

IL-4, IL-13, and dexamethasone augment fibroblast proliferation in asthma

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Background: IL-4 and IL-13 have been shown to be critical for expression of the asthma phenotype in a murine model and may modulate human fibroblast function.

Objective: We hypothesized that IL-4 and IL-13 would increase airway fibroblast proliferation and reduce the ability of dexamethasone to decrease this proliferation.

Methods: Six subjects with severe asthma, 5 subjects with mild asthma, and 5 healthy subjects underwent bronchoscopy with endobronchial biopsy. Biopsy specimens were placed in Dulbecco modified Eagle medium and cultured, and only fibroblasts from the first and second passages were evaluated. Cells were incubated with IL-4 (50 ng/mL), IL-13 (10 ng/mL), and the combination for 48 hours in the presence and absence of dexamethasone, 10^{-7} mol/L, and 10^{-8} mol/L. Fibroblasts were also incubated with IFN- γ at 50 ng/mL to assess the response of a T_H1 cytokine on proliferation.

Results: Fibroblast proliferation, determined by ³H-thymidine incorporation, was significantly increased by IL-4 in subjects with mild asthma as compared with IL-4 in subjects with severe asthma and healthy subjects ($P = .003$), IL-13 ($P = .011$), and the combination ($P = .004$). Dexamethasone also increased proliferation in the group with mild asthma as compared with the group with severe asthma and the healthy group (10^{-7} mol/L, $P = .02$; 10^{-8} mol/L, $P = .02$). IFN- γ did not significantly alter airway fibroblast proliferation.

Conclusion: IL-4, IL-13, and dexamethasone all significantly increased fibroblast proliferation in subjects with mild asthma. (J Allergy Clin Immunol 2001;107:602-6.)

Key words: Asthma, airway remodeling, fibroblast, IL-4, IL-13

Airway remodeling in asthma has received attention as a significant alteration of the airways due to derangements in wound repair after injury. Analysis of airway biopsy specimens from patients with asthma has revealed evidence of significant changes in airway structure in addition to increased airway inflammation as compared with analysis of specimens from control subjects. Specifically, one such change is subepithelial basement membrane thickening, which has been shown in some studies to correlate with bronchial hyperresponsiveness.¹ This morphologic change is believed to be due to increased

Abbreviations used

PDGF: Platelet-derived growth factor

collagen deposition, particularly types I, III, and V, produced by airway fibroblasts adjacent to the collagen layer.² These fibroblasts, which also include myofibroblasts, produce collagen and have been shown to produce specific cytokines that may attract inflammatory cells to the airway and perpetuate inflammatory and fibrotic processes.^{3,4} These processes, although still being defined, are believed to be detrimental to the host in that they may lead to fixed airflow limitation.⁵⁻⁷

The cytokines IL-4 and IL-13 are part of the T_H2 subset of cytokines, which are involved in the regulation of B cells and Ig isotype switching. IL-13, unlike IL-4, acts primarily in the monocyte population to regulate surface antigen expression, antibody-dependent cellular cytotoxicity, and cytokine synthesis.⁸⁻¹⁰ It has been shown to reduce glucocorticoid binding affinity in monocytes but not in T lymphocytes.¹¹ We have previously shown that in nocturnal asthma, IL-4 and IL-13 in combination reduced steroid responsiveness and glucocorticoid receptor β expression of alveolar macrophages at 4 AM.¹² In addition, both cytokines have been shown to bind to a human lung fibroblast cell line and induce proliferation.^{13,14} It is not known whether these receptors are present in fibroblasts from patients with asthma or whether these cytokines modulate fibroblast function in this disease process.

Corticosteroids are first-line therapy in patients with asthma more severe than mild intermittent.¹⁵ However, it is unclear whether corticosteroids affect the remodeling process. In regard to fibroblast function, corticosteroids may either reduce or increase fibroblast proliferation and also may exhibit differential effects on other aspects of fibroblast function.¹⁶ Some investigators have shown a decrease in proliferation by corticosteroids,^{17,18} whereas others have shown an increase.¹⁹⁻²² The same issue may be true for collagen production and contractile properties of airway fibroblasts.²³ For instance, Skold et al²³ have shown that glucocorticoids augment contraction of collagen gels by inhibition of prostaglandin E₂. In vivo, it is unclear whether inhaled corticosteroids reduce subepithelial collagen deposition significantly because the data are conflicting.^{24,25} We hypothesized that IL-4 and IL-13 would increase airway fibroblast proliferation in patients with asthma and reduce the ability of dexamethasone to decrease this proliferation.

