Interleukin-4 inhibits mitogen-induced proliferation of human airway smooth muscle cells in culture

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Hawker, Kristen M., Peter R. A. Johnson, J. Margaret Hughes, and Judith L. Black. Interleukin-4 inhibits mitogen-induced proliferation of human airway smooth muscle cells in culture. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L469–L477, 1998.—The increase in the amount of airway smooth muscle in the bronchial wall associated with asthma is partly due to hyperplasia. It is therefore important to determine which factors regulate growth and especially proliferation. In this study, we describe the effect of interleukin-4 (IL-4), a mast cell- and T lymphocyte-derived cytokine, on human airway smooth muscle proliferation as determined by [3H]thymidine uptake in the presence of fetal bovine serum (FBS), platelet-derived growth factor, basic fibroblast growth factor, and thrombin. IL-4 (5, 15, 50, and 150 ng/ml) significantly decreased 10% FBS-induced proliferation by 50, 73, 43, and 46%, respectively. The proliferative responses to platelet-derived growth factor (20 and 40 ng/ml), basic fibroblast growth factor (30 ng/ml), and thrombin (1 and 10 U/ml) were significantly reduced by 19, 21, 37, and 36%, respectively, in the presence of 50 ng/ml of IL-4. We investigated the effect of IL-4 and other known inhibitors of smooth muscle proliferation, namely PGE2, heparin, and forskolin, on intracellular cAMP concentrations. IL-4 (50 ng/ml) and heparin (100 U/ml) did not alter intracellular CAMP levels when cells were treated with 1 or 10% FBS. PGE2 (1 µM) and forskolin (10 µM) significantly increased CAMP concentration above the control value in nonproliferating cells (1% FBS treated) by 7- and 37-fold, respectively. The effect of IL-4 (50 ng/ml), PGE2 (1 µM), and forskolin (10 µM) on cyclin D1 protein expression in 10% FBS-stimulated human airway smooth muscle cells was also examined. PGE2 and forskolin did not significantly inhibit cyclin D1 expression. However, IL-4 decreased cyclin D1 expression by 21%. These results provide evidence that IL-4 decreases human airway smooth muscle cell proliferation via a mechanism that is cAMP independent and mediated, in part, by a decrease in cyclin D1 protein expression.

A characteristic pathological feature of chronic asthma is an increase in the mass of airway smooth muscle that results from an increase in cell number (hyperplasia) combined with an increase in cell size (hypertrophy) (7). A study by Lambert et al. (17) suggested that if airway smooth muscle contractile properties are maintained, increased airway smooth muscle mass in asthma may be the single most important contributing factor to exaggerated airway narrowing. Thus it is critical to elucidate the factors that may modify the proliferation of human airway smooth muscle cells in culture.

Numerous studies of airway smooth muscle proliferation have been performed in animal tissue. For example, in a variety of animal species, an increase in airway smooth muscle proliferation has been observed in response to leukotriene D4 (39), histamine (26), insulin-like growth factor-I (15), interleukin (IL)-1β, IL-6 (5), and epidermal growth factor (31). Because human airway smooth muscle cells in asthmatic lungs are exposed to a number of inflammatory mediators, it is important to investigate the effects of these mediators on muscle growth and division. Proliferation occurs in response to histamine (20), thrombin, epidermal growth factor, basic fibroblast growth factor (bFGF) (30), and platelet-derived growth factor (PDGF) (13), whereas dexamethasone (30), salbutamol (35), tumor necrosis factor (TNF)-α (32), heparin, PGE2 (13), and vasoactive intestinal peptide (20) inhibit mitogen-induced proliferation of human airway smooth muscle cells in culture.

IL-4 is a T lymphocyte (23)-, mast cell (1)-, and basophil (4)-derived cytokine that is able to regulate a wide range of immune responses. IL-4 plays an essential role in the regulation of IgE synthesis (6). It has also been shown to enhance the expression of vascular cell adhesion molecule-1 on the surface of human bronchial tissue (11). In addition to its proinflammatory effects, we hypothesized that IL-4 may also be able to modify the proliferative responses of human airway smooth muscle cells in culture. IL-4 has effects on a variety of structural cells in that it can potentiate proliferation of endothelium (34) and skin fibroblasts (21) and decrease proliferation of adult human astrocytes (8) and human vascular smooth muscle (37).

