IL-5 Increases Expression of 5-Lipoxygenase-Activating Protein and Translocates 5-Lipoxygenase to the Nucleus in Human Blood Eosinophils

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IL-5 Increases Expression of 5-Lipoxygenase-Activating Protein and Translocates 5-Lipoxygenase to the Nucleus in Human Blood Eosinophils

Andrew S. Cowburn, Stephen T. Holgate, and Anthony P. Sampson

Cysteinyl-leukotrienes are potent bronchoconstrictor mediators synthesized by the 5-lipoxygenase (5-LO) pathway. Eosinophilopoietic cytokines such as IL-5 enhance cysteinyl-leukotriene synthesis in eosinophils in vitro, mimicking changes in eosinophils from asthmatic patients, but the mechanism is unknown. We hypothesized that IL-5 induces the expression of 5-LO and/or its activating protein FLAP in eosinophils, and that this might be modulated by anti-inflammatory corticosteroids. Compared with control cultures, IL-5 increased the proportion of normal blood eosinophils immunostaining for FLAP (65 ± 4 vs 34 ± 4%; \( p < 0.0001 \)), enhanced immunoblot levels of FLAP by 51 ± 14% (\( p = 0.03 \)), and quadrupled ionophore-stimulated leukotriene C\(_4\) synthesis from 5.7 to 20.8 ng/10\(^6\) cells (\( p < 0.02 \)). IL-5 effects persisted for 24 h and were abolished by cycloheximide and actinomycin D. The proportion of FLAP\(^+\) eosinophils was also increased by dexamethasone (\( p < 0.0001 \)). Neither IL-5 nor dexamethasone altered 5-LO expression, but IL-5 significantly increased 5-LO immunofluorescence localizing to eosinophil nuclei. Compared with normal subjects, allergic asthmatic patients had a greater proportion of circulating FLAP\(^+\) eosinophils (46 ± 6 vs 27 ± 3%; \( p < 0.03 \)) and a smaller IL-5-induced increase in FLAP immunoactivity (\( p < 0.05 \)). Thus, IL-5 increases FLAP expression and translocates 5-LO to the nucleus in normal blood eosinophils in vitro. This is associated with an enhanced capacity for cysteinyl-leukotriene synthesis and mimics in vivo increases in FLAP expression in eosinophils from allergic asthmatics. The Journal of Immunology, 1999, 163: 456–465.
inflammation in the asthmatic airway. A mechanism for bronchoconstriction resulting from eosinophil cys-LT synthesis in eosinophils in asthmatic patients and provide evidence that IL-5 may induce the expression of 5-LO or FLAP in human eosinophils are unknown. We hypothesized that dexamethasone may alter 5-LO or FLAP expression in normal blood eosinophils and/or modulate changes induced by IL-5.

We report that in normal blood eosinophils, IL-5 significantly increases FLAP expression and translocates 5-LO to the nucleus, which is accompanied by a 4-fold increase in ionophore-stimulated cys-LT synthesis. Changes in FLAP expression peak at 6 h, persist for 24 h, and are abolished by actinomycin D and cycloheximide, showing dependence on gene transcription and de novo protein synthesis. FLAP expression is also enhanced by dexamethasone alone or in combination with IL-5. In allergic asthmatic patients, a decrease in FLAP expression was observed compared to normal donors. These results suggest that FLAP expression is regulated by IL-5 and dexamethasone, and that these drugs may play a role in modulating FLAP expression in human eosinophils.

Materials and Methods

Materials

The following were purchased: actinomycin D, aminoethylcarbazole, BSA fraction V, bromophenol blue, calcium ionophore A23187, cycloheximide, dexamethasone, dinitrophenylated swine anti-rabbit IgG, and streptavidin-biotin-HRP conjugate (Life Technologies, Paisley, U.K.); human rIL-5 (R&D Systems, Abingdon, U.K.); Diff-Quik (Baxter International, Inc., Westwood, MA); acetone, HEPES, sodium azide, and Tween-20 (Merck, Luton, U.K.); PBS and HBSS (Gibco, Paisley, U.K.); actinomycin D, aminoethylcarbazole, and streptavidin-biotin-HRP conjugate (Life Technologies, Paisley, U.K.); acetone, HEPES, sodium azide; and Tween-20 (Becton Dickinson, New Jersey, U.S.).

Cytochalasin D was purchased from a commercial source. The viability of eosinophils was assessed by trypan blue exclusion.

