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Multiple \( \beta_1 \) Integrins Mediate Enhancement of Human Airway Smooth Muscle Cytokine Secretion by Fibronectin and Type I Collagen

Qi Peng, Dilys Lai, Trang T.-B. Nguyen, Vivien Chan, Takeshi Matsuda, and Stuart J. Hirst

Altered airway smooth muscle (ASM) function and enrichment of the extracellular matrix (ECM) with interstitial collagen and fibronectin are major pathological features of airway remodeling in asthma. We have previously shown that both fibronectin and collagen types I, III, and V enhance ASM proliferation in vitro, but their action on ASM secretory function is unknown. Here, we examined the effects of fibronectin and collagen types I, III, and V on IL-1\( \beta \)-dependent secretory responses of human ASM cells, and characterized the involvement of specific integrins. Cytokine production (eotaxin, RANTES, and GM-CSF) was evaluated by ELISA, RT-PCR, and flow cytometry. Function-blocking integrin mAbs and RGD (Arg-Gly-Asp)-blocking peptides were used to identify integrin involvement. IL-1\( \beta \)-dependent release of eotaxin, RANTES, and GM-CSF was enhanced by fibronectin and fibrillar and monomeric type I collagen, with similar changes in mRNA abundance. Collagen types III and V had no effect on eotaxin or RANTES release but did modulate GM-CSF. Analogous changes in intracellular cytokine accumulation were found, but in <25% of the total ASM cell population. Function-blocking Ab and RGD peptide studies revealed that \( \alpha_\beta_1 \), \( \alpha_\beta_3 \), and \( \alpha_\beta_4 \) integrins were required for up-regulation of IL-1\( \beta \)–dependent ASM secretory responses by fibronectin, while \( \alpha_\beta_2 \) was an important transducer for type I collagen. Thus, fibronectin and type I collagen enhance IL-1\( \beta \)–dependent ASM secretory responses through a \( \beta_1 \) integrin–dependent mechanism. Enhancement of cytokine release from ASM by these ECM components may contribute to airway wall inflammation and remodeling in asthma. The Journal of Immunology, 2005, 174: 2258–2264.
ASM. We compared IL-1β/TNF-α-dependent eosinophil-activating
cytokine release from human ASM cells that were cultured
either on plastic (Pl) or fibronectin or on varying forms of collagen.
Moreover, we characterized the integrin heterodimer subunits
expressed by ASM and their involvement in modulating secretory
responses.

Materials and Methods

Isolation and culture of human ASM cells

Human ASM cells were obtained in accordance with procedures approved
by the Guy’s and St. Thomas’ Hospitals’ Research Ethics Committee from
the lobar or main bronchi of 45 nonasthmatic patients (mean age, 64 ±
4 years; range, 28–78 years; 24 male, 21 female) undergoing lung resection
for carcinoma of the bronchus using methods described previously (13).
Fluorescent immunocytochemical and flow cytometric techniques con-
ﬁrmed that near-confluent, FBS-deprived human ASM cells (passage 2)
staing (∼95%) for smooth muscle-speciﬁc α-smooth- and calponin (17).
Cells at passages 3–5 were used in all experiments.

Surface coating with ECM proteins

Lyophilized human plasma ﬁbronectin (Sigma-Aldrich), rat monomeric
type I collagen (ICN Chemicals), and human ﬁbrillar collagen types I, III,
and V (Chemicon International) were reconstituted in sterile PBS. ECM
proteins (0.1–10 μg/ml) diluted in PBS were adsorbed to tissue culture
plasticware overnight at 37°C as previously described (17). Excess un-
bound ECM protein was removed by aspiration and washing with PBS.
Unoccupied protein-binding sites were blocked with 0.1% BSA for 30 min.

