Mast Cell Fibroblastoid Differentiation Mediated by Airway Smooth Muscle in Asthma

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Mast cell microlocalization to the airway smooth muscle (ASM) bundle is a key feature of asthma, but whether these mast cells have an altered phenotype is uncertain. In this paper, we report that in vivo, mast cells within the ASM bundle, in contrast to mast cells in the bronchial submucosa, commonly expressed fibroblast markers and the number of these cells was closely related to the degree of airway hyperresponsiveness. In vitro human lung mast cells and mast cell lines cultured with fibronectin or with primary human ASM cells acquired typical fibroblastic markers and morphology. This differentiation toward a fibroblastoid phenotype was mediated by ASM-derived extracellular matrix proteins, independent of cell adhesion molecule-1, and was attenuated by α5β1 blockade. Fibroblastoid mast cells demonstrated increased chymase expression and activation with exaggerated spontaneous histamine release. Together these data indicate that in asthma, ASM-derived extracellular matrix proteins mediate human mast cell transition to a fibroblastoid phenotype, suggesting that this may be pivotal in the development of airway dysfunction in asthma. The Journal of Immunology, 2010, 185: 6105–6114.
**Immunohistochemistry**

Sequential 2-μm sections were cut from glycolmethacrylate-embedded bronchial biopsies and stained using mAbs against α-smooth muscle actin (α-SMA; Dako, Ely, U.K.), mast cell tryptase (Dako), the fibroblast marker Thy-1 (Ab1) (Calbiochem, San Diego, CA), and appropriate isotype controls (Dako). Thy-1-tryptase* (fibroblast), Thy-1+tryptase+ (mast cell) and Thy-1+tryptase+ cells (fibroblastoid mast cell) were enumerated per square millimeter ASM by colocalization (7, 13, 15). The whole biopsy was assessed, and a minimum area of 0.1 mm² was considered assessable, as described previously (7). Double immunostaining for tryptase and Thy-1 fibroblast marker was performed using the EnVision Doublestain Kit (Dakocytomation, Carpinteria, CA) according to the manufacturer’s instructions. Thy-1 expression was developed with peroxidase and 3,3-diaminobenzidine tetrahydrochloride (brown reaction product) and for tryptase alkaline phosphatase and fast red (red reaction product). Sections were then counterstained with hematoxylin and mounted in an aqueous mounting medium (BDH Chemicals, Poole, U.K.). Appropriate isotype controls were performed in which the primary Abs were replaced by irrelevant mouse mAb of the same isotype and at the same concentration as the specific primary mAb.

**Cell culture and culture**

Pure ASM bundles were isolated from bronchoscopic samples (n = 12; 10 asthmatic subjects, 2 nonasthmatic) and from lung resection (n = 10). Primary ASM was cultured and characterized as previously described (21). Cells used were passage 2–5. Primary fibroblasts were isolated from bronchial biopsies, characterized for the fibroblast markers Thy-1 and 1B10 (AbCam, Cambridge, U.K.), and used between passage 6 and 8 (n = 13). The human lung mast cells (HLMCs) were isolated and cultured from nonasthmatic lung (n = 19), as previously described (21). The human mastocytoma cell line (HMC-1) was a generous gift from Dr. J. Butterfield (Mayo Clinic, Rochester, MN), and was cultured as described previously (21).

**Adhesion, elongation, and differentiation assays**

**Mast cells and ECM proteins.** HMC-1 cells (1 × 10⁶ cells/well) were seeded onto six-well plates uncoated or coated with 5–40 μg/ml fibronectin (Sigma-Aldrich, Poole, Dorset, U.K.) or 60 μg/ml collagen (Inamed Biomaterials, Fremont, CA) in ITS Media (ASM media with 1% ITS+3 supplement [Sigma-Aldrich] in place of FBS). After 6 d, nonadherent cells were collected and counted. The percentage of elongated adherent HMC-1 cells was calculated from five random high-powered fields. Adherent cells were then harvested using Accutase (Insight Biotechnology, Wembley, London, U.K.) and the percentage of adherence determined as the proportion of adherent cells/total cells.