In the present study, we investigated the effect of IL-4 on the proliferation of human airway smooth muscle cells in culture. IL-4 was studied in the presence and absence of four known proliferative agents. Proliferation occurred, as expected, in the presence of the higher concentrations of fetal bovine serum (FBS) and thrombin and all concentrations of PDGF and bFGF. We examined the effect of IL-4 on proliferation in response to these four stimuli. Contrary to our expectations, IL-4 inhibited proliferation of human airway smooth muscle and was effective against all four mitogens. The possible mechanisms of IL-4-induced inhibition were also investigated.

METHODS

Cell Culture

Human lung was obtained from patients undergoing either surgical resection for carcinoma or lung transplantation for
emphysema. Collection and use of lung specimens was approved by the Human Ethics Committee of the University of Sydney. Segments of bronchus were dissected free from the surrounding parenchyma in ice-cold carbonated Krebs–Henseleit solution, pH 7.4 (composition in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 25.5 NaHCO3, and 11.1 d-glucose). The epithelium was removed to expose the underlying bands of smooth muscle, which were then gently separated from the underlying connective tissue in small bundles (smooth muscle cell explants). The smooth muscle cell explants were washed three times with DMEM containing 20 U/l of penicillin, 20 µg/ml of streptomycin, and 2.5 µg/ml of amphotericin B. The explants were transferred into 25-mm² flasks (~10 explants/flask) and covered with a minimal amount of DMEM containing 20 U/l of penicillin, 20 µg/ml of streptomycin, 2.5 µg/ml of amphotericin B, and 10% FBS. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2-95% air. The medium was replenished every 5 days for the first 10–20 days, and within this time, smooth muscle growth occurred. The cells were passaged (1:3 split) with a solution of trypsin [0.5% wt/vol in Hanks’ balanced salt solution (HBSS)] containing 1 mM EDTA. The cells were maintained in DMEM free of penicillin, streptomycin, and amphotericin B and supplemented with 10% FBS. Cells were passaged once they reached confluence, and cells at passages 3–5 were used for experimentation.

Cell Characterization

Morphology. Human airway smooth muscle cells were examined with a light microscope (×40 magnification) to identify specific morphological characteristics. Immunohistochemistry. Immunohistochemical identification of smooth muscle specific α-actin was observed in cultures from all patients as previously described (32). Background staining was determined by omitting the primary antibody, and the cells were visualized with a fluorescence microscope.

Proliferation Assays

[3H]thymidine uptake. Cells were harvested by trypsinization and were resuspended in DMEM containing 10% FBS. The cells were plated at a density of 1 × 104 cells/cm² in 96-well cluster plates and incubated for 7 days to achieve cell confluence. The cells were growth arrested by incubation in 1% FBS in DMEM for 24 h.

In a separate series of experiments, cell cycle status was confirmed by flow cytometry. Confluent cells from three separate patients were treated with 1% FBS in DMEM for 24 h before being permeabilized with 0.075% Triton X-100 in DMEM. The DNA was labeled with propidium iodide, and cell cycle analysis was performed on 104 cells. The majority of cells (80 ± 2%) were found to be in the G0/G1 phase of the cell cycle, the remainder being in the S and G2/M phases. Thus we established that cells incubated for 24 h in 1% FBS were growth arrested.

