HANNELE HASALA

Role of Histamine in the Regulation of Human Eosinophil Apoptosis

With Special Reference to Antihistamines and the Role of c-Jun N-terminal Kinase in Human Eosinophil Apoptosis

ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

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

I Hasala H, Malm-Erjefält M, Erjefält J, Giembycz MA, Zhang X, Moilanen E and Kankaanranta H (2005): Ketotifen Induces Primary Necrosis of Human Eosinophils. J Ocular Pharmacol Ther 21:318-327.

II Hasala H, Moilanen E, Janka-Junttila M, Giembycz MA and Kankaanranta H (2006): First-generation Antihistamines Diphenhydramine and Chlorpheniramine Reverse Cytokine-afforded Eosinophil Survival by Enhancing Apoptosis. Allergy Asthma Proc, in press.

III Hasala H, Zhang X, Saarelainen S, Moilanen E and Kankaanranta H (2006): c-Jun N-terminal Kinase Mediates Constitutive Human Eosinophil Apoptosis. Pulm Pharmacol Ther, in press.

IV Hasala H, Giembycz MA, Janka-Junttila M, Moilanen E and Kankaanranta H (2006): Histamine Reverses IL-5-Afforded Human Eosinophil Survival by Inducing Apoptosis: Pharmacological Evidence for a Novel Mechanism of Action of Histamine. Submitted for publication.

ABBREVIATIONS

AC	adenylate cyclase
Ac-IETD-CHO	Ac-Ile-Glu-Thr-Asp-CHO, caspase 8 inhibitor
Ac-LEHD-CHO	Ac-Leu-Glu-His-Asp-CHO, caspase 9 inhibitor
8-Br-cAMP	8-Bromoadenosine-3’,5’-cyclic monophosphate
cAMP	cyclic adenosine 3’,5’-monophosphate
cGMP	cyclic guanosine 3’,5’-monophosphate
D609	*O*-(Octahydro-4,7-methano-1*H*-inden-5-yl)carbonopotassium dithioate, phosphatidylcholine-specific phospholipase C -inhibitor
DMSO	dimethyl sulfoxide
DPPE	N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethan-amide, H_{IC}-antagonist
EC_{50}	the concentration that produces 50% of a compound’s maximal effect
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EPO	eosinophil peroxidase
FADD	Fas associated death domain
FEV_1	forced expiratory volume in 1 s
GM-CSF	granulocyte/macrophage colony-stimulating factor
GRE	glucocorticoid response element
H_{IC}	intracellular histamine “receptor”
5-HT	5-hydroxytryptamine (serotonin)
IgE	immunoglobulin E
IL	interleukin
Jak	janus kinase
JNJ7777120	1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine, H_4-antagonist
JNK	c-Jun N-terminal kinase

L-JNKI1	c-Jun N-terminal kinase peptide inhibitor, L-stereoisomer
L-TAT	Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro, L-stereoisomer
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MMPCI	7-methyl-2-[(4-methylpiperazin-1-yl)carbonyl]-1H-indole, H_4-antagonist
NF-κB	nuclear factor kappa B
OUP-16	(-)-2-Cyano-1-methyl-3-{(2R,5R)-5-[1H-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine, H_4-agonist
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
Q-VD-OPh	N-(2-Quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl ketone, broad-spectrum inhibitor of caspases 1, 3, 8, 9, 10 and 12
RANTES	regulated upon activation, normal T-cell expressed and secreted
Rp-8-CPT-cAMPS	8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, RP-isomer, sodium salt
SEM	standard error of mean
SP600125	Anthra(1,9-*cd*)pyrazol-6(2H)-one, JNK inhibitor
STAT	signal transducer and activator of transcription
TEM	transmission electron microscopy
Th2	T helper 2 –type lymphocyte
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor

U73122	1-[6-[[(17-*β*)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione, phospholipase C inhibitor
Z-Asp-CH_2-DCB	benzyloxycarbonyl-Asp-CH_2-OC(*O*)-2,6-dichlorobenzene, pan-caspase inhibitor
Z-D(OMe)QMD(OMe)-FMK	Z-Asp-Gln-Met-Asp-fluoromethylketone, caspase 3 inhibitor
Z-VE(OMe)ID(OMe)-FMK	Z-Val-Glu-Ile-Asp-fluoromethylketone, caspase 6 inhibitor

ABSTRACT

Eosinophilic inflammation represents an essential element in the pathogenesis of allergic conditions such as asthma, allergic rhinoconjunctivitis and atopic eczema. Histamine, released from inflammatory cells in response to allergen challenge, is regarded as a key mediator in the development of allergic symptoms. The activation and degranulation of eosinophils in the airways evoke epithelial tissue injury and airway remodelling. With regard to the clearance of eosinophils, apoptosis or programmed cell death is considered as a major mechanism in the resolution of eosinophilic inflammation. Antihistamines (aka histamine H_1 receptor antagonists) are widely used in the treatment of allergic disorders although little is known about their effects on eosinophil functions.

The present study was designed to evaluate the possible effects of histamine and antihistamines on human eosinophil apoptosis. In addition, the role of c-Jun N-terminal kinase (JNK) in the regulation of constitutive or glucocorticoid- or antihistamine-induced eosinophil apoptosis was elucidated. Isolated human blood eosinophils were cultured *in vitro*, after which the rate of apoptosis was assessed by flow cytometric analyses of the relative DNA content or Annexin-V binding or by morphological analyses by bright field microscopy or transmission electron microscopy.

Histamine partly reversed interleukin (IL)-5-induced human eosinophil survival by enhancing apoptosis via a mechanism that did not apparently involve the activation of any of the currently known histamine receptor subtypes. Two antihistamines, diphenhydramine and chlorpheniramine, also promoted human eosinophil apoptosis in the presence of IL-5 whereas ketotifen was observed to induce primary eosinophil necrosis. In addition, it was observed that JNK does seem to mediate constitutive human eosinophil apoptosis but not to selectively mediate glucocorticoid-induced human eosinophil apoptosis. JNK was also found to be involved in antihistamine-induced apoptosis of eosinophils.

In conclusion, the present results suggest that histamine may play an anti-inflammatory role in allergy by enhancing eosinophil apoptosis under the influence of survival-prolonging cytokines. This pro-apoptotic role of histamine may be mediated through a novel, hitherto unidentified histamine receptor. Furthermore, direct regulation of eosinophil longevity via H_1 receptor-independent mechanisms may also be involved in the therapeutic actions of antihistamines in the treatment of allergy.

TIIVISTELMÄ

Eosinofiilinen tulehdus on tärkeä tekijä allergisten sairauksien, kuten astman, allergisen nuhan ja silmätulehduksen sekä atooppisen ihottuman, synnyssä. Myös histamiini, jota vapautuu tulehdussoluista allergeenialtistuksen jälkeen, on tärkeä allergiaoireiden synnyssä. Eosinofiilisten valkosolujen aktivaatio ja solunsisäisten jyvästen sisällön vapautuminen solunulkoiseen tilaan aiheuttaa epiteelivauriota ja hengitysteiden rakenteen pysyviä muutoksia. Apoptoosia eli ohjelmoitua solukuolemaa pidetään keskeisenä mekanismina, jolla eosinofiilejä poistuu kudoksista ja eosinofiilipainotteinen tulehdus lievittyy. Antihistamiineja eli histamiinin H_1-reseptorin salpaajia käytetään yleisesti allergisten sairauksien hoidossa, vaikka niiden vaikutuksista eosinofiileihin tiedetään hyvin vähän.

Tämän tutkimuksen tavoitteena oli arvioida histamiinin ja antihistamiinien mahdollisia vaikutuksia eosinofiilisten valkosolujen apoptoosiin. Lisäksi selvitettiin c-Jun N-terminaalisen kinaasin (JNK) merkitystä ihmisen eosinofiilien apoptoosin säätelyssä. JNK:n tärkeyttä arvioitiin sekä spontaanisti tapahtuvassa solukuolemassa että glukokortikoideilla tai antihistamiineilla stimuloidussa solukuolemassa. Vapaaehtoisten henkilöiden verestä eristettyjä eosinofiilejä viljeltiin koeolosuhteissa, jonka jälkeen ohjelmoidun solukuoleman määrää arvioitiin eri menetelmillä. Käytettyjä menetelmiä olivat suhteellisen DNA:n määrän tai Annexin-V:n sitoutumisen mittaaminen virtaussytometrillä sekä solujen morfologian arviointi valomikroskoopin tai läpäisyelektronimikroskoopin avulla.

Histamiinin havaittiin osittain kumoavan interleukiini-5:n (IL-5:n) eosinofiilejä hengissäpitävää vaikutusta tehostamalla eosinofiilien apoptoosia mekanismilla, joka ei näyttänyt välittyvän minkään tunnetun histamiinreseptorin kautta. Antihistamiinit difenhydramiini ja kloorifeniramiini lisäsivät samoin eosinofiilien apoptoosia IL-5:n läsnäollessa, kun taas ketotifeeni sai aikaan eosinofiilien nekroottisen solukuoleman. Lisäksi havaittiin, että JNK välittää

spontaanisti tapahtuvaa eosinofiilien ohjelmoitua solukuolemaa mutta ei selektiivisesti glukokortikoidien tehostamaa apoptoosia. JNK-kinaasilla näyttää myös olevan merkitystä antihistamiineilla aikaansaadussa eosinofiilisten valkosolujen apoptoosissa.

Yhteenvetona havaittiin, että histamiinilla voi olla myös tulehdusta hillitsevä vaikutus allergiassa sen tehostaman eosinofiiliapoptoosin takia. Tämä saattaa välittyä uuden, toistaiseksi tuntemattoman histamiinireseptorin kautta. Suora eosinofiilien solukuoleman säätely saattaa myös olla yksi joidenkin antihistamiinien terapeuttisista vaikutuksista allergian hoidossa, joka välittyy H_1-reseptorista riippumattomalla mekanismilla.

INTRODUCTION

The eosinophil has traditionally been considered as the primary cell responsible for host defence against parasites. However, eosinophils have also been acknowledged to modulate innate and adaptive immunity and to play a major pathogenetic role in allergic diseases due to their recruitment, accumulation, activation and defective clearance. Asthma and allergic conditions are characterized by prominent eosinophilic inflammation, which has the potential to cause airway mucosal damage through the degranulation of eosinophils and the subsequent release of various inflammatory mediators.

Apoptosis or programmed cell death is considered as an important mechanism in the resolution of inflammation. Failure in the apoptotic process of eosinophils has been associated with pulmonary and allergic disease states. Apoptosis is a controlled process of cell suicide that allows the quiet removal of senescent cells or those cells whose continued survival would be detrimental to the organism. Unlike necrosis, in which the integrity of the cell membrane is lost and the cytosolic constituents of the cell are released into the extracellular space causing inflammation and tissue damage, apoptotic cells are phagocytozed intact without the induction of inflammation. *In vitro*, eosinophils undergo apoptosis in the absence of the survival-prolonging cytokines interleukin (IL)-3, IL-5 and granulocyte/macrophage colony-stimulating factor (GM-CSF).

Histamine is an important physiological amine that has many physiological and pathological functions. In addition to the regulation of gastric acid secretion and neurotransmitter release, histamine mediates the immediate hypersensitivity reaction. Furthermore, it has been reported to regulate chronic inflammation and the immune response. The effects of histamine are mediated through at least four distinct cell surface G-protein-coupled receptors H_{1-4} that have all been claimed to be expressed in eosinophils.

Antihistamines (aka histamine H_1 receptor antagonists) are a commonly used and effective treatment for allergic disorders. In addition to their antagonism at the H_1 receptors, antihistamines are often claimed to have additional anti-inflammatory properties. However, the effects of histamine and antihistamines on eosinophil longevity are poorly understood and surprisingly little studied. The present study focused on elucidating the possible effects of histamine and antihistamines on constitutive and IL-5-inhibited human eosinophil apoptosis and on establishing the signaling cascades behind the possible effects.

The c-Jun N-terminal kinase (JNK) is an intracellular kinase involved in the pathways of apoptosis. JNK has been proposed to play a role in nitric oxide and glucocorticoid-induced human eosinophil apoptosis. The current study aimed at clarifying the role of JNK as a mediator of human eosinophil apoptosis and investigated whether JNK could mediate glucocorticoid- or antihistamine-induced apoptosis or acted more as a general mediator of constitutive human eosinophil apoptosis.

To summarize, the present study aimed at elucidating the role of histamine and different histamine receptors in the regulation of human eosinophil apoptosis. Furthermore, the importance of JNK as a mediator of eosinophil apoptosis was investigated.

REVIEW OF THE LITERATURE

1 Asthma and Allergy

Asthma and atopy are chronic inflammatory diseases that have created a worldwide and increasing public health burden. Atopy is manifested in several clinical disease states including atopic eczema, allergic rhinitis and allergic conjunctivitis. Moreover, atopy constitutes a major risk for asthma. In the United States, more than 11 million people were reported as experiencing an asthma attack during the year 2002 and more than 5% of children under the age of 18 have suffered from asthma attacks (National Heart Lung and Blood Institute 2003). Despite our current greater understanding of the mechanisms of asthma and the development of therapeutic approaches capable of reducing morbidity and improving the quality of life of asthmatic persons, asthma is still responsible for 2 million annual emergency department visits, 0.5 million hospitalizations and 4500 deaths in the United States (National Heart Lung and Blood Institute 2003). In Finland, the number of patients suffering from asthma or asthma-like symptoms has increased, especially among children and young people. Currently, approximately 6% of adults are believed to have asthma and 4-7% of children are being treated by physicians due to diagnosed asthma while another 4-7% suffer from asthma-like symptoms (Finnish Expert Panel 2000 and 2006). However, despite the increase in asthma prevalence across the 1960s or 1970s to 1990s, more recent evidence suggests that this increase has reached a plateau or even reversed in the developed countries (Rees 2005, von Hertzen and Haahtela 2005). Earlier detection and improved treatment of asthmatics as well as environmental factors appear to be involved in this current declining trend in asthma prevalence as the genetic susceptibility elements have not apparently changed (Rees 2005, von Hertzen and Haahtela 2005).

1.1 Definitions of Asthma and Allergy

Asthma is defined as a *"chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells"* (National Heart Lung and Blood Institute 2003). In susceptible individuals, the chronic inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night and in the early morning. These symptoms are usually associated with variable airflow obstruction that is at least partly reversible, either spontaneously or after treatment. In addition, the chronic asthmatic inflammation generates an associated increase in the existing airway hyperresponsiveness to a variety of stimuli (National Heart Lung and Blood Institute 2003). The complex asthmatic inflammatory process can be divided into fairly distinct components responsible for the various clinical consequences seen in asthma. These include IgE-mediated acute allergic reaction responsible for immediate bronchoconstriction, chronic inflammation causing exacerbations and non-specific bronchial hyperreactivity, and airway remodelling, which evokes the persistent and irreversible obstruction of the airways (Bousquet et al. 2000).

Asthma is by far a single condition but a heterogeneous group of different clinical phenotypes. In general terms, asthma can be divided into non-allergic (intrinsic) asthma and allergic (extrinsic) asthma, which results from immunological reactions and can further be divided into IgE-mediated asthma and the presumably small group of non-IgE-mediated allergic asthma (Humbert et al. 1999, Johansson et al. 2004). IgE-mediated allergic asthma is characterized by infiltration of the bronchial mucosa with T helper 2 (Th2) –type lymphocytes and eosinophils as well as circulating IgE-antibodies and positive skin prick tests to common aeroallergens (i.e. atopy) (Humbert et al. 1999). In contrast, patients with non-allergic asthma are non-atopic (i.e. their skin prick tests are negative, their serum IgE concentrations are within the normal range and they have no history of allergic diseases) (Humbert et al. 1999). However, the immunopathological mechanisms and inflammatory changes appear to be very similar in both forms of asthma (Humbert et al. 1999, Johansson et al. 2004).

Atopy is defined as a tendency to become sensitized and to produce IgE antibodies in response to ordinary exposures to allergens, which are usually proteins. Atopy itself is not a disease but atopic persons are susceptible to develop the symptoms of eczema, rhinoconjunctivitis or asthma (Johansson et al. 2004). Allergy is a hypersensitivity reaction initiated by specific immunological mechanisms and most commonly the offending antibody belongs to the IgE isotype. These patients are said to have IgE-mediated allergy (Johansson et al. 2004). Allergic diseases include asthma, rhinitis, conjunctivitis, eczema, urticaria and anaphylaxis (Johansson et al. 2004). In this thesis, the terms atopy and allergy are used as synonyms to describe IgE-mediated allergic disease conditions.

1.2 Pathogenesis of Asthma and Allergic Diseases

In atopic individuals, an imbalance between T helper (Th) –type 1 and 2 lymphocytes exists, favouring the generation of Th2 cytokines. During sensitization, contact of Th2 cells with an allergen leads to the production of several cytokines including IL-4, IL-5 and IL-9, which stimulate B-lymphocytes, eosinophils and mast cells. B-cells produce IgE, which is captured on the cell surface of mast cells, basophils and eosinophils by its receptors. During subsequent encounters with the allergen, cross-linking of the allergen with allergen-specific IgE stimulates the release of various harmful substances including histamine, proteases, leukotrienes and cytokines generating the immediate, type I hypersensitivity reaction (Pearlman 1999, Broide 2001, Barrios et al. 2006). This type I hypersensitivity reaction (i.e. early-phase reaction) is responsible for generating the immediate symptoms and signs of allergic diseases such as sneezing, nasal itch, rhinorrhea and congestion of the nose, the epiphora and itching of the eyes, or bronchoconstriction, mucus secretion and vasodilatation in asthma (Bachert 1998, Pearlman 1999, Bousquet et al. 2000, Christodoulopoulos et al. 2000, Keane-Myers 2001, Stahl et al. 2002, Calder and Lackie 2004). Mast cells are considered to play a central role in initiating the typical changes encountered during an early-phase allergic response (Bachert 1998, Pearlman 1999, Broide 2001).

In the late-phase reaction occurring between 6 to 9 h after allergen challenge, the recruitment and activation of eosinophils, CD4+ T-cells, basophils, macrophages and neutrophils are crucial (Pearlman 1999, Bousquet et al. 2000, Christodoulopoulos et al. 2000). However, an alternative view on the mechanism of the late-phase reaction in the airways has been presented in which a direct interaction between activated T cells and airway smooth muscle cells was proposed to be more important (Kay 2005). The recruitment of inflammatory cells is triggered by Th2 cytokines as well as by the release of mast cell cytokines during the early-phase response. The recruitment of peripheral blood inflammatory cells such as eosinophils, lymphocytes and monocytes into inflamed airways results from adhesive interactions between these circulatory inflammatory cells and endothelial cells in response to the production of inflammatory mediators, cytokines and chemokines, and the expression of cell surface adhesion molecules such as CD11a, CD11b, CD18, intercellular adhesion molecule -1 and vascular cell adhesion molecule -1. The recruitment of cells into the bronchial walls is associated with their priming and activation, which is dependent not only on certain on cytokines such as IL-5 and GM-CSF but also on chemokines such as RANTES and eotaxin (Pearlman 1999, Bousquet et al. 2000). The accumulation of predominantly eosinophils into the airway tissue induces, through the release of various eosinophil products, the late-phase clinical responses of congestion, increased mucus production and bronchial contraction (Pearlman 1999). In allergic rhinitis, the late-phase response is characterized by a recurrence of sneezing, rhinorrhea and increased nasal air flow resistance (Christodoulopoulos et al. 2000). The recurrence of ocular symptoms including itching, chemosis, lacrimation, mild conjunctival hyperemia and eyelid oedema also occur during the late-phase response of ocular allergy (Keane-Myers 2001).

In addition to the immediate and late-phase allergic reactions, chronic airway inflammation is a key feature in asthma. Eosinophils play a critical role in this condition through the release of pro-inflammatory mediators, cytotoxic granule contents and cytokines resulting in vascular leakage, mucus hypersecretion, smooth muscle contraction, epithelial shedding and bronchial

hyperresponsiveness (Giembycz and Lindsay 1999, Bousquet et al. 2000, Lee et al. 2004, Kay 2005). In addition to the eosinophilic inflammation, all cells of the airways, including T-cells, mast cells, macrophages, epithelial cells, fibroblasts and bronchial smooth muscle cells are involved and become activated in chronic airway inflammation (Bousquet et al. 2000). Furthermore, activated neutrophils are present, especially in severe asthma (Ennis 2003). Eosinophils are also involved in airway remodelling through the release of growth factors, elastase and metalloproteases (Bousquet et al. 2000, Kay et al. 2004, Humbles et al. 2004, Kay 2005). In asthma, the remodelled phenotype includes increased deposition of several extracellular matrix proteins in the basement membrane and bronchial mucosa, hyperplasia of smooth muscle cells as well as goblet cells and the formation of new blood vessels, which generate permanent structural changes and irreversible obstruction in the asthmatic airway (Kay et al. 2004). It is currently believed that early and intensive intervention with anti-inflammatory treatment extinguishes the chronic inflammatory process and thus prevents the formation of permanent changes and damage. Allergic rhinitis is also a chronic inflammatory condition characterized by modification of the histological and functional structure of the tissue, leading to remodelling (Christodoulopoulos et al. 2000). In ocular allergy, the acute allergic eye diseases include seasonal and perennial allergic conjunctivitis whereas chronic allergic conjunctivitis manifests as vernal keratoconjunctivitis or atopic keratoconjunctivitis, which may even lead to loss of vision through corneal destruction (Keane-Myers 2001).

Despite advances in our understanding of the importance of the eosinophil in the pathogenesis of allergy and asthma, the role of eosinophils in the late-phase reaction has recently been questioned based on ambiguous findings on studies made with anti-IL-5 antibodies (Kay 2005). A single dose of a monoclonal antibody to IL-5 was reported to significantly decrease blood and sputum eosinophils but not to influence the late asthmatic response or airway hyperresponsiveness to histamine (Leckie et al. 2000). Triplicate infusions of anti-IL-5 antibody were reported to completely deplete blood eosinophils but only to halve the amount of eosinophils in the airways or blood marrow. Moreover, no effects were observed on the eosinophil major basic protein (MPB)

content in the bronchial mucosa or on the clinical measures of asthma (i.e. airway hyperresponsiveness, forced expiratory volume in 1 s (FEV_1), peak flow measurements) (Flood-Page et al. 2003a). Further evidence against the role of eosinophils in the late-phase allergic reaction has emerged from studies in human skin, in which the kinetics of eosinophil accumulation can be separated from the time course of the late-phase reaction. In addition, the significant reduction in eosinophil numbers by anti-IL-5 monoclonal antibody infusions did not affect the magnitude of allergen-induced cutaneous swelling and oedema (Phipps et al. 2004). In contrast, the importance of eosinophils in the remodelling processes is supported by studies with anti-IL-5 antibodies as triplicate infusions of a monoclonal antibody to IL-5 reduced the deposition of extracellular matrix proteins both in the bronchial mucosa and in skin biopsies (Flood-Page et al. 2003b, Phipps et al. 2004).

The presence of one allergic disease entity is frequently associated with other forms of allergy. Moreover, allergic rhinitis is considered as a risk factor for asthma and often precedes asthma in the atopic march (i.e. in the natural course of atopic disorders) (Spergel 2005). A link between atopic dermatitis and asthma has also been proposed (Spergel 2005). Despite the clinical differences between the various phenotypes of allergy, the underlying pathogenetic mechanisms appear to be very similar. Today, a considerable amount of evidence has demonstrated an unquestionable link between allergic upper airway disease (i.e. allergic rhinitis) and inflammation of the lower airways (i.e. asthma) indicative of a single, unified or united airways disease (Passalacqua et al. 2001, Togias 2003, Passalacqua et al. 2004, Spergel 2005). Thus, the treatment of allergic diseases and asthma should not be limited only to the nose or bronchi but rather should be targeted to the entire respiratory tract. The concept of a united airways disease therefore yields relevant diagnostic and therapeutic implications.

1.3 Treatment of Asthma and Allergic Diseases

Regular use of inhaled corticosteroids (aka glucocorticoids) is the mainstay of current asthma management both in adults and children with persistent asthma (Finnish Expert Panel 2000 and 2006, National Heart Lung and Blood Institute 2003, Kankaanranta et al. 2004, Rees 2006). Glucocorticoids are the most effective anti-inflammatory therapy for asthma. By alleviating inflammation, glucocorticoids attenuate bronchoconstriction, airway hyperresponsiveness and prevent remodelling. In addition, short-acting β_2-agonists are used to achieve immediate bronchodilatation during asthma attacks. Long-acting β_2-agonists are generally used as first choice add-on therapy for patients who do not achieve adequate control of asthma with low or moderate doses of inhaled corticosteroids (Finnish Expert Panel 2000 and 2006, National Heart Lung and Blood Institute 2003, Kankaanranta et al. 2004, Rees 2006). Leukotriene receptor antagonists or theophylline may be useful alternatives for patients unable or unwilling to inhale corticosteroids and have an additive effect with low or moderate doses of steroids (Finnish Expert Panel 2000 and 2006, National Heart Lung and Blood Institute 2003, Kankaanranta et al. 2004, Rees 2006). Although antihistamines as such have not been shown to be beneficial in the treatment of asthma, it has been suggested that cetirizine delays the onset or even prevents the development of asthma in atopic children (Warner and ETAC Study Group 2001).

Antihistamines (aka H_1 receptor antagonists) are a common and effective medication in the treatment of allergy. Antihistamines may be used topically or systemically as oral preparations. Corticosteroid nasal sprays are a safe and effective form of treatment in chronic allergic rhinitis (Christodoulopoulos et al. 2000). Especially in patients with coexistent allergic rhinitis and asthma, a combination of inhaled and nasal corticosteroids may provide better disease control (Rees 2006). In addition to antihistamines and glucocorticoids, allergic rhinitis may also be treated with oral leukotriene receptor antagonists or topical mast cell stabilizers (cromoglicates). Allergic conjunctivitis may be controlled with topical eye drop preparations of antihistamines, mast cell stabilizers or steroids in addition to oral antihistamines (Keane-Myers 2001).

2 Eosinophils

2.1 Eosinophil Development

Eosinopoiesis, or eosinophil production, occurs almost exclusively in the bone marrow from pluripotential stem cells, which first differentiate into a hybrid precursor with shared properties of basophils and eosinophils. These cells then separate into an eosinophil lineage generating the end-stage eosinophilic granulocytes (Giembycz and Lindsay 1999, Rothenberg and Hogan 2006). The bone marrow of healthy individuals contains approximately 3% eosinophils or their precursors, of which 37% are mature, non-dividing eosinophils, and the remainder consist of promyelocytes/myelocytes and metamyelocytes, which exist in storage compartments (Giembycz and Lindsay 1999). The time consumed from the last cell division in the bone marrow until the appearance of mature eosinophils in the blood is approximately 2.5 days (Giembycz and Lindsay 1999). Eosinophils have been estimated to have a half-life of 18 h in the circulation, which may be prolonged if there is an increase in the concentration of circulating survival-prolonging cytokines or due to the saturation of sites through which eosinophils migrate into tissues (Giembycz and Lindsay 1999).

In the regulation of eosinophil development, a variety of cytokines and other mediators, including interleukin (IL)-3, IL-4, IL-5, IL-6, IL-11, IL-12, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor (GM-CSF), eotaxin, stem cell factor and leukaemia inhibitory factor, play a role (Giembycz and Lindsay 1999). However, three cytokines, IL-3, IL-5 and GM-CSF, are known to be of particular importance in stimulating the proliferation and differentiation of eosinophils in the bone marrow (Lampinen et al. 2004, Rothenberg and Hogan 2006). The release of eosinophils from the bone marrow into the blood stream is mainly promoted by IL-5 (Giembycz and Lindsay 1999, Lampinen et al. 2004, Rothenberg and Hogan 2006).

In the circulation, eosinophils account for only 1-3% of leukocytes. However, the eosinophil is primarily a tissue cell, i.e. there are approximately 500 times as many eosinophils present in the tissues than are circulating in blood (Lampinen et al. 2004). Eosinophil priming in the blood is stimulated by IL-3, IL-5 and GM-CSF. A critical step in the extravasation of eosinophils is their adhesion to vascular endothelium, which is enhanced by IL-1, IL-4, tumour necrosis factor (TNF)-α and interferon-γ (Lampinen et al. 2004). A variety of cytokines and other mediators act as chemotactic factors for eosinophils. The most important eosinophil chemoattractants are IL-5, IL-8, RANTES, eotaxin, eotaxin-2, eotaxin-3, monocyte chemotactic proteins 3 and 4, and TNF-α (Lampinen et al. 2004, Rothenberg and Hogan 2006).