**Mast cell coculture with ASM.** ASM cells (5 × 10⁶ cells/60 mm dish for HLMC coculture; 2.5 × 10⁶ cells/T75 flask for HMC-1 cocultures) were incubated at 37˚C for 48 h, prior to incubation with ITS media at 37˚C for 24 h. HLMCs or HMC-1 cells were then added in ITS media at a 1:1 ratio to Transwell membranes (0.4 μm; Corning, Corning, NY) inserted into wells containing ASM cells (1 × 10⁶ cells/well in six-well plates, incubated at 37˚C for 24–48 h, prior to incubation with ITS media for 24 h) or supernatant from ASM incubated in ITS media for 24 h or ITS media alone. After 6 d, the percentage of adhesion was calculated.

**HMC-1 cells were stained in ITS media at a 1:4 ratio to Transwell membranes (0.4 μm; Corning, Corning, NY) inserted into wells containing ASM cells (1 × 10⁶ cells/well in six-well plates, incubated at 37˚C for 24–48 h, prior to incubation with ITS media for 24 h) or supernatant from ASM incubated in ITS media for 24 h or ITS media alone.** After 6 d, the percentage of adhesion was calculated.

Following ECM/coculture assays, cells were stained for mast cell/fibroblast markers, where appropriate, by flow cytometry. Elongation of mast cells in coculture with ASM was also visualized by immunofluorescence.

**Flow cytometry**

HMC-1 cells were stained, where appropriate, with Abs to Thy-1, 1B10, Collagen 1 (Chemicon, Temecula, CA), CD117 (BD Pharmingen, San Diego, CA), chymase (Chemicon, Hampshire, U.K.), and tryptase (Dako), or their appropriate isotype controls indirectly labeled with FITC (Dako) or allophycocyanin (R&D Systems) and analyzed by one-color or two-color flow cytometry (in coculture samples) on a FACSCanto (BD Biosciences, Oxford, U.K.). Mast cells were distinguished from ASM by prelabeling with CellTrace CFSE prior to coculture (according to manufacturer’s instructions, Invitrogen Molecular Probes, Paisley, Scotland, U.K.) or by staining with CD117-RPE (Dako).

**Immunofluorescence**

Cells were stained, where appropriate, for surface Thy-1, 1B10, or appropriate isotype controls; indirectly labeled with FITC or allophycocyanin; and counterstained with DAPI (Sigma). Mast cells were distinguished from ASM cells by prelabeling with CellTrace CFSE or by staining with CD117-RPE and indirect labeling with NorthernLights-RPE (R&D Systems). Double immunofluorescence was performed on cocultured ASM and mast cells and mast cells adherent to fibronectin using biotinylated tryptase mAb (Promega, Madison, WI) and chymase mAb (Chemicon, Temecula, CA). The biotinylated Ab binding was detected using Streptavidin-Texas Red, and chymase Ab binding was detected using secondary FITC-labeled Ab. Sections were counterstained with DAPI. The slide was then mounted with fluorescent mounting medium and analyzed.

**Assessment of cell contraction by collagen gel analysis**

Following culture in fibronectin-coated six-well plates over 6 d, the extent of collagen gel contraction by HMC-1 cells was assessed. A total of

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Table I. Clinical characteristics of subjects

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<td>BD response (%)§</td>
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*Mean (SE).
†Geometric mean (95% CI).
§Median (IQR).
BD, bronchodilator; BDP, beclomethasone dipropionate; ICS, inhaled corticosteroids.
FIGURE 1. Mast cells in the ASM bundle express fibroblastic markers. Representative photomicrographs of a bronchial biopsy from an asthmatic (original magnification ×400) stained for isotype control, illustrating the epithelium and lamina propria (A), and sequential sections of the ASM bundle (B–D), with arrows highlighting the same cells across the three sections stained for α-SMA (B), mast cell tryptase (C), and the fibroblast marker Thy-1 (Ab1) (D). The arrowhead and the long and short arrows illustrate cells that are Thy-1+tryptase− (fibroblast), Thy-1−tryptase+ (mast cell), and Thy-1+tryptase+ (fibroblastic mast cell), respectively. Single staining of Thy-1+ (brown, black arrowhead) and double staining of Thy-1+ and tryptase+ of mast cells (red staining, white arrowhead, E). Dot plot of the number of mast cells per square millimeter of ASM (F) and Thy-1+ cells per square millimeter of ASM (G) in subjects with or without asthma. Horizontal bars represent the median, p < 0.05, Kruskal–Wallis test, for all across-group comparisons; the p value for Dunn’s posttest is given on the figure. H, Percentage of mast cells colocalizing with Thy-1+ cells in subjects with or without asthma. Mean (SEM), p < 0.05 ANOVA; p value for Tukey’s post hoc test is given on the figure. Correlation of mast cells per square millimeter of ASM (I) and Thy-1+ mast cells per square millimeter of ASM, with methacholine PC20 FEV1; correlation coefficient and p value are given on the figure.
1.25 × 10^5 HMC-1 cells were resuspended in 144 μl ITS media ± stimulus, with 299 μl collagen, 37 μl 10× DMEM (Invitrogen), and 20 μl sodium bicarbonate (Invitrogen), and added to 24-well plates. The mixture was left to polymerize into gels at 37°C for 90 min prior to detachment from the well; 500 μl ITS media ± stimulus was added and incubated over 5 d. Gel surface area was measured using ImageJ (http://rsb.info.nih.gov/ij) by a blinded observer. Collagenase (1.9 mg/ml; Sigma-Aldrich) for 20 min at 37°C was used to extract HMC-1 cells from the gels, to further characterize mast cell differentiation.

