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The Relationship Between Allergen-Induced Tissue Eosinophilia and Markers of Repair and Remodeling in Human Atopic Skin

Simon Phipps,* Sun Ying,* Arun Wangoo,* Yee-Ean Ong,* Francesca Levi-Schaffer,† and A. Barry Kay‡,

Several in vitro studies suggest that eosinophils may play a role in fibrosis, remodeling, and repair processes associated with IgE-mediated hypersensitivity. However, the relationship in vivo, between allergen-induced tissue eosinophilia and markers of repair has yet to be established in human atopic subjects. Using the allergen-induced cutaneous late-phase reaction as a model of allergic inflammation, we have tested the hypothesis that eosinophil-derived TGF-β1 and IL-13 are temporarily associated with myofibroblast formation and deposition of tenascin and procollagen I. Biopsies were taken from atopic volunteers at 1, 3, 6, 24, 48, and 72 h after intradermal allergen challenge and were examined by immunohistochemistry. Following the peak of the late-phase reaction (6 h) there were persisting TGF-β1* eosinophils, α-smooth muscle actin* myofibroblasts, tenascin immunoreactivity, and procollagen-I* cells 24–48 h postchallenge. Direct evidence of generation of repair markers was obtained by coculture of eosinophils and fibroblasts. This resulted in α-smooth muscle actin immunoreactivity that was inhibitable by neutralizing Abs to TGF-β as well as production of tenascin transcripts and protein product. TGF-β1 and IL-13 also induced tenascin expression. We conclude that TGF-β1 and IL-13, provided partially by eosinophils, contribute to repair and remodeling events in allergic inflammation in human atopic skin. The Journal of Immunology, 2002, 169: 4604–4612.

Eosinophils are principally regarded as pro-inflammatory cells in the pathogenesis of allergic disease and asthma through the release of lipid mediators, cytokines, chemokines, and highly charged basic proteins (1). However, in allergen challenge situations such as the late asthmatic reaction, eosinophils persist after the resolution of airway narrowing. This has led to speculation that eosinophils may also play a role in wound healing, remodeling, and the development of postinflammatory fibrosis, especially as a tissue eosinophilia and eosinophil degranulation are associated with several fibrotic syndromes (2–4). It is well documented that human eosinophils express the potent fibrogenic factor TGF-β1, and in coculture systems this cell type stimulates fibroblast proliferation, collagen synthesis, and lattice contraction (5–7). In addition, the Th2-like cytokines IL-4 and IL-13, also expressed by eosinophils, up-regulate fibroblast chemokine and matrix protein expression (8, 9) and weakly induce a myofibroblastic phenotype (9). Furthermore, the selective overexpression of IL-13 in murine clara cells causes subepithelial matrix deposition (10). Eosinophils also express other growth factors and cytokines that modulate mesenchymal cells, including fibroblast growth factor-2 (FGF-2)* (11), nerve growth factor (12), vascular endothelial growth factor (13), and IL-4 (14).

Collagen deposition and remodeling events are usually considered to be chronic processes that, in the context of asthma, are the result of long term exposure to Ag. However, Gizycki et al. (15) observed an increase in myofibroblast formation 24 h after inhalational challenge in atopic asthmatics, suggesting that fibroblast activation can occur shortly after allergen exposure, possibly as a result of eosinophil-derived TGF-β1. Thus, further in vivo evidence in man is required regarding the relationship among eosinophil infiltration, allergic inflammation, and specific allergen-induction of markers of repair. For these reasons we have tested the hypothesis that in allergic inflammation in vivo in man, eosinophil infiltration is temporarily associated with fibroblast activation (as shown by the formation of α-smooth muscle (α-SM) actin myofibroblasts) as well as the deposition of procollagen I and the matrix cellular protein tenascin.

We have used the allergen-induced cutaneous late phase reaction (LPR) as our experimental model because this has been used extensively to study the cellular and molecular characteristics of allergic inflammation. Biopsies have been taken at time points after intradermal injection of allergen and examined by immunohistochemistry. In addition we have used fibroblast cultures to show that eosinophil-associated TGF-β1 and IL-13 induce the formation of tenascin over a similar time course as that observed in vivo.