2.2 Elimination of Eosinophils

The number of eosinophils in the blood and tissues is largely determined by the balance between eosinophil maturation, recruitment and removal (Simon et al. 1997, Kankaanranta et al. 2005). Even though extravasation and increased recruitment of eosinophils may be considered as the principal cause of tissue eosinophilia at inflamed sites, decreased elimination of the cells can also be a contributing factor (Lampinen et al. 2004). Normally, eosinophils are eliminated from the tissues via apoptosis, or programmed cell death. The most important cytokines prolonging eosinophil survival are IL-3, IL-5 and GM-CSF (Giembycz and Lindsay 1999, Lampinen et al. 2004, Kankaanranta et al. 2005). With regard to the resolution of eosinophilic inflammation, eosinophil apoptosis and its induction are regarded as a major mechanism (Haslett 1999, Kankaanranta et al. 2005, Bianchi et al. 2006). It has been shown that eosinophil apoptosis is delayed in patients with asthma, inhalant allergy and atopic dermatitis (Wedi et al. 1997, Kankaanranta et al. 2000). Moreover, the appearance of apoptotic eosinophils has been reported in the lung tissue *in vivo* (Kodama et al. 1998, Druilhe et al. 1998, Vignola et al. 1999, Duez et al. 2001, Ohta et al. 2001, Uller et al. 2005). Apoptotic eosinophils have also clearly been demonstrated to exist in the airway lumen (Woolley et al. 1996, Erjefält and Persson 2000, Uller et al. 2001, Duncan et al. 2003, Uller et al. 2005). However, alternative routes of eosinophil

clearance have been proposed as significant apoptosis of eosinophils in inflamed airway tissues has not yet been incontestably demonstrated *in vivo* and thus the presence of apoptotic eosinophils in the lung tissue remains a matter of speculation (Erjefält and Persson 2000, Erjefält 2005). Transepithelial migration of eosinophils into the airway lumen has been suggested as an effective and non-injurious alternative to apoptosis for the effective clearance of airway tissue eosinophils (Erjefält and Persson 2000, Uller et al. 2001, Erjefält et al. 2004, Erjefält 2005). The luminal eosinophils may then be cleared through cytolysis, apoptosis or mucociliary clearance (Erjefält and Persson 2000, Erjefält 2005). Alternatively, airway eosinophils may be eliminated via cytolysis or necrosis directly in the tissue (Erjefält and Persson 2000, Erjefält 2005). In nasal polyps, the clearance of eosinophils has been reported to occur through cytolysis and paraepithelial migration instead of apoptosis (Uller et al. 2004). However, eosinophil removal has also been reported to occur via apoptosis in nasal polyps (Simon et al. 1997, Davidsson et al. 2000).

2.3 Eosinophil Functions

A prominent feature of the eosinophil is the presence of many spherical or ovoid granules. Four distinct populations of granules have been identified, i.e. secondary granules, small granules, primary granules and lipid bodies (Fig. 1) (Giembycz and Lindsay 1999). The primary granules are the principal sites of Charcot-Leyden crystals. The secondary granules consist of a prominent crystalline core composed of major basic protein (MBP). Eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN) are located within the non-crystalloid matrix of the secondary granules along with a number of cytokines, such as IL-2, IL-4, IL-5, GM-CSF and TNF-α. Lipid bodies represent a site of lipid mediator biosynthesis generating e.g. leukotrienes and prostanoids, and provide a major store for arachidonic acid. Small granules provide a store for proteins such as arylsulfase B and acid phosphatase (Giembycz and Lindsay 1999). In summary, the eosinophil has the capacity to produce a variety of inflammatory mediators including cytotoxic granular proteins, membrane-derived lipids, chemokines, cytokines, fibrogenic

and growth factors, neuropeptides and reactive oxygen species, which have the potential to cause mucosal damage and to modulate allergic inflammation (Kay 2005, Rothenberg and Hogan 2006).

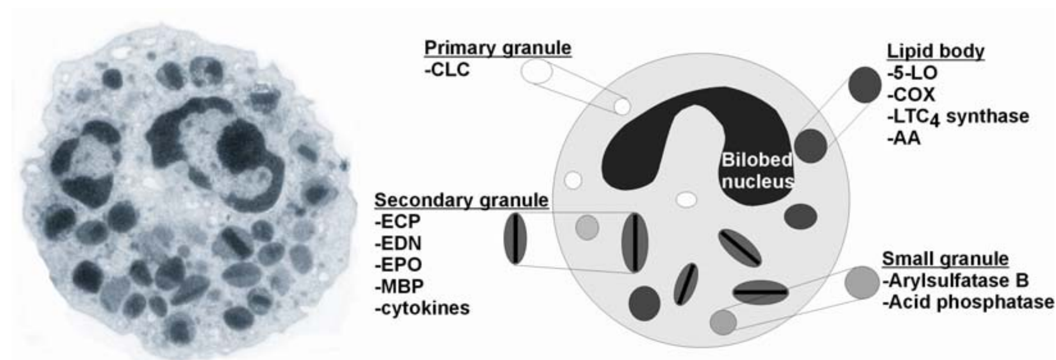

Figure 1. *Cardinal structures of the human eosinophil. Shown on the left is a transmission electron microscope picture of a viable, isolated human eosinophil. On the right, the typical bilobed nucleus and the four main granules and their contents of eosinophils are depicted. CLC, Charcot-Leyden crystals; 5-LO, 5-lipoxygenase; COX, cyclooxygenase;* LTC_4*, leukotriene* C_4*; AA, arachidonic acid. (Modified from Giembycz and Lindsay 1999)*

The eosinophil has traditionally been considered to be involved in host protection against parasitic helminths. However, as primary sites of eosinophil accumulation include the gastrointestinal tract and the respiratory tract, the eosinophil has long been acknowledged as playing an important pathogenetic role in allergic diseases, asthma and inflammatory bowel disease. Moreover, the eosinophil has also been recognized to modulate innate and adaptive immunity (Adamko et al. 2002, Lampinen et al. 2004, Rothenberg and Hogan 2006). The activation state of tissue eosinophils varies from the resting state through partially activated or primed cells up to fully activated degranulating cells (Adamko et al. 2002). Degranulation is considered as the end activation of eosinophils. Eosinophils are able to release their granular contents by four different mechanisms. In necrosis or cytolysis, eosinophils lose the integrity of their plasma membrane and release the contents of their granules into the surrounding tissues thus possibly evoking toxic effects or inflammation. In

piecemeal degranulation, numerous small vesicles appear in the cytoplasm while the crystalloid secondary granules appear to gradually lose their matrix components and crystalline cores. Gradual emptying of the secondary granules is thought to occur. In granule exocytosis, the crystalloid granules fuse directly with the plasma membrane prior to releasing their contents to the outside of the cell. In compound exocytosis, the secondary granules fuse with each other prior to release from the cell through a single fusion pore (Adamko et al. 2002). Piecemeal degranulation and cytolysis have been established as the major cellular processes through which eosinophils release their cytotoxic granule contents into diseased tissues (Erjefält and Persson 2000, Erjefält et al. 2001).

Due to the capacity of the eosinophil to release its cytotoxic granule contents into the surrounding tissue in inflammation, the process of apoptosis or programmed cell death provides a non-inflammatory solution for eosinophil clearance since apoptosis occurs without the release of the cellular contents into the tissues (Saraste and Pulkki 2000, Ziegler and Groscurth 2004). Thus, eosinophils that have undergone apoptosis either spontaneously or after induction by pharmacological agents are phagocytozed and eliminated without further exacerbating the inflammation.

3 Histamine

3.1 History of Histamine

Histamine, 2-(4-imodazole)-ethylamine, which is synthesized from L-histidine by histidine decarboxylase, was discovered nearly a century ago in 1910 by Barger and Dale and found to be capable of constricting guinea pig ileum. However, histamine had been synthesized as a chemical product already a couple of years prior to this, in 1907. Its smooth muscle-stimulating and vasodepressor activities were demonstrated soon after its characterization. The presence of histamine in normal tissues was demonstrated 17 years later and histamine was first observed to exist in the lung. The connection between histamine and

anaphylaxis was thereafter rapidly found. The association between histamine and mast cells was established in 1952 and the link to basophils in 1972. The first antihistamine (H_1-antagonist) was discovered in 1937 and introduced into therapeutic use 5 years later. However, the H_1 receptor was not discovered until 1966. Further research revealed the H_2 and H_3 receptors in 1972 and 1983, respectively (Bachert 1998, MacGlashan 2003, Jutel et al. 2005, Akdis and Simons 2006). In 1975, cimetidine, the first safe and effective H_2-antagonist was developed and introduced into the treatment of peptic ulcer. The Nobel Prize in Physiology or Medicine for 1988 was awarded to Sir James W. Black for his discoveries concerning the H_2 receptor and its antagonists (The Nobel Assembly at the Karolinska Institute 1988). More recent landmarks in the history of histamine have included the cloning of the $H_{1\text{-}3}$ receptor genes in the 1990s (Bachert 1998, MacGlashan 2003, Jutel et al. 2005, Akdis and Simons 2006). Recently, a fourth histamine receptor, the H_4, was discovered and cloned (Oda et al. 2000, Nakamura et al. 2000, Zhu et al. 2001, Nguyen et al. 2001, Liu et al. 2001, Morse et al. 2001). Histamine is one of the most intensively studied molecules in medicine. Although substantial knowledge about the metabolism, receptors, signal transduction and physiological and pathological effects of histamine has accumulated, many aspects concerning the complex interactions of histamine, its receptors and other receptors and mediators still remain to be elucidated.

3.2 Effects of Histamine

Histamine is produced by multiple cell types throughout the body, including central nervous system neurons, gastric mucosa enterochromaffin cells, mast cells, basophils and lymphocytes, and plays an important role both in normal human physiology as well as in pathological conditions (Bakker et al. 2002, Jutel et al. 2005). Although originally considered as a mediator of acute inflammatory and immediate hypersensitivity reactions, histamine is now acknowledged to affect chronic inflammation and to regulate several essential events in the immune response. Histamine is involved in cell proliferation and differentiation, haematopoiesis, embryonic development, cognition and memory, the cycle of

sleep and awake, gastric acid secretion and in energy and endocrine homeostasis (Jutel et al. 2005).

Through the H_1 receptor, histamine induces the immediate symptoms of the allergic response, such as pruritus, pain, flushing and dyspnoea as well as the pathophysiological changes behind the symptoms, which include vasodilatation, increased vascular permeability, hypotension, bronchoconstriction and stimulation of airway vagal afferent nerves. Histamine also modulates allergic inflammation via the H_1 receptor by increasing the release of inflammatory mediators, the expression of adhesion molecules and the chemotaxis of eosinophils and neutrophils (Akdis and Simons 2006). The histamine H_2 receptor is responsible for the increase in gastric acid secretion, vascular permeability, hypotension, flushing, bronchodilatation and mucus production in the airways (Akdis and Simons 2006). The H_2 receptor also participates in the regulation of inflammation and the immune response in a complex manner. For example, the H_2 receptor has been reported to decrease eosinophil and neutrophil chemotaxis, to inhibit IL-12 production by dendritic cells, to increase IL-10 production in dendritic and T cells, to induce the development of Th2 or tolerance-inducing dendritic cells, to increase humoral immunity but to decrease cellular immunity, to suppress Th2 cells and cytokines and to inhibit neutrophil activation and degranulation (Jutel et al. 2005, Akdis and Simons 2006). Thus, the H_2 receptor possesses various anti-inflammatory properties. The histamine H_3 receptor is mainly a pre-synaptic nervous system receptor, which decreases the release of histamine, dopamine, 5-HT, noradrenaline and acetylcholine (Akdis and Simons 2006). The functions of the newly identified H_4 receptor remain largely to be elucidated, however, it has been shown to increase Ca^{2+} influx in human eosinophil as well as to increase eosinophil chemotaxis. The high expression of the H_4 receptors in the bone marrow and peripheral haematopoietic cells predicts a role for the H_4 receptor in immune regulatory functions (Jutel et al. 2005, Akdis and Simons 2006).

3.3 Histamine Receptors and Signaling Cascades

The effects of histamine are mediated through at least four distinct cell surface G-protein-coupled receptors denoted as histamine H_{1-4} receptors (Bakker et al. 2002, Jutel et al. 2005, Akdis and Simons 2006). The histamine H_1 receptor is expressed in lymphocytes, eosinophils, neutrophils, dendritic cells, endothelial and epithelial cells, airway and vascular smooth muscle cells and nerve cells. Through the $G_{q/11}$-coupled H_1 receptor, phospholipase C (PLC) is activated and this leads to the activation of protein kinase C (PKC) and an increase in the intracellular Ca^{2+} concentration (Fig. 2). In addition, for example cyclic guanosine monophosphate (cGMP), phospholipase D, phospholipase A_2 and nuclear factor kappa B (NF-κB) have been reported to be activated (Bakker et al. 2002, Jutel et al. 2005, Akdis and Simons 2006). Through the histamine H_2 receptor, which is coupled to G_s-protein, stimulation of adenylate cyclase (AC) is achieved, which leads to an increase in cyclic adenosine monophosphate (cAMP) levels and further to activation of protein kinase A (PKA) (Fig. 2). Moreover, a role for c-Fos, c-Jun and PKC has also been implicated in the transduction of H_2 receptor-mediated effects (Bakker et al. 2002, Jutel et al. 2005, Akdis and Simons 2006). Stimulation of the $G_{i/o}$-coupled histamine H_3 and H_4 receptors inhibits the activity of AC, decreases the production of cAMP and suppresses the activity of PKA (Fig. 2). In addition, elevated intracellular Ca^{2+} concentrations have been reported after stimulation of these receptors. The H_3 receptor has also been suggested to activate mitogen-activated protein kinases (MAPK) (Bakker et al. 2002, Jutel et al. 2005, Akdis and Simons 2006).

In addition to the four unequivocally characterized G-protein-coupled cell surface histamine H_{1-4} receptors, histamine has also been described to possess an intracellular binding site in haematopoietic cells, which is denoted the H_{IC} receptor (MacGlashan 2003). The signaling cascades initiated by the H_{IC} receptor are not currently understood, however, it has been speculated that histamine and the H_{IC} receptor itself may act as second messengers (MacGlashan 2003). In conclusion, despite the fact that our understanding of the effects and signal transduction cascades of histamine has already increased tremendously

during the last few decades, it is evident that several interesting issues remain unresolved with respect to histamine and its receptors. Furthermore, it has been proposed that additional histamine receptors still remain to be discovered (Hough 2001).

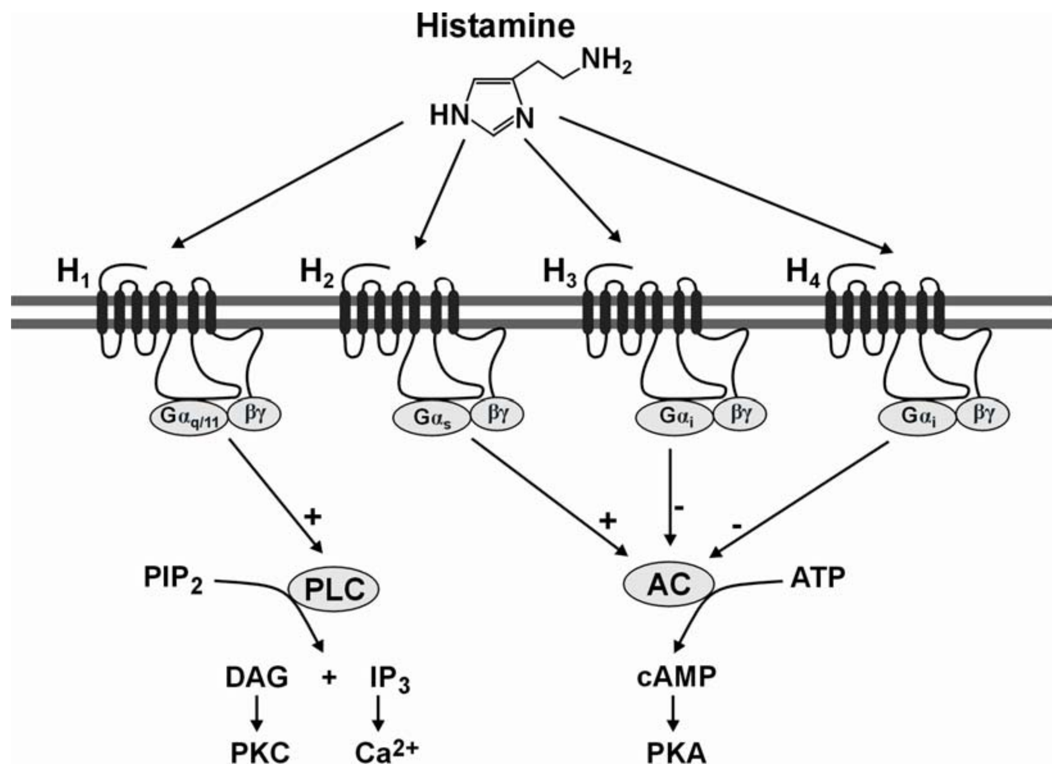

Figure 2. *Histamine receptors and their main intracellular signal transduction cascades. PIP_2, phosphatidylinositol(4,5)-bisphosphate; IP_3, inositol(1,4,5)-triphosphate; DAG, 1,2-diacylglycerol; ATP, adenosine triphosphate. (Modified from Bakker et al. 2002)*

3.4 Histamine and Eosinophils

Eosinophils have been reported to express each of the histamine $H_{1\text{-}4}$ receptor subtypes (Giembycz and Lindsay 1999, Oda et al. 2000, Liu et al. 2001, Morse et al. 2001, Jutel et al. 2005, Akdis and Simons 2006). However, evidence pointing to the existence of the histamine H_3 receptors on human eosinophils (Raible et al. 1992 and 1994, Giembycz and Lindsay 1999) is now considered to be incorrect.

The novel histamine receptor discovered in eosinophils in the 1990s (Raible et al. 1992 and 1994) has now been identified as the H_4 subtype (Oda et al. 2000, Hough 2001, Liu et al. 2001, Morse et al. 2001, Zhu et al. 2001, O'Reilly et al. 2002, Buckland et al. 2003, de Esch et al. 2005). Furthermore, human eosinophils have recently been shown not to express the H_3 receptor subtype (Nakayama et al. 2004). As a conclusion, human eosinophils are currently considered to express the H_1, H_2 and H_4 subtypes of histamine receptors.

Histamine has been found to affect or modulate several essential functions of eosinophils. Allergen-induced accumulation of eosinophils in the airways, nose and skin can be inhibited by histamine H_1 receptor antagonists (Jutel et al. 2005, Akdis and Simons 2006). Histamine also influences the migration of eosinophils: at high concentrations, histamine was previously suggested to inhibit eosinophil chemotaxis via the H_2 receptor but at low concentrations, histamine can enhance chemotaxis via the H_1 receptor (Jutel et al. 2005, Akdis and Simons 2006). However, it has recently been suggested that the receptor responsible for the selective recruitment and chemotaxis of eosinophils by histamine is the newly identified H_4 subtype (O'Reilly et al. 2002). In addition, histamine mediates cell shape changes and upregulation of adhesion molecules as well as induces actin polymerization and intracellular calcium mobilization in eosinophils through the H_4 receptor (Buckland et al. 2003, Ling et al. 2004). Furthermore, via stimulation of the H_2 receptor, histamine can inhibit eosinophil degranulation and the consequent elevation in intracellular cAMP levels (Ezeamuzie et al. 2000). At present, the effects of histamine on eosinophil apoptosis are unknown albeit it has been suggested that histamine does not affect eosinophil survival in the absence of cytokines (Levi-Schaffer et al. 1998). Thus, studies evaluating the effects of histamine on eosinophils in addition to examining other relationships between histamine and eosinophils are warranted.

4 Cell Death

4.1 Apoptosis and Necrosis

Cell death is differentiated into two main forms: apoptosis and necrosis. Necrosis or cytolysis occurs frequently in situations with tissue ischemia and hypoxia, which depletes the cellular energetic pools. Other causes of necrotic cell death include heat stress and toxic agents (Ziegler and Groscurth 2004, Szabo 2005). In necrosis, the cell membrane becomes permeable, organelles dilate, the nucleus disintegrates and eventually the cell swells and ruptures (Fig. 3). The early leakage of the cellular contents into the extracellular space may trigger massive inflammation in the surrounding tissue (Ziegler and Groscurth 2004, Szabo 2005). Necrosis is generally considered not to be programmed but instead to be a rather accidental form of cell death as it is accompanied by rapid destruction of the plasma membrane, cytoplasmic structures and the nucleus (Ziegler and Groscurth 2004, Szabo 2005). However, recent observations suggest that necrosis may also have certain regulated features (Szabo 2005).

A key difference between the two forms of cell death is that during necrosis, the membrane integrity breaks down and cytosolic constituents are released into the extracellular space through the damaged plasma membrane whereas during apoptosis, cells shrink and their nuclei condense, resulting in their encapsulation into discrete apoptotic bodies, which are phagocytozed by macrophages (Saraste and Pulkki 2000, Ziegler and Groscurth 2004, Szabo 2005). The specific morphological and biochemical hallmarks of apoptosis include chromatin condensation, nuclear fragmentation, cellular shrinkage, membrane blebbing and formation of densely packaged apoptotic bodies containing the cellular organelles and nuclear fragments (Fig. 3). One of the earliest signs of apoptosis is the externalization of phosphatidylserine, which provides an “eat-me”-signal for phagocytes (Saraste and Pulkki 2000, Ziegler and Groscurth 2004). Apoptosis, or programmed cell death, is a regulated, energy-dependent form of cell death. Several hours are usually required from the initiation of apoptosis to

the final cellular fragmentation although the time schedule is dependent on the cell type, the initiating stimulus and the apoptotic pathway. Apoptosis occurs without any accompanying inflammation due to the containment of cellular constituents by an intact membrane and the subsequent engulfment of apoptotic bodies by phagocytes (Ziegler and Groscurth 2004). However, if the cellular remnants cannot be phagocytozed due to dysfunctional or disturbed phagocyte system, apoptotic cells will then undergo a form of degradation that resembles necrosis (i.e. the cell disintegrates and its contents are released into the surrounding tissue); this is called secondary necrosis and the process initiates an inflammatory reaction (Ziegler and Groscurth 2004, Erjefält 2005).

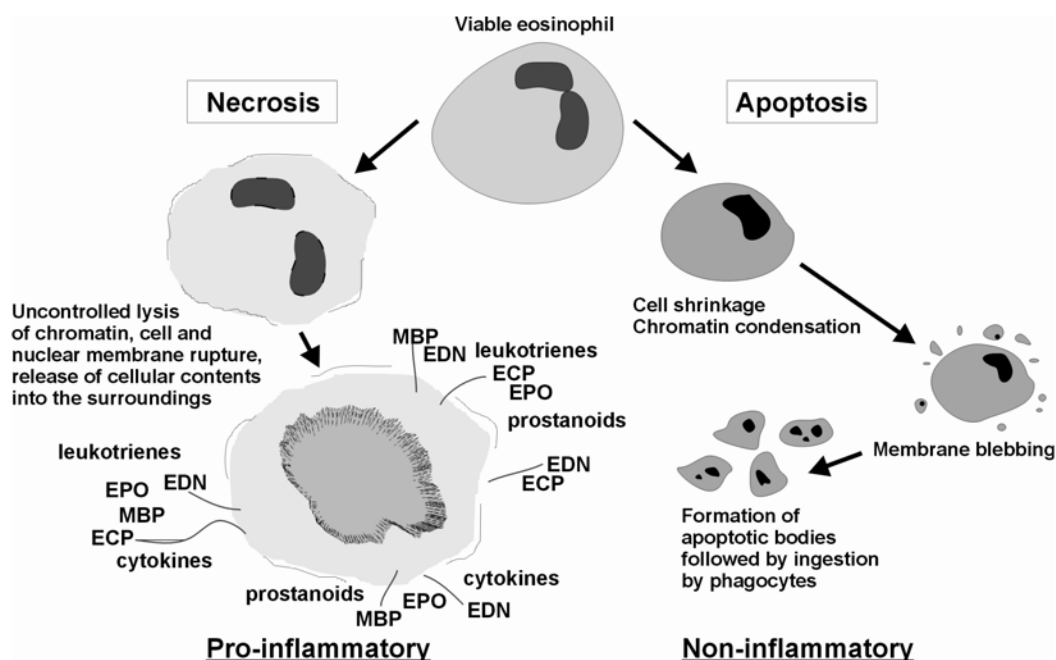

Figure 3. *Schematic presentation of apoptosis and necrosis.*

4.2 Pathways of Apoptosis

In general, apoptosis is an important physiological phenomenon by which cellular homeostasis can be regulated. Apoptosis is a controlled process of cell suicide that allows the removal of senescent cells or those whose continued survival would be detrimental to the organism. During the fetal period, apoptosis is a critical means of regulating embryonic development. In contrast, apoptosis is disturbed during tumour formation and the progression of cancer. The mechanisms and pathways of apoptosis differ between various cell types complicating our understanding of this process. Unlike many other cell types in which apoptosis must be induced, eosinophils cultured *in vitro* rapidly undergo spontaneous apoptosis, which can be inhibited by certain cytokines, e.g. IL-3, IL-5 and GM-CSF.

4.2.1 Caspases

Activation of the caspase cascade is believed to be the most central step in apoptosis (Thornberry 1998, Thornberry and Lazebnik 1998, Saraste and Pulkki 2000, Ziegler and Groscurth 2004, Harwood et al. 2005). Caspases are cysteine aspartate specific proteases that cleave many intracellular proteins containing aspartic acid residues. Caspases are formed constitutively and are normally present as inactive proenzymes. The full enzymatic activity is induced after cleavage at specific internal aspartate residues (Thornberry 1998, Thornberry and Lazebnik 1998, Saraste and Pulkki 2000). During apoptosis, caspases are activated in a self-amplifying cascade. Two functional subgroups of caspases have been characterized: the initiators and the executors. Activation of the upstream, initiator caspases 2, 8 and 9 by pro-apoptotic signals initiates the apoptotic pathway and leads to the proteolytic activation of downstream, effector or executor caspases 3, 6 and 7 (Saraste and Pulkki 2000, Ziegler and Groscurth 2004, Harwood et al. 2005). The effector caspases cleave a set of important proteins and thus, initiate and execute the apoptotic degradation phase including DNA fragmentation and other typical morphological features of apoptosis (Saraste and Pulkki 2000).

Activation of the caspase cascade may be initiated via two different pathways: by ligation of the death receptors such as Fas and TNF receptors (the extrinsic apoptotic pathway) or by mitochondrial damage and by the Bcl-2 protein family (the intrinsic apoptotic pathway). Caspase 8 appears to be crucial in the death receptor –mediated pathways whereas caspase 9 functions as the most upstream caspase in the mitochondrial apoptotic pathway. Caspase 8 activation requires an association of pro-caspase 8 with its cofactor, the Fas-associated death domain (FADD) whereas caspase 9 activation is dependent on a cytosolic complex including cytochrome C, which is released from the mitochondrial transmembrane space into the cytosol, and apoptosis protease-activating factor 1 (Fig. 4). Caspase 8 can also regulate mitochondrial apoptosis by the cleavage of Bid, which facilitates cytochrome C release. Thus, mitochondria also amplify the receptor-mediated apoptosis (Thornberry and Lazebnik 1998, Saraste and Pulkki 2000, Cho and Choi 2002, Harwood et al. 2005).

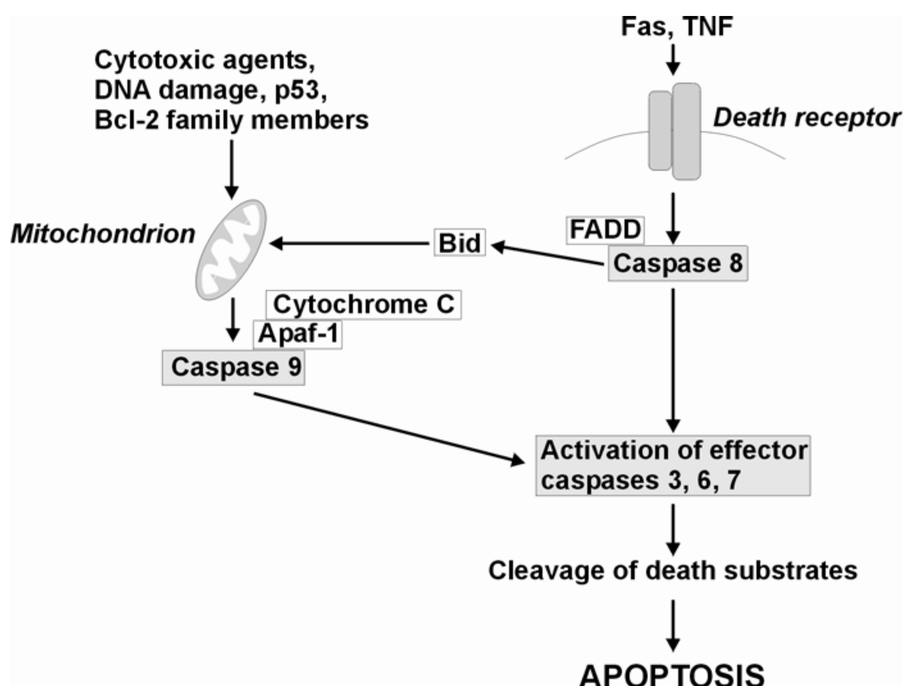

Figure 4. *Major caspase pathways leading to apoptosis. Apaf-1, apoptosis protease-activating factor 1.*

Although the caspase cascades mediating human eosinophil apoptosis have been poorly characterized, the presence of caspases 3, 6, 7, 8 and 9 in eosinophils has been described. Spontaneous eosinophil apoptosis appears to involve the processing caspases 3, 6, 7, 8 and 9, and IL-5 has been demonstrated to prevent caspase activation (Zangrilli et al. 2000, Dewson et al. 2001, Kankaanranta et al. 2005, Kankaanranta et al. 2006).

Apoptosis may be regulated by inhibiting caspases by proteins such as a family of inhibitors of apoptosis proteins, which inhibit effector caspases, c-FLIP (cellular FLICE (Fas-associating protein with death domain-like interleukin-1-converting enzyme) inhibitory protein that binds and inhibits e.g. FADD and caspase 8), p53 (a baculovirus protein) and CrmA (a cowpox virus protein that inhibits initiator caspases) (Thornberry and Lazebnik 1998, Cho and Choi 2002). The exact role of these proteins in the regulation of human eosinophil apoptosis remains unresolved.

