

Glucocorticoid-induced surface expression of annexin 1 blocks β_2 -integrin adhesion of human eosinophils to intercellular adhesion molecule 1 surrogate protein

Jie Liu, MD, PhD,^{a,b} Xiangdong Zhu, MD,^a Saori Myo, MD, PhD,^a Anissa T. Lambertino, BS,^a Chang Xu, BS,^a Evan Boetticher, MS,^a Nilda M. Muñoz, MS,^a Masaaki Sano, MD,^a Martha Cordoba, MD,^a Jonathan Learoyd, BS,^a Angelo Meliton, MD,^a Malcolm Johnson, PhD,^c and Alan R. Leff, MD^{a,b} Chicago, Ill, and Uxbridge, United Kingdom

Background: Glucocorticoids attenuate the population of eosinophils and T lymphocytes in asthmatic airways. The decrease in airway eosinophilia is caused both by accelerated cell death and by induction of blockade of integrin adhesion. In this study, we examined the hypothesis that annexin 1 surface expression, which is upregulated by the glucocorticoid receptor, prevents integrin adhesion essential to cell migration by blocking intracellular translocation of cytosolic group IV phospholipase A₂ (cPLA₂).

Objective: To examine the relationship of the glucocorticoid on annexin 1 expression and the effect of blockade of annexin 1 activity on adhesion of human eosinophils *in vitro*. To determine the relationship between annexin 1 surface expression and nuclear membrane translocation of cPLA₂.

Methods: Eosinophils isolated from human peripheral blood were pretreated with fluticasone propionate (FP), and β_2 -integrin adhesion was measured after stimulation with IL-5 or eotaxin. Effects of FP on cPLA₂ expression, phosphorylation, and translocation were determined. The role of annexin 1 was examined by using annexin 1 blocking antibody and/or mimetic peptides.

Results: Fluticasone propionate decreased stimulated eosinophil adhesion and caused 4-fold increase in annexin 1 expression on the plasma membrane. Inhibition of adhesion by FP was blocked with annexin 1 blocking antibody. Annexin 1 N-terminal mimetic peptide also blocked β_2 -integrin adhesion. Translocation of cPLA₂ to the nuclear membrane was

significantly blocked by incubation with FP. Blockade was reversed with annexin 1 blocking antibody.

Conclusion: Blockade of β_2 -integrin adhesion by glucocorticoid is regulated by annexin 1, which blocks cPLA₂ translocation to nuclear membrane. (J Allergy Clin Immunol 2005;115:493-500.)

Key words: Eosinophils, adhesion molecules, glucocorticoids, annexin 1, cPLA₂ translocation

Migration of eosinophils into specific tissue sites is a hallmark of allergic diseases such as asthma. Eosinophil recruitment may contribute to sustained airway inflammation by releasing tissue-damaging agents such as major basic protein, eosinophil peroxidase, lipid mediators, and chemotactic cytokines.¹

Glucocorticoids have been reported to reduce the numbers of circulating and tissue eosinophils in asthmatic airways. However, the precise mechanism by which glucocorticoids reduce tissue eosinophilia has not been established. Inhibition of cytokine-stimulated eosinophil survival is one of the important actions of glucocorticoid.²⁻⁴ *In vitro* studies examining the direct effect of glucocorticoid on adhesion molecule expression and functions have not yielded definitive data. Hartnell et al.⁵ found that dexamethasone suppressed IL-3-stimulated upregulation of CD11b expression in human eosinophils at 24 hours. Lim et al.⁶ have found that prolonged exposure (24-48 hours) of murine eosinophils to dexamethasone reduced CD11b surface expression and chemotaxis caused by eotaxin. By contrast, Kaiser et al.⁷ found that pretreatment of human eosinophils, cultured endothelial cells, or both with 10⁻⁷ mol/L budesonide did not inhibit either baseline adhesion or adhesion caused by the leukocyte activators fMLP (formyl-Met-Leu-Phe) or platelet-activating factor. We recently reported that cytosolic group IV phospholipase A₂ (cPLA₂) is the critical enzyme in the regulation of integrin-mediated eosinophil adhesion and that cPLA₂-mediated adhesion is regulated by its downstream production of platelet-activating factor.^{8,9} The role of cPLA₂ in airway infiltration of granulocytes also has been demonstrated *in vivo*.¹⁰⁻¹² However, the effect of glucocorticoids on cPLA₂ and cPLA₂-mediated eosinophil adhesion is currently unknown.

To explore the mechanism of anti-inflammatory effect of glucocorticoid, we examined the role of annexin 1, a

From ^athe Section of Pulmonary and Critical Care Medicine, Department of Medicine, and the Departments of Neurobiology, Pharmacology and Physiology, Pediatrics, Anesthesia, and Critical Care, and ^bthe Committees on Clinical Pharmacology and Pharmacogenomics, Cell Physiology and Molecular Medicine, Division of the Biological Sciences, University of Chicago; and ^cGlaxoSmithKline, Uxbridge.

Supported by National Heart, Lung, and Blood Institute grant HL-46368; National Heart, Lung, and Blood Institute HL-56399 (Dr Leff) and AI52109 (Dr Zhu); the University of Chicago GlaxoSmithKline Center of Excellence in Asthma; and an individual grant from GlaxoSmithKline, Research Triangle Park, NC.

Received for publication July 22, 2004; revised October 21, 2004; accepted for publication November 1, 2004.

Available online January 10, 2005.

Reprint requests: Alan R. Leff, MD, Section of Pulmonary and Critical Care Medicine, Department of Medicine, MC6076, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637. E-mail: aleff@medicine.bsd.uchicago.edu.