Quantification of histamine release by mast cells
Histamine was measured by a sensitive radioenzymatic assay, as described previously, and corrected for cell number (23).

ELISA
PGD2 expression in cocultured cell supernatants was measured by ELISA (Cayman Chemical, Ann Arbor, MI).

Statistical analysis
Statistical analysis was performed using GraphPad Prism 4 (GraphPad, San Diego, CA). Parametric data are presented as mean ± SEM, log normally distributed data were log transformed and expressed as geometric mean (95% confidence interval), and nonparametric data are presented as median (interquartile range [IQR]). Comparisons across groups was analyzed by ANOVA and post hoc Tukey’s test or by Kruskal–Wallis testing with post hoc Dunn’s pairwise comparisons for nonparametric data. Between-group comparisons were made by paired and unpaired t tests, as appropriate. Differences were considered significant when p < 0.05.

Results
Mast cells express fibroblast markers in the ASM bundle in asthma
To determine the localization of mast cells, fibroblasts, and cells coexpressing markers for both cell types in the airway wall, bronchial biopsies from 16 healthy controls and 26 subjects with asthma were studied. The clinical characteristics of these subjects are shown in Table I. Representative photomicrographs of mast cells (tryptase⁺ cells) and fibroblasts (Thy-1 [Ab1]⁺ cells) within the ASM bundle are shown in Fig. 1.† The proportion of mast cells that coexpressed fibroblast markers (tryptase⁺/Thy-1⁺ cells) was increased in the ASM bundle (18 ± 3%) compared with the lamina propria (2.9 ± 0.6%; p < 0.0001), and in the ASM bundle the proportion was increased in mild-moderate (22 ± 3%) and severe asthma (22 ± 4%), compared with healthy controls (4 ± 2%; p = 0.0002 ANOVA, p < 0.01 asthma groups versus controls, Tukey’s post hoc comparison) (Fig. 1F).

In the 21 asthmatic subjects who underwent bronchial challenge, the number of mast cells within the ASM bundle was significantly inversely correlated with the degree of AHR (r = −0.45; p = 0.04; Fig. 1F). There was no significant correlation between the number of fibroblasts in the ASM bundle and AHR (data not shown). The number of mast cells that coexpressed fibroblast markers was strongly correlated with the degree of AHR (r = −0.61; p = 0.004; Fig. 1F). There was no significant correlation between FEV1 percentage predicted and the number of mast cells, fibroblasts, or fibroblastoid mast cells in the ASM bundle (data not shown).

Mast cells adhere to ECM and undergo morphological transition
We therefore considered whether mast cells differentiate to a fibroblastoid phenotype in the presence of ECM proteins. Indeed, as illustrated (Fig. 1C). Double immunostaining for Thy-1 and tryptase on the same section are shown in Fig. 1E, where the Thy-1 cells are stained individually and double stained with tryptase⁺ mast cells within the ASM bundles (n = 3 severe asthmatics). The median (IQR) mast cell number within the ASM bundle was increased in mild-moderate (6.8 [9.8] cells/mm² ASM) and severe asthma (6.2 [16.4] cells/mm² ASM) compared with healthy controls (1.7 [4.4] cells/mm² ASM; p = 0.001 Kruskal–Wallis, p < 0.01 asthma groups versus controls, Dunn’s pairwise comparison) (Fig. 1F).