The effect of increasing concentrations of IL-4 (1.5, 5, 15, 50, and 150 ng/ml) was examined on cells exposed to 10% FBS. In addition, the effect of IL-4 (50 ng/ml) on proliferation induced by three known smooth muscle mitogens, PDGF (20 and 40 ng/ml), bFGF (30 ng/ml), and thrombin (0.3, 1, and 10 U/ml), was examined. The adherent cells were washed three times with 100 µl of HBSS that was then replaced with 100-µl aliquots of DMEM containing FBS (10%), PDGF (20 or 40 ng/ml), bFGF (30 ng/ml), or thrombin (0.3, 1, 10 U/ml) alone or in combination with IL-4 (50 ng/ml) in triplicate wells. Each treatment (PDGF, bFGF, and thrombin) was assessed in the presence of 1% FBS. The cells were then incubated at 37°C for 19 h. After this incubation period, all cells were incubated for a further 5 h with 1 µCi of [methyl-3H]thymidine ([3H]Tdr; specific activity 20 mCi/mmol). The stock solution of [3H]Tdr was diluted in HBSS to give a final concentration of 1 µCi/well. Cell proliferation was then assessed by placing the culture plates in a −20°C freezer. After 24 h, the cells were harvested, and [3H]Tdr uptake was determined as previously described (13). Viable cell counts. Human airway smooth muscle cells were plated at a density of 1 × 104 cells/cm² in 24-well cluster plates and incubated for 7 days in DMEM containing 10% FBS. The cells were growth arrested in 1% FBS in DMEM for 24 h and then washed three times with 500 µl of HBSS. The HBSS was replaced with 500-µl aliquots of DMEM containing 10% FBS in the presence or absence of IL-4 (50 ng/ml) in duplicate wells, and the cultures were incubated for a further 24, 48, or 72 h. The cells were harvested with a trypsin-EDTA solution and counted with a hemocytometer. Cell viability was determined with trypan blue exclusion; cells that stained blue were counted as nonviable.

cAMP Radioimmunoassay

Human airway smooth muscle cells were seeded at a density of 1 × 104 cells/cm² in six-well cluster plates and grown for 7 days in DMEM containing 10% FBS. Cells were growth arrested in 1% FBS in DMEM for 24 h. The medium was aspirated and replaced with DMEM containing 1% FBS and 100 µM 3-isobutyl-1-methylxanthine, and the cells were incubated at 37°C for 1 h. Cells were treated with either 1 or 10% FBS in DMEM alone or in combination with IL-4 (50 ng/ml), PGE2 (1 µM), heparin (100 U/ml), or forskolin (10 µM) in duplicate wells for 1.5 h. The medium was aspirated and replaced with 3 ml of 100% ethanol. The culture wells were dried overnight in a 25°C oven, and the cells were reconstituted in DMEM. The cAMP assay was performed as previously described (19). Briefly, 20-µl aliquots of sample were acetylated by the addition of 10 µl of triethylamine-acetic anhydride (5:2 vol/vol) and then mixed. Acetylation proceeded within a few seconds, and the reaction was rapidly deactivated by the addition of distilled water. After 20 min, 400 µl of antiserum (diluted 1:8,000 in buffer) were added, followed by adenosine 3’,5’-cyclic phosphoric acid 2’-O-succinyl 3’-[125I]-iodotyrosine methyl ester [125I-ScAMP; 100 µl · 10,000 counts/min (cpm), specific activity 2.2 mCi/mmol]. The buffer solution (pH 6.2) used for antiserum incubations consisted of sodium acetate (50 mM), calcium chloride dihydrate (25 mM), sodium azide (0.02% wt/vol), and bovine serum albumin (0.1% wt/vol). Samples were incubated overnight at 4°C. Ice-cold polyethylene glycol 6000 (1 ml of 20% wt/vol in distilled water) was then added, followed by 100 µl of normal bovine γ-globulin solution (0.5% wt/vol in distilled water), and the tubes were mixed and centrifuged (2,400 g) for 20 min at 4°C. The supernatants were discarded, and the radioactivity of the remaining pellet was counted to determine the bound fraction.