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METHODS

Subjects

Six subjects with asthma that was considered severe by American Thoracic Society criteria, 5 subjects with mild asthma by American Thoracic Society criteria, and 5 healthy subjects were recruited from the general Denver community. All subjects with asthma met diagnostic criteria for asthma,²⁶ exhibiting a methacholine PC₂₀ of less than 8 mg/mL, and were receiving inhaled β_2 -agonists only. The healthy group had no history of asthma and no bronchial hyperresponsiveness (methacholine PC₂₀ > 25 mg/mL). Exclusion criteria included use of cromolyn, nedocromil, inhaled or oral corticosteroids, leukotriene modifiers, and/or theophylline within the previous 6 weeks; an upper respiratory tract infection within the previous 6 weeks; immunotherapy within the previous 3 months; history of cigarette use; and significant nonasthma pulmonary disease or other medical illnesses. Informed consent was obtained from all patients for this institutional review board–approved protocol.

Protocol

Subjects underwent bronchoscopy with endobronchial biopsy as previously described.²⁷ The site of biopsy was randomized to either the right or left lower lobe, and 5 to 6 endobronchial biopsy specimens were taken from the tertiary carinae of the right or left lower lobes under direct visualization. Before the bronchoscopy, spirometry was performed before and after intravenous administration of 0.18 mg of albuterol from a metered dose inhaler and 0.4 mg atropine. Xylocaine (4%) was used to anesthetize the upper airway, and Xylocaine (1%) was applied to the laryngeal area, trachea, and orifice of the right lower or left lower lobe bronchi through the bronchoscope. Supplemental oxygen was administered throughout the procedure along with monitoring of heart rate and oxygen saturation. Subjects' vital signs were monitored in our laboratory after the procedure, and subjects were discharged from the laboratory when their FEV₁ was 90% of the prebronchoscopy, postbronchodilator value.

Fibroblast culture

Biopsy specimens were cut into several smaller pieces (~5–6 per biopsy) and placed into wells (2 pieces per well) of a 24-well tissue culture plate containing Dulbecco modified Eagle medium supplemented with FBS (10%), streptomycin (100 μ g/mL), penicillin (10,000 U/mL), and gentamicin (100 μ g/mL). This medium does not support growth of other cells, such as epithelial and endothelial cells. The tissue pieces were incubated at 37°C/5% CO₂ and cultured until cell growth was established at greater than 50% confluency (~8–20 days), with the media changed every 2 to 3 days. On reaching greater than 50% confluency, the tissue pieces were removed, and the cells were trypsinized, counted, tested for viability, stained for identification, and plated in 96-well plates for proliferation experiments and in 15 $\times$ 60-mm culture dishes for subsequent cell passages (Becton Dickinson, Franklin Lakes, NJ). All cells were stained with monoclonal mouse anti-human antibodies against fibroblast antigen (Ab-1; Calbiochem, San Diego, Calif), vimentin (DAKO, Carpinteria, Calif), and α -smooth muscle actin (DAKO), to confirm fibroblast, myofibroblast, and mesenchymal cell specificity, respectively. First-passage cells generated directly from the biopsy tissue were stained on cytospin slides, whereas second-passage fibroblasts were grown directly onto 18-mm coverslips (Fisher Scientific, Pittsburgh, Pa) that were placed in the culture dishes on repassage. Only cells from the first (200,000–400,000) and second (700,000–1,000,000) passages were used for experiments, given the potential concern for changes in cell function, including enhanced senescence, that may occur after several passages.²⁸

Measurement of fibroblast proliferation

For the cytokine and dexamethasone modulation studies, the cells were seeded at a density of 10⁴ cells/well in 96-well culture plates in Dulbecco modified Eagle medium and 0.5% FBS. A proliferation dose-response study was performed in 2 subjects with mild asthma and a human lung fibroblast cell line (Clonetics, Walkersville, Md). The concentrations of IL-4 used were 1, 10, 20, 30, 40, 50, and 100 ng/mL. The concentrations of IL-13 used were 10, 20, 30, 40, 50, and 100 ng/mL. After 48 hours, ³H-thymidine (1 mCi/mL) was added to the cells and incubated for an additional 24 hours. The cells were harvested onto glass fiber filter plates, and ³H-thymidine incorporation was measured. There was no significant proliferation dose response for IL-4 and IL-13, although all concentrations increased proliferation above unstimulated control.