Cell stimulation and application of blocking mAbs

Near-conﬂuent, FBS-deprived cells from ﬂasks were seeded (5000 cells/cm²)
in DMEM containing 1% FBS on Pl or ECM substrate (0.1–10
μg/ml)-precoated plasticware and left overnight at 37°C. In some experi-
ments, cells in suspension were pretreated for 30 min at room temperature
with integrin function-blocking mAbs (1 μg/ml) or isotype-matched con-
trol Abs with continuous rolling before seeding. After attachment,
cells were washed twice with FBS-free RPMI 1640 (containing 25 mM
HEPES, 2 mM L-glutamine, 100 U/ml penicillin/100
μg/ml streptomycin), and then stimulated for 24 h in RPMI 1640 with 1 ng/ml recombinant human IL-1β or 10 ng/ml recombinant human TNF-α. Soluble RGD inte-
grin-blocking peptides (10 μM) or a negative control peptide were added
directly to adherent cells 30 min before stimulation with cytokines. Integrin
function-blocking anti-human mouse mAbs (Chemicon International, un-
less stated otherwise) were anti-α1 (clone FB12), anti-α2 (clone P1E6),
anti-α3 (clone P1B5), anti-α5 (clone P1D6), anti-αv (clone L230, gift from
Dr. D. Daniel Marshall (John Vane Science Center, London, U.K.)/21), anti-β1
(clone 6S6), anti-αvβ1 (clone LM609), and anti-β3 (clone 2E11). Mouse-
puriﬁed or isotype-matched IgG (Chemicon International) was used as a
nonimmune control.

Flow cytometric labeling of surface integrins and intracellular
cytokines

Cell surface integrins were localized by binding of the above anti-human
integrin subunit mAbs. Near-conﬂuent growth-arrested cells on Pl were
harvested using trypsin/EDTA, washed twice in PBS (200 × g for 5 min),
resuspended, and ﬁxed with 200 μl of 4% formaldehyde (methanol-free,
EM-grade; Polysciences) for 30 min on ice in round-bottom FACS tubes.
Fixed cells (20,000/tube) were resuspended in PBS containing 3% FBS and
left overnight at 37°C as previously described (17). Excess un-
occupied protein-binding sites were blocked with 0.1% BSA for 30 min.

Statistical analysis

Data are mean ± SEM from cells cultured from n patient donors. Data
were compared using one- or two-way ANOVA, where appropriate, fol-
lowed by Bonferroni’s t test post hoc to evaluate statistical differences
between treatment groups (SigmaStat; SPSS). A p value of <0.05 was
considered signiﬁcant.

Results

Regulation of ASM secretory responses by ECM proteins

IL-1β (1 ng/ml) induced marked release of eotaxin, RANTES, or
GM-CSF from human ASM cells when cultured on Pl (Fig. 1),
conﬁrming previous observations (13). After culture in plates pre-
coated with ﬁbronectin or ﬁbrillar type I collagen, IL-1β-stimu-
lated cells were signiﬁcantly more secretory compared with culture
on Pl (p < 0.05–0.01; Figs. 1 and 2). A similar concentration-
dependent increase in IL-1β-dependent secretory capacity was
found with monomeric type I collagen (p < 0.05–0.01; Fig. 2),
which did not differ from the up-regulation found with ﬁbrillar
type I collagen (p > 0.05, two-way ANOVA; Fig. 2). Up-regu-
lation of IL-1β-dependent eotaxin or RANTES release was not
found with culture on either type III or V collagen (p > 0.05, n =
5; data not shown), although in the same samples there was a
concentration-dependent decrease (52 ± 3% at 10 μg/ml) in GM-
CSF with type V collagen and an increase in GM-CSF release
(195 ± 13% at 10 μg/ml) with type III collagen compared with
IL-1β-stimulated cells on Pl (p < 0.05–0.01, n = 5–6; data not
shown). Additionally, ﬁbronectin and type I collagen (10 μg/ml)
bind GM-CSF as previously described (22). In all experiments, release of
eotaxin, RANTES, or GM-CSF from cells cultured on these ECM
substrates (1 or 10 μg/ml) in the absence of IL-1β/TNF-α did not
differ from release by unstimulated cells on Pl (p > 0.05, n = 6–7
for each ECM; Figs. 1 and 2).
Cytokine gene regulation by ECM proteins