Western Blot Analysis

Human airway smooth muscle cells were seeded at a density of 1 × 104 cells/cm² in 175-cm² tissue culture flasks in DMEM containing 10% FBS. After 7 days, the cells were growth arrested in 1% FBS for 24 h. The cells were either left untreated or stimulated with 10% FBS in DMEM alone or in combination with the growth inhibitors IL-4 (50 ng/ml), PGE2 (1 µM), or forskolin (10 µM) for 5 h. The cells were washed three times in ice-cold PBS and harvested by cell scraping.
Cells were separated into cytosolic and then nuclear fractions with the following method. Initially, the cells were incubated for 5 min in 150 µl of a solution containing HEPES (10 mM), KCl (10 mM), MgCl2 (1.5 mM), dithiothreitol (DTT) (1 mM), aprotinin (1 µg), leupeptin (1 µg), phenylmethysulfonyle fluoride (2 µg), and Triton X-100 (0.2% vol/vol). The samples were centrifuged at 2,000 g for 2 min, and the supernatant (cytosolic fraction) was discarded. The remaining pellet, which contained cell nuclei only, was resuspended in 30 µl of a solution containing HEPES (20 mM), KCl (0.4 mM), MgCl2 (1.5 mM), EDTA (0.2 mM), DTT (1 mM), glycerol (25% vol/vol), aprotinin (0.1 µg), leupeptin (0.1 µg), and phenylmethysulfonyle fluoride (0.2 µg). After 30 min, the cells were centrifuged at 16,000 g at 4°C for 5 min. The supernatant was collected as the nuclear fraction and stored at −20°C. The samples (10 µg of total protein) were combined with a gel loading buffer [SDS (2% wt/vol), glyceral (7.5% vol/vol), Tris·HCl (31.25 mM, pH 6.8), bromophenol blue (0.0025%, wt/vol), and DTT (200 mM)] and then heated at 100°C for 5 min before being separated by electrophoresis on SDS-10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane that was blocked overnight in a solution containing glycine (1 M), nonfat milk powder (5% wt/vol), and FBS (5%) at 4°C. The membrane was washed three times in rinsing solution (0.05% Tween 20 in PBS) before being incubated at room temperature with anti-human cyclin D1 monoclonal antibody (diluted 1:400 in rinsing solution). The membrane was again washed three times in rinsing solution before being incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (diluted 1:40,000 in wash solution). The membrane was then washed three times and developed with an enhanced chemiluminescence detection kit. Autoradiography was performed immediately, and the blots were quantified with computerized densitometry.

**RESULTS**

**Smooth Muscle Cell Identification**

Examination of the cultures with light microscopy revealed that the cells were elongated and spindle shaped, with central prominent nuclei, and cell confluence resulted in a “hill-and-valley” formation. These are all well-known characteristics of smooth muscle cells in culture (25). The identity of the smooth muscle cells was verified with immunohistochemistry. Greater than 98% of cells from each patient displayed positive immunohistochemical staining for smooth muscle-specific α-actin. Elongated, linear fibers within the cell were positively stained. This pattern of staining was observed for all patients.

**Mitogen-Induced Proliferation**

Increasing concentrations of FBS (1, 5, and 10%) resulted in corresponding increases in [3H]Tdr uptake (Table 1). Both 5 and 10% FBS significantly increased [3H]Tdr uptake above control condition (1% FBS) by 3.3- and 5.6-fold, respectively (P < 0.05; n = 3 patients). As shown in Table 1, PDGF (20 and 40 ng/ml) significantly increased [3H]Tdr uptake above the control condition (1% FBS) by 3.3-

<table>
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<tr>
<th>FBS, %</th>
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<tbody>
<tr>
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<td>243 ± 28</td>
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<tr>
<td>5</td>
<td>947 ± 112*</td>
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<tr>
<td>10</td>
<td>1,362 ± 224*</td>
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<th>PDGF, ng/ml</th>
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<td>413 ± 28</td>
</tr>
<tr>
<td>20</td>
<td>1,377 ± 111*</td>
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<tr>
<td>40</td>
<td>2,072 ± 126*</td>
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<td>2,587 ± 522*</td>
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<tr>
<td>10</td>
<td>3,646 ± 879*</td>
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</table>

Values are means ± SE; n, no. of patients; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor. *Significant increase above control response, P < 0.05.
and 7.0-fold, respectively ($P \leq 0.05; n = 4$ patients). All concentrations of bFGF (1, 3, 10, and 30 ng/ml) significantly increased $[^{3}H]$Tdr uptake above the control condition (1% FBS; $P \leq 0.05; n = 4$ patients), with an 8.1-fold increase occurring at a concentration of 10 ng/ml. Thrombin significantly increased $[^{3}H]$Tdr uptake above the control condition (1% FBS) at all concentrations tested (0.3, 1, and 10 U/ml) by 3.0-, 3.5-, and 4.9-fold, respectively ($P \leq 0.05; n = 3$ patients; Table 1).

