Airway smooth muscle remolds pericellular collagen fibrils: implications for proliferation

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Schultiga M, Ong SC, Soon L, Zal F, Harris T, Stewart AG. Airway smooth muscle remolds pericellular collagen fibrils: implications for proliferation. Am J Physiol Lung Cell Mol Physiol 298: L584–L592, 2010. First published January 15, 2010; doi:10.1152/ajplung.00312.2009.—Airway wall remodeling comprises a broad range of structural changes including increases in the volume of airway smooth muscle (ASM) and fibrillar collagen. The impact of fibrillar collagen remodeling on ASM proliferation was investigated. Human ASM cultured on type I fibrillar collagen remodeled the collagen substrate by both degradation (collagenolysis) and formation of networks comprised of thicker reticular collagen fibrils (fibrillogenesis). In cultures maintained on fibrillar collagen, the levels of matrix metalloproteases (MMPs) -1 and -14 mRNA and active MMP-2 were higher than in cultures maintained on non-fibrillar type I collagen (gelatin) or plastic. Although there was no apparent increase in cytotoxicity or apoptosis, the number of ASM was lower on fibrillar collagen than on gelatin or plastic for control conditions. Furthermore, maintenance on fibrillar collagen attenuated basic fibroblast growth factor-stimulated increases in cell number and the percentage of cells entering S-phase. In cultures maintained on fibrillar collagen, the MMP inhibitor ilomastat (2.5 μM) 1 attenuated collagenolysis, 2 enhanced fibrillogenesis, and 3 inhibited proliferation. In contrast, knockdown of the β1-integrin gene in ASM maintained on fibrillar collagen led to an increase in proliferation and reduced MMP-1 and -14 expression. Thus, ASM remodel the pericellular environment by degrading collagen fibrils and spinning them into larger collagen assemblies. Moreover, the collagen fibrils limit proliferation and activate autocrine MMPs in a β1-integrin-dependent manner, suggesting a potential negative feedback on modeling executed through fibrillar collagen activation of β1-integrins.

asthma; extracellular matrix; integrin; matrix metalloprotease; proliferation

PATIENTS WITH SEVERE ASTHMA (5–10% of all asthmatics) show extensive tissue remodeling of the airways that amplifies airway hyperresponsiveness (AHR) (20). The remodeling compromises hyperplasia of airway smooth muscle (ASM) and fibroblasts, increased deposition of extracellular matrix (ECM) in the bronchial wall, mucous cell metaplasia, and increased vascular density (23). In asthma, mesenchymal cells undergo activation to a myofibroblast phenotype that secretes structural proteins, including collagen and fibronectin (5). In the remodelled airway, the ECM is expanded by collagen, not only in the subepithelial layer, but also around and within smooth muscle bundles (35). Fibrils composed of type I and III collagen have a high tensile strength that maintains the structural integrity of the airway wall by counter-balancing distending forces (11). In asthma, the increase in fibrillar collagen causes the airway wall to become less distensible (43). Increases in collagen as well as ASM hyperplasia and hypertrophy contribute to airway wall thickening that is linked with AHR (2). Current therapies for asthma including glucocorticoids, β2-adrenoceptor agonists, leukotriene receptor antagonists, and theophylline, appear to have a limited impact on remodeling as remodeling is evident in severe asthma despite the use of these therapeutic agents (3, 39). The need for an effective anti-remodeling treatment to reduce airway reactivity and symptoms has long been recognized (38).