The number of fibroblasts was also increased in the ASM bundle in mild-moderate (4.4 [11.5] cells/mm² ASM) and severe asthma (6.5 [10.2] cells/mm² ASM), compared with controls (1.5 [2.5] cells/mm² ASM; p = 0.003 Kruskal–Wallis, p < 0.05 asthma groups versus controls) (Fig. 1G). The proportion of mast cells that coexpressed fibroblast markers (tryptase⁺/Thy-1⁺ cells) was increased in the ASM bundle (18 ± 3%) compared with the lamina propria (2.9 ± 0.6%; p < 0.0001), and in the ASM bundle the proportion was increased in mild-moderate (22 ± 3%) and severe asthma (22 ± 4%), compared with healthy controls (4 ± 2%; p = 0.0002 ANOVA, p < 0.01 asthma groups versus controls, Tukey’s post hoc comparison) (Fig. 1H).

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FIGURE 2. HMC-1 cells that adhere to fibronectin develop fibroblastic morphology. A. Representative images of HMC-1 cells cultured with serum-free media over 6 d without fibronectin (i) or cultured on fibronectin (40 μg/ml) (ii); original magnification ×40. The black arrow indicates the area of detailed morphology, of elongated HMC-1 cells shown in iii. B and C. HMC-1 cell adhesion on fibronectin, the percentage of elongated (B) (n = 4 or 5 experiments) and adhering (C) HMC-1 cells (n = 3–6 experiments) on fibronectin over 6 d. The percentage of elongated/adhering cells was calculated as described in Materials and Methods. Data are presented as mean ± SEM. Comparisons for adhering cells were made against cells without fibronectin (0), using the paired t test. *p < 0.05; **p < 0.01; ***p < 0.001. The p value for the paired t test of the effect of α5β1 inhibition, compared with its isotype control, is given on the figure.

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we found that in vitro the morphology of the HMC-1 changed over 6 d in the presence of plasma fibronectin, with cells becoming elongated (Fig. 2A). The percentage of elongated cells was related to the concentration of fibronectin in a dose-dependent manner (Fig. 2B). The number of elongated cells was greatest in the presence of fibronectin 40 μg/ml. Therefore this concentration of fibronectin was used throughout the study. At this concentration, HMC-1 cells adhered avidly to fibronectin after 6 d (81 ± 5%, n = 8; p < 0.001), and adhesion was markedly inhibited by anti-α5β1 blocking Ab (4.3 ± 0.4% versus isotype control, 78 ± 2%; p = 0.0005; n = 3; Fig. 2C).

Previous reports have shown HMC-1 cells to adhere to collagen at a maximum concentration of 60 μg/ml (6). At this concentration we found that the proportion of HMC-1 cells that adhered to collagen after 6 d was 59 ± 2% (n = 5; p < 0.001), and the proportion of elongated cells was increased (44 ± 2.4%; n = 4; p < 0.001).

**Mast cells adherent to ECM express fibroblast markers**

The fibroblast markers Thy-1 and 1B10 were highly expressed on cultured fibroblast cells, as evidenced by immunofluorescence (Fig. 3A) and by flow cytometry (Fig. 3B). The proportion of fibroblast cells that expressed Thy-1 was 67 ± 7% (p = 0.001; n = 13), and 1B10 was 26 ± 5 (p = 0.003; n = 7) (Fig. 3C).

HMC-1 cells cultured for 6 d without fibronectin (control), HMC-1 cells cultured with fibronectin that did not adhere (non-adherent cells) and those cells that adhered (adherent cells) were analyzed by flow cytometry for surface Thy-1, IB10, CD117, and intracellular tryptase expression. 1B10 expression was significantly increased in the adherent HMC-1 cells (ΔGMFI 21 ± 2%; n = 3) compared with control (6.7 ± 1.76; p = 0.002). HMC-1 cells cultured with or without fibronectin did not significantly express Thy1 (n = 5; data not shown).

