating ASM cells *in vitro*, also suggesting an alteration in muscle phenotype consistent with active remodeling. Indeed, HB-EGF may be a potential biomarker for the active stage of ASM remodeling.

Previous studies have addressed the relationship between the assessment of proliferation by using the 2 markers that we have used and have shown that they are correlated in normal tissue.¹⁷ However, in the setting of abnormal and highly proliferative tumor tissue, this correlation has not been found,¹⁸ raising questions about possible nonspecificity under some pathological circumstances. However, the 2 markers were correlated, and the relationship between the 2 extrapolated to or close to 0, suggesting that there is not a nonspecific signal detected by one over the other. The greater sensitivity of PCNA suggested to us that it was a more appropriate marker to use in the setting of potentially low-frequency events in ASM. The literature has supported the idea that excess ASM is predominantly attributable to hyperplasia,⁵ although in some subjects there may also be a contribution from hypertrophy.⁶

The argument for a hyperplastic increase in ASM has been based on the finding of a proportionate increase in nuclei in ASM to the

area increase.^{5,32} Earlier studies have documented an increase in ASM in the airways of resected lungs, whereas recent studies have addressed ASM remodeling in proximal bronchial biopsies. There is general agreement between the 2 approaches, perhaps because the increase in muscle is associated with a progressive encroachment by muscle on the subepithelial compartment.^{3,4,9} The increase in ASM is therefore detected by these partial thickness biopsies. Using a similar analysis and appropriate stereological techniques, Woodruff et al³² reported that ASM in bronchoscopic biopsies from airways of patients with mild to moderate asthma shows no evidence of hypertrophy and argued that the increase in muscle must therefore have been attributable to hyperplasia.

There is to date no reported evidence of an excess of proliferating cells in the ASM. Indeed, a recent study failed to find any increase in proliferating ASM cells and found only an increase in proliferating cells within the lamina propria.³³ An earlier study addressing airway remodeling noted that there was no evidence of Ki 67 immunoreactivity in ASM in subjects with asthma of varying severity, including subjects with severe asthma who appear to be have been comparable to those we have studied.³⁴ The basis for the different findings is not apparent. Our study therefore demonstrates a dynamic remodeling of ASM with hyperplasia in subjects with severe asthma. These findings are not only of interest from a conceptual standpoint but also important from a therapeutic point of view and support the idea that