To determine the effects of a T_H1 cytokine on airway fibroblast proliferation, we performed a proliferation dose-response study in 2 subjects with mild asthma and a human lung fibroblast cell line (Clonetics) using IFN- γ . The concentrations of IFN- γ evaluated were 100, 200, 300, 400, 500, 600, and 1000 U. Because there was no significant dose response with either subject or the cell line, fibroblasts from the remaining subjects of the mild asthma group and the healthy group were incubated with IFN- γ at 300 U (50 ng/mL). After 48 hours, ³H-thymidine (1 mCi/mL) was added to the cells and incubated for an additional 24 hours. The cells were harvested onto glass fiber filter plates, and ³H-thymidine incorporation was measured.

Incubation with cytokines and dexamethasone

Given the lack of a proliferation dose response with IL-4 and IL-13, the concentrations of cytokines chosen were those used by Doucet et al¹⁴ in their study of IL-4 and IL-13 effects on a human lung fibroblast cell line. IL-4 at 50 ng/mL and IL-13 at 10 ng/mL and the combination were added to each well in triplicate (R&D, Minneapolis, Minn). The fibroblasts were also incubated with the cytokines in the conditions described above in the presence of dexamethasone to achieve concentrations of 10⁻⁷ mol/L and 10⁻⁸ mol/L and dexamethasone alone. These concentrations were chosen because they represent the physiologic range of dexamethasone relative to cortisol given their difference in potency. After 48 hours, ³H thymidine (1 mCi/mL) was added to the cells and incubated for an additional 24 hours. The cells were harvested onto glass fiber filter plates, and ³H-thymidine incorporation was measured. Results are expressed as percentage of unstimulated control.

Statistical analysis

The main outcome variable was fibroblast proliferation at baseline (unstimulated control) and after exposure to IL-4, IL-13, dexamethasone, and the combination of cytokines and dexamethasone. The data were analyzed with a mixed-effects model with fixed effects for group, cytokine, and dexamethasone concentrations; the interactions between dexamethasone, cytokines, and groups; plus a random subject effect.^{29–31} If an interaction was significant, pairwise comparisons of least squares means were made within groups and within times. This statistical analysis, which is a repeated-measures design, initially provides *P* values when all 3 groups are compared with one another. Contrasts are then performed to evaluate differences between pairs, and adjustments are made for multiple comparisons. This method was chosen given that repeated measures under different conditions were performed within each group, and a mixed-effects model allows evaluation of all the conditions simultaneously. When *P* values were significant, contrasts were performed to evaluate significance between groups. Within-group comparisons were also performed with means within one group, in which a specific condition is compared with an unstimulated control for that group.

TABLE I. Subject characteristics

	Healthy group	Mild asthma group	Severe asthma group	P value
Sex (M/F)	3/2	0/5	4/2	—
Age (y)	32.4 ± 3.4	32.2 ± 3.7	39.8 ± 4.7	.13
Medicines	None	β ₂ -Agonists	β ₂ -Agonists	—
FEV ₁ (L)	3.8 ± 0.4	2.7 ± 0.2	2.4 ± 0.2	.009
FEV ₁ (% predicted)	99.8 ± 4.8	80.8 ± 1.6	65.6 ± 2.6	.0001

TABLE II. Results of proliferation of airway fibroblasts incubated with IL-4, IL-13, IFN-γ, and dexamethasone

	Healthy group (n = 5)	Mild asthma group (n = 5)	Severe asthma group (n = 6)	Statistical condition	P value*
Baseline proliferation [†]	3384 ± 1136	1386 ± 451	705 ± 177	H > M and S	.09
Effects of cytokines and DEX [‡]					
IL-4 (50 ng/mL)	136 ± 10	191 ± 19	77 ± 5	M > H > S	.003
IL-13 (10 ng/mL)	124 ± 12	177 ± 15	11 ± 12	M > S and H	.011
IL-4 and IL-13	130 ± 17	196 ± 23	82 ± 10	M > S and H	.004
IFN-γ (300 U or 50 ng/mL)	89 ± 1	126 ± 33	ND		.43
DEX 10 ⁻⁷ mol/L	100 ± 10	192 ± 31	67 ± 22	M > H, M > S	.02/.09
DEX 10 ⁻⁸ mol/L	100 ± 10	177 ± 22	58 ± 50	M > S and H	.02
IL-4, IL-13, and DEX 10 ⁻⁷ mol/L	115 ± 13	243 ± 53	103 ± 4	M > S and H	.07
IL-4, IL-13, and DEX 10 ⁻⁸ mol/L	120 ± 17	246 ± 45	190 ± 66	M > S and H	.10

H, Healthy; M, mild asthma; S, severe asthma; DEX, dexamethasone; ND, not done.

*The depicted P value for the designated statistical condition in which the results of the 3 groups were compared by mixed-effects model (see Methods).