The accumulation of types I and III collagen in the remodelled airway wall may have an important effect on ASM function (6). These collagens form fibrils comprising supramolecular aggregates of collagen that are stabilized by interactions between their helical domains (41). Collagen types I and III also exist as nonfibrillar forms that do not contribute structurally to airway wall integrity, but retain cell signaling activity. The GFOGER motif of the characteristic triple helix of nonfibrillar type I collagen binds with high affinity to the integrins α1β1, α2β1, α10β1, and α11β1 (13, 25). Furthermore, proteolytic cleavage of the helix reveals “matricryptic” integrin-binding sites such as the short RGD peptide sequence (10). Nonfibrillar type I collagen appears to stimulate ASM proliferation (30), survival (16), and cytokine release (32) by an integrin-dependent manner. Less is known of the effects of the native fibrillar form of collagen on ASM function. The α2β1 integrin, unlike the α1β1 integrin, is a functional receptor for type I collagen fibrils involved in several aspects of matrix remodeling, including the formation of collagen fibrils (24) and the generation of isometric forces in tethered collagen lattices (46). In addition, the α3β1 integrin has been shown to mediate the antiproliferative response of fibrillar collagen on arterial smooth muscle cells, which become arrested in the G1 phase of the cell cycle (26).

The matrix metalloproteases (MMPs) are a family of zinc-containing endopeptidases that degrade ECM components including collagen. The triple helix of types I and III collagen is proteolyzed primarily by the collagenolytic MMPs (MMP-1, 8, 13, and 14), which cleave individual collagen subunits more readily than those assembled in fibrils (33). ASM expresses MMP-1,-2, -9, -12, and -14 (also known as MT1-MMP1) (12) in a manner that is modulated by inflammatory cytokines (31). Abnormal expression or activation of MMPs in the airways may contribute to asthma pathophysiology. Levels of MMP-1 mRNA (7) and MMP-9 activity (17) are higher in the sputum/ BALF of asthmatics and are associated with a reduced bronchodilator response. The levels or activity of MMP-1 in the airway wall (34) and MMP-9/12 in ASM (1, 45) are higher in asthmatics than in nonasthmatics. In murine models of allergic inflammation and remodeling, both MMP-9 gene deletion (29)
and the MMP inhibitor doxycycline (18) show antifibrotic effects. These observations suggest that airway remodeling in asthma is associated with an increase in the expression/activity of MMPs and that their inhibition may be beneficial in treating asthma. However, the mechanism by which MMPs contribute to airway wall remodeling remains unclear. In the present study, we focus on the possibility that MMP-mediated conversion of fibrillar to nonfibrillar collagen in the microenvironment of ASM enhances proliferation.

**METHODS**

**Cell culture.** Human ASM cultures were established using bronchi obtained from macroscopically normal resections of lung transplant patients (Alfred Hospital, Melbourne, Australia) as described previously (15). Tissue specimens were obtained with approval from the University of Melbourne’s ethics committee (HREC980168X). Nineteen separate specimens were obtained for this study from donors of 14–74 years of age (12 male, 3 female, 4 information not available). The indications for resection/diagnoses were: bronchial carcinoma (3 donors), asthma (3 donors), and α1-antitrypsin deficiency (1 donor). A diagnosis was not available for the other donors. For each experiment, separate primary cultures established from a minimum of four different donors (n = 4) were used with cells between the 4th and 13th passage. Cultures were not tested for contamination by mycoplasma.

In most experiments, cells were seeded onto six-well plates (0.6 × 10^4 cells cm⁻²) coated with either bovine gelatin (Sigma) or fibrillar collagen prepared from rat-tail tendons (9). The wells of culture dishes were coated by adding collagen (0.5 mg ml⁻¹) in solution (0.1 ml cm⁻²) and incubating for 1 h before excess solution and unbound collagen was aspirated. Cells were incubated at 37°C in air containing 5% CO₂ and maintained in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% v v⁻¹ (ml per 100 μl) nonessential amino acids (Sigma), 5% v v⁻¹ heat-inactivated fetal calf serum, and 2.5 μg ml⁻¹ amphotericin. Twenty-four hours before the addition of 300 pM bFGF (Promega, Madison, WI), cells were incubated in serum-free DMEM containing 0.25% wt/v bovine serum albumin and 1% v v⁻¹ insulin-transferrin-selenium containing supplement (Monomeda A, CSL). In selected experiments, the MMP inhibitor ilomastat (2.5 μM, Calbiochem) in DMSO was added to the media. The final concentration of DMSO was 0.1% v v⁻¹.