To examine whether up-regulation of IL-1β-dependent secretory responses by ECM substrates involved increased gene activation, cytokine mRNA levels were examined in human ASM cells cultured on fibronectin. Consistent with previous reports, treatment of cells growing on Pl with IL-1β (1 ng/ml) for 16 h increased the mRNA abundance for eotaxin, RANTES, and GM-CSF (p < 0.05–0.01; Fig. 3) (10, 11). A further increase in eotaxin and GM-CSF mRNA occurred in IL-1β-stimulated cells after culture on 10 μg/ml fibronectin (p < 0.05; Fig. 3). An apparent increase in IL-1β-dependent RANTES mRNA was also found, but this failed to reach significance. No change in 18S rRNA between the treatments was found (Fig. 3). In two separate experiments, a similar enhancement of IL-1β-dependent eotaxin, RANTES, and GM-CSF mRNA abundance at 16 h was found after culture on fibrillar type I collagen (10 μg/ml), compared with stimulation on Pl (not shown).

Modulation of intracellular cytokine labeling by ECM proteins

To investigate whether enhancement of cytokine production by ECM substrates occurred in all ASM cells or in a subset, intracellular cytokine levels were examined by flow cytometry in fixed saponin-permeabilized cells. In preliminary studies, positive labeling for eotaxin, RANTES, or GM-CSF was detected in 2% of the total population after stimulation with IL-1β for 24 h, which increased to 4–5% after culture on 10 μg/ml fibronectin or fibrillar type I collagen (n = 2). Given these low levels of labeling, cells in subsequent studies were costimulated with maximally effective concentrations of IL-1β (1 ng/ml) and TNF-α (10 ng/ml), shown previously in ELISA studies to act synergistically to induce release of GM-CSF and RANTES (10, 13). Optimal numbers of GM-CSF (12.2 ± 3.7%)-, RANTES (19.03 ± 3.72%)-, or eotaxin (11.4 ± 3.41%)-labeled cells occurred with stimulation for 24 h (data not shown). Kinetics for GM-SCF and RANTES accumulation were similar, consistent with our previous ELISA findings in IL-1β-stimulated cells (13). In keeping with the current ELISA (Figs. 1 and 2) and mRNA findings (Fig. 3), culture on fibronectin or fibrillar type I collagen further increased the number of IL-1β/TNF-α-stimulated eotaxin-, RANTES-, or GM-CSF-positive cells by ~1.5-fold (p < 0.05–0.01 compared with stimulated cells on Pl; Fig. 4). Likewise, culture on type III collagen increased IL-1β/TNF-α-stimulated GM-CSF (p < 0.05) but not RANTES labeling, which overall resembled closely the ELISA findings with collagen type III (described above).

Attenuation of fibronectin or collagen I-enhanced eotaxin release from cultured human ASM cells by integrin specific blocking mAbs

Having defined a probable role for ECM substrates in the regulation of cytokine production from human ASM cells, subsequent
experiments examined possible integrin receptors that could mediate this response. Flow cytometry of cells on Pl confirmed that α5 and β1 integrin subunits were universally expressed and that ∼50–60% of cells had detectable surface α1, α2, α3, and α5 integrin subunits and ∼50% expressed the α1β1 heterodimer (Fig. 5A). To exclude possible underestimation of the number of labeled cells due to cleavage of Ab recognition sites by the trypsin used to remove the cells from the culture flasks, we compared this profile of integrin subunit expression in cells harvested using PBS-containing EDTA (0.5 mM for 20 min) and found no differences, which was further supported by examination of cultured human ASM cells in situ by reflected UV light immunofluorescence microscopy (T. T.-B. Nguyen and S. J. Hirst, unpublished observations).