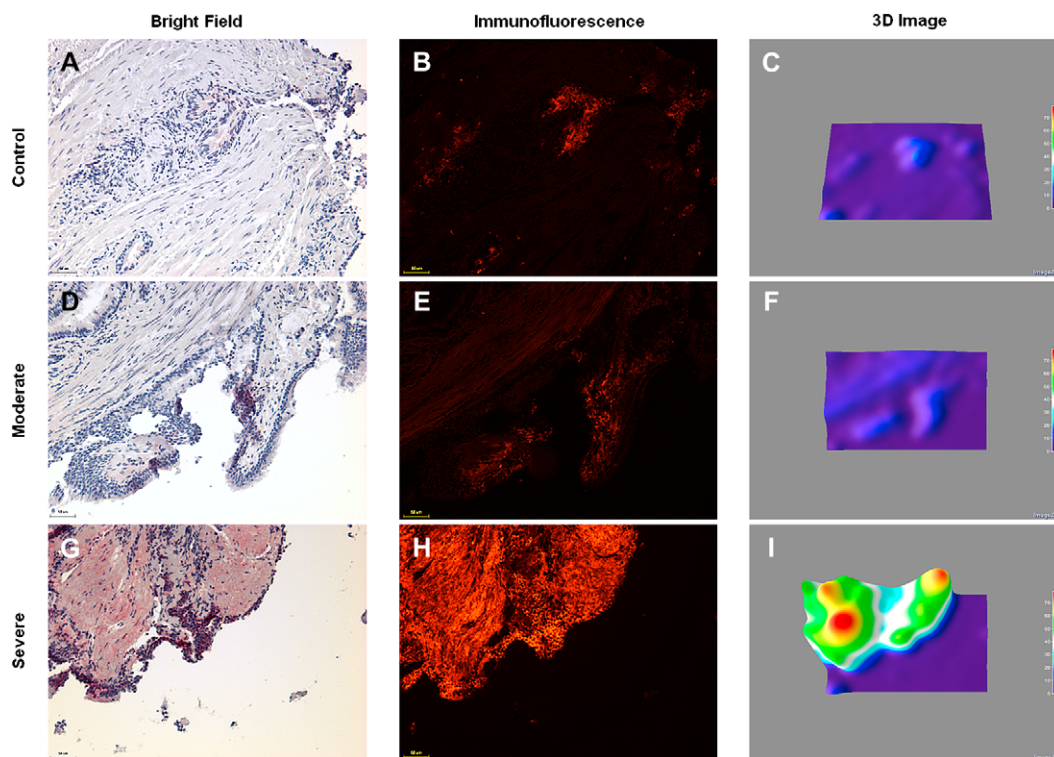


FIG 5. Immunohistochemical staining of HB-EGF in human airways. HB-EGF immunoreactivity was detected by immunohistochemical stains (red staining) in inflammatory cells, epithelial cells, and ASM bundles as shown in bright field (A, D, G) and their corresponding immunofluorescent images (B, E, H) and 3-dimensional (3D) images, which are shown according to thermo map (C, F, I). Scale bar = 50 μ m.

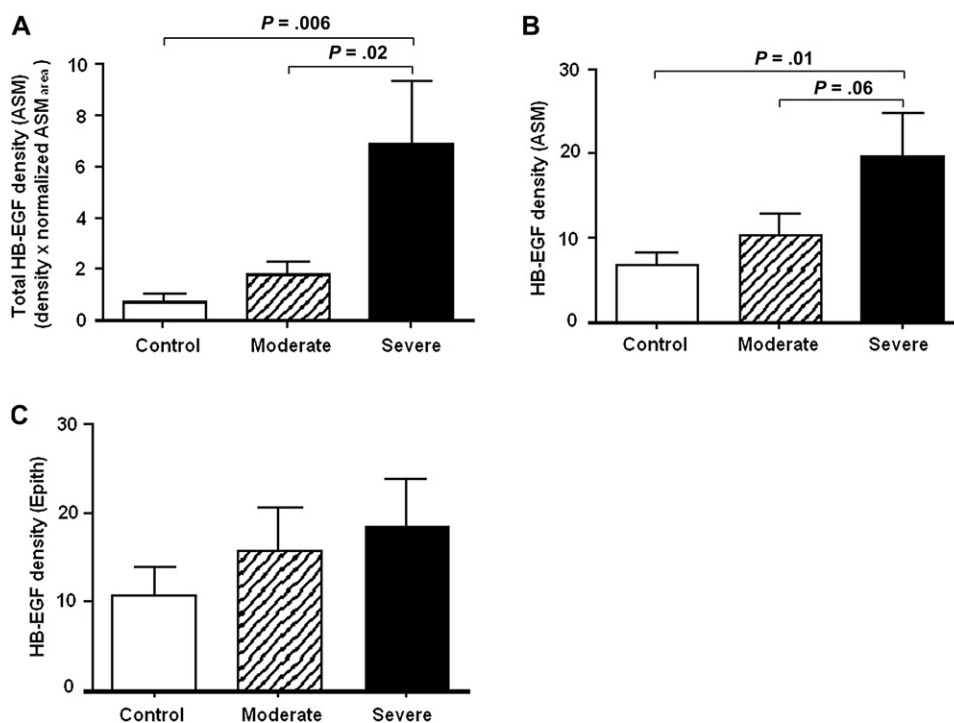


FIG 6. Quantification of HB-EGF protein in airway tissues. Total HB-EGF protein expression in ASM was significantly increased in patients with severe asthma ($P = .02$ vs moderate; $P = .006$ vs control; A). There was also a significant increase in the HB-EGF density of expression within ASM in severe asthma compared with control subjects ($P = .01$, B). Epithelial HB-EGF protein immunoreactivity was comparable among all groups (C). Bars show mean $\pm$ SE ($n = 8$ /group).

leads to pulmonary fibrosis.⁴³ Therefore, HB-EGF is a plausible candidate that could account for a variety of features of airway remodeling, including that of ASM in asthma.