4.2.2 Death Receptors

The extrinsic apoptotic pathway is initiated by ligation of death receptors of the TNF receptor (TNFR) family, including TNF-R1, Fas/APO-1/CD95, DR3, DR4, the p75 nerve growth factor receptor and CD40, by their ligands such as TNF and Fas ligand, which induces the trimerization of the ligand-bound receptors leading to the enhancement of the interaction between the death domains of the receptors and the cytoplasmic death domain-containing proteins. TNF-R1 associates with the TNFR-associated death domain protein whereas Fas acts in conjunction with FADD. FADD can physically associate with caspase 8 and activate it directly. In TNF-related apoptosis, the TNFR-associated death domain protein recruits FADD before the activation of caspase 8. In addition to FADD-caspase 8 activation, Fas can also interact with and activate Daxx, which in turn associates with an upstream MAPK kinase kinase (MAPKKK) called the apoptosis signal-regulating kinase 1 in the JNK and p38 MAPK pathways. Thus, Fas may induce apoptosis through the persistent activation of JNK and p38 (Leist and Jäättelä 2001, Kim 2002, Cho and Choi 2002, Harwood et al. 2005).

Eosinophils have been reported to express the death receptor CD95 (Fas/APO-1) on their surface (Tsuyuki et al. 1995, Hebestreit et al. 1996, Druilhe et al. 1996, Luttmann et al. 1998), which evokes apoptosis upon ligation by agonistic antibodies or the natural Fas ligand (Tsuyuki et al. 1995, Matsumoto et al. 1995, Hebestreit et al. 1996, Druilhe et al. 1996, Luttmann et al. 1998). Interferon γ and TNF-α have been suggested to increase the expression of Fas receptors on eosinophils (Luttmann et al. 1998, Luttmann et al. 2000) whereas the cytokines IL-3, IL-5 or GM-CSF do not modulate the constitutive expression of Fas receptors (Luttmann et al. 1998). IL-5 and interferon γ have been reported to inhibit Fas-induced apoptosis of eosinophils (Matsumoto et al. 1995, Letuve et al. 2001) whereas glucocorticoids further enhance this process (Druilhe et al. 1996). The signaling cascades mediating Fas-induced apoptosis remain largely unknown. However, tyrosine phosphorylation and the Lyn tyrosine kinase have been suggested to be involved in Fas-induced eosinophil apoptosis (Simon et al. 1998) in addition to activation of caspases 3 and 8 (Zangrilli et al. 2000, Letuve et al. 2001) and the loss of mitochondrial membrane potential (Letuve et al. 2001). Nitric oxide, cAMP and cGMP have all been reported to reverse Fas-mediated eosinophil apoptosis (Hebestreit et al. 1998).

4.2.3 Mitochondria and the Bcl-2 Protein Family

The intrinsic apoptotic pathway is governed by mitochondria. Upon the initiation of apoptosis by an appropriate stimulus, pro-apoptotic Bcl-2 family proteins such as Bad, Bid, Bax and Bim facilitate the loss of mitochondrial membrane integrity, which results in the leakage of mitochondrial proteins such as cytochrome C and apoptosis protease-activating factor 1 into the cytoplasm. These proteins form a complex (the apoptosome) with pro-caspase 9, which leads to the activation of the initiator caspase 9 (Vander Heiden and Thompson 1999, Leist and Jäättelä 2001, Cho and Choi 2002, Harwood et al. 2005). The regulatory counterparts in the mitochondria include anti-apoptotic members of the same Bcl-2 protein family such as Bcl-x_L and Bcl-2, which inhibit the release of cytochrome C. Eventually, the ratio of death and survival signals sensed by the Bcl-2 family proteins is thought to determine whether the cell will live or

will undergo apoptosis (Vander Heiden and Thompson 1999, Leist and Jäättelä 2001, Denecker et al. 2001, Harwood et al. 2005).

With regard to eosinophils, the role of the Bcl-2 family proteins in eosinophil apoptosis remains ambiguous as the literature addressing the matter is inconsistent. However, human eosinophils have been reported to express Mcl-1, Bcl-x_L and Bax (Druilhe et al. 1998, Dibbert et al. 1998, Zangrilli et al. 2000, Dewson et al. 2001, Zhang et al. 2003). There is a controversy about whether Bcl-2 is expressed in eosinophils (Ochiai et al. 1997, Druilhe et al. 1998) or not (Dibbert et al. 1998, Zangrilli et al. 2000, Dewson et al. 2001), whether spontaneous eosinophil apoptosis is associated with a decrease in Bcl-x_L expression and whether incubation with IL-5 or GM-CSF results in enhancement of Bcl-x_L levels (Dibbert et al. 1998) as this has not been confirmed by subsequent studies (Druilhe et al. 1998, Dewson et al. 2001).

4.3 Modulation of Eosinophil Survival

In allergic diseases and asthma, eosinophils infiltrate the inflamed tissues and are thought to induce epithelial tissue injury and tissue remodelling (Gleich 2000, Kay et al. 2004, Kay 2005). To avoid the accumulation of activated eosinophils, their recruitment must be balanced by efficient cell clearance mechanisms. In this regard, elimination of eosinophils through apoptosis and subsequent engulfment of apoptotic cells by phagocytes is considered to be essential (Kankaanranta et al. 2005). Therefore, modulation of eosinophil survival by regulating apoptosis provides an alternative for controlling eosinophilic inflammation. In the following sections, factors modulating eosinophil survival are reviewed except for the drug groups of antihistamines and glucocorticoids, which are reviewed in detail in section 5.

4.3.1 Cytokines GM-CSF, IL-3, IL-5

GM-CSF, IL-3 and IL-5 are the key cytokines for eosinophil survival inhibiting eosinophil apoptosis both *in vitro* and *in vivo* (Lopez et al. 1986, Begley et al. 1986, Rothenberg et al. 1988, Valerius et al. 1990, Yamaguchi et al. 1991, Tai et al. 1991, Simon et al. 1997, Giembycz and Lindsay 1999, Lampinen et al. 2004, Kankaanranta et al. 2005). As reviewed earlier in section 2, GM-CSF, IL-3 and IL-5 also affect a plethora of other features in the development and recruitment of eosinophils besides their anti-apoptotic role. The signaling pathways involved in cytokine-induced eosinophil survival are complex and not fully understood. However, the receptors for these cytokines have a unique α-chain and a common β-chain, which is essential for signal transduction. Upon activation, cytokine receptors form dimers and phosphorylate tyrosine residues. The multiple signaling events include adaptor proteins such as Shc and Grb-2, Ras-MAPK pathways and Jak (janus kinase)-STAT (signal transducer and activator of transcription) pathways (Fig. 5) (Geijsen et al. 2001, Kankaanranta et al. 2005). IL-5 is also thought to inhibit eosinophil apoptosis by inhibiting Bax translocation to mitochondria and thus attenuating cytochrome C release from the mitochondria (Kuwano et al. 2005).

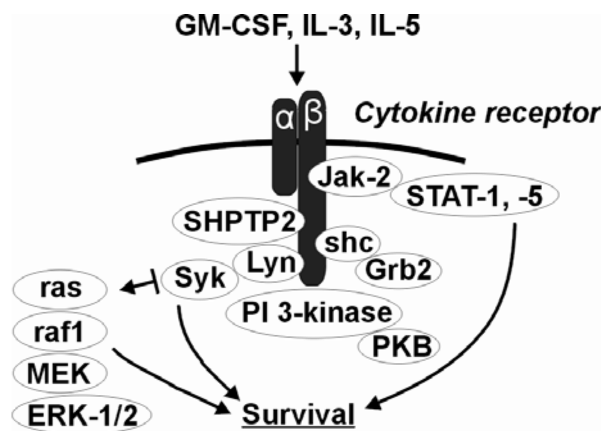

Figure 5. *The intracellular signaling pathways initiated by the survival-prolonging cytokines GM-CSF, IL-3 and IL-5 in human eosinophils. SHPTP2, Src homology 2 domain-containing protein-tyrosine phosphatase; Syk, spleen tyrosine kinase; MEK, MAPK kinase; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; Grb2, growth factor receptor-bound protein 2. (Modified from Kankaanranta et al. 2005)*

4.3.2 Tyrosine Kinases

Stimulation of non-receptor tyrosine kinases such as Lyn, Syk and Jak is the earliest detectable signaling response seen after activation of cytokine receptors in inflammatory cells (Wong 2005). Activation of tyrosine kinases evokes multiple downstream signaling cascades, including phosphoinositide 3-kinase, MAPK and NF-κB, which may lead to changes in cell proliferation, survival and recruitment (Wong 2005). Cytokine-induced dimerization of their receptors activates especially the family of Janus kinases (Jak1, Jak2, Jak3 and TYK2), which leads to further recruitment and activation of a family of transcription factors termed STAT. Phosphorylated STATs form dimers and translocate into the nucleus from the cytoplasm to regulate selective gene expression (Wong 2005).

The exact tyrosine signaling cascades mediating human eosinophil apoptosis remain unknown at present. However, the Lyn and Syk intracellular tyrosine kinases have been demonstrated to mediate the effects of IL-5 and GM-CSF on eosinophil survival (Pazdrak et al. 1995b, Yousefi et al. 1996, Pazdrak et al. 1998, Stafford et al. 2002, Kankaanranta et al. 2005). In addition, at least Jak2 has incontestably been shown to be activated after stimulation with IL-5 or GM-CSF in human eosinophils and IL-5 has also been reported to induce STAT1 and STAT5 activation in human eosinophils (Pazdrak et al. 1995a, van der Bruggen et al. 1995, Bates et al. 1996, Pazdrak et al. 1998, Caldenhoven et al. 1999, Bhattacharya et al. 2001, Kankaanranta et al. 2005). Therefore, the development of tyrosine kinase inhibitors, which may enhance eosinophil apoptosis, appears to represent a novel strategy for the treatment of asthma and allergic disorders (Wong 2005, Adcock et al. 2006). In fact, many tyrosine kinase inhibitors, e.g. inhibitors of spleen tyrosine kinase and Jak2, have already been observed to inhibit eosinophilia in animal models of asthma (Wong 2005). However, despite the encouraging results in animal and clinical trials with certain inhibitors, many tyrosine kinases including Jak and spleen tyrosine kinase are involved in such a variety of signaling pathways and are so widely distributed in the immune

system that they may be impractical targets for drug development and their long-term safety may pose a problem (Adcock et al. 2006).

4.3.3 Mitogen-Activated Protein Kinases

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases characteristically activated by dual phosphorylation in kinase cascades. Three major MAPK subfamilies have been identified in mammalian cells: the extracellular signal-regulated kinase (ERK), the p38 MAPK and the c-Jun N-terminal kinase (JNK), which is also known as stress-activated MAP kinase (SAPK) (Davis 2000, Dong et al. 2002). MAP kinases are widely involved in the immune response, from the initiation phase of innate immunity to activation of adaptive immunity and finally, to cell death (Dong et al. 2002). Both p38 and JNK have been proposed to play a role in the signaling pathways of apoptosis (Davis 2000). It has been demonstrated that p38 MAPK activity is involved in the inhibition of human eosinophil apoptosis (Kankaanranta et al. 1999).

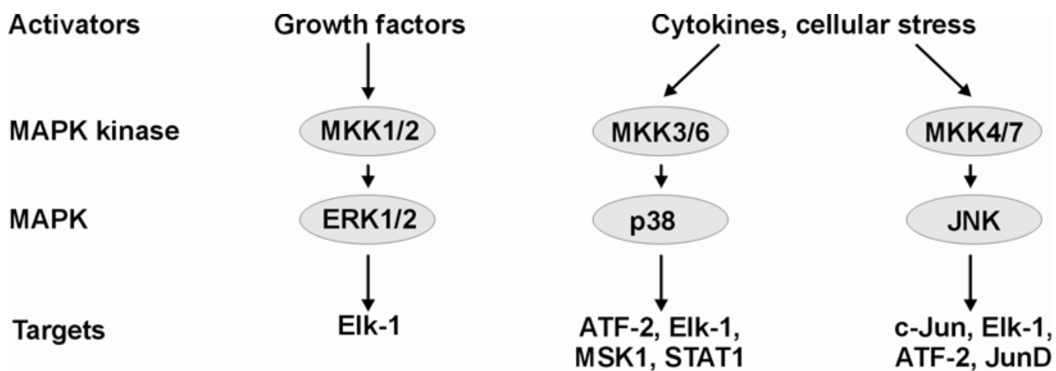

Figure 6. *A simplified scheme of the MAPK pathways. MKK, MAPK kinase; ERK, extracellular signal-regulated kinase; ATF-2, activating transcription factor 2; MSK2, mitogen- and stress-activated protein kinase 2.*

The JNK signaling pathway can be stimulated by e.g. genotoxic stress, heat shock, osmotic shock, metabolic stress and cytokines (Fig. 6) (Davis 2000, Barr and Bogoyevitch 2001). JNK is activated by dual phosphorylation at the

tripeptide motif Thr-Pro-Tyr by MKK4 or MKK7, which are MAP kinase kinases (MAPKK) that are, in turn, activated by MAP kinase kinase kinases (MAPKKKs), such as apoptosis signal-regulating kinase 1, mixed lineage kinases, TAK1 and TPL-2 (Davis 2000, Mielke and Herdegen 2000, Barr and Bogoyevitch 2001, Cho and Choi 2002, Dong et al. 2002). JNK may be activated both in the nucleus and in the cytoplasm. In MAPKKK activation, the Rho family GTPases, including Rac and Cdc42, have been found to play a role, especially in JNK activation caused by receptor tyrosine kinases (Davis 2000, Dong et al. 2002). The activation of JNK by cytokine receptors appears to be mediated by the TRAF group of adaptor proteins, however, the TRAF adaptor proteins may be involved in the activation of the JNK pathways through multiple stimuli (Davis 2000). Signal transduction along the MAPK pathways may be facilitated by scaffold proteins, of which the JNK interacting protein group appears to be important for the JNK pathway (Davis 2000, Cho and Choi 2002, Dong et al. 2002). The substrate proteins of JNK are mainly transcription factors including c-Jun, JunB, JunD, activating transcription factor 2 and Elk-1 but JNK may also phosphorylate a variety of cytoplasmic substrates or mitochondrial proteins (Davis 2000, Mielke and Herdegen 2000, Barr and Bogoyevitch 2001, Weston and Davis 2002, Cho and Choi 2002, Dong et al. 2002).

Three different JNK-encoding genes have been identified in mammals: JNK1, JNK2 and JNK3. These genes are alternatively spliced to create ten different JNK isoforms, which are either 46 kDa or 55 kDa in size. JNK3 is selectively expressed in the neuronal tissue, the heart and the testis, whereas JNK1 and 2 are ubiquitously expressed (Davis 2000, Mielke and Herdegen 2000, Barr and Bogoyevitch 2001, Dong et al. 2001). The exact functional roles and downstream targets of the different JNKs have not been established. However, JNK1 seems to preferentially mediate apoptosis whereas JNK2 may play a more critical role in proliferation, Th cell differentiation and cytokine production (Dong et al. 2001). The 55 kDa sized JNK2 has also been suggested to be the principal active JNK isoform in tumours (Tsuiki et al. 2003). In addition, 55 kDa JNK2 isoforms have been found to have significant autophosphorylation activity in primary glial tumours resulting in constitutive activation without the need for

upstream kinases (Tsuiki et al. 2003, Cui et al. 2005). The alternative splicing of the JNK genes may influence the substrate specificity of the JNK isoforms by altering the ability of JNK to interact with the docking sites of its substrates (Davis 2000, Barr and Bogoyevitch 2001). It has been suggested that JNK2 (55 kDa) binds and phosphorylates c-Jun more efficiently than JNK1 (46 kDa) (Kallunki et al. 1994, Sluss et al. 1994).

The precise role of JNK in mediating human eosinophil apoptosis remains uncharacterized. However, JNK has been suggested to play a role in nitric oxide (Zhang et al. 2003) and glucocorticoid-induced (Gardai et al. 2003) apoptosis of human eosinophils although the data concerning the importance of JNK in mediating glucocorticoid-induced apoptosis is rather controversial as JNK inhibition by antisense oligodeoxynucleotides was reported not to affect apoptosis (Zhang JP et al. 2000). Additionally, JNK has recently been reported to mediate orazipone-enhanced human eosinophil apoptosis (Kankaanranta et al. 2006).

4.3.4 TNF-α

Tumour necrosis factor-α is a pro-inflammatory cytokine produced by several cell types, mainly macrophages and monocytes, which mediates its effects on cells via two distinct receptors, TNF-RI and TNF-RII (Vandenabeele et al. 1995). Peripheral human eosinophils have been shown to express both of these receptors (Matsuyama et al. 1998). The binding of TNF to its receptor initiates a variety of complex intracellular signal transduction cascades, including the activation of caspase 8 via TNFR-associated death domain protein and FADD interactions as described earlier in section 4.2, the activation of JNK and further of activator protein 1, as well as the activation of the NF-κB pathway (Wajant et al. 2003).

In asthmatic patients, the levels of TNF-α have been found to be elevated in the bronchoalveolar lavage fluid (Broide et al. 1992). The observed effects of TNF-α on human eosinophil apoptosis appear to be controversial as TNF-α has been

reported to both enhance (Tsukahara et al. 1999, Peacock et al. 1999, Temkin and Levi-Schaffer 2001) and attenuate (Valerius et al. 1990, Ward et al. 1999) eosinophil survival. However, most reports seem to support the proposal that TNF-α induces human eosinophil survival (Kankaanranta et al. 2005). The mechanism of TNF-α-enhanced eosinophil survival appears to involve both of the TNF receptor subtypes as well as the activation of NF-κB and the production of GM-CSF (Temkin and Levi-Schaffer 2001, Kankaanranta et al. 2005).

4.3.5 Other Agents

β_2-agonists, which are commonly used to relieve bronchoconstriction in asthma, have been found to inhibit human eosinophil apoptosis (Kankaanranta et al. 2000b, Kankaanranta et al. 2005). Of the other agents used in the treatment of asthma, theophylline has been reported to have similar but also controversial effects (Adachi et al. 1996, Yasui et al. 1997, Momose et al. 1998, Kankaanranta et al. 2000b, Chung et al. 2000, Takeuchi et al. 2002, Kankaanranta et al. 2005). Theophylline is a phosphodiesterase inhibitor, which increases intracellular cAMP concentrations by reducing the degradation of cAMP. A cell-permeant cAMP analogue, dibutyryl-cAMP, has been observed to prolong eosinophil survival by inhibiting apoptosis but possibly to reverse the cytokine-afforded eosinophil survival (Kankaanranta et al. 2005). In summary, cAMP-elevating agents seem to prolong eosinophil survival although some controversy remains. Cysteinyl leukotrienes are important mediators of airway inflammation (Holgate et al. 2003), and leukotriene receptor antagonists are used in the treatment of asthma and allergy. It has been suggested that leukotrienes induce eosinophil survival and that antagonism of cysteinyl leukotriene receptors increases eosinophil apoptosis but entirely opposite findings have also been reported suggesting that leukotrienes do not influence human eosinophil apoptosis (Lee et al. 2000, Murray et al. 2003, Kankaanranta et al. 2005).

Oxygen-dependent mechanisms have been implicated in the regulation of eosinophil apoptosis (Wedi et al. 1999). It has been shown that exogenous H_2O_2 enhances spontaneous human eosinophil apoptosis as well as reverses IL-5-

induced survival (Kankaanranta et al. 2002). Thus, reactive oxygen species may play a role in regulating eosinophil apoptosis.

Nitric oxide concentrations are elevated in the exhaled air of patients with asthma and alveolitis (Alving et al. 1993, Kharitonov et al. 1994, Lehtimäki et al. 2001, Kharitonov and Barnes 2006). Recently, it has been reported that nitric oxide can reverse IL-5-evoked survival of human eosinophils through a mechanism that involves caspases and the activation of JNK but is independent of cGMP (Zhang et al. 2003). Earlier, nitric oxide has been reported to disrupt Fas receptor-mediated apoptosis of eosinophils probably via cGMP (Hebestreit et al. 1998). The effects of nitric oxide on constitutive eosinophil apoptosis remain obscure and contradictory (Kankaanranta et al. 2005). Hence, nitric oxide seems to have a complex role in the regulation of human eosinophil apoptosis.

4.4 Importance of Apoptosis in Inflammation

Apoptosis is essential for tissue homeostasis and for normal development in multicellular organisms. Granulocyte apoptosis provides a potential control point in the physiological resolution of innate immune responses. There is increasing evidence that cellular processes of apoptosis can be dysregulated by pathogens and that delayed apoptosis, which results in the prolonged survival of inflammatory cells, is important in the persistence of tissue inflammation (Bianchi et al. 2006). The key role of eosinophil apoptosis in the resolution of eosinophilic inflammation is now largely acknowledged (Haslett 1999, Kankaanranta et al. 2005, Bianchi et al. 2006). However, airway eosinophils may also be cleared by alternative, non-apoptotic mechanisms including transepithelial migration into the airway lumen and eosinophil cytolysis (Erjefält 2005). In conclusion, granulocyte apoptosis represents a fascinating area of research that has advanced our understanding of inflammation and its resolution and may provide novel therapeutic approaches for the treatment of inflammatory lung disease and allergic disorders. However, thus far, only few clinical trials in humans have been undertaken by using modulators of apoptosis (de Souza and Lindsay 2005).

5 Drugs

5.1 Antihistamines

Antihistamines (aka histamine H_1 receptor antagonists) are commonly used to treat allergic symptoms. These drugs are divided into three groups, the first-, second- and third-generation antihistamines. In contrast to first-generation antihistamines, the second- and third-generation antihistamines are non-sedating as they do not penetrate into the central nervous system through the blood-brain-barrier due to their greater water-solubility. In addition to antagonism of the H_1 receptors, many first-generation antihistamines also possess anticholinergic properties, which accounts for their therapeutic effects on motion sickness but also for some adverse effects including drying of the mouth. In addition to sedation and the anticholinergic effects of the first-generation antihistamines, the most common adverse effects of antihistamines include cardiac arrhythmias due to lengthening of the QT-interval. In summary, antihistamines generally represent a well-tolerated and safe drug group, of which the second- and third-generation antihistamines have many advantages compared to the older antihistamines.

5.1.1 Mechanism of Action

Antihistamines bind to histamine H_1 receptors preventing the binding of the natural ligand histamine to its receptors and the subsequent H_1-mediated effects of histamine in the allergic response. These include increased vascular permeability, vasodilatation, flushing, mucus secretion, pruritus and smooth muscle contraction. Thus, antagonism of the H_1 receptors has traditionally been thought to be mainly responsible for the beneficial, symptom-relieving effects of antihistamines on itchy skin, weal and flare responses in urticaria, nasal blockage, sneezing, itching and discharge in rhinitis (Walsh 2005).

Antihistamines are often claimed to have additional anti-inflammatory properties that may be either H_1-dependent or -independent. Some H_1-antagonists have been reported to affect the function of inflammatory cells, e.g. the chemotaxis and adhesion of eosinophils (Church 1999). Cetirizine is the most extensively investigated currently available oral antihistamine, and together with its active enantiomer levocetirizine, it has been observed to possess several anti-inflammatory activities such as the inhibition of the expression or release of leukotriene B4, TNF-α, intercellular adhesion molecule 1 and IL-8 (Walsh 2005). In addition, several other antihistamines including azelastine, levocabastine, oxatomide, terfenadine, loratadine and fexofenadine have been reported to reduce the expression of intercellular adhesion molecule 1 in ocular and nasal challenge models (Ciprandi et al. 2003b). Recently, levocetirizine has been shown to inhibit eosinophil adhesion to vascular cell adhesion molecule 1 (Wu et al. 2005) whereas fexofenadine has been shown to inhibit the expression of intercellular adhesion molecule 1 on eosinophil surface (Vancheri et al. 2005). Cetirizine and levocetirizine have also been demonstrated to inhibit transepithelial migration of eosinophils *in vitro* (Thomson et al. 2002). *In vivo*, levocetirizine has been observed to decrease the numbers of eosinophils and neutrophils, to lower the levels of IL-8 and to attenuate nasal symptoms during the pollen season (Walsh 2005). Thus, antihistamines seem to possess a variety of anti-inflammatory actions that may have relevance in allergic inflammation. Moreover, cetirizine has even been reported to delay, or even prevent, the development of asthma in children with atopic dermatitis (Warner and ETAC Study Group 2001) and to reduce new sensitisations in allergic children (Ciprandi et al. 2003a).

5.1.2 Effects on Eosinophil Survival

Until recently, the effects of antihistamines on eosinophil longevity have been surprisingly scantly studied despite the critical role of eosinophils in the pathogenesis of allergic diseases and the importance of antihistamines in the treatment of allergy. Recently, oxatomide has been shown to induce human eosinophil apoptosis both in the absence and presence of IL-5 (Domae et al.

2003). Fexofenadine, at high concentrations, was reported to reverse IL-5-induced eosinophil survival by enhancing apoptosis (Vancheri et al. 2005). In addition, desloratadine has been observed to inhibit eosinophil survival, although apoptosis was not specifically measured in that study (Mullol et al. 2006). Ketotifen has also been observed to decrease eosinophil viability in the presence of IL-5 (Hossain et al. 1994). Cetirizine and/or levocetirizine have been reported to affect the survival or apoptosis of eosinophils at high concentrations but to be ineffective at lower concentrations (Sedgwick and Busse 1997, Thomson et al. 2002).

5.2 Glucocorticoids

Glucocorticoids are the most effective and commonly used drug group for the treatment of asthma. Furthermore, glucocorticoids are widely used to treat a variety of other inflammatory disorders due to their diverse anti-inflammatory properties. In addition to their beneficial effects on inflammation and the immune response, glucocorticoids also extensively influence the metabolism of bones, muscles, connective tissue, carbohydrates, lipids and proteins as well as the electrolyte and fluid balance. The most commonly known adverse effects of glucocorticoids include osteoporosis, muscle and skin atrophy, peptic ulcer, hypertension, hyperglycemia, diverse psychic effects, glaucoma, cataract and increased susceptibility to infections.

5.2.1 Mechanism of Action

The classical model of the mechanism of action of glucocorticoids includes glucocorticoid penetration into the cell and binding to cytosolic glucocorticoid receptors. Inactive glucocorticoid receptors are bound to a protein complex that includes two heat shock proteins (hsp 90). Upon glucocorticoid binding to its receptor, the heat shock proteins dissociate, exposing nuclear localization signals. Upon activation, the glucocorticoid receptors form dimers, enter the nucleus and bind to DNA at sites termed glucocorticoid response elements

(GRE) in the promoter regions of steroid-responsive target genes. This interaction modulates gene transcription and results in either induction or repression of the gene (Fig. 7). Positive GREs increase transcription resulting in increased messenger RNA and protein synthesis whereas negative GREs (nGRE) decrease gene transcription (Adcock 2000, Adcock 2001, Barnes 2001b, Adcock et al. 2004, Hayashi et al. 2004, Ito et al. 2006, Barnes 2006). However, nGREs have only rarely been demonstrated and are not a common feature of the promoter region of the inflammatory genes that are suppressed by glucocorticoids when they are used in the treatment of allergic diseases. Thus, other mechanisms of action exist, which are responsible for the inhibition of inflammatory protein synthesis by glucocorticoids in controlling inflammation (Adcock 2000, Barnes 2001b).

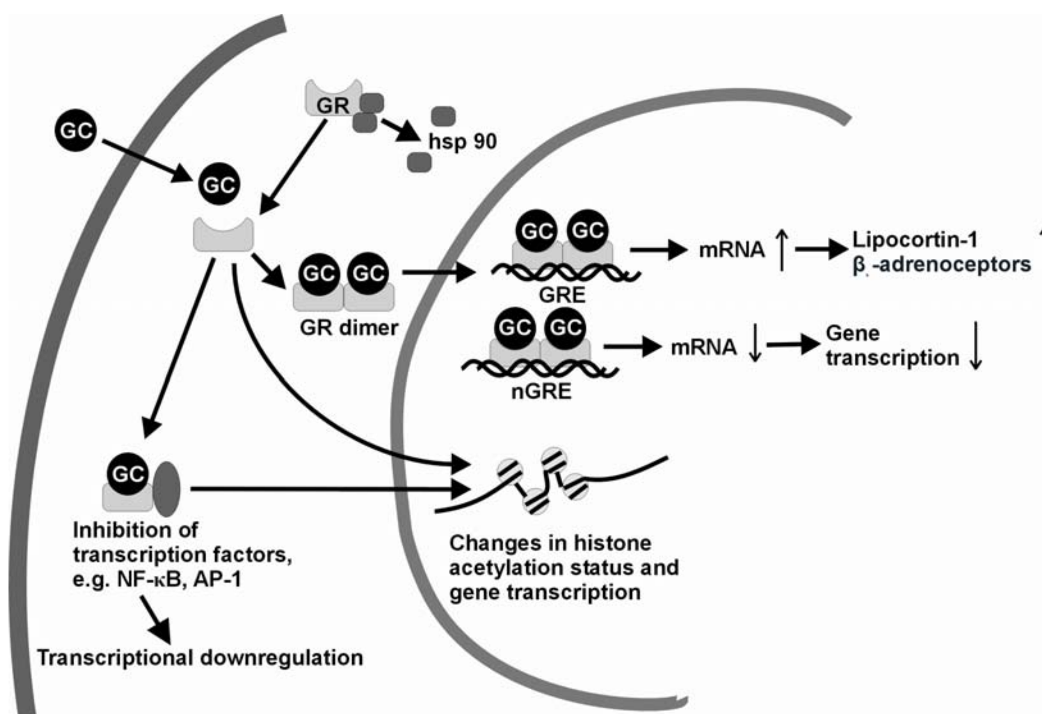

Figure 7. *Mechanisms of action of glucocorticoids. GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; nGRE, negative glucocorticoid response element; hsp, heat shock protein; mRNA, messenger ribonucleinic acid.*

Activated glucocorticoid receptors interact with several activated transcription factors and inhibit their effects (Fig. 7). This represents one mechanism by which glucocorticoids are thought to downregulate the transcription of inflammatory genes and to decrease the production of inflammatory mediators, such as cytokines, adhesion molecules, inflammatory enzymes and receptors. These transcription factors normally switch on inflammatory genes by recruiting transcriptional coactivator proteins and by modifying chromatin structure e.g. at the level of histone acetylation. The transcription factors include NF-κB and activator protein 1, which are important regulators of the inflammatory genes in asthmatic airways. Glucocorticoids may also act downstream of the binding of the pro-inflammatory transcription factors to DNA, e.g. on chromatin structure, on histone acetylation and on coactivator molecules (Adcock 2000, Adcock 2001, Barnes 2001b, Adcock et al. 2004, Hayashi et al. 2004, Ito et al. 2006, Barnes 2006).