**Scanning electron microscopy.** Cells were seeded into the wells of glass, eight-chambered slides coated either with type I fibrillar collagen or gelatin. ASM were allowed to attach to the slides and grow in the presence of serum before being maintained for an additional 3 days in the absence of sera. The samples were fixed and mounted as previously described (36) and viewed under a Phillips XL 30 CP scanning electron microscope (FEI, Hillsboro, OR).

**Cell harvest, enumeration, and cell cycle analysis.** Cells were harvested by digestion first with Clostridium collagenase (0.25 mg ml⁻¹, Worthington Biochemicals) in PBS and then with trypsin (0.125 wt v⁻¹)/EDTA (0.02% wt v⁻¹) in PBS. The conditioned media were collected and stored at −20°C for zymography and Western blotting. Cell suspensions were divided for cell enumeration, fluorescence-activated cell sorting (FACS), and total RNA extraction. For cell enumeration, cells were resuspended in an appropriate volume of

![Figure 1](http://ajplung.physiology.org/)

**Fig. 1.** Airway smooth muscle (ASM) remodel pericellular fibrillar collagen. Scanning electron microscopy images of cultures of ASM maintained on type I fibrillar collagen (A and B); cultures maintained on gelatin (D and E); and cell-free fibrillar collagen (C) and gelatin (F) substrates. Small arrows show denuded regions of the substratum composed of fine collagen fibrils. Large arrows show networks of thicker reticular collagen fibrils associated with cells maintained on fibrillar collagen, but not gelatin.
2% v⁻¹ fetal calf serum in PBS containing trypan blue (0.2% wt v⁻¹), and both viable and nonviable cells were counted in duplicate with the aid of a hemocytometer. For cell cycle analysis, cells were fixed overnight in 70% v⁻¹ ethanol in PBS before staining with propidium iodide (50 μg ml⁻¹) in PBS containing 0.1% v⁻¹ Triton X-100 for 24 h. FACS was performed using a FACSCalibur (Becton Dickinson) instrument, and the percentages of cells in S phase were determined by ModFit LT 3.0 flow cytometry modeling software (Verity Software House).

Western analysis. For Western blotting of MMP-1 in conditioned media, supernatants were loaded in volumes corresponding to equivalent cell numbers and subjected to electrophoresis on a 12% acrylamide gel. Proteins were electroblotted onto Hybond nitrocellulose membrane (Amersham Biosciences) at 100 V for 1 h in chilled transfer buffer (20% v⁻¹ methanol; 25 mM Tris; 192 mM glycine). Membranes were blocked with 5% skim milk in TBS-T (10 mM Tris, 75 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h before an overnight incubation at 4°C with a mouse monoclonal antibody against MMP-1 (Calbiochem), diluted 1:500, in 3% bovine serum albumin. Blots were washed with TBS-T before incubation with secondary antibody (sheep anti-mouse IgG, Chemicon, diluted 1:2,000 in 5% skim milk/TBS-T) for 1 h at room temperature. After additional TBS-T washes, antigen was detected by enhanced chemiluminescence (Amersham Biosciences). Bands were visualized and quantified by densitometry using the Kodak IS4000 imaging system.

Gelatin zymography. Cell supernatants were diluted 1:4 with serum-free DMEM and loaded onto a 10% wt v⁻¹ acrylamide gel containing 2.5 mg ml⁻¹ gelatin. Samples were subjected to SDS-PAGE in the absence of 2-mercaptoethanol. Zymography was carried out as previously described (36).