0091-6749/\$30.00

© 2005 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2004.11.010

Abbreviations used

ALXR: Lipoxin A₄ receptor
 cPLA₂: Cytosolic group IV phospholipase A₂
 EPO: Eosinophil peroxidase
 FP: Fluticasone propionate
 ICAM-1: Intercellular adhesion molecule 1
 TBS-T: TRIS-buffered saline plus Tween-20

37-kd glucocorticoid-regulated protein. Annexin 1 has been implicated in the regulation of phagocytosis, cell signaling, proliferation, and inflammation.¹³ Studies in several animal inflammation models have shown that annexin 1 inhibits neutrophil inflammatory function.¹³⁻¹⁵ However, the effect of annexin 1 on eosinophil function has not been characterized.

In this study, we postulated that increased cell surface expression of annexin 1 caused by glucocorticoids was the mechanism of blockade of eosinophil adhesion to β_2 -integrin counterligand. We found that fluticasone propionate (FP), a highly potent, lipid-soluble glucocorticoid,¹⁶ inhibited human eosinophil adhesion *in vitro* after 12 hours of incubation. Inhibition was not caused by a downregulation of CD11b integrin or decrease of cPLA₂ expression or phosphorylation, but corresponded with inhibition of intracellular cPLA₂ translocation to the nuclear membrane. Both inhibition of adhesion and cPLA₂ translocation by FP were prevented by antiannexin 1 blocking antibody.

METHODS

Materials

IL-5 and eotaxin were purchased from R & D (Minneapolis, Minn). Eosinophil isolation materials were obtained from Miltenyi Biotec (Sunnyvale, Calif). Polystyrene 96-well microtiter plates were obtained from Costar (Cambridge, Mass). S⁵⁰⁵ phosphorylation specific cPLA₂ antibody was purchased from Cell Signaling Technology (Beverly, Mass). Phosphorylation nonspecific polyclonal anti-cPLA₂ and antiannexin 1 (against N-terminus, N19) were obtained from Santa Cruz Biotech (Santa Cruz, Calif). Antiannexin 1 monoclonal antibody was obtained from Transduction Laboratory (Lexington, Ky). Annexin 1 N-terminus mimetic peptides Ac2-12 and Ac2-26 were obtained from Phoenix Pharmaceuticals (Belmont, Calif). FP was provided by GlaxoSmithKline (Uxbridge, United Kingdom).

Isolation of human eosinophils

Human eosinophils were isolated from mildly atopic volunteers according to a protocol approved by the University of Chicago Institutional Review Board. Informed consent was obtained from all volunteers in this study before participation. We defined atopy through a questionnaire used in the National Heart, Lung and Blood Institute Asthma Genetics Project. Patients with symptoms or a history of asthma were excluded. The study included a total of 16 individuals age 20 to 45 years, 7 male subjects and 9 female subjects. For this study, we used the National Institutes of Health questionnaire only, because the objective was to obtain eosinophils from donors not having asthma or taking asthma or allergy drugs. None of the subjects

had received any medication for at least 1 month before the study. All components of the project were in compliance with the University of Chicago and US government guidelines for studies in which donors are not participating subjects.

Eosinophils were isolated by a modification of the negative immunomagnetic selection technique.¹⁷ The method is based on percoll centrifugation (density, 1.089 g/mL) to isolate granulocytes. After hypotonic lysis of red blood cells, immunomagnetic depletion of neutrophils is accomplished by a magnetic cell separation system using anti-CD16-coated MACS (magnetic cell sorting) particles. Eosinophil purity of $\geq 98\%$ was routinely obtained. Cells were kept on ice until use.

Pretreatment eosinophils with FP, antiannexin 1 antibody, or annexin 1 mimetic peptides

Fresh isolated eosinophils were washed 2 times with sterilized RPMI-1640 medium. Cells then were resuspended with RPMI-1640 containing 10% FBS, 1% antibiotics, and 10 pg/mL IL-5. FP with different concentrations was added to each vial. Antiannexin 1 antibody or IgG isotype control was added to certain vials according to experimental design. Cells then were incubated for 12 to 24 hours at 37°C. In studies of the effect on annexin 1 peptide fragments, fresh cells were exposed to different concentration of either Ac2-26 or Ac2-12 at 37°C for 3 hours before activation by IL-5 or eotaxin.

Eosinophil adhesion assay

For these studies, we used BSA as the surrogate ligand for intercellular adhesion molecule 1 (ICAM-1). We have demonstrated previously that BSA replicates fully and specifically all ligand-specific binding and blocking properties for β_2 -integrin in human eosinophils.¹⁸ Plates with 96 flat-bottom wells were coated with 50 μ L 10 μ g/mL BSA dissolved in 0.05 mol/L NaHCO₃ coating buffer (15 mmol/L NaHCO₃, 35 mmol/L Na₂CO₃; pH 9.2). Coating proceeded overnight at 4°C. Before use, 200 μ L heat-inactivated FBS was added to each well for 1 hour at 37°C to block nonspecific binding. The wells then were washed twice with HBSS. Eosinophils, 10⁴/100 μ L, which had been incubated with FP or buffer control for 12 to 24 hours, were washed once with HBSS/0.1% gelatin and then were added to each well of the BSA-coated microplates.

Adhesion was assessed as residual eosinophil peroxidase (EPO) activity of adherent cells.¹⁹ Serial dilutions of the original cell suspension were added to the empty wells to generate a standard curve. Then, 100 μ L EPO substrate (1 mmol/L H₂O₂, 1 mmol/L O-phenylenediamine dihydrochloride, and 0.1% Triton X-100 in TRIS buffer; pH 8.0) was added to the wells. After 20 minutes of incubation at room temperature, 50 μ L 4 N H₂SO₄ was added to stop the reaction. Absorbance was measured at 490 nm in a microplate reader (Thermomax; Molecular Devices, Menlo Park, Calif). All assays were performed in duplicate. Data storage and analysis were facilitated by the use of computer software interfaced with the reader (Softmax; Molecular Devices). The detection of EPO by this assay was linear between concentrations of 10³ cells/well and 10⁴ cells/well, as determined by a standard curve. No EPO activity was detected in the cell-free reaction supernatants after 30 minutes of incubation, confirming that EPO was not present because of spontaneous eosinophil degranulation.

