



XIANZHI ZHANG

Regulation of Human Eosinophil and
Neutrophil Apoptosis

with Special Reference to Asthma and
Effect of Glucocorticoids and Nitric Oxide



ACADEMIC DISSERTATION

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Supervised by
Docent Hannu Kankaanranta
University of Tampere
Professor Eeva Moilanen
University of Tampere

Reviewed by
Professor Kjell Alving
Karolinska Institutet, Stockholm
Professor Tari Haahtela
University of Helsinki

Distribution



University of Tampere
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 215 6055
Fax +358 3 215 7685
taju@uta.fi
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ABSTRACT

Eosinophils are important inflammatory cells involved in the pathogenesis of asthma and exacerbations of chronic obstructive pulmonary disease (COPD). Accumulation and activation of neutrophils at the inflamed site is involved in the pathogenesis of COPD, severe asthma and asthma exacerbations. Granulocyte apoptosis is considered to be a critical feature in the resolution of inflammation. Inhaled glucocorticoids are widely used in the treatment of asthma and COPD, although their role in the latter is controversial.

The present study was designed to investigate the effects of glucocorticoids on human eosinophil and neutrophil apoptosis. In addition, the difference in eosinophil apoptosis between asthmatic patients and healthy individuals and the effects of nitric oxide (NO) on eosinophil apoptosis were studied.

Isolated human peripheral blood eosinophils and neutrophils were cultured *in vitro* and apoptosis assessed by flow cytometric analysis of relative DNA content, annexin-V binding, and by morphological analysis.

Apoptosis of peripheral blood eosinophils from asthmatic patients was delayed as compared with that of cells from healthy individuals. The difference may be partly but not fully explained by the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) by the eosinophils. β_2 -agonists inhibited human eosinophil apoptosis *in vitro*.

Inhaled glucocorticoids, beclomethasone, budesonide, fluticasone and mometasone, had opposite effects on eosinophil and neutrophil apoptosis: they enhanced constitutive eosinophil apoptosis, but inhibited neutrophil apoptosis. These effects of glucocorticoids occurred at clinically achievable drug concentrations and were mediated via glucocorticoid receptor (GR). Glucocorticoids completely reversed tumor necrosis factor (TNF)- α -mediated eosinophil survival and to a lesser extent that induced by interleukin (IL)-5. In contrast, they further enhanced the inhibitory effect of GM-CSF on neutrophil apoptosis.

NO reversed IL-5-mediated eosinophil survival. The mechanism of action of NO involved activation of c-Jun-N-terminal kinase (JNK) and caspase in a cyclic guanosine 3': 5'-

monophosphate (cGMP)-independent manner. NO had no effect on signal transducer and activator of transcription (STAT) 5 phosphorylation in IL-5-treated cells.

Taken together, the present results suggest that there is a defect in eosinophil apoptosis in asthma. Furthermore, direct regulation of eosinophil apoptosis is probably involved in the anti-inflammatory mechanisms of glucocorticoids in asthma therapy. Increased generation of NO in asthmatic airways may regulate eosinophil survival.

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-V.

- I Kankaanranta H., Lindsay M.A., Giembycz M.A., Zhang X., Moilanen E., Barnes P.J., 2000. Delayed eosinophil apoptosis in asthma. *J Allergy Clin Immunol* 106:77-83.
- II Zhang X., Moilanen E., Kankaanranta H., 2000. Enhancement of human eosinophil apoptosis by fluticasone propionate, budesonide and beclomethasone. *Eur J Pharmacol* 406: 325-332.
- III Zhang X., Moilanen E., Kankaanranta H., 2001. Beclomethasone, budesonide and fluticasone propionate inhibit human neutrophil apoptosis. *Eur J Pharmacol* 431:365-371
- IV Zhang X., Moilanen E., Adcock I.M., Lindsay M.A., Kankaanranta H., 2002. Divergent effect of mometasone on human eosinophil and neutrophil apoptosis. *Life Sci* 71:1523-1534.
- V Zhang X., Moilanen E., Lahti A., Hämäläinen M., Giembycz M.A., Barnes P.J., Lindsay M.A., Kankaanranta H., 2003. Regulation of eosinophil apoptosis by nitric oxide: role of c-Jun-N-terminal kinase and signal transducer and activator of transcription 5. *J Allergy Clin Immunol* 112:93-101.

ABBREVIATIONS

| | |
|------------------------|---|
| 1400W | <i>N</i> - [3-(aminomethyl) benzyl] acetamidine, selective iNOS inhibitor |
| 5-LO | 5-lipoxygenase |
| AP-1 | activator protein-1 |
| Apaf | apoptosis protease-activating factor |
| AT | antitrypsin |
| ATP | adenosine triphosphate |
| BAL | bronchoalveolar lavage |
| BHR | bronchial hyperreactivity |
| cAMP | cyclic adenosine 3': 5'- monophosphate |
| Carboxy-PTIO | 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. potassium salt, nitric oxide scavenger |
| cGMP | cyclic guanosine 3': 5'- monophosphate |
| CLC | charcot-leyden crystals |
| C_{max} | maximal plasma concentration |
| cNOS | constitutively expressed NOS (nNOS and eNOS) |
| COPD | chronic obstructive pulmonary disease |
| COX | cyclooxygenase |
| DED | death effector domain |
| EC₅₀ | the concentration of a drug producing 50 % of its own maximal effect |
| ECP | eosinophil cationic protein |
| EDN | eosinophil-derived neurotoxin |
| EDRF | endothelium-derived relaxing factor |
| eNOS | endothelial nitric oxide synthase |
| EPO | eosinophil peroxidase |
| ERK | extracellular-regulated kinase |
| FADD | Fas-associated death domain |
| FLICE | Fas-associating protein with death domain-like interleukin-1-converting enzyme |
| FLIP | Fas-associating protein with death domain-like interleukin-1-converting enzyme (FLICE)-inhibitory protein |
| G-CSF | granulocyte colony-stimulating factor |

| | |
|-----------------|--|
| GEA 3175 | 1,2,3,4-oxatriazolium,-3-(3-chloro-2-methylphenyl)-5-[[4-methylphenyl) sulfonyl] amino]-, hydroxide inner salt, nitric oxide donor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GR | glucocorticoid receptor |
| HBSS | Hank's balanced salt solution |
| HOCl | hypochlorous acid |
| IBMX | isobutylmethylxanthine, phosphodiesterase inhibitor |
| ICAM | intercellular adhesion molecule |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| Jak | janus kinase |
| JNK | c-Jun-N-terminal kinase |
| LIF | leukemia inhibitor factor |
| L-JNKI1 | c-Jun N-terminal kinase peptide inhibitor 1, L-stereoisomer |
| L-NIO | N-iminoethyl-L-ornithine, nitric oxide synthase inhibitor |
| L-NMMA | N ^G -nitro-L-arginine methyl ester, nitric oxide synthase inhibitor |
| LPS | lipopolysaccharide |
| LT | leukotriene |
| L-TAT | Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro, L-stereoisomer |
| MAP | mitogen-activated protein |
| MBP | major basic protein |
| MIP | macrophage inflammatory protein |
| mRNA | messenger ribonucleic acid |
| Mst | mammalian sterile 20-like kinase |
| NF-κB | nuclear factor kappa B |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| ODQ | 1H-[1,2,4] oxadiazolo [4,3, -α] quinoxalin-1-one, inhibitor of guanylate cyclase |
| PAF | platelet-activating factor |

| | |
|----------------|--|
| PARP | poly (ADP-ribose) polymerase |
| PBS | phosphate buffered saline |
| PDE | phosphodiesterase |
| ROS | reactive oxygen species |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SCF | stem cell factor |
| SDS | sodium dodecyl sulfate |
| SHPTP-2 | SH2 protein tyrosine phosphatase 2 |
| SNAP | S-nitroso- <i>N</i> -acetylpenicillamine, nitric oxide donor |
| STAT | signal transducer and activator of transcription |
| TGF | transforming growth factor |
| TNF | tumor necrosis factor |
| TRAIL | tumor necrosis factor-related apoptosis-inducing ligand |
| VCAM | vascular cell adhesion molecule |

INTRODUCTION

Eosinophils and neutrophils are major effector cells in host defense against parasites and bacteria. Over-recruitment, uncontrolled activation or defective removal of these cells, however, plays a prominent role in the initiation and propagation of chronic inflammatory disease. Asthma and chronic obstructive pulmonary disease (COPD) are the most common pulmonary inflammatory diseases. There is an increasing body of evidence indicating that eosinophils play a critical role in the pathogenesis of asthma and exacerbations of chronic obstructive pulmonary disease (COPD). Accumulation and activation of neutrophils at the inflamed site is involved in the pathogenesis of COPD, severe asthma and asthma exacerbations.

Granulocyte apoptosis is considered to be a key feature in the resolution of inflammation. Unlike necrosis, which destroys cell membrane integrity and releases the contents of the dying cell in an uncontrolled and harmful manner, the apoptotic cell is phagocytosed intact without release of its contents. While apoptotic eosinophils are present in asthmatic airways, it is not known whether eosinophil apoptosis is differentially regulated in patients with asthma as against healthy controls. *In vitro*, eosinophils and neutrophils undergo apoptosis in the absence of survival-prolonging cytokines. Interleukin (IL)-5 has been proposed to be the most important survival-prolonging cytokine for eosinophils. The exact mechanisms involved in the regulation of constitutive eosinophil apoptosis and cytokine-afforded survival are at present incompletely understood.

Glucocorticoids are the most potent anti-inflammatory agents for the treatment of asthma, but their role in COPD is controversial. The target cells of glucocorticoids include eosinophils, neutrophils, macrophages, T-lymphocytes, mast cells and other inflammatory cells. Glucocorticoids suppress various vascular and cellular mediators in the inflammatory response, and have been reported to regulate apoptosis in some cell types. The present study focused on the pharmacological regulation of eosinophil and neutrophil apoptosis by glucocorticoids.

Nitric oxide (NO) regulates various physiological and pathophysiological processes. There is evidence to suggest that exhaled NO is increased in asthmatic patients and is reduced after treatment with glucocorticoids. However, the relationship between NO and eosinophil function

has not been established. In the present study, the mechanisms involved in the regulation of eosinophil apoptosis by NO were investigated.

REVIEW OF THE LITERATURE

1 Asthma

Airway inflammation is a general feature of asthma, cystic fibrosis, bronchopulmonary dysplasia and chronic obstructive pulmonary disease (COPD) (Tiddens et al. 2000). Airway inflammation has been widely demonstrated in all forms of asthma (Fahy et al. 2000). An association between the extent of inflammation and the clinical severity of asthma has been shown (Louis et al. 2000), although some controversy remains (Haley and Drazen 1998).

Our understanding of allergy might be taken to start from 1819, when John Bostock described *catarrhus aestivus* or hay fever, and our understanding of the cellular and molecular mechanisms of allergic disease has substantially increased in the past 30 years (Holgate 1999). Atopy is acknowledged to be a major risk factor for asthma. Atopy is also implicated in syndromes such as atopic dermatitis and hay fever (Anderson and Morrison 1998). Inhalation of a specific allergen evokes acute- and late-phase reactions in atopic (allergic) asthmatics (Bousquet et al. 2000). The prevalence of asthma has, moreover, increased in recent years (Smith et al. 1997); almost half of the populations in the West evince sensitization to one or more environmental allergens (Holgate 1999). According to current estimates of asthma prevalence, 14 to 15 million persons suffer from asthma in the United States (Smith et al. 1997) and there are almost 5 million American children diagnosed with asthma (McGovern 2002). The prevalence of asthma attacks in the previous 12 months was reported to be 0.67% in rural Beijing adults (Chan-Yeung et al. 2002) and the 12-month prevalence of asthma in Beijing was 2.2% among 13-14 years old children (The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee 1998). The prevalence of physician-diagnosed asthma in Helsinki has recently been estimated to be 6.2% (Pallasaho et al. 2002).

1.1 Definitions of asthma

The guideline of the Global Strategy for Asthma Management and Prevention by Global Initiative for Asthma (GINA) (NHLBI 2002) states that asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to

recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment (NHLBI 2002). The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli (Bousquet et al. 2000).

Asthma is not a single condition but a heterogeneous collection of clinical phenotypes. It comprises a spectrum of diseases ranging from paroxysms of coughing, wheezing, and dyspnoea occurring periodically and with symptom-free periods to severe persistent asthma where symptoms are continuously present (Anderson and Morrison 1998). Allergic asthma is characterized by infiltration of the bronchial mucosa with eosinophils and T helper (Th) 2 -type cells, circulating specific immunoglobulin (Ig) E antibodies and positive skin test reactions to common aeroallergens, together with hyperresponsiveness, defined as an increased sensitivity to bronchoconstrictors such as histamine or cholinergic agonists (Humbert et al. 1999, Johansson et al. 2001, Renauld 2001). In contrast, non-allergic asthmatics are negative in skin tests, have no allergy history and normal range serum total IgE concentrations (Humbert et al. 1999).

1.2 Pathogenesis of asthma

The course of allergic asthma involves the development of allergen-specific response to inhalants or other allergens, a process normally starting during childhood and in a subset of individuals resulting in the development of a Th2-polarized immunological memory which increases the risk of allergic respiratory disease (Prescott et al. 1999). In asthma, pulmonary inflammation is characterized by mucosal edema, epithelial damage, increased neuronal responsiveness, bronchoalveolar eosinophilia, increased mucus secretion and decreased mucociliary clearance (Fahy et al. 2000). The inflammatory infiltrate in both allergic and non-allergic asthma consists of mast cells, eosinophils, macrophages, lymphocytes, neutrophils and plasma cells (Anderson and Morrison 1998, Hamid et al. 2003). Inflammatory cytokine production in the airway wall in the absence of inflammatory stimulation is relatively low. However, in asthmatics, eosinophils and other inflammatory cells as well as structural cells (airway epithelial cells, fibroblasts, endothelial cells and smooth muscle cells) produce a wide

range of cytokines and mediators, most probably as part of the tissue's response to chronic inflammation (Barnes et al. 1998a, Smart and Kemp 2002).

Increased numbers of eosinophils in the airways constitute a key feature in asthma (Bousquet et al. 1990, Laitinen et al. 1991, Oosterhoff et al. 1995, Keatings et al. 1997a, Bousquet et al. 2000, Gaga et al. 2000, Wardlaw et al. 2000, Wardlaw 2001). Eosinophil accumulation and activation within the airway mucosa is thought to cause tissue injury, contraction of airway smooth muscle and increased bronchial responsiveness (Oddera et al. 1998, Ward et al. 2002).

1.2.1 The biology of eosinophils

Eosinophils are end-stage cells derived from the bone marrow; ancillary sites of production include the spleen, thymus and lymph nodes (Giembycz and Lindsay 1999). The bone marrow from normal individuals contains about 3% eosinophils, of which 37% are mature and non-dividing granulocytes, while the remainder are promyelocytes/myelocytes (37%) and metamyelocytes (26%) which exist in "storage" compartments (Giembycz and Lindsay 1999). The time taken from the last mitosis until they appear as mature cells in the blood is about 2.5 days. Their lifetime in the bloodstream is thought to be 6-12 hours, whereafter they migrate into tissues, preferentially in submucosal sites (Giembycz and Lindsay 1999).

In the development of a hematopoietic stem cell into a multipotential progenitor, a variety of cytokines and other factors are required, including interleukin (IL)-6, IL-11, IL-12, granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF; CD117) and leukemia inhibitory factor (LIF). SCF, IL-3, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF) and eotaxin then further promote the development of multipotential cells into eosinophil progenitors. IL-5 and possibly eotaxin then promote the terminal stages of maturation and release of eosinophils into the bloodstream (Palframan et al. 1998, Giembycz and Lindsay 1999, Bandeira-Melo et al. 2001).

A typical feature of eosinophils is the presence in them of numerous spherical or ovoid granules. Four distinct populations of granules (secondary granules, small granules, lipid bodies and primary granules) have been recognized. The secondary granules consist of a crystalline core composed of major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil

peroxidase (EPO) and eosinophil-derived neurotoxin (EDN), located within the noncrystalloid matrix (Figure 1). These granular proteins are potent histotoxic substances (Giembycz and Lindsay 1999). Eosinophils generate pro-inflammatory lipid mediators, e.g. leukotrienes (LT), prostanoids and platelet-activating factor (PAF). They are, further, reported to be a source of several growth factors and regulatory or proinflammatory cytokines (Moqbel et al. 1994), including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-12, tumor necrosis factor (TNF)- α , GM-CSF, transforming growth factor (TGF)- α and TGF- β , together with the chemokines eotaxin, macrophage inflammatory protein (MIP)-1 α and RANTES (Figure 1) (Weller 1991, Giembycz and Lindsay 1999).

The process of migration of eosinophils through the endothelium into tissues is under complex regulation mediated by the binding of adhesion receptors to their ligands or counter-structures on the post-capillary endothelium (Walsh 1999). Several cell adhesion molecules have been implicated in eosinophil adherence to the cytokine-stimulated vascular endothelium, including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin and L-selectin (Broide and Sriramarao 2001).

Compared with peripheral blood cells, tissue eosinophils can be considered to be in a more activated state (Moqbel et al. 1994). Degranulation is the end result of the full activation of the eosinophil. Eosinophils can release their secretory granule contents by cytolysis (necrosis), piecemeal degranulation, granule exocytosis and compound exocytosis (Adamko et al. 2002). During cytolysis they release intact or disrupted granules and may thus cause toxic effects on the surrounding cells. Erjefält and co-workers (1999) have reported that piecemeal degranulation, characterized mainly by the presence of partly or completely empty secondary granules, is the principal mode of exocytotic release from tissue eosinophils in nasal biopsy samples from allergic individuals. "Classical degranulation" (granule exocytosis) with extrusion of membrane-free specific granules whose membranes have first fused with the cell membrane and compound exocytosis are unusual in eosinophilic inflammation during allergen challenge (Erjefält and Persson 2000, Adamko et al. 2002).

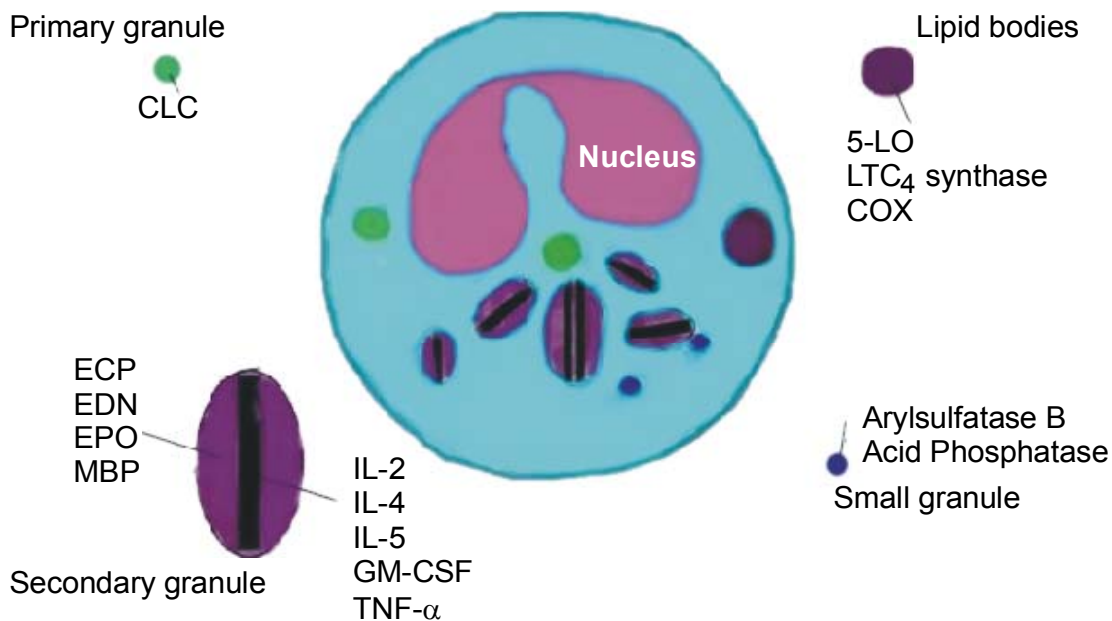


Figure 1. Cardinal structures of a human eosinophil. Shown are the typical bi-lobed nucleus and four main granules. The primary granule is the principle site of charcot-leyden crystals (CLC). MBP, ECP, EDN and EPO reside within secondary granules along with a number of cytokines. Lipid bodies contain enzymes such as 5-lipoxygenase (5-LO), cyclooxygenase (COX) and LTC₄ synthase. Small granules store proteins such as arylsulfatase B and acid phosphatase. Modified from Giembycz and Lindsay (1999).