RNA extraction and RT-PCR. Cell pellets for mRNA analysis were stored at −80°C until use. Cells were lysed and RNA purified using RNeasy miniprep kits (Qiagen) or Trizol (Invitrogen) according to the manufacturers’ instructions. Reverse transcription of total RNA and the subsequent RT-PCR using an ABI Prism 7900HT sequence detection system (Applied Biosystems) with the relevant forward and reverse primers were conducted as previously described (36). The integrin β1 (ITGB1) primers used in this study were 5’-GAT GCC ATC ATG C-3’ (forward primer) and 5’-TCC AGC CAA TCA GTG ATC CAC-3’ (reverse primer). The threshold cycle determined for each gene was normalized against that obtained for 18S ribosomal RNA, which was included as internal control.

β1-integrin mRNA knockdown. Cells in logarithmic phase of growth were transfected in antibiotic-free serum containing DMEM with either 30 nM ITGB1 or negative control (medium GC content) Stealth siRNA duplex oligonucleotides (Invitrogen) using RNAiMax Lipofectamine (Invitrogen). The two ITGB1 sequences used in this study were 1) 5’-AUA AUG UUC CAU CUG ACU AUAG GTT GCC ATG G-3’ and 2) 5’-UGG UGC GAG UGU GGU GCC UGC UGU AAAG UGC A-3’.

Measuring integrin surface expression. ASM cells maintained on plastic in six-well plates were dissociated by incubation with Ca²⁺- and Mg²⁺-free Hanks’ buffer salt solution (HBSS) containing 0.02% wt/v⁻¹ EDTA. Cells were washed twice in FACS buffer (PBS, 0.2% wt/v⁻¹ bovine serum albumin) before being labeled with either the mouse IgG1 clone AK-7 (Becton Dickinson), used to detect α₁ β₁ integrin expression (14), or mouse IgG1 clone MOPC-21 (Becton Dickinson), used as an isotype control. Cells were then labeled with biotinylated-anti-mouse-IgG (Jackson Laboratories) before staining with streptavidin-phyceroerythin (AV/PE, BD Biosciences). Cells were analyzed by flow cytometry (FACScalibur, BD Biosciences) using Cellquest software (BD Biosciences). FL2-H (log) channel histograms were generated (1 × 10⁶ cells/scan), and data were expressed as mean fluorescence units.

Collagen degradation. Cells were seeded into the wells of 96-well microplates (4,000 cells/well) coated with FITC-labeled type I fibri-bilar collagen (Molecular Probes, Invitrogen) as described previously (36). The labeled collagen is so densely saturated with FITC fluorophore that fluorescence is quenched in the native cross-linked fibrillar form. However, the quenching is diminished upon degradation resulting in an increase in fluorescence. Cells were incubated for 4 h in the presence of serum and then for a further 72 h in serum-free DMEM.

Fig. 2. Fibrillar collagen modulates collagenolytic matrix metalloprotease (MMP) production and activity. A and B: levels of MMP-1 and MMP-14 mRNA for ASM maintained on fibrillar collagen or gelatin, as analyzed by RT-PCR (n = 4; included 1 culture of known asthma status; passage median 10). C and D: levels of MMP-1 protein and active MMP-2 in the conditioned media of ASM maintained on fibrillar collagen, gelatin, or plastic substrate (n = 7–8; no cell lines of known asthma status; passage median 7). Levels of MMP-1 and active MMP-2 were analyzed by Western immunoblotting and gelatin zymography, respectively (see insets for representative blots/zymographs), and quantitated by densitometry. *P < 0.05, **P < 0.01 by Bonferroni post hoc test.
The fluorescence in individual wells comprising both cell-associated and supernatant fluorescence (excitation wavelength 485 nm and excitation wavelength 535 nm) was determined using a Fluorostar Optima plate reader (BMG Labtechnologies). Fluorescence was corrected for background by subtracting the fluorescence values of FITC-type I collagen-coated wells maintained under identical conditions in the absence of cells.