There is increasing evidence that glucocorticoids affect chromatin structure. The DNA in chromosomes is wound around histone molecules in the form of nucleosomes. Acetylation of the lysine residues of the core histones by histone acetyltransferases results in the unwinding of DNA, which open the chromatin structure, allowing gene transcription. Deacetylation of histones by histone deacetylases increases the winding of DNA, which leads to repressed transcription of inflammatory genes. Activated glucocorticoid receptors may bind to several transcription corepressor molecules that associate with proteins that have histone deacetylase activity, resulting in the deacetylation of histones and the repression of inflammatory genes (Fig. 7) (Adcock 2000, Adcock 2001, Barnes 2001b, Adcock et al. 2004, Hayashi et al. 2004, Ito et al. 2006, Barnes 2006). The importance of the histone acetylation status in asthma has been recognized, and glucocorticoids have been demonstrated to switch off inflammatory genes in asthma through the inhibition of histone acetyltransferase activity, which is markedly increased in asthma, and more importantly, by the recruitment of histone deacetylase 2 to the activated inflammatory gene complex (Barnes et al. 2005, Barnes 2006). Glucocorticoids may also regulate protein

expression at the level of messenger RNA stability (Korhonen et al. 2002, Ito et al. 2006).

Glucocorticoids are by far the most important and effective therapy available for asthma. Even small doses of inhaled glucocorticoids are effective in suppressing asthmatic inflammation, although many different types of inflammatory cells and mediators are involved in the pathophysiology of asthma. Glucocorticoids appear to suppress asthmatic inflammation by switching off inflammatory genes by targeting transcription factors and their ability to induce modulations in histone acetylation and chromatin remodelling (Adcock et al. 2004, Barnes et al. 2005, Barnes 2006). The multiple effects of glucocorticoids in controlling asthma and allergic inflammation include increased synthesis of anti-inflammatory proteins and β_2-adrenoceptors, decreased synthesis of cytokines, chemokines, inflammatory enzymes (e.g. inducible nitric oxide synthase, cyclooxygenase-2) and inflammatory receptors and decreased expression of adhesion molecules. Glucocorticoids also affect cell survival. The target cells include macrophages, T-lymphocytes, mast cells, dendritic cells, endothelial and epithelial cells, neutrophils and eosinophils (Adcock 2000, Barnes 2001b).

5.2.2 Effects on Eosinophil Survival

Glucocorticoids markedly reduce the survival of eosinophils by inducing apoptosis (Wallen et al. 1991, Lamas et al. 1991, Hallsworth et al. 1992, Meagher et al. 1996, Druilhe et al. 1996, Hagan et al. 1998, Nielson and Hadjokas 1998, Zhang X et al. 2000, Barnes 2001b, Zhang et al. 2002, Kankaanranta et al. 2005) whereas the opposite effects have been described with neutrophils (Meagher et al. 1996, Zhang et al. 2001, Barnes 2001b, Zhang et al. 2002, Kankaanranta et al. 2005). The apoptosis-enhancing effect of glucocorticoids on spontaneous eosinophil apoptosis is clear and significant. However, the effects of glucocorticoids on cytokine-promoted eosinophil survival are not as clear: glucocorticoids do partly reverse cytokine-induced eosinophil survival but the effect of glucocorticoids seems to be abolished by high concentrations of cytokines (Kankaanranta et al. 2005). With regard to

asthma, clinically relevant concentrations of inhaled glucocorticoids have been reported to promote human eosinophil apoptosis, both constitutive apoptosis and that occurring in the presence of low concentrations of cytokines (Hagan et al. 1998, Zhang X et al. 2000, Zhang et al. 2002). The mechanism of action of glucocorticoid-induced eosinophil apoptosis is thought to involve glucocorticoid receptors and a loss in mitochondrial membrane potential with subsequent cytochrome C release into the cytosol (Druilhe et al. 2003, Kankaanranta et al. 2005).

6 Purpose of the Study

Given the critical role of eosinophils and histamine in the pathogenesis of allergic diseases, the importance of apoptosis in the resolution of inflammation, the role of JNK in apoptosis, and the common use of antihistamines and glucocorticoids in the treatment of allergy and asthma as reviewed above, the present study was conducted to examine the role of histamine, antihistamines and JNK as regulators of human eosinophil apoptosis.

AIMS OF THE STUDY

The general aim of the present study was to investigate the role of histamine in the regulation of human eosinophil apoptosis as both histamine and eosinophils are key players in allergy and asthma. The aims of the study also included investigating whether H_1 receptor antagonists (aka antihistamines) or other antagonists or agonists of the histamine H_{2-4} receptors could modulate eosinophil longevity as well as elucidating the role of c-Jun N-terminal kinase (JNK) in human eosinophil apoptosis.

The following hypotheses were tested:

1. Histamine affects human eosinophil apoptosis.

2. Histamine modulates eosinophil apoptosis through its G-protein-coupled cell surface receptors.

3. H_1 receptor antagonists (chlorpheniramine, diphenhydramine, ketotifen) modulate human eosinophil survival.

4. A signaling pathway involving the activation of c-Jun N-terminal kinase constitutes a general regulator of constitutive human eosinophil apoptosis.

MATERIALS AND METHODS

1 Blood Donors (I-IV)

The blood donors consisted of healthy or atopic volunteers with either normal or slightly elevated blood eosinophil counts. The recruited volunteers included friends, relatives and patients from the clinic of Respiratory Medicine of Tampere University Hospital (Tampere, Finland). Patients with hypereosinophilic syndrome were excluded from the studies. The blood donors were allowed to take their usual medication normally and the medication was documented. The most commonly used medications were inhaled glucocorticoids and β_2-agonists, antihypertensive medication, statins and analgesics. Many healthy blood donors were not taking any medications. None of the patients were taking oral glucocorticoids. However, the blood donors were not further subdivided according to their medication. Before donating blood, the subjects gave written informed consent to the study protocol approved by the Ethics committee of Tampere University Hospital (Tampere, Finland).

2 Eosinophil Isolation and Culture (I-IV)

Eosinophils were isolated under sterile conditions. In brief, venous blood (100 ml) from volunteers was collected into 20 ml of acid citrate dextrose anticoagulant and hydroxyethyl starch solution. After sedimentation for 40-60 min, the leukocyte rich cell pellet was laid onto Ficoll and centrifuged at 2000 rpm for 30 min at room temperature. The mononuclear cells were discarded after which the granulocyte rich cell pellet was resuspended and washed in Hank's balanced salt solution. Contaminating erythrocytes were eliminated by hypotonic lysis. Granulocytes were then washed twice with RPMI 1640 (with 2% fetal bovine serum and 5 mM ethylenediaminetetraacetic acid), counted and resuspended in RPMI 1640 (with 2% fetal bovine serum and 5 mM ethylenediaminetetraacetic acid). Eosinophils were separated from neutrophils

by using immunomagnetic anti-CD16 antibody conjugated beads that retain the CD16-positive neutrophils in the magnetic field whereas the CD16-negative eosinophils are eluted through a column in the magnetic field. The separated eosinophils were next washed with RPMI 1640 (Dutch modification with 10% fetal bovine serum and antibiotics) after which the cells were counted with bright field microscopy under Kimura stain (0.05% Toluidine blue, 0.03% Light green, 3% (v/v) Saponin, 20 mM phosphate buffer). The purity of the eosinophil population was >99% (n=80) with few, if any, contaminating basophils, neutrophils or lymphocytes. The isolated eosinophils were then resuspended at $1x10^6$ cells/ml in RPMI 1640 (Dutch modification with 10% fetal bovine serum and antibiotics) and cultured at +37ºC and 5% CO_2 for the indicated time in the absence and presence of the test compounds as indicated. The viability of freshly isolated eosinophils was high: after a 60 min incubation, 98% of the cells were viable as analyzed by flow cytometry in propidium iodide (PI)-staining (n=8, data not shown).

3 Determination of Cell Viability by Flow Cytometry

3.1 Relative DNA Fragmentation in Propidium Iodide –Staining (I-IV)

The number of apoptotic cells was assessed by measuring the relative DNA content by flow cytometry (FACScan, Becton Dickinson, San Hose, CA) of propidium iodide (PI)-stained eosinophils. Briefly, after a 40 h culture, eosinophils were suspended in 300 µl of hypotonic fluorochrome solution (25 µg/ml propidium iodide in 0.1% sodium citrate and 0.1% Triton-X 100) and incubated at +4ºC for 60 min before flow cytometric analyses. The excitation and emission wavelengths were 488 nm and 550 nm, respectively. Endonuclease-catalysed DNA fragmentation is regarded as a specific feature of apoptosis, therefore, the cells depicting decreased relative DNA content were considered to have undergone apoptosis (Nicoletti et al. 1991, Kankaanranta et al. 2000a).

3.2 Annexin-V FITC - Propidium Iodide –Counterstaining (III)

To assess the amounts of early and late apoptotic, necrotic and viable cells, Annexin-V binding and analysis by flow cytometry were performed according to the instructions of the manufacturer and as previously reported (Zhang et al. 2002). In short, eosinophils cultured for 18 h were washed in phosphate buffered saline solution and resuspended in 195 µl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Subsequently, 5 µl of Annexin-V fluorescein isothiocyanate solution (Annexin-V FITC, containing 50 mM Tris, 100 mM NaCl, 1% bovine serum albumin, 0.02% sodium azide, pH 7.4) was added to the cell suspension prior to an incubation of 10 min at room temperature. The cells were washed and resuspended in binding buffer containing 10 µg/ml PI after which the flow cytometric analyses were performed immediately. The cells showing positive Annexin-V-staining were considered as being apoptotic.

3.3 Flow Cytometric Determination of Primary Necrosis (I)

The percentage of primary necrotic cells was evaluated by flow cytometry of PI-stained cells after 60 min incubation at +37ºC in 5% CO_2. The cells showing uptake of PI after 60 min were assumed to be necrotic as fast cell membrane breakdown is a feature typical of necrotic cell death (Kankaanranta et al. 2000a). Eosinophils were suspended in 250 µl Hank’s balanced salt solution with 20 µg/ml PI, incubated at room temperature for 5 min and analyzed immediately by flow cytometry.

4 Morphological Analysis

4.1 Bright Field Microscopy (I-IV)

Eosinophil morphology was assessed by bright field microscopy. For morphological analysis, eosinophils cultured for 40 h (+37ºC, 5% CO_2) were spun onto cytospin slides (500 rpm, 5 min) and stained with May-Grünwald-Giemsa after fixation in methanol. Cells showing the characteristic apoptotic morphology including cell shrinkage, nuclear coalescence and chromatin condensation were considered to have undergone apoptotic cell death (Fig. 8) (Kankaanranta et al. 2000a).

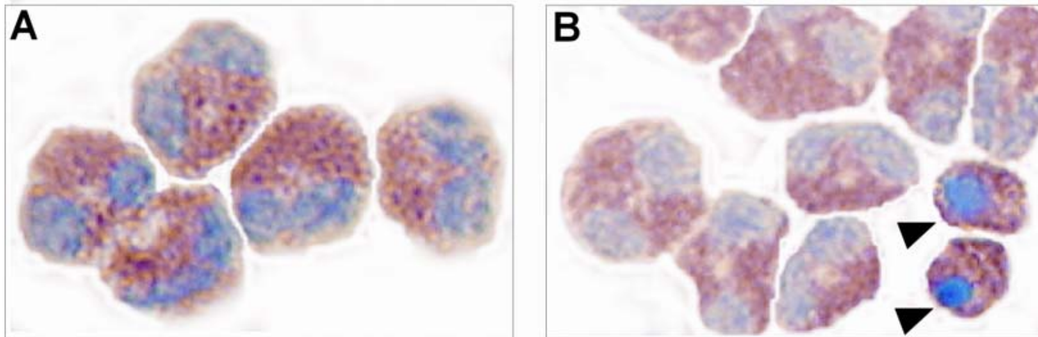

Figure 8. *The morphology of May-Grünwald-Giemsa-stained human eosinophils. In A), the normal morphology of viable eosinophils is shown whereas in B), two apoptotic eosinophils (arrowheads) can be seen among viable cells.*

4.2 Histological Assessment of Eosinophil Ultrastructure by Using Transmission Electron Microscopy (I)

The degranulation and viability status of eosinophils cultured for 1 and 40 h (+37ºC, 5% CO_2) was assessed by detailed ultrastructural examination with transmission electron microscopy (TEM) (Malm-Erjefält et al. 2004). Briefly, the cell suspensions were fixed in a 1:2 volume ratio of 4% paraformaldehyde in

0.1 M phosphate buffered saline (yielding a 2.7% total concentration of fixative) for 60 min at room temperature. After centrifugation, the cell pellet was fixed in 3% paraformaldehyde/1% glutaraldehyde in phosphate buffered saline overnight. The cell suspensions were then added to microcentrifuge tubes (3-5x10^6 cells/tube) and centrifuged at +4ºC for 10 min at 2600 rpm. The obtained pellets were gently embedded in warm (+40-50ºC) 3% agarose in phosphate buffered saline and post-fixed in 1% osmium tetroxide for 60 min, dehydrated in graded acetone solutions and embedded in Polybed 812. Thin (1 μm) plastic sections were cut on an ultratome (Ultracut E, Leica, Germany) and cell-rich areas were selected for further electron microscopical analysis. Ultrathin sections (90 nm) were cut and placed on 200-mesh, thin-bar copper grid and stained with uranyl acetate and lead citrate. The specimens were examined by using a Philips EM10 transmission electron microscope (Philips, Eindhofen, Netherlands). Individual eosinophils were evaluated at x 8000 magnification. Eosinophils were divided into sub-groups based on morphological criteria (Erjefält and Persson 2000), defined in the following categories: Viable eosinophils: Preserved integrity of cytoplasmic and nuclear membranes, normal nuclear euchromatin and heterochromatin. Eosinophil cytolysis: Presence of chromatolysis, cell membrane blebs, loss of plasma membrane integrity and partly dissolved cytoplasm. Apoptotic eosinophils: Presence of chromatin condensation, preserved plasma membrane and non-dilated organelles. Evaluation of eosinophil degranulation was also performed. Eosinophils were assessed for structural changes due to piecemeal degranulation, e.g. ragged loss of core material, coarsening of the granular matrix, or more or less empty granules.

5 Immunoblot Analysis (II-III)

To evaluate protein expression, immunoblot analysis was performed. Eosinophils were suspended at 1x10^6 cells/ml and cultured at +37ºC. At the indicated time points, eosinophils were collected by centrifuging the samples at 12000 rpm for 15 s. The cell pellet was lysed by boiling for 5 min in 30 μl Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate, 0.025% bromophenol blue and 5% β-mercaptoethanol). The

samples were then centrifuged for 10 min at 12000 rpm after which the debris was carefully removed. Samples were stored at -20ºC until the Western blot analysis. The protein sample (30 µl) was loaded onto 10% sodium dodecyl sulphate-polyacrylamide electrophoresis gel and electrophoresed for 2 h at 100 V in a buffer containing 95 mM Tris-HCl, 960 mM glycine and 0.5% sodium dodecyl sulphate. The separated proteins were transferred to Hybond™ enhanced chemiluminescence (ECL™) nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) with a semidry blotter at 2.5 mA/cm^2 for 60 min in a buffer containing 25 mM Tris-base, 192 mM glycine and 20% methanol. After transfer, the membranes were blocked with 5% bovine serum albumin in TBS/T (20 mM Tris base, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 60 min at room temperature. Proteins were labelled in an overnight incubation at +4ºC in the blocking solution with specific rabbit polyclonal primary antibodies for human c-Jun (1:1000) or c-Jun N-terminal kinase (JNK, 1:250) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), for serine- 63-phosphorylated c-Jun (1:1000) (Cell Signaling Technology, Inc., Beverly, MA, USA) and for phosphorylated JNK (1:1000) (Thr183/Tyr185, Thr221/Tyr223) (Upstate, Lake Placid, NY, USA). The membranes were subsequently washed 4x with TBS/T for 5 min, incubated for 30 min at room temperature with horseradish peroxidase-conjugated polyclonal goat anti-rabbit secondary antibody (1:1000-1:2000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in the blocking solution and washed 4x with TBS/T for 5 min. Bound antibody was detected by using SuperSignal® West Dura chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). The chemiluminescent signal was quantified by using the FluorChem™ software version 3.1.

6 Caspase Activity Assay (IV)

Caspase 3/7, 8 and 9 activities in eosinophils were assessed by using Caspase-Glo® 3/7, 8, and 9 assays (Promega Corp., Madison, USA) according to the instructions of the manufacturer. Briefly, human eosinophils were cultured in

RPMI 1640 medium (Dutch modification, with 10% fetal bovine serum and antibiotics) in the absence and presence of IL-5 (1 pM) and histamine (100 μM) for 16 h. Equal volumes of Caspase-Glo® 3/7, 8 or 9 –reagents were added after which the samples were incubated for 60 min at room temperature before measurement of luminescence. The background luminescence of the medium control was deducted from the results.

7 Statistics

The results are expressed as the mean ± the standard error of mean (SEM). Apoptosis is expressed as an apoptotic index (number of apoptotic cells/total number of cells, i.e. apoptotic index 0.1 means that 10% of the cells are apoptotic) (II-IV). In histological assessment of eosinophil ultrastructure by TEM (I), the results are expressed as percentage of cells displaying signs of necrosis from two separate donors. Cell death in the relative DNA fragmentation assay (I) is expressed as the percentage of cells showing a hypodiploid DNA content indicative of apoptosis. Primary necrosis in the relative DNA fragmentation assay (I) is expressed as percentage of primary necrotic cells taking up propidium iodide. Statistical significance of the results was calculated by paired t-tests or by analysis of variance for repeated measures supported by the Dunnett test by using GraphPad Instat software (GraphPad Software, San Diego, CA, USA). If the data was not matched, ordinary analysis of variance was used followed by Dunnet multiple comparisons test. Differences were regarded as statistically significant when $p<0.05$. The concentration-response data of histamine-induced apoptosis, the EC_{50} (the effective concentration of a compound that produces 50% of its maximal effect) value and the 95% confidence interval were analyzed by using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) (IV).

8 Materials (I-IV)

The reagents used in this study were obtained as follows: Caspase-Glo® 3/7, 8, 9 assays (Promega Corp., Madison, WI, USA); cirazoline hydrochloride, clobenpropit dihydrobromide, clonidine hydrochloride, D609, DPPE fumarate (N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethanamide fumarate) and U73122 (Tocris Cookson Ltd., Avonmouth, UK); anti-CD16 microbeads and the magnetic cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany); human recombinant IL-5 (R&D system Europe, Abingdon, UK); Ficoll-Paque (Pharmacia AB, Uppsala, Sweden); antibiotics and RPMI 1640 (Dutch modification) (Gibco BRL, Paisley, UK); Hank's balanced salt solution and RPMI 1640 (BioWhittaker, Verviers, Belgium); fetal bovine serum (Euroclone, Pero, Italy); Z-Asp-CH_2-DCB (Peptide Institute, Inc., Osaka, Japan); May-Grünwald, Z-DQMD-FMK, Z-VEID-FMK, Ac-IETD-CHO, Ac-LEHD-CHO and Q-VD-OPh (Merck, Darmstadt, Germany); Giemsa (J.T. Baker, Deventer, Holland); Annexin-V-FITC kit (Bender medSystems, Vienna, Austria); L-JNKI1 (c-Jun N-terminal kinase peptide inhibitor, L-stereoisomer) and L-TAT control peptide (Alexis Corp., Läufelfingen, Switzerland); SP600125 (anthra(1,9-*cd*)pyrazol-6(2H)-one, JNK inhibitor) (Calbiochem, San Diego, CA, USA); c-Jun and JNK1 antibodies and goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); phospho-c-Jun (Ser63) antibody (Cell Signaling Technology, Inc., Beverly, MA, USA); phospho-JNK antibody (Thr183/Tyr185, Thr221/Tyr223) (Upstate, Lake Placid, NY, USA); lead citrate and uranyl acetate (VWR International, Stockholm, Sweden); osmiumtetroxide (AGAR Scientific, Stanstedt, Essex, England); paraformaldehyde (TAAB Laboratories Equipment, Aldermaston, Berkshire, England); phosphate buffered saline (without calcium and magnesium) (Life Technologies, Paisley, UK) and Polybed 812 (Polysciences, Eppleheim, Germany). (-)-2-Cyano-1-methyl-3-{(2R,5R)-5-[1H-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine (OUP-16) was a kind gift from Prof. Atsushi Yamatodani and Dr. Shinya Harusawa from the University of Osaka, Japan. H4-antagonist 7-methyl-2-[(4-methylpiperazin-1-yl)carbonyl]-1H-indole (MMPCI) was a kind gift from Dr. Michael Peck from UCB Pharma (Brussels, Belgium). Acetone, agarose, 8-Br-

cAMP, bovine serum albumin, cimetidine, chlorpheniramine maleate, dexamethasone, diphenhydramine hydrochloride, (±)-adrenaline hydrochloride, glutaraldehyde, histamine, 5-hydroxytryptamine (5-HT) hydrochloride, JNJ7777120 (1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine), ketotifen fumarate, mepyramine (pyrilamine maleate), methysergide maleate, pertussis toxin, phentolamine hydrochloride, propidium iodide, Rp-8-CPT-cAMPS, thioperamide maleate and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. The mechanisms of action of the compounds used in this study are listed in Table 1.

Adrenaline, chlorpheniramine, cimetidine, cirazoline, clonidine, D609, diphenhydramine, histamine, 5-HT, mepyramine and thioperamide were dissolved in RPMI 1640 medium (Dutch modification). 8-Br-cAMP, clobenpropit, dexamethasone, DPPE, Rp-8-CPT-cAMPS, L-JNKI1 and L-TAT were dissolved in Hank's balanced salt solution. JNJ7777120, ketotifen, 7-methyl-2-[(4-methylpiperazin-1-yl)carbonyl]-1H-indole (MMPCI), OUP-16, methysergide, U73122, SP600125 and all of the caspase inhibitors (Z-DQMD-FMK, Z-VEID-FMK, Ac-IETD-CHO, Ac-LEHD-CHO, Q-VD-OPh and Z-Asp-CH_2-DCB) were dissolved in dimethyl sulfoxide (DMSO). The final concentration on DMSO in culture was 0.5% except for Z-Asp-CH_2-DCB for which the final concentration of DMSO within the cells was 1.0%. 0.5-1% DMSO was not found to affect eosinophil viability as assessed by bright field microscopy (n=6, data not shown). Similar concentrations of DMSO were added to the control cultures. Stock solution of phentolamine was prepared in ethanol. The final concentration of ethanol in the culture was 0.1%, and a similar concentration of ethanol was added to the control cultures. 0.1% ethanol did not affect eosinophil viability as assessed by flow cytometry (n=4, data not shown). Pertussis toxin was dissolved in 50% (v/v) glycerol containing 50 mM Tris, pH 7.5, 10 mM glycine and 0.5 M NaCl. A similar solution was also added to the control, and was found not to affect eosinophil apoptosis as assessed by flow cytometry (n=5, data not shown).

Table 1. Mechanisms of action of the compounds used in the present study.

Compound	Mechanism of action
Agonists or antagonists of the histamine receptors	
Chlorpheniramine	H_1-antagonist
Cimetidine	H_2-antagonist
Clobenpropit	H_3-antagonist (<10 pM) / H_4-agonist (>1 nM)
Diphenhydramine	H_1-antagonist
DPPE	H_{IC}-antagonist
Histamine	Natural ligand for histamine receptors
JNJ7777120	H_4-antagonist
Ketotifen	H_1-antagonist
Mepyramine	H_1-antagonist
MMPCI	H_4-antagonist
OUP-16	H_4-agonist
Thioperamide	H_3-antagonist (<100nM) / H_4-antagonist (>1 μM)
Agonists or antagonists of other amine receptors	
Adrenaline	Non-selective adrenoceptor agonist
Cirazoline	α_1-adrenoceptor agonist
Clonidine	α_2-adrenoceptor agonist
Phentolamine	Non-selective α-adrenoceptor antagonist
5-HT	Natural ligand for 5-HT-receptors
Methysergide	$5\text{-HT}_{1,2,7}$-antagonist
Inhibitors or activators of different signal transduction pathways	
8-Br-cAMP	Cell permeant cAMP analogue
Rp-8-CPT-cAMPS	PKA-inhibitor
D609	Phosphatidylcholine-specific PLC-inhibitor
U73122	General PLC-inhibitor
Pertussis toxin (PTX)	Inactivates $G_{i/o}$-protein
Caspase inhibitors	
Ac-IETD-CHO	Caspase 8 inhibitor
Ac-LEHD-CHO	Caspase 9 inhibitor
Z-Asp-CH_2-DCB	Pan-caspase inhibitor
Z-D(OMe)QMD(OMe)-FMK	Caspase 3 inhibitor
Z-VE(OMe)ID(OMe)-FMK	Caspase 6 inhibitor
Q-VD-OPh	Inhibitor of caspases 1, 3, 8, 9, 10, 12
Others	
Dexamethasone	Glucocorticoid
L-JNKI1	JNK inhibitor peptide, L-stereoisomer
L-TAT	Control peptide for L-JNKI1
SP600125	JNK inhibitor

SUMMARY OF THE RESULTS

1 Constitutive Eosinophil Apoptosis

Isolated human eosinophils cultured (+37°C, 5% CO_2) for 40 h in cytokine-deprived conditions underwent spontaneous apoptosis with an apoptotic index of 0.58 ± 0.02 (mean ± SEM, n=126, Fig. 9, I-IV) as assessed with the relative DNA fragmentation assay in PI-staining. When eosinophils were stained by May-Grünwald-Giemsa and visualized by bright field microscopy, the apoptotic index of spontaneously dying eosinophils after a 40 h culture was found to be 0.38 ± 0.06 (n=18, I-IV). After a shorter 18 h incubation, the apoptotic index of isolated human eosinophils was 0.23 ± 0.02 (n=8, III) as evaluated by flow cytometry in Annexin-V FITC – PI –counterstaining.

2 Effect of IL-5 on Eosinophil Survival

IL-5 enhanced human eosinophil longevity by inhibiting apoptosis. The maximal anti-apoptotic effect of IL-5 was achieved at a 10 pM concentration although a smaller concentration of 1 pM was also very potent, inhibiting apoptosis by 66% as compared to untreated eosinophils (Fig. 9). The apoptotic indices of eosinophils cultured for 40 h with 1 pM and 10 pM concentrations of IL-5 were 0.20 ± 0.01 (n=120, I, II, IV) and 0.09 ± 0.01 (n=22, I-II), respectively, as measured by the relative DNA fragmentation assay in PI-staining (Fig. 9). When morphological analysis was performed, the apoptotic indices were 0.08 ± 0.02 and 0.03 ± 0.00 (n=6, I-II) for IL-5 concentrations of 1 pM and 10 pM, respectively.

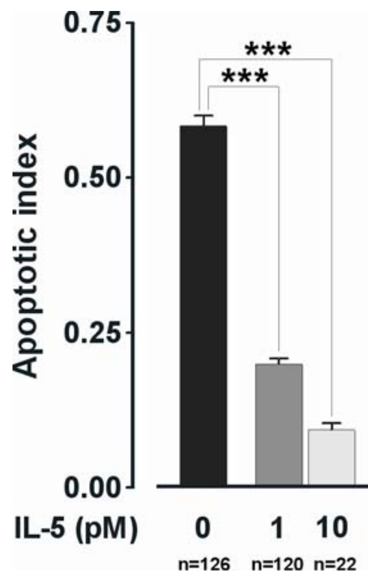

Figure 9. *Effect of IL-5 on human eosinophil apoptosis. The apoptotic indices of eosinophils cultured for 40 h in cytokine-deprived conditions and with IL-5. Apoptosis was evaluated by the relative DNA fragmentation assay. Each data point represents the mean ± SEM. *** indicates p<0.001 as compared to spontaneous eosinophil apoptosis.*

3 Effect of Histamine on Eosinophil Survival (IV)

3.1 Effect of Histamine on Human Eosinophil Apoptosis

Culture of human eosinophils for 40 h in the presence histamine (0.01 to 100 µM) was found to partially reverse eosinophil survival induced by IL-5 (1 pM) through enhancement of apoptosis (Fig. 10B). The increase in human eosinophil apoptosis by histamine was concentration-dependent and reached a maximum of approximately 40% at histamine concentrations of 10-100 µM (Fig. 10D). The EC_{50} value for the enhancement of eosinophil apoptosis by histamine was 0.56 µM (95% confidence interval = 0.33-0.97 µM; Fig. 10D insert). 100 µM concentration of histamine was chosen for further studies due to its maximal pro-apoptotic capacity combined with lower variability than that of 10 µM histamine. The apoptotic indices as analyzed by the relative DNA fragmentation assay were 0.19 ± 0.01 and 0.26 ± 0.01 (n=83, $p<0.01$) for eosinophils cultured in the

presence of IL-5 (1 pM) without and with histamine (100 μM), respectively. The form of cell death was further confirmed to be apoptosis by bright field microscopy. The apoptotic indices were 0.08 ± 0.02 and 0.13 ± 0.07 in the absence and presence of histamine (100 μM), respectively ($n=6$, $p<0.01$).