In the current study, we examined whether HB-EGF was associated with airway with smooth muscle hyperplasia. We first demonstrated that HB-EGF was upregulated *in vitro* in ASM cells stimulated to proliferate with the mitogenic stimulus FBS and therefore may be a potential biomarker of active remodeling of this tissue. However, stimulation of the cells with FBS was insufficient to lead to release of the ligand into the medium. Then we examined HB-EGF expression in the asthmatic and control airways by *in situ* hybridization and by immunohistochemistry. HB-EGF was expressed in both the airway epithelium and ASM in human airway tissues but was upregulated only in the ASM of the subjects with severe asthma. Both the density of expression and total expression of HB-EGF (corrected for normalized ASM area) within ASM were increased both at mRNA and protein levels, particularly in subjects with severe asthma. Although epithelial expression was readily detectable at both the mRNA and protein levels, the expression was not different among groups, consistent with a study indicating that although EGFR levels were increased in asthma, this was not linked to changes in expression of its activating ligands in the bronchial epithelium.²¹ It is possible that the fact that all subjects were on high-dose inhaled corticosteroid treatment may have modified the epithelial expression but perhaps not the deeper ASM tissue. It is also possible that ASM characteristics in asthma may differ from healthy control subjects; asthmatic ASM is reported to proliferate more rapidly in response to mitogenic stimuli,⁴⁴ supporting such an idea.

In conclusion, we have demonstrated that PCNA and Ki 67 are highly correlated markers of proliferation both *in vivo* and *in vitro*, but PCNA is a more sensitive marker of proliferation. There is an increase in the proliferation rate of ASM cells in subjects with severe asthma, indicating that remodeling is not an end-stage phenomenon but remains an active process even in longstanding asthma. Hyperplasia of ASM accounts at least in part for the increase in ASM mass in asthma. We also demonstrated a gradient of HB-EGF expression at both the mRNA and protein levels in the airways of subjects ranging from controls without asthma to severe asthma. Epithelial expression of HB-EGF, although detectable, did not vary significantly among groups, whereas ASM expression was related to disease severity. These results demonstrate ongoing ASM cell hyperplasia in asthma and suggest that HB-EGF is a potential biomarker of active remodeling of ASM.

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Key messages

- PCNA and Ki 67 are correlated markers of proliferation, but PCNA has superior sensitivity.
- PCNA demonstrates an excess of proliferating ASM cells in subjects with severe asthma associated with HB-EGF expression, consistent with active proliferation.

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METHODS

Subjects

Subjects were recruited at the Montreal Chest Institute and the Sacré-Coeur Hospital and had experienced no exacerbations during the 12 weeks preceding the study.

Fiberoptic bronchoscopy and tissue processing

For ISH, biopsy specimens were fixed immediately in 4% paraformaldehyde in 0.1 mol/L PBS for 4 hours. After fixation, tissues were stored overnight at 4°C in diethylpyrocarbonate-treated PBS. Tissues were then processed and subsequently embedded in paraffin. For immunohistochemical stains, specimens were fixed immediately in 10% buffered formalin, stored overnight at room temperature, processed, and then embedded in paraffin. Sections of 5- μ m thickness (Leica microtome model 2040, VWR International, Mississauga, ON, Canada) were used.

Co-localization of proliferating cells to ASM in airway tissue

Proliferation of ASM cells was detected by coimmunolocalization of PCNA or Ki 67 and α -SMA. Staining was preceded by high-temperature epitope unmasking in antigen retrieval solution (Vector Laboratories, Burlingame, Calif) and permeabilization in 0.2% Triton X-100 (Sigma-Aldrich, St Louis, Mo). Sections were then blocked with either 20% horse serum (PCNA) or 20% goat serum (Ki 67) (Vector Laboratories) in universal blocking solution (Dako Cytomation, Mississauga, ON, Canada), and mouse mAb to PCNA (Calbiochem, La Jolla, Calif) or rabbit mAb to Ki 67 (Abcam, Cambridge, Mass) was detected with biotinylated horse antimouse IgG or biotinylated goat antirabbit mouse IgG, respectively, avidin/biotin-alkaline phosphatase complex, and BCIP/NBT chromogen substrate (Vector Laboratories). Double-immunostaining with anti- α -SMA mAb was developed with Vector Red and was followed by methyl green (Sigma-Aldrich) counterstain.