**Statistical analysis.** Data are presented as the means \( \pm \) SE for \( n \) individual experiments. Each experiment was repeated in a minimum of four separate cultures obtained from four different donors (\( n = 4 \), as annotated in specific experiments). All data were statistically analyzed using GraphPad Prism 4.0 (Graphpad, San Diego, CA). In most cases, either one- or two-way ANOVA with repeated measures was used to analyze the data, and treatment groups were compared with Bonferroni post hoc tests. A \( P \) value of \( < 0.05 \) was considered to be statistically significant.

**RESULTS**

**ASM remodels extracellular fibrillar collagen.** Ultrastructural changes to an ECM enriched in type I fibrillar collagen were examined by scanning electron microscopy. ASM seeded on fibrillar collagen and maintained for 4 days were observed growing on a dense mesh of fine collagen fibrils that were denuded in regions (Fig. 1, A and B). The mesh of collagen fibrils comprising the substratum remained intact in the absence of cells (Fig. 1C) and was not observed in cultures exposed to the nonfibrillar form of type I collagen (gelatin) (Fig. 1, D and E). In cultures of ASM maintained on fibrillar collagen, larger and thicker fibrils in a reticular conformation and associated with both the cell and surrounding matrix were observed (Fig. 1B). This network of thicker fibrils was also not observed in the absence of cells (Fig. 1C) or when cells were maintained on gelatin (Fig. 1E).

**Fibrillar collagen enhances MMP expression and activation.** The effects of type I fibrillar collagen on the expression and activation of MMPs by ASM were examined. Levels of mRNA for the collagenolytic MMPs, MMP-1 and MMP-14 (MT1-MMP), were 2.5-fold higher in ASM cultured on fibrillar collagen compared with gelatin under basal conditions (Fig. 2, A and B). In addition, the conditioned media of ASM maintained on fibrillar collagen contained significantly higher levels of pro-MMP-1 protein compared with gelatin (Fig. 2C). No active MMP-1 was detected in the conditioned media of ASM regardless of exposure to the different substrata. Gelatinase activity in the conditioned media was analyzed by gelatin zymography (Fig. 2D). The levels of active MMP-2 were significantly higher in cultures maintained on fibrillar collagen than gelatin or plastic.

**MMP inhibition enhances formation of thicker fibrillar collagen bundles.** Electron microscopy revealed that the fibrillar collagen substratum of ASM cultures maintained in the presence of thicker reticular fibrils in the presence of ilomastat.
ence of the MMP inhibitor ilomastat (2.5 μM) was more compact and less denuded (Fig. 3, C and D) than that of vehicle-treated cultures (Fig. 3, A and B). Furthermore, the reticular fibrils associated with the cells and surrounding matrix for the ilomastat treatment group were thicker and more abundant (Fig. 3). Figure 3D shows a reticular collagen bundle in the presence of ilomastat that appears to be formed by smaller fibrils twisted around each other.

Ilomastat attenuates ASM-mediated collagenolysis. The effect of ilomastat on pericellular degradation of fibrillar collagen by ASM was measured using native FITC-labeled type I collagen. Fluorescence resulting from ASM-mediated degradation of FITC-collagen comprising the substratum was lower in the presence of the MMP inhibitor ilomastat (2.5 μM) (Fig. 4).

Fibrillar collagen suppresses ASM proliferation. ASM were maintained on fibrillar collagen, gelatin, or plastic in complete growth medium for 3 days and then in the absence of serum for an additional 3 days before cell enumeration. The percentage of viable cells, as assessed by trypan blue exclusion, was similar for all matrices examined (>95%). However, the total number of viable cells on fibrillar collagen under control conditions was lower by 20–30% than for the other substrata (Fig. 5). Although relatively lower, the number of viable cells on fibrillar collagen was still 2.6-fold greater than that at seeding, suggesting that proliferation did occur on fibrillar collagen, albeit at a slower rate than the other substrata. In the presence of bFGF (300 pM) for 48 h (added to culture media 4 days after seeding), an increase in cell number on gelatin and plastic, but not on fibrillar collagen, was observed (Fig. 5).