The panel of blocking mAbs was also used to investigate the specific involvement of integrin subunits in the enhanced ASM secretory responses elicited by fibronectin and type I collagen. Preliminary flow cytometry studies showed enhancement of IL-1β/TNF-α-dependent intracellular eotaxin and RANTES labeling was reduced by 1 μg/ml β1 integrin subunit-blocking mAb (p < 0.05, Table I). Likewise, in ELISA studies, blocking the binding of matrix factors to α5 or β1 integrin subunits abolished enhancement of IL-1β-dependent eotaxin release by either fibronectin or fibrillar type I collagen (p < 0.05 compared with control IgG1, Fig. 5, B and C). Function-blocking mAbs (1 μg/ml) to α5 or α6 subunits had a similar effect, but only against enhancement by fibronectin (p < 0.05 compared with control IgG1). Abs against α5 or β1 subunits were without effect (p > 0.05 compared with control IgG1). However, the α1β1 heterodimer-specific blocking mAb (1 μg/ml) abolished enhancement of eotaxin release by fibronectin (p < 0.05 compared with control IgG1). A similar profile of neutralization was obtained when IL-1β-dependent RANTES release by either fibronectin or type I collagen was examined in supernatants in place of eotaxin (data not shown, n = 4).

To further support a role for integrins in enhanced secretory signals by the ECM, preincubation of cells with 10 μM soluble RGD-blocking peptides (Gly-Arg-Gly-Asp-Ser (GRGDS) or Gly-Arg-Gly-Asp-Thr-Pro (GRGDTP)), but not the negative control Gly-Arg-Ala-Asp-Ser-Pro (GRADSP), was found to abolish the enhancement of IL-1β-dependent eotaxin release by fibronectin (p < 0.05, Fig. 5D). This result is in keeping with a role for α5β1, α1β1, and α1β5 integrin-mediated secretory signals, though α3β1, α1β1, α1β3, α1β5, and α3β1 are also known to be RGD sensitive (23).

Discussion

In this present study, we demonstrate selected airway wall ECM components that are increased in asthma, such as collagen and fibronectin (5–9), have the capacity to up-regulate ASM cell secretory responses. Although absolute values for the amounts of ECM substrates in contact with ASM in the asthmatic airway are
unknown, we report that culture on fibronectin, fibrillar, or monomeric type I collagen at concentrations similar to those found to enhance ASM cell proliferative and survival responses (17–19), provided a transcriptionally regulated enhancement of the IL-1β-dependent cytokine secretory signal. ELISA studies indicated that enhancement by fibronectin was mediated through multiple β1 integrins expressed by ASM including α3β1, α5β1, and αvβ5, but also by αvβ3, as function-blocking mAbs directed against these integrin subunits or to the αvβ3 heterodimer prevented enhancement of IL-1β-dependent eotaxin release by fibronectin, as did blocking a fibronectin recognition site with soluble RGD peptides. Similarly, enhancement by fibrillar collagen type I appeared to involve the αvβ5 integrin. Furthermore, flow cytometric analysis indicated these events may involve a minority of cultured ASM cells because intracellular cytokine expression elicited by IL-1β/TNF-α and its potentiation by the ECM could only be detected in <25% of the total population; even under conditions considered to induce maximal cytokine release when measured by ELISA (10, 13).

Although the involvement of specific ECM components in up-regulation of airway and vascular smooth muscle responses such as attachment, proliferation, migration, and survival is well established (17, 18, 24, 25), up-regulation of cytokine secretory capacity by fibronectin or collagen ECM substrates to our knowledge has not previously been reported in mesenchymal cells from the lung or elsewhere. In ELISA studies, we found that overnight culture of human ASM cells in plates precoated with fibronectin, fibrillar, or monomeric denatured type I collagen increased IL-1β-dependent cytokine release compared with similarly stimulated cells cultured on PL. Collagen types III and V had no effect on secretion, suggesting enhancement of cytokine release requires these ECM substrates to be in the polymerized form present after coating (Q. Peng and J. S. Hirst, unpublished observations).

The extent of enhancement of cytokine release was consistent across each of the substrates examined with GM-CSF being the most increased (~200%) followed by eotaxin (~150%) and RANTES being the least susceptible (~130%). In keeping with this

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Integrin-dependent enhancement of IL-1β-stimulated eotaxin release by fibronectin and fibrillar type I collagen. A. Flow cytometric analysis of integrin expression by human ASM cells cultured on PL (n = 6). Also shown is the effect of integrin function-blocking mAbs on enhancement of IL-1β-dependent eotaxin release (ELISA) by either fibronectin (B; FN) or collagen (C; Col) type I (10 μg/ml). D. The effect of soluble RGD integrin-blocking peptides (10 μM) compared with a negative control RAD peptide (n = 3–4). *p < 0.05 denotes significant reduction in eotaxin release compared with IgG1 or absence of blocking peptide.