Human SM cell cultures

Primary cultures of human ASM cells were prepared from lung transplant specimens. In brief, tissue digestion was performed by gently shaking the tissues in HBSS (in mmol/L: 5 KCl, 0.3 KH₂PO₄, 138 NaCl, 4 NaHCO₃, and 5.6 Na₂HPO₄) containing collagenase type IV (0.4 mg/mL), elastase (0.38 mg/mL), and soybean trypsin inhibitor (1 mg/mL) at 37°C for 90 minutes. The dissociated cells were collected by filtering through 125- μ m Nytex mesh (Small Parts, Miami Lakes, Fla) followed by centrifugation. The pellet was reconstituted in growth medium consisting of Dulbecco modified Eagle medium–Ham's F-12 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin, and cells were plated in a 25-cm² flask. Confluent cells were detached with a 0.025% trypsin solution containing 0.02% EDTA. Cells were grown in 6-well culture dishes for protein and RNA extraction. The growth of cells was arrested 72 hours before the experiment by incubating them with 0.1% FBS solution.

Cytospin and fixation

Human ASM cells were incubated with 10% FBS, 0.1% FBS, or 0.1% FBS treated with 10 ng/mL recombinant human HB-EGF (reference no. 259-HE; R&D Systems) for 4 or 24 hours. Cells were trypsinized, washed twice in a wash/staining buffer (3% heat-inactivated FBS, 0.09% sodium azide in PBS), and resuspended at 1.10⁶ cells/mL. Two cytopspins were performed from each cell suspension (150 μ L each) for 4 minutes at 1000 rpm. Cells were immediately fixed in 4% paraformaldehyde solution in 0.1 mol/L PBS at 4°C for 15 minutes, followed by treatment with methanol at –20°C for 15 minutes, and immunostaining for PCNA or Ki 67 was then performed.

Immunostaining for PCNA or Ki 67 using cytopspins of human ASM cells

Proliferation of human ASM cell was detected by immunostaining for PCNA or Ki 67. After fixation, cells were permeabilized in 0.2% Triton X-100 (Sigma-Aldrich). Cytopspins were then blocked with either 20% horse serum

(PCNA) or 20% goat serum (Ki 67; Vector Laboratories) in universal blocking solution (Dako Cytomation), and mouse mAb to PCNA (Calbiochem) or rabbit mAb to Ki 67 (Abcam) was detected with biotinylated horse antimouse IgG or biotinylated goat antirabbit mouse IgG, respectively, avidin/biotin-alkaline phosphatase complex, and BCIP/NBT chromogen substrate (Vector Laboratories), followed by methyl green (Sigma-Aldrich) counterstain.

HB-EGF transcript expression

Human ASMC were cultured in 10% FBS or 0.1% FBS for 3 and 6 hours. After trypsinization, cells were lysed and RNA was purified with a commercially available kit (Rneasy; Qiagen, Valencia, Calif) following the manufacturer's instruction. RNA integrity was verified with the Experion electrophoresis station (BioRad, Hercules, Calif). HB-EGF mRNA expression was examined by real-time PCR.

Reverse transcription and quantitative PCR

Reverse transcription was done with 500 ng RNA for each sample. Quantitative PCR was performed with 2 μ L cDNA per reaction with 12.5 μ L 2x QuantiTect SYBR Green PCR (Qiagen), 0.375 μ L forward and reverse primers (0.3 μ mol/L), and 9.75 μ L ribonuclease-free water for a total volume of 25 μ L per well (see Table E2 for primer sequences and PCR product length). The samples were amplified in StepOnePlus real-time PCR system (Applied Biosystems, Foster City, Calif). A negative control (RNase free water) was added the quantitative PCR run. Relative mRNA expression was calculated by using the $\Delta\Delta$ cycle threshold method. Individual data were normalized for their corresponding housekeeping gene (ribosomal S9). PCR product lengths were verified on an agarose gel.