[†]Baseline proliferation is expressed as mean ± SEM in counts per minute of incorporated ³H-thymidine during 48 hours of incubation without cytokines or dexamethasone.

[‡]Results for effects of cytokines and dexamethasone are expressed as mean ± SEM of percentage increase from unstimulated control fibroblast cultures.

All tests were 2 sided and conducted at the 5% significance level. Data are expressed as mean ± SEM because the data were normally distributed, as determined by the Shapiro-Wilk test.³²

RESULTS

Subjects

Subject characteristics are shown in Table I.

Proliferation

Immunostaining with vimentin (DAKO), Ab-1 (Calbiochem), and α-smooth muscle actin (DAKO) confirmed the fibroblast phenotype where cells were 100% vimentin, more than 95% Ab-1 positive, and less than 5% α-smooth muscle actin positive. The baseline proliferation without stimulation or exposure to cytokines and dexamethasone was greatest in the healthy group, but not significantly so ($P = .09$, Table II). To take into account differences in baseline proliferation and evaluate proliferation after exposure to cytokines and dexamethasone, we present the data as a percentage of the unstimulated control.

Analysis of proliferation between the 3 groups revealed that IL-4 and IL-13, each alone and in combination, significantly increased proliferation in the mild asthma group as compared with the severe asthma and healthy groups. Specifically, proliferation after exposure to IL-4 was significantly greater in the mild asthma group as compared with the severe asthma and the healthy groups ($P = .003$) (Table II). Proliferation after exposure to IL-13 was also significantly greater in the

mild asthma group ($P = .011$) (Table II). The combination of IL-4 and IL-13 also increased fibroblast proliferation significantly in the mild asthma group as compared with the severe asthma and healthy groups ($P = .004$) (Table II).

When each group was analyzed separately, within-group comparisons revealed a significant effect by IL-4, IL-13, and the combination of IL-4 and IL-13 in both the mild asthma group ($P = .002$ when compared with unstimulated control) and healthy group ($P = .03$), but not in the severe asthma group ($P = .81$).

Analysis of proliferation between the 3 groups with the mixed-effects model also revealed a significant group by steroid interaction at $P = .009$. Specifically, dexamethasone at 10⁻⁷ and 10⁻⁸ mol/L each significantly increased proliferation in the mild asthma group as compared with the severe group and healthy group. Proliferation after exposure to dexamethasone at 10⁻⁷ mol/L was significantly higher in the mild asthma group than in the healthy group ($P = .02$). However, proliferation in the mild asthma group was greater than in the severe asthma group, but not significantly so ($P = .09$) (Table II). Dexamethasone at 10⁻⁸ mol/L increased fibroblast proliferation in the mild group as compared with both the severe asthma and healthy groups ($P = .02$) (Table II).

When each group was analyzed separately, within-group comparisons revealed that dexamethasone at both concentrations increased proliferation significantly in the mild asthma group ($P = .0008$ for both concentrations as compared with unstimulated, control), but not in the healthy

group ($P = .71$). Dexamethasone at both concentrations decreased proliferation in the severe asthma group, and this effect trended toward significance ($P = .06$).

The combination of dexamethasone and IL-4/IL-13 also increased proliferation primarily in the mild asthma group, but the differences between the 3 groups trended toward significance: IL-4/IL-13 + dexamethasone 10^{-7} mol/L, $P = .07$ (Table II); and IL-4/IL-13 + dexamethasone 10^{-8} mol/L, $P = .10$ (Table II). The same trends were observed when IL-4 and IL-13 were used separately with dexamethasone: IL-4 + dexamethasone 10^{-7} mol/L, $P = .12$; IL-4 + dexamethasone 10^{-8} mol/L, $P = .07$; IL-13 + dexamethasone 10^{-7} mol/L, $P = .09$; and IL-13 + dexamethasone 10^{-8} mol/L, $P = .18$.

Exposure of airway fibroblasts with IFN- γ did not significantly increase proliferation in either the mild asthma or healthy groups ($P = .43$, Table II).

DISCUSSION

We have shown that in mild asthma, dexamethasone augmented fibroblast proliferation and did not diminish the ability of cytokines IL-4 and IL-13 to increase proliferation. The augmentation of proliferation did not occur to the same extent in the severe asthma and healthy groups. IFN- γ , a T_H1 cytokine, had no effect on fibroblast proliferation.

