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Enhancement of human eosinophil apoptosis by fluticasone propionate, budesonide, and beclomethasone

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Abstract

Beclomethasone, budesonide, dexamethasone, and fluticasone propionate enhanced human eosinophil apoptosis in a concentration-dependent manner in vitro as assessed by flow cytometric analysis and morphological analysis. The order of potency was fluticasone propionate (EC_{50} 3.7 ± 1.8 nM) ≈ budesonide (EC_{50} 5.0 ± 1.7 nM) > beclomethasone (EC_{50} 51 ± 19 nM) > dexamethasone (EC_{50} 303 ± 40 nM). Hydrocortisone, prednisolone, and prednisone (up to 1 μM) did not induce any significant increase in eosinophil apoptosis. The apoptosis promoting effects of glucocorticoids on eosinophils were reversed by an antagonist of glucocorticoid receptor mifepristone. The survival-prolonging effect of tumor necrosis factor (TNF)-α was reversed by dexamethasone and fluticasone (1 μM). In contrast, fluticasone, and dexamethasone (1 μM) did not reverse the survival-prolonging effects of interleukins-3 and -5 or granulocyte-macrophage colony-stimulating factor (GM-CSF). The results suggest that fluticasone and budesonide induce eosinophil apoptosis at clinically achievable drug concentrations via an effect on glucocorticoid receptor.

Keywords: Apoptosis; Eosinophil; Fluticasone propionate; Budesonide; Beclomethasone

1. Introduction

Asthma is believed to be an inflammatory disease, which is characterized by the accumulation and activation of eosinophils in the bronchial mucosa of airways (Barnes, 1996; Giembycz and Lindsay, 1999). Eosinophils are involved in the development of airway hyper-responsiveness through the release of their granule proteins and other mediators. It is now well established that apoptosis or programmed cell death of eosinophil is an important feature in the resolution of asthmatic inflammation (Anderson, 1996; Simon, 1998; Simon et al., 1997). Unlike necrosis, that losses cell membrane integrity and releases the dying cell's contents in an uncontrolled and possibly harmful manner, apoptotic cell is phagocytosed intact without release of its contents (Walsh, 1997; Ohta and Yamashita, 1999). Recently, apoptotic eosinophils have been characterized in asthmatic airways (Druilhe et al., 1998; Vignola et al., 1999). The number of eosinophils in the asthmatic lung is elevated and is inversely correlated with the number of apoptotic eosinophils (Druilhe et al., 1998; Vignola et al., 1999).

Glucocorticoids are potent anti-inflammatory agents for the treatment of allergic diseases such as bronchial asthma (Demoly and Chung, 1998; Barnes et al., 1998). Glucocorticoids affect the numbers and activation of inflammatory cells in the airways. These target cells include eosinophils, macrophages, T-lymphocytes, mast cells, dendritic cells, neutrophils, endothelial and epithelial cells (Barnes et al., 1998). Inhaled glucocorticoids have been reported to reduce the numbers of circulating and tissue eosinophils in asthma (Barnes et al., 1998). The exact mechanisms of how inhaled glucocorticoids achieve these are unknown. The induction of eosinophil apoptosis may be one of the anti-inflammatory mechanisms of glucocorticoids in asthma or allergy. In patients with asthma, oral (prednisolone) and inhaled glucocorticoids (beclomethasone) in an open non-placebo-controlled study reduced the number of eosinophils

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in sputum with a concomitant increase in eosinophil apoptotic index (Woolley et al., 1996). In another study, in steroid-treated (steroid quality and quantity not specified) patients with asthma, the eosinophil numbers in bronchial biopsies were reduced and the apoptotic index was higher than in untreated asthma patients (Druilhe et al., 1998). Dexamethasone and methylprednisolone at high (usually 1 μM or higher) concentrations have been reported to reduce eosinophil survival or induce apoptosis in vitro (Lamas et al., 1991; Wallen et al., 1991; Adachi et al., 1996; Meagher et al., 1996). These evidences suggest that glucocorticoids may work via induction of eosinophil apoptosis. Furthermore, there is evidence that topical glucocorticoids inhibit eosinophil survival in the presence of a suboptimal concentration of interleukin-5 (Hagan et al., 1998; Stellato et al., 1999). However, it is not known whether the inhaled glucocorticoids (i.e., fluticasone propionate, budesonide, and beclomethasone), which are currently used in the treatment of asthma, directly enhance constitutive apoptosis of human eosinophils at clinically achievable drug concentrations. In the present study, firstly, we investigated the effects of beclomethasone, budesonide, and fluticasone on human eosinophil apoptosis. For comparison, dexamethasone, hydrocortisone, prednisolone, and prednisone were included. Secondly, the role of glucocorticoid receptor in the effects of beclomethasone, budesonide, and fluticasone was determined. Thirdly, their abilities to reverse the anti-apoptotic effects of cytokines such as interleukin-3, interleukin-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF)-α, when used at concentrations producing maximal or near maximal anti-apoptotic effect, were studied.