Western blot analysis

Annexin 1 expression. Eosinophils (2×10^6 /group) were preincubated with FP (10^{-8} - 10^{-7} mol/L) for 24 hours at 37°C. After washing once with PBS, cells were centrifuged for 1 minute at 400g. The pellets were lysed in 50 μ L lysis buffer (20 mmol/L TRIS-

HCl, 30 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 50 mmol/L NaF, 40 mmol/L NaCl, 5 mmol/L EDTA; pH 7.4) containing 1% Nonidet P-40, 10 $\mu\text{g}/\text{mL}$ leupeptin, 5 $\mu\text{g}/\text{mL}$ aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L Na_3VO_4 , and 0.5% deoxycholic acid (Sigma Chemical, St Louis, Mo). After 10 minutes on ice, cells were sonicated. Then cells were centrifuged at 500g for 1 minute. The pellets, which are mainly nuclear component of the cells, were resuspended with 50 μL boiling buffer and boiled for 5 minutes. The supernatants were centrifuged again at 100,000g for 1 hour. Eight microliters of loading buffer was added to the supernatant, which is the cytoplasm fraction, and the mixture was boiled for 5 minutes. The pellets, which are the membrane fraction, were resuspended with 50 μL boiling buffer and boiled for 5 minutes. Then 8 μL loading buffer was again added and boiled for another 5 minutes. All 3 cell fractions were kept at -20°C after boiling.

cPLA₂ phosphorylation. Eosinophils (2×10^6 / group) were stimulated with either IL-5 or eotaxin for indicated times, and the reaction was stopped by a brief centrifugation. The pellets were lysed in 80 μL lysis buffer. After 20 minutes on ice, samples were centrifuged at 500g for 1 minute to remove nuclear and cellular debris. Afterward, 14 μL $6 \times$ loading buffer was added to the collected supernatants, boiled for 5 minutes and then saved at -20°C .

Samples were subjected to SDS-PAGE by using 10% acrylamide gels under reducing condition (15 mA/gel). Electrophoresis of proteins from the gels to polyvinylidene fluoride membrane was achieved using a semidry system (400 mA, 60 minutes). The membrane was blocked with 1% BSA for 30 minutes, then incubated with primary antibodies overnight at 4°C . For testing annexin 1 expression, 50 ng/mL monoclonal antiannexin 1 antibody was used. For testing phosphorylation and total cPLA₂, 1:1000 polyclonal antiphosphorylated cPLA₂ and 1 $\mu\text{g}/\text{mL}$ monoclonal anti-cPLA₂ antibody were used. The membranes then were washed 3 times for 20 minutes with TRIS-buffered saline plus Tween-20 (TBS-T). Antirabbit or mouse immunoglobulin conjugated with horseradish peroxidase was diluted 1:3000 in TBS-T and incubated with polyvinylidene fluoride membrane for 60 minutes. The membranes were again washed 3 times with TBS-T and assayed by an enhanced chemiluminescence system (Amersham, Arlington Heights, Ill).

Immunofluorescence flow-cytometry

Eosinophils were preincubated with 10^{-7} to 10^{-6} mol/L FP for 24 hours and then stimulated with 10 ng/mL IL-5, 300 ng/mL eotaxin, or buffer control for 30 minutes. The concentration of FP (10^{-7} mol/L to 10^{-6} mol/L) used in these *in vitro* studies corresponds with that achieved in human airways *in vivo*.²⁰ Eosinophils then were centrifuged at 400g for 10 minutes, and the pellets were resuspended in PBS/0.5% BSA. Aliquots of 5×10^5 eosinophils were incubated with 10 μL of the mAb CD11b (Bear 1), or mAb against annexin 1 (Transduction Laboratory, Lexington, Ky). After 2 washes, the cells were incubated with an excess of fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin for 20 minutes at 4°C . The cells were washed twice, resuspended in 1% paraformaldehyde, and kept at 4°C until analyzed. Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, Calif). Fluorescence intensity was determined on at least 5000 cells from each sample.

Immunofluorescence microscopy

After stimulation by IL-5, eosinophils (1×10^6) were centrifuged and resuspended in PBS. Eosinophils then were transferred to 8-well Permanox slides (Nalge Nunc International Corp, Naperville, Ill) and incubated for 20 minutes. An equal volume of 4% paraformaldehyde then was added to each well. After 20 minutes of fixation, the paraformaldehyde solution was aspirated, and 100% methanol was added to permeabilize the cells. After 1 wash with PBS, 4%

parabenzoquinone was added to each well for 20 minutes to quench the autofluorescence. After a washing step, cells were incubated with 30 $\mu\text{g}/\text{mL}$ mAb against cPLA₂ (Santa Cruz Biotech) for 60 minutes at room temperature. Then cells were incubated with 20 $\mu\text{g}/\text{mL}$ BODIPY FL-conjugated goat antimouse secondary antibody (Molecular Probes, Eugene, Ore) for 60 minutes at room temperature. After the final wash, the chamber well was detached from the slide. Slides were mounted by Mount Medium (Biomed, Foster City, Calif) and viewed by fluorescence microscopy on a Zeiss Axoplan microscope (magnification $\times 1000$; Carl Zeiss, Thornwood, NY).