In the presence of the anti-α5β1 blocking Ab, HMC-1 cells did not express 1B10 (Fig. 3F). We were unable to compare 1B10 expression in adherent versus nonadherent HMC-1 cells in the presence of the blocking Ab, as it markedly inhibited adhesion and

**FIGURE 3.** HMC-1 cells adherent to fibronectin develop fibroblastic markers Thy-1 and 1B10; fibroblast marker expression was confirmed by cultured fibroblast cells by immunofluorescence (A) and flow cytometry (B). The example fluorescent histograms represent populations of Thy-1 and 1B10 positive cells (black line) plotted with the corresponding isotype control (gray line). C, Percent positive analysis of fibroblast cells demonstrates surface Thy-1 (n = 13 experiments) and 1B10 (n = 7 experiments) expression. D, Representative histograms of surface 1B10, CD117, intracellular collagen I, tryptase, and chymase expression on HMC-1 cells cultured with serum-free media over 6 d without fibronectin, versus nonadherent cells and adherent cells with fibronectin. E and F, The ΔGMFI of CD117 (n = 5 experiments) and tryptase expression (n = 5 experiments) (E) and 1B10 expression with and without α5β1 fibronectin blocking Ab (n = 3) (F) in nonadherent versus adherent HMC-1 cells cultured on fibronectin over 6 d. The ΔGMFI was determined by the difference between the total binding expression of total GMFI minus the total binding of matched isotype control for 1B10, CD117, and tryptase. G, 1B10 expression was also confirmed by immunofluorescence on adherent HMC-1 cells on fibronectin (40 μg/ml) over 6 d. Data presented as mean ± SEM. Statistical differences were assessed using the t tests and p values as shown. Original magnification ×400.
the collagen gels without stimulation over 5 d (Fig. 3G).

In the presence of collagen (60 μg/ml) for 6 d, adherent
HMC-1 cells demonstrated increased 1B10 expression (ΔGMFI
79 ± 5; n = 3) compared with nonadherent cells (33 ± 9; p = 0.025) and HMC-1 cells alone (28 ± 6; n = 3).

Mast cell fibroblastoid transition causes collagen gel contraction

We considered whether during fibroblastoid transition in a three-
dimensional matrix mast cells undergo shape change that induces
contraction. Therefore, to test whether the HMC-1 cells that were
nonadherent to fibronectin in two-dimensional culture after
6 d have the capacity to undergo fibroblastic transition in a three-
dimensional matrix, we cultured adherent and nonadherent HMC-1
cells in collagen gels for 5 d.

We confirmed that after 6 d culture with fibronectin, the 1B10
expression was increased in the adherent versus nonadherent HMC-1
cells (ΔGMFI [95% confidence interval] 233 [140–325]; p = 0.003;
n = 3) (Fig. 4A). Following culture in collagen gels for a further
5 d, there was no difference in 1B10 expression between the adherent
and the nonadherent HMC-1 cells (Δ GMFI [95% confidence
interval] 43 [−26–112]; p = 0.18), but the change from baseline in 1B10 expression in the nonadherent cells was significantly increased compared with that in the adherent cells (280;
95% CI [142–418]; p = 0.005) (Fig. 4A). This observation suggests
that the nonadherent mast cells in the two-dimensional culture had indeed undergone fibroblastoid transition in collagen gels.

This HMC-1 fibroblastoid transition was associated with a significant
reduction in the size of the collagen gel. Representative
gels are as shown (Fig. 4B). After 5 d the mean ± SEM percentage
size of gel compared with baseline was 66 ± 4% (n = 7) for
nonadherent cells versus 82 ± 4% for adherent cells (p = 0.025)
(Fig. 4C). This finding illustrates that the mast cells had undergone
concomitant changes in cell shape and expression of fibroblast
markers in the collagen gels.

Mast cell fibroblastoid transition in coculture with ASM

To investigate the effects of mast-cell-ASM interactions on mast
cell phenotype, we extended our model to a coculture system. Primary
HLMCs and HMC-1 cells cocultured with primary ASM cells over
7 d changed their cell morphology to become more elongated (Fig.
5A). This change in cell morphology was also confirmed by immuno-
fluorescence (Fig. 5B). The percentage of tryptase/chymase-
positive cells increased significantly after 10 d (27 ± 5.8%; p = 0.05;
n = 13; Fig. 5C, 5D) compared with cells in coculture (9.85 ± 5.3%;
n = 7) and cells adherent to fibronectin (10.1 ± 3.3%; n = 6; p = 0.02)
over 1 d.

In contrast to HMC-1 cells, HLMCs expressed 1B10 in the absence
of fibronectin and/or ASM (13 ± 5%; n = 3), whereas the percentage
of Thy-1–positive HLMCs in the absence of fibronectin and ASM was low (4.7 ± 1.7%). Therefore, Thy-1 expression by mast cells was studied in coculture with ASM cells.