1.2.2 Eosinophils in asthma

Histopathological studies of airway inflammation in asthma patients have found an increase in the numbers of eosinophils (Bousquet et al. 1990, Laitinen and Laitinen 1991, Laitinen et al. 1991, Humbert et al. 1999, Wardlaw 1999, Bousquet et al. 2000, Jeffery et al. 2000). In addition, the eosinophils showed evidence of activation with increased release of MBP, ECP and other granular proteins such as EDN and EPO, which have also been held to have a major role in the disorder (Persson and Erjefalt 1997). The resultant phenomena of mucosal edema, vascular congestion, bronchoconstriction, increased mucus production and impaired ciliary function are thought to be a consequence of the interaction and activation of inflammatory cells, including eosinophils (Oddera et al. 1998, Ward et al. 2002).

Studies using bronchoalveolar lavage (BAL) or induced sputum have shown that the main cellular marker of airway inflammation in both atopic and non-atopic asthma is the eosinophil

(Wardlaw et al. 1988, Fahy et al. 1993, Smith and Deshazo 1993, Oosterhoff et al. 1995, Oddera et al. 1998, Humbert et al. 1999, Gaga et al. 2000, Bousquet et al. 2000, Jayaram et al. 2000, Rytala et al. 2000, Broide and Sriramarao 2001, Metso et al. 2001). Eosinophils are extremely rare in induced sputum in normal subjects (Belda et al. 2000), whereas, they are enriched in the airways of asthmatic subjects as compared to neutrophils (Wardlaw et al. 2000), giving a 20 –200-fold increase in the eosinophil/neutrophil ratio (Wardlaw 1999). Eosinophils and their granule products have also been found in abundance in the airways in *postmortem* specimens from patients who died of asthma (Filley et al. 1982). Even in very mild asthma there is evidence of airway inflammation and the most obvious feature of the inflammatory response is an increased number of eosinophils in BAL fluid without increase in neutrophil count (Wardlaw et al. 2000). BAL eosinophilia ranged from 1-30% (the normal being less than 1%) (Wardlaw et al. 2000). Increased numbers of eosinophils in the airways have also been reported in occupational (Saetta et al. 1992, Frew et al. 1995) and aspirin-sensitive asthma (Nasser et al. 1996).

The presence of eosinophils in BAL fluid is associated with symptomatic asthma and in general there is a correlation between the clinical severity of asthma and the degree of airway eosinophilia (Wardlaw et al. 2000). Bronchial hyperreactivity (BHR) entails increased sensitivity in asthmatics to irritant inhaled stimuli (Wardlaw et al. 2000). BHR and eosinophilic inflammation generally in fact occur together, but the reason for BHR is still largely unknown and under debate (Wardlaw et al. 2000). The two conditions may be independently regulated but closely interrelated (Rosi et al. 1999, Leckie et al. 2000, Buttner et al. 2003, Flood-Page et al. 2003). Earlier studies suggested that peripheral blood eosinophil counts have an inverse correlation with lung function both in clinical disease and after allergen challenge (Wardlaw et al. 2000), although blood eosinophils may be an insensitive and imprecise marker of tissue inflammatory responses (Pizzichini et al. 1997, Leckie et al. 2000, Buttner et al. 2003, Flood-Page et al. 2003).

As in asthma, eosinophils are also characteristically implicated in seasonal and perennial rhinitis (Christodoulopoulos et al. 2000), nasal polyposis (Lamblin et al. 1999), idiopathic eosinophilic syndromes and atopic dermatitis (Wardlaw et al. 2000, Greenfeder et al. 2001, Leiferman 2001, Trautmann et al. 2001). Accumulation of eosinophils also occurs in several other disorders such as parasitic infections and cancer (Rothenberg 1998, Gleich 2000).

1.2.3 Neutrophilic inflammation in asthma

The role of neutrophils in asthma is less clear than that of eosinophils. Neutrophil counts are usually similar in induced sputum of the control subjects and patients with mild-moderate asthma (Keatings et al. 1996, Taha et al. 2001), in BAL (Bousquet et al. 1991, Lacoste et al. 1993, Boulet et al. 1993) and bronchial biopsies (Jeffery et al. 1989, Bradley et al. 1991, Lacoste et al. 1993, Wenzel et al. 1997, Wenzel et al. 1999, Bousquet et al. 2000, Ennis 2003). Neutrophilic inflammation may nonetheless also be important in the pathophysiology of asthma, especially in more severe asthma (Woodruff and Fahy 2002). There is increased evidence to suggest that the numbers of neutrophils in severe asthma are raised (Wenzel et al. 1997, Jatakanon et al. 1999, Louis et al. 2000, Ordonez et al. 2000). Neutrophils have been found to be increased in some patients who died within hours after an asthma exacerbation (Sur et al. 1993). Neutrophil numbers and their activation status are also increased during exacerbations of asthma (Fahy et al. 1995). Asthma severity likewise appears to correlate with the degree of neutrophilia in sputum and bronchial specimens (Wenzel et al. 1997, Jatakanon et al. 1999). Jatakanon and co-workers (1999) reported significantly increased neutrophil numbers in the sputum of patients with severe asthma (53.0 %) as compared with mild cases (35.4 %) and normal control subjects (27.7 %). However, it remains to be established whether the increased numbers of neutrophils in severe asthma are involved in its pathogenesis or whether this is an iatrogenic or even treatment-related phenomenon. Neutrophilia has also been described in occupational asthma (Leigh and Hargreave 1999).

2 Chronic obstructive pulmonary disease

COPD is a major cause of chronic morbidity and mortality throughout the world (Pauwels et al. 2001). It is currently the fourth commonest cause of death in the world and further increases in the prevalence and mortality of the disease can be predicted in the coming decades (Pauwels et al. 2001).

2.1 Definitions of chronic obstructive pulmonary disease

There is as yet no precise agreement on a definition of COPD. The National Heart, Lung and Blood Institute/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) workshop defines COPD as a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases (Pauwels et al. 2001). The inflammatory process contributes to the pathogenesis of chronic cough and sputum production (chronic bronchitis), peripheral airway obstruction and emphysematous destruction of the lung parenchyma which define COPD. Chronic bronchitis (mucous hypersecretion) is defined by the presence of chronic cough and a recurrent increase in bronchial secretions sufficient to cause expectoration. Emphysema is defined by permanent, destructive enlargement of airspaces distal to the terminal bronchiole without obvious fibrosis (Jeffery 1999).

2.2 Pathogenesis of chronic obstructive pulmonary disease

COPD is characterized by chronic inflammation throughout the airways, parenchyma and pulmonary vasculature (Pauwels et al. 2001). In addition to inflammation, an imbalance of proteinases and antiproteinases in the lung and oxidative stress are thought to be important in the pathogenesis of COPD (Pauwels et al. 2001). Inflammatory cells, e.g. neutrophils, macrophages and T lymphocytes, are reported to be increased in COPD (Pauwels et al. 2001). There may also be an increase in the numbers of eosinophils in some patients, particularly during exacerbations of COPD (Saetta et al. 1994). Activated inflammatory cells in COPD release a variety of mediators, e.g. LTB₄ (Hill et al. 1999), IL-8 (Keatings et al. 1996, Yamamoto et al. 1997) and TNF- α (Keatings et al. 1996), which are capable of damaging lung structures and/or sustaining neutrophilic inflammation.

Cigarette smoking is believed to be involved in initiating the pathogenic process of COPD (Fiel 1996). Previous studies have shown that the habit is associated with an airway inflammatory process (Saetta 1999). Cigarette smoking causes an inflammatory process in the central airways, peripheral airways and lung parenchyma, even in smokers with normal lung function (Saetta 1999). Smokers evince increased inflammation in the alveolar walls as compared with non-smokers, its level correlating significantly with the destruction of the alveolar walls

attached to the airways (alveolar attachments) (Saetta 1999). In bronchial biopsies obtained from central airways, smokers have an increased number of macrophages and T lymphocytes as compared with nonsmokers, while neutrophils, eosinophils and mast cells are similar in the two groups (Saetta et al. 1993). In contrast, an increased number of neutrophils has been reported in BAL fluid (Riise et al. 1995, Keatings et al. 1996, Peleman et al. 1999) and in induced sputum (Bhowmik et al. 1998, Metso et al. 2001) in patients with COPD.

2.2.1 The biology of neutrophils

Neutrophils are polymorphonuclear leukocytes which are major effector cells in the immune system and play an essential role in the pathogenesis of many inflammatory lung disorders other than COPD (Dallegrì and Ottonello 1997, Mecklenburgh et al. 1999). A prominent neutrophilic inflammatory component has been found in diseases involving tissue infection by extracellular bacteria and various noninfectious diseases such as adult respiratory distress syndrome, some types of immune-complex alveolitis and other conditions including glomerulonephritides, acute phases of rheumatoid arthritis, gouty arthritis, ulcerative colitis, certain forms of dermatosis, i.e. psoriasis and vasculitides (Dallegrì and Ottonello 1997). In these noninfectious conditions, neutrophils are thought to play a crucial role in the development of tissue injury which, when persistent, can lead to irreversible tissue destruction with consequent organ dysfunction (Dallegrì and Ottonello 1997).

Neutrophils are produced in the bone marrow from myeloid precursor cells, the production of which is regulated by G-CSF and GM-CSF (Smith 1994). Neutrophils differentiate in the bone marrow for approximately 7-10 days before being released into the circulation (Stockley 2002). They constitute 50-70 % of the total circulating leukocytes and build the first line of defence against infectious agents penetrating the body's physical barriers (Smith 1994). Neutrophils contain more than 40 hydrolytic enzymes and toxic molecules in their granules (Table 1) and can generate a variety of oxidants. Neutrophils are also a major source of cytokines and chemokines.

Once recruited at tissue sites of inflammation, active neutrophils as well as eosinophils generate superoxide (O_2^-) anions (Dallegrì and Ottonello 1997, Giembycz and Lindsay 1999). O_2^- may react with NO, which generates peroxynitrite, a strong microbicidal and cytotoxic compound

(Nathan 1992, Schmidt and Walter 1994). Moreover, O_2^- can be rapidly dismutated to hydrogen peroxide (H_2O_2). Neutrophils convert H_2O_2 to the more powerful oxidant hypochlorous acid (HOCl), which exerts a variety of undesirable effects; e.g. it induces depletion of adenosine triphosphate (ATP) with consequent cell dysfunction and cell necrosis. HOCl also inactivates α_1 -antitrypsin (AT), a glycoprotein able to diffuse easily from the circulation into inflamed tissues. As AT is the specific inhibitor of neutrophil elastase, its inactivation by HOCl favors the uncontrolled proteolytic activity of the enzyme (Dallegrì and Ottonello 1997).

Table 1. Contents of human neutrophil granules.

| Azurophil granules | Specific granules | Gelatinase granules | Secretory vesicles |
|---------------------------|-----------------------------------|----------------------------|---------------------------|
| α_1 -antitrypsin | TNF-receptor | Gelatinase | Alkaline phosphatase |
| β -glucuronidase | Cytochrome b_{558} | FMLP-receptor | FMLP-receptor |
| Azurocidin | FMLP-receptor | Cytochrome b_{558} | Complement receptor 1 |
| Cathepsin G | Laminin-receptor | Lysozyme | Cytochrome b_{558} |
| Defensins | Collagenase | | Urokinase-receptor |
| Lysozyme | Gelatinase | | |
| Myeloperoxidase | Lysozyme | | |
| Elastase | Lactoferrin | | |
| | Vitamin B_{12} -binding protein | | |
| | Vitronectin-receptor | | |

Modified from Dallegrì and Ottonello (1997).

If the target is represented by phagocytosable particles, i.e. opsonized microorganisms, binding of the target results in its engulfment and the formation of an intracytoplasmic phagosome. This allows killing and digestion of phagocytosed microorganisms, with minimal risk to the surrounding tissue. However, an extracellular release of destructive granule constituents and oxygen metabolites can occur during the formation of the phagosome, particularly when the neutrophil is faced with a large number of targets or a target too substantial to be ingested. This event is generally responsible for neutrophil-mediated tissue injury during phagocytosis (Mecklenburgh et al. 1999)

2.2.2 Neutrophils in chronic obstructive pulmonary disease

Increased numbers/levels of neutrophils and neutrophil-activating inflammatory mediators such as IL-8 and LTB₄, as well as neutrophil-derived oxidants and proteases, are found in the airway lumen in COPD (Jeffery 1999). Prominent neutrophilia is observed in smokers with severe COPD (Peleman et al. 1999). Neutrophil numbers are not only increased in smokers but also correlate with the degree of airflow limitation (Di Stefano et al. 1998). Also in surgical specimens prominent neutrophilia is present in the bronchial glands of smokers who develop symptoms of chronic bronchitis and chronic airflow limitation as compared with asymptomatic smokers with normal lung function (Saetta et al. 1997). Groups under Saetta (1994) and Fabbri (1998) have also reported an increased number of neutrophils during exacerbations of chronic bronchitis. It is possible that the neutrophilia represents a response to an altered milieu, perhaps infectious, in the airways of these subjects. Keatings and associates (1996) reported that bronchial secretions contain increased numbers of neutrophils in subjects with COPD. In addition, subjects with α_1 -AT deficiency, who are at high risk of developing emphysema, have an increased number of neutrophils in the distal airways (Hubbard et al. 1991). Neutrophils may thus play a major role in the pathogenesis of COPD.

3 Cell death

3.1 Apoptosis and necrosis

Two common forms of cell death have been described in vertebrate tissues. Necrosis is most often seen when cells die from severe and acute injury such as ischemia, sustained hyperthermia, or chemical trauma (Cohen 1993, Denecker et al. 2001). During necrosis, the mitochondrial shape and function are altered and the cells are unable to maintain homeostasis (Cohen 1993). The plasma membrane loses its ability to regulate osmotic pressure, so that the cell eventually swells and ruptures. This leads to a total disintegration of the cell and the release of the cytoplasmic contents to the surrounding tissue (Figure 2).

In contrast, apoptosis is a form of cell death which is demonstrably different from the necrotic process. Apoptosis is often equated with programmed cell death. The important physiological

role of apoptosis is that it occurs during development and maintenance of tissue homeostasis. Apoptosis is also involved in pathologic conditions such as neurodegenerative diseases, acquired immunodeficiency syndrome, cancer and autoimmune and inflammatory diseases (Simon 2000). Apoptotic cells or bodies are phagocytosed by macrophages or neighboring cells, thus avoiding an inflammatory response such as occurs during necrosis (Haslett 1999) (Figure 2).

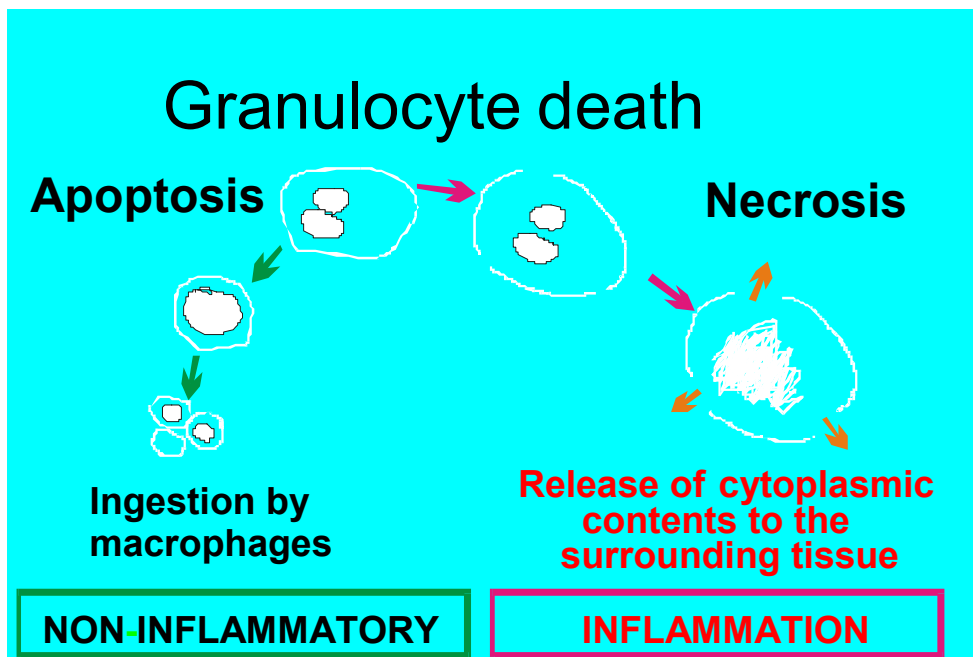


Figure 2. Apoptosis and necrosis

3.1.1 Morphological and biochemical hallmarks of apoptosis

There are thought to be many common features of apoptosis in nearly all cell types (Kinloch et al. 1999). The onset of apoptosis is characterized by shrinkage of cell and nucleus and condensation of nuclear chromatin. Subsequently, the nucleus progressively condenses and breaks up. The cell detaches from the surrounding tissue (Saraste and Pulkki 2000). The plasma membrane in apoptosis becomes ruffled and blebbed. The extensions separate and the plasma membrane forms a separate membrane around the detached solid cellular material. These apoptotic bodies are sealed and maintain cellular organelles and fragments of nucleus. Intact structures such as membranes and mitochondria, are well preserved within the bodies. An early step in apoptosis *in vivo* is that phagocytic cells recognize the apoptotic bodies and subsequently phagocytize them. Apoptotic bodies/cells can be recognized inside these cells and

degraded. If the fragmented cell is not phagocytosed it will finally undergo swelling and lysis, an event referred to as “secondary necrosis” (Kinloch et al. 1999, Saraste and Pulkki 2000). *In vitro*, eosinophils cultured in the absence of cytokines undergo a marked volume decrease (Beauvais et al. 1995a) and typical features of apoptosis such as nuclear coalescence and nuclear chromatin condensation can be seen (Haslett 1999, Kankaanranta et al. 2000). Subsequently, as apoptosis progresses, the nucleus is totally disintegrated, and “late apoptotic secondary necrotic” cells can be seen, where the plasma membrane is still relatively intact and the granules are preserved inside the cells. These cells are smaller in size as compared with normal eosinophils (Kankaanranta et al. 2000).

Apoptosis is characterized by degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180–200 base pairs (bp) in many cell types (Bortner et al. 1995, Saraste and Pulkki 2000). Internucleosomal fragmentation has been demonstrated with well-characterized apoptotic morphology in a wide variety of situations and cell types, although apoptosis may also occur without internucleosomal DNA fragmentation (Bortner et al. 1995, Kinloch et al. 1999).

The appearance of phosphatidylserine in the outer leaflet of the plasma membrane appears to be a universal phenomenon in apoptotic cells (Fadok et al. 1992, Martin et al. 1995b, Vermes et al. 1995, Kagan et al. 2003). In eukaryotic cells, phosphatidylserine is predominantly confined to the inner cell membrane leaflets, whereas choline-containing phospholipid is mainly located in the outer leaflet (Kagan et al. 2003). During apoptosis, phosphatidylserine is relocated to the outer layer of the plasma membrane (Fadok et al. 1992, Allen et al. 1997, Martin et al. 1995b). Phosphatidylserine exposure is dependent on extracellular Ca^{2+} and occurs downstream of the activation of caspases (Kagan et al. 2003).

3.2 Apoptosis pathways

3.2.1 Caspases

The degradation or cleavage of various intracellular proteins during apoptosis is a common feature of this physiological cell death process. Regulation of caspase activity is believed to be central in the process (Martin and Green 1995a, Philchenkov 2003).

Caspases are cysteine proteases which cleave their substrate proteins specifically behind an aspartate residue (Thornberry and Lazebnik 1998). They are constitutively expressed and are normally present as inactive proenzymes (Wolf and Green 1999). After cleavage at specific internal aspartate residues, full enzymatic activity is induced. Caspases are activated during apoptosis in an amplifying cascade (Thornberry and Lazebnik 1998). They can be divided into two functional subgroups, initiator and executioner. Activation of upstream (initiator) caspases such as caspases-2, 8, 9 and 10 by a pro-apoptotic signal leads to proteolytic activation of the downstream (executioner or effector) caspases-3, 6 and 7 (Saraste and Pulkki 2000).

Two major pathways of caspase activation have been characterized. One is initiated by ligation of death receptors such as Fas and TNF receptors, the other is regulated by the Bcl-2 protein family and mitochondrial damage (Ashkenazi and Dixit 1998, Marsden and Strasser 2003). Activation of procaspase-8 seems to be crucial in the death receptor-mediated pathways, which require association of procaspase-8 with the Fas-associated death domain (FADD) (Srinivasula et al. 1996, Thornberry and Lazebnik 1998). In contrast, caspase-9 is considered central to the mitochondrial pathway, which involves cytochrome c and apoptosis protease-activating factor (Apaf)-1 (Thornberry and Lazebnik 1998) (Figure 3).

The caspase cascades in eosinophils are not known in detail. Freshly purified eosinophils display detectable amounts of procaspases-3, 8 and 9 (Letuve et al. 2001, Dewson et al. 2001, Daigle and Simon 2001b). Daigle and Simon (2001b) suggest that caspases-3 and 8 do not play a critical role in the regulation of eosinophil apoptosis. However, Dewson and co-workers (2001) report that caspases-3, 7, 8 and 9 are activated during spontaneous eosinophil apoptosis.