Determination of eosinophil survival after incubation with FP

To determine whether FP affected eosinophil survival, trypan blue exclusion was assessed in eosinophils incubated with various concentration of FP. We have demonstrated in previous investigations that trypan blue exclusion at ≤ 24 hours corresponds identically to viability assessment obtained with propidium iodide. Further studies with terminal deoxynucleotidyl assay also confirm that there is no loss of viability in eosinophils cultured under these conditions.⁹ Aliquots of 10^4 eosinophils were incubated for 12 to 24 hours at 37°C with 10^{-10} to 10^{-6} mol/L concentration of FP in the presence of 10 pg/mL of IL-5. Eosinophils then were centrifuged at 400g, and pellets were resuspended in 10 μL HBSS. An equal volume of 0.01% trypan blue was added, and the numbers of eosinophils, excluding trypan blue staining cells, were counted in a hemacytometer.

Statistical analysis

All data are expressed as means $\pm$ SEMs. Differences between groups were assessed by paired *t* test. Where more than 2 groups were compared, differences among groups were assessed by 1-way ANOVA. Where differences were found, comparisons among groups were made by the Fisher least protected difference test. Statistical significance was claimed where $P < .05$.

RESULTS

Effect of FP on stimulated eosinophil adhesion

To establish whether FP affected integrin-mediated adhesion, *in vitro* adhesion assay using plated ICAM-1 surrogate (BSA) was performed in the presence of IL-5 or eotaxin after a short incubation time with FP (12-24 hours). This time was used to insure maximal cell viability and absence of cell apoptotic effects. In the absence of IL-5 or eotaxin, preincubation of eosinophils with FP had no effect on adhesion. IL-5 induced $23.8\% \pm 2.2\%$ eosinophil adhesion and eotaxin induced $17.5\% \pm 3.3\%$ adhesion after 30 minutes of stimulation. The nonspecific adhesion for untreated cells, which is 2% to 4%, was always subtracted from the observed total adhesion. Pretreatment of eosinophils with FP blocked IL-5-stimulated eosinophil adhesion in a concentration-dependent and time-dependent manner (Fig 1, A). The concentration for FP causing maximal inhibition of adhesion ($\sim 40\%$) was 10^{-7} mol/L. FP inhibited IL-5-induced eosinophil adhesion to $66.5\% \pm 7.2\%$ of stimulated control at 12 hours and $57.6\% \pm 6.1\%$ at 24 hours ($P < .01$ for all comparisons vs stimulated controls). Similar inhibition was observed in eotaxin-stimulated cells (Fig 1, B). FP at

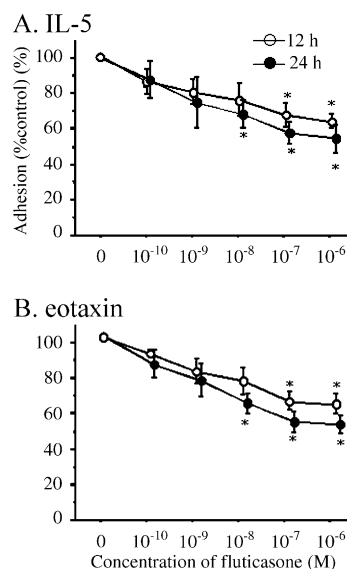


FIG 1. Effect of FP on stimulated adhesion of eosinophils to plated BSA. **A.** Concentration-dependent and time-dependent effect of FP on IL-5-stimulated eosinophil adhesion. Eosinophils were preincubated with indicated concentrations of FP for 12 to 24 hours and then stimulated by IL-5 for 30 minutes. Eosinophil adhesion was measured by residual eosinophil peroxidase activity ($n = 5$). Results are presented as the means $\pm$ SEMs. Background adhesion ($2.1\% \pm 0.3\%$) was subtracted from each data point in each experiment. **B.** Concentration-dependent and time-dependent effect of FP on IL-5-stimulated eosinophil adhesion. Eosinophil adhesion was measured as described.

10^{-7} mol/L inhibited eotaxin-induced eosinophil adhesion to $64.5\% \pm 4.8\%$ at 12 hours and $52.0\% \pm 6.0\%$ at 24 hours ($P < .01$ for all comparisons vs stimulated controls). The decreased adhesion by FP was not related to its effect on eosinophil survival. FP or control incubation alone had no effect on trypan blue exclusion at 12 or 24 hours, and inhibitory effects were fully preventable at 24 hours in subsequent studies. At 24 hours, cell surface expression of annexin 1 was greatly augmented.

Reversal of FP-induced inhibition of eosinophil adhesion by antibody against N-terminal of annexin 1

We next examined the potential role of annexin 1 in the regulation of adhesion of eosinophil to β_2 -integrin. The annexin antibody (anti-N19) is a polyclonal antibody (IgG) generated from the functional N-terminus of annexin. Anti-N19 (10 μ g/mL) alone had no effect on eosinophil adhesion induced by either IL-5 or eotaxin. However, the same concentration of anti-N19 significantly (Fig 2; $P < .05$) reversed the inhibitory effect of FP (10^{-7} mol/L) on eosinophil adhesion (FP vs FP + anti-N19). In the control experiments, IgG (10 μ g/mL or higher) failed to reverse the FP-caused inhibition of adhesion (FP vs FP + IgG). There was a significant difference between anti-N19 and IgG in restoring eosinophil adhesion ($P < .05$; FP + anti-N19 vs FP + IgG).

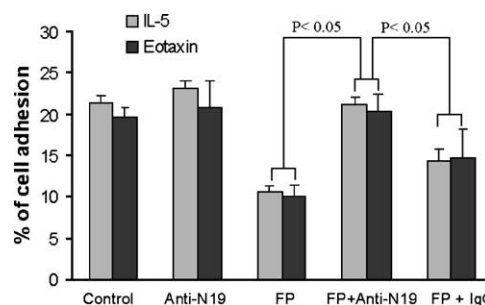


FIG 2. Effect of annexin 1 antibody on FP-induced inhibition of eosinophil adhesion. Eosinophils were preincubated with either FP (10^{-7} mol/L) or polyclonal antibody against annexin 1 N terminus (anti-N19; 10 μ g/mL) alone or together for 24 hours. In a parallel control, rabbit IgG was used to replace anti-N19 annexin 1 antibody. Eosinophil adhesion was elicited by either IL-5 (10 ng/mL) or eotaxin (300 ng/mL) and was measured as residual eosinophil peroxidase activity ($n = 7$). Percentage of cell adhesion is net adhesion after subtraction of background of adhesion of untreated cells.