2. Materials and methods

2.1. Eosinophil purification

Eosinophils were isolated under sterile conditions using a modification of the method of Hansel et al. (1989). Briefly, a buffy-coat preparation (50 ml) from normal individuals was collected into 10 ml of acid citrate dextrose anticoagulant and hydroxyethyl starch solution. White blood cell pellet was laid onto Ficoll and centrifuged at $700 \times g$ for 30 min at 20°C. The mononuclear cell layer was discarded and the phagocyte pellet was resuspended and washed in Hanks' balanced salt solution (HBSS). Contaminating red blood cells were lysed by hypotonic lysis. The remaining granulocytes were washed, counted and resuspended in 300 μl of RPMI 1640 (2% fetal calf serum and 5 mM EDTA). Eosinophils were purified by using immunomagnetic anti-CD16 antibody conjugated beads. Cells mixed with beads were incubated at 4°C for 40 min before loading onto a separation column positioned within a magnetic field and washed with 40 ml RPMI 1640. The eluted eosinophils were washed and counted using microscopic examination in Kimura stain, and the purity of eosinophil population was $>99\%$. The cells were resuspended at 1×10^6 cells/ml, cultured (37°C, 5% carbon dioxide) for 18 h (cytokine-deprived) or 40 h (cytokines present) in PRMI 1640 (Dutch modification) with 10% fetal serum plus antibiotics in 96-well plates.

2.2. Flow cytometry

Eosinophil apoptosis was determined by propidium iodide staining of DNA fragmentation and flow cytometry (FACScan, Becton Dickinson, San Jose, CA) as previously described (Kankaanranta et al., 1999, 2000a). Briefly, the cells were suspended in 200 μl of hypotonic fluorochrome solution (propidium iodide 25 μg/ml in 0.1% sodium citrate and 0.1% Triton X-100), protected from light and incubated overnight at 4°C before flow cytometric analysis. The cells showing decreased relative DNA content were considered as apoptotic.

2.3. Morphological analysis

Eosinophils were spun onto cytospin slides (1000 rpm, 7 min) and stained with May-Grünwald–Giemsa after fixation in methanol. The cells showing the typical features of eosinophil apoptosis such as cell shrinkage, nuclear coalescence (shift from bilobed to monolobed nucleus) and nuclear chromatin condensation were considered as apoptotic (Kankaanranta et al., 2000a). When the percentages of apoptotic cells obtained by morphological analysis and flow cytometric analysis of relative DNA content were compared, a significant correlation between the two methods was observed ($r = 0.968$, $P < 0.00001$, $n = 132$).

2.4. Determination of DNA fragmentation

Oligonucleosomal DNA fragmentation was analyzed by agarose gel DNA electrophoresis as previously described (Kankaanranta et al., 1999, 2000a). Eosinophils (2 ml of 1×10^6/ml in RPMI 1640) were cultured for indicated time. The cell pellet ($350 \times g$, 7 min) was suspended in 0.5 ml of digestion buffer (100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg/ml proteinase K). The samples were incubated at 50°C for 12 h. The solution was firstly extracted with phenol/chloroform/isoamyl alcohol (25:24:1; v:v:v), buffered with Tris–EDTA buffer (pH 8.0). Following a further chloroform/isoamyl alcohol (24:1; v:v) extraction, DNA was precipitated with 2.5 M ammonium acetate and two volumes of ethanol at −20°C for at least 24 h. The DNA precipitates were recovered by centrifugation at $12\,000 \times g$ for 30 min. After drying, DNA was dissolved in TE buffer (10 mM Tris–HCl, 5 mM EDTA, pH 8.0), mixed with orange G and loaded into wells of 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out in 40 mM Tris-

base, 1.1 mM glacial acetic acid and 1 mM EDTA, pH 8.0. After electrophoresis, gels were visualized by ultraviolet light and photographed.