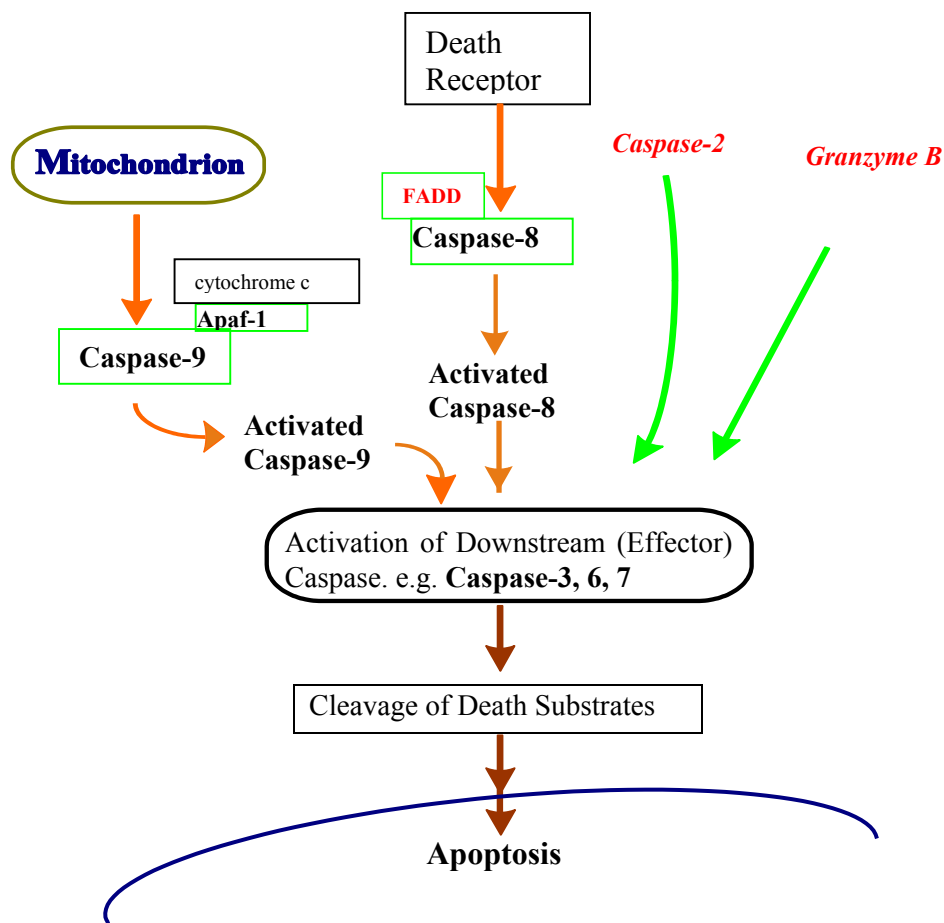


Figure 3. Caspase pathways to apoptosis.

Proteins such as IAPs (inhibitors of apoptosis proteins), FLIPs (a family of inhibitors of FLICE (Fas-associating protein with death domain-like interleukin-1-converting enzyme) proteins), CrmA (a 38-kDa cytokine response modifier protein of the cowpox virus) and p35 prevent apoptosis at the level of caspases and inhibit aberrant cell death (Bortner and Cidlowski 2002). This variety of different regulatory proteins used to inhibit specific caspase activities depends on the particular stimuli employed to initiate apoptosis. The role of these proteins in the regulation of eosinophil apoptosis is not clear (Bureau et al. 2002, Gardai et al. 2003).

3.2.2 Apoptosis through death receptors

The death receptors of the TNF receptor (TNFR) family include TNFR1, Fas (CD 95, APO-1), DR3/WSL, DR6 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/APO-2L receptors (TRAIL-R1/DR4, TRAIL-R2/DR5) (Zimmermann and Green 2001). Both eosinophils and neutrophils have been reported to express functional death receptors (e.g. TNF, Fas and TRAIL receptors) on their surface (Luttmann et al. 1998, Daigle and Simon 2001a). TNF- α , Fas and TRAIL induce apoptosis in many cell types, including neutrophils (Salamone et al. 2001, Li et al. 2003, Maianski et al. 2003). In contrast, TNF- α and TRAIL have been reported to enhance eosinophil survival while Fas induce apoptosis in eosinophils (Luttmann et al. 1998, Daigle and Simon 2001a).

Fas is a cell surface protein intimately involved in the regulation of apoptosis. It has been reported to be expressed on several cell types such as T and B lymphocytes (Miyawaki et al. 1992), neutrophils and eosinophils (Hebestreit et al. 1996, Liles et al. 1996, Luttmann et al. 1998). Activation of Fas with Fas ligand, FasL (Luttmann et al. 2000) or with cross-linking antibodies (Matsumoto et al. 1995, Hebestreit et al. 1996, Druilhe et al. 1996) can induce eosinophil apoptosis. Fas ligation with soluble FasL leads to clustering of the death domain of CD 95, initiating a chain of events which culminates in apoptosis. FADD also contains a death effector domain (DED) which binds to an analogous domain in procaspase-8, leading to its autoactivation (Reed 2000). The effects of TNF- α on eosinophil apoptosis are described in greater detail on page 38.

3.2.3 Mitochondria

The mitochondria play a notable role in the regulation of apoptosis. The mitochondrial pathway delivers apoptotic signals caused by various cytotoxic agents, aberrant oncogene expression and p53 (Saraste and Pulkki 2000). A key feature of this pathway is the release of cytochrome C and its binding to the Apaf-1 and the formation of “apoptosome”, which recruits and activates procaspase-9. This results in the processing and activation of other caspases, finally leading to execution of the cell as described above (Green 1998, Wolf and Green 1999, Saraste and Pulkki 2000). A number of cell death proteins such as Bid have also been located in the mitochondria, where they are released upon apoptotic stimulation to activate downstream effectors of the

death process (Bortner and Cidlowski 2002). Additionally, several proteins, e.g. Bcl-2, have been identified which prevent apoptosis specifically at the level of the mitochondria (Bortner and Cidlowski 2002).

The mitochondrion is also the major site for the generation of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxides and radicals (Kannan and Jain 2000). Disruption of the mitochondrial respiratory chain can result in overproduction of ROS, leading to oxidative stress and activation of apoptotic mediators (Green 1998). The release of calcium from the mitochondria by oxidants also occurs in some models of apoptosis. This increase in intracellular calcium may result in stimulation of calcium-dependent enzymes such as nucleases and proteases, to trigger apoptosis (Chakraborti et al. 1999).

Eosinophils contain mitochondria in small numbers and generate mitochondrial membrane potential from hydrolysis of ATP rather than from respiration, as indicated in a study using mitochondrial respiratory inhibitors and mitochondrial uncouplers. The authors proposed that during the differentiation of eosinophils mitochondrial respiration is lost, whereas the other central role of mitochondria, the induction of apoptosis, is retained (Peachman et al. 2001). Recently, loss of mitochondrial membrane potential has been proposed to play a significant role in mediating glucocorticoid-induced eosinophil apoptosis (Letuve et al. 2002, Gardai et al. 2003).

3.2.4 Bcl-2 family members

Bcl-2 is the founding member of a family of proteins with similar sequence identity belonging to a subgroup of anti-apoptotic proteins which includes Bcl-X_L, Bcl-w, Mcl-1, and A-1. This family also includes a subgroup of pro-apoptotic proteins such as Bax, Bak and Bok as well as the so-called “BH3-only” members (Bid, Bad, Bim, Bik, Noxa and Puma), which contain only the BH3 minimal death domain. Together these proteins function primarily at the level of the mitochondria either to prevent or to enhance apoptosis (Adams and Cory 1998, Borner 2003, Opferman and Korsmeyer 2003).

The mechanism by which members of the Bcl-2 family regulate apoptosis has not been completely elaborated. It has been suggested that the “BH3-only” molecules activate and

require the multidomain pro-apoptotic proteins Bax and Bak to release cytochrome c and kill the cells. In contrast, anti-apoptotic Bcl-2 or Bcl-X_L would sequester “BH3-only” molecules and thus block the proapoptotic cascade (Opferman and Korsmeyer 2003).

Druilhe and associates (1998) reported that a high amount of Bax is expressed in freshly purified peripheral blood eosinophils and Mcl-1 expression is increased in interferon (IFN)- γ -treated umbilical-cord-blood eosinophils (defined as immature cells). However, the role of the Bcl-2 family proteins in eosinophil apoptosis or its prevention is not clear and the data reported are partly conflicting (Dibbert et al. 1998, Dewson et al. 1999, Chung et al. 2000, Zangrilli et al. 2000).

3.3 Modulation of eosinophil apoptosis and survival

The eosinophil possesses a considerable array of histotoxic substances, including cytotoxic granule proteins, cytokines and lipid mediators, which contribute to the initiation and maintenance of the allergic inflammatory response. Eosinophil apoptosis is fundamental to the maintenance of a viable immune system (Cohen 1999) and there is an evolving hypothesis that the tissue load of eosinophils in allergic and asthmatic diseases is related to inhibition of or defects in the apoptotic process (Walsh 2000). Eosinophils rapidly undergo apoptosis unless provided with support from eosinophil growth factors such as IL-3, GM-CSF or IL-5 (Wardlaw 2001).

3.3.1 Granulocyte-macrophage colony-stimulating factor, interleukin-3 and -5

GM-CSF, IL-3 and IL-5 are cytokines important for eosinophil survival. They keep eosinophils alive under *in vitro* (Begley et al. 1986, Lopez et al. 1986, Rothenberg et al. 1988, Valerius et al. 1990, Tai et al. 1991, Yamaguchi et al. 1991) and *ex vivo* (Simon et al. 1997) conditions. In culture IL-3, IL-5 and GM-CSF prolong eosinophil survival for up to 2 weeks as well as inhibit apoptosis (Yamaguchi et al. 1991). A study in nasal polyps shows that IL-5 is able to enhance eosinophil survival at tissue level (Simon et al. 1997).

IL-5 modulates the proliferation of various hematopoietic cell lineages, as well as the differentiation, activation and recruitment of eosinophils into inflamed tissues (Clutterbuck et

al. 1989, Dvorak et al. 1989, Clutterbuck and Sanderson 1990, Egan et al. 1996, de Groot et al. 1998, Giembycz and Lindsay 1999, Adamko et al. 2002). In humans, IL-5 is relatively selective for eosinophils and basophils, whereas in the mouse it also acts on B-lymphocytes (Greenfeder et al. 2001). In contrast, IL-3 and GM-CSF promote maturation in number of lymphocyte lines in addition to preventing eosinophil apoptosis (Giembycz and Lindsay 1999, Adamko et al. 2002). The accumulation of eosinophils in the airway mucosa of patients with asthma has been attributed to both increased sequestration and increased longevity. IL-3 and IL-5 have also been shown to enhance eosinophil margination on the endothelial cells of capillaries by the upregulation of adhesion molecules on eosinophils (Giembycz and Lindsay 1999).

3.3.2 Tyrosine kinases and signal transducer and activator of transcription

The signaling pathways involved in growth factor-induced eosinophil survival are complex and include a number of signal transduction molecules. The receptors for IL-3, IL-5 and GM-CSF have a common β -chain and a unique α -chain (van der Bruggen and Koenderman 1996, Giembycz and Lindsay 1999, Geijsen et al. 2001). The β -chain is essential for signal transduction and thus explains the overlapping activities of these cytokines. Cytokine receptors depend on dimerization for their activation, and upon dimerization, multiple tyrosine residues in the β -chain become phosphorylated (Geijsen et al. 2001). In general, the signaling events include the phosphorylation of tyrosine kinases, adapter proteins such as Shc and Grb-2, Ras-MAP kinase pathways, and Jak (janus kinase)- STAT (signal transducer and activator of transcription) pathways (Giembycz and Lindsay 1999). Activation of tyrosine kinases appears to be cell type-dependent (van der Bruggen and Koenderman 1996).

The Lyn and Syk intracellular tyrosine kinases constitutively associate at a low level with the IL-3/IL-5/GM-CSF receptor β subunit in human eosinophils (Yousefi et al. 1996). Stimulation with IL-5 or GM-CSF results in a rapid and transient increase in the amount of Lyn and/or Syk associated with the β subunit of the IL-3/IL-5/GM-CSF receptor (Pazdrak et al. 1995, Yousefi et al. 1996). The latter authors, using an antisense oligodeoxynucleotide technique, indicated a requirement of Lyn and Syk tyrosine kinases for the prevention of apoptosis by IL-5 in human eosinophils. Similarly, Pazdrak and colleagues (1998) reported that Lyn is critical for the antiapoptotic effect of IL-5, showing that Lyn antisense oligodeoxynucleotides reverse the effect of IL-5 on eosinophil survival. Recently, a group under Stafford (2002) reported that the

Src family tyrosine kinases Lyn, Hck, Lck but not Fyn, were expressed in eosinophils. Of these kinases, Lyn was associated with IL-5 R α under basal conditions, but this association was not increased by stimulation with IL-5. In contrast, IL-5 induced IL-5 R α tyrosine phosphorylation upon ligand binding and the association of the β -chain receptor subunit and Lyn was increased after stimulation with IL-5. In addition, Lyn kinase phosphorylates both IL-5 α and β -chain receptor subunits *in vitro* (Stafford et al. 2002).

Jak is a family of cytoplasmic tyrosine kinases associated with cytokine receptors and playing a major role in cytokine signaling. The members of this family include Jak1, Jak2, Jak3 and Tyk2 (Kisseleva et al. 2002). These tyrosine kinases phosphorylate a group of nuclear factors, termed STATs (Wong et al. 2002, Kisseleva et al. 2002). Jak2 has been reported to be activated by stimulation with IL-5 or GM-CSF in human eosinophils (van der Bruggen et al. 1995, Bates et al. 1996, Ogata et al. 1997, Pazdrak et al. 1998, Ogata et al. 1998). Ogata and associates (1998) reported that Jak2 and Jak1 constitutively associate with IL-5 α and β -chain receptor, respectively, and are activated upon IL-5 stimulation. IL-5-induced dimerization of IL-5R subunits caused Jak2 activation. In fact, blockade of Jak2 activity by tyrphostin AG490 has been reported to reverse the survival-prolonging action of IL-5 in rat or human eosinophils (Pazdrak et al. 1998, Chang et al. 2000, Ishihara et al. 2001). It has been demonstrated that IL-3 and GM-CSF induce the activation of STAT5 homologues in various myeloid cell types (Azam et al. 1995, Gouilleux et al. 1995). IL-5 has been reported to induce STAT1 or STAT1 α activation (van der Bruggen et al. 1995, Pazdrak et al. 1995, Ogata et al. 1997) and STAT5 activation in rat eosinophils, HL60 –eosinophilic cells and human eosinophils (van der Bruggen et al. 1995, Ogata et al. 1997, Ogata et al. 1998, Caldenhoven et al. 1999, Bhattacharya et al. 2001, Ishihara et al. 2001). Whether STATs mediate human eosinophil survival and /or apoptosis remains as yet unknown.

3.3.3 Mitogen-activated protein kinases and mammalian sterile 20-like kinase 1

Mitogen-activated protein (MAP) kinases are serine and threonine kinases which can be activated by phosphorylation in kinase cascades. The members include ERK (extracellular-regulated kinase) 1, 2, 5 and 6, JNK/SAPK, and various isoforms of p38 (Cross et al. 2000, Arbabi and Maier 2002, Dong et al. 2002). Activation of the Ras-Raf-1-MEK-MAP kinase pathway in human eosinophils was first proposed by Pazdrak and co-workers (1995), and it was

confirmed by Bates and co-workers (1996) that IL-5 activates a 45 kDa MAP kinase, which most probably corresponds to ERK1, in human eosinophils. Since then activation of both ERK1 and ERK2 has been reported in eosinophils after stimulation by IL-5 (Bracke et al. 1998, Kankaanranta et al. 1999). Recently, the role of the small-molecular-weight G-protein Ras in the activation of ERKs was confirmed by the use of a dominant-negative H-Ras (Hall et al. 2001). Also activation of SH2 protein tyrosine phosphatase 2 (SHPTP-2) and adapter proteins Shc and Grb-2 has been reported in response to IL-5 stimulation (Pazdrak et al. 1997, Bates et al. 1998). SHPTP2 antisense oligonucleotides have been observed to block ERK activation and reverse IL-5-afforded eosinophil survival (Pazdrak et al. 1997). However, the role of MAP kinases in the regulation of eosinophil apoptosis remains controversial (Wong et al. 2000, Hall et al. 2001, Wong et al. 2002). Recently the functional roles of MAP kinases in eosinophils have been characterized using pharmacological inhibitors for ERK1/2 (PD98059) and p38 (SB203580). PD98059 did not block the eosinophil survival induced by IL-5 or GM-CSF, indicating that ERK1/2 are not critical for the anti-apoptotic signal (Kankaanranta et al. 1999, Miike et al. 1999). Adachi and colleagues (2000) reported that neither of the MAP kinases (ERK1/2 and p38) is necessary for the maintenance of eosinophil survival by IL-5. In contrast, inhibition of Ras with a dominant-negative H-Ras resulted in repression of ERK activity and reversal of IL-5-afforded eosinophil survival (Hall et al. 2001). Results from our group have shown that activation of p38 MAP kinase but not that of ERKs prevents spontaneous apoptosis in eosinophils (Kankaanranta et al. 1999). In contrast, p38 MAP kinase seems not to mediate the survival-prolonging activity of IL-5 (Kankaanranta et al. 1999).

The JNK signal transduction pathway is preferentially activated in response to environmental stress and by the engagement of several classes of cell surface receptors, including cytokine receptors, serpentine receptors and receptor tyrosine kinases (Davis 2000). JNK targets are mainly transcription factors, including c-Jun, ATF2, Elk1, c-myc, or p53 (Weston and Davis 2002). Earlier, NO was reported to disrupt JNK mediated Fas-signaling in human eosinophils (Hebestreit et al. 1998). However, the study in question, no direct effect of Fas on JNK activation was shown. Also, sodium salicylate has been reported to induce JNK and p38 MAP kinase activation in human eosinophils as well as to induce apoptosis, but the effects of sodium salicylate on apoptosis could not be reversed by inhibiting JNK and p38 with antisense oligodeoxynucleotides or with the specific p38 MAP kinase inhibitor SB203580 (Wong et al. 2000).

Recently, De Souza and co-workers (2002) have reported that human eosinophils express Mst1 and Mst2 (Ste20-like kinases, mammalian sterile 20-like kinase 1 and 2). Constitutive eosinophil and neutrophil apoptosis were temporally associated with the activation of a 36-kDa and a 34-kDa myelin basic protein kinase, respectively. In addition, a constitutively active 63-kDa myelin basic protein kinase was detected in eosinophils. This p36 kinase activity was identified as being Mst1 and Mst2 and the p36 kinase activity in eosinophils was identified as emanating from the N-terminal catalytic fragment of Mst1. Mst1 and Mst2 were expressed in eosinophils but not in neutrophils. The p36 kinase activation was increased upon exposure to Fas, but attenuated in the presence of IL-5 in eosinophils. Furthermore, catalase and the general caspase inhibitor inhibited spontaneous and Fas-induced activation of the p36 MBP kinase. It was thus concluded that caspase- and H₂O₂-mediated cleavage of Mst1 and the subsequent release of the 36-kDa catalytic fragment have a role in eosinophil apoptosis.

3.3.4 Tumor necrosis factor- α

TNF- α is a proinflammatory cytokine, the concentration of which has been found to be increased in the bronchoalveolar lavage fluid of asthmatic patients (Broide et al. 1992). TNF- α is synthesized by mast cells, neutrophils, monocytes, lymphocytes, macrophages, natural killer (NK) cells, eosinophils and fibroblasts during inflammatory reactions (Camussi et al. 1991, Giembycz and Lindsay 1999, Barnes 2001a).

The effects of TNF- α on target cells are mediated by two receptors: TNF-RI (p55) and TNF-RII (p75), which are present on nearly all cell types tested (Vandenabeele et al. 1995). Eosinophils have been shown to express both of these receptors (Matsuyama et al. 1998). The binding of TNF- α to its receptor complexes activates a variety of biochemical pathways which transfer the signal to transcription factors and other regulatory proteins. Transcription factors known to be activated by TNF- α include nuclear factor- κ B (NF- κ B) and activator protein (AP)- 1 (Montgomery et al. 1991, Westwick et al. 1994).