**IL-4 Inhibition of Mitogen Response**

IL-4 (5, 15, 50, and 150 ng/ml) significantly inhibited the increase in $[^{3}H]$Tdr uptake induced by 10% FBS by 50, 73, 43, and 46%, respectively ($P \leq 0.05; n = 3$ patients). IL-4 (1.5 ng/ml) did not significantly decrease 10% FBS-stimulated $[^{3}H]$Tdr uptake (Fig. 1). A concentration of 50 ng/ml was chosen to investigate the response to PDGF, bFGF, and thrombin because the inhibition achieved at this concentration displayed the lowest coefficient of variation. IL-4 (50 ng/ml) significantly decreased the response to PDGF (20 and 40 ng/ml) by 19 and 21%, respectively ($P \leq 0.05; n = 4$ patients; Fig. 2) and decreased the response to bFGF by 37% ($P \leq 0.05; n = 3$ patients; Fig. 3). At two concentrations of thrombin (1 and 10 U/ml), IL-4 significantly decreased $[^{3}H]$Tdr uptake by 36 and 57%, respectively ($P \leq 0.05; n = 3$ patients; Fig. 4).

**Viable Cell Counts**

The cell viability assay (trypan blue exclusion) confirmed that IL-4 (50 ng/ml) was not producing a nonspecific cytotoxic effect. On day 2, an increase in cell number above the control condition (1% FBS) was observed in response to 10% FBS. The number of cells in IL-4-treated wells was 24% lower than in wells receiving 10% FBS alone ($P \leq 0.05; n = 3$ patients). However, in the presence and absence of treatment with IL-4, cell viability was not significantly different...
and was 96.0 ± 0.6 and 95.0 ± 0.5%, respectively (Table 2). Furthermore, on day 3, the number of cells in the IL-4-treated wells was 14% lower than in wells receiving 10% FBS alone; however, cell viability remained approximately the same (95.0 ± 1.4 and 93.0 ± 2.1%, respectively).

cAMP Radioimmunoassay

Addition of IL-4 (50 ng/ml) or heparin (100 U/ml) to human airway smooth muscle cells stimulated with either 1 or 10% FBS did not significantly increase the intracellular cAMP concentration above the control value (Table 3). PGE2 (1 mM) significantly increased cAMP levels above the control value in cells stimulated with 1% FBS by 7-fold (P < 0.05; n = 3 patients), and despite a 2.3-fold increase above the control value in 10% FBS-stimulated cells, this was not significant. Forskolin (10 µM) significantly increased the intracellular cAMP concentration in human airway smooth muscle cells stimulated with 1 or 10% FBS by 37- and 16-fold, respectively (P < 0.05; n = 3 patients).

Western Blot Analysis

A 1.8-fold increase in cyclin D1 protein expression above the control value was evident 5 h after treatment with 10% FBS (P < 0.05; n = 4 patients). IL-4 (50 ng/ml) significantly decreased the expression of cyclin D1 in cells stimulated with 10% FBS by 21% (P < 0.05; n = 4 patients). In the presence of 10% FBS, PGE2 (1 mM) and forskolin (10 µM) both decreased cyclin D1 expression by 25%; however, this was not significant (Fig. 5). A representative experiment is shown in Fig. 6.