2.5. Materials

Beclomethasone, budesonide, dexamethasone, hydrocortisone, mifepristone, prednisone, prednisolone, and propidium iodide were purchased from Sigma (St. Louis, MO, USA). Other reagents were obtained as follows: fluticasone propionate (GlaxoWellcome, County Durham, UK), antibiotics, fetal calf serum, RPMI 1640 (Gibco, Paisley, Scotland, UK), anti-CD16 microbeads and magnetic cell separation system (Miltenyi Biotec, Surrey, UK), human recombinant interleukin-3, interleukin-5, GM-CSF, TNF-α (R&D system Europe, Abringdon, UK), May-Grünwald (Merck, Darmstadt, Germany) and Giemsa (J.T. Baker, Deventer, Holland). Dexamethasone was dissolved in HBSS. Stock solutions of all other steroids (50 mM) were prepared in ethanol. The final concentration of ethanol in the culture was 0.2% and was found not to affect constitutive apoptosis or cytokine-afforded survival in eosinophils ($n = 36$, data not shown).

2.6. Statistics

Results were expressed as mean ± S.E.M. Apoptosis is expressed as apoptotic index (number of apoptotic cells/total number of cells). EC_{50} was defined as the concentration of drug producing 50% of its own maximal effect. Statistical significance was calculated by analysis of variance for repeated measures supported by Student–Newman–Keuls multiple comparisons test or Dunnet's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. The effects of glucocorticoids on spontaneous eosinophil apoptosis

When eosinophils were cultured in cytokine-deprived conditions for 18 h, the apoptotic index was 0.12 ± 0.01 ($n = 42$) as assessed by flow cytometry measuring the relative DNA content in propidium iodide-stained cells. Beclomethasone and dexamethasone significantly enhanced the constitutive eosinophil apoptosis at 0.1–1 μM and 1 μM drug concentrations, respectively (Fig. 1(A), (B)). Budesonide and fluticasone promoted eosinophil apoptosis at the concentration range of 10–1000 nM (Fig. 1(A)). The EC_{50} values for enhancement of apoptosis were 3.7 ± 1.8, 5.0 ± 1.7, 51 ± 19 and 303 ± 41 nM for fluticasone, budesonide, beclomethasone, and dexamethasone, respectively. The order of potency of these glucocorticoids was fluticasone ≈ budesonide > beclomethasone > dexamethasone. Fluticasone and budesonide were significantly more potent than beclomethasone ($P < 0.05$) and dexamethasone ($P < 0.001$). There was no statistically significant difference in the potency or in the maximal