The effect of TNF- α on human eosinophil apoptosis is somewhat controversial. TNF- α has been reported to enhance eosinophil survival *in vitro* (Tsukahara et al. 1999, Peacock et al. 1999, Temkin and Levi-Schaffer 2001), although an opposite effect has also been described (Valerius et al. 1990, Ward et al. 1999). It has been reported that the survival-promoting effect of TNF- α

was mediated by the production of the autocrine survival cytokine GM-CSF, via activation of both TNF receptors, TNF-RI and TNF-RII. In addition, the activation and nuclear translocation of NF- κ B was found to be an essential step in the TNF- α -induced GM-CSF production by eosinophils (Temkin and Levi-Schaffer 2001). In contrast, inhibition of NF- κ B activation has recently been reported to unmask the ability of TNF- α to induce human eosinophil apoptosis (Ward et al. 1999, Fujihara et al. 2002). Our unpublished data suggest that TNF- α prolongs human eosinophil survival by activating both TNF receptor subtypes and NF- κ B, but that the mechanism does not involve production of GM-CSF (Kankaanranta et al. 2001)

3.3.5 Other cytokines

TGF- β is a pleiotropic cytokine produced by macrophages, monocytes (Assoian et al. 1987), eosinophils (Wong et al. 1991) and other cell types (Duvernelle et al. 2003). TGF- β abrogates the survival-promoting activity of IL-3, IL-5, GM-CSF and IFN- γ on eosinophils (Alam et al. 1994, Atsuta et al. 1995). The mechanism of TGF- β action in eosinophils may involve inhibition of IL-5-signaling at the level of tyrosine phosphorylation of Lyn, Jak2, MAP kinases and STAT1 nuclear factor (Pazdrak et al. 1995). IFN- α (Valerius et al. 1990) and IFN- γ (Valerius et al. 1990, Ochiai et al. 1997, Druilhe et al. 1998) have been reported to enhance the viability of eosinophils or to reduce eosinophil apoptosis.

IL-4, a pleiotropic cytokine with both stimulatory and inhibitory effects on other inflammatory cells (Barnes et al. 1998a), has been reported to induce apoptosis in eosinophils (Wedi et al. 1998) or to have no effect (Hoontrakoon et al. 2002).

IL-9 promotes eosinophilia *in vivo* (McLane et al. 1998, Temann et al. 1998, Dong et al. 1999). Gounni and associates (2000) report that human eosinophils express IL-9 receptor on their surface and that IL-9 significantly inhibits eosinophil apoptosis.

IL-10 is produced by both Th1 and Th2 cells and acts more generally to reduce T-cell activation and cytokine synthesis (Koulis and Robinson 2000). IL-10 is also produced by eosinophils (Nakajima et al. 1996) and other inflammatory cells such as macrophages and B-cells (Koulis and Robinson 2000). IL-10 has been reported to have no direct effect on the survival of

eosinophils (Ward et al. 1997), but it has reduced LPS-induced eosinophil survival (Takanashi et al. 1994).

IL-12 has been observed to increase constitutive eosinophil apoptosis (Nutku et al. 2001) or to have no effect on constitutive eosinophil apoptosis and IL-5-mediated cell survival (Wedi et al. 1998).

IL-13 has also been shown to prolong eosinophil survival *in vitro* (Luttmann et al. 1996).

IL-15 has been found to reduce apoptosis in eosinophils in the presence or absence of TNF- α *in vitro* and the mechanism appeared to involve autocrine production of GM-CSF (Hoontrakoon et al. 2002). However, cytokines such as IL-9, IL-13 and IL-15 are less potent than IL-5 in promoting eosinophil survival.

IL-1 β , IL-2 and IL-8 have been shown not to alter constitutive eosinophil survival (Valerius et al. 1990, Wedi et al. 1998, Hoontrakoon et al. 2002).

3.3.6 Cyclic adenosine 3': 5'- monophosphate-elevating agents

Theophylline is a phosphodiesterase (PDE) inhibitor which enhances cellular cyclic adenosine 3': 5'- monophosphate (cAMP) concentrations by reducing its degradation. Theophylline is commonly used in the treatment of bronchial asthma (Somerville 2001). Recent studies have suggested that theophylline also has anti-inflammatory effects (Simon 2000). It has been reported to inhibit eosinophil survival in the absence of cytokines *in vitro* and partially or completely suppress the IL-5-induced prolongation of eosinophil survival (Adachi et al. 1996, Yasui et al. 1997, Momose et al. 1998). Some other PDE inhibitors (IBMX, rolipram) have been reported to reduce constitutive eosinophil apoptosis (Yasui et al. 1997).

A cell-permeant cAMP analogue, dibutyryl-cAMP, has been observed to prolong eosinophil survival by inhibiting apoptosis (Hallsworth et al. 1996, Yasui et al. 1997, Peacock et al. 1999, Chang et al. 2000, Ward et al. 2002). Prostaglandin E₂ suppresses eosinophil apoptosis, an effect probably mediated by interaction with the EP₂ receptor and an increase in the levels of cAMP (Peacock et al. 1999). Prostaglandin D₂ has been reported to slightly inhibit eosinophil

apoptosis (Gervais et al. 2001) or to increase the rate of eosinophil apoptosis (Ward et al. 2002). The reason why theophylline has the opposite effect on eosinophil apoptosis as compared with other PDE inhibitors and cAMP analogues remains for the present unknown. However, the effects of cAMP-elevating agents on eosinophil survival may depend on prior exposure to GM-CSF (Hallsworth et al. 1996). Chang and co-workers (2000) suggest that the effect of cAMP on eosinophil apoptosis requires the activation and subsequent nuclear localization of protein kinase A and may be partly mediated through the activation of MAP kinase but not of Jak2 (using their inhibitors PD98059 and AG490, respectively). A study by Hebestreit and co-workers (1998) showed that increased cAMP and cyclic guanosine 3': 5' monophosphate (cGMP) concentrations prevent Fas receptor-mediated eosinophil apoptosis. Nielson and Hadjokas (1998) have reported that the β_2 -agonists isoproterenol and salbutamol that increase intracellular cAMP concentrations blocked steroid-induced eosinophil apoptosis.

3.3.7 Lipopolysaccharide

Lipopolysaccharide (LPS) is a Gram-negative bacterial constituent present in the environment. Inhalation of LPS can induce bronchoconstriction in asthmatics and increase bronchial reactivity (Liu 2002). LPS has been observed to enhance eosinophil survival in a dose-dependent manner, and this effect was largely inhibited by an anti-GM-CSF neutralizing antibody, suggesting that it is due to LPS-induced GM-CSF production in eosinophils (Takanashi et al. 1994, Saitou et al. 1997).

3.3.8 Other agents

Cyclosporins A and H have been reported to induce apoptosis in mouse and rat eosinophils (Kitagaki et al. 1996) but not in human eosinophils (Temkin and Levi-Schaffer 2001, Kitagaki et al. 1997). Galectin-9 belongs to the galectin family, which is a member of a newly named growing family of β -galactoside-binding animal lectins (Wada and Kanwar 1997). It evinces potent chemotactic activity on eosinophils but not on neutrophils, lymphocytes or monocytes (Matsumoto et al. 1998). Saita and associates (2002) report that galectin-9 is involved in human eosinophil apoptosis, but has differential effects on eosinophils from patients with hypereosinophilic disease (partially suppress apoptosis) and normal subjects (increase apoptosis). Macrolide antibiotics (Adachi et al. 1996) and tranilast (N- (3,4-

dimethoxycinnamoyl) anthranilic acid) (Cheng et al. 2001) have been found to reduce IL-5-induced-eosinophil survival by inducing apoptosis. Oxatomide is an antiallergic drug which blocks histamine H₁ receptor (Marone et al. 1999). Recently, oxatomide has been reported to promote eosinophil apoptosis and suppress IL-5-mediated eosinophil survival (Domae et al. 2003). Levi-Schaffer and co-workers (1998) have reported that histamine did not influence eosinophil viability in the absence of mast cell sonicate, and that the antihistaminic drugs pyrilamine (anti-H₁) and cimetidine (anti-H₂) did not influence eosinophil viability in the presence of mast cell sonicate. Sodium salicylate has been seen to induce apoptosis in human peripheral blood eosinophils (Wong et al. 2000). Staphylococcal exotoxins inhibit spontaneous eosinophil apoptosis and counteract anti-Fas mAb-induced apoptosis (Wedi et al. 2002). Sodium arsenite is known to disturb the oxygen metabolism in mitochondria. There was a significant increase in the rate of eosinophil apoptosis with low concentrations of arsenite. Furthermore, Fas- and sodium arsenite-induced apoptosis as well as constitutive eosinophil apoptosis could be delayed by specific antioxidants such as glutathione and N-acetyl cysteine, which would imply a role for oxygen-dependent mechanisms in the regulation of eosinophil apoptosis (Wedi et al. 1999). Recently, the role of oxygen metabolites in the regulation of both constitutive and cytokine-promoted eosinophil survival was re-elucidated and it was shown that exogenous H₂O₂ is able to reverse IL-5-afforded eosinophil survival by inducing apoptosis as well as to enhance constitutive apoptosis. Constitutive eosinophil apoptosis has been seen to be inhibited by a reduction in intracellular levels of H₂O₂ by catalase (Kankaanranta et al. 2002, De Souza et al. 2002). Recombinant soluble ICAM-1 has prolonged eosinophil survival in a concentration-dependent manner and β_2 integrin seemed to be involved (Chihara et al. 2000). Lidocaine and its analogues have been reported to inhibit IL-5-mediated eosinophil survival (Okada et al. 1998). Cysteinyl leukotrienes are important molecules which promote airway inflammation (Holgate et al. 2003). Leukotrienes (LTB₄, LTC₄ and LTD₄) have been reported to induce eosinophil survival, and blockade of cysteinyl leukotriene receptor or inhibition of leukotriene synthesis increase constitutive eosinophil apoptosis and reverses GM-CSF-afforded survival (Lee et al. 2000). However, a recent study by Murray and co-workers (2003) has shown leukotrienes (LTB₄, LTC₄ and LTD₄) to have no effect on eosinophil apoptosis.

CD 137 is one of the non-death receptors of the TNF receptor superfamily. Heinisch and co-workers (2001) have reported that CD137 stimulation alone had no effect on eosinophil apoptosis. However, when CD137-expressing eosinophils were stimulated with anti-CD137

monoclonal antibody and GM-CSF (or IL-5), but not IFN- γ , the effect of the survival-promoting cytokine was blocked (Heinisch et al. 2001). Eosinophil apoptosis has also been reported to be increased by ligation of CD69 (Walsh et al. 1996) and CD 45 (Blaylock et al. 1999).

3.4 Neutrophil apoptosis

In vitro studies have identified a variety of agents which modulate neutrophil apoptosis. G-CSF (Begley et al. 1986), GM-CSF (Begley et al. 1986, Brach et al. 1992, Colotta et al. 1992, Lee et al. 1993, Murray et al. 1997, Coxon et al. 1999, Moulding et al. 1998), hypoxia (Hannah et al. 1995), IFN- γ (Colotta et al. 1992), IL-1 β (Colotta et al. 1992), IL-13 (Girard et al. 1996), LTB₄ (Hebert et al. 1996, Murray et al. 1997) and LPS (Colotta et al. 1992, Lee et al. 1993) have been reported to enhance neutrophil survival or inhibit neutrophil apoptosis.

TNF- α has been observed either to induce, to delay, or to have no effect on neutrophil apoptosis (Colotta et al. 1992, Murray et al. 1997, Ward et al. 1999, Yamashita et al. 1999, Salamone et al. 2001). Neutrophils cultured with TNF- α alone evince a low but significant increase in the number of apoptotic cells (Salamone et al. 2001). Results on the effects of IL-6 on neutrophil apoptosis are likewise conflicting; it has been reported either to induce (Afford et al. 1992), not to affect (Brach et al. 1992), or to delay apoptosis (Colotta et al. 1992, Biffl et al. 1995). IL-4 has been reported to enhance neutrophil survival (Girard et al. 1997) or to have no effect (Brach et al. 1992).

IL-3, IL-8, TGF- β , formyl-methionyl-leucyl-phenylalanine (FMLP), rhC5a (Brach et al. 1992, Colotta et al. 1992, Murray et al. 1997) and IL-10 (Ward et al. 1997) have been reported not to affect constitutive neutrophil apoptosis.

3.5 Granulocyte apoptosis in resolution of inflammation

Persistent inflammatory responses may arise from failure or inefficiency of the mechanisms normally responsible for resolution of inflammation and restoration of tissue homeostasis (Haslett 1999). Apoptosis and clearance of apoptotic cells is believed to be involved in resolving inflammation in the lungs and other organs (Grigg et al. 1991, Savill et al. 1992, Cox et al. 1995, Haslett 1999, Walsh et al. 2003, Heasman et al. 2003). The appearance of apoptotic

eosinophils in lung tissue is a matter of debate. Groups under Druilhe (1998) and Vignola (1999), using the TUNEL (in situ end labeling of fragmented DNA) method, have found apoptotic eosinophils in bronchial biopsy specimens from asthmatic patients. Kodama and associates (1998), using electron microscopy and TUNEL methods, observed eosinophils migrating into lung tissue to undergo apoptosis within the subepithelium following ovalbumin challenge in mice. Ohta and colleagues (2001) have also reported that local administration of anti-Fas antibody induced apoptosis in eosinophils infiltrated into the airways in a murine model. In this model apoptotic eosinophils were also found in the lung tissue. A group under Duez (2001) found the number of apoptotic cells to be significantly lower in the lung in Fas-deficient mice than in Fas-sufficient mice. The appearance of apoptotic eosinophils in the lung has been questioned by Uller and co-workers (2001). They reported absence of apoptotic eosinophils in the lung tissue of Sephadex-challenged mice (allergic model) treated with steroids. It is nonetheless clear that apoptotic eosinophils occur in the airway lumen (Woolley et al. 1996, Erjefalt and Persson 2000, Uller et al. 2001).

There may thus be alternative eosinophil clearance pathways such as eosinophil cytolysis and/or eosinophils dwelling in inflamed airway tissue eliminated by migration into the airway lumen (Persson et al. 1999, Erjefalt and Persson 2000, Uller et al. 2001). The luminal eosinophils are still capable of undergoing cytolysis and thus releasing the cell contents to the surrounding space (Persson et al. 1999). The features of eosinophil cytolysis and generation of eosinophil granules in eosinophilic diseases have been described by Persson and Erjefält (1997). They found that free eosinophil granules appeared in diseased tissue close to important target cells and organs, and eosinophil granules might also abound in the airway lumen (Persson and Erjefalt 1997). The presence of eosinophil granules may be the result of non-apoptotic death (named eosinophil cytolysis by Erjefält and co-workers) associated with eosinophil activation and degranulation and disappearance of eosinophil cells (Persson and Erjefalt 1997). Thus, one of the main pathogenetic events in asthmatic inflammation may be the delayed or prevented apoptosis and the uncontrolled activation and cytolytic death of eosinophils with the subsequent release of eosinophil contents to the surrounding tissue. Currently, little evidence is available to show whether eosinophil apoptosis is differentially regulated in asthma. Wedi and colleagues (1997) have shown that the apoptosis of peripheral blood eosinophils isolated from patients with inhalant allergy is delayed as compared with that

of eosinophils from healthy controls. However, the report did not address the question whether the patients were actually suffering from clinically defined asthma.

Macrophages are thought to be one of the most important and efficient cells in the recognition and engulfment of apoptotic cells (Aderem 2002). Proinflammatory mediators such as cytokines, proteolytic enzymes, eicosanoids and reactive oxygen and nitrogen derivatives are released in response to the ingestion of particles (Walsh et al. 1999). Ingestion of apoptotic neutrophils by macrophages without stimulating pro-inflammatory activities of phagocytosing cells thereby provides a removal mechanism for senescent neutrophils which can limit the leakage of toxic compounds of dying cells (Whyte et al. 1993, Hart et al. 1997). In *in vitro* experiment conditions, intact senescent neutrophils have been reported to be recognized and cleared by macrophages (Savill et al. 1989, Hart et al. 1997). *In vitro* experiments also suggest that in the absence of macrophages, apoptotic neutrophils undergo secondary necrosis after a few hours, and later disintegrate and discharge their own contents (Savill et al. 1989). Ishii and co-workers (1998) report that apoptotic neutrophils were engulfed by macrophages during the resolution of acute pulmonary inflammation in rats. Also fibroblasts have been demonstrated to recognize apoptotic neutrophils (Hall et al. 1994). Cytokines are involved in the process of phagocytosis. Proinflammatory cytokines such as GM-CSF increase the proportion of macrophages taking up apoptotic neutrophils and also increase the number of apoptotic neutrophils taken up by each macrophage. IFN- γ , IL- β , TNF- α and TGF- β 1, but not IL-4 and IL-6, also enhance phagocytosis (Ren and Savill 1995).

Apoptotic eosinophils are also recognized and phagocytosed by macrophages (Stern et al. 1992, Stern et al. 1996), thus allowing intact eosinophils to be removed from tissue and avoiding an unexpected proinflammatory response. Tsuyuki and co-workers have observed that Fas receptor activation *in vivo* leads to the recognition of murine lung eosinophils by lung macrophages (Tsuyuki et al. 1995). Also human small-airway epithelial cells have been reported to recognize and ingest apoptotic eosinophils (Walsh et al. 1999).

4 Inhaled glucocorticoids

Glucocorticoids are the most commonly used agents in the treatment of asthma. Inhaled glucocorticoids have become the mainstay of treatment in persistent asthma. Glucocorticoids have a variety of anti-inflammatory effects on inflammatory and tissue cells.

4.1 Mechanism of glucocorticoids action

The basic mechanism of glucocorticoid action is that they penetrate into the cell and bind to glucocorticoid receptor (GR) molecules in the cytoplasm (Pedersen and O'Byrne 1997, Barnes et al. 1998b, Demoly and Chung 1998). The glucocorticoid-GR complex acts as a transcription factor, binding to specific DNA sites in the nucleus. Within the nucleus, GR induces gene transcription by binding to specific DNA sequences known as glucocorticoid response elements (GREs) in the promoter-enhancer regions of steroid-responsive genes. Binding to negative GRE sites results in negative regulation of transcription (Figure 4). However, negative GREs have been described only for relatively few genes (Newton 2000, Leung and Bloom 2003). The glucocorticoid-GR complex may also directly or indirectly interact with transcription factors such as NF- κ B and AP-1, resulting in a transcriptional downregulation (transrepression) (Newton 2000, Leung and Bloom 2003). By these mechanisms glucocorticoids inhibit the transcription of several pro-inflammatory cytokines and chemokines in asthma (Newton 2000, Leung and Bloom 2003). They may, however, also work via mechanisms not involving the GR, i.e. interaction with cell-membrane receptors or direct physicochemical interaction with the plasma membrane (Buttgereit and Scheffold 2002).

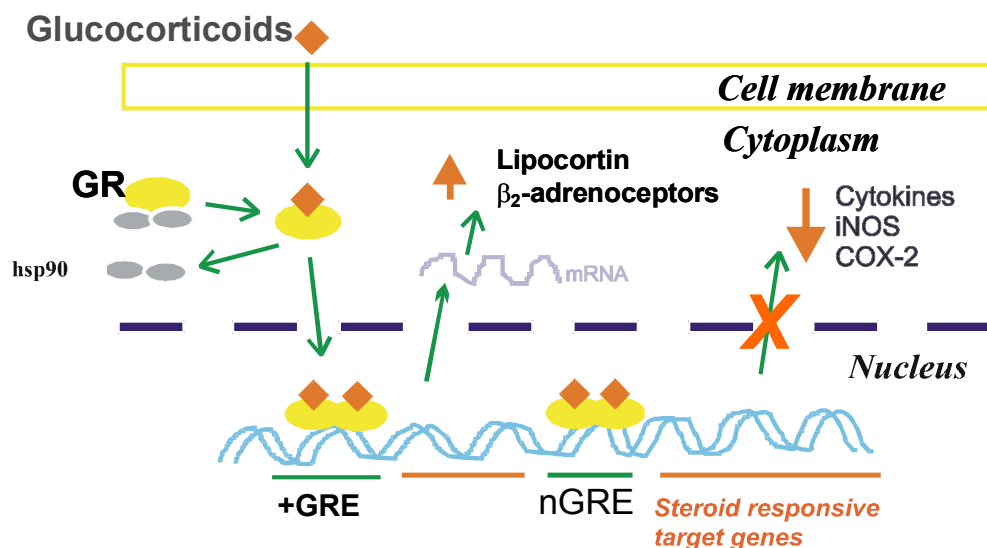


Figure 4. Classical model of glucocorticoid action. Modified from Barnes (1998a).

The basic unit of chromatin is the nucleosome, where DNA is wrapped around core histone proteins. The functional consequence of chromatin packaging is to limit access of transcription factors to the DNA. There is evidence that gene transcription is associated with an increase in histone acetylation resulting in unwinding of the DNA, whereas hypoacetylation is correlated with reduced transcription (Johnson and Turner 1999, Leung and Bloom 2003). Recently, Ito and co-workers (2000) have reported that GR recruitment of histone deacetylase 2 inhibits IL-1 β -induced histone H4 acetylation on lysines 8 and 12. These data suggest that anti-inflammatory glucocorticoids interact with transcriptional activation of inflammatory genes by inhibiting histone acetylation.

Another mechanism of action of glucocorticoids has recently been described (Newton et al. 1998, Korhonen et al. 2002, Staples et al. 2003), showing that dexamethasone regulates protein expression at the level of mRNA stability.

