

Eosinophil-fibroblast interactions induce fibroblast IL-6 secretion and extracellular matrix gene expression: Implications in fibrogenesis

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Background: Eosinophils are frequently associated with tissue remodeling and fibrosis in allergic and other diseases and animal models. Their close physical proximity to fibroblasts at sites of tissue remodeling strongly implicates them in fibrogenesis, including subepithelial fibrosis and airway remodeling characteristic of asthma.

Objective: To identify the mediators and characterize the mechanisms underlying the fibrogenic activities of eosinophils.

Methods: A coculture system of blood eosinophils or eosinophil cell lines with normal fibroblasts was used to assess their ability to induce a fibrogenic fibroblast phenotype, including IL-6 secretion and mRNA expression, and induction of genes involved in extracellular matrix production and homeostasis. The mediators of these responses were identified by using transwell barrier cocultures, eosinophil-conditioned media, and cytokine-specific antibody neutralization.

Results: Eosinophil-fibroblast coculture induced potent fibroblast IL-6 secretion and mRNA expression, responses further enhanced by IL-5. The soluble nature of the eosinophil-derived mediators was demonstrated by using eosinophil-fibroblast coculture in the presence of permeable transwell barriers, and fibroblast culture in eosinophil-conditioned media, indicating that cell contact was not required. Induction of fibroblast IL-6 expression was accompanied by increased expression of fibronectin and the extracellular matrix regulatory genes plasminogen activator inhibitor 1 and tissue inhibitor of metalloproteinase 1. Antibody neutralization identified the principal eosinophil-derived mediator of

fibroblast IL-6 expression as IL-1 β (>60%), with lesser contributions from IL-1 α , IL-4, and TGF- β (10% to 20%). **Conclusion:** Eosinophils express at least 2 potent mediators (IL-1 β and TGF- β) that induce a fibrogenic fibroblast phenotype, strongly supporting a role for the eosinophil in the dysregulation of extracellular matrix homeostasis and consequent tissue remodeling and fibrosis in eosinophil-associated diseases. (J Allergy Clin Immunol 2005;116:796-804.)

Key words: Eosinophil, fibrosis, IL-1 α , IL-1 β , IL-5, IL-6, TGF- β , fibronectin, PAI-1, TIMP-1

Tissue fibrosis is characterized by the pathologic accumulation of extracellular matrix (ECM) in response to tissue damage, a variety of infectious processes, and dysregulated host inflammatory responses. Eosinophils are hypothesized to play a significant role in mediating tissue remodeling and fibrosis in eosinophil-associated diseases that involve pathological tissue fibrosis. For example, eosinophil-associated diseases such as asthma,¹ eosinophil myalgia syndrome,² eosinophilic endomyocardial fibrosis,³ idiopathic pulmonary fibrosis,⁴ scleroderma,² and eosinophilic esophagitis⁵ all involve tissue remodeling and fibrosis. Flood-Page et al⁶ have shown that a reduction in bronchial mucosal eosinophils induced by anti-IL-5 antibody (mepolizumab; GlaxoSmithKline, Middlesex, United Kingdom) significantly decreased the expression of the ECM proteins tenascin, lumican, and type III collagen in the bronchial reticular basement membrane of patients with asthma, and recent studies by Humbles et al⁷ using the murine asthma model in eosinophil-deficient mice further support a role for the eosinophil in allergic airways remodeling. However, the mechanisms by which eosinophils and their cytokine and granule protein mediators of inflammation alter normal fibroblast function and phenotype leading to tissue fibrosis have not been fully elucidated.

We previously reported that eosinophil granule major basic protein (MBP) synergizes with TGF- β or IL-1 primed human lung fibroblasts to induce significant increases in gene transcription and secretion of members of the IL-6 family of inflammatory cytokines including IL-6, IL-11, and leukemia inhibitory factor (LIF).⁸ Evidence strongly implicating IL-6 in tissue remodeling and fibrogenesis includes findings that its expression is highly

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Abbreviations used

ECM:	Extracellular matrix
ECP:	Eosinophil cationic protein
EDN:	Eosinophil-derived neurotoxin
MBP:	Eosinophil granule major basic protein
NF- κ B:	Nuclear factor- κ B
PAI-1:	Plasminogen activator inhibitor-1
TIMP:	Tissue inhibitor of metalloproteinase

correlated with fibrosis in tissues such as the liver,⁹ skin,¹⁰ and lung,¹¹ the development of subepithelial fibrosis in transgenic mice with targeted overexpression of IL-6 in the lung,¹² and reduction of fibrosis in IL-6 null (knockout) mice.¹³ TGF- β -induced fibroblast secretion of IL-6, in what is described as an autocrine loop, is implicated in the production of collagens, tissue inhibitor of metalloproteinases (TIMPs), and glycosaminoglycans in fibrogenesis.¹⁴ Growth factors and cytokines such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and TNF- α , and cytokines including IL-1 β , IL-4, IL-11, and IL-13, have also been implicated in studies of airway remodeling, or fibrogenesis in various tissues including lung, skin, kidney, and liver.¹⁵⁻¹⁷ Although many of these cytokines are expressed by eosinophils,¹⁸ their participation in eosinophil-mediated fibrogenesis has not been thoroughly investigated.