The effect of corticosteroids on fibroblast function is controversial. Warshamana et al²² have shown in a human lung fibroblast cell line and early-passage rat fibroblasts that dexamethasone increased fibroblast proliferation. The proliferative effect of corticosteroids was blocked by a polyclonal antibody to the AB isoform of platelet-derived growth factor (PDGF) α . Dexamethasone also upregulated PDGF receptor α mRNA and protein. The authors concluded that dexamethasone-induced fibroblast proliferation is mediated in part by PDGF-AA, another isoform of PDGF that binds to the PDGF receptor α . Dube et al,²⁸ in fibroblasts obtained from a group of subjects with mild asthma and a group of healthy subjects, determined that dexamethasone also increased proliferation in airway fibroblasts over control. The proliferation observed in the mild asthma group was 73.9% above control, as compared with that observed in our subjects with mild asthma, who demonstrated proliferation at 91% above control. The subjects studied by Dube et al had mild asthma by description, but pulmonary function was not reported. In addition, they also showed that PDGF-BB, also an isoform of PDGF, increased proliferation in asthmatic fibroblasts 25.3% above control, without any significant change in the fibroblasts from healthy control subjects. In our study, corticosteroids did not affect fibroblast proliferation significantly in the healthy control subjects, trended toward decreasing proliferation in the severe asthma group, and increased proliferation in the mild asthma group. Therefore it is not clear whether corticosteroids significantly attenuate the remodeling process in asthma, if we are to use fibroblast proliferation as a surrogate measurement. In fact, these

data suggest that corticosteroids may enhance proliferation and wound repair as part of the remodeling process, particularly in mild asthma. Clearly, there are other consequences of remodeling that include smooth muscle hypertrophy/hyperplasia, collagen expression, and mucous gland hyperplasia, which were not assessed in this study. In addition, we are using an in vitro model, which may not precisely mimic asthma in vivo. However, the fibroblasts were obtained from well-characterized subjects with and without asthma, and only the first and second passages were evaluated.

Each of the cytokines was able to significantly augment proliferation of airway fibroblasts in the mild asthma and healthy groups, with the greatest effect in the mild asthma group. There was no significant effect of either cytokine or the combination on fibroblasts from the severe asthma group. A shared receptor for IL-4 and IL-13 has been demonstrated in a lung fibroblast cell line and thus may provide a mechanism by which both cytokines can augment proliferation without acting synergistically when the 2 are combined.^{13,14} Doucet et al¹⁴ evaluated 3 different types of lung fibroblasts (ICIG7, CCL202, and FPA) and found that they all express functional but different IL-4/IL-13 receptors. All expressed the IL-4R α and IL-13R α_1 subunits. The CCL202 and FPA fibroblasts also expressed the IL-13R α_2 and IL-2R γ subunits, suggesting the presence of a heterotrimeric receptor (IL-4R α /IL-13R α /IL-2R γ) able to bind IL-4 and IL-13. The presence of these receptors may have implications beyond fibroblast proliferation because IL-4 and IL-13 have each been shown to increase production of the proinflammatory cytokines IL-6 and monocyte chemoattractant protein 1 by airway fibroblasts.^{13,14} The lack of effect of IFN- γ , a T_H1 cytokine, on airway fibroblast proliferation suggests that the fibroblasts may exhibit differential responses to T_H1 versus T_H2 cytokines. These observations are preliminary, and further study of this differential response is required for validation.

It is of interest that the greatest augmentation of fibroblast proliferation by cytokines and dexamethasone occurred in the subjects with mild asthma. It is possible that the fibroblasts from subjects with severe asthma, exposed to presumed persistent inflammation and elevated cytokine levels in vivo, were unable to respond further to cytokine exposure in vitro. Perhaps the receptor number or function has changed in these patients, resulting in reduced responses. Similarly, the receptors may be intact and functional in the healthy control subjects; thus their fibroblasts are responsive to cytokines in vitro. It is also possible that receptor number or function changed in vitro, resulting in reduced or increased effects. Although the healthy group's fibroblasts responded to cytokine stimulation, they did not respond to dexamethasone. Thus there may be a mechanism unique to mild asthma that allows enhanced response to these particular cytokines and to corticosteroids. Further study of cytokine receptors and the modulation of fibroblast production by corticosteroids are needed to elucidate the mechanisms driving these observations.

In summary, we have shown that dexamethasone, IL-4, IL-13, and the combination of corticosteroid and cytokines augment airway fibroblast proliferation in patients with mild asthma, as compared with subjects with severe asthma and healthy control subjects. Because corticosteroids are recommended as first-line therapy for asthma more severe than mild intermittent, further study regarding steroid and cytokine modulation of fibroblast function is required to determine whether patients experience long-term benefits with the available therapies for asthma.

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