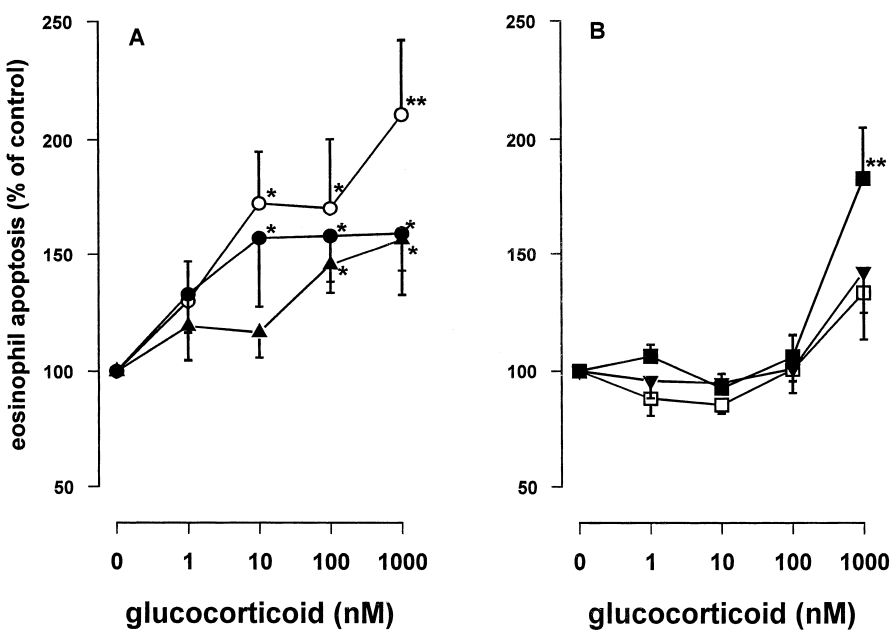

Fig. 1. The effects of (A): beclomethasone (▲), budesonide (○), and fluticasone (●); and (B): dexamethasone (■), hydrocortisone (□), and prednisolone (▼) on constitutive apoptosis of cytokine-deprived human eosinophils. Eosinophils were cultured in the absence or presence of glucocorticoids for 18 h and apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained eosinophils. Each data point represents the mean ± S.E.M. of 5–6 independent determinations using eosinophils from different donors. * indicates $P < 0.05$, ** indicates $P < 0.01$ as compared with the respective solvent control.

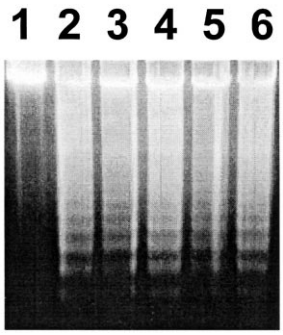

Fig. 2. The effects of beclomethasone, budesonide, and fluticasone (each at 1 μM) on oligonucleosomal DNA fragmentation in human eosinophils cultured for 18 h in the absence of any cytokines. Lanes are as follows: (1) fresh cells, (2) fluticasone, (3) solvent control, (4) budesonide, (5) solvent control, and (6) beclomethasone. See Section 2 for further details. A representative of two similar experiments.

effect between fluticasone and budesonide. When morphological criteria for apoptosis was used, beclomethasone, budesonide, and fluticasone (all at 1 μM) induced 2.0 ± 0.4, 1.9 ± 0.5 and 2.1 ± 0.4-fold increase in the number of apoptotic cells, respectively ($n = 6$). Also, the analysis of DNA fragmentation by agarose gel electrophoresis revealed an increase in DNA fragmentation in eosinophils cultured with beclomethasone, budesonide, fluticasone as compared with the solvent control (Fig. 2). Prednisolone and hydrocortisone had no significant effects on eosinophil apoptosis even at 1 μM drug concentration (Fig. 1B). As

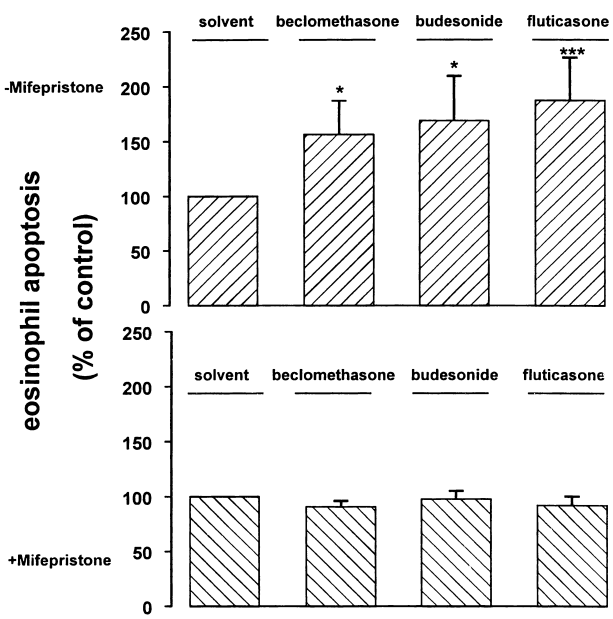

Fig. 3. The effect of mifepristone (10 μM) on the induction of eosinophil apoptosis by beclomethasone, budesonide, and fluticasone (all at 1 μM) during 18-h culture. Apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained eosinophils. Each data point represents the mean ± S.E.M. of seven independent determinations using eosinophils from different donors. * indicates $P < 0.05$, *** indicates $P < 0.001$ as compared with the respective control without mifepristone.