Materials and Methods

Volunteers for cutaneous time-course study

Ten atopic subjects who were not receiving oral corticosteroids were recruited from the Allergy Clinic of the Royal Brompton Hospital, Imperial College staff, or by advertisement. No subject had taken aspirin within the

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3 Abbreviations used in this paper: FGF-2, fibroblast growth factor; AP, alkaline phosphatase; APAAP, alkaline phosphatase/anti-alkaline phosphatase; LPR, late phase reaction; RBM, reticular basement membrane; α-SM, α-smooth muscle.
before week 12 of the study. Inclusion criteria were as follows: 1) age of 18–55 years, 2) history of seasonal and/or perennial allergic rhinitis and/or asthma, 3) absence of any other illness, and 4) positive skin prick test (wheat diameter, ≥5 mm) to grass pollen, house dust mite, cat dander, (all from ALK, Horsholm, Denmark) in the presence of a positive histamine and negative vehicle control. All patients had a positive radioallergosorbent test, and the median total serum IgE concentration was 398.5 IU/ml (range, 65–1102). All subjects gave informed consent, and the study was approved by the Royal Brompton and Harefield Trust ethics committee.

Study design and processing of specimens
All injections were performed with a 29-gauge needle and a 0.5-ml plastic syringe. Using this method, 30 biological units of allergen (mixed grass, cat dander, or house dust mite) was injected intradermally into individual sites on the extensor aspect of the forearms of each subject. An additional site was injected with a similar volume of diluent. Macrophagic responses were measured at 1, 3, 6, 24, 48, and 72 h by evaluating skin induration by resistance to the movement of a sharpened pencil point with which the reaction was outlined. Permanent sticky tape records of the outlines of the responses at all time points were then made. A 4-mm disposable biopsy punch was used to take a biopsy from the center of each reaction after using 1% plain lignocaine for local anesthesia. The control site injected with diluent was biopsied at 24 h. In this way each patient served as his/her own control. The biopsies were immediately fixed in 4% paraformaldehyde and were washed in 15% PBS-buffered sucrose (Sigma-Aldrich, Poole, U.K.), embedded in OCT (optimal cutting temperature; Miles, Elkhart, IN), and snap-frozen in isopentane precooled in liquid nitrogen. Cryostat sections (∼8 μm) were cut from biopsies, mounted onto SuperFrost Plus slides, dried overnight at 37°C, and stored with silica gel at −80°C until use (all from VWR, Dagenham, U.K. unless otherwise stated).

Histochemistry and immunohistochemistry
Eosinophil accumulation was determined by Congo red, an elective stain for eosinophils (16), in tissue sections as described previously (17). Briefly, sections were washed in PBS for 5 min, then incubated in 0.5% Congo red (Sigma-Aldrich) in ethanol/0.1 M glycine (1/1) for 5 min at room temperature. The slides were rinsed in 70% ethanol until the background became clear, then mounted in Glycergel (VWR) and snap-frozen in isopentane precooled in liquid nitrogen. Cryostat sections (∼8 μm) were cut from biopsies, mounted onto SuperFrost Plus slides, dried overnight at 37°C, and stored with silica gel at −80°C until use (all from VWR, Dagenham, U.K. unless otherwise stated).

Immunocytofluorescence
Primary human dermal fibroblasts were obtained from biopsies of normal skin from informed consent volunteers who underwent minor skin surgery. The biopsies were placed as explants, and fibroblasts were obtained and cultured as described below. The human fetal lung fibroblasts (MRC-5) were obtained from European Collection of Cell Cultures (Ref. no. 97112601; Salisbury, U.K.). Fibroblasts were routinely maintained in DMEM supplemented with 10% (v/v) FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) and supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO2. Cells were passaged by trypsinization using 0.05% trypsin/0.02% EDTA (Sigma-Aldrich) and seeded into slide chambers, 12-well plates, or 96-well microtiter plates (VWR) at 5 × 105, 10 × 105, or 2 × 106 cells/well, respectively. Fibroblasts were cultured for 3 days until ~70% confluent before treatment with human eosinophils or exogenous cytokine as described below. Following treatment, fibroblasts were washed with PBS and stored at −80°C in situ, collected as cytospins or cell pellets, or lysed for RNA extraction.