Fibrillar collagen suppresses ASM proliferation. ASM were cultured on type I fibrillar collagen, gelatin, or plastic for 6 days (first 3 in serum, last 3 serum-free) were harvested for cell enumeration. bFGF (300 pM) was added at day 4. The cell density on plastic control was 2.1 ± 0.5 × 10^4 cells cm⁻². *P < 0.05, ***P < 0.01 (n = 8 different primary cultures established from separate donors; no cell line of known asthma status; passage median 7).

**Fig. 5.** Fibrillar collagen suppresses increases in ASM number. ASM cultured on type I fibrillar collagen, gelatin, or plastic for 6 days (first 3 in serum, last 3 serum-free) were harvested for cell enumeration. bFGF (300 pM) was added at day 4. The cell density on plastic control was 2.1 ± 0.5 × 10^4 cells cm⁻². *P < 0.05, ***P < 0.01 (n = 8 different primary cultures established from separate donors; no cell line of known asthma status; passage median 7).

**Fig. 6.** Fibrillar collagen suppresses bFGF-stimulated cell transition into S phase. ASM cultured on fibrillar collagen or gelatin were incubated with bFGF (300 pM) for 24 h before harvesting for cell FACS analysis. Representative FACS profiles (A) and the relative number of cells (B) in S Phase (*P < 0.05, n = 4 different primary cultures established from separate donors; no cell line of known asthma status; passage median 7).

**Fig. 7.** Ilomastat suppresses ASM proliferation on fibrillar collagen. The number of ASMs maintained on fibrillar collagen or gelatin, in the presence of DMSO vehicle or ilomastat (2.5 μM). *P < 0.05 (n = 4; no cell line of known asthma status; passage median 8).
The ASM cell lines used in this study were established from tissue resections of patients that had either undergone lobectomy or lung transplant from a range of lung/airway diseases including asthma. The potential of the disease status of the cell lines to influence our findings was mitigated by the use of primary cultures derived from at least four donors for each experiment. To exclude the possibility that the use of asthmatic ASM would impact our main findings, the cell proliferation experiment (Fig. 5) was repeated with four asthmatic cultures under similar culture conditions. In the asthmatic cultures, bFGF induced a 36 ± 3% increase in cell number on plastic, but no change on fibrillar collagen (P > 0.05). These latter observations paralleled the findings in nonasthmatic cultures (35 ± 5% increase on plastic; no change on fibrillar collagen). Nevertheless, in most of our experiments, no more than one asthmatic culture was used.

FACS cell cycle profiles of ASM maintained on fibrillar collagen showed no loss of symmetry of the G0-G1 DNA peak, indicating that the rates of apoptosis were very low and similar to those of cells maintained on gelatin (Fig. 6A). For cells maintained on fibrillar collagen, incubation with bFGF (300 pM) for 24 h did not cause a significant increase in the proportion of cells that transited into S phase (Fig. 6B), unlike the 2.1-fold increase observed for those maintained on gelatin.

Ilomastat suppresses ASM cell proliferation on fibrillar collagen. The effect of MMP inhibition for ASM proliferation on fibrillar collagen was investigated. Cell number was lower in the presence of ilomastat (2.5 μM) compared with vehicle control for cultures maintained on fibrillar collagen (Fig. 7). Ilomastat had no significant effect on cell number of cultures maintained on gelatin.

β1-integrin gene knockdown enhances ASM proliferation and attenuates MMP expression on fibrillar collagen. The role of the β1-integrin in mediating the effects of fibrillar collagen on MMP expression (Fig. 2) and proliferation (Fig. 3 and 4) of ASM was investigated by reducing the levels of ITGB1 mRNA transcript for the β1-integrin subunit. Levels of mRNA for ITGB1 in ITGB1 siRNA transfected cells were lower, by at least 66%, compared with the respective transfection control for cells on either fibrillar collagen or gelatin (Fig. 8A). The reduced expression of ITGB1 reversed both the antiproliferative effects (Fig. 8B) and the MMP-increased expression elicited by fibrillar collagen (Fig. 8, C and D). It should be noted that a reduction in ITGB1 mRNA corresponded with a significant decrease in the surface expression of the α2β1 integrin, as shown by FACs analysis (Fig. 9).