Isolation of human eosinophils

All eosinophil donors were volunteers and gave informed consent to participate in the study, which was approved by the Southport and South West Hampshire joint research ethics committee. Thirteen normal eosinophil donors were recruited; they had no previous clinical history of asthma or allergy, negative skin-prick tests to common allergens, and eosinophil counts in peripheral blood that were <5% of the total leukocytes. None was taking any medication. Five mild to moderate allergic asthmatic donors (one man and four women; mean age, 27 ± 2 yr) were recruited; they had appropriate clinical history, two or more skin tests to common allergens, and a mean eosinophil count in peripheral blood of 5.8% (range, 2–14%). Mean forced expiratory volume in one second (FEV1) was 79.3% of the predicted (range, 70–85%), and the geometric mean provocation concentration of inhaled histamine required to reduce FEV1 by 20% was 3.5 mg/ml (range, 0.13–16). All asthmatic patients were using inhaled β2 agonists as required, and one patient was receiving low dose inhaled flutic- sone (250 µg twice daily), but none was receiving systemic corticosteroids, theophylline, cromones, or leukotriene modifiers.

Peripheral blood eosinophils were immunomagnetically purified as previously described (41). Briefly, 100 ml of blood was collected into tubes with lithium heparin and was diluted with an equal volume of PBS containing 2% heat-inactivated FCS. Diluted blood was carefully layered in 25-ml aliquots onto isotonic Percoll (15 ml; density, 1.082 g/ml) and was centrifuged at 400 × g for 30 min at room temperature. Following centrifugation, the upper layer containing mononuclear cells was discarded, and cells were resuspended in phosphate-buffered saline with 1% sodium azide. Eosinophils were purified by negative selection using a magnet (Innovatis, West Sussex, U.K.), with a purity of 98–99% as assessed by Kimura and Diff-Quik staining and by immunostaining for eosinophil cationic protein detected with the EG2 mAb (Pharmacia Biosystems, Milton Keynes, U.K.) as described above. The remaining cells were neutrophils.

Eosinophil culture

Purified eosinophils were suspended at 0.75 × 106 cells/ml in RPMI 1640 culture medium containing 1% serum-free supplement (insulin-transferrin-sodium selenite, penicillin (100 IU/ml), and streptomycin (100 µg/ml) and were stimulated in costar ultra low attachment 24-well plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere with 5% CO2. Cells were stimulated with or without rIL-5 (10 ng/ml) for 0–24 h. In some experiments cells were pretreated with dexamethasone (1 µM), cycloheximide (10 µM), or actinomycin D (2 µg/ml) for 2 h before further culture with or without rIL-5 (10 ng/ml) or vehicle. Eosinophils were aspirated at various time points for immunocytochemistry, SDS-PAGE/Western blotting, or cys-LT enzyme immunoassay. The viability of eosinophils remained >95% for 24 h as measured by trypan blue exclusion.

Enzyme immunoassay of ionophore-stimulated cys-LT synthesis

Cultured eosinophils were centrifuged at 180 × g for 5 min and resuspended in 1 ml of RPMI 1640 culture medium containing serum-free supplement and antibiotics as described above. Cells were stained with trypan blue to assess cell viability and were counted to determine the recovery of cells from the plate. Eosinophils (105 or 106 cells) were stimulated for 0 and 15 min with calcium ionophore A23187 (1 µM) at 37°C in a shaking water bath, and the reaction was terminated by the addition of ice-cold methanol. Methanolic suspensions were incubated at 4°C overnight to extract intracellular LTs, and supernatants were removed and stored at −20°C. Supernatants were evaporated in vacuo and resuspended in assay buffer before Biotrak LTC4/ LTC4 and LTC4/ LTC4 enzyme immunoassay in accordance with the manufacturer’s instructions (Amersham). The enzyme immunoassay was calibrated with standard LTC4 from 0.75–48 pg/well. The polyclonal antiserum cross-reacts with LTC4 (100%), LTD4 (100%), LTE4 (70%), and their 11-trans isomers, but negligibly with LTB4 (0.3%), PGE2, PGD2, PGE2, 6-keto-PGF1α, thromboxane B2, and glutathione (<0.006%). Cys-LT synthesis was expressed as ng/106 cells.

Immunocytochemistry for 5-LO and FLAP

Eosinophils (5 × 106) were cytocentrifuged (Shandon Southern Products, Runcorn, U.K.) on glass slides at 450 rpm for 5 min, air-dried for 24 h, and stored at −20°C for a maximum of 2 wk. Cytospins were fixed in anhydrous aceton for 15 min and allowed to dry for 10 min. Cells were treated with sodium azide (0.1%) and hydrogen peroxide (0.3%) for 30 min to irreversibly inhibit endogenous peroxidase, confirmed by the lack of color development when aminoethylcarbazole (AEC; 0.03%) chromagen was added to washed cytospins in the absence of Abs. Nonspecific background staining was minimized by blocking with BSA (1%, w/v) in DMEM. Cytospins were incubated with primary Ab to 5-LO or FLAP (1:750 dilution)
for 1 h before the addition of biotinylated swine anti-rabbit IgG (1/300) for 1 h. Immunostaining was visualized with streptavidin-biotin-HRP conjugate (1/200) using AEC (0.03%) in acetate buffer (pH 5.2) as chromagen. All washes were conducted in Tris-buffered saline (TBS; 50 mM) with 0.1 M HEPES at pH 7.6, and all Abs were diluted in HEPES-buffered TBS with 0.1% BSA. Control slides were routinely performed in the absence of primary Abs or in the presence of an irrelevant polyclonal Ab and always showed no color development.