Western immunoblotting
The degree of α-SM actin expression in response to coculture with human eosinophils or TGF-β1 was analyzed by immunoblotting. Fibroblast pellets were resuspended in RIPA lysis buffer (PBS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) containing 0.1% SDS, 1 mM PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM sodium orthovanadate on ice for 30 min (all from Sigma-Aldrich). Cell lysates were collected by microcentrifugation (Eppendorf, Sunderland, U.K.), mixed with an equal volume of sample buffer (50 mM Tris, 400 mM glycine, 0.2% SDS, and 10% glycerol, pH 8.3), and boiled for 5 min at 95°C. Rainbow m.w. markers and cell extracts (15 × 10⁶ cells/lane) were loaded onto a 10% SDS gel and electrophoresed for 1 h at 150 V in a mini-gel system. Separated proteins were transferred onto a polyvinylidene difluoride membrane in transfer buffer containing 20% methanol for 1 h. Membranes were blocked for 1 h in blocking buffer (5% fat-free milk powder in PBS/Tween (0.05%)), then incubated overnight at 4°C with anti-α-SM
actin Ab (250 ng/ml) in blocking buffer under constant agitation. After three 5-min washes, the membrane was incubated with HRP-conjugated goat anti-mouse (1/4000 dilution; DAKO) in blocking buffer for 30 min at room temperature and subsequently developed with ECL detection reagent (Amersham, Little Chalfont, U.K.) for 5 min. X-ray film (Calumet, Milton Keynes, U.K.) was exposed to chemiluminescence reagent, and the film was developed (all reagents from Bio-Rad unless stated).

**Semiquantitative RT-PCR for tenascin-C**

Total RNA was extracted from the MRC-5 fibroblasts using RNeasy minicolumns (Qiagen, Crawley, U.K.) and eluted in diethylpyrocarbonate-treated H2O. RNA quality and quantity were assessed by ethidium bromide-agarose gel electrophoresis and by relative absorbance at 260 vs 280 nm. cDNA was synthesized from 200 ng total RNA. The gene-specific primers were as follows: tenascin-C: forward primer, 5'-TGAAACAAAT CACAGCCCG-3'; reverse primer, 5'-CAGTGGAAACCAGTTAAGCCTGG-3'; and β-actin: forward primer, 5'-TCTCTTGGCATCCAGAATC-3'; reverse primer, 5'-GAACGATTGGCGTGGAGAT-3'. Tenascin-C primers showed 100% alignment with human mRNA for tenascin-C only as checked with GenBank and EMBL sequence databases. The predicted sizes of tenascin-C and β-actin DNA products were 333 and 315 bp, respectively. The PCR amplification mixture consisted of 10 μL PCR buffer, 1.25 mM MgCl2, 1 U Taq polymerase (Promega, Southampton, U.K.), 200 μM deoxynucleosidetriphosphates (dATP, dGTP, dCTP, and dTTP), 0.3 μM of each primer, 10 μL appropriate dilution of cDNA, made up to 50 μL with sterile distilled water. Amplification was conducted for 37 cycles in a DNA thermal cycler (Hybaid, U.K.) under the following reaction conditions: 94°C for 5 min for first cycle, then 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and the last cycle at 72°C for 10 min. PCR products (15 μl) were separated by electrophoresis on a 2% agarose gel containing ethidium bromide. For visualization of the bands, the gel was photographed under UV light. The photographs were scanned, and bands were quantified using Gel Works Intermediate software (Ultra-Lumi, Claremont, CA). Results are expressed as arbitrary units.