Table 1
The effects of dexamethasone (1 μM) and fluticasone (1 μM) on human eosinophil apoptosis with interleukin-3 (100 pM), interleukin-5 (10 pM) and GM-CSF (7 pM)

	Apoptotic index			
	Control	Dexamethasone	Control	Fluticasone
Medium	0.51 ± 0.14	0.65 ± 0.10	0.46 ± 0.12	0.66 ± 0.09
Interleukin-3	0.15 ± 0.03	0.12 ± 0.03	0.22 ± 0.05	0.20 ± 0.06
Interleukin-5	0.08 ± 0.02	0.11 ± 0.02	0.19 ± 0.03	0.19 ± 0.01
GM-CSF	0.10 ± 0.02	0.11 ± 0.02	0.14 ± 0.04	0.18 ± 0.04

The apoptotic index is shown after 40-h incubation as determined by flow cytometric analysis of relative DNA content of propidium iodide stained cells. Cytokines and steroids were added simultaneously. Values are the mean ± S.E.M. of three (fluticasone) or four (dexamethasone) duplicate experiments with cells isolated from different donors.

expected, prednisone, an inactive pro-drug of prednisolone, did not affect eosinophil apoptosis ($n = 6$, data not shown). None of the glucocorticoids affected eosinophil apoptosis at concentrations of 0.1–100 pM ($n = 5$–6, data not shown).

3.2. The effect of mifepristone on eosinophil apoptosis induced by glucocorticoids

To investigate whether the effects of glucocorticoids on eosinophil apoptosis are mediated via glucocorticoid receptor, we studied the effect of mifepristone, an antagonist of the glucocorticoid receptor (Agarwai, 1996). Mifepristone alone did not significantly affect the constitutive eosinophil apoptosis as assessed by flow cytometry ($n = 7$, data not shown). The apoptosis-promoting effects of beclomethasone, budesonide, and fluticasone (all at 1 μM) on eosinophils were reversed by mifepristone (10 μM) as assessed by flow cytometry measuring the relative DNA content of propidium iodide stained cells (Fig. 3). Similar

Table 2
The effects of fluticasone (1–1000 nM) on human eosinophil apoptosis with interleukin-5 (10 pM)

	Apoptotic index	
Fluticasone (nM)	Added before interleukin-5	Added after interleukin-5
0	0.10 ± 0.03	0.12 ± 0.04
1	0.11 ± 0.03	0.13 ± 0.04
10	0.12 ± 0.03	0.14 ± 0.04
100	0.11 ± 0.03	0.13 ± 0.03
1000	0.12 ± 0.03	0.13 ± 0.03

The apoptotic index is shown after 40-h incubation as determined by flow cytometric analysis of relative DNA content of propidium iodide stained cells. The experiments were arranged to two sets. In the first set of experiment, fluticasone was added 60 min before interleukin-5 in which case the apoptotic index in the solvent control (without fluticasone and interleukin-5) was 0.43 ± 0.04. In the second set of experiment, fluticasone was added 60 min after interleukin-5 in which case the apoptotic index in the solvent control (without fluticasone and interleukin-5) was 0.46 ± 0.07. Values are the mean ± S.E.M. of five duplicate experiments with cells isolated from different donors.

results were obtained when apoptosis was analyzed using morphological criteria ($n = 6$, data not shown).

3.3. The effects of glucocorticoids on cytokine-afforded eosinophil survival

When the incubation time was increased to 40 h, the apoptotic index was 0.49 ± 0.04 ($n = 27$). Interleukin-5 promoted eosinophil survival in a concentration-dependent manner during a 40-h incubation by delaying apoptosis ($n = 6$, data not shown). The maximal anti-apoptotic effect of interleukin-5 was obtained at 10 pM concentration. In order to investigate whether glucocorticoids reverse interleukin-5-induced eosinophil survival, the effects of dexamethasone and fluticasone were studied in the presence of interleukin-5 (10 pM). Dexamethasone and fluticasone (1 μM), added simultaneously with interleukin-5, did not reverse interleukin-5-afforded eosinophil survival during 40-h incubation (Table 1). A possibility exists that if glucocorticoid and interleukin-5 are added at the same time, the action of glucocorticoid is not fast enough to inhibit the signaling events initiated by interleukin-5. Thus, the effect of fluticasone (1–1000 nM) was studied by incubating the cells for 1 h with interleukin-5 or fluticasone, where after fluticasone and interleukin-5 were added, respectively. However, independently of the order of addition of fluticasone and interleukin-5, fluticasone did not reverse interleukin-5-induced cell survival (Table 2). Interleukin-3, GM-CSF and TNF-α also promote eosinophil survival in vitro by inhibiting apoptosis. Their maximal or near maximal anti-apoptotic effects were obtained at 100-, 7- and 600-pM concentrations, respectively. Similarly to interleukin-5, fluticasone and dexamethasone (1 μM) did not reverse interleukin-3 or GM-CSF-induced eosinophil survival. (Table 1). In contrast, both fluticasone and dexamethasone (1 μM) completely reversed TNF-α (600 pM)-induced cell survival (Fig. 4).