Immunocytochemistry was quantified by a blinded observer using a light microscope with oil immersion at x1000 magnification. Positive cells were defined as AEC-stained intact nucleated cells, excluding areas of smearing damage due to cytocentrifugation, and were expressed as a percentage of a minimum of 100 cells in randomly chosen fields. The repeatability of blind counting calculated as previously described (42) was 10.4% for 5-LO (n = 13) and 13.1% for FLAP (n = 15).

**Immunofluorescent confocal microscopy**

Cytocentrifuge preparations were fixed for 20 min in 4% paraformaldehyde in TBS (50 mM; pH 7.6) and washed three times with HEPES-buffered TBS. Cells were permeabilized with methanol at 4°C for 7 min and washed again three times with TBS/HEPES. The slides were incubated with 1/750 dilutions of the 5-LO and FLAP primary Abs, washed, incubated for 60 min with biotinylated swine anti-rabbit IgG (1/300), and washed again. Ab binding was detected by a 20-min incubation with extravidin-FITC (1/200 dilution in HEPES-buffered TBS) before counterstaining with the nuclear stain propidium iodide. Immunofluorescence was assessed using a Leica laser scanning confocal microscope equipped with an argon/krypton 3-μm fiberoptic laser (488/568/647 nm) with a Leitz high resolution DMR BE optical microscope (Leitz, Rockleigh, NJ). The intensity of 5-LO and FLAP immunofluorescence localizing to the nucleus was determined by the amount of cross-over between the extravidin-FITC channel (emission wavelength, 520 nm) and the propidium iodide channel (600 nm), and displayed as arbitrary gray scale units from 0 (lowest) to 256 (highest), using the SCANware software package (Leica, Milton Keynes, U.K.). For statistical analysis, the spectrum was divided into four intensity bands: 1 (0–49 U), 2 (50–99 U), 3 (100–149 U), and 4 (150–256 U), and the statistical analysis were compared by Mann-Whitney t test). The effect of IL-5 on cys-LT synthesis was abolished by preincubation with the transcription inhibitor actinomycin D (Act D; 2 μg/ml; p < 0.01, by unpaired t test).

**Results**

**Effect of IL-5 on A23187-stimulated cys-LT synthesis from normal eosinophils**

The capacity of intact eosinophils to release cys-LTs when stimulated for 15 min with calcium ionophore A23187 (1 μM) was examined by enzyme immunoassay in freshly isolated cells and in cells cultured in the presence and the absence of IL-5 (10 ng/ml) for 6 h. Freshly isolated eosinophils generated median cys-LT levels of 4.0 ng/10⁶ cells (range, 2.1–10.2; n = 10), and this did not change significantly after 6 h culture without IL-5 (5.7 ng/10⁶ cells; range, 2.0–27.4; n = 10; p > 0.10). Culture with IL-5 for 6 h increased median A23187-stimulated cys-LT synthesis 3.7-fold to 20.8 ng/10⁶ cells (range, 1.8–68.0; n = 10), significantly greater than the vehicle control value (p < 0.02, by Wilcoxon’s test; Fig. 1). The IL-5-induced increase in cys-LT synthesis was abolished by actinomycin D (median, 1.58 ng/10⁶ cells; range, 1.3–4.0; n = 4; p < 0.01, vs IL-5 alone, by Mann-Whitney test; Fig. 1).

**Effect of IL-5 on the proportion of normal eosinophils immunostaining for FLAP and 5-LO**

Immunomagnetically purified normal eosinophils were cultured for up to 24 h in the presence or the absence of rhIL-5 (10 ng/ml). Representative photomicrographs of eosinophils obtained from