**In situ cell ELISA**

Fibroblasts grown in a 96-well microtiter plate were treated to various concentrations of TGF-β1 (100 pm), IL-4 (10–1000 pm), IL-5 (10–1000 pm), IL-13 (10–1000 pm), or TGF-β2 (1000 pm; all human recombinant proteins; R&D Systems) for 24 h, then washed three times with PBS and left to air-dry overnight. Eight replicates were performed for each treatment group. Wells were blocked with PBS containing 2% BSA and 0.05% Tween 20 for 2 h at 37°C, then incubated with 100 μl anti-tenascin Ab at 10 μg/ml in PBS containing 0.1% saponin and 10% normal human serum overnight at room temperature. After labeling, the plate was washed three times for 5 min each time with PBS/Tween and incubated with an AP-conjugated goat anti-mouse Ab (DAKO) for 2 h. After extensive washing, the chromogenic substrate, p-nitrophenyl phosphate (5 mM, pH 10.0; DAKO), diluted in 100 mM sodium bicarbonate/carbonate buffer (pH 10), was added to produce color, and absorbance was read at 405 nm after 10-min development in a microtiter plate reader (Titer-Tek Multiskan; Labsystems, Basingstoke, U.K.). Absorbance values obtained with an irrelevant mouse IgG1 monoclonal were similar to those found for wells treated with substrate only.

**Statistical analysis**

Data were analyzed using a statistical software package (Minitab Release 13.1; Minitab, State College, PA). The Wilcoxon signed rank test was used to analyze changes in the numbers of immunoreactive-positive cells in response to allergen. Data from in vitro studies were analyzed by Student’s t test. A value of p > 0.05 was accepted as nonsignificant.

**Results**

**Eosinophils and the LPR**

All subjects (n = 10) exhibited an allergen-induced, but not dinitro-induced, cutaneous LPR, with mean diameters (SEM) of 34.7 ± 2.5 (1 h), 45.1 ± 5.3 (3 h), 62.1 ± 7.5 (6 h), 47.5 ± 8.2 (24 h), 30.4 ± 6.7 (48 h), and 8.8 ± 3.8 (72 h) mm (Fig. 1). At 72 h the LPR had completely subsided in five of the 10 volunteers.

At allergen challenge sites, there was a significant eosinophil accumulation within 1 h (p < 0.01; 54.3 ± 17.0 cells/mm²), in contrast to diluent challenge sites where there were very few Congo red⁺ eosinophils (0.4 ± 0.2 cells/mm²). The mean number of Congo red⁺ eosinophil infiltration peaked at 6 h (163.4 ± 42.5 cells/mm²) and plateaued out to 48 h. At 72 h the intensity of eosinophil accumulation decreased slightly (63.7 ± 22.4 cells/mm²), but remained significantly elevated (p < 0.01; Fig. 1) compared with diluent challenge. Unlike the LPR, which had resolved, all subjects had tissue eosinophilia at the 72 h point. No significant correlation was found between eosinophil numbers and size of LPR.

**TGF-β1 and IL-13**

The kinetics of TGF-β1 expression showed a pattern similar to that observed for eosinophils (Fig. 2A). At diluent-challenged sites very few cells were immunoreactive for TGF-β1 (17.7 ± 3.4 cells/mm²), whereas after allergen challenge the numbers of TGF-β1⁺ cells increased markedly at 1 h (120.1 ± 20.7 cells/mm²), was maximal at 6 h (204.7 ± 47.1 cells/mm²), and then declined progressively. TGF-β1⁺ cells were still evident at 72 h (30.6 ± 13.6 cells/mm²), but at levels similar to that found at the diluent site. At 1, 3, 6, 24, 48, and 72 h the percentages of eosinophils that were TGF-β1⁺ were 30, 25, 37, 48, 30, and 22%, respectively. Thus at 24 h eosinophils represented approximately one-half of the inflammatory cells found to be TGF-β1⁺. Other cellular sources of TGF-β1 included neutrophils (earlier time points; data not shown). The allergen-induced LPR was also associated with an increase in the number of IL-13⁺ cells (Fig. 2B). This was apparent even at 1 h (21.5 ± 6.2 cells/mm²; p = 0.014) and peaked at 24 h (39.8 ± 6.3 cells/mm²; p = 0.006). Significant numbers persisted up to 72 h (9.6 ± 1.5 cells/mm²; p = 0.008). Double staining with Congo red revealed that appreciable numbers of IL-13⁺ cells were eosinophils and ranged from 8.5 ± 2.7% (48 h) to 19.5 ± 7.8% (3 h; data not shown). The relative contribution of eosinophils to the total number of IL-13-immunoreactive cells was considerable given the high number present within the tissue and the comparatively low numbers of IL-13⁺ cells. Other inflammatory cells expressing IL-13 were neutrophils (earlier time points) and lymphocytes (particularly at 24 h). Examples of Congo red⁺ eosinophils and TGF-β1⁺ cells are shown in Fig. 3, A and B.
Myofibroblasts