Table 2. Effect of FBS and FBS + IL-4 on cell count and viability over 3 consecutive days

<table>
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<th>Treatment</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tr>
<td></td>
<td>Cell count, 10^4</td>
<td>Cell viability, %</td>
<td>Cell count, 10^4</td>
<td>Cell viability, %</td>
</tr>
<tr>
<td>1% FBS</td>
<td>3.7 ± 0.6</td>
<td>95 ± 1.8</td>
<td>3.7 ± 0.4</td>
<td>89 ± 2.3</td>
</tr>
<tr>
<td>10% FBS</td>
<td>3.7 ± 0.6</td>
<td>95 ± 1.8</td>
<td>5.4 ± 0.9</td>
<td>90 ± 3.1</td>
</tr>
<tr>
<td>10% FBS + 50 ng/ml IL-4</td>
<td>3.7 ± 0.6</td>
<td>95 ± 1.8</td>
<td>5.8 ± 1.6</td>
<td>93 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE from duplicate culture wells from 3 patients. Cells were grown for 7 days before treatment. IL-4, interleukin-4.

*Significant difference from 10% FBS-stimulated cells, P < 0.05.

Table 3. Effect of IL-4, PGE2, heparin, and forskolin on intracellular cAMP concentration in human airway smooth muscle cells stimulated with FBS

<table>
<thead>
<tr>
<th></th>
<th>cAMP, µg/ml</th>
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<tr>
<td></td>
<td>Control (0 ng/ml)</td>
</tr>
<tr>
<td>1% FBS</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>10% FBS</td>
<td>3.4 ± 2.2</td>
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Values are means ± SE from duplicate culture wells from 3 patients. Significant increase above control response of log-transformed data, P < 0.05.

DISCUSSION

This study has shown that IL-4 can decrease the proliferative response induced by the known human airway smooth muscle mitogens FBS, PDGF, and bFGF. The largest inhibitory effect occurred against 10% FBS in the presence of 15 ng/ml of IL-4. Other concentrations of IL-4 (5, 50, and 150 ng/ml) also significantly inhibited the proliferative response induced by 10% FBS. In the presence of 50 ng/ml of IL-4, a decrease in proliferation was also observed against PDGF (20 and 40 ng/ml), bFGF (30 ng/ml), and thrombin (1 and 10 U/ml). IL-4 (50 ng/ml) did not alter intracellular cAMP levels. However, IL-4 significantly inhibited cyclin D1 protein expression. These results indicate that IL-4-induced inhibition of proliferation is cAMP independent and mediated, in part, by a decrease in cyclin D1 protein expression.

Because IL-4 inhibited smooth muscle cell proliferation caused by FBS, PDGF, bFGF, and thrombin, the possibility arose that IL-4 was exerting a nonspecific cytotoxic effect. However, this proved not to be the case because cell counts combined with trypan blue exclusion showed that even 3 days after treatment, although IL-4-treated wells contained fewer cells, cell viability in
L474  IL-4 INHIBITS HUMAN AIRWAY SMOOTH MUSCLE PROLIFERATION

Fig. 5. Cyclin D1 protein expression as determined by densitometry. Cells were either left unstimulated (1% FBS) or stimulated with 10% FBS in presence of absence of growth inhibitors IL-4 (50 ng/ml), PGE2 (1 µM), and forskolin (10 µM) for 5 h. Values are means ± SE; n = 4 experiments, 3 separate patients. Significant difference (P < 0.05) from: *control (unstimulated cells); †10% FBS.

Both the IL-4-treated and nontreated groups was not significantly different. Thus it is unlikely that IL-4-induced inhibition of proliferation was due to a nonspecific cytotoxic effect.

We hypothesized that IL-4-induced inhibition of human airway smooth muscle was mediated via a rise in cAMP. Intracellular cAMP levels were measured after treatment with IL-4 (50 ng/ml) and the known inhibitors of human airway smooth muscle proliferation, heparin, PGE2 (13), and forskolin (35). IL-4 and heparin did not alter intracellular cAMP levels above the control level. However, PGE2 and forskolin significantly increased cAMP levels in growth-arrested cells. Forskolin also increased cAMP levels in proliferating cells. These results provide evidence that IL-4-induced inhibition of human airway smooth muscle proliferation is not cAMP dependent.