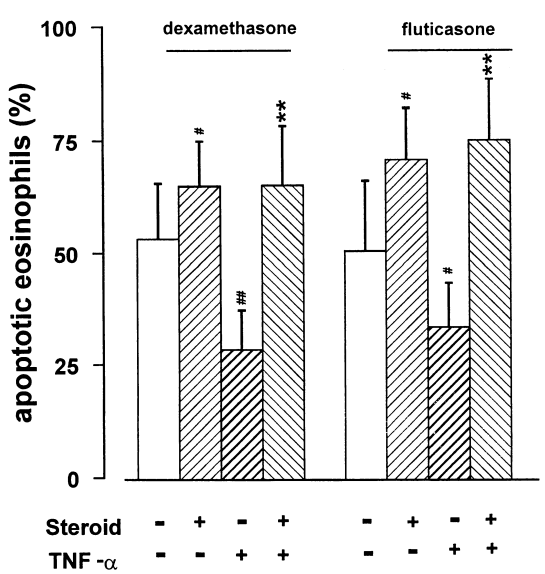

Fig. 4. The effects of dexamethasone (1 μM) and fluticasone (1 μM) on TNF-α (600 pM)-induced eosinophil survival during 40-h incubation. Each data point represents the mean ± S.E.M. of four independent determinations using eosinophils from different donors. ** indicates $P < 0.01$ as compared with the respective control with TNF-α and without steroid. # indicates $P < 0.05$, ## indicates $P < 0.01$ as compared with the respective control without TNF-α and steroids.

4. Discussion

Glucocorticoids exert their potent anti-inflammatory effect by modulating the function of multiple target cells relevant in asthma. Eosinophils are believed to be an important target cell of glucocorticoid action (Giembycz and Lindsay, 1999). Glucocorticoids promote eosinophil apoptosis in the absence of cytokines in vitro (Meagher et al., 1996). In the present study, we compared the commonly used inhaled glucocorticoids for their relative potency to induce eosinophil apoptosis in the absence of added cytokines. Beclomethasone, budesonide, dexamethasone, and fluticasone produced a 1.5- to 2.1-fold increase in the number of apoptotic eosinophils in culture. Fluticasone and budesonide were the most potent glucocorticoids in promoting constitutive eosinophil apoptosis.

The EC_{50} values found in the present study for the enhancement of eosinophil apoptosis were 3.7, 5.0 and 51 nM for fluticasone propionate, budesonide, and beclomethasone, respectively. The maximal budesonide concentration in plasma (C_{max}) has been reported in a range of 2.2–5.6 nM after inhalation of 1000 μg via Turbuhaler® (Thorsson et al., 1994). Furthermore, after inhalation, the tissue concentration of budesonide in lung was shown to be an average 8-fold higher than its plasma concentration. After inhalation of 1600 μg, the budesonide concentration varied in the range of 2–10 nmol/kg in the lung tissue (Thorsson, 1995). The C_{max} of fluticasone has been reported to vary from 0.18–0.74 nM after inhalation of 200–1000 μg via metered dose inhaler or dry powder inhaler (Meibohm et al., 1998). In another study the C_{max} was in the range of 0.4–2 nM after repeated inhalation of fluticasone (1000 μg, b.i.d.) for 7 days (Thorsson et al., 1997). However, fluticasone is highly lipophilic and has significantly higher volume of distribution than budesonide. The important consequence of this is that only a small fraction of the amount of the drug in the body is found in plasma and that the plasma concentration of fluticasone does not correlate with its effects (Thorsson et al., 1997; Barnes et al., 1998). In fact, the tissue drug concentration has been reported to vary in the range of approximately 20–44 nmol/kg in central airways after inhalation of 1000 μg of fluticasone. More importantly, these high fluticasone concentrations could be measured in the lung even 15 h after the inhalation (Högger and

Rohdewald, 1998). Thus, the increase in the rate of eosinophil apoptosis by budesonide and fluticasone is obtained at clinically achievable drug concentrations. This is also supported by our recent study where the delayed rate of apoptosis in peripheral blood eosinophils isolated from patients with asthma seemed to be reversed by inhalation therapy consisting mainly of budesonide and fluticasone (Kankaanranta et al., 2000b).