There was a small, but significant, increase in the numbers of single α-SM actin myofibroblast-like cells at 24 and 48 h when allergen challenge sites were compared with diluent (p < 0.02; Fig. 2C). This persisted to 72 h. Fig. 3C also shows an example of immunohistochemistry of α-SM actin in a cutaneous LPR. These single α-SM actin+ cells were independent of any vascular or glandular structure and were found to reside mostly in the lower dermis. Pericytes localized around blood vessels were also α-SM actin+, but were excluded from cell counts. A moderate increase in the numbers of cells expressing procollagen I was observed at 24 h compared with diluent-challenged sites (6.6 ± 1.7 cells/mm²) after allergen challenge (10.0 ± 2.5 cells/mm²), peaked significantly at 48 h (16.0 ± 4.2 cells/mm²; p = 0.006), and declined slightly at 72 h (9.4 ± 3.2 cells/mm²; Fig. 2D). At diluent-challenged sites a small number of immunoreactive cells was also observed, indicating low level turnover of collagen. Other structures, such as eccrine sweat glands, blood vessels, smooth muscle, and hair follicles, were likewise immunoreactive for α-SM actin and procollagen I in both control and allergen-challenged sites.

Tenasin expression

In diluent-challenged sites very few single cells were found to be positive for tenasin-C. Similarly, at 1 and 3 h following allergen challenge little or no immunoreactivity was evident. At 6 h a number of fibroblast-like cells, fusiformic in shape and distributed predominantly in the lower dermis, were immunoreactive for tenasin. In biopsies obtained at 24 and 48 h after challenge, the number of tenasin+ cells was significantly increased compared with that at 3 h (p < 0.01) and was still elevated at 72 h (Fig. 4A). The temporal pattern of tenasin expression at both the endothelial and eccrine sweat gland basement membrane and in and around the arrector pilorum muscle closely matched that observed at the single-cell level (Fig. 4, B and C). Tenasin expression was significantly up-regulated around basement membranes at 24 and 48 h compared with that at 3 h (p < 0.01).

Eosinophil-induced myofibroblast formation in vitro

Culture of primary human dermal fibroblasts (Fig. 5A) with human eosinophils (Fig. 5B) resulted in a marked increase in α-SM actin+ cells (green signal). These cells showed the hallmark morphological features of myofibroblasts, appearing larger in size with irregular projections and bundles of actin filaments and stress fibers. When light was collected from both the red and green channels of the confocal microscope, eosinophils appeared orange, indicating strong autofluorescence (Fig. 5C). To quantify the effect of eosinophils on myofibroblast formation, fibroblasts were harvested by trypsinization and stained for α-SM actin expression. Eosinophils obtained from various donors (n = 3–8) induced the formation of...
Eosinophils and Tissue Remodeling

Even at relatively low eosinophil concentrations, i.e., one eosinophil to every two fibroblasts, there was a significant increase in the number of myofibroblasts ($p < 0.01$). This effect was maximal at a 1:1 ratio between the two cell types. Increasing the concentration of eosinophils to fibroblasts resulted in a decrease in myofibroblast formation in a cell-dependent manner in all experiments, although an effect was still apparent at this ratio (1:16). Fibroblast viability, as determined by trypan blue, was >90% at all points.