In the current work, we have characterized eosinophil-fibroblast interactions in terms of the eosinophil-derived mediators responsible for fibroblast expression and secretion of the profibrogenic cytokine IL-6, and increased expression of fibronectin and inhibitors of matrix metalloproteinases, responses that can lead to the pathologic dysregulation of ECM homeostasis and tissue fibrosis. We have identified IL-1 β , IL-1 α , TGF- β , and possibly IL-4 as eosinophil-secreted mediators that may be responsible for the tissue remodeling and fibrosis seen in many eosinophil-associated diseases.

METHODS

Purification of blood eosinophils

Eosinophils were purified by magnetic-activated cell sorting as described previously¹⁹ from the blood of normal, nonallergic, healthy donors with informed consent according to guidelines established by the Institutional Review Board of the University of Illinois at Chicago. The purity and viability of the eosinophils was routinely >98%.

Eosinophil-fibroblast cocultures, culture supernatants, and eosinophil-conditioned media

Two different human fibroblast sources (skin and lung) were used to confirm eosinophil coculture findings in more than 1 tissue type. Primary foreskin fibroblasts, isolated as described previously,²⁰ and the lung-derived fibroblast cell line CCL-202 (ATCC) were grown in Eagle's Minimum Essential Medium supplemented with 10% FBS, 1 mmol/L L-glutamine, 1% vitamins (Gibco, Carlsbad, Calif), and 1% penicillin/streptomycin (Gibco). Purified blood eosinophils, the

eosinophil-committed myeloblast cell line AML14, and the differentiated eosinophil myelocyte cell line AML14.3D10²¹ were cultured in RPMI 1640 media supplemented with 8% FBS, 2 mmol/L sodium pyruvate, 1 mmol/L L-glutamine, 50 μ mol/L β -mercaptoethanol, and 1% penicillin/streptomycin. Unless otherwise stated, all coculture experiments were performed as follows: AML14, AML14.3D10 (0.5×10^6 /mL), or purified blood eosinophils (1×10^6 /mL) were added to confluent cultures of lung or skin fibroblasts and cocultured with or without IL-5 (2.5 ng/mL) for 24 hours. Culture supernatants were harvested and centrifuged at 300g for 5 minutes to remove any residual eosinophils, and the cell-free fibroblast culture supernatants were assayed for IL-6 by using an IL-6 ELISA kit (R & D Systems, Minneapolis, Minn). Eosinophil conditioned media were prepared from purified blood eosinophils (1×10^6 /mL), AML14 myeloblasts, or AML14.3D10 eosinophils (5×10^5 /mL) cultured for 24 hours at 37°C in the presence or absence of IL-5 (2.5 ng/mL).

Transwell barrier cultures

Experiments were performed by using Transwell plates (Costar; Coming Inc., Coming, NY) with 0.4- μ m pores. Fibroblasts were seeded and cultured in EMEM complete media without Transwell inserts in 6-well tissue culture plates to confluence. Transwell inserts containing 5×10^5 AML14.3D10 eosinophils/mL in RPMI complete media were cocultured with the confluent fibroblasts at 37°C for 24 hours, and the supernatant below the transwell plate was collected and assayed for fibroblast IL-6 secretion by ELISA.

Antibody neutralization of eosinophil-conditioned media

Antibodies were mixed with eosinophil-conditioned media and incubated at room temperature for 1 hour before adding the conditioned media to confluent fibroblast cultures. The antibodies included neutralizing antigen affinity purified antibodies (R & D Systems; α FGF [basic], α IL-1 α , α GM-CSF, α NGF), neutralizing protein G-affinity purified IgG (R & D Systems; α TGF- β , α IL-1 β , α TNF- α , α GM-CSF, α IL-4); Western blotting protein G purified antibodies (generated in-house; α MBP, α eosinophil-derived neurotoxin [EDN; rabbit], α eosinophil cationic protein [ECP]), and α EDN (mouse monoclonal; kindly provided by Drs Gerald Gleich and Hirohito Kita). Fibroblast culture supernatants were harvested after 24 hours and assayed for IL-6 secretion by ELISA.

RT-PCR analyses

Total fibroblast RNA was isolated for standardized semiquantitative RT-PCR by using Ultraspec RNA Isolation kits (Biotex, Houston, Tex). cDNA synthesis was performed by using a First Strand cDNA Synthesis Kit (Pharmacia, Piscataway, NJ). The linear range for amplification conditions (both cycle number and cDNA input) for each set of primer pairs was first determined to establish linear relationships between RNA (cDNA) input and amplified PCR product (see Table E1 in the Online Repository in the online version of this article at www.jacionline.org). The composition of the PCR reaction mixtures and amplification protocols for each primer set are available in the Online Repository (see Methods in the online version of this article at www.jacionline.org). The expression levels for all genes of interest were normalized to the glyceraldehyde-3-phosphate dehydrogenase signal for data analysis and presentation (see Methods in the Online Repository in the online version of this article at www.jacionline.org).