Effect of annexin 1 mimetic peptides on eosinophil adhesion

We further investigated the role of annexin 1 in eosinophil adhesion by using specific annexin 1 mimetic peptides. The annexin 1 N-terminus peptide, Ac2-26, inhibited eosinophil adhesion to plated BSA in a concentration-dependent manner (Fig 3, A). At the maximal concentration used in the experiment (30 μ mol/L), adhesion caused by 10 ng/mL IL-5 was decreased from $18.6\% \pm 2.1\%$ to $8.2\% \pm 0.9\%$ ($P < .01$). The inhibition caused by 30 μ mol/L Ac2-26 was not observed when cell were coincubated with 10 μ g/mL anti-N19 (Fig 3, A). By contrast, a shorter fragment of annexin 1 N-terminus mimetic peptide, Ac2-12, had no inhibitory effect on eosinophil adhesion (Fig 3, B).

Effect of FP on annexin 1 expression on eosinophils

We then examined the effect of FP on annexin 1 expression in eosinophils. Western blot analysis of cell-fractional components demonstrated that annexin 1 mainly is distributed in cytoplasm, with some in the nuclear component and a small fraction on the cytoplasmic membrane (Fig 4, A). Treatment of FP at 10^{-8} mol/L to 10^{-7} mol/L caused a significant increase of annexin 1 on the cytoplasmic membrane and a slightly increase on nuclear membrane. However, the change of annexin 1 level in cytoplasm was not substantial (Fig 4, A and B). The total amount of annexin 1 in whole cell lysates was not changed after treatment with FP at 10^{-8} mol/L or 10^{-7} mol/L at 24 hours.

The effect of FP on the plasma membrane expression of annexin 1 in eosinophils was confirmed further by flow cytometry. Annexin 1 on the eosinophil surface membrane was increased in a concentration-dependent manner when eosinophils were incubated with FP. In the presence of

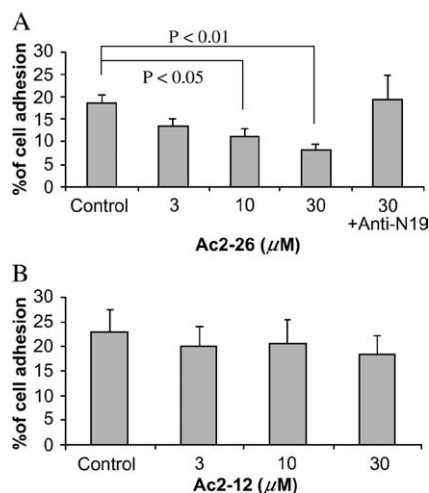


FIG 3. Effect of annexin 1 mimetic peptides on eosinophil adhesion. Eosinophils were preincubated with either peptide Ac2-12 or Ac2-26 for 3 hours before stimulation by IL-5 (10 ng/mL). Eosinophil adhesion was measured by residual eosinophil peroxidase activity ($n = 4$).

10^{-7} mol/L FP, the membrane annexin 1 level was 4-fold that measured in the absence of FP. Fig 4, C, is a representative histogram showing that FP caused a concentration-dependent upregulation of surface annexin 1 expression, as indicated by the right shift of the curve.

Effects of FP on CD11b surface expression

We previously found that stimulated ligand-specific adhesion to plated ICAM-1 or BSA is mediated by CD11b/CD18. As an initial control, we first determined whether the decreased adhesion caused by FP resulted from decreased surface expression of CD11b. Incubation with FP (10^{-7} mol/L) for 24 hours did not affect the basal expression of CD11b (Fig 5, A, left). Stimulation by either IL-5 or eotaxin caused an increased expression of CD11b, indicating by a right shift of the curve (Fig 5, A, middle and right). FP did not affect the amplitude of the CD11b upregulation caused by either IL-5 or eotaxin (Fig 5, A, middle and right). Furthermore, incubation for 24 hours with FP did not alter the extent of stimulated CD11b increase compared with freshly isolated, untreated eosinophils (data not shown).

Effects of FP on cPLA₂ protein expression and phosphorylation

We have shown previously that cPLA₂ activation is required for IL-5-stimulated eosinophil adhesion.⁸ To determine the mechanism by which FP blocks eosinophil adhesion, we examined the effect of FP on cPLA₂ expression and Ser⁵⁰⁵ phosphorylation by Western blot analysis. The total expression of cPLA₂ in eosinophils was not significantly changed when eosinophils were incubated with FP (10^{-7} mol/L) for 24 hours. Both IL-5 and eotaxin caused an increase of cPLA₂ phosphorylation. There was no significant difference in cPLA₂ phosphor-

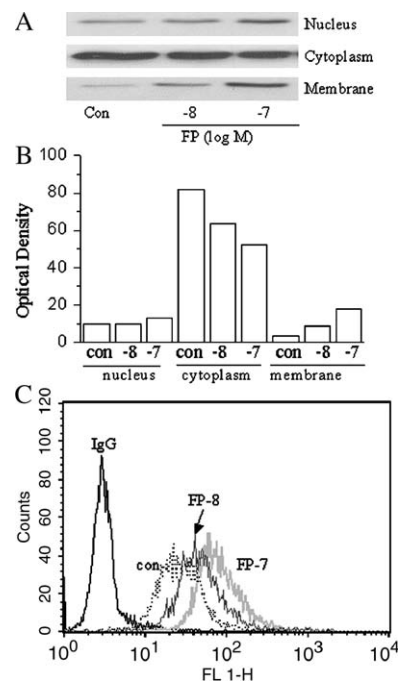


FIG 4. Effect of FP on annexin 1 expression in eosinophils. **A**, Effect of FP on annexin 1 translocation. Eosinophils were pretreated with FP (10^{-8} – 10^{-7} mol/L) for 24 hours, and annexin 1 expression was detected by Western blots in different cellular compartments after centrifugations as described in Methods. **B**, Densitometric analysis of annexin 1 expression in cell fractions. **C**, Membrane annexin 1 expression was examined by immunofluorescence flow cytometry. Representative histogram is shown ($n = 4$). The antiannexin 1 antibody is mAb generated from whole sequence of annexin 1.

ylation between the cells treated with FP and those incubated with control buffer after activation with IL-5 (Fig 5, B) or eotaxin (Fig 5, C).