To track mast cells in coculture with ASM over 6–15 d, mast cells were labeled with the fluorescent marker CFSE, a stable
dye that is not passed between cells upon adhesion. With use of
flow cytometry, CFSE-labeled mast cells were gated and analyzed
for Thy-1 expression. Example flow cytometry dot plots and his-
tograms are as shown, illustrating Thy-1 expression by HLMCs, HMC-1 cells, and in cells cocultured with ASM (Fig. 5E).

Following coculture with ASM, HLMC Thy-1 expression was in-
creased compared with control after 7 d (22 ± 5%; p = 0.005; n = 6)
and 15 d (45 ± 8%; p = 0.004; n = 6) (Fig. 5F). A small, albeit
significant, increase in Thy-1 expression was observed for HMC-1
cells (9 ± 2%; p = 0.011; n = 7) cocultured with ASM for 6 d,
compared with cells alone (Fig. 5G). Similarly, Thy-1 expression by
HMC-1 cells cultured with ASM separated by a Transwell insert or
with ASM-conditioned media was increased, compared with that in
controls. This increased expression was reduced in comparison with
that of HMC-1 cells in coculture with ASM (Fig. 5G; p < 0.05).

HMC-1 cell Thy-1 expression was not modulated by TGFβ1 or
2 (0–10 ng/ml) (data not shown).

Mast cell fibroblastoid transition in coculture with ASM is
mediated via α5β1

We considered whether this mast cell fibroblastoid transition was
dependent upon mast cell-ASM adhesion or mast cell ECM-ASM
interactions. The percentage adhesion of HMC-1 cells to ASM after
6 d coculture was 57 ± 7%, n = 4. In contrast to short-term culture
(14), this HMC-1 adhesion to ASM after long-term culture was
not inhibited by preincubation with the CADM1 blocking Ab
(58 ± 9% versus isotype control 57 ± 9%; n = 4; p = 0.67) but was
markedly inhibited by α5β1 blockade (26 ± 4% versus isotype
control 68 ± 6%; p = 0.0008; n = 6) (Fig. 6A). The blockade
of CADM1 and α5β1 in concert was not different to α5β1 alone
(p = 0.49) (Fig. 6A).

In coculture with ASM and in the presence of the α5β1 blocking
Ab, the proportion of Thy-1+ HMC-1 cells (6.4 ± 4.3%; n = 4)
and HLMCs (4.9 ± 1.4%; n = 5) was significantly decreased.
FIGURE 5. Mast cell-ASM coculture promotes fibroblastoid differentiation. A, Representative photomicrographs of ASM cells cultured alone with serum-free media (i), over 7 d with HLMCs (ii, iii), or HMC-1 cells over 6 d (iv) (original magnification ×40). The black arrow indicates the area in ii that is shown in detail in iii. B, CD117 expression of HLMCs cultured on ASM cells over 3 and 7 d, showing elongated cells at day 7 (nuclei stained blue). Original magnification ×200. C, Immunofluorescence staining demonstrating HLMCs cultured on fibronectin for 1 d (i–iii) and cocultured on ASM cells over 10 d (iv–vi); i and iv, double isotype control; ii and v, red trypase+ve HLMCs; iii and vi, double stained HLMCs for trypase (red) and chymase (green) (n = 6–13). Original magnification ×400. D, Percentage of trypase/chymase-positive HLMCs cultured alone (1 d; n = 6) and with ASM (1–10 d; n = 6–13). E, Representative dot plots for ASM alone and ASM cultured with HLMCs (prelabeled with CFSE FITC). Labeled CFSE HLMCs were gated and analyzed for Thy-1 expression, using the allophycocyanin secondary Ab. Shown are representative flow cytometric histograms for HLMCs and HMC-1 cells prelabeled with CFSE and stained for Thy-1–allophycocyanin (black line) plotted with the corresponding isotype control (gray line) over 6 d culture. F, Percentage of Thy-1–positive HLMCs cultured alone (7–15 d; n = 10 donors) and with ASM for 7 d (n = 6) and 15 d (n = 6). G, Percentage of Thy-1–positive HMC-1 cells cultured alone (n = 3–5), with ASM (n = 7), on inserts over ASM cells (n = 4), and with ASM cell supernatants (n = 4). Data are presented as mean ± SEM. Statistical differences were assessed using ANOVA and t tests as appropriate, and p values are as shown.
compared with their isotype controls (27.4 ± 7.5% and 13.4 ± 3.1%, respectively; p < 0.05) (Fig. 6B). Blockade of CADM1 did not affect Thy-1 expression by HLMCs (15.8 ± 3.9% versus 17.7 ± 1.3%; p = 0.59).