**FIGURE 1.** Effect of IL-5 on ionophore-stimulated cys-LT synthesis in normal eosinophils. Total cys-LTs (LTC₄, LTD₄, and LTE₄) were assessed by Biotrak enzyme immunoassay of supernatants of normal human eosinophils 15 min after the addition of the calcium ionophore A23187 (1 μM). Cys-LTs synthesis is expressed as nanograms per million eosinophils, and horizontal bars indicate median values. Culture for 6 h with IL-5 (10 ng/ml; n = 10) significantly increased A23187-stimulated cys-LT synthesis compared with that after culture with the vehicle control (p < 0.02, by paired t test). The effect of IL-5 on cys-LT synthesis was abolished by preincubation with the transcription inhibitor actinomycin D (Act D; 2 μg/ml; p < 0.01, by unpaired t test).
FIGURE 2. Effect of IL-5 on FLAP and 5-LO immunoreactivity in normal eosinophils. Representative photomicrographs (×400) showing immuno-cytochemical and immunofluorescent staining for 5-LO and FLAP in cytocentrifuge preparations of immunomagnetically purified blood eosinophils from one normal donor. Immunocytochemical staining is shown for FLAP after 6-h culture (A; vehicle control), for FLAP after 6-h culture with IL-5 (B; 10 ng/ml), for 5-LO vehicle control (C), for 5-LO after 6-h culture with IL-5 (D; 10 ng/ml), and for control (E; irrelevant primary Ab). AEC immunostaining shows red against blue Mayer’s hematoxylin counterstain. Laser scanning confocal microscopy was used to quantify 5-LO immunoreactivity in eosinophil nuclei by displaying the intensity of cross-over between extravidin-FITC-labeled 5-LO immunofluorescence (520 nm) and fluorescence associated with the nuclear dye propidium iodide (600 nm). After culture for 6 h, normal eosinophil nuclei predominantly have low levels of nuclear 5-LO immunofluorescence (F) compared with significantly higher levels after culture with IL-5 (10 ng/ml; G), as indicated on the 256-color scale.
one normal donor and immunostained for FLAP and 5-LO following 6-h incubation with and without IL-5 are shown in Fig. 2, A–E. When freshly isolated, the mean (±SEM) proportion of eosinophils immunopositive for FLAP was 26.5 ± 2.7% (n = 13), and in the absence of IL-5 this did not change significantly at any time point up to 24 h (36.5 ± 6.5%; n = 13; p > 0.15, by paired t test; Fig. 3). When cultured with IL-5 (10 ng/ml), the proportion of eosinophils immunostaining for FLAP rose significantly at 2 h (p = 0.018 vs vehicle control, by paired t test) and at 6 h (p = 0.027), and reached a plateau of 65.3 ± 4.1% (n = 13) at 6 h, significantly higher than the control value (33.5 ± 4.4%; n = 13; p < 0.0001, by paired t test). The proportion of FLAP⁺ eosinophils remained significantly higher than that in control cells at 24 h (58.0 ± 4.0 vs 36.5 ± 6.5%; n = 13; p = 0.005; Fig. 3).

The protein synthesis inhibitor cycloheximide (CHX; 10 μM) added 2 h before IL-5 abolished the increase in FLAP⁺ eosinophils at 6 h (28.0 ± 2.5%; n = 5; p = 0.002 vs IL-5 alone) and at 24 h (23.2 ± 3.4%; n = 5; p = 0.002; Fig. 3). CHX did not reduce the proportion of FLAP⁺ cells significantly below that in control cells cultured without IL-5. Addition of the transcription inhibitor actinomycin D (Act D; 2 μg/ml) 2 h before IL-5 abolished the increase in FLAP⁺ eosinophils at 4 h (21.4 ± 6.0%; n = 5; p = 0.01 vs IL-5 alone) and at 6 h (26.0 ± 7.3%; p = 0.003).

In contrast to the low proportion of FLAP⁺ cells at baseline, the mean (±SEM) proportion of freshly isolated eosinophils immunostaining for 5-LO was 87.8 ± 2.0% (n = 13). In the absence of IL-5, the proportion of 5-LO⁺ cells remained stable up to 6 h (83.9 ± 2.0%; n = 13; p > 0.05), falling slightly, but significantly, at 24 h (75.9 ± 2.7; n = 13; p < 0.001 vs freshly isolated cells; Fig. 3). Culture of eosinophils with IL-5 (10 ng/ml) did not alter the proportion of 5-LO⁺ eosinophils at time points up to 6 h (82.2 ± 2.0%; n = 13; p > 0.15) or at 24 h (78.8 ± 2.7%; n = 13; p = 0.4) compared with that in vehicle controls (Fig. 3). The presence of IL-5 did not prevent the small but significant fall in the proportion of 5-LO⁺ eosinophils at 24 h.

Cultures of eosinophils for 2 h with CHX (10 μM) before the addition of IL-5 (10 ng/ml) produced small but significant decreases in the proportion of 5-LO⁺ cells at 6 h (72.2 ± 2.5%; n = 5; p = 0.012, by unpaired t test) and at 24 h (67.4 ± 1.9%; n = 5; p = 0.004) compared with IL-5 alone (n = 13), and also compared with the vehicle control (p = 0.005 and p = 0.02, respectively; Fig. 3). Preincubation of eosinophils with actinomycin D (2 μg/ml) for 2 h before IL-5 treatment reduced the proportion of 5-LO⁺ eosinophils at 6 h (43.6 ± 11.5%; n = 5) compared with IL-5 alone (p < 0.03, by unpaired t test) and compared with vehicle control (p < 0.03).