Effects of FP on cPLA₂ translocation

To determine further the mechanism of FP-caused inhibition of adhesion, we examined the effect of FP and antiannexin 1 antibody on intracellular distribution of cPLA₂ by immunofluorescent staining (Fig 6). cPLA₂ was diffusely distributed in the cytoplasm of unstimulated eosinophils (Fig 6, A). When cells were stimulated with IL-5 or eotaxin, cPLA₂ redistributed to the perinuclear region and formed bright rings around the nuclear (Fig 6, B). When cells were preincubated with FP (10^{-7} mol/L) for 24 hours, much less cPLA₂ redistribution was observed (Fig 6, C). However, when cells were incubated with FP (10^{-7} mol/L) plus anti-N19 annexin 1 antibody (10 μg/mL), the stimulants (IL-5 or eotaxin) caused redistribution of cPLA₂ similar to that in the control cells (Fig 6, D).

DISCUSSION

In this study, we investigated the effect of a potent glucocorticoid (FP) on the β_2 -integrin-dependent adhesion

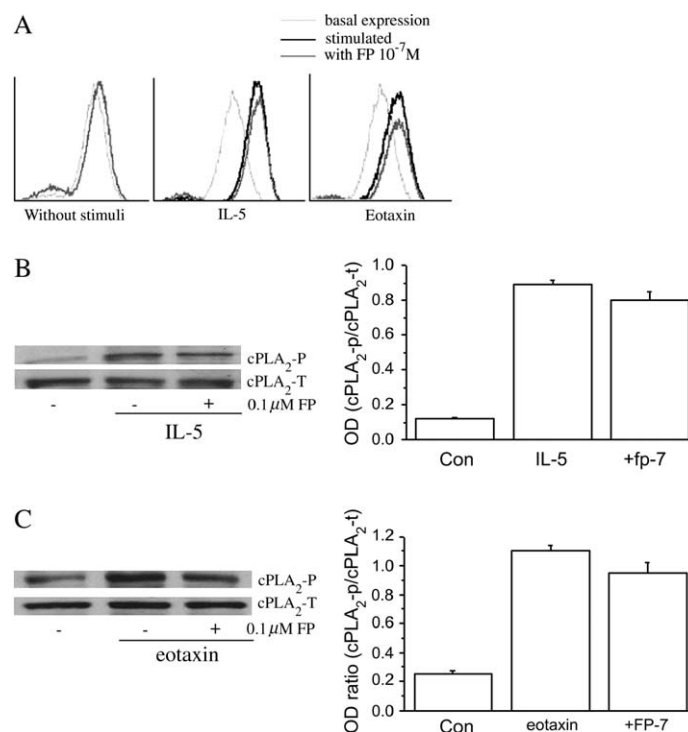


FIG 5. Effects of FP on CD11b expression and cPLA₂ phosphorylation. Eosinophils were incubated with FP for 24 hours and then stimulated with or without IL-5 (10 ng/mL) or eotaxin (300 ng/mL) for 30 minutes. **A**, CD11b surface expression was measured by flow cytometry as described in Methods. The figure represents the results from 3 experiments. **B**, Lack of effect of FP on cPLA₂ phosphorylation caused by IL-5. Total and phosphorylation form of cPLA₂ was measured by Western blot. The figure represents the results from 3 experiments. The *right panel* is the densitometric analysis of these 3 experiments. **C**, Western blot analysis was performed to determine cPLA₂ phosphorylation (*left*), and densitometric analysis from 3 experiments was used to collate the data (*bar graph, right*).

in human eosinophils *in vitro*. We find that (1) FP blocks stimulated eosinophil adhesion to plated BSA in a concentration-dependent and time-dependent manner, (2) this blockade likely is caused by blockade of cPLA₂ translocation, and (3) FP increases membrane expression of annexin 1, which appears to downregulate cPLA₂ nuclear membrane translocation, an essential step for integrin adhesion.⁸

In initial studies and previous studies using similar times for incubation, full eosinophil viability was maintained as long as 24 hours in the presence of a minimum amount of IL-5 (10 pg/mL) that had no effect on eosinophil activation, such as adhesion or adhesion molecular upregulation.^{18,20} Hence, FP decreased eosinophil adhesion caused by IL-5 or eotaxin at 12 to 24 hours without affecting cell viability. Experiments were performed for these relatively short times to minimize the possibility of interactions between cell viability and viable downregulation of the adhesive process (trypan blue exclusion was unaltered and annexin 1 surface expression was substantial at 24 hours). Hence, the partial inhibition likely represents only part of the maximally achievable effect over longer periods. It is also likely that blockade of adhesion is not the only mechanism accounting for the ability of glucocorticoids to reduce eosinophilic infiltration in asthma. Cell death and apoptosis, which did not

occur with these short incubation times, are likely to account for augmented reduction in airway inflammation. Finally, although decreased inflammation results from the use of inhaled corticosteroids, some degree of infiltration generally persists. Our data accordingly reflect the fact that glucocorticoids may reduce eosinophilic inflammation—even at short intervals—but may not fully abolish it.