**Effect of Anti-TGF-β**

To investigate the eosinophil-derived factor responsible for eosinophil-induced myofibroblast formation, cultures were incubated in the presence or the absence of anti-TGF-β Ab at 0.1, 1, and 10 μg/ml. Fibroblasts cultured in the presence of eosinophils showed a marked up-regulation in the level of α-SM actin expression (lane 2), in contrast to fibroblasts grown in medium alone, where a band was barely visible (lane 1). Treatment with the neutralizing Ab to active TGF-β significantly inhibited eosinophil-induced fibroblast α-SM actin expression in a dose-dependent manner as determined by both Western blot (Fig. 7A) and immunocytochemistry (Fig. 7B; $p < 0.01$). To control against a nonspecific Ab effect, fibroblasts were incubated with an irrelevant IgG2a Ab (lane 4). This control Ab was without effect. Eosinophils alone did not express α-SM actin (data not shown).

**Tenascin Gene and Protein Expression**

MRC-5 fetal lung fibroblasts were treated with eosinophils to determine the effect on the expression of tenascin gene and gene product. MRC-5 fibroblasts constitutively expressed low levels of tenascin mRNA. As observed with α-SM actin expression, relatively low eosinophil concentrations were found to up-regulate tenascin gene expression (Fig. 8A), with a 1:1 ratio between the two cell types inducing the most marked up-regulation. At this ratio, eosinophils up-regulated tenascin transcripts at 3 h (Fig. 8B) and significantly up-regulated protein after a 24-h coculture (Fig. 8C; $p < 0.02$). Eosinophils alone did not express either gene or gene product for tenasin (data not shown).

**Effect of Cytokines on Fibroblast Expression of Tenascin**

Dermal fibroblasts were incubated in the absence or the presence of various concentrations of IL-4, IL-5, IL-13, TGF-β1, and FGF-2 for 24 h. Supernatants were removed, and fibroblasts were examined for tenascin expression in situ by ELISA. Under control conditions, fibroblasts constitutively expressed tenascin (Fig. 9). Treatment with IL-13 induced a marked up-regulation in the expression of tenascin in a dose-dependent manner. Both IL-4 and TGF-β1 (100 pM) also up-regulated the expression of tenascin compared with medium alone. In contrast, IL-5 had no effect at 100 pM, whereas FGF-2 decreased basal tenascin expression at 1000 pM.

**Discussion**

In this study we have shown that intradermal allergen challenge into skin sites of atopic individuals induced several characteristics associated with tissue remodeling, including activation of mesenchymal cells (fibroblasts, endothelial cells, smooth muscle cells) and increased expression of the matrix proteins procollagen I and tenascin. Moreover, we were able to delineate the temporal pattern of this allergen-induced remodeling and showed that the peak of eosinophil- and TGF-β1-immunoreactive cells (6 h after allergen challenge) preceded the formation of myofibroblast-like cells and their associated matrix proteins, which were prominent at later time points when allergic inflammation (i.e., the LPR) was resolving (24 and 48 h). The function of eosinophils in these events was

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**FIGURE 4.** The time course of tenascin-C immunoreactivity on skin sites after allergen challenge in atopic subjects. Diluent-challenged sites (Dil) at 24 h were used as controls. The results are expressed as the number of fibroblast-like (as observed by their elongated nuclei and highlighted with arrows) tenascin positive cells (A) or tenascin basement membrane (B; mean ± SEM) per square millimeter of skin biopsy (n = 10). A semiquantitative estimation was performed with a score from 0 (no staining) to 3 (intense staining) to determine SM immunoreactivity. Eosinophils are highlighted with arrowheads. C. Photomicrographs of connective tissue sections of skin from various subjects (C3 (intense staining) to determine SM immunoreactivity. Eosinophils are quantitatively estimated with a score from 0 (no staining) to 3 (intense staining).
repair processes associated with allergic tissue injury. Thus, repair and tissue remodeling during resolution of the inflammation (Fig. 1) led us to hypothesize that eosinophils may play a role in and tissue eosinophils at later time points after allergen challenge finding was observed by Wong and colleagues (3) in a hamster model of cutaneous wound healing. In expression in situ. A similar binding to TGF-activation of fibroblasts may be partly under the control of infiltrating TGF-β1+/IL-13+ leukocytes, particularly the eosinophil.