Effect of IL-5 on 5-LO and FLAP protein levels in normal eosinophils

SDS-PAGE/Western blots for FLAP and 5-LO were performed on equal amounts of total cellular protein obtained from freshly isolated cells and after culture in the presence and absence of IL-5 (10 ng/ml) for 6 h, the latter corresponding to the time point at which the greatest changes in FLAP were observed immunocytochemically. The expression of FLAP in freshly isolated cells (120,400 ± 23,100 density units; n = 5) was unchanged after 6 h of culture without IL-5 (121,600 ± 18,100; p > 0.8), but was significantly increased by 6 h of culture with IL-5 (178,300 ± 36,300; n = 5; p = 0.031, by paired t test), a mean increase of 59.9 ± 13.6% (Fig. 4). In contrast, densitometric analysis confirmed that the total levels of 5-LO protein in freshly isolated cells were not altered by culture for 6 h in either the presence or the absence of IL-5.

Effect of IL-5 on the subcellular localization of FLAP and 5-LO in normal eosinophils

The amount of FLAP and 5-LO localizing to eosinophil nuclei was assessed by the intensity of extravidin-FITC-labeled immunofluorescence (520 nm) which colocalized to fluorescence associated with the nuclear dye propidium iodide (600 nm). As expected, immunofluorescent staining for FLAP localized overwhelmingly to eosinophil nuclei at baseline and was not altered by IL-5 treatment. In contrast, treatment with IL-5 for 6 h significantly increased immunofluorescence for 5-LO in the nucleus (Fig. 2, F and G). In freshly isolated eosinophils from four normal donors, peak 5-LO immunofluorescence in the nucleus was of low intensity (bands 1 and 2) in 96.5 ± 0.9% of cells, and this remained stable during culture for 6 h (89.8 ± 4.4%). However, after culture for 6 h with IL-5 (10 ng/ml), the proportion of eosinophil nuclei with
Effect of IL-5 on colocalization of 5-LO immunofluorescence to eosinophil nuclei. Normal eosinophils were cultured in the presence of FLAP and 5-LO (10 ng/ml) for 6 h, and extravidin-FITC-labeled 5-LO immunofluorescence (520 nm) was colocalized to fluorescence associated with the nuclear dye propidium iodide (600 nm) using a Leica laser scanning confocal microscope. The intensity of colocalization was assessed with the SCANware software package and arbitrarily divided into bands 1 (lowest intensity) to 4 (highest intensity). IL-5 treatment significantly increased the proportion of eosinophils with nuclear 5-LO immunofluorescence in the higher intensity bands compared with that in the vehicle control group.

FIGURE 5. Effect of IL-5 on colocalization of 5-LO immunofluorescence to eosinophil nuclei. Normal eosinophils were cultured in the presence and the absence of FLAP of IL-5 (10 ng/ml) for 6 h, and extravidin-FITC-labeled 5-LO immunofluorescence (520 nm) was colocalized to fluorescence associated with the nuclear dye propidium iodide (600 nm) using a Leica laser scanning confocal microscope. The intensity of colocalization was assessed with the SCANware software package and arbitrarily divided into bands 1 (lowest intensity) to 4 (highest intensity). IL-5 treatment significantly increased the proportion of eosinophils with nuclear 5-LO immunofluorescence in the higher intensity bands compared with that in the vehicle control group.

5-LO immunofluorescence in intensity band 1 fell significantly from 50.9 to 21.8% (p = 0.025, by paired t test), whereas corresponding increases were seen in the proportion of nuclei in the higher intensity bands 2 (from 38.9 to 56.8%; p = 0.02), 3 (from 10.1 to 23.0%; p = 0.18), and 4 (from 0 to 10.7%; p = 0.028; Fig. 5).

Effect of dexamethasone on FLAP and 5-LO immunostaining and on cys-LT synthesis in normal eosinophils

Culture with dexamethasone (1 μM) alone significantly increased the proportion of eosinophils immunostaining for FLAP from 27.8 ± 4.1 (vehicle control) to 45.3 ± 4.8% after 2 h (n = 8; p < 0.002, by paired t test), and from 35.3 ± 4.0 to 67.8 ± 4.2% after 6 h (n = 8; p < 0.0001; Fig. 6). Dexamethasone had no effect on the proportion of eosinophils expressing 5-LO at any time point up to 6 h (71.5 ± 3.9%) compared with control (77.3 ± 4.4%; n = 6; p > 0.4; Fig. 6).

Eosinophils from five normal donors were also pretreated with dexamethasone (1 μM) for 2 h before the addition of IL-5 (10 ng/ml) for a further 22 h. The combination of dexamethasone and IL-5 increased the proportion of FLAP+ cells to 81.4 ± 3.4% (n = 5) after 4 h, significantly greater than that with IL-5 alone (69.6 ± 3.8%; n = 5; p = 0.04). The effects of IL-5 and dexamethasone in combination and of IL-5 alone were both significantly greater than those of the vehicle control (44.4 ± 3.0%; n = 5; p < 0.005). In contrast, the proportion of eosinophils immunostaining for 5-LO was not affected by the combination of dexamethasone and IL-5 (88.0 ± 3.1%; n = 5) compared with IL-5 alone (88.2 ± 2.8%; n = 5; p > 0.9) or compared with vehicle control (86.0 ± 1.5%; n = 5; p > 0.3).