We tested the hypothesis that FP-induced inhibition of eosinophil adhesion was mediated by annexin 1. In our study, the inhibition of adhesion caused by FP was reversed when cells were coincubated with both FP and antiannexin 1 blocking antibody. We further used 2 synthetic peptides (Ac2-12 and Ac2-26) that were generated according to the natural amino acid sequence of the annexin 1 N-terminus to investigate the effect of exogenous annexin 1 on eosinophil adhesion. It has been shown in several inflammation models using neutrophils that the longer form of the peptide, Ac2-26, consistently mimics the anti-inflammatory effect of glucocorticoids, whereas the shorter form, Ac2-12 peptide, does not.²¹⁻²⁴ We found that Ac2-26 inhibited cell adhesion in human eosinophils in a concentration-dependent manner and that the maximal inhibition was similar to that produced by FP. Ac2-12, a shorter peptide fragment, had no effect on adhesion. The antibody-blocking and peptide-mimicking effects, as well as the 4-fold increase in annexin 1 membrane expression

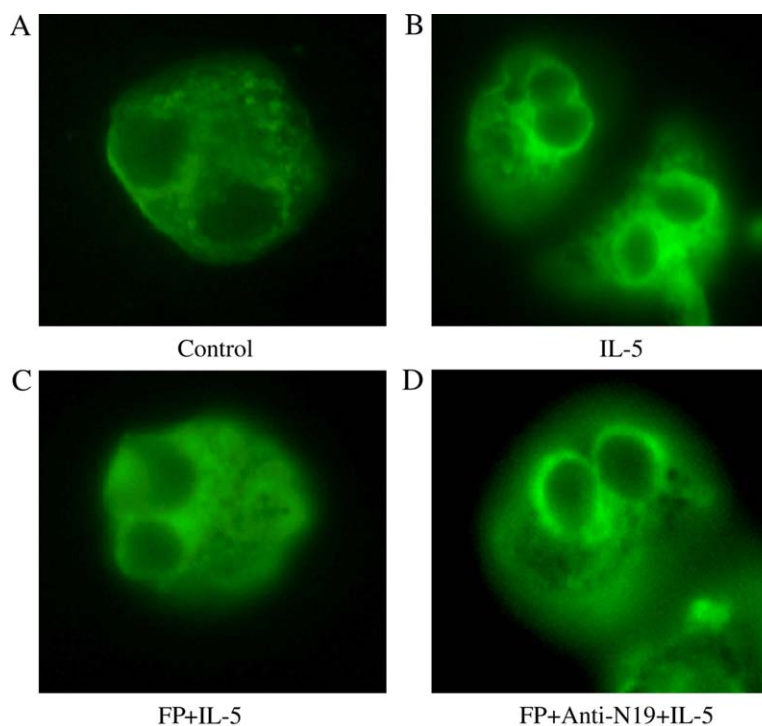


FIG 6. Effects of FP and antiannexin 1 antibody on cPLA₂ translocation caused by IL-5. Eosinophils were preincubated with FP (10^{-7} mol/L) $\pm$ antiannexin 1 antibody (anti-N19; 10 μ g/mL) for 24 hours. IL-5 (10 ng/mL) was used to stimulate cPLA₂ translocation. cPLA₂ translocation was measured by immunocytochemistry using anti-cPLA₂ mAb. The representative cells are shown from 3 experiments. **A**, Nonstimulated control cells (no FP). **B**, Stimulated control cells (no FP). **C**, Pretreatment with FP. **D**, Pretreatment with FP and anti-N19.

caused by FP, suggest that annexin 1 is very likely involved in the inhibition of adhesion caused by FP.

We have reported previously that cPLA₂ and its lysophospholipid products are essential for both β_1 -integrin-dependent and β_2 -integrin-dependent adhesion of eosinophils. Inhibition of cPLA₂ by annexin 1 through a protein-protein interaction has been reported.²⁵ We therefore examined the effect of FP on cPLA₂ activation in the presence of antibody against N-terminal annexin 1. We found there was no significant decrease of cPLA₂ phosphorylation. However, the cPLA₂ translocation was significantly inhibited by FP (10^{-7} mol/L). We found that blocking annexin 1 N-terminus by specific antibody indeed prevented inhibition of cPLA₂ translocation caused by FP. Considering the large molecular weight of our antibody, we presume that the antibody-annexin binding occurs on the cell surface. Accordingly, translocation of annexin 1 to the plasma membrane must further effect an outside-in signal to prevent cPLA₂ translocation. The nature of this signal was not defined in these studies. Recent studies have shown that annexin 1 is an endogenous ligand for 2 structurally similar receptors: formyl peptide receptor and lipoxin A₄ receptor (ALXR).^{23,24,26} By interacting with these 2 receptors, especially the ALXR, annexin 1 inhibited neutrophil adhesion and infiltration, thereby reducing neutrophil recruitment to inflammation sites in these studies. Because both receptors are also found in eosinophils, it is likely that these

receptors may also be responsible for delivering the signal to downregulate the cPLA₂ translocation and cell adhesion when FP increases annexin 1 expression on the eosinophil membrane.

Activation of ALXR by lipoxin A₄ and its stable analogue has been shown to block both airway hyperresponsiveness and eosinophil-mediated pulmonary inflammation in animal models.²⁷ Thus, increasing eosinophil membrane annexin 1, which can interact with ALXR,^{23,26} may serve as another mechanism for glucocorticoid-mediated anti-inflammatory effects. This mechanism may imply a new therapeutic approach for asthma and other allergic diseases. Our data suggest that annexin 1 stimulation of the plasma membrane of inflammatory cells may cause at least partial blockade of integrin adhesion in eosinophilic (and likely other cellular) inflammation. This also suggests possible alternative therapeutic or augmenting approaches to corticosteroid therapy for airway inflammation.