Statistical analyses

Results are expressed as means $\pm$ SDs unless otherwise indicated. The differences between means were assessed for statistical significance by using 1-way ANOVA, followed by post hoc analyses using

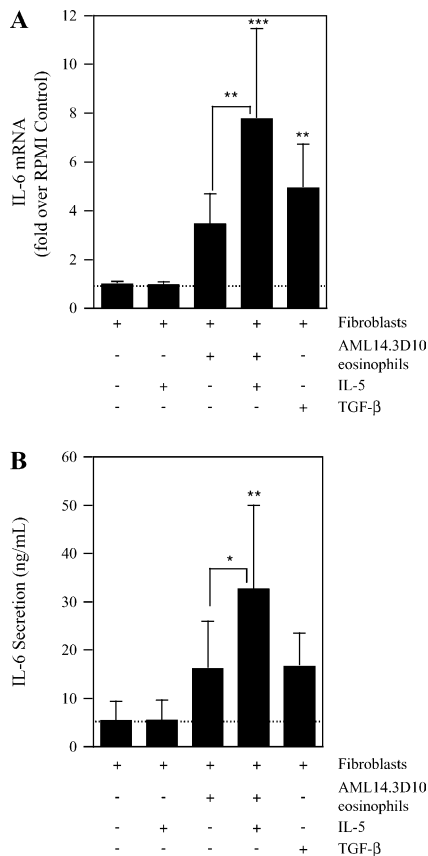


FIG 1. Coculture of AML14.3D10 eosinophils with fibroblasts induces fibroblast IL-6 secretion and IL-6 mRNA expression. Fore-skin fibroblasts were cocultured with or without AML14.3D10 eosinophils (5×10^5 cells/mL) in the presence or absence of IL-5 (2.5 ng/mL) for 24 hours. Fibroblast RNA was isolated and used for semiquantitative RT-PCR analysis of IL-6 mRNA (**A**) and IL-6 secretion measured in the cell-free supernatants by ELISA (**B**). Data in (**A**) are presented as the fold induction over fibroblast IL-6 mRNA expression in the absence of AML14.3D10 cells or IL-5. * $P \leq .05$; ** $P \leq .01$ and *** $P \leq .001$ for comparisons with the untreated fibroblast control; comparisons between groups are indicated by the brackets.

the least significant difference test for comparisons between any 2 means. A P value $\leq .05$ was considered statistically significant.

RESULTS

AML14.3D10 eosinophils induce fibroblast expression of IL-6

We previously reported that MBP synergized with IL-1 α or TGF- β to potentially induce fibroblast transcription and secretion of IL-6, and the IL-6 family members IL-11 and LIF.⁸ To determine whether direct eosinophil-fibroblast interactions induce this response, AML14.3D10 eosinophils were cocultured with normal human skin fibroblasts for 24 hours, and the induction of both IL-6 expression (mRNA) and secretion was determined (Fig 1). Eosinophil-fibroblast coculture induced a significant increase in IL-6 mRNA expression (3.4-fold; Fig 1, A) concomitant with marked fibroblast IL-6 secretion (Fig 1, B), and both of these stimulatory effects were further

enhanced (6-fold and 8-fold, respectively; $P < .01$) by the addition of IL-5 to the cocultures. For comparison, TGF- β_1 , a strong inducer of IL-6 gene expression and secretion, induced 3-fold to 5-fold increases in IL-6 mRNA (Fig 1, A) and IL-6 secretion (Fig 1, B), comparable with that obtained by coculture of fibroblasts with AML14.3D10 eosinophils, but less than that induced by AML14.3D10 eosinophils in the presence of IL-5. To confirm that fibroblasts were the principal if not sole source of the secreted IL-6 in the cocultures, the AML14.3D10 eosinophils were collected both before and after coculture and recultured for an additional 24 hours, and culture supernatants were assayed for IL-6 secretion by ELISA and IL-6 mRNA expression by RT-PCR. No detectable secretion of IL-6 or expression of IL-6 mRNA by the AML14.3D10 eosinophils was observed (data not shown).

To determine whether the induction of IL-6 secretion by fibroblasts in eosinophil-fibroblast cocultures is an eosinophil-specific response, and to rule out the possibility that this response was unique to skin and not other fibroblast cell types, the lung fibroblast line CCL-202 was cocultured with either AML14.3D10 eosinophils or its undifferentiated parental myeloblast line, AML14. The AML14 myeloblast line, which does not express any eosinophil-specific proteins other than a functional IL-5R, failed to induce any lung fibroblast IL-6 secretion whatsoever, compared with a 3-fold induction of IL-6 secretion by the AML14.3D10 eosinophils (see Fig E1 in the Online Repository in the online version of this article at www.jacionline.org), indicating that the fibroblast IL-6 response is not a nonspecific effect of the coculture system and is not specific to the type of fibroblast, and importantly, that factors expressed by more mature terminally differentiated eosinophils are required.

Blood eosinophils are potent inducers of fibroblast IL-6 secretion

To determine whether the IL-6 inducing activity of the AML14.3D10 eosinophil line reflects the functions of terminally differentiated blood eosinophils, we compared their abilities to induce IL-6 secretion by fibroblasts. Both the blood and AML14.3D10 eosinophils induced significant IL-6 secretion compared with fibroblast controls (Fig 2). The IL-6 secretion induced by coculture with purified blood eosinophils was significantly greater than that induced by the AML14.3D10 eosinophil cell line or TGF- β_1 . Consistent with earlier experiments, addition of IL-5 to the cocultures further augmented eosinophil-induced fibroblast IL-6 secretion (2.7-fold for AML14.3D10, and 2.1-fold and 5.6-fold for blood eosinophils from 2 different normal donors; Fig 2, A and B). Blood eosinophils showed considerable donor variability in eosinophil-fibroblast induction and IL-5 amplification of fibroblast IL-6 secretion as indicated by a comparison of the levels of IL-6 secretion in Fig 2, A and B. These results indicate that AML14.3D10 and mature blood eosinophils have both constitutive and IL-5-inducible capacity to stimulate fibroblast IL-6 secretion and gene expression,