The apparent dissociation between the magnitude of the LPR and tissue eosinophils at later time points after allergen challenge (Fig. 1) led us to hypothesize that eosinophils may play a role in repair and tissue remodeling during resolution of the inflammatory reaction. Several reports have suggested a role for eosinophils in repair processes associated with allergic tissue injury. Thus, TGF-β1 expression was prominent in nasal polyps and increased in airway biopsies from asthmatic subjects (20–22). TGF-β1, through specific binding to TGF-β receptor type II (23, 24), is a predominant differentiation factor of the myofibroblast phenotype (25, 26). Fibroblast activation by Th2 cytokines may be TGF-β dependent, since IL-4 up-regulated TGF-β1 mRNA and product by eosinophils, and animal models of IL-13-induced fibrosis suggest that these effects are mediated indirectly via activation of a TGF-β pathway (27, 28).

Of interest was the discordance between the total eosinophils and TGF-β1+ eosinophils at the different time points, i.e., more eosinophils were TGF-β1+ at 24 and 48 h than at 1, 3, or 6 h (Fig. 2A). Thus, tissue eosinophils appear to up-regulate their TGF-β1 expression in situ. A similar finding was observed by Wong and colleagues (3) in a hamster model of cutaneous wound healing. In contrast, IL-13 expression was significantly elevated at all time points, but peaked at 24 h, confirming our earlier findings (Fig. 2B) (29). However, in the present study with sampling at earlier time points, we observed significant increases in the numbers of infiltrating IL-13+ leukocytes as early as 1 h following allergen challenge. Moreover, the percentage of eosinophils positive for IL-13 protein ranged between 8 and 20% (data not shown), suggesting that eosinophils represent a substantial source of IL-13. Other cellular sources of IL-13 included mast cells, neutrophils, and lymphocytes. Thus, the expression for both TGF-β1 and IL-13 ceased the formation of myofibroblasts (Fig. 2C), suggesting that the allergen-induced up-regulation of these potent differentiation factors may induce the transformation of fibroblasts to myofibroblasts in vivo.

We have also shown that intradermal allergen challenge induces the formation of α-SM actin-immunoreactive myofibroblast-like cells. Myofibroblasts have also been reported after allergen-induced late asthmatic reactions (15) and may contribute to the thickened subepithelial reticular basement membrane (RBM) evident within the airways of asthmatics, since there is a correlation between the thickness of the RBM and the number of myofibroblasts beneath it (30). Our skin model allowed us to study allergen-induced myofibroblast formation over a prolonged time course (Fig. 2C). We found that cell numbers increased at 24 h and were still significantly elevated at 48 h compared with diluent control. Similarly, we observed a significant increase in the numbers of fibroblast-like procollagen-I+ cells at 48 h (Fig. 2D) suggesting that active remodeling and fibroblast activation persist several hours after resolution of the LPR. In the context of allergic inflammation in the lung, our data indicate that this structural cell may persist long after the height of the LPR and may be responsible for the increased deposition of new matrix proteins.

Tenascin, a highly regulated member of the matricellular family, is expressed during development and growth and in response to injury (31, 32). We have observed a significant up-regulation in the expression of tenascin within fibroblast-like cells, in and around bundles of smooth muscle and at the vascular smooth muscle basement membrane in response to allergen challenge (Fig. 4). The FIGURE 5. Eosinophil-induced dermal fibroblast transformation to a myofibroblast-like phenotype. Fibroblasts were grown on slide chambers and cocultured in the absence (A) or the presence (B) of 0.1 × 10⁶ eosinophils for 24 h. Myofibroblast-like cells (α-SM actin+) registered in green. The eosinophils show intense autofluorescence and appear orange when viewed in both the red and green channels (C).
temporal pattern of tenascin expression at all three sites was identical and increased prominently at 24 h, at which time the gross LPR was declining. Single tenascin-immunoreactive cells, predominantly located in the lower dermis and fusiformic in shape, were evident at 6 h, peaked at 24 h, and remained elevated out to 72 h. The increased expression of tenascin at the basement membrane of eccrine glands and blood vessels may affect cell trafficking. Tenascin has been demonstrated to act as a permissive substrate to prevent or allow cell migration (33), and tenascin knockout mice exhibit prolonged influx and retention of polymorphonuclear leukocytes (34). We suggest that tenascin may function to coordinate the inflammatory cell infiltrate, since the peak expression of tenascin around blood vessels was observed at 24 h, when the numbers of tissue granulocytes at the site of inflammation were in decline compared with 6 h (35). Moreover, the temporal expression of tenascin suggests that 1) repair mechanisms are in place and become activated to control the allergic inflammation; and 2) these processes may result from interactions between eosinophils and other inflammatory cells with mesenchymal cells. In support of these findings, we have shown that the selective depletion of allergen-induced tissue eosinophilia following treatment with anti-IL-5 led to a significant decrease in tenascin formation in MRC-5 fibroblasts after cessation of exposure.