4.1.1 Cellular effects of glucocorticoids

Steroids have a number of anti-inflammatory effects on inflammatory and structural cells in pulmonary and airway diseases (Table 2).

Table 2 The effects of glucocorticoids on inflammatory and structural cells

| Cells | Effects | References |
|------------------------|---|--|
| Macrophage | mediators and cytokines ↓ | (Martinet et al. 1992, Standiford et al. 1992, Linden and Brattsand 1994) |
| Eosinophils | mediators ↓, numbers (survival) ↓ | (Kita et al. 1991) (Lamas et al. 1991, Wallen et al. 1991, Adachi et al. 1996, Meagher et al. 1996) |
| T-lymphocytes | cytokines ↓ proliferation ↓ number ↓ | (Barnes 1998a, Melis et al. 2002) |
| Mast cells | mucosal mast cell number ↓ | (Laitinen et al. 1992, Yoshikawa and Tasaka 2000) |
| Dendritic cells | numbers in nasal mucosa ↓ | (Holm et al. 1995) |
| Neutrophils | numbers ↓ mediators ↓ numbers ≈ or ↑ | (Confalonieri et al. 1998) (Inoue et al. 1999) |
| Endothelial cells | leak ↓ adhesion molecules ↓ | (Boschetto et al. 1991, Erjefalt and Persson 1991) |
| Epithelial cells | cytokines and mediators ↓ number ↓ proliferation ↓ | (Kwon et al. 1994a) (Kwon et al. 1994b, Dorscheid et al. 2001) |
| Submucosal gland cells | mucus secretion ↓ | (Shimura et al. 1990) |

4.2 Effects of glucocorticoid on asthma and chronic obstructive pulmonary disease

Glucocorticoids provide the most effective anti-inflammatory treatment currently available for asthma (Haahtela et al. 1991, Laitinen et al. 1992, Barnes et al. 1998b, Haahtela 2002). The importance of early introduction of inhaled glucocorticoids as first-line treatment for subjects with mild disease has recently been recognized (Haahtela et al. 1991, Laitinen et al. 1992, Haahtela et al. 1994, Barnes 1998b, Haahtela 1999). Patients with mild to severe asthma respond to inhaled glucocorticoids as assessed by reduced asthma symptoms and improved lung function as well as reduced bronchial hyperreactivity and eosinophilic inflammation (Barnes et al. 1998b).

The role of glucocorticoids in the treatment of COPD is, in contrast, controversial (Barnes 2003). Recent studies suggest that inhaled corticosteroids have no beneficial effect on the rate of progressive decline of lung function in patients with COPD (Vestbo et al. 1999, Pauwels et al. 1999), although a slight effect on COPD exacerbations has been reported (Burge et al. 2000). It has been suggested that the benefit of steroids in COPD is restricted to effects on the eosinophilic component in the inflammation (Barnes 2002). In addition, some studies suggest that the quality of life of COPD patients is improved during steroid treatment (Postma and Kerstjens 1999). It has been recommended that most patients with COPD should not be treated

with inhaled corticosteroids in view of the significant risk of systemic adverse effects, these including skin bruising and osteoporosis (Kerstjens et al. 2001, Barnes and Woolcock 1998c).

4.3 Glucocorticoid effects on human eosinophils apoptosis

It has been shown that glucocorticoids inhibit the formation of eosinophil-rich colonies *in vitro* in bone marrow colony assays. This may be a result of tempering the production and/or release of IL-5 and other eosinopoietic factors from other cells (Giembycz and Lindsay 1999). However, in some experimental conditions glucocorticoids have been reported to enhance eosinophil production from bone marrow cells (Gaspar et al. 2000).

Inhaled glucocorticoids have been reported to reduce the numbers of circulating and tissue eosinophils in asthma (Barnes et al. 1998b). In an open non-placebo-controlled study oral (prednisolone) and inhaled glucocorticoids (beclomethasone) reduced the number of eosinophils in sputum with a concomitant increase in eosinophil apoptotic index (Woolley et al. 1996). In another study, the eosinophil numbers in bronchial biopsies from steroid-treated patients with asthma were reduced and the apoptotic index was higher than in untreated asthma patients (Druilhe et al. 1998). The induction of eosinophil apoptosis may be one of the mechanisms whereby glucocorticoids reduce eosinophil counts. However, there is only limited direct evidence of the effect of glucocorticoids on eosinophil apoptosis or survival. High concentrations of glucocorticoids have been reported to reduce eosinophil survival or induce apoptosis *in vitro*, and most of the relevant data are limited to dexamethasone (Lamas et al. 1991, Wallen et al. 1991, Druilhe et al. 1996, Kitagaki et al. 1996, Meagher et al. 1996, Nielson and Hadjokas 1998, Nittoh et al. 1998). However, it remains unknown whether these effects are obtained at clinically relevant drug concentrations.

Glucocorticoids suppress the synthesis of survival-prolonging cytokines and inhibit eosinophil migration, and may thus reduce blood and tissue eosinophilia. In addition, glucocorticoids have been shown to inhibit many of the effects of these cytokines on mature eosinophils. However, the relationship between glucocorticoids and eosinophils involves synergistic as well as antagonistic aspects, and might depend on the maturation stage of the target cells. Glucocorticoids can actually potentiate the eosinopoietic actions of GM-CSF and IL-5 in the bone marrow (Gaspar et al. 2000). It has been claimed that glucocorticoids reverse IL-3/IL-

5/GM-CSF-afforded eosinophil survival (Hallsworth et al. 1992, Schleimer and Bochner 1994). However, this conception is based on limited data. High concentrations of dexamethasone (10^{-7} - 10^{-6} M) and methylprednisolone (10^{-6} M) have been reported to partly reverse IL-5-afforded survival, but the effect of steroids falls off as the concentration of IL-5 increases (Wallen et al. 1991, Adachi et al. 1996).

More recently, the effects of several currently used inhaled or systemic steroids on cytokine-afforded eosinophil survival were studied and they were found to reduce it significantly, but the effect of glucocorticoids was reduced or abolished by higher concentrations of cytokines (Hagan et al. 1998). Another study investigated the effects of glucocorticoids on eosinophil survival induced by epithelial cell-conditioned media obtained by using epithelial cells isolated from nasal mucosa or nasal polyps. The cytokine(s) mediating the prolonged eosinophil survival was not identified. Interestingly, glucocorticoids were able to reverse eosinophil survival induced by epithelial cell-conditioned media from nasal mucosa, while eosinophil survival induced by epithelial cell conditioned media from nasal polyps seemed to be much more resistant to glucocorticoids (Mullol et al. 1997). However, in both of these studies the mode of eosinophil death was not analyzed.

The mechanism whereby steroids enhance eosinophil apoptosis remains largely unknown, but is thought to involve GR (Meagher et al. 1996, Nittoh et al. 1998), loss of mitochondrial membrane potential and the antioxidant protein Mn superoxide dismutase (Letuve et al. 2002, Gardai et al. 2003) and activation of JNK, together with a decrease in the amount of antiapoptotic protein X-linked inhibitor of apoptosis (Gardai et al. 2003).

4.4 Glucocorticoid effects on human neutrophil apoptosis

Oral and topical glucocorticoids reduce tissue eosinophilia in a wide range of eosinophilic conditions in a dose-dependent manner. In contrast, they have little effect on neutrophils. In some studies in patients with asthma and COPD, glucocorticoids have been reported not to reduce, but even to increase, neutrophil numbers in induced sputum (Hoshino and Nakamura 1996, Inoue et al. 1999, Keatings et al. 1997a, Keatings et al. 1997b). Glucocorticoids are relatively ineffective in most respiratory diseases characterized by neutrophilic inflammation (Keatings et al. 1997b). Dexamethasone (10^{-8} - 10^{-6} M) and budesonide (10^{-8} - 10^{-6} M) have

been shown to inhibit human neutrophil apoptosis (Cox 1995, Liles et al. 1995, Kato et al. 1995, Meagher et al. 1996, Cox and Austin 1997) and this effect appears to be mediated via GR.

5 Nitric oxide

Before the 1980s, nitric oxide (NO) was known as a noxious gas found in air pollution, cigarette smoke and automobile exhaust. In 1987, endothelium-derived relaxing factor (EDRF) was found to be NO (Ignarro et al. 1987, Palmer et al. 1987). NO has many physiological and pathophysiological functions in the body, for example regulation of vascular tone, platelet aggregation and the immune response (Moilanen et al. 1999). In general, NO is produced at high levels in the immune system and has host-protective effects during infection and tissue-damaging effects during autoimmune responses (Moilanen et al. 1999, Bogdan 2001).

5.1 Nitric oxide and the immune response

NO is synthesized from the amino acid L-arginine by enzymes called NO synthases (NOS). These enzymes are essential constituents (constitutive NOS) of a wide variety of cells and they are also generated *de novo* (inducible NOS) in response to certain stimuli, including various inflammatory mediators. The constitutive NOS are of two major types, endothelial and neuronal. Endothelial NOS (eNOS or type III NOS) are bound to cell membranes, e.g. in vascular endothelial cells, platelets, myocardium and endocardium, mast cells and neutrophils. Neuronal NOS (nNOS or type I NOS) are present in the cytosol of neuronal cells and in skeletal muscle. Inducible NOS (iNOS or type II NOS) have been detected in many cell types, including inflammatory and epithelial cells (Becherel et al. 1994, Robbins et al. 1994, Lundberg et al. 1995, Moilanen et al. 1997).

The biological effects of NO can be divided into cGMP-mediated and cGMP-independent mechanisms. Although NO can affect cellular functions through posttranslational modifications of proteins directly (nitrosylation and nitration) and indirectly (methylation and ribosylation), its main physiological signaling pathway is held to be the activation of guanylyl cyclase, the formation of cGMP and concomitant protein phosphorylation via the activation of protein

kinase G (Pieper et al. 1999). NO might play a role not only in regulating cell respiration but also in augmenting the generation of ROS by mitochondria, and thus triggering mechanisms of cell survival or death (Moncada and Erusalimsky 2002).

5.2 Nitric oxide and asthma

Endogenous NO has been suggested to play an important role in the physiological regulation of the airways (Kharitonov and Barnes 2003). In the early 1990s, Alving and colleagues (1993) first reported increased amounts of NO in exhaled air in asthmatics compared with normal subjects, a finding confirmed by other studies (Kharitonov et al. 1994, Persson et al. 1994). The increased concentration of NO in air exhaled by asthmatic patients has been shown to decrease toward normal concentrations after treatment with steroids (Massaro et al. 1995, Kharitonov et al. 1996b, Kharitonov et al. 1996a, van Rensen et al. 1999, Lehtimäki et al. 2001a, Lehtimäki et al. 2001b, Kharitonov et al. 2002). Furthermore, there is evidence that the concentration of exhaled NO is associated with asthma severity (Stirling et al. 1998). Moreover, the concentration of NO derivatives in induced sputum has been observed to be significantly higher in patients with asthma than in normal control subjects (Kanazawa et al. 1997). The increase in the exhaled NO derived from the bronchial compartment has been shown to accompany eosinophilic inflammation and to correlate with other indices of inflammation in asthma (Jatakanon et al. 1998, Mattes et al. 1999, Lehtimäki et al. 2001a, Lehtimäki et al. 2001b). Thus, measurement of exhaled NO amounts provides a reliable and effective marker reflecting airway inflammation in asthma (Kharitonov and Barnes 2000).

5.3 Nitric oxide and eosinophils

There are few data on the effect of NO on eosinophil functions and survival, and NO production by activated eosinophils remains controversial. Arock and associates have reported that the NOS inhibitor N^G-monomethyl-L-arginine inhibited IgE/antiIgE-stimulated production of TNF- α and the NO donor SIN-1 increased TNF- α levels in human bone marrow eosinophil cultures (Arock et al. 1994). The presence of iNOS in human eosinophils has been detected by molecular techniques (del Pozo et al. 1997). NO has been reported to disrupt Fas receptor signaling in human eosinophils, suggesting that it may affect eosinophil survival (Hebestreit et al. 1998). Beauvais and co-workers (1995b) reported that the NO donors sodium azide and

hydroxylamine as well as cGMP analogues prolong human eosinophil survival. Subsequently Beauvais and Joly (1999) reported that some other NO-donors (SIN-1, SNAP, S-nitroso-L-cysteine and NOC-18) did not support eosinophil survival, but rather induced apoptosis or necrosis. However, the addition of hematin with small concentrations of NO-donors to produce a nitroxyl heme resulted in increased eosinophil survival. Furthermore, they noted that the NO-donors S-nitroso-L-cysteine and NOC-18 reduced the survival of IL-5 primed eosinophils. In contrast, a group under Peacock et al. (Peacock et al. 1999) reported that NO donors SNAP (1 and 100 μ M) and DETANONOate did not alter the rates of spontaneous eosinophil apoptosis.

AIMS OF THE STUDY

The general aim here was to study the regulation of human eosinophil apoptosis in asthma and the effect of glucocorticoids and NO on eosinophil or neutrophil cell death. The specific undertakings were:

1. To examine whether peripheral blood eosinophil apoptosis differs between asthma patients and healthy individuals.
2. To investigate the effects of inhaled glucocorticoids, beclomethasone, budesonide, fluticasone and mometasone, on constitutive human eosinophil and neutrophil apoptosis as well as the role of GR in the glucocorticoid effect.
3. To characterize the effects of glucocorticoids on eosinophil and neutrophil apoptosis in the presence of survival-prolonging cytokines.
4. To clarify the effect of NO on apoptosis in IL-5-treated or cytokine-deprived human eosinophils, and to elucidate the mechanisms of action of NO.

MATERIAL AND METHODS

1 Patients and donors

1.1 Patient characterization (I)

The study cohort comprised 16 healthy individuals (11 men, ages 33 ± 2 years) and 34 patients with asthma (27 men, ages 34 ± 2 years). Of the asthmatic patients, 21 were not on corticosteroids, 13 were using regular inhaled corticosteroids. All asthma patients not on inhaled corticosteroids used intermittent β_2 -agonist medication (salbutamol 100 μg 1-2 puffs as needed). They were asked to refrain from β_2 -agonist use overnight (i.e., for 10 hours) prior to sampling. Patients on regular inhaled corticosteroids used beclomethasone (mean 933 ± 353 μg , $n = 3$), budesonide (mean 866 ± 256 μg , $n = 7$), or fluticasone (mean 667 ± 167 μg , $n = 3$). One patient on fluticasone was also using oral prednisolone (5 mg daily). Of those patients using inhaled corticosteroids, 3 also used regular inhaled salmeterol (50 μg twice daily) and 2 regular formoterol (12 μg twice daily). Patients on regular inhaled glucocorticoids were allowed to take their regular medication (both steroids and β_2 -agonists) 1 hour before sampling. All patients not using steroids had either mild intermittent or mild persistent asthma and of those on inhaled steroids 5 were rated as having mild persistent, 7 as having moderate persistent, and 1 as having severe persistent asthma according to the 1997 classification (Murphy et al. 1997). In experiments evaluating the effects of β_2 -agonists, theophylline and dibutyryl-cyclic AMP and in the washout experiments with GM-CSF, IL-5 and salbutamol, eosinophils from healthy volunteers were used. All subjects gave informed consent.

1.2 Donor characterization (II-V)

The buffy-coat preparations were made from the blood of normal healthy donors obtained from the Finnish Red Cross blood transfusion service center (Tampere).

1.3 Ethical aspects (I-V)

The study protocol was approved by the ethical committees of Tampere University Hospital (Tampere, Finland) and Royal Brompton Hospital Ethics Committee (London, UK). Permission

for the use of buffy-coat preparations was obtained from the ethical committee of the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland).

2 Eosinophil purification and culture

Eosinophils were isolated under sterile conditions. Briefly, venous blood from healthy or asthmatic individuals (I) or a buffy-coat preparation (50 ml) from normal individuals (II, IV, V) was collected into 10 ml of acid citrate dextrose anticoagulant and hydroxyethyl starch solution. The white blood cell pellet was laid on Ficoll and centrifuged at 700g for 30 min at 20 °C. The mononuclear cell layer was discarded and the phagocyte pellet resuspended and washed in Hank's balanced salt solution (HBSS). Contaminating red blood cells were removed by hypotonic lysis. The remaining granulocytes were washed twice with RPMI 1640. Following counting and resuspension in 300 µl of RPMI 1640 (2% fetal calf serum and 5 mM EDTA), eosinophils were purified using immunomagnetic anti-CD16 antibody conjugated beads to remove CD16-positive neutrophils. 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, the purity of the eosinophil population being > 99 %. The main contaminating cells were mononuclear cells and neutrophils. The eosinophils were resuspended at 1×10^6 cells /ml, cultured (37 °C, 5 % CO₂) for the indicated time in RPMI 1640 (Dutch modification) with 10 % fetal calf serum and antibiotics (I, II, IV, V). The cells for testing the effect of NOS inhibitor were cultured in L-arginine, nitrate and nitrite-free Dulbecco's MEM supplemented with 100 µM L-arginine (for nitrite production and NOS inhibitor cultures), 10% fetal calf serum and antibiotics (V).

3 Neutrophil isolation and culture

Granulocytes were isolated as above to obtain neutrophils. These were washed twice with RPMI 1640. The neutrophils were counted using microscopic examination in Kimura stain, and the purity of the neutrophil population being > 98 %. The cells were resuspended at 2×10^6 cells /ml and cultured for the indicated time (37 °C; 5 % CO₂) in RPMI 1640 (Dutch modification) with 10 % fetal calf serum and antibiotics (III, IV).

4 Determination of eosinophil and neutrophil apoptosis and viability by flow cytometry

4.1 Propidium iodide staining (I-V)

Apoptosis was determined by propidium iodide staining of DNA fragmentation and flow cytometry (FACScan, Becton Dickinson, San Jose, CA). Briefly, after culture for the time indicated, eosinophils 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 prior to flow cytometric analysis. Neutrophils were washed in phosphate buffered saline (PBS) solution, fixed by 70% ethanol and incubated on ice for 30 min. The pellet was resuspended in propidium iodide solution (25 µg/ml in PBS) and measured by flow cytometry. The excitation and emission wavelengths were 488 nm and 550 nm. Cells showing decreased relative DNA content were considered apoptotic (Kankaanranta et al. 2000).

4.2 Annexin-V (IV-V)

Annexin-V binding and analysis by flow cytometry were performed according to the instructions of the manufacturer as previously described (Sandstrom et al. 2000). Briefly, the cells (2×10^5) were washed in PBS solution and suspended in 195 µl of binding solution (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). Five µl of annexin-V fluorescein isothiocyanate (annexin-V-FITC) (solution containing 50 mM Tris, 100 mM NaCl, 1% bovine serum albumin, 0.02% Sodium Azide, pH 7.4) was added and the cell suspension incubated at room temperature for 10 min. The cells were then washed and resuspended in binding buffer containing 10 µg/ml propidium iodide and analyzed by flow cytometry. Cells showing positive staining with annexin-V were considered to be apoptotic.

5 Determination of DNA fragmentation (II, V)

Oligonucleosomal DNA fragmentation was analyzed by agarose gel DNA electrophoresis as previously described (Kankaanranta et al. 1999, Kankaanranta et al. 2000). Eosinophils ($2 \text{ ml of } 1 \times 10^6/\text{ml}$ in RPMI 1640) were cultured for the time indicated. The cell pellet (350 g, 7 min) was suspended in 0.5 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg/ml proteinase K, pH 8.0). Samples were

incubated at 50 °C for 12 h. The solution was first extracted with phenol: chloroform: isoamyl alcohol (25:24:1; v:v:v), buffered with Tris-EDTA buffer (pH 8.0). Following 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 12000 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, the gels were visualized by ultraviolet light and photographed.

6 Morphological analysis (II-V)

Eosinophils or neutrophils were spun onto cytospin slides (500g, 7 min) and stained with May-Grünwald-Giemsa after fixation in methanol. Cells showing features typical of apoptosis, i.e. cell shrinkage, nuclear coalescence and nuclear chromatin condensation were considered apoptotic (Haslett 1997, Kankaanranta et al. 2000).

7 Measurement of nitrite concentrations (V)

Nitrite was measured as previously described by adding 100 µl of Griess reagent to 100 µl samples of medium (Green et al. 1982). The optical density at 546 nm was measured by a microplate reader using sodium nitrite as standard.

8 RNA extraction and reverse transcriptase polymerase chain reaction (V)

Eosinophils stimulated with the compounds of interest were pelleted by centrifugation after the desired time of incubation. Cell pellets were washed twice with PBS, lysed and purified using QIAshredder™ (Qiagen Inc., Santa Clarita, CA). Thereafter RNA was extracted using an Rneasy kit for isolation of total RNA (Qiagen Inc., Santa Clarita, CA). Synthesis of cDNA from extracted RNA and subsequent amplification of cDNA were performed with a GeneAmp ThermoStable tTth Reverse Transcriptase RNA PCR Kit (Perkin-Elmer, Roche Molecular Systems Inc, Branchburg, NJ.). First strand cDNA was synthesized using sequence-specific

downstream primer for human iNOS or human β -actin, which was used as reference. The upstream primer was added to the reaction mixture at the beginning of PCR amplification.