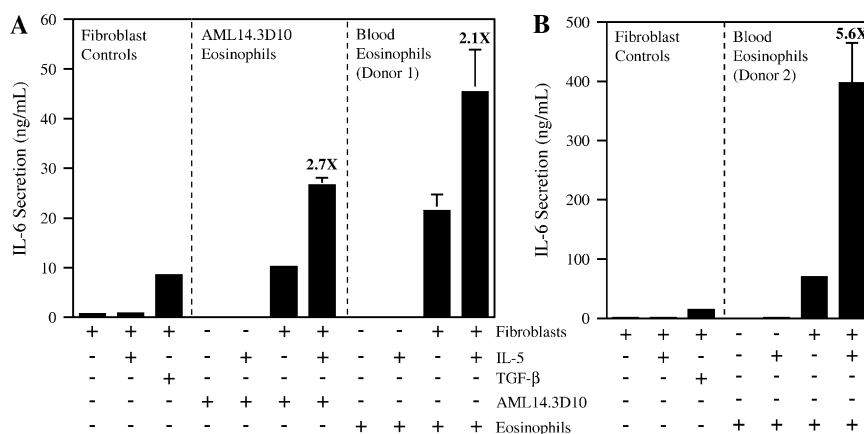


FIG 2. Purified blood eosinophils induce potent IL-6 secretion by fibroblasts comparable to or greater than that induced by AML14.3D10 eosinophils. Skin fibroblasts were cocultured for 24 hours with 5×10^5 AML14.3D10 eosinophils/mL or 1×10^6 blood eosinophils/mL from 2 different normal donors (**A** and **B**) in the presence or absence of IL-5 (2.5 ng/mL), and culture supernatants assayed for IL-6 by ELISA. As controls, supernatants from confluent fibroblasts cultured with or without IL-5 (2.5 ng/mL) or TGF- β (1 ng/mL) alone for 24 hours and conditioned media (CM) from 24-hour cultures of AML14.3D10 or blood eosinophils alone were included. The means for duplicate determinations are shown. The fold increase in IL-6 secretion induced by inclusion of IL-5 in the cocultures is indicated at the top of the bars.

validating the AML14.3D10 eosinophil line as a model for primary blood eosinophils to study eosinophil-fibroblast interactions relevant to fibrogenesis.

Eosinophil-fibroblast contact is not required for induction of fibroblast IL-6 expression

To determine whether direct contact of eosinophils with fibroblasts was necessary for induction of fibroblast IL-6 secretion, confluent lung fibroblasts were cultured in presence or absence of IL-5 with AML14.3D10 eosinophils for 24 hours by using transwell membrane barriers (Fig 3, A). The results showed that AML14.3D10 eosinophils induced significant IL-6 secretion when cocultured either in the presence or absence of the transwell membrane barrier, with somewhat reduced IL-6 levels for the transwell barrier, and the induction was similarly enhanced by the addition of IL-5 under both conditions (Fig 3, A). Furthermore, culture of lung fibroblasts with AML14.3D10-conditioned media or coculture with AML14.3D10 eosinophils induced comparable IL-6 secretion, which was again significantly augmented by the addition of IL-5 (Fig 3, B). The combined results from the transwell barrier and conditioned media experiments demonstrated that eosinophil-fibroblast contact was not required for induction of fibroblast IL-6 expression, suggesting that the eosinophil-derived mediators were soluble in nature.

To characterize further the mechanism by which eosinophils induce increased steady-state levels of fibroblast IL-6 mRNA, we determined whether eosinophil-conditioned media stimulate the activation of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), 2 principal regulators of IL-6 gene transcription.²² NF- κ B and AP-1 activity was determined by gel mobility shift assay using nuclear extracts of lung fibroblasts cultured for 3 hours with AML14.3D10 eosinophil-conditioned

media and DNA probes containing the NF- κ B and AP-1 binding sites in the IL-6 promoter. Results showed increased fibroblast nuclear NF- κ B (primarily p50 homodimers) and AP-1 DNA binding activities, consistent with eosinophil-induced activation of fibroblast IL-6 gene transcription (see Fig E2 in the Online Repository in the online version of this article at www.jacionline.org).

Coculture of eosinophils and fibroblasts induces fibroblast expression of genes involved in ECM maintenance

To determine whether eosinophil-fibroblast interactions induce increased expression of ECM proteins and/or regulators of ECM turnover and homeostasis, the expression levels of type I collagen, fibronectin, plasminogen activator inhibitor-1 (PAI-1), and TIMP-1 mRNAs were evaluated in foreskin fibroblasts (Fig 4). Coculture of confluent foreskin fibroblasts with AML14.3D10 eosinophils induced small but statistically significant increases in the expression of mRNAs for PAI-1 (1.6-fold; Fig 4, A), fibronectin (1.5-fold; Fig 4, B), and TIMP-1 (1.4-fold; Fig 4, C) compared with fibroblasts alone, with increases comparable with or in some cases greater than that induced by TGF- β ₁. In contrast, collagen type 1A2 (Fig 4, D) and 1A1 (data not shown) gene expression was not significantly induced by fibroblast coculture with AML14.3D10 eosinophils or by TGF- β ₁. The addition of IL-5 to the co-cultures did not significantly increase expression of these fibroblast ECM genes, aside from a small increase for TIMP-1.