However, when different concentrations (0.01-100 μM) of histamine were applied to eosinophils cultured in cytokine-deprived conditions, no alterations in the rate of apoptosis were observed (Fig. 10A, 10C). In the relative DNA fragmentation assay, the apoptotic indices were 0.59 ± 0.02 and 0.61 ± 0.02 ($n=83$, $p>0.05$) in the absence and presence of histamine (100 μM), respectively. Consistently, histamine (100 μM) did not modulate human eosinophil apoptosis in the absence of cytokines when morphological analysis was performed (the apoptotic indices were 0.41 ± 0.06 and 0.50 ± 0.07 without and with histamine, respectively, $n=6$, $p>0.05$).

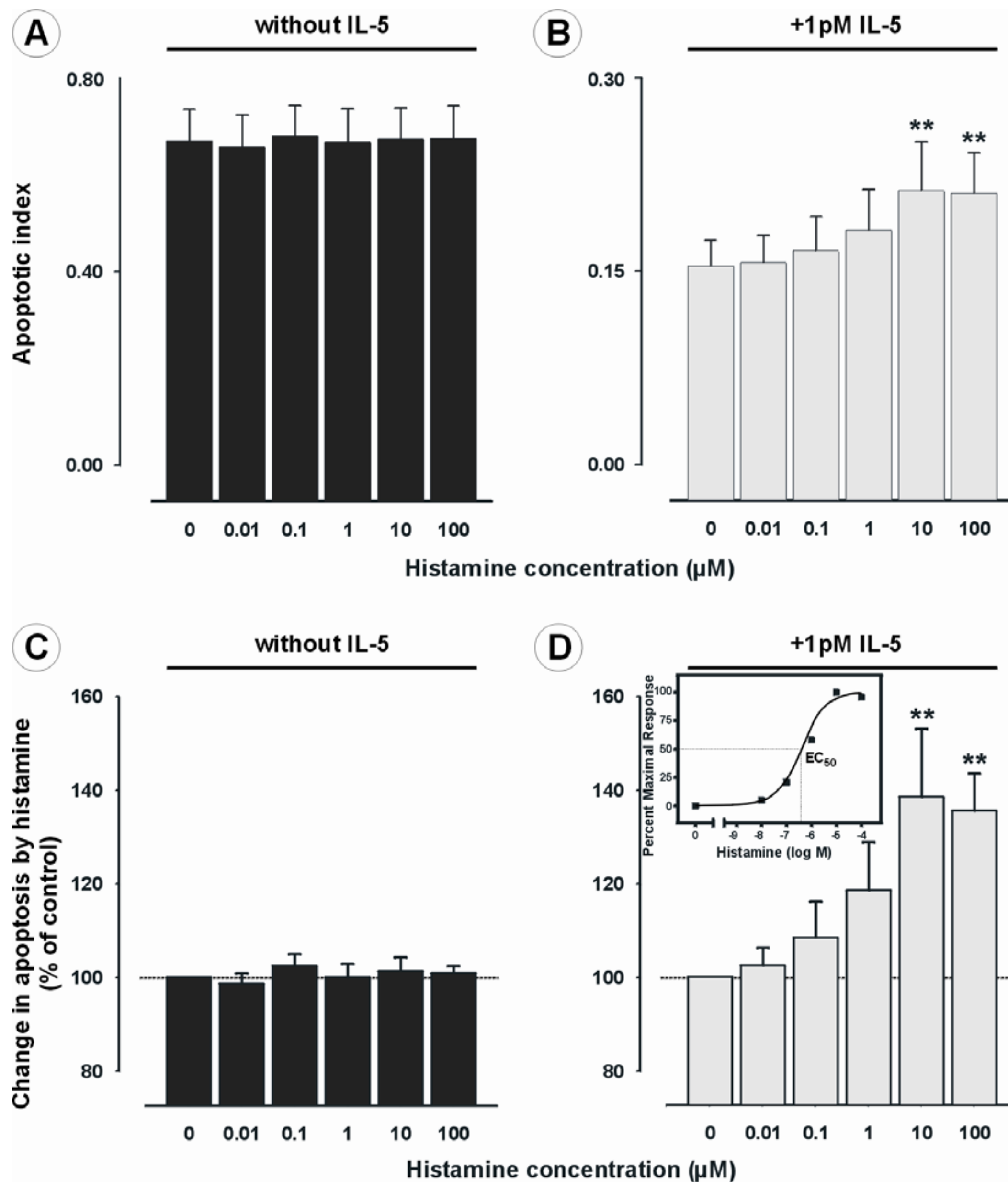

Figure 10. *Effect of histamine on human eosinophil apoptosis. The apoptotic indices of eosinophils cultured for 40 h with histamine in the absence (A) and presence (B) of IL-5 (1 pM). Percentage changes in apoptosis by different histamine concentrations in the absence (C) and presence (D) of IL-5 (1 pM). In D), the insert shows the concentration-response curve of histamine-induced apoptosis. Apoptosis was analyzed by the relative DNA fragmentation assay. Each data point represents the mean ± SEM of n=6 independent determinations using eosinophils from different donors. ** indicates $p<0.01$ as compared with the respective control.*

3.2 Signaling Pathways of Histamine-Induced Eosinophil Apoptosis

3.2.1 Caspases

To elucidate the role of caspases in histamine-induced reversal of IL-5-afforded survival of human eosinophils, the activities of caspases 3/7, 8 and 9 were evaluated by using Caspase-Glo® 3/7, 8 and 9 assays. After a 16 h culture, all caspases studied were found to be active, and the activities could markedly be reduced by IL-5 (1 pM) (n=6, $p<0.01$). Addition of histamine (100 µM) to the cultures slightly enhanced the activity of caspases 3/7 and 9 (n=6, $p<0.05$) but not of caspase 8 (n=6, $p>0.05$) in the presence of IL-5.

To further assess the importance of different caspases on human eosinophil apoptosis induced by histamine, the effects of various caspase-inhibitors were investigated. The broad-range inhibitor of caspases 1, 3, 8, 9, 10 and 12, Q-VD-OPh, and the caspase 6 inhibitor Z-VEID-FMK both attenuated histamine-induced human eosinophil apoptosis in the presence of IL-5 (Fig. 11). In addition, a similar effect was seen in the absence of IL-5 (Fig. 11). In contrast, inhibitors of caspases 3 (Z-DQMD-FMK), 8 (Ac-IETD-CHO) and 9 (Ac-LEHD-CHO) did not exert any effects on eosinophil apoptosis (Fig. 11), implying that even though caspases 3/7 and 9 are activated by histamine, they do not participate in mediating eosinophil apoptosis. Surprisingly, the pan-caspase inhibitor, Z-Asp-CH_2-DCB, markedly potentiated the apoptosis-enhancing effect of histamine in the presence of IL-5 but, as expected, inhibited apoptosis in the absence of IL-5 (Fig. 11). The mechanism of this phenomenon remains obscure. These results suggest that caspase 6 and one or more of caspases 1, 10 or 12 may play a role in mediating histamine-induced apoptosis of IL-5-treated eosinophils. However, the same caspases seem to mediate apoptosis of human eosinophils also in the absence of IL-5 as inhibition of the same caspases decreases apoptosis also in IL-5-deprived conditions.

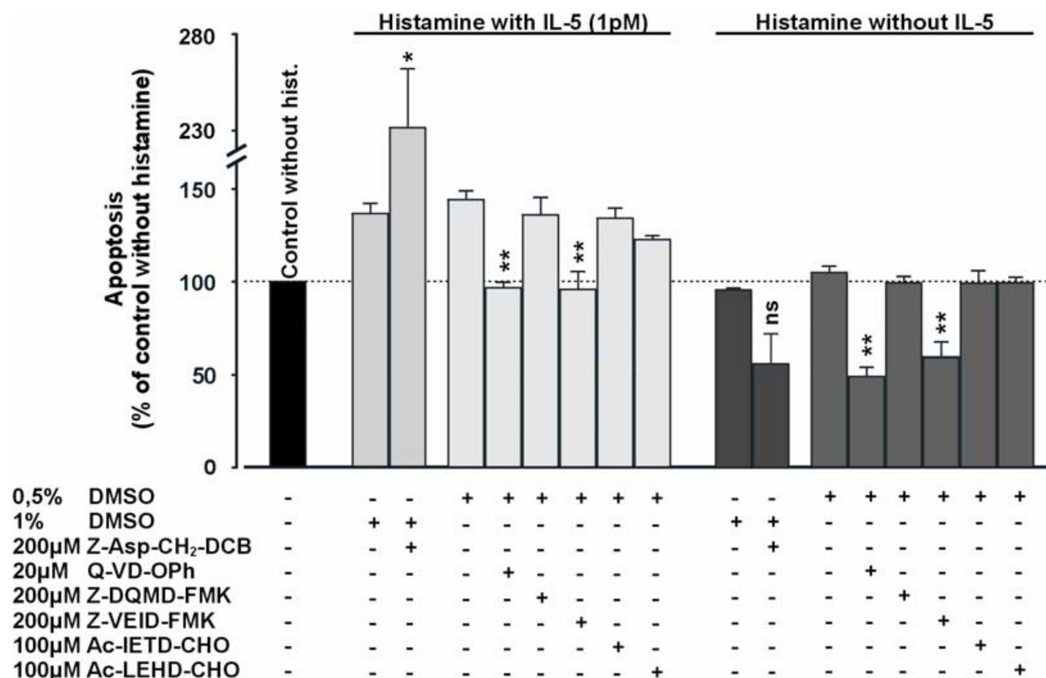

Figure 11. *Effect of caspase-inhibition on histamine-induced human eosinophil apoptosis. Percentage changes in apoptosis by histamine (100 µM) in the presence and absence of different caspase inhibitors and IL-5 (1 pM). Apoptosis was measured by the relative DNA fragmentation assay. Each data point represents the mean ± SEM of n=6 independent determinations using eosinophils from different donors. * indicates p<0.05, ** indicates p<0.01 and ns indicates p>0.05 as compared with the respective control (1% DMSO for Z-Asp-CH$_2$-DCB and 0.5% DMSO for the other caspase inhibitors).*

3.2.2 Histamine Receptors

In search for the receptor mediating histamine-induced reversal of IL-5-inhibited human eosinophil apoptosis, antagonists or agonists for each known histamine receptor subtype were employed. A role of histamine receptors H_1, H_2 and H_3 was excluded as mepyramine (1 µM, an H_1-antagonist), cimetidine (10 µM, an H_2-antagonist) or thioperamide (10 nM, an H_3-antagonist) did not reverse the apoptosis-promoting effect of histamine either alone or in combinations (Table 2, n=6).

A fourth histamine receptor (H_4) was recently identified and cloned by several independent research groups (Oda et al. 2000, Nakamura et al. 2000, Zhu et al.

2001, Nguyen et al. 2001, Liu et al. 2001, Morse et al. 2001) and was shown to be expressed in eosinophils (Oda et al. 2000, Liu et al. 2001, Morse et al. 2001). To evaluate whether the effect of histamine on eosinophil apoptosis in the presence of IL-5 is mediated through the newly discovered H_4 receptor, the effects of various H_4 receptor antagonists and agonists were studied. In addition to antagonizing the histamine H_3 receptor subtypes, thioperamide also functions as an H_4-antagonist in the low micromolar range. However, thioperamide failed to reverse the apoptosis-promoting effect of histamine on IL-5-induced survival of human eosinophils (Table 2, n=6). Two other H_4 receptor antagonists, JNJ7777120 and MMPCI, were also applied with analogous effects to thioperamide, i.e. without the reversal of the histamine effect (Table 2, n=6). To confirm that the histamine H_4 receptor subtype was not the receptor responsible for the enhancement of apoptosis by histamine, an H_4-agonist OUP-16 was tested. OUP-16 did not mimic the effects of histamine (Table 2, n=7) confirming that the newly identified H_4 receptor subtype was not involved in mediating the histamine-induced reversal of IL-5-afforded human eosinophil survival. Consistently, clobenpropit (an H_3-antagonist/ H_4-agonist) did not alter the rate of human eosinophil apoptosis (Table 2, n=5).

As the four unequivocally characterized histamine H_{1-4} receptor subtypes were not found to mediate the pro-apoptotic effect of histamine in the presence of IL-5, the importance of an intracellular binding site for histamine was evaluated. Histamine has been described to possess an intracellular "receptor" denoted the histamine H_{IC} receptor and DPPE functions as a cell permeant H_{IC}-antagonist (MacGlashan 2003). DPPE was not found to reverse the histamine-effect (Table 2, n=6). For the H_{IC} receptor to be involved, histamine would have to be either synthesized by eosinophils or taken up by active transport as histamine is not a cell permeable mediator since it is charged. However, this matter was not further examined as the H_{IC} receptor did not seem to represent a solution to the puzzle of which receptor was mediating the histamine effect.

3.2.3 Different Intracellular Signal Transduction Pathways

As none of the currently characterized histamine receptor subtypes apparently mediated the ability of histamine to enhance human eosinophil apoptosis under the influence of IL-5 and to support a receptor-mediated mechanism, the role of various intracellular signal transduction cascades was evaluated. Many receptors including the histamine H_3 and H_4 receptors are $G_{i/o}$-protein coupled receptors and pertussis toxin (PTX) can inactivate $G_{i/o}$. Pretreatment of eosinophils with PTX at concentrations 0.1, 0.5 and 1.0 µg/ml for 90 min at +37°C did not affect eosinophil apoptosis in the absence or presence of histamine (100 µM) and IL-5 (1 pM) (Table 2, n=5).

Cyclic AMP is an important intracellular second messenger for many receptors including the histamine receptors $H_{2\text{-}4}$. Whether a cell permeant cAMP analogue, 8-Br-cAMP, would affect human eosinophil apoptosis similarly to histamine was assessed. This would have indicated that the histamine-induced apoptosis was being mediated through stimulation of adenylate cyclase and increased cAMP formation. However, 8-Br-cAMP decreased constitutive eosinophil apoptosis but had no effect on cell survival in the presence of IL-5 (Table 2, n=4). Protein kinase A (PKA) is an intracellular kinase activated by cAMP. To clarify the role of the cAMP-PKA-pathway, the effect of a PKA-inhibitor Rp-8-CPT-cAMPS was studied but no effects were found on eosinophil apoptosis (Table 2, n=4). These results suggest that cAMP and PKA are not responsible for mediating the ability of histamine to promote IL-5-inhibited human eosinophil apoptosis.

To evaluate the possible role of phospholipase C (PLC) in human eosinophil apoptosis, we tested two pharmacological inhibitors of PLC, D609 and U73122. D609 had no effects on human eosinophil apoptosis whereas U73122 was found to inhibit constitutive eosinophil apoptosis as well as decreasing apoptosis in the presence of histamine without IL-5 but not to reverse the apoptosis-enhancing effect of histamine on IL-5-treated eosinophils (Table 2, n=4). These results suggest that PLC is not an important enzyme in histamine-induced apoptosis but may have a role in the general regulation of human eosinophil apoptosis.

3.2.4 Other Amine Receptors

As relatively high concentrations of histamine were required to enhance human eosinophil apoptosis, we evaluated the possibility that other amine receptors, namely those activated by 5-HT and catecholamines, would be involved in the pro-apoptotic ability of histamine. Serotonin has recently been suggested to play a role in asthma (Cazzola and Matera 2000, Barnes 2001a) and to act as an eosinophil chemoattractant via the 5-HT_{2A}-receptor (Boehme et al. 2004). Methysergide, a nonselective 5-HT_1-, 5-HT_2- and 5-HT_7-receptor antagonist (n=5), or the natural ligand for the 5-HT-receptors, serotonin (n=4), did not alter the rate of histamine-induced eosinophil apoptosis or did not mimic the apoptosis-promoting effect of histamine on IL-5-afforded eosinophil survival, respectively (Table 2). These results indicate that histamine does not induce human eosinophil apoptosis in the presence of IL-5 by activating 5-HT-receptors.

To resolve whether the pro-apoptotic ability of histamine is mediated via stimulation of α-adrenoceptors, which have been suggested to be expressed in eosinophils (Giembycz and Lindsay 1999), we evaluated the effect of phentolamine, a non-selective α-adrenoceptor antagonist. Phentolamine did not reverse the induction of apoptosis by histamine in the presence of IL-5 (Table 2, n=4). To further exclude the involvement of α-adrenoceptors, the effects of adrenaline (a non-selective agonist), cirazoline (a selective α_1-agonist) and clonidine (an α_2-agonist) were tested on apoptosis of human eosinophils in the absence and presence of IL-5. None of these adrenergic agonists increased apoptosis (Table 2, n=4), which further suggests that the apoptosis-enhancing effect of histamine on IL-5-induced survival of human eosinophils is not mediated through stimulation of α-adrenoceptors.

Table 2. *Summary of the effects of histamine receptor antagonists and agonists and other compounds used to elucidate the signaling pathway of histamine-induced reversal of IL-5-induced human eosinophil survival.*

	Effect on apoptosis in the presence of		
Compound	**Medium**	**IL-5**	**IL-5 + Histamine**
Agonists or antagonists of the histamine receptors			
Mepyramine (1 μM)	NS	NS	NS
Cimetidine (10 μM)	NS	NS	NS
Thioperamide (10 nM)	NS	NS	NS
Thioperamide (up to 10 μM)	↓	↓	↓ (No REV)
JNJ7777120 (up to 10 μM)	NS	NS	NS
MMPCI (up to 100 μM)	↓	NS	NS
OUP-16 (up to 10 μM)	NS	NS	not tested
Clobenpropit (up to 30 nM)	NS	NS	NS
DPPE (up to 30 μM)	↓	NS	NS
Inhibitors or activators of different signal transduction pathways			
PTX (up to 1 μg/ml)	NS	NS	NS
8-Br-cAMP (1 mM)	↓	NS	not tested
Rp-8-CPT-cAMPS (10 mM)	NS	NS	NS
D609 (10 μM)	NS	NS	NS
U73122 (10 μM)	↓	NS	NS
Agonists or antagonists of other amine receptors			
Methysergide (up to 10 μM)	NS	NS	NS
5-HT (up to 100 μM)	NS	NS	not tested
Phentolamine (10 μM)	↓	NS	NS
Adrenaline (up to 1 μM)	NS	NS	not tested
Cirazoline	NS	NS	not tested
Clonidine	↓	NS	not tested

NS indicates that no significant effects were seen on apoptosis, ↓ indicates a statistically significant decrease in apoptosis whereas No REV indicates that the compound did not reverse the apoptosis-promoting effect of histamine on IL-5-induced eosinophil survival. The effects of the compounds are shown in the presence of medium control, on 1 pM IL-5-induced survival and/or on 100 μM histamine-induced reversal of 1 pM IL-5-afforded eosinophil survival after a 40 h incubation. Apoptosis was analyzed by the relative DNA fragmentation assay.

4 Effects of Antihistamines on Eosinophil Survival

4.1 Effects of Diphenhydramine and Chlorpheniramine on Eosinophil Apoptosis (II)

Antihistamines (aka histamine H_1 receptor antagonists) are a common and effective medication for the treatment of allergic diseases. Previous reports have recently suggested that antihistamines are capable of attenuating human eosinophil longevity (Sedgwick and Busse 1997, Domae et al. 2003, Vancheri et al. 2005, Mullol et al. 2006). To clarify why both histamine and antagonists of the H_1 receptor seemed to decrease the survival of human eosinophils, the effects of two first-generation antihistamines chlorpheniramine and diphenhydramine were studied on spontaneous and IL-5-inhibited human eosinophil apoptosis. Diphenhydramine and to a lesser extent chlorpheniramine could reverse eosinophil survival induced by IL-5 through promotion of apoptosis (Fig. 12) when apoptosis was evaluated by the relative DNA fragmentation assay. When eosinophil morphology was used as the indicator of apoptosis, the effects of diphenhydramine (1 mM) were similar to those observed in the flow cytometric analyses ($n=6$, $p<0.01$). However, chlorpheniramine did not increase the number of cells showing the typical morphological features of apoptosis in the presence of IL-5 ($n=6$, $p>0.05$).

The effects of antihistamines on constitutive human eosinophil apoptosis were more complex as diphenhydramine (1 mM) tended to increase spontaneous eosinophil apoptosis both in the relative DNA fragmentation assay ($n=6$, $p>0.05$) and also in the morphological analysis ($n=6$, $p<0.01$) whereas chlorpheniramine (100 μM) decreased constitutive apoptosis both in the relative DNA fragmentation assay ($n=6$, $p<0.01$) as well as in morphological analysis ($n=6$, $p>0.05$).

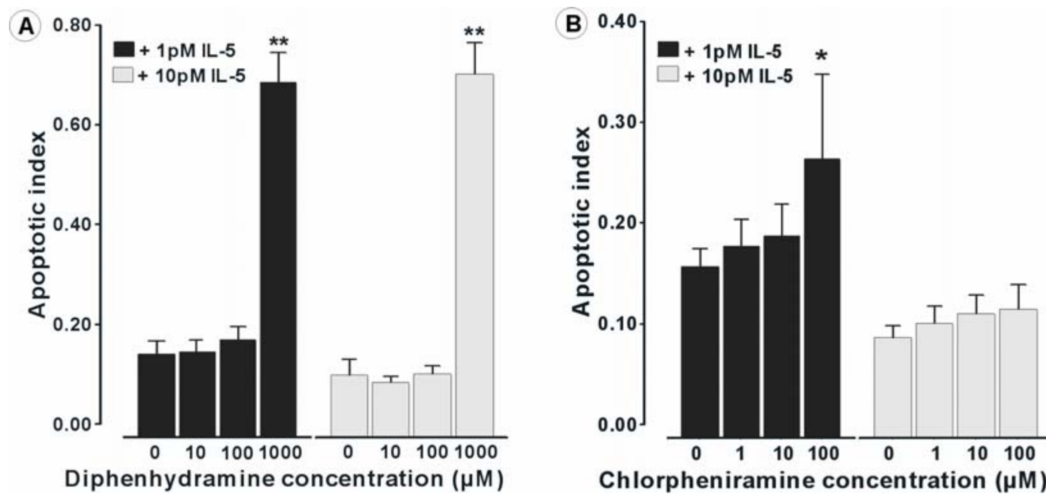

Figure 12. *Antihistamines reverse IL-5-induced eosinophil survival. The effects of (A) diphenhydramine (10-1000 µM) and (B) chlorpheniramine (1-100 µM) on eosinophil apoptosis in eosinophils cultured for 40 h IL-5 (1-10 pM). Apoptosis was assessed by the relative DNA fragmentation assay. * indicates p<0.05 and ** indicates p<0.01 as compared with the respective control. Each data point represents the mean ± SEM of n=6 independent measurements using eosinophils from different donors. (Reprinted with permission from: Hasala et al. 2006, Allergy Asthma Proc, in press. ©OceanSide Publications, Inc.)*

4.2 Effect of Ketotifen on Eosinophil Survival (I)

Ketotifen is an antihistamine most commonly used as a topical eye drop preparation to treat allergic conjunctivitis. After a 40 h culture, ketotifen (1 mM) reversed IL-5 (1-10 pM) –induced eosinophil survival by increasing the number of cells showing hypodiploid DNA content which is indicative of apoptosis as measured by the relative DNA fragmentation assay (n=4, p<0.01). However, the flow cytometry histograms of DNA in PI-stained cells were not typical of apoptotic cells. To test the possibility of primary necrosis, eosinophils were incubated for 60 min, stained with PI in isotonic conditions and analyzed immediately with flow cytometry. In the presence of IL-5 (10 pM), ketotifen (1 mM) unexpectedly increased the uptake of PI, this being indicative of primary necrosis (n=4, p<0.05). Therefore, the effect of ketotifen in increasing DNA breakdown and cell death in the presence of IL-5 in flow cytometric analysis seemed at least in part to be due to induction of primary necrosis instead of

apoptosis, which occurs with diphenhydramine and chlorpheniramine. To elucidate the form of cell death in ketotifen-treated eosinophils, May-Grünwald-Giemsa-stained cells were analyzed by bright field microscopy. Ketotifen (1 mM) was found to reverse IL-5-afforded eosinophil survival and the form of cell death was proposed to be primary necrosis since numerous swollen cells with a bursting nucleus and a broken plasma membrane were seen among few normal and apoptotic eosinophils (n=6).

To further confirm the form of eosinophil death after ketotifen treatment, transmission electron microscopical (TEM) analyses were performed. Eosinophils analyzed after incubation with IL-5 (10 pM) for 1 and 40 h were highly viable and only a few scattered cells displaying the ultrastructural signs of necrosis or apoptosis could be observed (Fig. 13A, 13B). In contrast, incubation of eosinophils for 1 and 40 h with IL-5 and ketotifen (1 mM) resulted in markedly increased necrotic cell death, characterized by chromatolysis as well as bleb formation or rupture of the cell membrane (Fig. 13C, 13D). In addition, the granules of ketotifen-treated eosinophils demonstrated a marked loss of their content irrespective of the cell viability status, which is indicative of piecemeal degranulation. After an incubation for 40 h with IL-5 and ketotifen, virtually all of the eosinophils were in a state of advanced necrosis (the percentages of necrotic cells were 98.5% and 100% in two separate experiments).

The effect of ketotifen on constitutive human eosinophil apoptosis was also evaluated. By using hypodiploid DNA content as a marker, ketotifen (100 μM and 1 mM) was found to attenuate eosinophil apoptosis (n=4). To determine if ketotifen induces primary necrosis also under cytokine-deprived conditions, eosinophils were incubated for 60 min and the uptake of propidium iodide was analyzed by flow cytometry. Ketotifen (1 mM) significantly increased the percentage of eosinophils that took up PI in the absence of IL-5, which is indicative of primary necrosis (n=4, $p<0.05$). In morphological analysis, normal and primary necrotic eosinophils could be seen (n=6).

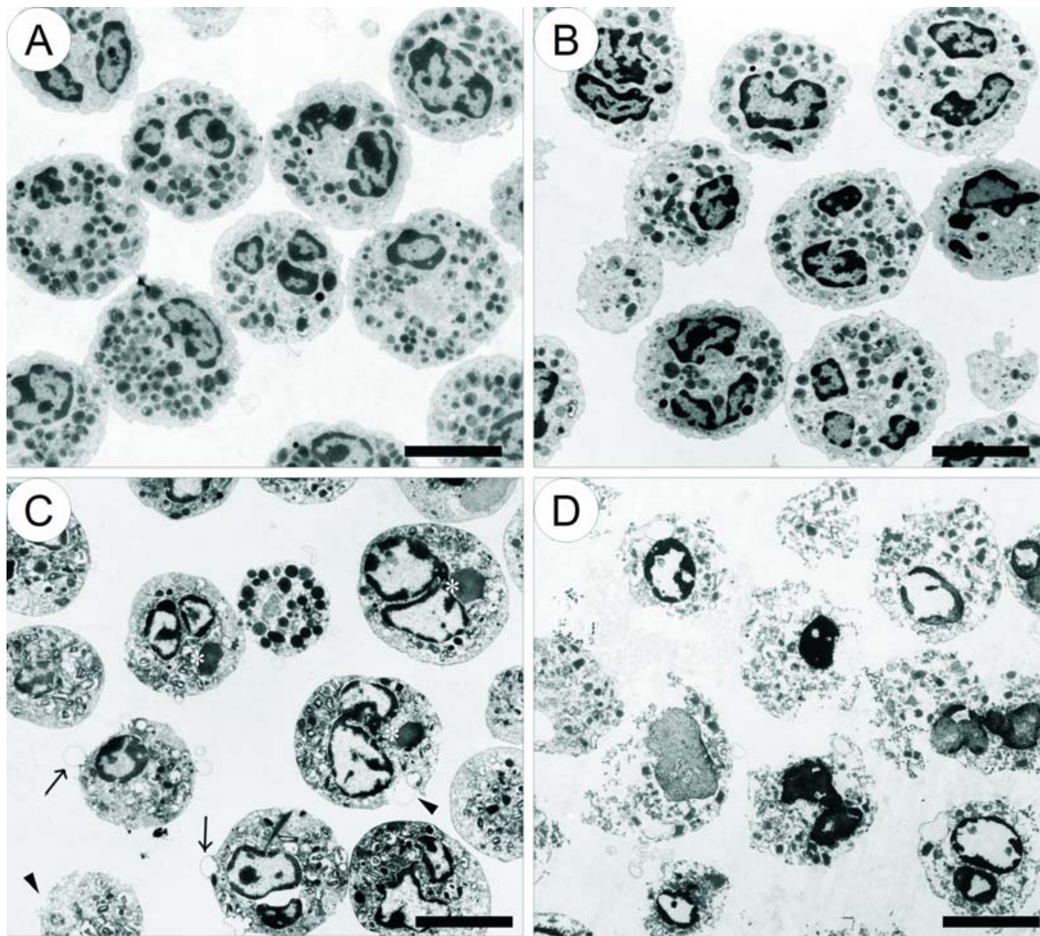

Figure 13. *Ketotifen induces primary necrosis in human eosinophils. Electron micrographs showing the representative ultrastructural morphology of eosinophils examined after incubation with 10 pM IL-5 (A and B) or 10 pM IL-5 and 1 mM ketotifen (C and D). As revealed after the 1 h (A) and 40 h (B) incubations, the IL-5-treated eosinophils displayed well-preserved membrane integrity and the characteristic chromatin pattern of viable eosinophils. After a 1 h incubation with IL-5 and ketotifen (C), many cells displayed chromatolysis (*) as well as bleb formation (arrows) or lost integrity (arrow heads) along the cell membrane. Virtually all cells were in the stage of advanced necrosis and dissolution after a 40 h incubation with IL-5 and ketotifen (D). The figures are representative of two essentially identical experiments. Scale bars A-D = 10 µm. (Reprinted with permission from: Hasala et al. 2005, J Ocular Pharmacol Ther 21:318-327. ©Mary Ann Liebert, Inc., publishers)*

To evaluate to role of the H_1 receptor in ketotifen-induced eosinophil necrosis, the effects of an H_1-antagonist, mepyramine, were tested on eosinophil survival in the absence and presence of IL-5 (1 pM) at a mepyramine concentration (1 µM) that is known to antagonize the H_1 receptors. Mepyramine had no effects on

eosinophil viability either in the absence or the presence of IL-5 (n=4) as determined by the relative DNA fragmentation assay, which suggests that pro-necrotic effects similar to those of ketotifen are not a common feature for the entire drug-group of antihistamines. Furthermore, ketotifen seems to induce primary eosinophil necrosis through a mechanism independent of the H_1 receptors.