The histamine concentration in the media corrected for cell number following coculture of HLMCs with ASM for 7 d was significantly reduced in the presence of the anti-α5β1 blocking Ab compared with its isotype control (2.4 ± 0.5 μg/10^6 cells versus 3.2 ± 0.6 μg/10^6 cells; p = 0.02; n = 6) (Fig. 6C). However, IgE–anti-IgE activation of HLMCs prior to incubating with ASM did not significantly reduce histamine release (Fig. 6C). PGD2 release by HLMCs was not affected by anti-α5β1 (117.7 ± 47.2 ng/10^6 cells; p = 0.9) compared with IgG1 control (109.9 ± 18.8 ng/10^6 cells).

Discussion
This study is the first to demonstrate that mast cells localized within the ASM bundle in asthma express fibroblast markers. In vitro we determined that this mast cell fibroblastoid differentiation is ASM-derived ECM protein dependent. These fibroblastoid mast cells have an increased expression of chymase and are in a heightened state of activation, with increased constitutive release of histamine. Taken together, our findings suggest that ASM-derived ECM-mediated mast cell fibroblastoid transition is important in the development of airway dysfunction in asthma.

Our study provides further evidence to support the view that, in asthma, mast cell localization to the ASM bundle is important in the pathogenesis of AHR. Several studies have consistently reported increased mast cell numbers in the ASM bundle in asthma (7, 8, 13, 16, 17, 24, 25). Using stepwise logistic regression to explore the association between AHR and immunopathological features of asthma, we observed that mast cell infiltration of the ASM bundle was the strongest independent predictor of AHR (8).

In this paper, we extend these earlier studies to demonstrate that the association with AHR is stronger for the number of “fibroblastic” mast cells than for the total mast cells within the ASM bundle, suggesting that this fibroblastoid-mast cell transition may play a pivotal role in mast cell-ASM interactions and the development of disordered airway function.

Consistent with our in vivo findings, we confirmed in vitro that mast cells have the capacity to undergo fibroblastoid differentiation. Primary HLMCs and human mast cell lines changed their morphology to spindle-shaped cells in the presence of the ECM proteins fibronectin or collagen I. These mast cells expressed proteins usually associated with fibroblasts, confirmed with two different Abs using a combination of techniques. Importantly, although the mast cells acquired fibroblast-like features and increased chymase expression, there was no downregulation in their CD117 or β-tryptase expression. This fibroblastoid transition was replicated in coculture with ASM and was associated with a change in cell size and increased constitutive histamine release, but not PGD2. This fibroblastoid transition was associated with an increase in gel contraction, which may be a consequence of cell size and function. This finding was consistent with earlier observations determined by electron microscopy whereby mast cells within the ASM bundle were found to be smaller with reduced granule content, as a consequence of piecemeal degranulation (17).

We have reported previously that HLMCs and HMC-1 cells in vitro adhere to ASM, in part via CADM1 (14), and in coculture with ASM mast cells survive and proliferate (23). In contrast, we report in this paper that the mast cell transition toward a fibroblastoid phenotype was CADM1 independent. Indeed, this phenotypic transition was dependent on mast cell-ECM adhesion. One possible explanation for this apparent paradox is that CADM1 is