9 Immunoblot analysis (V)

Eosinophils were suspended at 10^6 cells/ml and cultured at 37 °C. The cells were harvested at the designated time points and centrifuged at 12000 g for 10 min. The cell pellet was lysed by boiling for 5 min in 30 μ l of Laemmli buffer (X6) and centrifuged at 10000 g and debris was carefully removed. Samples were then stored at -20 °C until immunoblot analysis. For this procedure, 30 μ l of each protein sample was separated by SDS-PAGE on 8-12% polyacrylamide mini-gel and electrophoresed for 2 h at 100 V in buffer containing 95 mM Tris-HCl, 960 mM glycine and 0.5% SDS. The separated proteins were transferred to nitrocellulose (Hybond™ ECL™) with semi-dry blotter at 2.5 mA/cm² for 45 min in buffer containing 25 mM Tris-base, 192 mM glycine and 20% methanol, blocked using 5% non-fat dry milk in TBS/T. Proteins were labeled using specific antibody (1:500) and subsequently detected using a 1:2000 dilution of horseradish peroxidase-linked anti-rabbit or anti-mouse IgG and imaged on film using ECL™ Western blotting detection systems.

After detection of phospho-STAT5 as above, the membrane was stripped at 50 °C for 30 min (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl) and washed with TBS/T. Total STAT5 protein was labeled using antibody against STAT5 and detected as above.

10 Materials

The materials used in the study were obtained as follows: 8-bromo-cAMP, 8-bromo-cGMP, antibiotics, beclomethasone monopropionate, budesonide, dibutyryl-cAMP, dibutyryl-cGMP, dexamethasone, hydrocortisone, mifepristone, prednisolone, prednisone, propidium iodide, salbutamol, theophylline, triton X-100 and zaprinast were from Sigma Chemical Co., St. Louis, MO, USA, fluticasone propionate, and salmeterol from Glaxo-Wellcome, Durham, U.K, GM-CSF, IgG₁ isotype control, IL-3, IL-5, IL-6, TNF- α and neutralizing antibodies for human IL-3, IL-5 and GM-CSF from R&D system Europe, Abingdon, U.K; Carboxy-PTIO, L-arginine, L-JNKI1, L-NIO, L-TAT control peptide, and SNAP were provided by Alexis Corp., Laüfelfingen, Switzerland, L-NMMA by Global External Scientific Affairs, Cheshire, U.K and

AC-DMQD-CHO, AC-IETD-CHO, AC-LEHD-CHO and Z-Asp-CH₂-DCB by Peptide Institute, Inc, Osaka, Japan. The Annexin V-FITC kit was from Bender medSystems, Vienna, Austria, anti-CD16 microbeads and magnetic cell separation system from Miltenyi Biotec Ltd., Surrey, U.K, Ficoll-Paque from Pharmacia AB, Uppsala, Sweden, GEA 3175 from GEA Ltd., Copenhagen, Denmark, Giemsa from J.T.Baker, Deventer, The Netherlands and IBMX from EGA-Chemie, Steinheim, Germany. Fetal calf serum, RPMI 1640 and L-arginine-free DMEM were obtained from Gibco BRL, Paisley, Scotland, U.K, LTB₄ from Calbiochem, La Jolla, CA, USA, May-Grünwald from Merck, Darmstadt, Germany, mometasone furoate from Schering-Plough, Kenilworth, LA, USA, ODQ from Tocris, Bristol, UK, polyclonal Mcl-1 antibody from PharMingen, San Diego, CA, USA; polyclonal Bax, JNK1 (FL) and STAT5b and monoclonal caspase-3 and pJNK antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, USA, monoclonal caspase-8 and Bcl-2 antibodies from Oncogene Research Products, Boston, MA, USA, monoclonal caspase-9 antibody from NeoMakers, Fremont, CA, USA, polyclonal phospho-STAT5 from New England Biolabs, Hitchin, UK and horseradish peroxidase-linked anti-rabbit or anti-mouse IgG from Amersham Life Science, Amersham, UK. 1400W was a kind gift from Dr. R.G. Knowles (Glaxo-Wellcome, Stevenage, UK).

SNAP and GEA 3175 were dissolved in DMSO, dexamethasone, β_2 -agonists and theophylline 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 of eosinophils or neutrophils. The final concentration of DMSO in the culture was 0.2-0.5% and was found not to affect apoptosis in eosinophils.

11 Statistics

Results are expressed as means \pm S.E.M. Apoptosis is expressed as apoptotic index (number of apoptotic cells/total number of cells) or percentage of apoptotic cells. EC₅₀ 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 test or Dunnet's *t*-test. Differences were considered significant at $P < 0.05$.

SUMMARY OF RESULTS

1 Spontaneous eosinophil and neutrophil apoptosis

When eosinophils isolated from blood from healthy donors were cultured in cytokine-deprived conditions for 18 h, the apoptotic index was 0.15 ± 0.01 (n=60, II, IV, V) as assessed by flow cytometry measuring the relative DNA content in propidium iodide-stained cells. Using morphological analysis, an apoptotic index of 0.16 ± 0.03 (n=32, II, IV, V) was obtained. When the incubation time was increased to 40 h, the index was 0.40 ± 0.03 as assessed by flow cytometry measuring the relative DNA content in propidium iodide-stained cells (n=12, IV).

When neutrophils were cultured in cytokine-deprived conditions for 16 h, the apoptotic index was 0.51 ± 0.02 (n=56, III, IV) as assessed by flow cytometry measuring the relative DNA content in propidium iodide-stained cells.

2 Effect of cytokines on eosinophil and neutrophil apoptosis

IL-5, GM-CSF and to a lesser extent also TNF- α promoted eosinophil survival in a concentration-dependent manner during 40 h incubation by delaying apoptosis (Figure 5). The maximal anti-apoptotic effect of IL-5 was obtained at a concentration of 1-10 pM (apoptotic index 0.12 ± 0.02 and 0.44 ± 0.13 , in the presence and absence of IL-5, respectively; n=5). The maximal anti-apoptotic effect of GM-CSF was obtained at a concentration of 7 pM (apoptotic index 0.04 ± 0.01 and 0.40 ± 0.01 , in the presence and absence of GM-CSF, n=3). The maximal anti-apoptotic effect of TNF- α was obtained at 600 pM (apoptotic index 0.31 ± 0.02 and 0.43 ± 0.03 , in the presence and absence of TNF- α , n=5). IL-3 (100 pM) also promoted eosinophil survival by delaying apoptosis (apoptotic index 0.15 ± 0.03 and 0.51 ± 0.14 , in the presence and absence of IL-3, respectively; n=4).

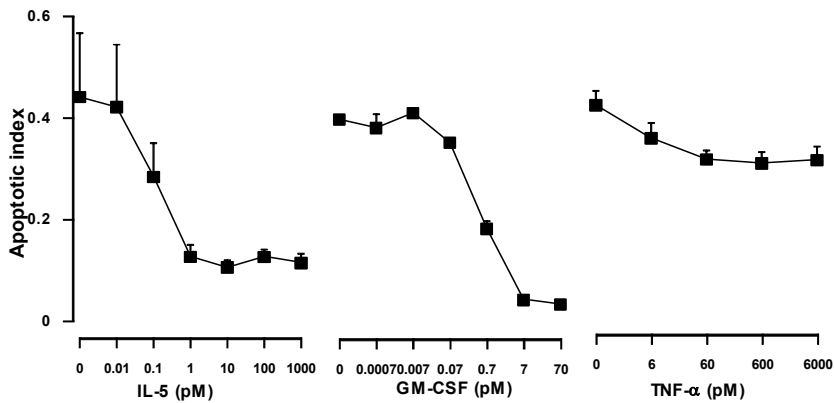


Figure 5. Effects of different cytokines on human eosinophil apoptosis. Eosinophils were cultured in the absence or presence of cytokines for 40 h and apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained eosinophils. Each data point represents the mean \pm S.E.M. of 3-5 independent determinations using eosinophils from different donors.

The maximal inhibitory effect of GM-CSF on neutrophil apoptosis was obtained at a concentration of 0.7 nM during 16 h incubation as assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained cells (III, IV). LTB₄ inhibited neutrophil apoptosis in a concentration-dependent manner within a concentration range of 0.1– 100 nM (IV). IL-6 (0.08-8 pM) did not significantly inhibit neutrophil apoptosis during 16 h incubation (IV).

3 Differences in eosinophil apoptosis between asthmatic patients and healthy controls (I)

Circulating eosinophils from asthmatic patients not taking steroid medication survived longer than those from healthy subjects (Figure 6). In contrast, the rate of apoptosis in eosinophils from patients concurrently taking steroids was higher than that of cells from patients not using steroids (Figure 6), and not different from that of cells from healthy subjects.

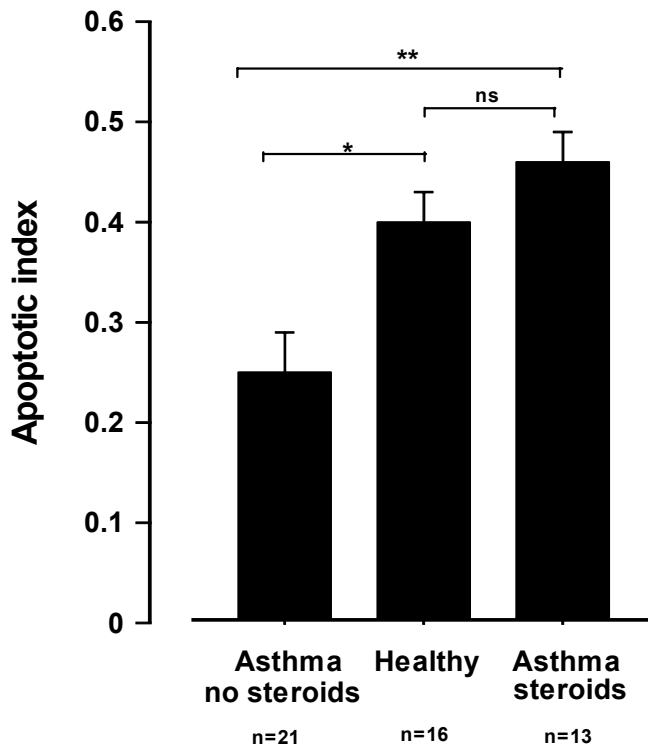


Figure 6. Delayed eosinophil apoptosis in asthma.

* indicates $P < 0.05$ and ** $P < 0.01$ as compared with asthma patients not taking steroids. Mean \pm S.E.M.

To assess whether endogenous IL-3, IL-5 and GM-CSF production contributed to the delayed eosinophil apoptosis in asthma patients, the effects of the corresponding neutralizing antibodies on eosinophil longevity were studied. Neutralization of GM-CSF, but not of IL-3 or IL-5, significantly increased ($P < 0.01$) the rate of apoptosis in eosinophils obtained from patients with asthma.

To assess whether β_2 -agonist medication could contribute to the differences observed, study was made of the effects of salbutamol, fenoterol and salmeterol on eosinophil apoptosis. All β_2 -agonists inhibited eosinophil apoptosis in a concentration-dependent manner (maximal inhibition $\approx 15\%$).

To establish whether prior exposure *in vivo* to IL-5, GM-CSF or β_2 agonists would explain the differences, eosinophils were incubated with GM-CSF, IL-5 and salbutamol for 2 to 3 hours,

followed by washout of the added compounds, and were subsequently cultured for 48 hours. Exposure to GM-CSF (7 pM) or IL-5 (10 pM) for 15 to 180 minutes was not a sufficient signal to prevent eosinophil apoptosis. In contrast, exposure to salbutamol (100 nM) for 120 minutes was sufficient to induce a slight but statistically significant ($P < 0.05$) decrease in eosinophil apoptosis.

The results show that eosinophil apoptosis is delayed in asthma and that the delay may be partly explained by production of GM-CSF. The *in vitro* effects of β_2 -agonists suggest that β_2 -agonists might also contribute to prolonged eosinophil survival through inhibition of apoptosis.

4 Effects of glucocorticoids on eosinophil and neutrophil apoptosis

In the further studies, the effects of the currently used inhaled glucocorticoids on human eosinophil and neutrophil apoptosis were measured. Glucocorticoids significantly enhanced constitutive eosinophil apoptosis (Figure 7, II, IV) in the absence of cytokines. The order of potency of glucocorticoids in enhancing eosinophil apoptosis was fluticasone \approx budesonide \approx mometasone $>$ beclomethasone $>$ dexamethasone. Fluticasone, beclomethasone, budesonide and mometasone produced 1.5-2.1-fold increases in the proportion of apoptotic eosinophils in culture and the degree of maximal enhancement did not differ among these compounds. The effects of budesonide and fluticasone were manifested at concentrations similar to those found in plasma after inhalation of the drug at doses clinically used (Table 3). The EC_{50} (the concentration of drug producing 50 % of its own maximal effect) values of mometasone and beclomethasone in the enhancement of human eosinophil apoptosis were approximately 4-58-fold higher than those measured in plasma after inhalation of the drugs (at clinical doses) (Table 3). Hydrocortisone, prednisolone and prednisone, the inactive pro-drug of prednisolone, had no significant effect on human eosinophil apoptosis at concentrations up to 1000 nM (II).

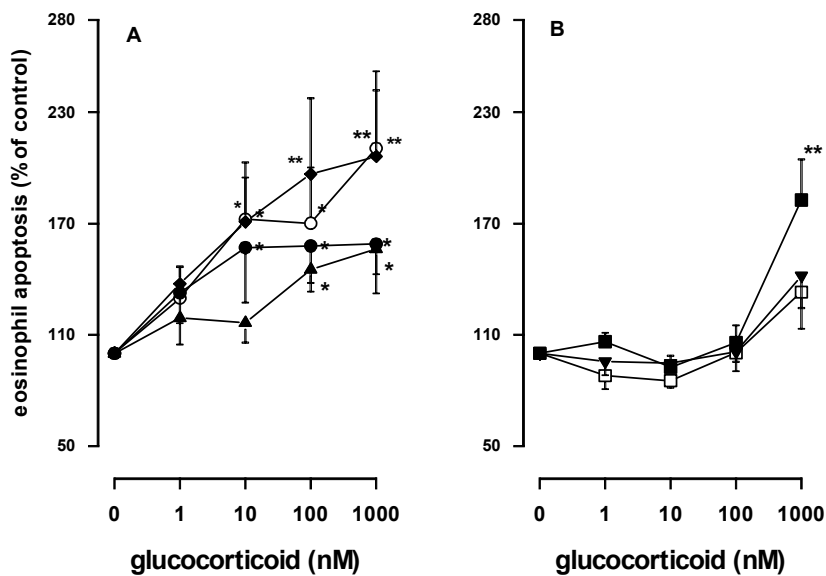


Figure 7. The effects of A. beclomethasone (▲), budesonide (○), fluticasone (●) mometasone (◆) and B. dexamethasone (■), hydrocortisone (□) and prednisolone (▼) on constitutive apoptosis in human eosinophils. Eosinophils were cultured in the absence or presence of glucocorticoids for 18 h and apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained eosinophils. Each data point represents the mean \pm 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 (data combined from II and IV).

Table 3 The EC₅₀ values of glucocorticoids in promoting eosinophil apoptosis and inhibiting neutrophil apoptosis

| Glucocorticoid | EC ₅₀ (nM) | | C _{max} (nM) in plasma (refs) | Relative receptor affinity | |
|----------------|---|--|--|----------------------------------|------|
| | Enhancement of eosinophil apoptosis | Inhibition of neutrophil apoptosis | | ref1 | ref2 |
| Beclomethasone | 51 | 20 | 2.3 (Harrison et al. 1999) | 76 | |
| Budesonide | 5.0 | 0.8 | 2.2-5.6 (Thorsson et al. 1994) | 935 | 258 |
| Dexamethasone | 565 | 8 | 455 (McCrea et al. 2003) | 100 | 100 |
| Fluticasone | 3.7 | 0.6 | 0.2-2 (Meibohm et al. 1998) | 1800 | 813 |
| Hydrocortisone | >1000 | 38 | 1000-1600 (Aalto-Korte and Turpeinen 1995) | 7.8 | |
| Mometasone | 5.6 | 0.17 | 0.096 (Bernstein et al. 1999) ^a | | 1235 |
| Prednisolone | >1000 | 13 | 500 (Jusko et al. 1996) | 12 | |

^a Mometasone is metabolized to several different metabolites, and the total concentration of these is estimated to be 1.5 nM in plasma after inhalation of 1 mg of mometasone furoate (Thonoor et al. 1999).

ref1 According to Högger and Rohdewald (1998).

ref2 According to Smith and Kreutner (1998)

Glucocorticoids significantly inhibited constitutive human neutrophil apoptosis (Figure 8, Table 3, III, IV). The order of potency of glucocorticoids in inhibiting neutrophil apoptosis was mometasone > fluticasone ≈ budesonide > dexamethasone > prednisolone > beclomethasone > hydrocortisone. At a drug concentration of 1 μM, the degree of inhibition of neutrophil apoptosis was about 50-60% and was not significantly different among the glucocorticoids tested except for prednisone. The inhibition of human neutrophil apoptosis by budesonide and fluticasone occurred at drug concentrations similar to those found in plasma after inhalation of the drugs (Table 3). The EC₅₀ values of beclomethasone and mometasone for inhibition of neutrophil apoptosis were approximately 2-9 times higher than those found in plasma after inhalation of the drugs (Table 3). In contrast, the EC₅₀ values of hydrocortisone and prednisolone for inhibition of neutrophil apoptosis were 26-42 times lower than the C_{max} (the maximal plasma concentration) values found in plasma after intravenous and oral administration of the drugs, respectively (Table 3).

The effects of glucocorticoids on eosinophil and neutrophil apoptosis were blocked by mifepristone (II, III, IV), an antagonist of the GR. In general, the order of the potency of glucocorticoids in eosinophil and neutrophil apoptosis roughly reflects that of the relative receptor affinity (Table 3).

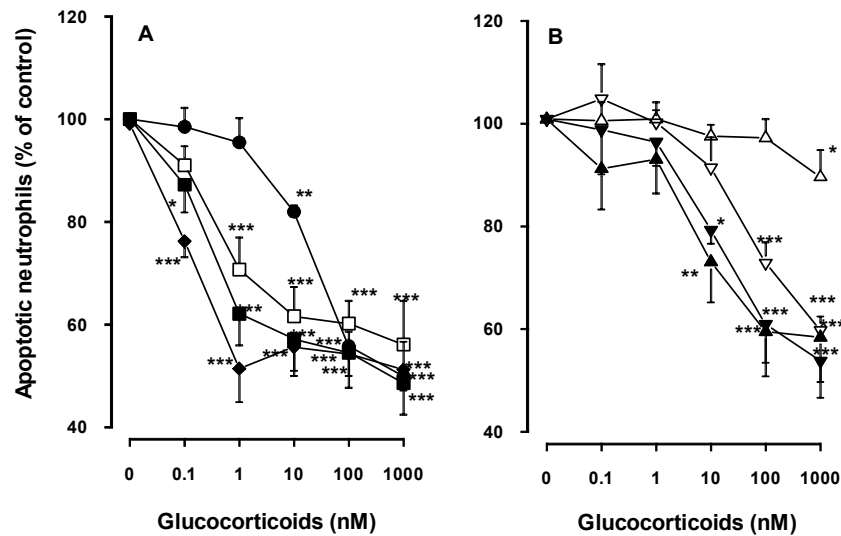


Figure 8. The effects of A. beclomethasone (●), budesonide (□), fluticasone (■), mometasone (◆) and B. dexamethasone (▲) hydrocortisone (▽), prednisolone (▼) and prednisone (Δ) on constitutive apoptosis in human neutrophils. Neutrophils were cultured in the absence or presence of glucocorticoids for 16 h and apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained neutrophils. Each data point represents the mean \pm SEM of five or six independent determinations using neutrophils from different donors. Results are expressed as percentage of control. Solvent control in the absence of glucocorticoids was set as 100%. * indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared with the respective solvent control. (data combined from III and IV).

5 Effects of glucocorticoids on eosinophil and neutrophil apoptosis in the presence of survival-prolonging factors

Dexamethasone, fluticasone and mometasone did not reverse IL-3, IL-5 or GM-CSF-afforded eosinophil survival when these cytokines were used at concentrations producing maximal inhibition of apoptosis (II, IV). Mometasone partially reversed the survival induced by submaximal concentrations of IL-5 (1 pM) (IV). However, dexamethasone, fluticasone and mometasone completely reversed TNF- α -induced cell survival (II, IV).

Dexamethasone, beclomethasone, budesonide, mometasone and fluticasone further enhanced the inhibitory effect of GM-CSF on neutrophil apoptosis (III, IV). Mometasone (1 μ M) further suppressed apoptosis of LTB₄-treated neutrophils during 16 h incubation (IV).