To support our in vivo findings we developed an in vitro coculture system and showed by use of immunofluorescence and immunoblot that human eosinophils induced the formation of myofibroblasts from primary human dermal fibroblasts. These cells showed the hallmark morphological features of myofibroblasts, appearing larger in size with irregular projections and bundles of the thickness of the RBM and the numbers of subepithelial fibroblasts after cessation of exposure.

To support our in vivo findings we developed an in vitro coculture system and showed by use of immunofluorescence and immunoblot that human eosinophils induced the formation of myofibroblasts from primary human dermal fibroblasts. These cells showed the hallmark morphological features of myofibroblasts, appearing larger in size with irregular projections and bundles of...
α-SM actin filaments (Fig. 5). Similar results were obtained with lung fibroblasts (data not shown). The effect was inhibitable by anti-TGF-β as previously observed by others (Fig. 7, A and B) (37, 38). Eosinophils at relatively low concentrations (1:1 ratio; <50 × 10^3/ml) without the addition of other factors, induced a 4-fold increase in the number of myofibroblasts (Fig. 6B), an effect that occurred rapidly (within 24 h; Fig. 6A), similar to that found in vivo (Fig. 2C) (15). The low numbers of eosinophils required for myofibroblast formation support the view that this cell is involved in thickening of the reticular basement membrane in asthma. Indeed, in a retrospective analysis both airway eosinophilia and epithelial RM thickening have been demonstrated to be apparent in children who later go on to develop asthma up to 4 yr before asthma is clinically expressed (39). Higher eosinophil/fibroblast ratios inhibited myofibroblast formation, possibly via release of FGF-2. FGF-2 is known to be expressed by blood (our unpublished observation) and tissue eosinophils (11) and to be an inhibitor of TGF-β-induced myofibroblast formation (37).

In an attempt to confirm our in vivo findings, we determined whether eosinophil coculture induced the expression of fibroblast tenascin. As observed in the diluent-challenged sites in vivo, fibroblasts constitutively expressed tenascin. However, eosinophil treatment led to a 2- to 3-fold increase in tenascin mRNA (Fig. 8A) and significantly increased tenasin protein at 24 h (Fig. 8B). Eosinophil coculture also increased transcripts for procollagen I and significantly up-regulated procollagen protein (data not shown). A number of eosinophilic cytokines may be responsible for this effect, with TGF-β1, IL-4, and IL-13 known to stimulate fibroblast collagen production (10). Consistent with previous reports, we showed that TGF-β1 (40, 41) and IL-4 (42) increased tenasin expression and for the first time demonstrated that addition of exogenous IL-13 induced a marked and dose-dependent increase in the expression of tenasin (Fig. 9). Furthermore, FGF-2 reduced basal tenasin expression. We have yet to ascertain whether FGF-2 inhibits TGF-β1- or IL-4-induced tenasin expression. However, Davidson et al. (43) have shown FGF-2 to be an inhibitor of TGF-β1-mediated collagen production in skin fibroblasts.

In conclusion, we propose that following allergen-induced, IgE-dependent mast cell degranulation there is infiltration of IL-13- and TGF-β1-positive eosinophils and other leukocytes, which leads to myofibroblast formation. This is followed by an increase in the expression of associated matrix proteins, procollagen I and tenasin. We speculate that resident structural cells become activated in a spatial and sequential pattern to produce matrix proteins such as tenasin and collagen, which modulate the inflammatory response, leading to its resolution.

References