![Image](http://ajplung.physiology.org/)

Fig. 8. Integrin β1 (ITGB1) gene knockdown modulates ASM proliferation and MMP expression on fibrillar collagen. ASM cells maintained on fibrillar collagen (fColl) or gelatin (Gel) substrate were transfected with control or ITGB1 siRNA (2 sequences used, i and ii). Cells were harvested 72 h posttransfection for analysis of levels of ITGB1 mRNA (A), cell number (B), and levels of mRNA transcript for MMP-1 (C) and MMP-14 (D). *P < 0.05, **P < 0.01, ***P < 0.001 (n = 4–7, cell lines included 1 of known asthma status; passage median 10).
supramolecular structures such as bundles, weaves, and layers vary greatly in diameter (20 – 500 nm) and form different fibronectin fibril formation (42). Collagen fibrils are known to involve integrin binding.

Collagen fibrillogenesis by mouse fibroblasts in muscle (28) and fibroblasts (24, 36), have also been shown to form the networks of thicker fibrils as they were not found for ASM proliferation have been identified. ASM modify the ultrastructure of an extracellular matrix composed of fibrillar collagen by both denaturing the collagen fibrils and spinning them into larger fibrils. The expression and activation of MMPs by ASM were stimulated by exposure to fibrillar collagen. Furthermore, the ECM remodeling likely involves MMP activity, as matrix ultrastructure and collagenolysis were modulated by pharmacological inhibition of MMP activity. ASM proliferation was suppressed when cultured on fibrillar collagen compared with culture on nonfibrillar type I collagen (gelatin) or plastic. This antiproliferative effect of fibrillar collagen was enhanced by ilomastat, a MMP inhibitor.

When ASM cells were maintained on a substratum composed of fine collagen fibrils, regions of the underlying matrix became denuded. Simultaneously, a network of larger and thicker collagen fibrils overlying both matrix and cells was formed. The denuded areas are consistent with an ASM-dependent depolymerization of fibrillar collagen (i.e., denaturation), which was also shown biochemically by ASM degrading FITC-labeled native type I collagen. Another explanation for the denuded regions is that the fibrils originally there were incorporated into the larger collagen assemblies. It seems that the collagen fibrils comprising the matrix substrata were used to form the networks of thicker fibrils as they were not found associated with cells cultured on gelatin, nor did they form in the absence of ASM. The formation of the larger collagen aggregates illustrates that ASM can modify the architecture of fibrillar collagen. Other cell types, including vascular smooth muscle (28) and fibroblasts (24, 36), have also been shown to polymerize collagen fibrils into larger supramolecular collagen assemblies. Collagen fibrillogenesis by mouse fibroblasts involves integrin binding (α2β1, α11β1) and is integrated with fibronectin fibril formation (42). Collagen fibrils are known to vary greatly in diameter (20–500 nm) and form different supramolecular structures such as bundles, weaves, and layers to suit differing roles and functions. The ability of ASM to increase the diameter of fibrils would increase tensile strength and resistance to collagenolysis by MMPs.