Identification of the soluble eosinophil-derived mediators that drive fibroblast IL-6 expression

Because TGF- β is a potent fibrogenic growth factor and eosinophils have been shown to express significant

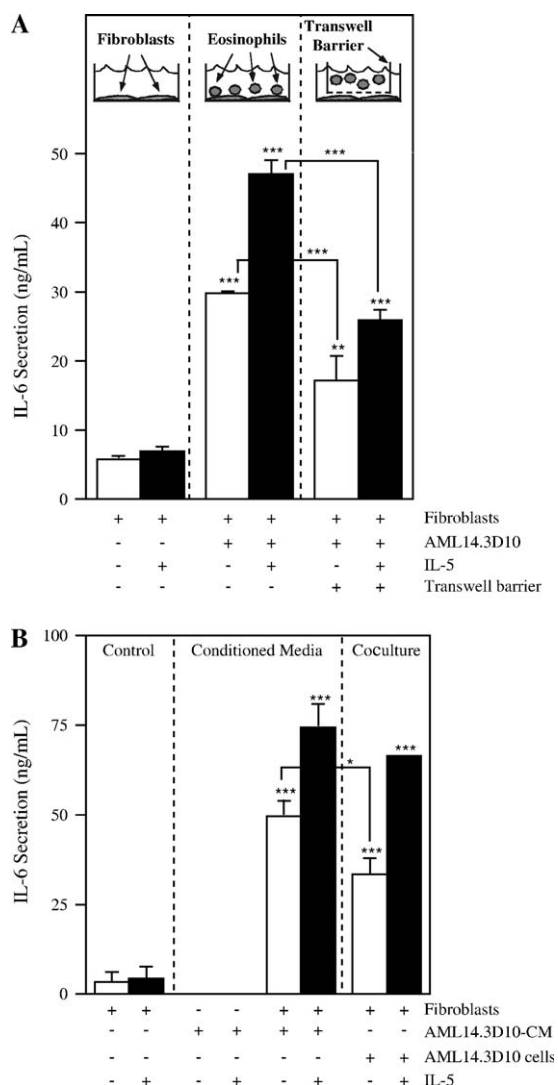


FIG 3. Eosinophil-fibroblast contact is not required for the induction of fibroblast IL-6 secretion. **A**, Confluent lung fibroblasts were cocultured for 24 hours with AML14.3D10 eosinophils at 5×10^5 cells/mL in the presence or absence of transwell barriers $\pm$ IL-5 (2.5 ng/mL). **B**, 24-Hour conditioned media from 5×10^5 AML14.3D10 cells/mL cultured in the presence or absence of IL-5 (2.5 ng/mL) were used to treat confluent lung fibroblasts for 24 hours. Fibroblasts cultured for 24 hours with RPMI 1640 media $\pm$ IL-5 (2.5 ng/mL) were included as controls, as were cocultures of AML14.3D10 eosinophils and fibroblasts ($\pm$ IL-5) for comparison with the AML14.3D10 conditioned media. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$ for comparisons with the untreated fibroblast control.

amounts of TGF- β ,^{1,23} we first determined its contribution to eosinophil-mediated activation of fibroblast IL-6 secretion using lung fibroblasts cultured with conditioned media from both blood and AML14.3D10 eosinophils. Neutralizing antibody to TGF- $\beta_{1,2,3}$ (capable of neutralizing all 3 TGF- β isoforms) reduced IL-6 secretion induced by both AML14.3D10-conditioned and blood eosinophil-conditioned media by only 20% and 15%, respectively (see Fig E3 in the Online Repository in the online version of this article at www.jacionline.org),

indicating that other eosinophil-secreted mediators must be responsible for the majority of the induction of fibroblast IL-6 gene expression and secretion.

In addition to TGF- β , several other growth factors and cytokines have been implicated directly or indirectly in tissue fibrosis, both in human diseases and animal models, including IL-1 β , IL-4, bFGF, TNF- α , and PDGF.^{4,15,24,25} Most of these factors have been reported to be expressed by eosinophils (see Lacy and Moqbel²⁶ for review). Their contributions as eosinophil-derived inducers of fibroblast IL-6 expression were determined in antibody neutralization experiments (Fig 5) performed by using both AML14.3D10 and blood eosinophil-conditioned media rather than coculture, because addition of the antibodies themselves induced significant eosinophil expression of IL-6 that prevented an assessment of fibroblast-expressed IL-6 (data not shown). As anticipated, TGF- $\beta_{1,2,3}$ contributed only partially ($<25\%$; $P < .001$) to the IL-6 secretion induced by AML14.3D10 and blood eosinophil-conditioned media (Fig 5, A and B). In contrast, IL-1 β secreted by both AML14.3D10 and blood eosinophils was the most potent inducer of lung fibroblast IL-6 expression, accounting for $>60\%$ of the IL-6 secreted by the lung fibroblasts. A smaller contribution (20% to 25%) from IL-1 α ($P < .001$), similar to that of TGF- β , was identified as well (Fig 5, A and B). Although a small but significant contribution from IL-4 ($P < .001$) and bFGF ($P < .001$) was observed with AML14.3D10 eosinophil-conditioned media (Fig 5, A), the contribution of these 2 factors was insignificant for blood eosinophil-conditioned media (Fig 5, B). The combination of anti-IL-1 α and anti-IL-1 β antibodies completely inhibited the induction of IL-6 secretion by AML14.3D10 eosinophil-conditioned media, whereas with blood eosinophil-conditioned media, the combined antibodies produced a 75% inhibition. We also tested other combinations of neutralizing antibodies including anti-IL-1 β with anti-TGF- $\beta_{1,2,3}$, anti-bFGF, and anti-TNF- α antibodies, but no other combination showed greater inhibition than the combination of anti-IL-1 α and anti-IL-1 β (data not shown).