Probe preparation

Radio-labeled cRNA probes coding for human HB-EGF were prepared from cDNA. cDNA was prepared by reverse transcription (SuperScript II reverse transcriptase; Invitrogen, Burlington, ON, Canada) from RNA extracted from human umbilical vein endothelial cells by using the RNeasy Mini Kit (QIAGEN).

cDNA was inserted into vectors (TOPO R vectors) linearized by EcoR I digestion. Antisense and sense RNA probes were prepared by *in vitro* transcription in the presence of 35S-labeled uridine triphosphate and either T7 (sense) or SP6 (antisense) polymerases. The percentage of the incorporation of radioisotope was in the range of 40% to 50%.

ISH

Paraffin-embedded sections from bronchoscopic biopsy specimens were permeabilized with Triton X-100 and proteinase K solution in TRIS containing EDTA. The samples were subsequently incubated with triethanolamine and acetic anhydride to prevent the nonspecific binding of the 35S-labeled cRNA probes. Prehybridization of the samples was carried out in formamide in standard saline citrate. Hybridization was carried out with the use of a hybridization mixture containing the appropriate sense or antisense probes (0.75 $\times$ 10⁶ counts per minute per slide). Posthybridization, we performed high-stringency washing of the samples. To remove any unbound RNA probes, the samples were washed with ribonuclease solution, dehydrated with increasing concentrations of ethanol, and air-dried. After this, the samples were dipped in Amersham (Baie d'Urfe, QC, Canada) LM-2 emulsion and then exposed for a period of 14 days. The autoradiographs were then developed, fixed, and counterstained with hematoxylin. The samples were examined by dark field microscopy.

Immunostaining for HB-EGF

Paraffin-embedded 5- μ m-thick sections of bronchial biopsy specimens were incubated with the primary antibody goat antihuman HB-EGF polyclonal antibody (R&D Systems) at 4°C overnight. The same concentration of isotype control IgG (R&D Systems) was used as a negative control. Sections were then incubated with the biotinylated rabbit anti-goat IgG as a secondary antibody, followed by the avidin/biotin-alkaline phosphatase complex. The signal was developed with Vector Red (Vector Laboratories) and followed by hematoxylin counterstaining.