In intact eosinophils from five normal donors, culture for 6 h with dexamethasone alone (1 μM) did not increase A23187-stimulated synthesis of cys-LTs (median, 12.6 ng/10⁶ cells; range, 4.1–38.2) when compared with that in freshly isolated cells (7.2 ng/10⁶ cells; range, 2.0–20.9; n = 5; p = 0.2, by Wilcoxon’s test) or compared with control cells cultured for 6 h (10.7 ng/10⁶ cells; range, 5.6–27.4; n = 10; p > 0.3). In contrast, in eosinophils from...
Whitney cys-LT synthesis was also apparent when compared by Mann–

Effect of IL-5 on FLAP and 5-LO immunostaining in eosinophils

In allergic asthmatic patients, the proportion of freshly isolated eosinophils immunostaining for FLAP was significantly higher in asthmatic patients (92.6 ± 1.3%; n = 5) and the normal subjects (87.8 ± 2.0%; n = 13) when freshly isolated (p > 0.05, by unpaired t test; Fig. 7) or after culture for 6 h (p > 0.3). The proportion of 5-LO+ eosinophils in asthmatic patients was not significantly altered by culture for 6 h with IL-5 (87.8 ± 2.9%) compared with that after culture with the vehicle control (88.2 ± 3.4%; n = 5; p > 0.8; Fig. 8). The proportion of 5-LO+ eosinophils was also not significantly altered by treatment with dexamethasone (1 μM; 87.3 ± 3.2%; n = 3) or cycloheximide (10 μM; 80.7 ± 7.0%; n = 3).

Discussion

We have demonstrated for the first time that treatment with the eosinophilopoietic cytokine IL-5 induces the expression of FLAP and translocates 5-LO to the nucleus in normal human blood eosinophils, associated with an enhanced capacity for cys-LT synthesis. In eosinophils from allergic asthmatic patients, elevated basal expression of FLAP and a diminished response to IL-5 suggest in vivo exposure to endogenous IL-5.

Dose- and time-dependent priming of cys-LT synthesis by IL-5 has been reported in human blood eosinophils purified by Percoll discontinuous density gradient centrifugation (17). We used negative immunomagnetic selection directed against FcγRIII (CD16), which is expressed on neutrophils but not on hypodense or nonmodense eosinophils (43), to provide highly purified populations of viable human blood eosinophils. Our data confirm that treatment of normal eosinophils with recombinant human IL-5 increases ionophore-stimulated cys-LT synthesis by 3.7-fold compared with that in cells cultured for 6 h without IL-5 (Fig. 1) and by more than 5-fold compared with that in freshly isolated cells. Priming of ionophore-stimulated cys-LT synthesis occurred at a concentration of IL-5 (10 ng/ml), similar to that shown to prime eosinophils for enhanced cys-LT synthesis in response to receptor-dependent stimuli (17), comparable to those detected in the serum of moderate to severe symptomatic asthmatics (34), and identical with the dissociation constant (Kₐ) of IL-5 for its high affinity receptor on human eosinophils (36). The effect of IL-5 on cys-LT synthesis was abolished by actinomycin D (Fig. 1), showing that at 6 h, priming of 5-LO pathway activity is dependent on gene transcription.

By immunocytochemical analysis, treatment with IL-5 was shown to significantly increase the proportion of normal eosinophils that immunostained for FLAP at 2, 4, and 6 h with autologous cells cultured without IL-5 (Figs. 2 and 3). In the absence of IL-5, the proportion of eosinophils immunostaining for FLAP remained stable in serum-free culture, and cycloheximide

![Figure 7](http://www.jimmunol.org/)

![Figure 8](http://www.jimmunol.org/)
treatment for 6 and 24 h did not reduce the proportion of FLAP⁺ cells below control levels (Fig. 3), indicating that the rate of degradation of FLAP in human eosinophils is low (t₁/₂, <24 h). That the increased proportion of FLAP⁺ eosinophils reflects a true increase in total FLAP protein per cell was confirmed by densitometric analysis of FLAP immunoblots at 6 h (Fig. 4) and by the complete blockade of IL-5-induced increases in FLAP immunostaining at 6 and 24 h in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3). FLAP is essential for LT synthesis in intact leukocytes (6), and its level of expression appears to be the rate-limiting factor in the synthesis of LT from endogenous arachidonate in activated alveolar macrophages (25). The evidence that IL-5 increases both FLAP expression and ionophore-stimulated cys-LT synthesis in eosinophils at 6 h and that both responses are abolished by actinomycin D suggests that transcriptional up-regulation of FLAP is an important factor contributing to increased 5-LO pathway activity in these cells. Additional contributions from other factors cannot be excluded, including increased PLA₂ activity and arachidonate availability. The activity of the terminal enzyme in the cys-LT pathway, LTC₄ synthase, is not altered by IL-5 or GM-CSF in an eosinophilic substrain of HL-60 cells (44).