### Table I. Blocking of fibronectin-enhanced and type I collagen-enhanced intracellular cytokine labeling by anti-β1 integrin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibronectin (% stimulated on PL) Ethaxin (% stimulated on PL)</th>
<th>RANTES</th>
<th>Type I Collagen (% stimulated on PL) Ethaxin (% stimulated on PL)</th>
<th>RANTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated/PL</td>
<td>21 ± 7.4</td>
<td>14 ± 7.7</td>
<td>21 ± 7.4</td>
<td>14 ± 7.7</td>
</tr>
<tr>
<td>IL-1β/TNF-α/PL</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IL-1β/TNF-α/ECM/IgG1</td>
<td>141 ± 21</td>
<td>179 ± 50</td>
<td>124 ± 6</td>
<td>157 ± 29</td>
</tr>
<tr>
<td>IL-1β/TNF-α/ECM/anti-β1</td>
<td>116 ± 14.2*</td>
<td>71 ± 14*</td>
<td>81 ± 20*</td>
<td>91 ± 9*</td>
</tr>
</tbody>
</table>

*Effect of blocking mAb (1 μg/ml) directed against the β1 integrin subunit on flow cytometric labeling of cotaxin and RANTES in human ASM cells cultured on fibronectin (10 μg/ml) or type I collagen (10 μg/ml) ECM following combined treatment with IL-1β (1 ng/ml) and TNF-α (10 ng/ml) for 24 h.*

*p < 0.05 compared with enhancement in presence of IgG1 control by one-way ANOVA. Means ± SEM are shown (n = 4).
enhancement of eosinophil-activating cytokine protein release by ECM substrates, analogous increases in these cytokines were found with techniques examining intracellular cytokine expression (flow cytometry) and mRNA abundance (RT-PCR), though the increase for RANTES mRNA failed significance, which may reflect either insufficient powering or saturation of the PCR. Enhancement of cytokine mRNA levels by fibronectin or type I collagen implies the increase in eotaxin, RANTES, and GM-CSF protein detected by the ELISA and flow cytometry studies involves increased cytokine gene transcription or stabilization of mRNA transcripts. Although not examined in the present study, it was recently shown that GM-CSF release from TNF-α-stimulated eosinophils in the presence of fibronectin involved mainly GM-CSF mRNA stabilization, which was dependent on ERK phosphorylation and the RNA binding proteins, YB-1 and HuR (27). Elsewhere, evidence suggests that growth factors can bind ECM components to form complexes that enhance subsequent growth factor activity through integrin collaboration with growth factor receptors. In the case of VEGF interacting with fibronectin to enhance endothelial cell migration, the amplified response was attributed to sustained ERK activation requiring activation of both the Flk-1 (VEGF receptor) and α5β3 (fibronectin receptor) (28). Whether similar mechanisms operate in human ASM cells remains an open question.

This study is among the first with ASM cells to demonstrate intracellular cytokine expression by flow cytometric techniques. The synthesis and release of proinflammatory mediators by ASM is a relatively new finding (29) and has almost without exception been estimated by ELISA- or RT-PCR-based methods in experiments that have not been designed to distinguish between signals generated from a subset of cells rather than the total population. Although the single labeling flow cytometry method used here demonstrated that culture on fibronectin or type I collagen potentiated intracellular cytokine expression (also type III collagen in the case of GM-CSF elaboration) in human ASM cells stimulated for 24 h with maximally effective concentrations of IL-1β and TNF-α in combination, overall numbers of positive-labeled cells generally did not exceed 25% of the total population indicating that only a subpopulation of human ASM cells could be induced to express eosinophil-activating cytokines under the current experimental conditions. It is possible that further increases in the frequency of positive cells may have been detected at time points beyond 24 h, though inclusion of the protein transport inhibitor brefeldin A either reduced labeling or had no effect and was therefore omitted (D. Lai and S. J. Hirst, unpublished observations). Technical difficulties associated with the high autofluorescence of human cultured ASM cells (Fig. 4A) precluded use of double- or triple-labeling strategies to examine whether those cells expressing eotaxin also expressed RANTES or GM-CSF, and so it remains possible that distinct multiple populations of cells exist with each capable of expressing one or more cytokine. Likewise, the current study did not ascertain whether culture on various ECM substrates increased expression levels in a single subpopulation of cells already expressing or recruited additional cells. Nevertheless, the data raise an important question concerning the possible heterogeneity of ASM cell cytokine expression, which has not previously been addressed.