6 Effects of nitric oxide on interleukin-5-mediated eosinophil survival (V)

Since exhaled NO and sputum eosinophils are raised in asthmatic subjects, a relationship between them is plausible. NO-donating compounds SNAP and GEA 3175 reversed IL-5-mediated eosinophil survival in a concentration-dependent manner by inducing apoptosis (Figure 9A). The NO-scavenging compound carboxy-PTIO abolished the apoptosis-inducing effect of SNAP in IL-5-treated eosinophils (Figure 9B), suggesting that the effect was mediated by NO and not the penicillamine moiety of SNAP. SNAP but not GEA 3175 increased constitutive eosinophil apoptosis.

The cell permeable cyclic nucleotide analogs, dibutyryl-cGMP and 8-bromo-cGMP failed to reverse IL-5-induced inhibition of eosinophil apoptosis. Neither the inhibitor of guanylate cyclase ODQ nor the inhibitor of PDEs IBMX nor the selective PDE₅ inhibitor zaprinast altered the effect of SNAP in the presence of IL-5. Stable and non-hydrolysable analogs of cAMP, dibutyryl-cAMP and 8-bromo-cAMP, failed to reverse IL-5-induced inhibition of eosinophil apoptosis. Taken together, the data suggest that the induction of human eosinophil apoptosis by NO in IL-5-treated cells is independent of cGMP and cAMP.

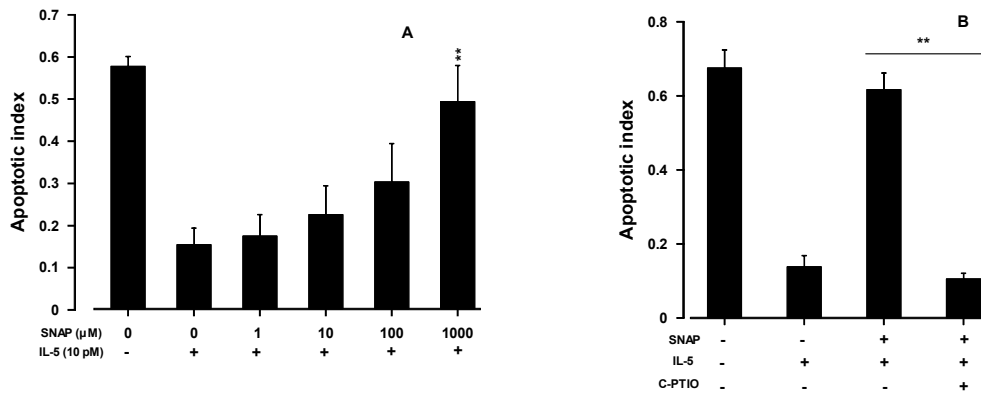


Figure 9. The effect of NO-donor SNAP (A, 1-1000μM) on apoptosis in IL-5 (10 pM)-treated eosinophils; ** indicates $P < 0.01$ as compared with the respective control in the absence of SNAP and in the presence of IL-5. B) Carboxy-PTIO (50 mM) abolished the apoptosis-inducing effect of SNAP (1000 μM) in IL-5 (10 pM)-treated eosinophils. Eosinophils were cultured for 40 h and apoptosis was assessed by flow cytometry measuring the relative DNA fragmentation in propidium iodide-stained cells; ** indicates $P < 0.01$ as compared with the respective control without C-PTIO. Each data point represents the mean \pm S.E.M. of 5-6 independent determinations using eosinophils from different donors. (Reprinted from Journal of Allergy and Clinical Immunology, 112, Zhang X et al., “Regulation of eosinophil apoptosis by nitric oxide: role of c-Jun-N-terminal kinase and signal transducer and activator of transcription 5.”, 93-101, (2003), with permission from the American Academy of Allergy, Asthma, and Immunology).

When IL-5-treated eosinophils were incubated in the presence of SNAP, a time-dependent increase in the activity of JNK was detected (Figure 10A). To evaluate the functional role of JNK activation in NO-induced apoptosis in IL-5-treated cells, a novel cell-permeable inhibitor peptide specific for JNK, L-JNKI1, was employed (Bonny et al. 2001). L-JNKI1 alone did not affect apoptosis in IL-5-treated eosinophils. However, L-JNKI1, but not the negative control peptide L-TAT, almost completely reversed SNAP-induced apoptosis in IL-5-treated eosinophils (Figure 10B).

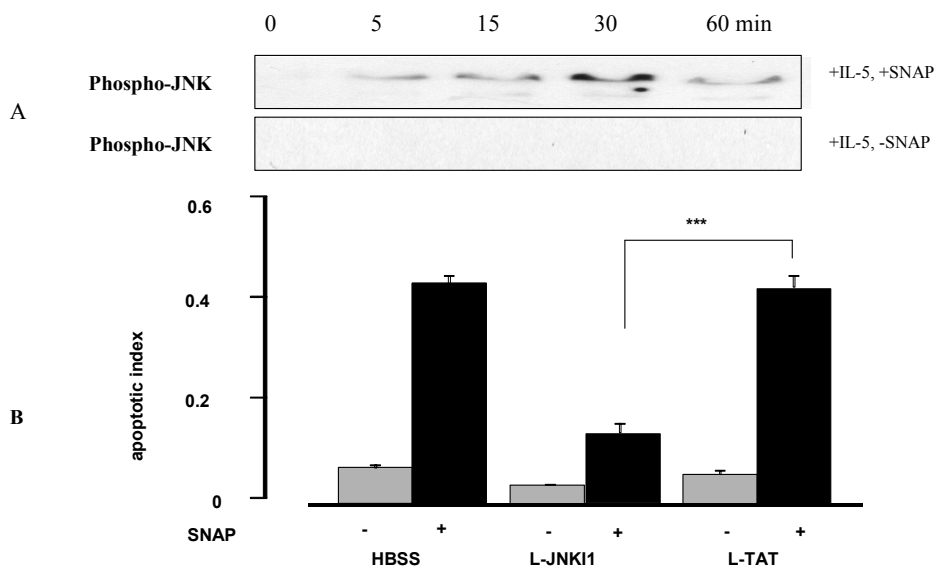


Figure 10. A) Effect of SNAP on phosphorylation of JNK. Eosinophils were incubated with IL-5 (10 pM) in the presence or absence of SNAP (1000 μM). The lanes show the JNK activation in SNAP-treated cells (upper lane), but not in the simultaneously prepared vehicle (DMSO 0.5 %)-treated cells (lower lane). A representative of three similar experiments is shown. B) The effect of the JNK inhibitor, L-JNKI1 and the negative control peptide L-TAT on SNAP (1000 μM)-induced apoptosis in IL-5-treated eosinophils. Each data point represents the mean ± S.E.M., n=4; *** indicates P<0.001 as compared with the respective SNAP-treated sample in the presence of L-TAT. (Reprinted from Journal of Allergy and Clinical Immunology, 112, Zhang X et al., “Regulation of eosinophil apoptosis by nitric oxide: role of c-Jun-N-terminal kinase and signal transducer and activator of transcription 5.”, 93-101, (2003), with permission from the American Academy of Allergy, Asthma, and Immunology).

When the eosinophils were incubated in the presence of 10 pM IL-5, STAT5 was phosphorylated in a time-dependent manner as determined using an anti-phospho-STAT5 antibody (Figure 11A). When the eosinophils were incubated in the presence of SNAP and IL-5 for 60 min, phosphorylation of STAT5 was not altered (Figure 11B). This suggests that NO does not affect the STAT5 phosphorylation or earlier IL-5 signaling steps in eosinophils.

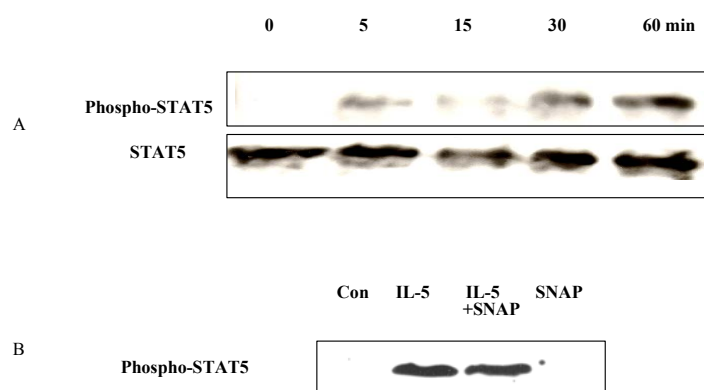


Figure 11. A) Time course of phosphorylation of STAT5. Eosinophils were incubated with IL-5 (10 pM) for the time indicated. STAT5 activation was detected using an antibody directed towards the phosphorylated (i.e. activated) form of STAT5 (upper lane). The lower lane shows the total STAT5 content in the same sample. B) The effect of SNAP on IL-5-induced phosphorylation of STAT5. Eosinophils were incubated with Medium (Con), IL-5 (10 pM), IL-5 (10 pM) + SNAP (1000 μ M) for 60 min. Phosphorylated STAT5 was detected as above by immunoblotting. Representatives of three experiments (A and B) with similar results are shown. (Reprinted from *Journal of Allergy and Clinical Immunology*, 112, Zhang X et al., “Regulation of eosinophil apoptosis by nitric oxide: role of c-Jun-N-terminal kinase and signal transducer and activator of transcription 5.”, 93-101, (2003), with permission from the American Academy of Allergy, Asthma, and Immunology).

No expression of Bcl-2 was detected, nor any change in the expression of Bax between the samples of freshly isolated cells and cells aged for 24 h in the absence or presence of IL-5 with or without SNAP.

The pan-caspase inhibitor Z-Asp-CH₂-DCB significantly reversed SNAP-induced apoptosis in IL-5-treated eosinophils during 40 h incubation. Caspase-3 appeared to be activated in the dying eosinophils cultured for 24 h, since the active 17-kDa subunit was detectable. IL-5 prevented the cleavage of procaspase-3. SNAP in the presence of IL-5 increased caspase-3 cleavage. However, the specific caspase-3 inhibitor (AC-DMQD-CHO) did not reverse the effect of SNAP in IL-5-treated eosinophils, suggesting that caspase-3 has no functional effect in NO-mediated eosinophil apoptosis. Furthermore, the specific inhibitors for caspase-8 and -9 had no effect on apoptosis induced by SNAP in IL-5-treated eosinophils.

Neither the NOS inhibitors (1400W, L-NIO, L-NMMA) nor L-arginine affected spontaneous eosinophil apoptosis. Consistently, spontaneous cell death did not induce any release of NO as measured as NO₂⁻. Similarly, IL-5 induced no production of NO. Correspondingly, no iNOS mRNA could be detected by reverse transcriptase polymerase chain reaction (RT-PCR) in eosinophils cultured for 0, 6 or 20 h in the absence or presence of 10 pM of IL-5.

DISCUSSION

1 Methodology

Apoptosis is defined as a form of programmed cell death (Savill 1997). Fragmentation of DNA is a typical feature of advanced apoptosis. In our study, apoptosis was measured by a relative DNA fragmentation assay in propidium iodide-stained cells. It is generally considered questionable to determine apoptosis by a single method, as other models of cell death cannot be reliably excluded (Gschwind and Huber 1997). In this series, we further assessed apoptosis with one to three of the following methods: double staining with propidium iodide and annexin-V and analysis by flow cytometry, analysis of DNA fragmentation by agarose gel electrophoresis (“DNA ladder”), and morphological assay by light microscopy.

Propidium iodide is taken up into the nucleus of apoptotic cells, where it stains DNA (Sandstrom et al. 2000). Thus propidium iodide staining and measurement of relative DNA fragmentation by flow cytometry can be used to measure the proportions of apoptotic cells in whole cell populations. Basically, the measurement of relative DNA fragmentation by flow cytometry in propidium iodide-stained cells detects apoptotic cells where DNA fragmentation (i.e. a typical hallmark of advanced apoptosis) occurs. Since, however, it may not detect cells undergoing changes in the early stages of apoptosis, this assay may underestimate the total number of apoptotic cells.

The anticoagulant annexin-V is a member of a family of structurally related proteins which exhibit Ca^{2+} -dependent phospholipid binding properties. Annexin-V binds to cells expressing phosphatidylserine on the outer layer of the cell membrane, which is a typical characteristic of apoptotic cells. Double staining with propidium iodide and Annexin-V assay is considered to be a sensitive and accurate means of detecting apoptosis as compared with other techniques (Walsh et al. 1998).

Electrophoretic separation of DNA (DNA ladder) is based on the generation of 180-200 bp multimers of DNA in apoptotic cells (Saraste and Pulkki 2000). In contrast, DNA from necrotic cells does not form a clear ladder pattern; the determination of the DNA ladder thus provides a classical method to demonstrate the presence of apoptotic cells.

Morphological criteria were used to evaluate the percentage of normal, apoptotic and necrotic cells. Eosinophils in the early and late stages of apoptosis can be identified. When the percentages of apoptotic cells obtained by morphological and flow cytometric analysis of relative DNA content were compared, a very high correlation between the two methods was observed ($r=0.968$, $P<0.00001$, $n=132$). However, there remains a theoretical possibility that in some experiments apoptosis was not detected by relative DNA fragmentation but might have been seen with other methods. In critical experiments we therefore used more than one method to analyze apoptosis.

Eosinophils are bone marrow-derived granulocytes normally not numerically prominent in either the peripheral blood or the tissues (Weller 1997). Neutrophils represent 50 to 70 % of the total circulating leukocytes. Isolation of sufficient eosinophils to process the experiments from one buffy-coat preparation from a healthy individual is a principal problem in eosinophil studies. Another major problem in human eosinophil studies is to obtain a pure eosinophil population. Eosinophil apoptosis is affected by cytokines such as IL-3, IL-5 and GM-CSF, and thus the presence of contaminating cells producing these cytokines could affect the results. We isolated human peripheral blood eosinophils by means of a CD-16 negative selection assay to a purity of > 99%. The main contaminating cells were mononuclear cells and neutrophils. We thus believe that the number of contaminating cells and their products have a negligible role in explaining the present results.

In the present series, we used isolated peripheral blood eosinophils. A recent study by Flood-Page and co-workers (2003) showed that the level of depletion of blood eosinophils is different from that of bone marrow and tissue eosinophils when anti-IL-5 antibody treatment is used. Their results suggest that blood eosinophils and tissue eosinophils may have different responsiveness to the survival-prolonging cytokine IL-5. We cannot thus exclude the possibility that some results obtained here may not apply to tissue eosinophils. However, at present it is very difficult, if not impossible, to elucidate detailed signaling mechanisms such as those presented in studies I-V in tissue eosinophils.

Another factor possibly affecting the interpretation of the results of these studies is that in the original communications II-V we used eosinophils isolated from healthy but not asthmatic individuals. The theoretical possibility thus exists that some signaling mechanisms may be

different in eosinophils isolated from patients with asthma. It might have been more sensible to use eosinophils isolated from patients with asthma throughout the study. The main reason for not doing so was that it is very difficult to find (eosinophilic) asthmatic patients not using steroid medication.

2 Delayed eosinophil apoptosis in asthma

Our study showed that apoptosis in peripheral blood eosinophils from asthma patients was delayed as compared with those from healthy subjects. The rate of apoptosis in eosinophils from patients concurrently taking steroids was higher than that of cells from patients not using steroids and not different from that of cells from healthy subjects. Wedi and co-workers (1997) report that prolonged peripheral blood eosinophil survival is a feature of inhalant allergy and extrinsic and intrinsic atopic dermatitis as compared with nonatopic subjects. Furthermore, in mild asthmatic patients demonstrating a late asthmatic response, the survival of peripheral blood eosinophils is prolonged after allergen challenge (Evans et al. 1996). Groups under Druilhe (Druilhe et al. 1998) and Vignola (Vignola et al. 1999) have reported that in bronchial biopsy specimens from patients with asthma there are more eosinophils and fewer apoptotic eosinophils as compared with healthy controls or patients with chronic bronchitis, respectively. Thus, our study contributes further data in showing that there is a functional defect in peripheral blood eosinophil apoptosis in patients with asthma. Similarly, a more recent study has suggested that eosinophils from atopic individuals display significantly higher viability as compared to non-atopic individuals in the absence of and in the presence of GM-CSF (Nopp et al. 2002).

The neutralizing antibody to GM-CSF increased constitutive apoptosis in the absence of added cytokines as compared with IgG₁ isotype control in eosinophils obtained from patients with asthma. In contrast, the neutralizing antibodies to IL-3 and IL-5 had no effect on constitutive apoptosis in patients with asthma. To validate our assay system, the ability of these neutralizing antibodies to reverse the effect of exogenously added cytokines was tested. The neutralizing antibodies to IL-3, IL-5 and GM-CSF reversed 36- 62% of the effect of the corresponding survival-prolonging cytokines when these cytokines were added at concentrations producing maximal anti-apoptotic effect. Thus, the delay in apoptosis may be partly explained by production of GM-CSF in eosinophils. Previously, GM-CSF has been reported to be present in

the supernatant of eosinophils from asthmatic patients following 36 h culture, whereas it was not detectable in the supernatant of eosinophils from healthy subjects (Iversen et al. 1997). However, the increase in the apoptotic index induced by GM-CSF neutralization was relatively small (0.05) as compared with the difference between asthmatic patients not using steroids and healthy control subjects (difference in apoptotic index 0.15). Thus, the delayed eosinophil apoptosis found in the present study may not be completely explained by the production of GM-CSF by eosinophils.

Another possible reason for the delayed eosinophil apoptosis in patients with asthma lay in the β_2 -agonists used in the treatment of their condition. To clarify whether β_2 -agonists affect eosinophil survival, their effects were studied *in vitro*. Fenoterol, salbutamol and salmeterol inhibited eosinophil apoptosis with EC_{50} values of 0.7 nM, 6.5 nM and 94 nM, respectively. C_{max} of salbutamol is 7.5-60 nM after oral or inhalation treatment (Jonkman et al. 1986, Powell et al. 1986, Hochhaus and Mollmann 1992). The C_{max} of fenoterol is 0.5-1.2 nM after inhalation. Thus, the inhibition of eosinophil apoptosis by salbutamol and fenoterol *in vitro* occurred at clinically relevant drug concentrations. The C_{max} of salmeterol is 0.36 nM after inhalation (Adkins and McTavish 1997). However, although the concentration of salmeterol required to inhibit apoptosis *in vitro* is higher than C_{max} in plasma, we cannot exclude the possibility that salmeterol might inhibit eosinophil apoptosis locally in the lung, as local concentrations in the lung may be higher than those in plasma. The maximal inhibitory effect of β_2 -agonists on eosinophil apoptosis was 19%. The apoptotic index of asthmatic patients was 38% lower than that of healthy controls. Thus the effect of β_2 -agonists found in *in vitro* studies does not suffice to explain the difference observed. Taken together, we show here that both the production of GM-CSF by eosinophils and exposure to β_2 -agonists may contribute to the prolonged eosinophil survival in asthma, but do not fully explain it.

Excessive β_2 -agonist use has been thought to be associated with increased risk of death or near death from asthma (Spitzer et al. 1992). One possible mechanism underlying this could be prevention of eosinophil clearance by inhibition of apoptosis. In severe airway eosinophilia, a 10% to 19% increase in eosinophil survival might result in a significant cumulative increase in the absolute numbers of eosinophils in the airways, resulting in a more severe inflammatory response and asthmatic symptoms. In fact, use of salbutamol has been associated with increased numbers of eosinophils in the lung even in patients with mild asthma (Gauvreau et al. 1997).

These results thus raise the concern that regular β_2 -agonist use in the absence of steroids might prolong eosinophil survival through inhibition of apoptosis and thus worsen eosinophilia in asthma patients.

3 Glucocorticoids, eosinophil and neutrophil apoptosis

Our study extends previous knowledge here by showing that currently used inhaled glucocorticoids, beclomethasone, budesonide, fluticasone and mometasone have opposite effects on human eosinophil and neutrophil apoptosis. Moreover, we showed that these effects occur at clinically relevant drug concentrations.

The EC_{50} values found here for the enhancement of eosinophil apoptosis were 3.7 nM for fluticasone propionate, 5.0 nM for budesonide, 5.6 nM for mometasone and 51 nM for beclomethasone. The C_{max} has been reported to lie in a range of 2.2-5.6 nM after inhalation of 1000 μ g of budesonide (Thorsson et al. 1994). Furthermore, after inhalation, the tissue concentration of budesonide in the lung was shown to be on an average eight-fold higher than its plasma concentration (Thorsson 1995). The C_{max} of fluticasone has been reported to vary from 0.2-2 nM after inhalation (Meibohm et al. 1998). However, the tissue drug concentration has been reported to vary in a range of approximately 20 – 44 nmol/kg in central airways after inhalation of 1000 μ g of fluticasone. More importantly, these high fluticasone concentrations could be measured in the lung even 15 h after inhalation (Högger and Rohdewald 1998). Thus, the increase in the rate of eosinophil apoptosis induced by budesonide and fluticasone is obtained at clinically achievable drug concentrations.