The steady state C_{max} of beclomethasone has been reported to be 2.3 nM after inhalation of 400 μg b.i.d for 2 weeks (Harrison et al., 1999). Approximately a 20-fold difference between the reported C_{max} and the EC_{50} value of the present study was found. Thus, beclomethasone may not produce a significant increase in the apoptosis of circulating eosinophils. However, it is difficult to estimate if beclomethasone would induce a significant increase in the rate of eosinophil apoptosis locally in the lung. In fact, this is supported by the finding of Woolley et al. (1996) that in an open non-placebo-controlled study, a 2-week treatment by inhaled beclomethasone in nine patients (of whom three also were treated with oral prednisolone) reduced the numbers of eosinophils in the sputum concomitantly with the increase in apoptotic index.

Our results are supported by the recent reports of Mullol et al. (1997), Hagan et al. (1998), and Stellato et al. (1999) showing a similar order of potency for the topical glucocorticoids in inhibiting eosinophil survival in the presence of a suboptimal concentration of IL-5 or when cultured in the presence of epithelial cell conditioned media. However, the mode of cell death (apoptosis or necrosis) was not analyzed in the above mentioned studies. Our results extend the existing data by showing that topical glucocorticoids decrease eosinophil survival by directly enhancing the constitutive eosinophil apoptosis as evidenced by flow cytometric analysis, morphological criteria and fragmentation of DNA.

The basic mechanism of glucocorticoid actions is that they penetrate into the cell and bind to glucocorticoid receptor molecules in the cytoplasm (Barnes et al., 1998; Demoly and Chung, 1998; Pedersen and O'Byrne, 1997). The glucocorticoid–glucocorticoid receptor complex acts as a transcription factor, binding to specific DNA sites in the nucleus. The glucocorticoid–glucocorticoid receptor complex may also directly interact with transcription factors such as nuclear factor-κB (NF-κB) and activator protein (AP)-1. By these mechanisms glucocorticoids inhibit the transcription of several pro-inflammatory cytokines and chemokines in asthma (Demoly and Chung, 1998). However, glucocorticoids may also work via mechanisms not involving the glucocorticoid receptor, i.e. interaction with cell-membrane receptors or direct physicochemical interaction with the plasma membrane (Högger and Rohdewald, 1998). Eosinophils have been shown to contain glucocorticoid receptor (McConnell and Howarth, 1998). Glucocorticoids differ in their receptor affinity, lipid solubility and other factors. The agents that have high glucocorticoid-receptor affinity, e.g. fluticasone propionate (relative receptor affinity 1800 when dexamethasone = 100) and budesonide (relative receptor affinity 935 when dexamethasone = 100) (Högger and Rohdewald, 1998) were more potent in increasing the rate of eosinophil apoptosis than beclomethasone (relative receptor affinity 76 when dexamethasone = 100) and dexamethasone. Mifepristone, an antagonist of the glucocorticoid receptor (Agarwai, 1996), completely reversed the effects of budesonide and fluticasone on eosinophil apoptosis. This suggests that budesonide and fluticasone modulate eosinophil apoptosis through glucocorticoid receptor. The EC_{50} of fluticasone increasing apoptosis was 3.7 nM, which is comparable to the reported EC_{50} value for binding of glucocorticoid (fluticasone)-receptor complex to DNA (0.72 nM). But it is significantly higher than drug concentrations that inhibit 50% of AP-1 or NF-κB binding to DNA (0.041 and 0.0089 nM, respectively) (Adcock and Barnes, 1996). This suggests that the increase in eosinophil apoptosis by fluticasone may be not mediated by inhibiting AP-1 or NF-κB binding to DNA.