Our data extend to human blood eosinophils the finding that FLAP expression is cytokine inducible in human neutrophils and monocytes and in monocytic cell lines (20, 23, 24, 45–47). However, the principal or exclusive 5-LO pathway product of neutrophils and monocytes is LTBr, which is not implicated in asthma. Eosinophils, in contrast, may be the predominant source of the bronchoconstrictor cys-LT in the persistent asthmatic lung (3, 4). Cys-LT, in turn, are potent and specific chemotaxitants for human eosinophils in vitro and in vivo (1). Up-regulation of FLAP expression in eosinophils by T cell- or mast cell-derived IL-5 may therefore represent a critical pathway underlying bronchoconstriction and chronic eosinophilic inflammation in the asthmatic airway.

In contrast to FLAP, 5-LO immunopositivity was observed in ~90% of normal eosinophils when freshly isolated, and this proportion did not change during culture for up to 24 h in either the presence or the absence of IL-5 (Fig. 3). The lack of effect of IL-5 on 5-LO was confirmed by densitometric measurements of 5-LO immunoblots at 6 h (Fig. 4). The small fall in 5-LO immunopositivity at 24 h in the presence of cycloheximide compared with that in the presence of IL-5 or vehicle alone (Fig. 3) confirms that degradation of 5-LO protein is measurable but slow (t₁/₂ >24 h), and that it is not altered by IL-5. These data are consistent with a previous t₁/₂ estimate for 5-LO of 26 h in mononuclear HL-60 cells (48). However, the lack of change in 5-LO expression in IL-5-treated eosinophils contrasts with the significant increases in 5-LO mRNA and protein observed in GM-CSF-treated neutrophils (21, 22), in monocytes exposed to GM-CSF and IL-3 (23), and in monocyte cell lines (24, 46). The difference in response may relate to the greater affinity of GM-CSF for its receptor compared with IL-5 (36) or to the different cell type examined in this study. Alternatively, changes in 5-LO expression may only become apparent after several days of treatment with IL-5, or accessory factors may be required (37, 46).

Although total levels of 5-LO protein were not changed by IL-5 treatment up to 24 h, laser scanning confocal microscopy showed that IL-5 significantly increased 5-LO immunofluorescence associated with eosinophil nuclei (Fig. 5). In resting human neutrophils and monocytes, 5-LO is largely cytosolic in distribution, but in cells that have undergone tissue recruitment and differentiation, a significant proportion of cellular 5-LO localizes to the nuclear euchromatin (26, 27). Redistribution of 5-LO to the nucleus also occurs in blood neutrophils and eosinophils during in vitro adherence (27, 49). In our study, increases in nuclear 5-LO immunofluorescence were not caused by adherence to the ultralow attachment microplates, as there was no significant difference in immunofluorescence between freshly isolated eosinophils and those cultured for 6 h in the absence of IL-5. Our data are the first to show that treatment of blood eosinophils with an eosinophilopoietic cytokine causes redistribution of 5-LO to the nucleus, and that this is accompanied by an increased capacity for cys-LT synthesis. 5-LO translocation is associated with enhanced LT synthesis in adherent and tissue-differentiated neutrophils and macrophages (26, 27), but with a reduction in cys-LT synthesis in eosinophils adhered to fibronectin for 2 h (49). The latter may reflect an early time point in eosinophil activation compared with that in our study or may indicate signal-specific transduction pathways for cytokines and adhesion molecules.

Binding of IL-5 to its receptor causes activation of cytosolic tyrosine kinases, including the Janus kinase JAK2, which activates the nuclear transcription factor Stat1α (50), and tyrosine kinase activity is essential for priming of eosinophil effector function by IL-5 (51). Although IL-5 priming is not Ca²⁺ dependent, low concentrations of calcium ionophore mimic IL-5 priming. In leukocytes activated by high concentrations of ionophore, a rapid translocation of 5-LO to the nuclear envelope occurs that also depends on tyrosine kinase activity and on a functional microtubule system and is associated with accumulation of phosphorylated 5-LO (52–54). We were not able to confirm the role of tyrosine kinases in IL-5-induced 5-LO translocation due to a deleterious effect of the tyrosine kinase inhibitor herbimycin A on eosinophil viability in overnight culture. However, taken together with these studies, our data suggest that IL-5 may initiate a slow tyrosine kinase-dependent redistribution of 5-LO to the eosinophil nucleus, and that this is associated with enhanced cys-LT synthesis in response to a subsequent stimulus.