Although we previously reported that purified MBP synergized with TGF- β or IL-1 α to induce IL-6 mRNA expression and secretion by lung fibroblasts primed with these factors,⁸ no inhibition of IL-6 secretion was observed with any of the rabbit antisera to the eosinophil granule cationic proteins (Fig 5, A and B), suggesting that MBP, EDN, and ECP were not involved in the induction of fibroblast IL-6 secretion under the conditions used in this coculture system, which were not likely to induce their significant secretion. Taken together, these findings indicate that IL-1 β and IL-1 α are 2 of the major factors secreted by eosinophils responsible for the eosinophil-mediated fibroblast IL-6 response, but do not rule out possible contributions by eosinophil granule proteins such as MBP in enhancing this response.⁸

On the basis of the antibody neutralization results, the effects of the individual factors on IL-6 secretion by lung fibroblasts were also examined. Confluent cultures of lung

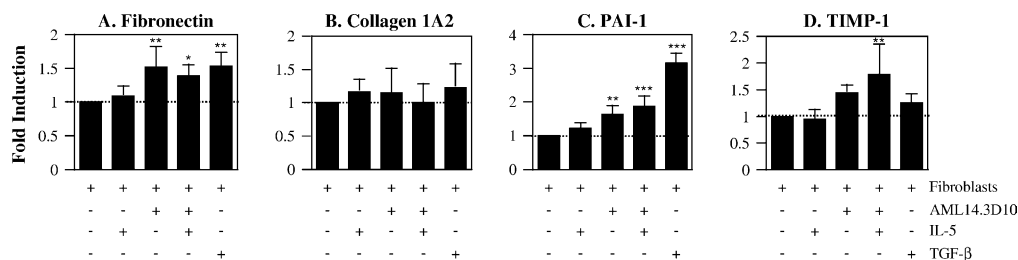
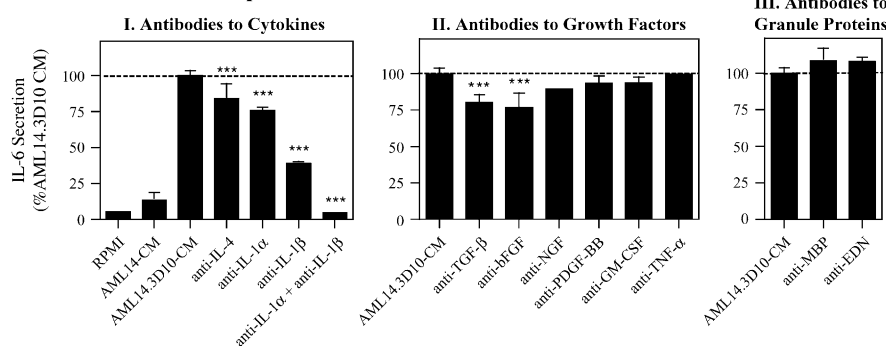


FIG 4. AML14.3D10 eosinophils upregulate fibroblast expression of mRNA for fibronectin, PAI-1, and TIMP-1. Foreskin fibroblasts were cultured for 24 hours in the absence (control) or presence of IL-5 (2.5 ng/mL) or TGF-β (1 ng/mL), or were cocultured with AML14.3D10 eosinophils at 5×10^5 cells/mL for 24 hours $\pm$ IL-5. Fibroblast RNA was analyzed by semiquantitative RT-PCR using linear amplification conditions and primers for (A) fibronectin, (B) collagen 1A2, (C) PAI-1, and (D) TIMP-1 as described in the Methods. Data were normalized to the signals obtained for GAPDH and expressed as the fold increases over the untreated fibroblast control sample. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$ for comparisons with the untreated fibroblast control.