5 Role of c-Jun N-terminal Kinase in Eosinophil Apoptosis

5.1 JNK and Constitutive Human Eosinophil Apoptosis (III)

To assess the importance of c-Jun N-terminal kinase in spontaneously occurring human eosinophil apoptosis, the effects of a novel cell-permeable JNK inhibitor peptide L-JNKI1 (Bonny et al. 2001, Bogoyevitch et al. 2005) were studied. L-JNKI1 (10 μM) decreased constitutive eosinophil apoptosis by 64% when apoptosis was assessed by using the relative DNA fragmentation assay (n=6, $p<0.01$). The negative control peptide L-TAT did not affect apoptosis as compared with the medium control (n=6, $p>0.05$). The flow cytometry histograms demonstrate the reduction in eosinophils with a decreased relative DNA content (hypodiploid DNA concentration) i.e. reduced apoptosis by L-JNKI1 as compared with the negative control L-TAT (Fig. 14A-B). When apoptosis was assessed by measuring phosphatidylserine expression on the outer cell membrane leaflet (i.e. Annexin-V binding), L-JNKI1 similarly decreased apoptosis (n=8, $p<0.001$, Fig. 14C-D). Consistently, L-JNKI1 also reduced the amount of eosinophils showing typical apoptotic morphology including cell shrinkage, chromatin condensation and nuclear coalescence (n=6, $p<0.01$, Fig. 14E-F).

To confirm the results obtained with the peptide JNK inhibitor L-JNKI1, we tested the effects of another JNK inhibitor, SP600125, on constitutive human eosinophil apoptosis. As anticipated, SP600125 (10 μM) reduced eosinophil

apoptosis by 19% when analyzed by the relative DNA fragmentation assay (n=7, p<0.05, data not shown).

To further enlighten the role of JNK in human eosinophil apoptosis, activation of JNK was assessed by calculating the ratio of phosphorylated JNK/total JNK by using antibodies directed against Thr183/Tyr185 and Thr221/Tyr223 phosphorylated (activated) JNK and total JNK, which recognize JNK at molecular weights of 46 and 55kDa. In eosinophils analyzed immediately after isolation, a spontaneously active JNK was observed (Fig. 15A). During spontaneous human eosinophil apoptosis, a slow increase in the phosphorylation of the 55 kDa JNK could be seen (Fig. 15A). In addition, an analysis was conducted of a downstream effector of JNK. c-Jun is an inducible transcription factor, which can be activated by phosphorylation at specific serine 63 and 73 residues only by JNK (Mielke and Herdegen 2000). The activity of c-Jun was analyzed by Western blotting using antibodies directed against Ser-63 phosphorylated c-Jun and total c-Jun. Similarly to the active JNK seen in freshly isolated human eosinophils, a phosphorylated c-Jun could be detected in eosinophils analyzed immediately after isolation. However, the extent of c-Jun phosphorylation at the serine 63 residue was not increased during constitutive apoptosis of eosinophils (n=3).

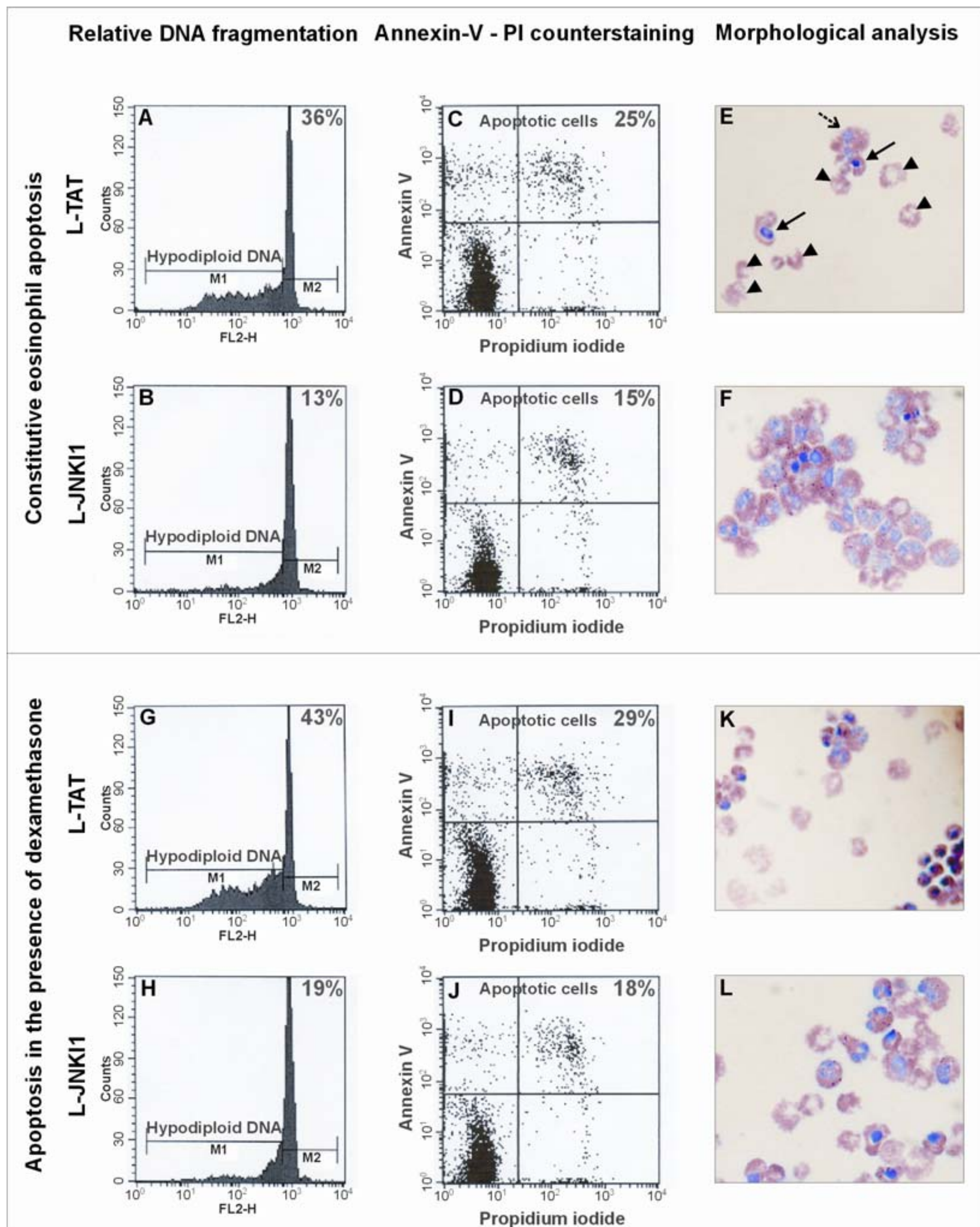

Figure 14. *The effect of L-JNKI1 on human eosinophil apoptosis. The results of one independent experiment are shown, this being representative of six essentially identical experiments of constitutive eosinophil apoptosis (A-F) and dexamethasone (1 µM) –induced apoptosis (G-L). The results were analyzed by the relative DNA fragmentation assay (A, B G, H), in which the cells showing a hypodiploid DNA concentration were considered as apoptotic, the Annexin-V assay (C, D, I, J), in which cells with increased Annexin-V-binding or both increased Annexin-V- and PI-binding were considered as apoptotic, and by morphological analysis (E, F, K, L), where the typical features of normal cells (dotted arrows), apoptotic (solid arrows) and late apoptotic (arrowheads) cells were assessed. The figure in the top right corner represents the percentage of apoptotic cells. (Reprinted with permission from: Hasala et al. 2006, Pulm Pharmacol Ther, in press. ©Elsevier Ltd.)*

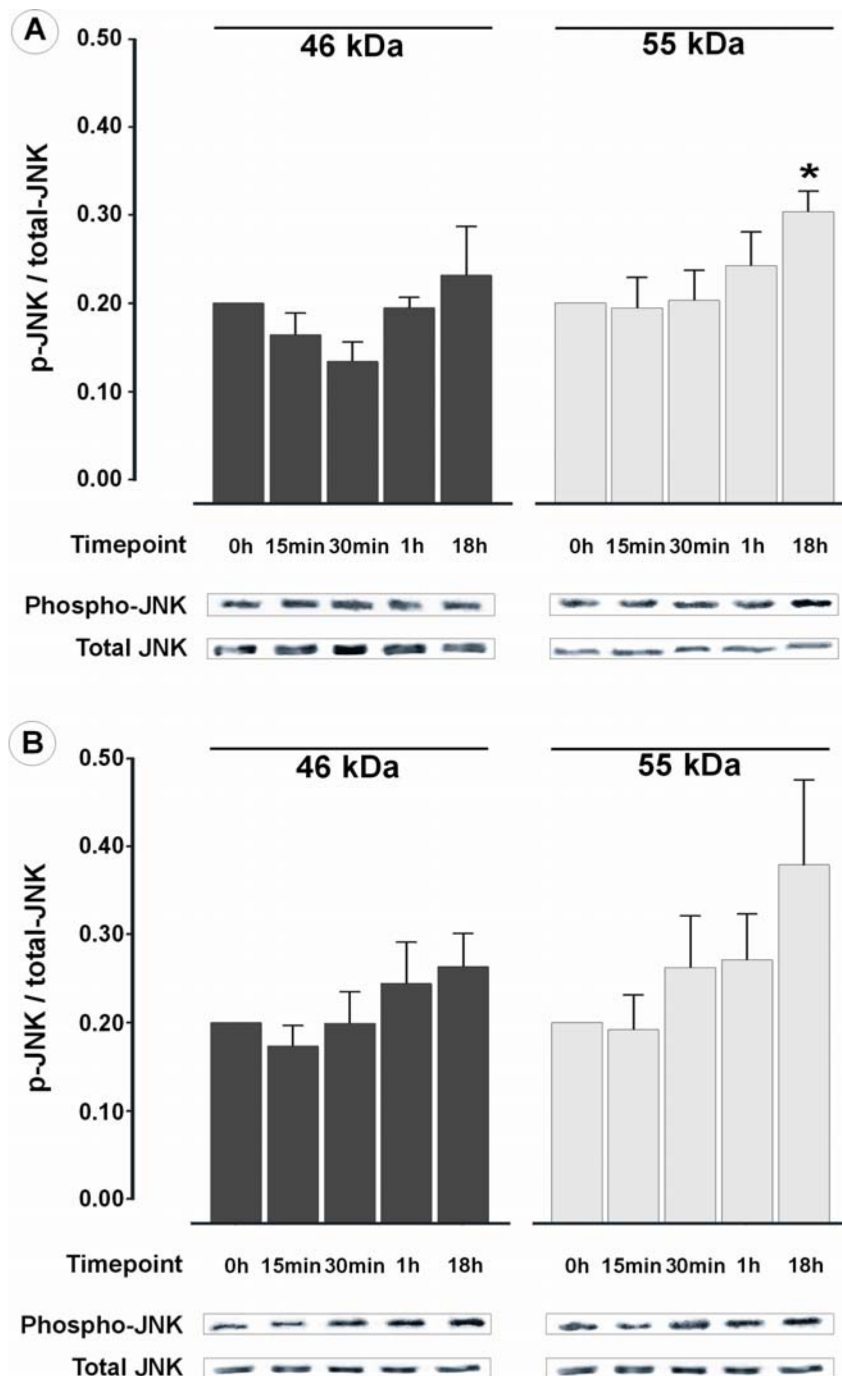

Figure 15. *The phosphorylation of JNK (46 and 55 kDa) during constitutive (A) and dexamethasone-enhanced (B) eosinophil apoptosis. The bars represent the ratio of phosphorylated JNK/total JNK from 4-6 independent Western blot analyses using eosinophils from different donors (mean ± SEM). The p-JNK/total JNK –ratio at time point 0 h is set as 0.20. * indicates $p<0.05$ as compared with the 0 h time point. The immunoblots are representative of n=4-6 essentially identical experiments. (Reprinted with permission from: Hasala et al. 2006, Pulm Pharmacol Ther, in press. ©Elsevier Ltd.)*

5.2 JNK and Glucocorticoid-Induced Eosinophil Apoptosis (III)

Culture of human eosinophils with dexamethasone (1 μM) increased the rate of apoptosis by approximately 20% (n=6, p<0.01) compared to constitutive apoptosis as analyzed by the relative DNA fragmentation assay. To evaluate whether glucocorticoid-induced human eosinophil apoptosis is mediated through the c-Jun N-terminal kinase, the effects of the JNK inhibitor L-JNKI1 were investigated. L-JNKI1 (10 μM), but not the negative control peptide L-TAT, decreased dexamethasone-induced eosinophil apoptosis by 53% (n=6, p<0.01). The flow cytometry histograms of dexamethasone-induced eosinophil apoptosis reveal the decrease in the number of cells with a hypodiploid DNA content indicative of reduced apoptosis by L-JNKI1 as compared with L-TAT (Fig. 14G-H). The results were similar whether this was assessed by Annexin-V binding (n=8, p<0.001, Fig. 14I-J) or by morphological analysis (n=6, p<0.05, Fig. 14K-L). Also the other JNK inhibitor, SP600125 (10 μM), decreased the rate of eosinophil apoptosis by 22% in the presence of dexamethasone as analyzed by the relative DNA fragmentation assay (n=7, data not shown).

Since the JNK inhibitor L-JNKI1 was found to delay human eosinophil apoptosis to a similar extent both in the presence and absence of dexamethasone, the ratio of how many fold dexamethasone had increased apoptosis in the absence and presence of L-JNKI1 was calculated. These fold-increased rates (1.25±0.17 and 1.34±0.15 in the absence and presence of L-JNKI1, respectively, n=4, p>0.05) of eosinophil apoptosis (apoptotic index in the presence of dexamethasone/apoptotic index in the absence of dexamethasone) showed that L-JNKI1 did not reverse the induction of apoptosis by dexamethasone, which suggests that the enhancement of eosinophil apoptosis by glucocorticoids does not seem to be mediated through activation of JNK. Similarly, SP600125 was not found to reverse the steroid-effect (n=5, data not shown).

To clarify the role of JNK in glucocorticoid-induced human eosinophil apoptosis, activation of JNK was assessed in the presence of dexamethasone. A non-significant tendency towards a slow activation of JNK could be seen in the

presence of dexamethasone (1 μM) (Fig. 15B). Since a statistically significant activation of JNK could be observed in the absence of dexamethasone (Fig. 15A), it is proposed that the c-Jun N-terminal kinase is slowly activated during human eosinophil apoptosis but glucocorticoids do not further increase JNK activity. When activation of c-Jun, a downstream effector of JNK, was assessed, dexamethasone (1 μM) did not increase c-Jun phosphorylation, which further suggests that dexamethasone does not induce further activation of JNK.

5.3 JNK and Antihistamine-Induced Eosinophil Apoptosis (II)

To determine whether antihistamine-induced human eosinophil apoptosis is mediated through activity of JNK, eosinophils were cultured for 40 h with IL-5 (10 pM) and in the absence and presence of diphenhydramine (1 mM) and L-JNKI1. L-JNKI1 markedly inhibited eosinophil apoptosis in the absence and presence of diphenhydramine in the relative DNA fragmentation assay (Fig. 16). In the morphological analysis, L-JNKI1 consistently decreased the number of cells showing the characteristic features of apoptosis (n=6). These results suggest antihistamine-induced reversal of IL-5-promoted eosinophil survival and enhancement of eosinophil apoptosis are at least in part mediated through the JNK pathway.

To further elucidate the role of the c-Jun N-terminal kinase in the antihistamine-induced human eosinophil apoptosis, activation of JNK was assessed by calculating the ratio of phosphorylated JNK/total JNK measured by Western blotting using antibodies that recognize JNK at molecular weights 46 and 55 kDa. In freshly isolated human eosinophils with IL-5 (10 pM), JNK was spontaneously active (n=4-6). A slow increase in the phosphorylation of the 55 kDa JNK isoform could be observed in eosinophils after incubation with diphenhydramine (1 mM) and IL-5 (10 pM). (n=6, $p<0.05$). This suggests that the c-Jun N-terminal kinase is slowly activated during antihistamine-induced reversal of cytokine-promoted human eosinophil survival and that increased JNK activity may participate in mediating the pro-apoptotic effect of antihistamines.

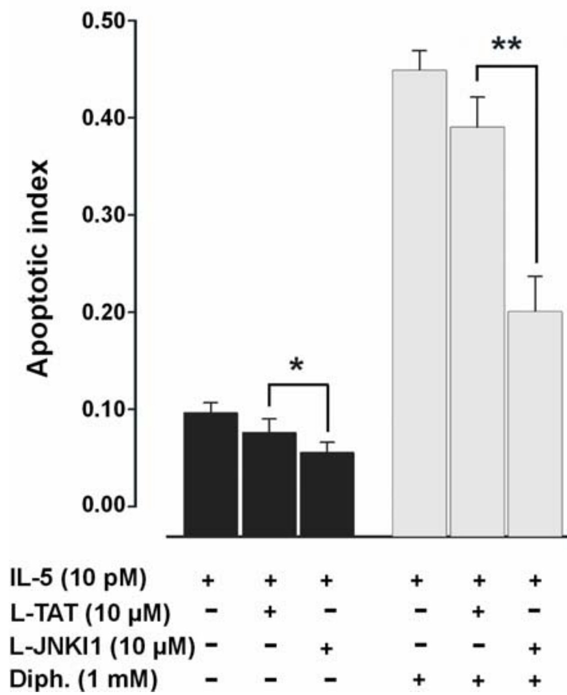

Figure 16. *The effect of L-JNKI1 on IL-5-inhibited human eosinophil apoptosis in the absence and presence of diphenhydramine (1 mM). Apoptosis was assessed by the relative DNA fragmentation assay. L-TAT is the control peptide for L-JNKI1. Hank's balanced salt solution was used as the negative control without peptides. * indicates p<0.05 and ** indicates p<0.01 as compared with the respective control. The values represent the mean ± SEM of six experiments with eosinophils from different donors. (Reprinted with permission from: Hasala et al. 2006, Allergy Asthma Proc, in press. ©OceanSide Publications, Inc.)*

DISCUSSION

1 Methodology

The present study was conducted solely by using primary human eosinophils, which are terminally differentiated, non-dividing cells that can only be cultured for a short time period. Primary human eosinophils are difficult to obtain and have thus been relatively little studied. Most studies have been made with malignant human or animal eosinophilic cell lines, which are easier to obtain and study. However, the receptors and the signaling pathways in cell lines may differ from those of primary cells, which may complicate the interpretation of the results. For the results to be applicable to human pathophysiology, the use of eosinophilic cell lines is not an option. Due to the use of primary human eosinophils, the findings of the current study can be applied to human physiology and allergic disease states, in which the eosinophil is known to play a crucial role.

In this study, eosinophils were isolated from the blood of healthy or atopic individuals, which also included asthmatics. It has previously been shown that apoptosis of blood eosinophils from asthmatic patients is delayed as compared to healthy persons (Kankaanranta et al. 2000b). Moreover, delayed eosinophil apoptosis has also been reported to characterize atopic dermatitis and inhalant allergy (Wedi et al. 1997). In general, eosinophils from atopic individuals display higher viability than eosinophils of non-atopic subjects (Nopp et al. 2002). It is therefore possible, even likely, that these differences in eosinophil longevity have influenced the present results to some extent. The spontaneous apoptotic indices may vary depending on the sample population. However, the rate of spontaneous eosinophil apoptosis varies between all individuals, whether they are atopic or not: the apoptotic indices after a 40 h culture vary from approximately 0.3 up to 0.8 between different individuals but are highly reproducible within each single individual (Kankaanranta et al. 1999). The reason for including both healthy and atopic persons in the study was that

sufficient amounts of eosinophils are difficult to obtain from healthy individuals. In general, only 2-10 x 10^6 eosinophils can be isolated from 100 ml of blood from healthy volunteers. On the other hand, the recruitment of atopic and asthmatic patients poses another problem. Thus, healthy individuals could not be excluded. However, the possibility cannot be excluded that some signaling mechanisms may differ in eosinophils of atopic or asthmatic patients as compared to eosinophils isolated from healthy individuals.

Another factor possibly affecting the interpretation of the results is the use of medication by the blood donors. The donors were allowed to take their normal medication without any breaks at the time of the blood collections. However, none of the blood donors were receiving systemic glucocorticoid medication, which undoubtedly would have influenced the numbers and behaviour of blood eosinophils (Druilhe et al. 2003, Kankaanranta et al. 2005). The most frequently used medications included inhaled glucocorticoids and β_2-agonists, which have been shown to modulate human eosinophil apoptosis (Kankaanranta et al. 2005). Inhaled glucocorticoids have been found to reduce cytokine-afforded survival and to enhance apoptosis of human eosinophils at clinically relevant drug concentrations (Hagan et al. 1998, Zhang X et al. 2000, Zhang et al. 2002). In contrast, β_2-agonists have been noted to inhibit human eosinophil apoptosis at the concentrations achieved after inhalation of the drug (Kankaanranta et al. 2000b). Other commonly used medications included drugs for hypertension and hypercholesterolemia as well as analgesics. Their possible effects on human eosinophils are unknown; therefore, it is impossible to speculate on whether the use of these medicaments may have influenced the results. However, during the eosinophil isolation process, the cells are repeatedly and efficiently washed to eliminate any confounding chemical substances. Therefore, we believe that after the isolation process, the cell suspension contains only minute traces of medicaments, if any. In conclusion, the medications of patients may represent confounding factors, which could not be standardized in the present study.

Normally, neutrophils make up from 40 to 75% whereas lymphocytes represent 25 to 50% of the total circulating blood leucocytes. Eosinophils account for only

1 to 6% of blood leucocytes. The numbers of eosinophils may be elevated due to allergic disorders, parasite infections and specific, relatively rare haematological or eosinophilic disease states such as hypereosinophilic syndrome or malignancies. Therefore, isolation of sufficient amounts of eosinophils, especially from healthy individuals, poses a problem. To overcome these limitations, patients with allergy and asthma have been included in these studies. However, no patients with hypereosinophilic syndrome or malignancies were included due to the possibility that malignant cells would possess different signaling mechanisms and might not respond to the survival or death signals in a similar manner to non-malignant cells.

In the present series of studies, isolated venous blood eosinophils were used. The possibility remains that not all of the results shown here apply to tissue eosinophils. It has previously been suggested that blood and tissue eosinophils may respond differently to the survival-prolonging cytokine IL-5 (Flood-Page et al. 2003a). However, due to ethical aspects and the extreme difficulties in obtaining bronchial mucosal or sputum eosinophils, this problem could not be overcome.

Another major problem complicating human eosinophil studies is how to gain a pure eosinophil population. In this study, a CD16-negative selection methodology was used to obtain eosinophils of >99% purity. If any, the contaminating cells comprised of neutrophils, basophils or lymphocytes. As eosinophil apoptosis is affected by many factors such as cytokines IL-3, IL-5 and GM-CSF, the presence of contaminating cells which could produce these factors might influence the results. However, the very levels of contaminating cells observed in this study are not believed to have affected the longevity of eosinophils.

To eliminate contaminating erythrocytes early in the isolation process, hypotonic lysis was used. In this technique, ice-cold distilled water is added to the cell pellet for 30 s after which the physiological osmolarity is restored by adding sodium chloride to a final concentration of 0.9%. It has recently been suggested

that eosinophil isolation including erythrocyte lysis yields eosinophils with granules with partial or complete loss of electron density, which is indicative of piecemeal degranulation (Malm-Erjefält et al. 2004). These granule abnormalities compared to whole blood baseline are introduced during erythrocyte lysis and further increased during the immunomagnetic separation step (Malm-Erjefält et al. 2004). However, the viability of eosinophils isolated by using different isolation protocols did not differ and was ≥98% (Malm-Erjefält et al 2004). Malm-Erjefält and co-workers concluded that for obtaining eosinophils with normal granule morphology for the studying of the early transformation of resting eosinophils into the degranulating phenotype found in diseased tissues, erythrocyte lysis should be avoided (Malm-Erjefält et al. 2004). However, as the present study mainly focused on analyzing apoptosis and not degranulation with the exception of the study I, which also included analysis of primary necrosis and degranulation of eosinophils, we believe that these granule abnormalities have a negligible role in explaining the present results. However, to overcome these limitations in the future, the isolation process could be modulated to favour isolation protocols evoking minimal granule alterations. For example, erythrocytes could be eliminated without lysis by centrifugation or lysed in 0.2% NaCl instead of distilled water (Malm-Erjefält et al. 2004).

Apoptosis is characterized by DNA fragmentation followed by endonuclease-catalyzed cleavage at the internucleosomal linker regions during the advanced stages of apoptosis (Kankaanranta et al. 2000a). In this study, the primary method to measure apoptosis was the relative DNA fragmentation assay in PI-staining in which cells with reduced DNA content in their nuclei are considered as being apoptotic. Propidium iodide is able to penetrate the broken plasma membranes of dead (i.e. necrotic) cells but unable to enter viable or apoptotic cells. However, to enable the entry of PI into apoptotic or live cells to stain DNA, PI was added in a hypotonic solution with detergent. Since fragmented DNA is a feature of apoptosis, less DNA is stained compared to normal live cells. This method allows the quantitative assessment of apoptosis in a relatively small amount of cells and the results have been shown to correlate well with the morphological features of apoptosis (Kankaanranta et al. 2000a). However, this

method does not discriminate between apoptotic and necrotic cells. Moreover, cells undergoing the early stages of apoptosis may not be detected with this method. As described in study I, we introduced a situation where this assay would have produced unreliable results unless the hypodiploid DNA peak was carefully analyzed. In study I, the results of the relative DNA fragmentation assay were further confirmed by measuring cell membrane permeability by propidium iodide uptake assay, and by using both bright field and transmission electron microscopy.

In general, it is thought unreliable to determine apoptosis by only a single method. The findings in study I further stress the recommendation that the occurrence of apoptosis should be analyzed by using more than one method. In this thesis, changes in the rate of apoptosis observed in the relative DNA fragmentation assay were confirmed with at least one of the following methods: Annexin-V-propidium iodide –counterstaining, flow cytometric determination of primary necrosis based on plasma membrane permeability to PI, morphological analysis by bright field microscopy or by ultrastructural analysis by transmission electron microscopy.

Annexin-V is a protein capable of Ca^{2+}-dependent phospholipid binding. Phosphatidylserine conversion to the outer cell membrane leaflet characterizes the early stages of apoptosis. Annexin-V binds to cells expressing phosphatidylserine on their outer cell membrane. Double staining with Annexin-V and PI enables the discrimination between viable cells (Annexin-V-negative and PI-negative), early apoptotic cells (Annexin-V-positive but PI-negative), late apoptotic aka secondary necrotic cells (Annexin-V-positive and PI-positive) and primary necrotic cells (Annexin-V-negative but PI-positive). Annexin-V binding combined with PI-uptake is considered as a very sensitive and reproducible early marker of eosinophil apoptosis (Walsh et al. 1998).

To evaluate the percentages of normal, apoptotic and necrotic eosinophils, morphological criteria were applied under May-Grünwald-Giemsa-staining in bright field microscopy. This method can also distinguish the different stages of

apoptosis. Chromatin condensation and disappearance of the normal bilobed nucleus replaced by an intensively stained rounded nucleus belong to the earliest morphological features of apoptosis (Kankaanranta et al. 2000a). Other characteristics of programmed cell death include cell shrinkage, condensation of the cytoplasm, membrane blebbing and the formation of apoptotic bodies (Kankaanranta et al. 2000a). Late apoptotic aka secondary necrotic cells appear as rounded, shrunken cells without a nucleus. In contrast, primary necrotic cells are swollen with a broken plasma membrane and a bursting nucleus (Kankaanranta et al. 2000a). In general, morphological analysis can be considered as a very reliable method although the number of analyzed cells is much lower than in flow cytometric analyses due to the considerable time and effort involved with this method.

Ultrastructural analysis of eosinophils by TEM is a validated method for both analyzing the morphological changes and determining the granulation status of eosinophils. Electron microscopy provides the most reliable method for recognizing viable, apoptotic and necrotic cells. However, it is expensive as well as time and labour -intensive. Therefore, electron microscopy functions best as a confirmatory method for results in which apoptosis cannot reliably be analyzed by other methods and for experiments aiming at analyzing the detailed ultrastructure and granulation status of the cells.

In the present study, the experiments were generally performed with four to six replicates. Corresponding amounts of replicates are common in pharmacological studies. However, when studies are made with n=4-6, the possibility of a type II statistical error remains (i.e. that small actual effects do not reach statistical significance and false negative results may be reported). Nonetheless, we believe that no bona fide results were ignored in this study given the relatively small deviations in the experiments and the additional fact that even statistically insignificant tendencies in the rates of apoptosis were noted and reported.