Integrins, which comprise αβ heterodimers are the principal receptors mediating multiple cell responses to ECM substrates (30). Their extracellular domains recognize short peptidic sequences (e.g., Arg-Gly-Asp (RGD) found on some ECM proteins (fibronectin and vitronectin)), while the intracellular domains are involved in the formation of focal adhesion complexes and downstream signaling events. Our data provide new information that ~50% of human ASM cells express the promiscuous vitronectin receptor, α5β1, and 65–70% of cells express α3β1 integrin required for type I collagen binding. These data confirm an earlier report showing similar expression patterns of multiple β1 integrin family subunits including α1, α3, and α5 (19). In keeping with this earlier report, α5 and β1 subunits were found to be universally expressed. ELISA studies with integrin function-blocking mAbs suggest that enhancement of IL-1β-stimulated eotaxin release by fibronectin involved multiple integrins including α5β1, α5β3, α3β1, and α5β2; while enhancement on type I collagen involved α2β1. The findings with fibronectin support the consensus that a single ECM substrate may ligate several integrin heterodimers. For example, α5β1, α5β3, α2β1, and α2β3, which are expressed on ASM cells are all known to bind fibronectin with varying affinities (31). Furthermore, the finding that in each case blocking Abs abolished enhancement of cytokine release by fibronectin or type I collagen, irrespective whether the target integrins were universally (α5 and β1) or partially expressed (α2 and α5), supports our hypothesis from the intracellular cytokine flow cytometry findings that the enhancement of eotaxin by fibronectin or type I collagen in the ELISA study likely involved only a subpopulation of human ASM cells that also express α3 and α5 integrin subunits.

Block of fibronectin-induced potentiation of IL-1β-dependent eotaxin release, as well as that by type I collagen, by α5 subunit neutralization was an unexpected finding as α3β1 is the major type I collagen receptor. The response to fibronectin may depend on autocrine type I collagen production, possibly via an IL-1β-dependent GM-CSF-mediated amplification loop, and GM-CSF has recently been shown to induce the production of type I collagen by ASM cells (32). Alternatively, collagen-binding regions are present in purified fibronectin (33) and other recent data in a cell-free system has revealed that reconstituted α2β1 heterodimers ligate both type I collagen and fibronectin (34).

In conclusion, we have demonstrated that interstitial ECM proteins such as fibronectin and type I collagen, among the most widely expressed proteins in the lung, enhance release of IL-1β-stimulated eotaxin, RANTES, or GM-CSF from human ASM cells. The underlying mechanism involves increased cytokine mRNA levels and appears to require multiple β1 integrins as well as α3β1 in the case of fibronectin. Although the relevance of these events has not been demonstrated in vivo, their significance is unlikely to be restricted to a culture environment. The fact that fibronectin and collagen are increased in asthmatic airways (5–9) adds weight to the hypothesis that such changes in the ECM environment surrounding ASM cell may favor not only enhanced survival (19) and growth (17, 18) responses, but also enhanced secretion during inflammation and remodeling.

Acknowledgments

We thank the thoracic surgeons, operating theater staff, and pathologists of Guy’s and St. Thomas’ Hospitals, London, for the supply of human lung tissue, and Dr. Maria B. Sukkar for invaluable initial advice in optimizing the flow cytometry protocol for intracellular cytokine staining in human ASM cells.

References


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