The genes for 5-LO and FLAP have been cloned and sequenced (55, 56). The 5-LO gene 5′-flanking region has the general characteristics of a constitutive gene (55), but reporter gene analysis showed regions exerting positive and negative transcriptional control, including GC-rich regions that bind the transcription factor Sp1, a recognition site for the phorbol ester-inducible transcription factor AP-2, and a recognition sequence for NF-κB (57), a transcription factor activated by a number of proinflammatory cytokines. In contrast, promoter analysis of FLAP revealed features characteristic of a highly inducible gene, including a TATA box and response elements for AP-2 and for glucocorticoids (56). Our results in eosinophils are consistent with studies in neutrophils, monocytes, and alveolar macrophages showing a greater inducibility of FLAP compared with 5-LO (20, 21, 25).

The effects of glucocorticoids on the 5-LO pathway are complex. In vitro, glucocorticoids may suppress eicosanoid synthesis in alveolar macrophages by inducing the expression of lipocortin-1, an inhibitor of cPLA₂ (58), but their effects in blood leukocytes are highly dependent on cell type and activation status, and there is little information in human eosinophils (59). Our data show a pronounced, paradoxical effect of in vitro treatment with dexamethasone (1 μM) in increasing FLAP (but not 5-LO) immunopositivity in normal blood eosinophils, both on its own (Fig. 6) and in combination with IL-5. The FLAP gene promoter (but not the 5-LO gene promoter) contains a glucocorticoid response element (56). Our data are consistent with the ability of glucocorticoids, at similar concentrations and over a similar time period, to increase FLAP mRNA and protein in human neutrophils and to augment the increases induced by GM-CSF (20).
In human blood monocytes and monocyte THP-1 cells, dexamethasone also increases ionophore-stimulated 5-LO pathway activity (39), and this is attributable to increased expression of FLAP and 5-LO, not to an effect on arachidonate availability. In our study, despite increasing FLAP expression, dexamethasone did not significantly change ionophore-stimulated cys-LOT synthesis in eosinophils from five normal subjects. The effects of dexamethasone on FLAP expression may be counterbalanced by inhibition of the activity and/or expression of proximal and distal enzymes in the 5-LO pathway, particularly cPLA2 (40, 58, 60). We cannot exclude the possibility that dexamethasone may fail to translocate 5-LO to the nucleus, and that 5-LO translocation may be required for priming of cys-LT synthesis. Overall, the lack of effect of dexamethasone on cys-LT synthesis on eosinophils in our experiments is consistent with the failure of systemic glucocorticoid therapy to inhibit in vivo leukotriene synthesis in normal and asthmatic patients (61, 62).

Blood eosinophils from asthmatic patients have a 5- to 10-fold enhanced capacity for cys-LT synthesis compared with normal cells (12). Our data show that a significantly greater proportion of freshly purified blood eosinophils from allergic asthmatic patients immunostain for FLAP (46%) compared with those from normal patients (27%; Fig. 7). Furthermore, IL-5-induced increases in FLAP immunostaining were significantly smaller in eosinophils from asthmatic patients (Fig. 8) than in normal eosinophils (Fig. 3). Although we did not examine cys-LT synthesis or 5-LO nuclear immunofluorescence in asthmatic eosinophils, our data suggest that in vivo priming of cys-LT synthesis in asthmatic patients (12) may involve the induction of FLAP expression following exposure to endogenous IL-5 or related cytokines. The rapid induction of FLAP in normal eosinophils by IL-5 (2–4 h) (Fig. 3) and the elevated levels of IL-5 and GM-CSF in the plasma of symptomatic moderate to severe asthmatics (34, 63) suggest that priming by IL-5 or related cytokines in vivo may occur within the systemic circulation. Alternatively or in addition, priming may occur locally within the pulmonary circulation or the bone marrow.

In summary, using immunocytochemistry, SDS-PAGE immuno blotting, immunofluorescent confocal microscopy, and ELISA, we have shown that an enhanced capacity for cys-LT synthesis in human blood eosinophils after 6-h culture with IL-5 is associated with significant induction of FLAP expression and increased localization of 5-LO to the nucleus. That IL-5 exerts its effects on cys-LT synthesis and FLAP expression via gene transcription and protein synthesis was confirmed with actinomycin D and cycloheximide. The changes in FLAP expression induced by IL-5 in vitro were rapid in onset and persisted up to 24 h, suggesting that exposure to excess IL-5 in vivo in the bone marrow, circulation, or lung may produce a marked increase in 5-LO pathway activity, leading to impaired lung function and further eosinophil migration.

In asthmatic patients, increased FLAP immunopositivity and a relatively refractory response to IL-5 in vitro suggest that these mechanisms operate in vivo to prime blood eosinophils for enhanced cys-LT release following physiological activation in the airway.

Acknowledgments

We thank Dr. J. Evans (Merck Frosst Canada Inc., Quebec, Canada) for the generous gift of Abs to 5-LO and FLAP.

References