**FIGURE 6.** Mast cell fibroblastoid differentiation promoted by ASM-derived ECM proteins is α5β1 dependent and is associated with mediator release. A, Percentage inhibition of HMC-1 cell adherence to ASM cells by adhesion blocking Abs directed against CADM1 (n = 4) and α5β1 integrin (n = 6) alone and in combination (n = 4), compared with appropriate isotype controls (IgY and IgG1). B, Percentage of Thy-1–positive HMC-1 cells (n = 4) and HLMCs (n = 3–5) cocultured with ASM for 6 d (HMC-1) and 7 d (HLMC) in the presence of anti-α5β1 mAb determined by flow cytometry. C, Constitutive and net IgE-dependent histamine release from HLMC was measured after 7 d coculture with ASM cells in the presence of the anti-α5β1 integrin or isotype control (IgG1) (n = 2 ASM cell donors and 4 HLMC donors). Data are presented as mean ± SEM. Statistical differences were assessed using the t tests and p values as shown.
important for the initial adhesion of mast cells to ASM and may initiate important cell signaling events, but that sustained adhesion of mast cells within the ASM bundle is maintained by mast cell interactions within a local ECM protein environment. This observation provides an explanation for the lack of effect we reported for blocking β-integrins on short-term adhesion for 30 min between ASM and mast cells, as well as providing a rationale for our inability to inhibit mast cell adhesion to ASM by CADM1 blockade after coculture for several days. Mast cell adhesion to fibronectin has been consistently reported (26–28), and in some circumstances, stem cell factor and IgE cross-linking promote mast cell adhesion to fibronectin (29, 30). We report in this paper that the development of fibroblastoid mast cells cultured with ECM proteins increased in a dose-dependent manner. This also occurred in coculture with ASM and was present, albeit to a substantially reduced extent, in the absence of direct contact with ASM when using Transwells and ASM supernatants and may have been mediated by soluble fibronectin. All of these effects were inhibited by α5β1 integrin blockade, implicating mast cell-fibronectin interactions. Importantly, fibronectin is increased in the ASM bundle in asthma deaths (19) suggesting that this ECM protein is important in asthma, particularly in severe disease, and therefore has the capacity to mediate important mast cell-ECM interactions. Consistent with this view, previous reports have shown that rat mast cells cocultured with fibroblasts (31) or fibronectin result in increased histamine release (26). Similarly, human mast cells that adhere to fibronectin become more oblong, with pronounced formation of filopodia (27). This adhesion is attenuated by TLR3 activation, which consequently abrogates mast cell attachment-dependent potentiation of IgE-mediated responses (28). Similarly, reduction in mast cell β1 integrin surface expression as a consequence of diminished surface receptor stability in rabaptin-5 knockdown also leads to inhibition of mast cell adhesion, migration, and activation (32). Perhaps of most importance, adhesion to fibronectin does not increase IgE-dependent histamine release from HLMCs (33), supported by data reported in this paper, in contrast to findings in rodent mast cells (34). This suggests that ASM-driven HLMC mediator release, which is in part dependent on this matrix protein, drives the chronic mast cell activation evident within the ASM bundle in asthma in vivo (17). Taken together, these data suggest that ASM-derived ECM-mast cell interactions are critical for the transition of mast cells to a fibroblastoid phenotype and are likely to be central for the functional effects of this altered mast cell phenotype.

Our findings lead us to propose a new paradigm for the development of disordered airway physiology in asthma whereby the location of the mast cell within the ASM bundle leads to important reciprocal phenotypic changes in both cell types, which are perpetuated via positive feedback. We have reported that in coculture IgE-independent mast cell release of β-tryptase results in an upregulation of α-SMA intensity mediated via autocrine TGF-β release (35). Consequently, the ASM undergoes a phenotypic switch to a more contractile phenotype. This is supported by a very strong correlation in vivo between the number of mast cells within the ASM bundle and the ASM α-SMA intensity. We found that TGF-β did not influence the mast cell phenotype. However, TGF-β is an important inducer of ECM protein production by ASM, in particular, fibronectin (36, 37). This increased release of fibronectin will in turn promote ASM synthetic function (38, 39) and mast cell activation (26, 40), as well as transition to fibroblastoid phenotype. This altered phenotype has the capacity to further drive the ASM to a more contractile phenotype, therefore propagating the cycle to promote AHR (35).

One potential criticism of our findings is the cross-sectional design of the bronchoscopy study, which does not allow us to determine whether the strong relationship between the number of activated fibroblastoid mast cells in the ASM bundle and AHR is causal or an epiphenomenon. To confirm that fibroblastoid mast cells and their products promote airway dysfunction, bronchoscopic evaluation is required as part of interventional studies of therapies that attenuate AHR. In addition, to date there is paucity of robust animal models of asthma in which mast cell infiltration of the ASM bundle is a feature. Thus there is a need for improved models and new therapies to further study the importance of this mast cell fibroblastoid transition in asthma. Whether this transition is reversible and the fibroblastoid mast cell phenotype is plastic is also important and warrants further study.

In conclusion, we report in this paper the first evidence that mast cells develop a fibroblastoid phenotype mediated via ASM-derived ECM proteins, dependent on the α5β1 integrin. This novel mast cell phenotype demonstrates increased mediator release of histamine, and its presence in the ASM bundle is closely related to AHR. This presents a new paradigm for asthma and underlines the importance of mast cell-ASM interactions as a therapeutic target.

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References