Similar to vascular smooth muscle cells (26) and lung fibroblasts (36), we found that the native form of type I collagen is antiproliferative for ASM from the time of seeding until the end of the experiment (first 3 days in serum, last 3 days in the absence of serum). ASM cell number increased on plastic, gelatin, and fibrillar collagen, but the increase was least on fibrillar collagen. Cell viability was greater than 95% on all matrices examined, excluding cytotoxicity as an explanation for the lower cell number on fibrillar collagen. Apoptosis was also excluded because nuclear DNA fragmentation was not apparent in cells cultured on fibrillar collagen. The inference that fibrillar collagen had an antiproliferative effect is supported by the observation that fewer cells on fibrillar collagen, compared with gelatin, transited into the S phase of cell cycle after stimulation with bFGF. The antiproliferative effect appears to be active rather than passive because cell numbers on fibrillar collagen were lower in comparison not only to gelatin, but also to plastic. A contribution of the larger collagen fibrils to the regulatory effect of fibrillar collagen on proliferation was not addressed in this study. However, an antiproliferative effect of these larger fibrils may explain why the inhibition of basal proliferation by the fibrillar collagen substrate was only modest compared with the almost complete inhibition of the later bFGF response. As the formation of the larger fibrils required a period of 3 days, these fibrils would not have been present during the initial serum exposure, but would have formed by the time of bFGF addition. An alternative explanation for the modest effect of the fibrillar collagen substrate under basal conditions is that the antiproliferative effect is mitogen dependent. Fibrillar collagen has previously been reported to have no effect on ASM proliferation in response to PDGF (30), the predominant mitogen in serum.

We showed that the inhibitory effect of fibrillar collagen for ASM proliferation involves a β1-integrin-dependent mechanism. Furthermore, MMP-1 and MMP-14 gene expression was also regulated by fibrillar collagen in a manner that involved...
β1-integrin signaling. However, the regulatory effect of β1-integrin on the level and activity of MMPs was not evaluated. The β1-integrin is a subunit of the αβ1 integrin receptor, which is activated by binding to the Asp-Gly-Glu-Ala tetrapeptide recognition sequence of fibrillar collagen (22). Fibrillar collagen inhibits the proliferation of vascular smooth muscle cells (26) and lung fibroblasts (44) and increases the expression and activity of collagenolytic MMPs by melanoma cells (21) in an αβ1 integrin-dependent manner. The "strait jacket" effect of ASM being embedded in a rigid microenvironment may be another reason why fibrillar collagen is antiproliferative. The β1-integrin serves as a mechanoreceptor in fibroblasts and vascular smooth muscle cells, regulating cell viability via the inositol 3-kinase (PI3K)/Akt signaling pathway (40). The insolubility and high tensile strength of fibrillar collagen may be permissive for tensional forces to be transmitted to adhering ASM through β1-integrin mechanoreceptors.

ASM are a source of MMPs in vivo (45) and in vitro (19, 37). We show that fibrillar type I collagen enhances both their production and activation. Furthermore, MMP inhibition by ilomastat also reduces ASM-mediated degradation of fibrillar collagen and enhances fibrillogenesis, suggesting a role for MMPs in the modification of fibrillar collagen ultrastructure by ASM. This effect on collagen ultrastructure may also explain why ilomastat, as shown in this study and that of Ceresa et al. (8), inhibits ASM proliferation in a fibrillar collagen-enriched extracellular environment. Inhibition of pericellular collagen-enolysis in situ may reduce ASM hyperplasia in airway wall remodeling by maintaining the antiproliferative effects of fibrillar pericellular collagen. It has been shown that administration of the MMP inhibitory antibiotic doxycycline reduces ASM hyperplasia (18) and airway hyperresponsiveness (27) in allergen-challenged mice, consistent with the operation of a counterregulatory effect of fibrillar collagen on ASM in inflamed airways. Interventions with selective MMP inhibitors could be beneficial to ameliorate airway remodeling.

This study shows ASM have the capacity to change tissue architecture by degrading fibrillar collagen as well as spinning collagen into larger supramolecular assemblies. The native fibrillar form of type I collagen directs ASM into a nonproliferative phenotype that can be reversed as a consequence of autocrine MMP activity. These findings are compatible with the possibility that MMPs may contribute to ASM hyperplasia by diminishing the counterregulatory effects of pericellular fibrillar collagen.

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DISCLOSURES

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