A. AML14.3D10 Eosinophil Conditioned Media



B. Blood Eosinophil Conditioned Media

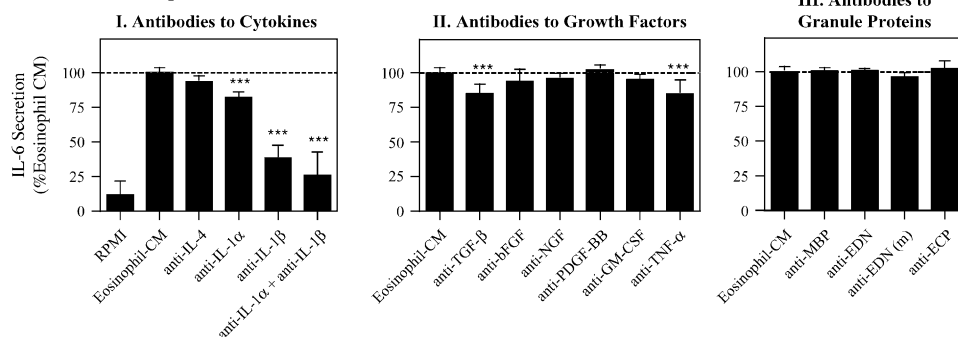


FIG 5. Inhibition of AML14.3D10 and blood eosinophil-induced lung fibroblast IL-6 secretion by neutralizing antibodies to candidate fibrogenic factors. Confluent lung fibroblasts were cultured for 24 hours with or without 24 hour conditioned media from AML14.3D10 (A) or blood (B) eosinophils to which neutralizing antibodies to cytokines IL-1α (1 μg/mL), IL-1β (5 μg/mL), IL-4 (10 μg/mL); growth factors TGF-β (10 μg/mL), bFGF (2.5 μg/mL), NGF (1 ng/mL), PDGF-BB (50 μg/mL), GM-CSF (2 μg/mL), TNF-α (10 μg/mL); and eosinophil granule proteins MBP, EDN, ECP (rabbit antisera at 1:50 dilution or an EDN-specific mouse mAb at 45 μg/mL) were first added. IL-6 levels in culture supernatants were determined after 24 hours by ELISA, and secreted IL-6 expressed as a percentage of the IL-6 secreted by fibroblasts cultured with nonneutralized conditioned media. The data in (A) represent the means $\pm$ SDs of experiments performed with 2 different sets of AML14.3D10 conditioned media, with experiments performed in duplicate. The experiment in (B) used conditioned media from blood eosinophils of 2 to 3 different donors. *** $P \leq .001$ for comparisons with nonneutralized AML14.3D10 or blood eosinophil conditioned media controls.

fibroblasts were treated with recombinant factors including TGF-β₁, PDGF-BB, GM-CSF, NGF, bFGF, IL-1β, and IL-4 for 24 hours. IL-1β showed a robust induction of IL-6 secretion (>100-fold) compared with TGF-β and

IL-4, which stimulated smaller increases (~3-fold) over the untreated controls, whereas GM-CSF, PDGF-BB, bFGF, and NGF had no effect (see Fig E4 in the Online Repository in the online version of this article at

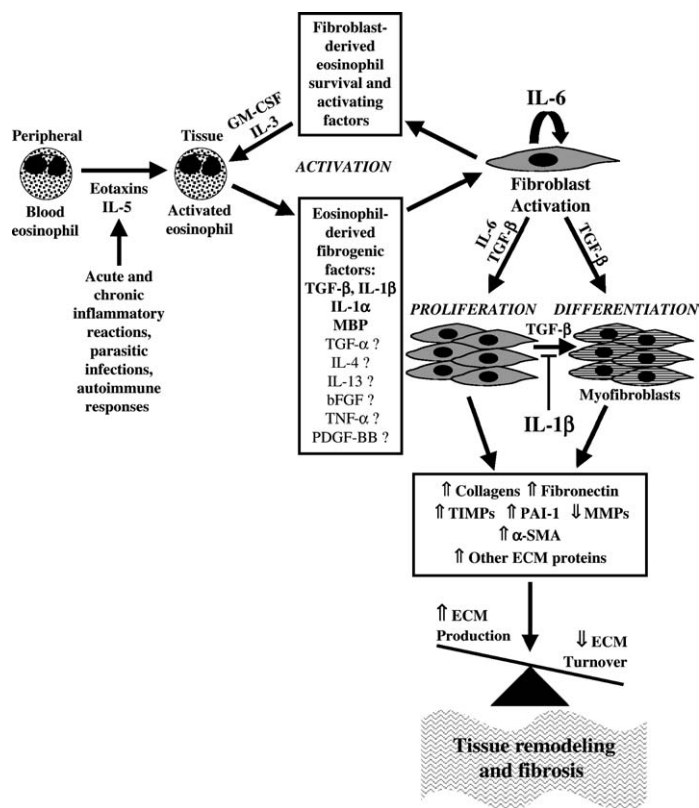


FIG 6. Model for the role of eosinophils in fibrogenesis. Eosinophil activation during recruitment, in part through interactions with fibroblasts and epithelial cells, leads to their expression of fibrogenic cytokines and growth factors: the particular combination of factors may depend on the nature of the eliciting inflammatory response. Fibroblast activation by eosinophil-derived factors such as TGF- β , IL-1 α , IL-1 β , and IL-4 supports fibroblast proliferation, myofibroblast differentiation, and dysregulation of ECM maintenance. These eosinophil-derived mediators activate fibroblasts through signaling cascades that in part involve fibroblast expression of IL-6 and TGF- β , and autocrine signaling by IL-6 may contribute to fibroblast proliferation and expression of TGF- β . IL-1 β may induce fibroblast proliferation and ECM protein production by a TGF- β -dependent mechanism³³ but antagonize myofibroblast differentiation by inducing myofibroblast apoptosis,³⁴ a response counterregulated by TGF- β .³⁵ The combinatorial effects of these signaling cascades include increased expression of ECM, decreased expression of matrix remodeling matrix metalloproteinases (MMPs), or increased expression of MMP inhibitors, outcomes which disrupt the fine balance between production and degradation of ECM, leading to its pathologic accumulation, tissue remodeling, and fibrosis.

www.jacionline.org). The potent induction of fibroblast IL-6 expression by IL-1 β supports the results obtained by using antibody neutralization of IL-1 β compared with the other factors present in eosinophil-conditioned media.

DISCUSSION

Although eosinophils are one of the principal inflammatory cells routinely associated with tissue remodeling and fibrosis, their pathogenetic role in the development of fibrotic diseases is not well understood.^{4,15,27} Tissue infiltration by eosinophils provides them with an opportunity for direct interactions with resident fibroblasts, which may in turn serve to support eosinophil survival, activation and secretion—for example, through their elaboration of GM-CSF or IL-3, promoting eosinophil expression of a variety of mediators with fibrogenic potential including TGF- β , TGF- α , IL-1, and IL-6⁴ (Fig 6).