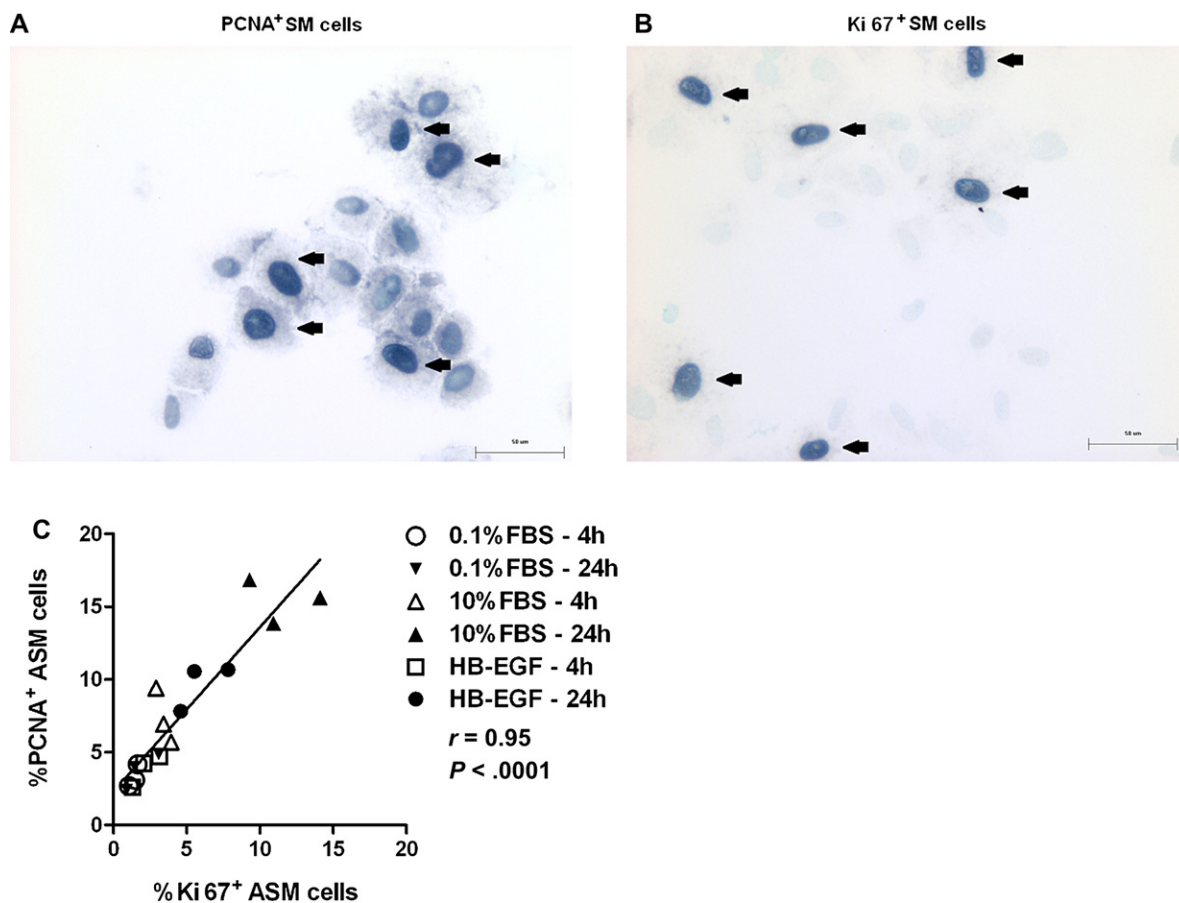


FIG E1. PCNA and Ki 67 correlation *in vitro*. Human ASM cells were incubated with 10% FBS, 0.1% FBS, or 0.1% FBS treated with 10 ng/mL recombinant HB-EGF for 4 or 24 hours. Cytospins were then performed and immunostained for PCNA or Ki 67 (**A** and **B**). Arrows indicate some of the PCNA⁺ cells. The proliferation indices of the 2 markers were then correlated (**C**). Data are mean cell percentages $\pm$ SE. Scale bar = 50 μ m.

TABLE E1. Clinical characteristics of control subjects and subjects with asthma

Groups	Age (y)	Sex (M: F)	Atopy (%)	Smoking	Treatment	FEV ₁ (L)*	FEV ₁ (%)*	FEV ₁ /FVC*	Eos (%)
Control	34.8 (5.7)	4:5	44.4	(-), Ex	0	4.0 (0.5)	112.0 (7.6)	84.5 (2.5)	0
Moderate	45.8 (1.6)	6:2	87.5	(-), Ex	βA (7/8), ICS (7/8), NCS (3/8), MK (1/8), OCS (0/8)	3.6 (0.3)	94.4 (5.9)	76.0 (0.4)	4.0 (2.0)
Severe	47.6 (3.2)	5:4	87.5	(-), Ex	βA (9/9), ICS (9/9), NCS (4/9), MK (3/9), OCS (1/9)	1.8 (0.3)	58.0 (8.8)	60.0 (4.5)	19.2 (8.8)

βA, β-Agonists; *Eos*, sputum eosinophils; *Ex*, exsmoker; *F*, female; *FVC*, forced vital capacity; *ICS*, inhaled corticosteroids; *M*, male; *MK*, montelukast; *NCS*, nasal corticosteroids; *OCS*, oral corticosteroids (prednisone).

Data expressed as mean (1 SEM).

*Postbronchodilator values are shown.

TABLE E2. Primer sequences

Target gene	Forward primer	Reverse primer	PCR product length
S9	TGCTGACGCTTGATGAGAAG	CGCAGAGAGAAGTCGATGTG	307 bp
HB-EGF	CAAGGGGCTAGGAAGAAGA	CGATGATGACCAGCAGACAGACA	245 bp