2 Histamine in the Regulation of Eosinophil Apoptosis

Histamine is an essential biogenic amine that plays a critical role in many physiological and pathological processes. Histamine was originally considered as a mediator of acute inflammatory and immediate hypersensitivity reactions but is now believed also to influence chronic inflammatory processes and to regulate the immune response (Jutel et al. 2005, Akdis and Simons 2006). Histamine is known to regulate or modulate several essential functions of eosinophils including eosinophil migration and allergen-induced accumulation of eosinophils in the airways (Jutel et al. 2005, Akdis and Simons 2006), cell shape change, upregulation of adhesion molecules, induction of actin polymerisation and intracellular calcium mobilization (Buckland et al. 2003, Ling et al. 2004) as well as being involved in eosinophil degranulation (Ezeamuzie and Philips 2000). Though both have important roles in the allergic response, the effects of histamine on eosinophil apoptosis remain scantly studied. It has been suggested that histamine does not effect eosinophil survival in the absence of cytokines (Levi-Schaffer et al. 1998). However, histamine has been suggested to induce human neutrophil apoptosis at high concentrations (Hur et al. 2003).

In study IV we demonstrated that histamine could partly reverse IL-5-promoted survival of human eosinophils by inducing apoptosis. This ability of histamine was not apparently mediated either through any of the known histamine receptors H_{1-4} or H_{IC} or through unspecific agonism at 5-HT-receptors or α-adrenoceptors. Moreover, the reversal of IL-5-inhibited eosinophil apoptosis by histamine did not seem to utilize the common intracellular second messenger pathways including cAMP, PKA or PLC but possibly involved caspase 6 and at least some of caspases 1, 10 or 12. Thus, the results raised the possibility that human eosinophils may express a novel, yet unidentified histamine receptor. Another possibility is that histamine promotes apoptosis through a non-histamine receptor that is not a 5-HT or alpha variant or that histamine acts by a mechanism that is non-receptor mediated. Based on our results under the experimental conditions described, we cannot exclude any of these explanations. However, the existence of another, yet undefined histamine receptor would

appear as the most likely alternative as it has been suggested that additional histamine receptor subtypes remain to be discovered (Hough 2001). Further studies need to address the question of the mechanism behind the reversal of IL-5-promoted eosinophil survival by histamine. Possible signaling transduction mechanisms might include non-G-protein-coupled cell surface receptors or MAPK or PKC pathways. Another alternative might be that histamine is actively transported into the cell and the nucleus and acts via some nuclear receptor.

The accretion in eosinophil apoptosis elicited by histamine in the presence of IL-5 reached a magnitude of approximately 40%, which is comparable to the partial reversal of cytokine-afforded eosinophil survival induced by glucocorticoids (Druilhe et al. 2003, Kankaanranta et al. 2005). However, the mechanism of action between these eosinophil ligands presumably differs as histamine does not enhance constitutive eosinophil apoptosis in the same way as glucocorticoids. One possibility also exists that the mechanism of action of histamine-induced human eosinophil apoptosis may be dependent on cytokine receptors or the signaling cascades initiated by them to some extent.

Mast cells are the primary source of histamine *in vivo* (Jutel et al. 2005). As histamine also seems to possess some anti-inflammatory actions, the question is raised whether it is disadvantageous to treat atopic patients with mast-cell stabilizing drugs such as β_2-agonists. Indeed, long-acting β_2-agonists have been reported to inhibit human eosinophil apoptosis (Kankaanranta et al. 2000b, Kankaanranta et al. 2005). It is tempting to speculate that the anti-apoptotic effects of long-acting β_2-agonists might be due to stabilization of mast cells and the subsequent inhibition of histamine release.

The EC_{50} value of histamine for the induction of eosinophil apoptosis under the influence of the survival-prolonging cytokine IL-5 was 0.56 μM. During inflammation, the local concentrations of histamine released from mast cells and basophils have been estimated to be in the millimolar range in the vicinity of the neighbouring cells (Adams and Lichtenstein 1979). Moreover, the concentrations of histamine used in nasal (Wuestenberg et al. 2004, Plevkova et al. 2006) and

inhalation (Koskela et al. 2005) challenges in diagnosing asthma and allergic diseases are many times higher than the present EC_{50} value. It is conceivable that similar levels of histamine, which induced apoptosis of cytokine-treated human eosinophils *in vitro*, can be achieved locally after the histamine challenges used in the diagnosis of asthma and allergic rhinitis, which may be relevant also *in vivo*. Thus, this novel, potentially anti-inflammatory ability of histamine represents yet another mechanism by which histamine may regulate the immune system. Whether the induction of IL-5-inhibited eosinophil apoptosis by histamine occurs *in vivo* at sites of allergic inflammation remains unresolved.

3 Antihistamines in the Regulation of Eosinophil Survival

Antihistamines (aka histamine H_1 receptor antagonists) constitute a common and effective medication for the treatment of allergic diseases. Antihistamines are often claimed to have additional anti-inflammatory properties other than those related to H_1-antagonism. To date, there are a few published studies reporting the effects of single antihistamines on human eosinophil viability. Oxatomide (Domae et al. 2003) and fexofenadine (Vancheri et al. 2005) have previously been shown to promote apoptosis of eosinophils. In addition, cetirizine (Sedgwick and Busse 1997) and desloratadine (Mullol et al. 2006) have been claimed to inhibit eosinophil survival although apoptosis was not specifically analyzed. However, contradictory findings of antihistamines have also been reported with smaller concentrations of cetirizine and levocetirizine, i.e. no effects on eosinophil survival (Thomson et al. 2002).

Our study extends the previous knowledge on the effects of antihistamines on human eosinophil longevity by demonstrating that two first-generation antihistamines, diphenhydramine and chlorpheniramine, could reverse the eosinophil survival induced by IL-5 via enhancement of apoptosis. Secondly, we observed that not all of the effects of antihistamines on eosinophil viability are necessarily desirable as is the case with ketotifen-induced primary eosinophil necrosis, which may aggravate the existing inflammatory response if it were to

occur under *in vivo* –conditions. This would evoke eosinophil cytolysis and the release of their potentially cytotoxic granule contents into the surrounding tissue.

The concentration of ketotifen (1 mM) found to induce primary necrosis of isolated human eosinophils in the present study is likely to be of clinical importance as it is at the same level as the drug concentration in eye drops (0.81 mM) used topically to treat conjunctival disease. In systemic use, however, the maximal plasma concentration of ketotifen does not reach millimolar concentrations. The concentrations of diphenhydramine (1 mM) and chlorpheniramine (100 μM) found to induce apoptosis in IL-5-stimulated eosinophils in the current study are relatively high but similar to those used in other studies (Sedgwick and Busse 1997, Vancheri et al. 2005). These concentrations could be achieved in topical preparations such as in nasal sprays or eye drops. Therefore, it could be opined that in allergic rhinitis or conjunctivitis, certain topically administered antihistamines would relieve the accumulation of nasal mucosal or conjunctival eosinophils through induction of apoptosis if those antihistamines that are capable of enhancing apoptosis were to be administered. After oral administration, the drug concentrations of diphenhydramine or chlorpheniramine in the plasma do not reach the high concentrations used in this study. However, tissue binding and accumulation often constitutes an important aspect of the pharmacokinetics of a drug. It is not known how high concentrations can actually be achieved locally in the tissue after oral administration of these drugs but does seem unlikely that even the local concentrations achieve millimolar levels. Thus, these results are not likely to apply to systemic use of antihistamines.

In relation to asthma, antihistamines are not an effective treatment for airway obstruction and are therefore not used to treat asthma as such. However, in a recent study evaluating the relationships among upper and lower airway function and nasal inflammation in patients with seasonal allergic rhinitis and asthma, nasal eosinophils were found to be correlated with nasal symptoms and airflow as well as with FEV_1 (Ciprandi et al. 2004). At present, the link between asthma and rhinitis (i.e the united airways disease concept) is gaining widespread

recognition (Passalacqua et al. 2001, Togias 2003, Passalacqua et al. 2004). It has been shown that treatment of allergic rhinitis with intranasal glucocorticoids has a favorable influence on the bronchial symptoms and can reduce asthma exacerbations (Passalacqua et al. 2001, Togias 2003, Passalacqua et al. 2004). Moreover, orally administered antihistamines may also improve the symptoms and decrease the occurrence of exacerbations of asthma (Passalacqua et al. 2001, Passalacqua et al. 2004). Thus, it may be conjectured that the reversal IL-5-induced survival of human eosinophils through antihistamine-enhanced apoptosis might have clinical implications in the treatment of asthma in addition to other allergic disorders.

Addressing the question of the mechanism through which antihistamines modulate human eosinophil longevity, it was hypothesized that their effects are non-H_1-receptor-mediated. Induction of primary necrosis by ketotifen was shown to be independent of the histamine H_1 receptors since the H_1-antagonist mepyramine did not have any effects on eosinophil survival as assessed by using the relative DNA-fragmentation assay. The observations that not all H_1 receptor antagonists affect eosinophil survival and that the concentrations required to reduce eosinophil viability were found to be higher than those needed for H_1-antagonism suggest that the effects of antihistamines on eosinophil survival are not a common feature for all members of this class of drugs and furthermore, they are not mediated through the H_1 receptor. Our results showing that activity of JNK at least partially mediates diphenhydramine-induced reversal of IL-5-inhibited apoptosis indicate that the c-Jun N-terminal kinase may play a role in mediating the apoptosis-promoting effects of antihistamines.

4 c-Jun N-terminal Kinase and Eosinophil Apoptosis

c-Jun N-terminal kinase belongs to a family of mitogen-activated protein kinases (MAPK) that are important in the differentiation, activation, proliferation, degranulation and migration of various inflammatory cells, airway smooth muscle and epithelial cells. JNK has been proposed to be an important mediator in the signaling pathways of apoptosis (Davis 2000). Recent studies have

suggested that nitric oxide and glucocorticoids may activate JNK in human eosinophils (Zhang JP et al. 2000, Gardai et al. 2003, Zhang et al. 2003). Activation of JNK by dexamethasone was associated with induction of eosinophil apoptosis, which could be attenuated by using a JNK inhibitor SP600125 (Gardai et al. 2003). However, the importance of JNK activation in glucocorticoid-induced eosinophil apoptosis remains controversial as no effects on dexamethasone-induced eosinophil apoptosis were observed by using JNK1/2 antisense phosphorothioate oligodeoxynucleotides (Zhang JP et al. 2000). A role for JNK has also been proposed in animal models of asthma (Eynott et al. 2003, Eynott et al. 2004, Nath et al. 2005). Recently, JNK inhibitors have attracted considerable interest as potential therapeutic targets for the treatment of asthma and COPD, and some JNK inhibitors are even undergoing in clinical trials (Adcock et al. 2006).

Our study shed new light on the importance of JNK in human eosinophil apoptosis. We demonstrated that JNK mediates constitutive eosinophil apoptosis and that disruption of the JNK pathway by a JNK inhibitor L-JNKI1 could attenuate apoptosis and lead to prolonged survival of human eosinophils. Studies with another JNK inhibitor, SP600125, supported these results. Active (i.e. phosphorylated) JNK and c-Jun were observed in freshly isolated eosinophils as well as a slight increase in the phosphorylation of the 55 kDa JNK isoform during the course of spontaneously occurring apoptosis, which support the role of JNK in the signaling pathway of constitutive human eosinophil apoptosis. Similar effects were seen in the presence of dexamethasone although no increased activity of JNK was detected during dexamethasone-enhanced eosinophil apoptosis. Moreover, JNK inhibition was not found to reverse the pro-apoptotic effect of dexamethasone. These results suggest that JNK may not be responsible for mediating the apoptosis-enhancing effects of dexamethasone on human eosinophils. In line with our results, it has previously been reported that the JNK inhibitor SP600125 could decrease dexamethasone-induced eosinophil apoptosis and a similar, although statistically non-significant (n=3), tendency was also detected in the absence of dexamethasone (Gardai et al. 2003).

In addition, we showed that JNK is active and that the JNK inhibitor L-JNKI1 could inhibit apoptosis during diphenhydramine-induced reversal of IL-5-afforded human eosinophil survival. This indicates that antihistamine-enhanced human eosinophil apoptosis involves the activity of the c-Jun N-terminal kinase. However, as L-JNKI1 inhibited human eosinophil apoptosis under the influence of IL-5 also in the absence of diphenhydramine and as we did not measure JNK activation only in the presence of IL-5 without diphenhydramine, the possibility cannot be excluded that JNK does not selectively mediate antihistamine-induced reversal of IL-5-afforded eosinophil survival but rather is a general mediator of human eosinophil apoptosis also in the presence of IL-5.

The signaling pathways that lead to the regulation of apoptosis after JNK activation remain largely unknown. However, JNK has been proposed to be critical in the regulation of the transcriptional activity of activator protein 1 and phosphorylation of the serine residues 63 and 73 of c-Jun by JNK has been observed to enhance the transcriptional activity of activator protein 1 (Davis 2000). In our study, despite the presence of serine 63 –phosphorylated c-Jun in freshly isolated eosinophils, we could not detect further activation (i.e. phosphorylation of serine 63 residues) of c-Jun although JNK itself was found to be slightly activated. One explanation for this phenomenon could be that the activities of JNK and c-Jun can be separately regulated in ways other than via the phosphorylation of serine 63 and 73 residues of c-Jun, which are strictly dependent on JNK activity (Mielke and Herdegen 2000). Moreover, JNK activation and even its nuclear translocation do not necessarily result in c-Jun phosphorylation as JNK is also able to catalyze the phosphorylation of other nuclear substrates (Mielke and Herdegen 2000). In addition, association of JNK with c-Jun does not necessitate the N-terminal phosphorylation of serine residues 63 and 73 as multiple other contacts exist between JNK and c-Jun (Mielke and Herdegen 2000). Further studies investigating JNK-initiated signaling cascades will be needed to elucidate the signaling pathways leading to the programmed cell death of human eosinophils. This knowledge may lead to new strategies for the treatment of allergy and asthma through increased understanding of the pathogenetic mechanisms underlying these disease states.

SUMMARY AND CONCLUSIONS

The present study was designed to elucidate the role of histamine as a regulator of human eosinophil apoptosis in relation to asthma and other allergic disorders and to clarify the importance of c-Jun N-terminal kinase in mediating eosinophil apoptosis. Isolated human blood eosinophils were used in these experiments.

The major findings and conclusions were:

1. Histamine reversed IL-5-afforded survival of human eosinophils by inducing apoptosis. Constitutive eosinophil apoptosis remained unaffected by histamine. The EC_{50} of histamine-induced eosinophil apoptosis was 0.56 μM, which is a clinically relevant concentration. The increased eosinophil apoptosis evoked by histamine reached a magnitude of 40%, which is comparable to the potency of the glucocorticoid-effect.

2. Based on studies with pharmacological agents, the ability of histamine to promote human eosinophil apoptosis in the presence of the survival-prolonging cytokine IL-5 could not be explained by any of the currently characterized histamine receptors or by non-specific activation of 5-HT-receptors or α-adrenoceptors on eosinophils. This suggests that human eosinophils may express a novel, yet unknown histamine receptor. Alternatively, histamine may mediate human eosinophil apoptosis through a receptor-independent mechanism.

3. First-generation antihistamines diphenhydramine and chlorpheniramine also reversed eosinophil survival by increasing the rate of apoptosis. The mechanism of action was proposed to be independent of histamine H_1 receptors but possibly to involve the activity of JNK. The possibility exists that the reversal IL-5-induced survival of human eosinophils by diphenhydramine or chlorpheniramine may have clinical implications in

the treatment of allergic rhinitis as well as asthma. The antihistamine ketotifen was observed to induce primary necrosis of human eosinophils and in that way to reverse the IL-5-enhanced eosinophil survival through an H_1-receptor-independent mechanism. The ketotifen concentration (1 mM) capable of inducing eosinophil necrosis was similar to the drug concentration present in topical eye drop preparations commonly used to treat allergic conjunctivitis. Therefore, it may be hypothesized that topical use of ketotifen may exacerbate the existing inflammatory response in the conjunctiva due to *in vivo* eosinophil cytolysis and the release of eosinophil granule contents.

4. Activity of JNK was shown to be important in mediating constitutive human eosinophil apoptosis. However, JNK seemed to play a negligible role in glucocorticoid-induced eosinophil apoptosis. Thus, it is suggested that activation of JNK is not confined to eosinophil apoptosis induced by certain drugs or compounds but rather is a general mediator of human eosinophil apoptosis. Based on our present results, inhibition of JNK activity may not be an optimal target to reduce lung tissue eosinophilia although JNK inhibitors have been a drug discovery target of considerable interest and are undergoing clinical trials.

5. The findings of this study extend our knowledge about the processes involved in the regulation of human eosinophil apoptosis. Individual antihistamines seem to differ in their capacity to modulate human eosinophil survival. Thus, knowledge of the anti-eosinophilic properties of antihistamines may be of importance in choosing the appropriate treatment for patients with asthma or other allergic disease states. Future studies should focus on resolving the detailed mechanisms of histamine- and antihistamine-induced human eosinophil apoptosis since this could serve as a basis for the development of novel therapies targeting the eosinophil.

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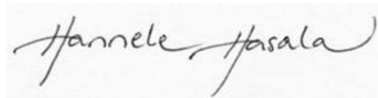

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Ketotifen Induces Primary Necrosis of Human Eosinophils

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ABSTRACT

Eosinophils are considered essential in the pathogenesis of allergy. Reduced eosinophil apoptosis is considered to be a key element in the formation of eosinophilia in allergic conditions. Antihistamines are widely used in the treatment of allergic disorders, but their effects on eosinophil apoptosis are poorly understood. The histamine H1-receptor antagonist, ketotifen, is available orally and as eye drops for the treatment of allergic symptoms. The aim of our study was to investigate the possible effect of ketotifen on constitutive eosinophil apoptosis and on interleukin (IL)-5-mediated eosinophil survival. Isolated peripheral blood eosinophils were cultured with or without the survival-prolonging cytokine IL-5 and ketotifen. Apoptosis was assessed by measuring the relative DNA content and by morphological analysis. Ketotifen was found to reverse eosinophil survival induced by interleukin-5. However, the flow cytometry histogram of DNA in propidium iodide–stained cells was not typical to apoptosis. Morphological analysis of the eosinophils by bright-field microscopy suggested that the effect of ketotifen was due to the induction of primary necrosis rather than apoptosis. Histological assessment of eosinophil ultrastructure by transmission electron microscopy confirmed signs of advanced necrosis. In summary, our results suggest that at clinically relevant drug concentrations, ketotifen induces primary necrosis in IL-5-treated human eosinophils.

INTRODUCTION

THE INCIDENCE of ocular allergy, as well as other atopic conditions, is increasing. Ocular allergy includes a spectrum of diseases with variable severity, from the relatively mild seasonal allergic conjunctivitis to the more severe and sight-threatening atopic ceratoconjunctivitis.[1,2] Allergic conjunctivitis is frequently associated with other forms of allergy, such as allergic rhinitis and asthma. Despite the clinical differences between various forms of allergy, the underlying pathogenic mechanisms are very similar. Ocular allergic inflammation is associated with IgE-mediated mast-cell activation in the conjunctiva followed by the release of mast-cell–derived mediators,

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such as histamine and cytokines, that recruit eosinophils and lymphocytes to the ocular surface.[1–3]

Eosinophils are absent from normal healthy conjunctival tissues, whereas high numbers are present in allergic conjunctiva. Increased levels of eosinophil granule contents, such as major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil cationic protein (ECP), have also been detected in tear specimens in ocular allergy.[1] Eosinophils, therefore, are important mediators in the pathogenesis of ocular allergy[1–3], as well as in other allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis.[4]

It has been demonstrated that eosinophil apoptosis is delayed in patients with asthma, inhalant allergy, or atopic dermatitis.[5,6] The numbers of granulocytes found in the blood and tissues are largely determined by the balance between cell maturation and death.[7] With regard to the clearance of eosinophils, apoptosis or programmed cell death has been suggested as a major mechanism in the resolution of eosinophilic inflammation.[8,9] Apoptosis is characterized by specific morphological and biochemical changes, including cell shrinkage, a shift from a bilobed to a monolobed nucleus, chromatin condensation, and endonuclease-catalyzed DNA breakdown followed by fragmentation of the cell into apoptotic bodies, which are phagocytozed intact without inducing inflammation.[8,10,11] The process of apoptosis is highly controlled and different from primary necrosis or cytolysis, which is characterized by uncontrolled lysis of chromatin, cell and nuclear membrane rupture, and the release of cellular granule contents into the surrounding tissues,[4,12] which may trigger or exacerbate the inflammatory response. In the absence of cytokines, eosinophils cultured *in vitro* undergo apoptosis, which can be inhibited by different cytokines, principally IL-3, IL-5, and granulocyte macrophage—colony stimulating factor (GM-CSF).[4,9,11]

Histamine released from mast cells in response to antigens is a key characteristic in allergic reactions. Antihistamines or histamine 1 (H1)-receptor antagonists are widely used in the treatment of allergic diseases. They may also have anti-inflammatory properties. The expression of H1 receptors in human eosinophils has been demonstrated.[4] Some H1 receptor antagonists are, at least *in vitro*, able to affect the function of inflammatory cells (e.g., the chemotaxis and adhesion of eosinophils).[13] There are, however, only few published studies on the effects of antihistamines on eosinophil viability. The H1-receptor antagonist, oxatomide, has been reported to promote eosinophil apoptosis and to suppress IL-5-induced eosinophil survival.[14]

The aim of this study was to examine whether the commonly used H1-receptor antagonist, ketotifen, influences constitutive eosinophil apoptosis or IL-5-afforded eosinophil survival.

METHODS

Eosinophil isolation and culture

Peripheral blood was obtained from volunteers with blood eosinophilia. Eosinophils were isolated under sterile conditions to a more than 99% purity, as previously described.[5,15–17] The cells were cultured for 40 h in the absence and presence of ketotifen and IL-5 (10 pM) (+37°C, 5% CO_2) in RPMI 1640 medium (Dutch modification) with 10% fetal calf serum and antibiotics, unless otherwise stated. The study protocol was approved by the Ethics committee of Tampere University Hospital (Tampere, Finland). Subjects gave informed consent before donating blood.

Flow cytometric determination of apoptosis by measuring the relative DNA content

The percentage of cells showing hypodiploid DNA content was assessed by measuring the relative DNA content by flow cytometry (FACScan, Becton Dickinson; San Hose, CA) of propidium iodide (PI)–stained cells.[5,15–17] Endonuclease-catalyzed DNA fragmentation is considered to be a specific feature of apoptosis.[18] The cells showing decreased relative DNA content were considered to be apoptotic. Eosinophils were suspended in 300 μL of hypotonic propidium iodide solution (25 μg/mL in 0.1% sodium citrate and 0.1% Triton X-100), protected from light and incubated at 4°C for 1 h before flow cytometric analysis.

Flow cytometric determination of primary necrosis

The percentage of primary necrotic cells was evaluated by flow cytometry of PI-stained cells after 1 h incubation at + 37°C, 5% CO_2. The cells showing uptake of PI after 1 h were assumed to be necrotic as fast cell-membrane breakdown

is a feature typical to necrotic cell death.[15] Eosinophils were suspended in 250 μL HBSS, 5 μL of 1 mg/mL PI was added, and the suspension was incubated at room temperature for 5 min before flow cytometric analysis.

Morphological analysis by bright field microscopy

Eosinophil morphology was assessed by bright-field microscopy. Eosinophils cultured for 40 h were spun onto cytospin slides (500 rpm, 5 min) and stained with May-Grünwald-Giemsa (MGG) after fixation in methanol. Cells showing the typical features of apoptosis, such as cell shrinkage, nuclear coalescence, and chromatin condensation, were considered as apoptotic.[15] Swollen cells having a broken plasma membrane and a bursting nucleus were considered to have undergone primary necrosis.

Histological assessment of eosinophil ultrastructure using transmission electron microscopy (TEM)

The degranulation and viability status of the eosinophils cultured for 1 and 40 h was assessed by detailed ultrastructural examination by transmission electron microscopy (TEM).[19] Briefly, the cell suspensions were primary fixed in a 1:2 volume ratio of 4% paraformaldehyde in 0.1 M of phosphate buffered saline (PBS; yielding a 2.7% total concentration of fixative) for 1 h at room temperature. After centrifugation, the cell pellet was secondarily fixed in 3% paraformaldehyde/1% glutaraldehyde in PBS over night. The cell suspensions were then added to microcentrifuge tubes ($3–5 \times 10^6$ cells/tube) and centrifuged at 4°C for 10 min at 2600 rpm (1500*g*). The obtained pellets were gently embedded in warm (40–50°C) 3% agarose in PBS and postfixed in 1% osmium tetroxide for 1 h, dehydrated in graded acetone solutions, and embedded in Polybed 812. One-micrometer-thick plastic sections were cut on an ultratome (Ultracut E; Leica, Germany), and cell-rich areas were selected for further electron microscopical analysis. Ultrathin sections (90 nm) were cut and placed on a 200-mesh, thin-bar copper grid and stained with uranyl acetate and lead citrate. The specimens were examined by a Philips EM10 transmission electron microscope (Philips; Eindhofen, The Netherlands).

The individual eosinophils were evaluated at ×8000 magnification. Based on morphological criteria, the eosinophils were divided into subgroups,[12] defined in the following categories: *Viable eosinophils*: Preserved integrity of cytoplasmic and nuclear membranes, normal nuclear euchromatin, and heterochromatin. *Eosinophil cytolysis (ECL)*: Presence of chromatolysis, cell membrane blebs, loss of plasma-membrane integrity, and partly dissolved cytoplasm. *Apoptotic eosinophils*: Presence of chromatin condensation, preserved plasma membrane, and nondilated organelles. Evaluation of eosinophil degranulation was also performed. The eosinophils were assessed for structural changes owing to piecemeal degranulation (PML) (e.g., ragged loss of core material, coarsening of the granular matrix, or more or less empty granules).

Materials

The following compounds were purchased from Sigma Chemical Company (St. Louis, MO): ketotifen fumarate and pyrilamine maleate (mepyramine), acetone, agarose, and glutaraldehyde. Lead citrate and uranyl acetate were from VWR International (Stockholm, Sweden). Other reagents were obtained as follows: human recombinant IL-5 (R&D System Europe; Abingdon, United Kingdom), propidium iodide (Tocris; Bristol, United Kingdom), anti-CD16 microbeads and the magnetic cell separation system (Miltenyi Biotec; Bergish Gladbach, Germany), Ficoll-Paque (Pharmacia AB; Uppsala, Sweden), antibiotics and RPMI 1640 (Dutch modification) (Gibco BRL; Paisley, United Kingdom), fetal calf serum, Hanks' balanced salt solution and RPMI 1640 (BioWhittaker; Verviers, Belgium), osmiumtetroxide (AGAR Scientific; Stansted, Essex, England), paraformaldehyde (EM grade; TAAB Laboratories Equipment; Aldermaston, Berkshire, England), Polybed 812 (Polysciences; Eppleheim, Germany), phosphate buffered saline (PBS, without calsium and magnesium, Life Technologies; Paisley, United Kingdom), May-Grünwald (Merck; Darmstadt, Germany), and Giemsa (J.T.Baker; Deventer, Holland). Ketotifen fumarate was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in culture was 0.5% and was found not to affect eosinophil viability, as assessed by bright-field microscopy (n = 6, data not shown). As the respective control for ketotifen, 0.5% DMSO was used. Mepyramine was dissolved in RPMI 1640 medium (Dutch modification).

Statistics

The results are expressed as the mean ± standard error of the mean, except for the TEM eosinophil viability assessments, where the results are expressed as the percentage of cells displaying signs of necrosis from two separate donors. Cell death in the relative DNA fragmentation assay is expressed as the percentage of cells showing hypodiploid DNA content suggesting apoptosis. Primary necrosis in the relative DNA fragmentation assay is expressed as the percentage of primary necrotic cells taking up propidium iodide. Statistical significance was calculated by analysis of variance for repeated measures supported by the Dunnett test or by paired t tests. Differences were regarded significant when $P < 0.05$.

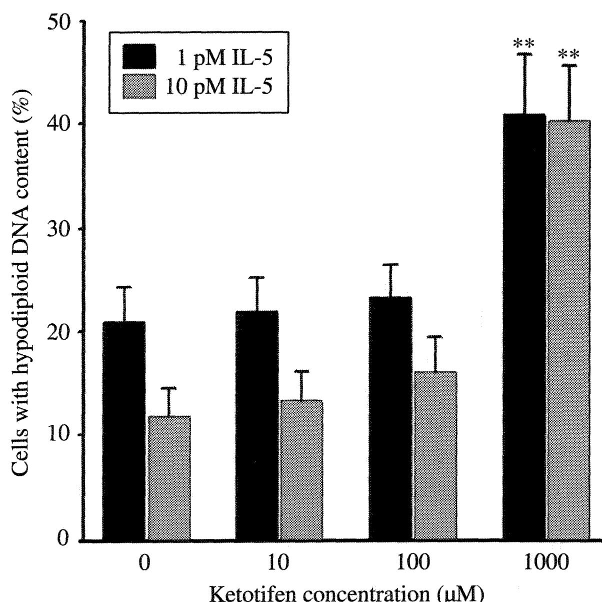

FIG. 1. Ketotifen increased the number of eosinophils with hypodiploid DNA amount in the presence of IL-5 (1 pM and 10 pM), as assessed by the relative DNA fragmentation assay suggesting an increase in apoptosis. However, the effect of 1 mM of ketotifen was mainly the result of the induction of primary necrosis and not apoptosis (see results and Table 1). **Indicates $P < 0.01$ as compared with the respective control. Results are expressed as the percentage of cells showing hypodiploid DNA content. Shown are the means ± standard error of the mean of $n = 4$ experiments with eosinophils from different donors.