We developed an *in vitro* coculture system to define more clearly the mechanisms by which eosinophil-fibroblast interactions may lead to tissue fibrosis. We demonstrated that coculture of eosinophils with fibroblasts stimulates fibroblast secretion of IL-6, with concomitant induction of IL-6, fibronectin, TIMP, and PAI-1 mRNA expression, all of which are implicated in tissue remodeling and maintenance of ECM homeostasis (Fig 6). PAI-1 (a serine protease inhibitor) and TIMP-1 (a metalloproteinase inhibitor) are 2 important inhibitors of ECM-degrading proteases whose overexpression has been shown to result in the accumulation of ECM proteins and fibrosis.²⁸ We have used the induction of IL-6 expression by fibroblasts as a measure of the eosinophil-induced fibrogenic response.⁸ Multiple lines of evidence indicate that IL-6 is a good marker of fibrosis. For example, the expression and secretion of IL-6 are well correlated with fibrosis in tissues such as the liver,⁹ skin,¹⁰ and lung,²⁹ providing support for its role in fibrogenesis. The

development of subepithelial fibrosis in transgenic mice with targeted overexpression of IL-6 in the lung,^{12,30} and significant reduction of fibrosis in IL-6 null (knockout) mice in a model of hepatic fibrosis,¹³ also strongly argue for an important role for IL-6 in tissue remodeling and fibrosis.

Although eosinophils have the capacity to express IL-6 themselves,²⁶ the IL-6 secreted in our coculture system, which was significantly enhanced by the addition of IL-5, was derived exclusively from the fibroblasts and not the cocultured eosinophils. Two lines of evidence support this point: (1) eosinophils salvaged from cocultures and recultured did not secrete any IL-6, and (2) both eosinophil cell line (AML14.3D10) and blood eosinophil-conditioned media induced fibroblast IL-6 secretion at levels comparable with that obtained by eosinophil-fibroblast coculture. The significant enhancement of fibroblast IL-6 secretion by IL-5 is likely a result of its ability to provide important signals that promote eosinophil priming, activation, and secretion of fibrogenic cytokines such as IL-1 and TGF- β . Studies by Phipps et al¹⁵ showed that IL-5 was likewise key to eosinophil-induced fibroblast to myofibroblast differentiation, a response attributed principally to eosinophil-derived TGF- β . The efficacy of the anti-IL-5 antibody mepolizumab in decreasing both the numbers of tissue eosinophils and the deposition of ECM proteins in the lung of patients with asthma⁶ and in allergen-induced late-phase reactions in the skin of atopic subjects¹⁵ further supports a role for IL-5 in eosinophil-induced fibrogenesis. Both studies support our current findings for IL-5 enhancement of eosinophil-induced fibroblast IL-6 expression, providing a strong link among IL-5, eosinophils, and fibrogenesis in allergic responses in the lung and skin (Fig 6).

Two findings, the induction of IL-6 secretion in the presence of a permeable transwell barrier, and the ability of eosinophil-conditioned media to induce IL-6 secretion, indicated that the IL-6-inducing factors were soluble in nature, leading to the demonstration of a principal role for IL-1. Although cell contact was not required for the induction of IL-6 expression in this coculture system, an additional physiologic role for eosinophil-fibroblast contact in fibrogenesis is likely—for example, for the activation of latent TGF- β via interaction of the TGF- β latency-associated peptide with the integrin α V β 6,³¹ and for eosinophil-induced fibroblast to myofibroblast differentiation, which was TGF- β -mediated, and involved cell contact during blood eosinophil-fibroblast coculture.¹⁵ In addition, the finding that eosinophil-induced IL-6 secretion was decreased ~50% in the presence of transwell barriers (Fig 3, A) suggests that eosinophil-fibroblast adhesion molecule/ligand interactions may amplify these responses.

Results with conditioned media from both blood eosinophils and the AML14.3D10 eosinophil line showed that IL-1 β rather than TGF- β was the principal mediator of IL-6 expression by both lung and skin fibroblasts. The contribution of IL-1 β toward the total fibrogenic activity of blood eosinophils, as measured by its ability to induce fibroblast IL-6 secretion, is surprising but not unexpected.

IL-1 β has been implicated in fibrosis in both human diseases and animal models. The most compelling evidence for the fibrogenic potential of IL-1 β was provided by Kolb et al,²⁵ who showed that transient adenovirus-mediated expression of IL-1 β in the rat lung caused increased local expression of the proinflammatory cytokines IL-6 and TNF- α , followed by severe progressive pulmonary fibrosis as determined by both increased numbers of myofibroblasts and extracellular deposition of collagens and fibronectin. However, the relative roles of TGF- β versus IL-1 β in tissue remodeling and fibrogenesis are still unclear, because both synergistic^{32,33} and antagonistic^{34,35} effects on fibroblast differentiation, proliferation, apoptosis, and production of ECM proteins have been reported. IL-1 β and TGF- β may induce 2 distinct, possibly counterregulatory fibrogenic pathways, with IL-1 β inducing fibroblast activation possibly through the autocrine activities of IL-6 (Fig 6), and the other involving TGF- β -mediated fibroblast to myofibroblast differentiation, an activity not yet demonstrated to be IL-6-dependent. These pathways, although not mutually exclusive, may be regulated through the differential expression of IL-1 β versus TGF- β by eosinophils or other inflammatory cells that express IL-1 β , especially because IL-1 β has been shown to induce fibroblast proliferation and matrix protein production by a TGF- β -dependent mechanism,³³ but to repress TGF- β -induced myofibroblast differentiation and expression of α -smooth muscle actin by selectively inducing apoptosis in myofibroblasts.^{34,35}

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