Interleukin-3, -5 and GM-CSF prolong eosinophil survival in vitro (Tai et al., 1991; Yamaguchi et al., 1991; Stern et al., 1992). These in vitro observations are corroborated by an in vivo study where interleukin-5 was shown to orchestrate the eosinophilia in human nasal polyps through its ability to inhibit apoptosis (Simon et al., 1997). Glucocorticoids such as dexamethasone (Lamas et al., 1991; Wallen et al., 1991) and some other glucocorticoids (Mullol et al., 1997; Hagan et al., 1998; Stellato et al., 1999) have been reported to inhibit the interleukin-5 or epithelial cell conditioned media–afforded survival of eosinophils. This survival inhibition by glucocorticoids has been reported to depend on the concentration of cytokines. In the presence of high concentrations of cytokines, the inhibitory effects of glucocorticoids on eosinophil survival were completely or almost completely abrogated (Lamas et al., 1991; Wallen et al., 1991; Hagan et al., 1998). Our study extends the existing data by showing that topical glucocorticoids do not reverse the inhibition of eosinophil apoptosis induced by interleukin-3, -5 and GM-CSF when added at concentrations producing maximal anti-apoptotic effect. Furthermore, a prior incubation of the cells for 1 h with fluticasone was insufficient to reverse interleukin-5-induced survival. This suggests that at concentrations producing maximal anti-apoptotic effect, the signal transduction pathway of interleukin-5 (for review, see Giembycz and Lindsay, 1999) does not include steroid-sensitive elements and, probably, is also different from that activated at lower cytokine concentrations.

TNF-α has recently been shown to prolong human eosinophil survival by inhibiting apoptosis (Tsukahara et al., 1999). This effect was proposed to be mediated, at least in part, via activation of p38 mitogen-activated protein kinase (Tsukahara et al., 1999). In some other cell types the anti-apoptotic effects of TNF-α have been re-

ported to be mediated by activation of NF-κB via TNF-receptor-1 or -2 and TNF-receptor associated factor 2-mediated signaling (Liu et al., 1996; Wallach et al., 1997; Darney and Aggarwal, 1997; Natoli et al., 1998). At the present, it remains unknown which signaling mechanisms are involved in the anti-apoptotic effects of TNF-α in human eosinophils. However, the finding that dexamethasone and fluticasone reversed the survival-prolonging effect of TNF-α, but not that of interleukin-3, -5 or GM-CSF indicates that the mechanisms by which TNF-α reduces eosinophil apoptosis are different from those triggered by interleukin-3, -5 and GM-CSF.

There is debate at the moment whether induction of eosinophil apoptosis in the lung tissue contributes to resolution of allergen-induced airway inflammation (Erjefält and Persson, 2000). It has been difficult to demonstrate apoptotic eosinophils even in steroid-treated animal or human airway tissue (Erjefält et al., 1998; Erjefält and Persson, 2000) and studies reporting the existence of apoptotic eosinophils in human airway tissue (Druilhe et al., 1998; Vignola et al., 1999) may suffer from methodological problems. Thus, alternative eosinophil clearance pathways such as eosinophil cytolysis and/or luminal entry may exist (Erjefält and Persson, 2000; Persson et al., 1999). In contrast, the occurrence of apoptotic eosinophil death in bronchial lumen has been detected without any doubt (Erjefält and Persson, 2000). The luminal eosinophils are not immunologically inactive, but are still capable of undergoing, e.g. cytolysis and thus release of the cell contents to the surrounding space (Persson et al., 1999). The present study suggests that inhaled glucocorticoids enhance human eosinophil apoptosis at clinically relevant drug concentrations. There is also evidence from an uncontrolled human study to suggest that glucocorticoid (oral and inhaled) treatment reduces the number of eosinophils in sputum with a concomitant increase in eosinophil apoptotic index (Woolley et al., 1996). These data suggest a possibility that inhaled glucocorticoids induce apoptosis in luminal eosinophils and thus prevent them from undergoing activation or cytolysis. Whether the induction of apoptosis by inhaled glucocorticoids occurs in airway tissue or lumen in vivo remains to be clarified.

In conclusion, the present results show that budesonide and fluticasone enhance constitutive apoptosis and reverse survival-prolonging action of TNF-α in human eosinophils at clinically achievable drug concentrations. Thus, these data suggest that direct regulation of eosinophil apoptosis is involved in the anti-inflammatory mechanisms of glucocorticoids in asthma therapy.

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