REFERENCES

1. Wong CK, Zhang J, Ip WK, Lam CW. Intracellular signal transduction in eosinophils and its clinical significance. *Immunopharmacol Immunotoxicol* 2002;24:165-86.
2. Zhang X, Moilanen E, Kankaanranta H. Enhancement of human eosinophil apoptosis by fluticasone propionate, budesonide, and beclomethasone. *Eur J Pharmacol* 2000;406:325-32.
3. Hagan JB, Kita H, Gleich GJ. Inhibition of interleukin-5 mediated eosinophil viability by fluticasone 17-propionate: comparison with other glucocorticoids. *Clin Exp Allergy* 1998;28:999-1006.

4. Walsh GM, Wardlaw AJ. Dexamethasone inhibits prolonged survival and autocrine granulocyte-macrophage colony-stimulating factor production by human eosinophils cultured on laminin or tissue fibronectin. *J Allergy Clin Immunol* 1997;100:208-15.
5. Hartnell A, Kay AB, Wardlaw AJ. Interleukin-3-induced up-regulation of CR3 expression on human eosinophils is inhibited by dexamethasone. *Immunology* 1992;77:488-93.
6. Lim LH, Flower RJ, Perretti M, Das AM. Glucocorticoid receptor activation reduces CD11b and CD49d levels on murine eosinophils: characterization and functional relevance. *Am J Respir Cell Mol Biol* 2000;22:693-701.
7. Kaiser J, Bickel CA, Bochner BS, Schleimer RP. The effects of the potent glucocorticoid budesonide on adhesion of eosinophils to human vascular endothelial cells and on endothelial expression of adhesion molecules. *J Pharmacol Exp Ther* 1993;267:245-9.
8. Zhu X, Munoz NM, Kim KP, Sano H, Cho W, Leff AR. Cytosolic phospholipase A2 activation is essential for beta 1 and beta 2 integrin-dependent adhesion of human eosinophils. *J Immunol* 1999;163:3423-9.
9. Sano A, Munoz NM, Sano H, Choi J, Zhu X, Jacobs B, et al. Inhibition of cPLA2 translocation and leukotriene C4 secretion by fluticasone propionate in exogenously activated human eosinophils. *Am J Respir Crit Care Med* 1999;159:1903-9.
10. Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, et al. Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature* 1997;390:618-22.
11. Myou S, Sano H, Fujimura M, Zhu X, Kurashima K, Kita T, et al. Blockade of eosinophil migration and airway hyperresponsiveness by cPLA2-inhibition. *Nat Immunol* 2001;2:145-9.
12. Nagase T, Uozumi N, Ishii S, Kume K, Izumi T, Ouchi Y, et al. Acute lung injury by sepsis and acid aspiration: a key role for cytosolic phospholipase A2. *Nat Immunol* 2000;1:42-6.
13. Roviezzo F, Getting SJ, Paul-Clark MJ, Yona S, Gavins FN, Perretti M, et al. The annexin-1 knockout mouse: what it tells us about the inflammatory response. *J Physiol Pharmacol* 2002;53:541-53.
14. Perretti M, Ahluwalia A, Harris JG, Goulding NJ, Flower RJ. Lipocortin-1 fragments inhibit neutrophil accumulation and neutrophil-dependent edema in the mouse: a qualitative comparison with an anti-CD11b monoclonal antibody. *J Immunol* 1993;151:4306-14.
15. Perretti M, Flower RJ. Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J Immunol* 1993;150:992-9.
16. Johnson M. Development of fluticasone propionate and comparison with other inhaled corticosteroids. *J Allergy Clin Immunol* 1998;101:S434-9.
17. Hansel TT, De Vries IJ, Iff T, Rihs S, Wandzilak M, Betz S, et al. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods* 1991;145:105-10.
18. Zhu X, Subbaraman R, Sano H, Jacobs B, Sano A, Boetticher E, et al. A surrogate method for assessment of beta(2)-integrin-dependent adhesion of human eosinophils to ICAM-1. *J Immunol Methods* 2000;240:157-64.
19. Nagata M, Sedgwick JB, Bates ME, Kita H, Busse WW. Eosinophil adhesion to vascular cell adhesion molecule-1 activates superoxide anion generation. *J Immunol* 1995;155:2194-202.
20. Myou S, Zhu X, Myou S, Meliton AY, Liu J, Boetticher E, et al. Additive blockade of beta 2-integrin adhesion of eosinophils by salmeterol and fluticasone propionate. *Eur Respir J* 2004;23:511-7.
21. Perretti M, Wheller SK, Choudhury Q, Croxtall JD, Flower RJ. Selective inhibition of neutrophil function by a peptide derived from lipocortin 1 N-terminus. *Biochem Pharmacol* 1995;50:1037-42.
22. Croxtall JD, Choudhury Q, Tokumoto H, Flower RJ. Lipocortin-1 and the control of arachidonic acid release in cell signalling. Glucocorticoids (changed from glucocorticoids) inhibit G protein-dependent activation of cPLA2 activity. *Biochem Pharmacol* 1995;50:465-74.
23. Gavins FN, Yona S, Kamal AM, Flower RJ, Perretti M. Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. *Blood* 2003;101:4140-7.
24. Walther A, Riehemann K, Gerke V. A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. *Mol Cell* 2000;5:831-40.
25. Kim S, Ko J, Kim JH, Choi EC, Na DS. Differential effects of annexins I, II, III, and V on cytosolic phospholipase A2 activity: specific interaction model. *FEBS Lett* 2001;489:243-8.
26. Perretti M, Chiang N, La M, Fierro IM, Marullo S, Getting SJ, et al. Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. *Nat Med* 2002;8:1296-302.
27. Levy BD, De Sanctis GT, Devchand PR, Kim E, Ackerman K, Schmidt BA, et al. Multi-pronged inhibition of airway hyper-responsiveness and inflammation by lipoxin A(4). *Nat Med* 2002;8:1018-23.