We investigated whether IL-4-induced inhibition of proliferation was associated with modulation of cyclin D1 expression. Proliferation was associated with a nearly twofold increase in cyclin D1 expression. IL-4 significantly inhibited cyclin D1 expression, whereas, although PGE2 (1 µM) and forskolin (10 µM) decreased cyclin D1 expression by 25 and 24.5%, respectively, this was not significant. These data indicate that IL-4-induced inhibition of proliferating human airway smooth muscle cells may be partially explained by a decrease in cyclin D1 protein expression.

IL-4 has been shown to decrease proliferation of other cell types, including human tumor cell lines (33), astrocytes (8), and vascular smooth muscle cells (37). In contrast, IL-4 induced the proliferation of endothelial cells (34) and fibroblasts (21). Concentrations used in these studies ranged from 0.1 to 200 ng/ml, a range similar to that used in the present study. It is evident, therefore, that the effects of IL-4 are particularly organ and cell dependent.

IL-4 is produced by Th-2 lymphocytes (23), human peripheral blood basophils (4), and human mast cells (1). The quantity of IL-4 released from human mast cells ranges between 6 and 13.2 ng/10⁶ lung mast cells. These in vitro concentrations are difficult to extrapolate to local concentrations that occur in vivo. However, with knowledge of the number of mast cells in close association with the smooth muscle, combined with periods of continual mast cell activation and degranulation, it can be hypothesized that significant concentrations of mast cell products (including IL-4) continually surround the airway smooth muscle. Furthermore, most of these products have the potential to either potentiate or inhibit human airway smooth muscle growth. The mast cell products histamine (20) and tryptase (3) are mitogens, whereas PGE2, heparin (13), and TNF-α (32), also mast cell products, inhibit proliferation of human airway smooth muscle cells. Histamine and tryptase (growth promoters) and heparin, IL-4, and TNF-α (growth inhibitors) are stored in mast cell granules and are released after IgE-mediated activation (1, 2, 38). Therefore, it seems unlikely that preferential release of growth promoters, rather than growth inhibitors, is the key to the increase in human airway smooth muscle proliferation that occurs in asthma. It is more likely that the balance between synthesis of growth promoters and growth inhibitors is altered by some as yet unknown mechanism.

IL-4 is just one of the cytokines that has been shown to either promote or inhibit airway smooth muscle proliferation in vitro. IL-1, IL-6 (5), and epidermal growth factor (31) potentiate proliferation of animal airway smooth muscle, and TNF-α (0.3–30 pM) has a small stimulatory effect on human airway smooth muscle proliferation. In addition, TNF-α (300 pM), like IL-4, can inhibit mitogen-induced proliferation of human airway smooth muscle cells (32).

The possible mechanism of IL-4-induced inhibition of proliferation was also addressed in this study. Various studies provide evidence that cAMP is a crucial step in the signal transduction pathway that results in inhibition of smooth muscle proliferation. Increased intracellular concentrations of cAMP elicited by PGE1 (22) or adenosine (14) result in a decrease in rat arterial smooth muscle proliferation. Tomlinson et al. (35) reported that salbutamol (a β2-adrenoceptor agonist) inhibits the proliferation of human airway smooth muscle, partly due to the elevation of intracellular...
cAMP levels. These findings show that, in vascular as well as in airway smooth muscle cells, activation of receptors that lead to increased cytosolic cAMP levels results in a corresponding decrease in mitogen-induced cell proliferation. There is evidence, however, for a dissociation between raised cAMP levels and inhibition of proliferation in airway smooth muscle cells. β-Hexosaminidase, which transiently increases cytosolic cAMP levels, induces proliferation in bovine airway smooth muscle (18); however, sustained increases in cAMP levels inhibited proliferation. In the present study, although the inhibitors of smooth muscle proliferation, forskolin and PGE2, increased cAMP concentrations, heparin and IL-4 did not. This provides evidence that inhibition of human airway smooth muscle proliferation can occur via a mechanism independent of cAMP production and that this is likely for IL-4-mediated inhibition.