The C_{max} of beclomethasone has been reported to be 2.3 nM after inhalation of 400 μ g b.i.d for two weeks (Harrison et al. 1999). An approximately 20-fold difference between the reported C_{max} and the EC_{50} value of the present study was found. Thus, beclomethasone may not significantly increase the apoptosis of circulating eosinophils. It might, however, induce a significant increase in the rate of eosinophil apoptosis locally in the lung. Such a conception is in fact supported by the finding that treatment with inhaled beclomethasone in asthmatic patients reduces the numbers of eosinophils in sputum concomitantly with the increase in apoptotic index (Woolley et al. 1996).

The plasma concentrations of mometasone are very low (0.096 nM) (Bernstein et al. 1999). This may be explained by the fact that mometasone furoate is rapidly and extensively metabolized. Due to this rapid metabolism plasma concentrations of mometasone may not correlate with its clinical effects. In a recent pharmacokinetic study the C_{\max} of mometasone furoate measured as ^3H -mometasone furoate and its metabolites in plasma was estimated to be 1.5 nM following administration of 1 mg by dry-powder inhalation (Thonoor et al. 1999). The EC_{50} of mometasone for eosinophil apoptosis in the present study was 5.6 nM. Thus, inhaled mometasone furoate may not produce a significant increase in the apoptosis of circulating eosinophils, but it is likely that concentrations of mometasone furoate found to enhance eosinophil apoptosis in the present study are achieved in the lungs *in vivo* following mometasone inhalation.

The EC_{50} values for the inhibition of neutrophil apoptosis are 0.8 nM for budesonide, 0.6 nM for fluticasone propionate and 0.17 nM for mometasone furoate. The inhibitory effects of these drugs on neutrophil apoptosis are thus obtained at concentrations found in the lungs during inhalation therapy. The EC_{50} of beclomethasone for inhibition of neutrophil apoptosis in the present study was 20 nM, which is 8.7-fold higher than the C_{\max} in plasma. However, it is conceivable that beclomethasone concentrations locally in the lung would be high enough to inhibit neutrophil apoptosis. Such a view is supported by the finding of Hoshino and Nakamura (Hoshino and Nakamura 1996) that treatment with inhaled beclomethasone increased the numbers of neutrophils in bronchial biopsies from patients with asthma. The EC_{50} values of prednisolone and hydrocortisone in inhibiting neutrophil apoptosis were 13 and 38 nM, respectively. The C_{\max} of prednisolone has been reported to be 500 nM after oral administration (Jusko et al. 1996). Intravenous hydrocortisone produced a C_{\max} of 1-1.6 μM (Aalto-Korte and Turpeinen 1995). Hence, prednisolone and hydrocortisone inhibit neutrophil apoptosis at drug concentrations achieved during drug treatment.

It is generally held that most, if not all, effects of glucocorticoids are mediated via the GR (Pedersen and O'Byrne 1997, Demoly and Chung 1998, Newton 2000, Barnes 2001b). The effects of glucocorticoids on eosinophil and neutrophil apoptosis were blocked here by a GR antagonist, mifepristone, showing that these effects are indeed mediated by the GR. Eosinophils have been shown to contain GR (McConnell and Howarth 1998). Synthetic glucocorticoids, again, differ in their affinity to GR. In the present study, the compounds having high GR

affinity were more potent inducers of eosinophil and inhibitors of neutrophil apoptosis than those showing lower affinity (See Table 3). However, GR affinity may not be the only factor determining the effects of glucocorticoids on eosinophils and neutrophils, as the ratio of EC₅₀ values for the modulation of eosinophil and neutrophil apoptosis were 2.5-6.3 for beclomethasone, budesonide and fluticasone, while the corresponding ratio for prednisolone and dexamethasone were 34 and 70, respectively. This, and the fact that neutrophil apoptosis was inhibited at lower glucocorticoid concentrations than eosinophil apoptosis, suggest that the GR-mediated effects may be differentially regulated in human eosinophils and neutrophils and that different glucocorticoid preparations may act differentially.

4 Glucocorticoids and asthma and chronic obstructive pulmonary disease

Glucocorticoids constitute the most effective therapy currently available for asthma (Barnes et al. 1998b, Haahtela 2002). They control the inflammation in asthma and several other chronic inflammatory diseases. In contrast, the role of glucocorticoids in the treatment of COPD remains controversial. More than 100 studies have investigated the effects of glucocorticoids on inflammation in this disorder (Postma and Kerstjens 1999), but the results are conflicting and there may exist differences in the mode of diagnosing asthma and COPD (especially co-existing asthma and COPD) in these studies. Glucocorticoids may have multiple cellular effects in controlling the inflammation in asthmatic airways, i.e. reducing the number and activation of several inflammatory cells in the airway mucosa and in BAL (Barnes 2001b).

There is evidence from an uncontrolled human study to suggest that glucocorticoid (oral and inhaled) treatment reduces the number of eosinophils in the sputum with a concomitant increase in eosinophil apoptotic index (Woolley et al. 1996), although there is at the moment debate as to whether induction of eosinophil apoptosis in the lung tissue contributes to resolution of allergen-induced airway inflammation (Erjefalt and Persson 2000, Uller et al. 2001). The present results suggest that inhaled glucocorticoids enhance human eosinophil apoptosis at clinically relevant drug concentrations, indicating a possibility that inhaled glucocorticoids induce apoptosis in eosinophils and thus prevent them from undergoing activation and/or cytolysis. Whether the induction of apoptosis by inhaled glucocorticoids *in vivo* occurs in airway tissue or lumen remains to be clarified.

In contrast, neutrophils may contribute to more severe forms of asthma. Severe asthma has been shown to be associated with sputum neutrophilia (Wenzel et al. 1997, Jatakanon et al. 1999, Louis et al. 2000). High sputum neutrophil counts were particularly evident in asthmatics who remained symptomatic despite treatment with inhaled glucocorticoids. The finding that currently used inhaled and oral glucocorticoids inhibit neutrophil apoptosis at clinically relevant drug concentrations may provide an explanation for the increased neutrophil numbers in severe asthma.

Neutrophils have been shown to be present in increased numbers in induced sputum in COPD (Jeffery 1999, O'Byrne and Postma 1999). A delay in constitutive apoptosis induced by glucocorticoids may increase the numbers of neutrophils in the airways of patients with COPD. Furthermore, during apoptosis neutrophils lose their capacity to degranulate (Haslett 1999). Modulation of apoptosis in neutrophils by glucocorticoids thus regulates their "functional longevity" (Cox 1995). Hence, the inhibition of constitutive neutrophil apoptosis by currently used inhaled or oral glucocorticoids may inhibit the course of resolution of the neutrophilic inflammation. This is one possible explanation for the limited efficacy of inhaled glucocorticoids in COPD.

Neutrophils are important cells in the process of inflammation. They represent 50 to 70 % of the total circulating leukocytes and constitute the "first line of defense" against infectious agents or "non-self" substances penetrating the body's physical barrier (Smith 1994). The fact that glucocorticoids prolong neutrophil survival by inhibiting apoptosis also provides a possibility to maintain adequate host defense during glucocorticoid therapy. Although this is an oversimplification, it might be reflected in the reduced number of exacerbations of COPD, known to be frequently caused by infectious agents (Wilson 1998), in patients treated with regular inhaled glucocorticoids (Paggiaro et al. 1998). Glucocorticoids are generally considered to lower the body's immunological defence mechanisms (Barnes 2001b). However, treatment of asthma with inhaled glucocorticoids is not considered to result in an increased susceptibility to infections. One could speculate that one possible explanation for this is that glucocorticoids do not reduce but rather increase the number of viable neutrophils. Similarly, the finding that glucocorticoids enhance neutrophil survival might explain why they can relatively safely be used as therapy adjunctive to oral antibiotics in the treatment of bacterial rhinosinusitis (Barlan

et al. 1997, Puhakka et al. 1998, Meltzer et al. 2000, Yilmaz et al. 2000, Dolor et al. 2001, Parikh et al. 2001, Nayak et al. 2002).

Taken together, our present findings suggest that the effect of glucocorticoids on eosinophil apoptosis may contribute to their anti-inflammatory action in the treatment of eosinophilic inflammatory diseases such as asthma. In contrast, the neutrophil survival-prolonging effect of glucocorticoids might contribute to the neutrophilic inflammation seen in severe asthma and afford one possible explanation for the poor efficacy of glucocorticoids in preventing disease progression in COPD.

5 Glucocorticoids, cytokines and eosinophil and neutrophil apoptosis

IL-5 may play an important role in eosinophil maturation, chemoattraction, activation and survival in asthmatic inflammation. There is evidence that IL-5 is expressed in lung tissues and inflammatory cells from patients with asthma (Barnes et al. 1998a). The inhibitory effects of glucocorticoids on eosinophil survival were completely or almost completely abrogated in the presence of maximal anti-apoptotic concentrations of IL-5. IL-5-afforded eosinophil survival was partly reversed by mometasone in the presence of low concentrations of IL-5 (1 pM) but not in the presence of higher concentrations. This would suggest that the signal transduction pathway of IL-5 may include steroid-sensitive elements activated at low but not at high concentrations of IL-5. Interestingly, glucocorticoids completely reversed TNF- α -afforded eosinophil survival independently of TNF- α concentration. This would imply that the mechanism of IL-5-induced eosinophil survival differs from that of TNF- α . Our own (Kankaanranta et al. 2001) and other (Ward et al. 1999) results show that TNF- α prolongs human eosinophil survival by activating NF- κ B. Meanwhile, glucocorticoids have been reported to inhibit NF- κ B activation (Wilson et al. 2001, Hart et al. 2000). In contrast, IL-5 has been seen not to activate NF- κ B (Wong et al. 2002). This may provide an explanation for the difference in survival mechanism between IL-5 and TNF- α .

GM-CSF has been reported to be expressed in the epithelium of subjects with chronic bronchitis (Vignola et al. 1997). GM-CSF and G-CSF have been shown to be the main survival-prolonging factors for neutrophils (Dibbert et al. 1999). Glucocorticoids are not able to reverse the effects of exogenously added GM-CSF on neutrophil apoptosis, but further enhance the

inhibitory effect of GM-CSF on neutrophil apoptosis, suggesting that the anti-apoptotic mechanisms of GM-CSF in neutrophils are insensitive to and at least partly distinct from those of glucocorticoids. It is possible that the slight enhancement of the anti-apoptotic effect of GM-CSF by inhaled glucocorticoids is of clinical relevance in some situations, but it is difficult to estimate the clinical net effect exerted by glucocorticoids on neutrophilic inflammation as they a) inhibit GM-CSF production, b) inhibit neutrophil apoptosis and c) enhance GM-CSF-afforded survival.

6 Nitric oxide and eosinophil apoptosis

NO has been found to be an important regulator of blood pressure and platelet aggregation, to act as a neurotransmitter in nervous systems and to regulate the immune response (Moilanen et al. 1999, Bogdan 2001, Vallance and Leiper 2002). In inflammation NO evinces either pro- or anti-inflammatory properties depending on the type and phase of inflammation (Moilanen et al. 1999). Similarly, NO appears to have a dual role in the regulation of apoptosis. It initiates apoptosis in various cell lines, such as macrophage cell lines B-cells, thymocytes and mast cells (Brune et al. 1999), whilst other studies have demonstrated inhibitory effects on apoptosis (Shen et al. 1998). In the present series, we found that exogenous NO induced apoptosis in eosinophil cultures in the presence of IL-5. Our results are in agreement with those of Beauvais and Joly (Beauvais and Joly 1999), where it was suggested that the NO donors S-nitroso-L-cysteine and NOC-18 reduce survival and induce apoptosis of IL-5-primed eosinophils. We used carboxy-PTIO to scavenge the NO released from SNAP and carboxy-PTIO reversed the effect of SNAP, suggesting that the effect was due to NO released and not to the penicillamin moiety of SNAP. Unfortunately, the effect of D-penicillamin itself was not studied.

Many of the cellular effects of NO are mediated through activation of guanylyl cyclase and production of cGMP. To establish whether NO-induced apoptosis is mediated by cGMP, we used several tools to modulate cGMP concentrations in the eosinophils. The cell-permeant cyclic nucleotide analogues, dibutyryl-cGMP and 8-bromo-cGMP failed to mimic the action of NO-donors. If the effect of NO on eosinophil apoptosis were mediated via the production of cGMP, this effect should be blocked by inhibiting guanylyl cyclase and potentiated by increasing the concentration of cGMP by inhibiting its breakdown via PDEs. However, neither an inhibitor of guanylyl cyclase (ODQ) nor a selective inhibitor of cGMP specific PDE₅

(zaprinast) nor a nonselective PDE inhibitor (IBMX) altered the effect of SNAP. These data suggest that the apoptotic effect of NO on IL-5-treated eosinophils is not dependent on cGMP.

An alternative mechanism whereby NO may induce cell death is the formation of peroxynitrite and/or other highly reactive radicals (Chung et al. 2001). Furthermore, it has been shown that NO directly inhibits mitochondria cytochrome c oxidase, resulting in the enhancement of ROS production (Moncada and Erusalimsky 2002, Yuyama et al. 2003). Another possibility is that NO can activate the nuclear enzyme poly (ADP-ribose) polymerase (PARP), which participates in DNA repair. However, excessive activation of PARP can deplete tissue stores of nicotamide adenine dinucleotide, the PARP substrate, with a resultant depletion of ATP, leading to cell death (Pieper et al. 1999). These alternative mechanisms of NO were not analyzed in this study.

Hebestreit and co-workers (1998) reported that NO is also able to inhibit Fas-induced eosinophil apoptosis. The effect of NO on Fas receptor signaling was held to be due to activation of soluble guanylyl cyclase, and dibutyryl-cGMP and dibutyryl-cAMP induced effect similar to those of NO-donors. Thus, the present results and those of Hebestreit's group suggest that NO can regulate human eosinophil apoptosis at two separate levels, namely by reversing IL-5-mediated inhibition of apoptosis in a cGMP independent manner, and by disrupting Fas-receptor signaling probably through cGMP. However, the mechanism by which NO disrupts Fas-signaling seems somewhat obscure, as cAMP was also able to reverse Fas-induced apoptosis.

We found that NO activates JNK in IL-5-treated human eosinophils. The effect of NO on apoptosis was reversed by specifically blocking JNK activation using a recently introduced L-TAT-conjugated inhibitory peptide. As the inhibitory peptide has been described as being specific to JNK, and the control L-TAT construct did not affect eosinophil apoptosis, we believe that this effect of the JNK inhibitor is specific for JNK. This would imply that the NO-induced apoptosis in human eosinophils involves activation of JNK. In mechanistic studies we also showed that STAT5 is phosphorylated in IL-5-treated human eosinophils, confirming the results of Hall and co-workers (2001). However, NO did not affect STAT5 phosphorylation in IL-5-treated cells, suggesting that early steps of IL-5 signaling leading to STAT5 activation are not affected by NO.

The effect of NO was apparently dependent on caspase activation, since it was blocked by a pancaspase inhibitor Z-Asp-CH₂-DCB. Caspase-3 is one of the effector caspases, activation of which is followed by the activation of caspase-8 and/or caspase-9 (Saraste and Pulkki 2000). We found some processing of caspase-3 but not of caspase-8 or 9 proenzymes by NO. However, studies with specific inhibitors indicated that the effect of NO was not mediated via activation of caspase-8, 9 or 3. The connection between caspases and JNK in inducing apoptosis is not very clear. It has recently been proposed that caspase inhibition might reduce dexamethasone-induced JNK activation, suggesting that caspases might activate JNK (Zhang et al. 2000).

The Bcl-2 proteins can be divided into two groups, anti-apoptotic proteins (such as Bcl-2, Bcl-X_L, Mcl-1) and proapoptotic proteins (such as Bax, Bad, and Bid) (Adams and Cory 1998). The role of Bcl-2 family proteins in eosinophil apoptosis or its prevention is not clear and the reported data are conflicting (Dibbert et al. 1998, Dewson et al. 1999, Zangrilli et al. 2000). To determine whether the anti-apoptotic effect of IL-5 could be attributed to changes in the expression of Bcl-2 family members, cell lysates were examined by immunoblotting for expression of the anti-apoptotic species Bcl-2 and the pro-apoptotic species Bax. We detected no expression of Bcl-2. In contrast, the pro-apoptotic species Bax was strongly expressed, but no changes were seen between the samples of freshly isolated cells and cells aged for 24 h in the absence or presence of IL-5 with or without SNAP. These results suggest that Bcl-2 or Bax are not involved in NO-induced apoptosis in IL-5-treated eosinophils.

The expression of NOS enzymes in human eosinophils is a matter of controversy (Arock et al. 1994, del Pozo et al. 1997). Eosinophils have been reported to contain the enzyme NOS and produce NO (del Pozo et al. 1997). We studied the effects of non-selective NOS-inhibitors, the selective iNOS inhibitor and NO substrate L-arginine, to ascertain whether endogenous NO production modulates eosinophil apoptosis. As these compounds are competitive inhibitors of NOS, the high concentrations (>1000 µM) of L-arginine present in RPMI 1640 could mask their effects. We thus used specially engineered L-arginine and NO₂⁻ free DMEM culture medium supplemented with 100 µM L-arginine. In general, the rate of apoptosis in this medium was higher than in RPMI 1640 medium. Neither the NOS inhibitors nor L-arginine affected spontaneous eosinophil apoptosis. In addition, we found no detectable amounts of iNOS mRNA

by RT-PCR in eosinophils cultured in the absence or presence of IL-5. This would imply that eosinophil-derived NO is not a major regulator of eosinophil apoptosis.

SUMMARY AND CONCLUSIONS

1 Apoptosis of peripheral blood eosinophils from asthma patients was delayed as compared with that of cells from healthy control subjects, whereas in patients treated with anti-inflammatory steroids it did not differ from that in healthy controls. Our results suggest that there is a functional defect in eosinophil apoptosis in asthma, which may be partly but not fully explained by production of GM-CSF in eosinophils. In addition, β_2 -agonists were seen to inhibit human eosinophil apoptosis *in vitro*.

2. Inhaled glucocorticoids beclomethasone, budesonide, fluticasone and mometasone enhanced constitutive eosinophil apoptosis. In contrast, they inhibited constitutive neutrophil apoptosis. In general, these effects occurred at clinically achievable drug concentrations. The order of potency of these glucocorticoids in the enhancement of eosinophil apoptosis was fluticasone \approx budesonide \approx mometasone $>$ beclomethasone $>$ dexamethasone. Hydrocortisone and prednisolone at up to 1 μ M concentrations had no statistically significant effect. In contrast, the order of potency of glucocorticoids in the case of neutrophil apoptosis was mometasone $>$ fluticasone \approx budesonide $>$ dexamethasone $>$ prednisolone $>$ beclomethasone $>$ hydrocortisone. These findings suggest that direct regulation of eosinophil apoptosis is involved in the anti-inflammatory mechanisms of glucocorticoids in asthma therapy. In contrast, inhibition of neutrophil apoptosis by glucocorticoids may be involved in their relative inefficiency in the treatment of COPD. The effects of glucocorticoids on eosinophil and neutrophil apoptosis were mediated by the GR.

3. Eosinophil apoptosis was inhibited by the survival-prolonging cytokines IL-3, IL-5, GM-CSF and TNF- α . Glucocorticoids reversed the effect of TNF- α but not that of IL-5 or GM-CSF when they were used at maximal anti-apoptotic concentrations. However, glucocorticoids partly reversed the effect of suboptimal concentrations of IL-5. Neutrophil apoptosis was inhibited by GM-CSF and LTB₄ and glucocorticoids further enhanced the effect of GM-CSF.

4. NO reversed the IL-5-mediated survival of eosinophils. The mechanism of action of NO involves activation of JNK and caspases in a cGMP-independent manner. NO did not affect STAT5 phosphorylation in IL-5-treated cells. The increased generation of NO in asthmatic airways may therefore offset the effect of IL-5 in prolonging eosinophil survival.

5. These findings extend our understanding of the mechanisms involved in the pathogenesis of asthmatic inflammation and may serve as a basis for the development of novel therapies which specifically target the eosinophil. Future studies based on the present findings should focus on the differences in signaling between eosinophils and neutrophils during the apoptotic process. The finding that NO reverses the survival-prolonging effect of IL-5 by a mechanism involving JNK raises interesting questions as to the role and specificity of JNK in the regulation of eosinophil apoptosis. Future studies should seek to provide information on whether the activation of JNK is confined to the eosinophil apoptosis induced by NO or is central to the apoptotic process in general.

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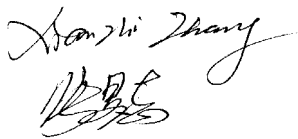
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A handwritten signature in black ink, appearing to read 'Xianzhi Zhang', with a stylized flourish below it.

Xianzhi Zhang

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ORIGINAL COMMUNICATIONS