Smooth muscle mitogens fall into two broad categories: 1) those with intrinsic, ligand-sensitive protein tyrosine kinase activity and 2) those with receptors that utilize GTP-binding proteins to initiate an intracellular signal cascade (reviewed in Ref. 24). It has been shown that FBS, PDGF, and bFGF induce proliferation by binding to receptors with tyrosine kinase activity (9, 12), and thrombin utilizes receptors that are coupled to GTP. The exact signal transduction pathway involved in initiating proliferation of human airway smooth muscle by these mitogens has not been fully established. Results from this study show that IL-4 not only inhibits the proliferative response of mitogens that bind to receptors with intrinsic tyrosine kinase activity but also inhibits mitogens that bind to receptors coupled to GTP such as thrombin. This would suggest that IL-4 is exerting its effect in the signal transduction pathway at a point distal to where the two pathways converge. For this reason, it seemed possible that the inhibitory effect of IL-4 may be evident at the level of the cell cycle regulatory proteins. Growing interest in the role that cell cycle machinery plays in cell proliferation has developed, and many cell cycle regulatory proteins have been elucidated. Cyclin D1 is an important protein in G1 progression (28) because the cell-regulatory retinoblastoma (RB) protein is phosphorylated by a complex formed by cyclin D1 and cyclin-dependent kinase (cdk). The unphosphorylated form of the RB protein binds to specific transcription factors, and this inhibits activation of genes that encode proteins that are essential for cell cycle regulation (reviewed in Ref. 40). Consequently, the cell cannot traverse the cell cycle, and cell division will not occur. When RB protein is phosphorylated by the cyclin D1-cdk complex, binding of the RB protein to transcription factors is inhibited, and this, in turn, allows the transcription of cell cycle regulatory proteins to take place. Cyclin D1 protein or mRNA is synthesized in response to a number of mitogens including PDGF (10), insulin-like growth factor (16), and 20% FBS (29) in vascular smooth muscle cells and by PDGF in bovine tracheal myocytes (41). Recently, in vascular smooth muscle cells stimulated with 10% FBS, IL-4 was shown to decrease the expression of both cyclin D1 and cdk2 (36). In this study, we further showed that IL-4 can inhibit human airway smooth muscle proliferation and that this may be partly explained by an inhibition of cyclin D1 synthesis. It is possible that IL-4 may also decrease the synthesis of other cell cycle progression factors such as cdk2 and that this may also account for part of the IL-4 inhibitory effect.

Although the receptor for IL-4 has been reported on cells of a hematopoietic lineage and some structural cells such as human fibroblasts (27), it is not known whether IL-4 receptors exist on human airway smooth muscle cells. It is interesting to note that, in human fibroblasts, IL-4 produced proliferation (21), whereas it caused inhibition in a structurally related cell (i.e., the smooth muscle cell) as seen in the present study. Whether the effect of IL-4 on human airway smooth muscle is initiated through an IL-4 receptor with signal transduction pathways identical to those of the IL-4 receptor on fibroblasts, through which IL-4 partly elicits a proliferative response, is not known.

This is one of the few reports that describes a functional effect of IL-4 on nonhematopoietic cells. Our study has shown that the regulation of airway smooth muscle cell growth in vitro is controlled by growth-promoting (e.g., PDGF, bFGF, and thrombin) and growth-inhibitory (e.g., IL-4) substances. It is therefore conceivable that these factors that are produced by smooth muscle cells, endothelial cells, fibroblasts, macrophages, T lymphocytes, and mast cells are likely to be important modulators of smooth muscle growth in vivo. Alterations in the expression of one or a combination of these mediators may provide the initiating signal for smooth muscle cycling, resulting in hyperplasia of the smooth muscle. Either an excess of growth factors or a deficit of growth-inhibitory substances could contribute to airway smooth muscle cell proliferation. Further identification of the endogenous mediators and the signal transduction pathways involved in the regulation of human airway smooth muscle growth may be important to determine the potential for reversal of structural changes associated with asthmatic airways.

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