RESULTS

Effect of ketotifen on IL-5-afforded eosinophil survival

In eosinophils cultured for 40 h without survival-prolonging cytokines, 69 ± 9% (n = 4) of the cells were apoptotic. IL-5 increased eosinophil survival significantly, and the percentages of apoptotic eosinophils (n = 4) were 21 ± 3% and 12 ± 3% when treated with 1 pM and 10 pM concentrations of IL-5, respectively (Fig. 1). Ketotifen (1 mM) reversed IL-5-induced eosinophil survival in the presence of both 1 pM and 10 pM IL-5 (Fig. 1), as measured by flow cytometric analysis of the relative DNA content of PI-stained eosinophils. However, the flow cytometry histograms of DNA in propidium iodide–stained cells were not typical of apoptosis.

To test the possibility of primary necrosis, eosinophils were incubated for 1 h, stained with PI in isotonic conditions and analyzed immediately with flow cytometer. In the presence of IL-5 (10 pM), ketotifen (1 mM) was found to increase the PI-uptake, suggesting an increase in the number of primary necrotic cells over twentyfold (n = 4, $P < 0.05$; Table 1). Based on these results, we concluded that the effect of ketotifen in increasing DNA breakdown and cell death in the presence of IL-5 in flow cytometric analysis may be, at least in part, the result of the induction of primary necrosis and not apoptosis.

After flow cytometric analyses, we wanted to confirm the form of cell death in ketotifen-treated eosinophils morphologically. When MGG-stained cells were analyzed under bright-field microscope, ketotifen (1 mM) was found to reverse IL-5-afforded eosinophil survival (n = 6; data not shown). The form of cell death was suggested to be primary necrosis, since numerous cells that

TABLE 1. INDUCTION OF PRIMARY NECROSIS IN EOSINOPHILS

	Primary necrotic eosinophils (%) taking up propidium iodide	
Ketotifen concentration	*No IL-5*	*+ 10 pM IL-5*
0	2 ± 0	2 ± 0
1 mM	47 ± 17*	49 ± 16*

The percentages of cells taking up propidium iodide after 1 h of incubation are shown, suggesting induction of primary necrosis. Propidium iodide–stained eosinophils were analyzed by flow cytometer. Each value represents the mean ± standard error of the mean of $n = 4$ with cells from different donors.

*Indicates $p < 0.05$, as compared to the respective control.

were swollen, that had a bursting nucleus and a broken plasma membrane, could be seen among few normal and apoptotic eosinophils (Fig. 2).

TEM analysis of eosinophil viability

After flow cytometric and light microscopical analyses, we wanted to further elucidate eosinophil viability and the form of eosinophil death after ketotifen treatment by transmission electron microscopy. Transmission electron microscopic analysis of eosinophils examined at baseline conditions or after incubation with 10 pM IL-5 for 1 and 40 h generally revealed a high viability, and only a few scattered cells displayed ultrastructural signs of necrosis or apoptosis (Fig. 3; A and B). In contrast, incubation of eosinophils for 1 and 40 h with 10 pM IL-5 and 1 mM ketotifen resulted in a marked necrotic cell death, characterized by chromatolysis, as well as bleb formation or rupture of the cell membrane (Fig. 3; C and D). In addition, regardless of cell viability, the specific granules of the ketotifen-treated eosinophils demonstrated a marked loss of their content (i.e., electron density). In many of the cells, large crystalline structures, indicative of so-called Charcot-Leiden crystals, were observed (Fig. 4). After 40 h of incubation with IL-5 and ketotifen, virtually all cells had signs of advanced necrosis (Fig. 5).

Effect of ketotifen on constitutive eosinophil apoptosis

The number of apoptotic eosinophils after 40 h of culture in IL-5-deprived medium amounted to 69 ± 9% (n = 4). Using hypodiploid DNA content as a marker, ketotifen at 100 μM and 1 mM decreased constitutive eosinophil apoptosis to 47 ± 9% (n = 4; $P < 0.05$) and 43 ± 6% (n = 4; $P < 0.01$, respectively. To determine if ketotifen induces primary necrosis under cytokine-deprived conditions, eosinophils were incubated for 1 h and uptake of propidium iodide was analyzed by flow cytometer. Ketotifen (1 mM) significantly (~twentyfold) increased the percentage of eosinophils that took up PI in the absence and presence of IL-5, which is indicative of primary necrosis (n = 4, $P < 0.05$; Table 1). In morphological analysis, normal and primary necrotic eosinophils could be seen (data not shown).

Effect of mepyramine on constitutive and IL-5-inhibited eosinophil apoptosis

To assess the role of the H1-receptor in ketotifen-induced eosinophil necrosis, we tested the effects of a pharmacologically well-characterized H1-receptor antagonist, mepyramine, on eosinophil survival in the absence and presence of 1 pM IL-5 at a mepyramine concentration (1 μM) that is known to antagonize the H1-receptors. In the presence of IL-5 (1 pM), mepyramine had no effect on eosinophil survival, and the percentages of apoptotic eosinophils were 20 ± 2% and 18 ± 3% (n = 4; $P > 0.05$) without and with mepyramine, respectively. In the absence of IL-5, the percentages of apoptotic eosinophils were 53 ± 6% and 50 ± 3% (n = 4) without and with mepyramine, respectively, as determined by flow cytometric analysis of the relative DNA content of PI-stained cells. In conclusion, the H1-antagonist, mepyramine, was not found to have any effects on eosinophil viability, as assessed by the

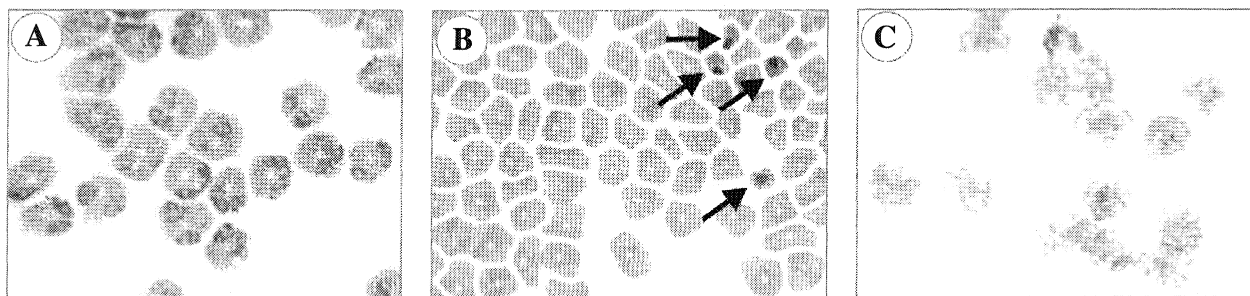

FIG. 2. The morphology of May-Grünwald–Giemsa–stained eosinophils with 10 pM IL-5 (**A**), 1 pM IL-5 (**B**), and 1 mM ketotifen + 10 pM IL-5 (**C**). In (**A**), shown are eosinophils displaying normal morphology. In (**B**), there are apoptotic eosinophils (arrows) showing chromatin condensation, a shift from bilobed to monolobed nucleus, and cell shrinkage in addition to normal eosinophils. In (**C**), cells, which are swollen, have a broken plasma membrane, and a bursting nucleus, suggesting primary necrosis, can be seen.

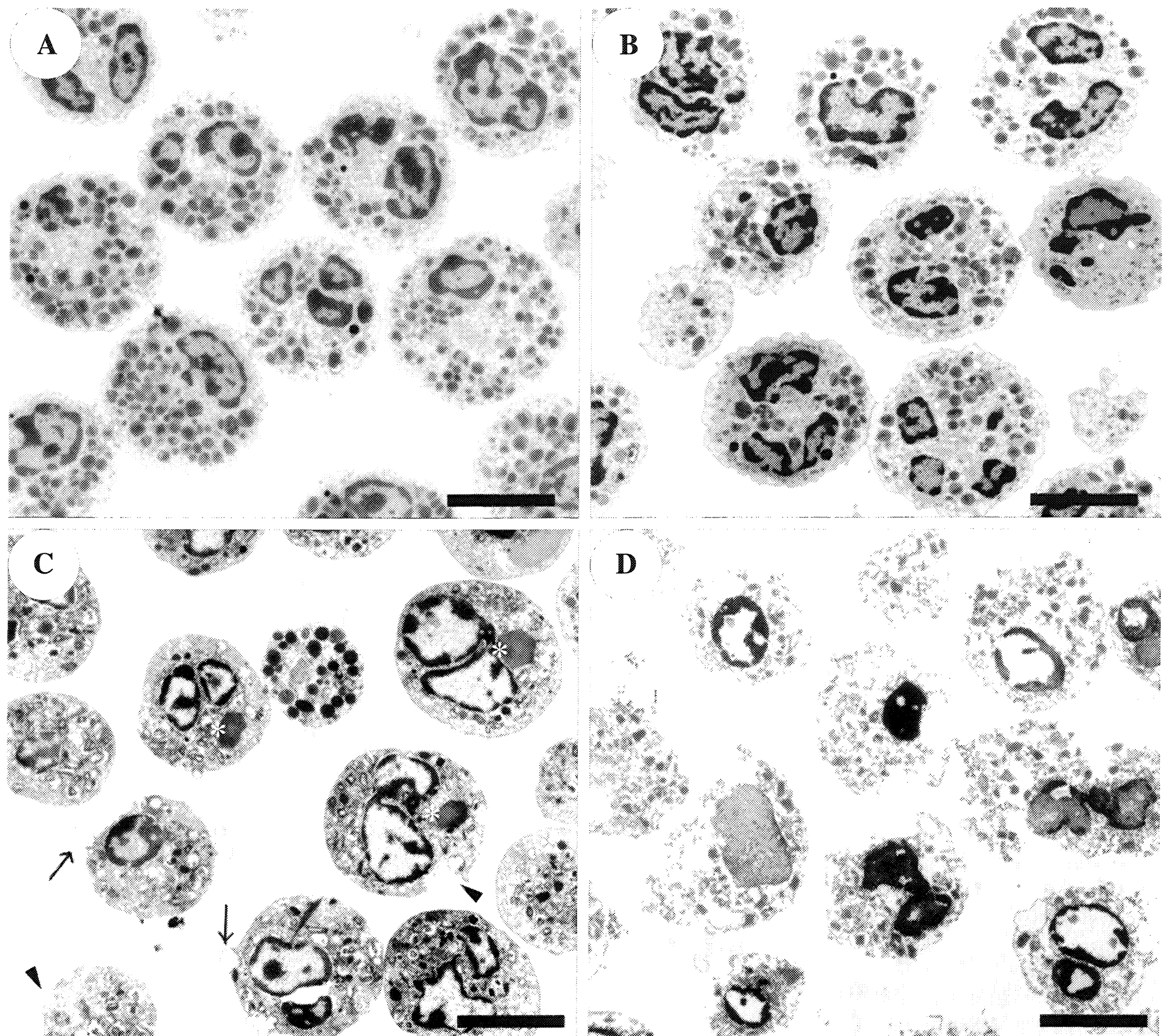

FIG. 3. Electron micrographs showing the representative ultrastructural morphology of eosinophils examined after incubation with 10 pM IL-5 (**A**) and (**B**) or 10 pM IL-5 + 1 mM ketotifen (**C**) and (**D**). As revealed by 1 h (**A**) and 40 h (**B**) of incubation, the IL-5-treated eosinophils displayed well-preserved membrane integrity and the characteristic chromatin pattern of viable eosinophils. After 1 h of incubation with 10 pM IL-5 + 1 mM ketotifen (**C**), many cells displayed chromatolysis (*) as well as bleb formation (arrows) or lost integrity (arrow heads) along the cell membrane. Virtually all cells were in the stage of advanced necrosis and dissolution after 40 h of incubation with 10 pM IL-5 and 1 mM ketotifen (**D**). Scale bars A–D = 10 μm.

relative DNA-fragmentation assay. This suggests that pronecrotic effects similar to ketotifen are not a common feature for the drug group of antihistamines and that ketotifen induces primary eosinophil necrosis through an H1-receptor-independent mechanism.

DISCUSSION

Histamine is a key player in allergic conditions, mainly through its actions mediated via the H1-receptor. H1-receptor antagonists or antihistamines are commonly used to treat allergic symptoms both systemically and as topical preparations, such as eye drops. Furthermore, it has convincingly been established that also the eosinophil has a crucial role in the pathogenesis of ocular allergy.[1–3] Silent and efficient elimination of the eosinophils by inducing apoptosis would theoretically be an attractive way to clear the conjunctival tissues of this important inflammatory cell. However, the effects of antihistamines on eosinophil apoptosis remain largely un-

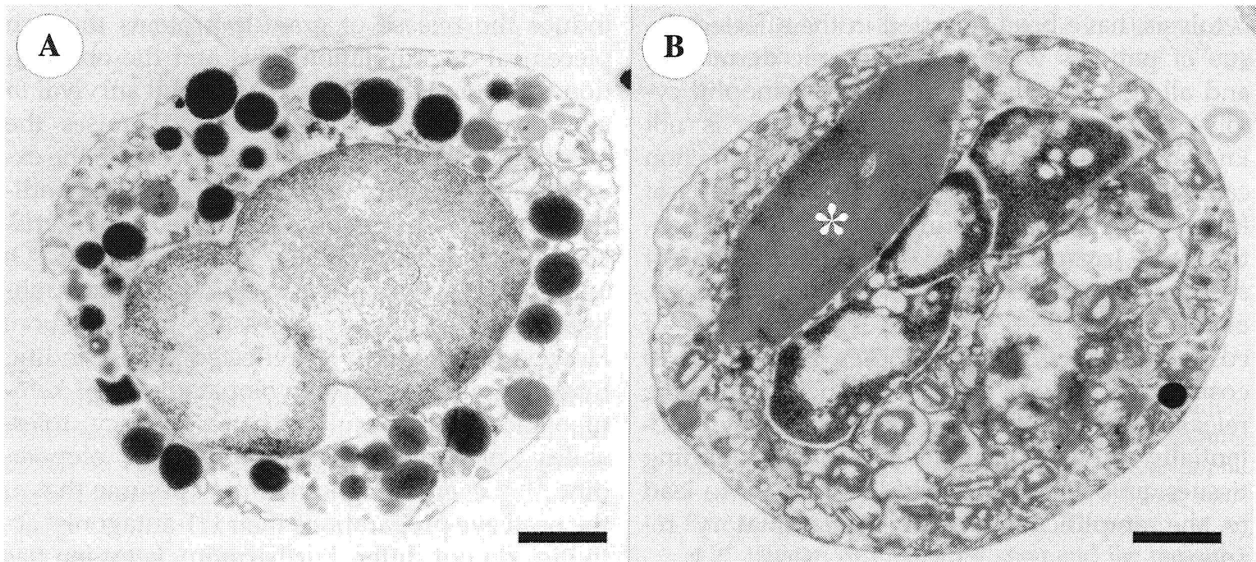

FIG. 4. Electron micrographs depicting distinct forms of necrosis at 1 h of incubation with 10 pM IL-5 and 1 mM ketotifen. In picture (**A**), an even dissolution of the chromatin is observed in a cell with well-preserved content of the granule material. In contrast, eosinophils with nuclear fragmentation (karyokexis*) have lost the granule material and typically contained large intracellular crystals (**B**). Scale bars A–B = 2 μm.

known. There are a few studies suggesting that some antihistamines increase eosinophil apoptosis[14] or decrease eosinophil survival.[20,21] In this study, we demonstrate that ketotifen reverses IL-5-induced survival of human eosinophils by inducing primary necrosis instead of apoptosis, as demonstrated by both bright-field microscopy and transmission electron microscopy. Furthermore, induction of primary necrosis by ketotifen seems to be non-H1-receptor-mediated, as the H1-receptor antagonist, mepyramine, does not affect eosinophil survival, as assessed by using the relative DNA-fragmentation assay. A direct consequence of the induction of primary eosinophil necrosis (or cytolysis) is that the eosinophils disintegrate and their granule contents are released into the surrounding tissue. Free eosinophil granules, indicative of eosinophil

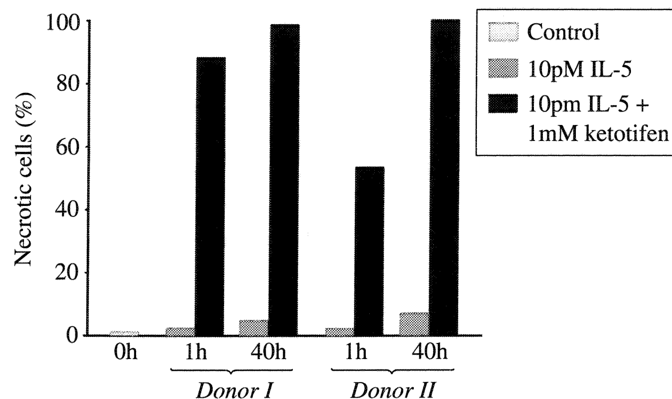

FIG. 5. Eosinophil viability as assessed by transmission electron microscopy (TEM). Freshly isolated eosinophils (0 h sample, no IL-5 or ketotifen, light grey column) and eosinophils cultured for 1 h or 40 h with 10 pM IL-5 (dark grey colums) show virtually no signs of primary necrosis as assessed by TEM. Eosinophils cultured for 1 h with 10 pM IL-5 and 1 mM ketotifen (black columns) show marked signs of necrosis. After 40 h of incubation with 10 pM IL-5 and 1 mM ketotifen, virtually all eosinophils are necrotic. Shown are the percentages of cells displaying clear signs of necrosis analyzed from cells of two different blood donors.

cytolysis, have been reported in the affected tissue of patients with asthma, atopic dermatitis, and allergic rhinitis.[22,23] Whether eosinophil cytolysis occurs in allergic conjunctivitis is not known, although occurrence of this phenomenon can be speculated based on scattered reports of free eosinophil granules in vernal keratoconjunctivitis.[24] Irrespective of the occurrence of eosinophil cytolysis under nontreated conditions, one might hypothesize that the induction of eosinophil cytolysis by ketotifen might lead to eosinophil cytolysis also *in vivo* and hence to the release of free eosinophil granules and their potentially cytotoxic contents into the surrounding tissues, an event that would be expected to lead to the amplification of the inflammatory response.

IL-5 increases human eosinophil survival by inhibiting apoptosis *in vitro*[4] and *in vivo*,[7] and is considered to be one of the main survival-prolonging cytokines for eosinophils *in vivo*. In this study, we used IL-5 to increase eosinophil survival and to inhibit apoptosis. Ketotifen induced the primary necrosis of eosinophils in the presence of IL-5. Whether IL-5 is the main survival-prolonging cytokine for eosinophils in allergic conjunctivitis is not known. However, the findings of this study seem to be of major clinical importance, as the mRNA levels and concentrations of IL-5 have been reported to be elevated in allergic and vernal conjunctivitis.[25–27]

The ketotifen concentration (1 mM) found to induce primary necrosis in isolated human eosinophils in this study is likely to be clinically relevant, as it is at the same level as the drug concentration in eye drops (0.81 mM) used topically to treat conjunctival disease. It has been reported that ketotifen ophthalmic solution is safe and well tolerated both in adult[28,29] and pediatric populations[30] in randomized, double-masked, and placebo-controlled studies, which may be the result of its inhibitory effects on eosinophil migration to the eyes.[31] Eosinophil cytolysis (ECL) and piecemeal degranulation (PMD, the occurrence of partly or completely empty granules in intact eosinophils) are the major cellular processes through which eosinophils release their cytotoxic granule proteins into human tissues in disease conditions.[12,22] The finding that the specific granules of ketotifen-treated eosinophils demonstrated a marked loss of their content (i.e., electron density) suggests that in addition to mere induction of primary necrosis, ketotifen may also induce the release of granule proteins through piecemeal degranulation. This and the observation that ketotifen reduces eosinophil survival *in vitro* by inducing primary necrosis raises the question whether ketotifen can exacerbate the existing inflammatory response in allergic conditions *in vivo* through the release of eosinophil tissue-toxic granule proteins, such as MBP, EPO, and ECP. We were not able to identify any published studies directly assessing this question. However, this might be reflected in the finding that comparisons between olopatadine and ketotifen most often result in better efficacy, tolerability, or patient preferability with olopatadine,[32–34] even though one might assume that at the used eye preparations their H1-antagonist activities do not differ. Furthermore, ketotifen has been found to stimulate histamine release at concentrations only slightly higher than those inducing maximal inhibition of histamine release.[35]

In our study, we have shown that ketotifen induces an increase in the number of cells showing hypodiploid DNA content when analyzed by relative DNA fragmentation assay. This assay is rapid, simple, and reproducible[18] and has been successfully used to analyse apoptosis in a number of cell types, including eosinophils.[15] Many commercial apoptosis assays are based on the same principle. Our results, however, introduce a case where this assay produces unreliable results, unless the hypodiploid peak in the DNA graph is carefully analyzed. These findings further stress the recommendation that the occurrence of apoptosis should be analyzed by using more than one method.[15] In this study, the results were further confirmed by measuring cell-membrane permeability by propidium iodide uptake assay, and by using both bright-field and transmission electron microscopy. Ultrastructural analysis of eosinophils by TEM is a validated method for both analyzing the morphological changes and determining the granulation status of eosinophils.[12,22]

Recently, eosinophil cytolysis has been identified as a pathogenic event in allergic inflammation, and cytolytic eosinophils, as well as their free granule contents, have been identified in the airways of patients with asthma.[12] However, there is a lack of *in vitro* models of eosinophil cytolysis, which has made studies on the signalling related to this potentially important phenomenon difficult.[12] We introduce ketotifen-induced cytolysis as a novel *in vitro* method that could be used

to study the signalling related to eosinophil cytolysis.

CONCLUSIONS

Taken together, we have, in this study, shown that at clinically relevant drug concentrations ketotifen reverses cytokine-afforded eosinophil survival by inducing primary necrosis rather than apoptosis. This may reduce the anti-inflammatory efficacy of ketotifen. Hence, further studies exploring the effect of ketotifen or other related drugs on eosinophil death modes *in vivo* are now highly warranted.

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First-generation Antihistamines Diphenhydramine and Chlorpheniramine Reverse Cytokine-afforded Eosinophil Survival by Enhancing Apoptosis

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Abstract

Background: Antihistamines or histamine H_1-receptor antagonists are commonly used to treat a variety of allergic symptoms. Eosinophils are considered to play an essential role in the pathogenesis of allergy. Reduced eosinophil apoptosis is thought to be an important element in the formation of eosinophilia in allergic conditions such as allergic rhinitis, atopic eczema and asthma. The aim of our study was to investigate the effects of two first-generation antihistamines diphenhydramine and chlorpheniramine on constitutive eosinophil apoptosis and on interleukin (IL)-5 –afforded eosinophil survival. The role of c-Jun N-terminal kinase (JNK) in mediating the effects of antihistamines on eosinophil apoptosis was also evaluated.

Methods: Apoptosis of isolated human eosinophils was assessed by measuring the relative DNA content of propidium iodide –stained cells and confirmed by morphological analysis. The activity of JNK was measured by Western blotting.

Results: Antihistamines were found to reverse the survival-prolonging effect of IL-5 in eosinophils by enhancing apoptosis. JNK was found to be activated slowly during diphenhydramine-induced eosinophil apoptosis. An inhibitor peptide specific for JNK, L-JNKI1, inhibited diphenhydramine-mediated eosinophil apoptosis.

Conclusions: Our results suggest that first-generation antihistamines diphenhydramine and chlorpheniramine reverse IL-5-afforded eosinophil survival, and that the enhanced apoptosis by antihistamines is mediated through activation of JNK. Reversal of IL-5-afforded eosinophil survival may thus contribute to the anti-allergic actions of diphenhydramine and chlorpheniramine.

Introduction

Eosinophils are thought to play an essential role in the pathogenesis of allergic diseases, such as asthma, allergic rhinitis and atopic dermatitis.[1-3] We and others have shown that eosinophil apoptosis is delayed in patients with asthma, inhalant allergy or atopic dermatitis.[4,5] Accumulation of eosinophils in the nasal mucosa is also a characteristic of allergic rhinitis.[3] The number of eosinophils found in the blood and tissues is largely determined by the balance between cell maturation and death.[6] In relation to the clearance of eosinophils from inflamed sites, apoptosis or programmed cell death has been suggested to be an important mechanism in the resolution of eosinophilic inflammation.[7,8] Apoptosis is a highly controlled process characterized by specific morphological and biochemical changes, such as cell shrinkage, a shift from bilobed to monolobed nucleus, chromatin condensation and endonuclease-catalysed DNA breakdown followed by fragmentation of the cell into apoptotic bodies, which are phagocytozed intact without the induction of inflammation.[7,9,10] Eosinophils cultured *in vitro* under cytokine-deprived conditions undergo apoptosis which can be inhibited by various cytokines such as IL-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF).[2] In allergic rhinitis, IL-5 has also been suggested to be critically involved in the bone marrow stimulation and the tissue recruitment of nasal eosinophils.[3]

Histamine released from mast cells in response to allergen stimulation is an essential mediator in allergy. Histamine exerts its effects through G-protein-coupled cell surface receptors of which four distinct subtypes (H_1, H_2, H_3, H_4) have been unequivocally defined.[11] Pharmacological studies have identified histamine H_1 receptors on human eosinophils.[1] Classical antihistamines (aka histamine H_1-receptor antagonists) are a common medication and are effective in the treatment of allergic diseases. They are also often claimed to have anti-inflammatory properties. Some H_1-receptor antagonists have been reported to affect the function of inflammatory cells, e.g. the chemotaxis and adhesion of eosinophils.[12] Cetirizine is the most extensively investigated currently available oral antihistamine, and together with its active enantiomer, levocetirizine, have been shown to have anti-inflammatory activities at therapeutically-relevant concentrations both *in vitro* and *in vivo*.[13] However, there are few published studies on the effects of antihistamines on eosinophil viability. Only oxatomide[14] and fexofenadine[15] have been reported to promote eosinophil apoptosis. In addition, we have recently shown that at clinically-relevant drug concentrations, ketotifen reverses IL-5-mediated human eosinophil survival by inducing primary necrosis instead of apoptosis.[16] The effects of other antihistamines on human eosinophil apoptosis remain unknown and further studies are warranted.

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that are activated by threonine and tyrosine phosphorylation. Three major MAPK subfamilies have been identified in mammals, the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and the p38 MAP kinase (p38 MAPK).[17] Both p38 MAPK and JNK have been proposed to participate in the signaling pathway of apoptosis.[17] We have previously shown that p38 MAPK activity is involved in the inhibition of apoptosis [18] and that JNK mediates nitric oxide (NO)-induced DNA breakdown in human eosinophils [19]. JNK has also been proposed to be involved in dexamethasone-induced eosinophil apoptosis.[20]

The objective of the present study was to examine whether first-generation H_1-receptor antagonists, diphenhydramine and chlorpheniramine, influence IL-5-afforded survival or constitutive apoptosis of human eosinophils and to study the possible role of JNK in any effects seen.

Materials and Methods

Eosinophil Isolation and Culture

One hundred millilitres of peripheral venous blood was obtained from volunteers with normal or slightly higher eosinophil counts. The volunteers were healthy or atopic. No subjects with hypereosinophilic syndrome were included. Before donating blood, the subjects gave written informed consent to the study protocol approved by the Ethics committee of Tampere University Hospital (Tampere, Finland). Eosinophils were isolated under sterile conditions to >99% purity as previously described.[4,18,19,21] The cells were cultured for 40 h (+37ºC, 5% CO_2) in the absence and presence of diphenhydramine (0.01-1 mM) or chlorpheniramine (1-100 µM) and IL-5 (1 pM or 10 pM) in RPMI 1640 medium (Dutch modification) with 10% fetal calf serum and antibiotics unless otherwise stated.

Determination of Apoptosis by Measuring the Relative DNA Content by Flow Cytometry

The percentage of apoptotic cells was assessed by measuring the relative DNA content by flow cytometry (FACScan, Becton Dickinson, San Hose, CA) of propidium iodide (PI) – stained cells.[4,18,19,21] Endonuclease-catalysed DNA fragmentation is considered to be a specific feature of apoptosis.[22] The cells showing decreased relative DNA content were considered to be apoptotic. Eosinophils were suspended in 300 µl of hypotonic propidium iodide solution (25 µg/ml in 0.1% sodium citrate and 0.1% Triton X-100), protected from light and incubated at 4ºC for 1 h before flow cytometric analysis.

Morphological Analysis

Eosinophil morphology was assessed by bright field microscopy. For morphological analysis, eosinophils cultured for 40 h were spun onto cytospin slides (500 rpm, 5 min) and stained with May-Grünwald-Giemsa (MGG) after fixation in methanol. Cells showing typical apoptotic morphology (cell shrinkage, nuclear coalescence and chromatin condensation) were considered to have undergone apoptosis.[21]

Western Blotting

Eosinophils were suspended at 10^6 cells/ml and cultured at 37ºC. At indicated time points, samples were centrifuged at 12000g for 15 s. The cell pellet was lysed by boiling in 30 µl of Laemmli sample buffer for 5 min. The sample was centrifuged at 12000g for 10 min after which the debris was carefully removed. The samples were stored at -20ºC until the Western blot analysis. The protein sample (30 µl) was loaded onto 10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 2 h at 100V. The separated proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) with semidry blotter at 2.5 mA/cm^2 for 60 min. After transfer, the membranes were blocked by 5% bovine serum albumin (BSA) in TBST (20 mM Tris base pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature and incubated with the specific primary antibody overnight at 4ºC in the blocking solution. Thereafter the membrane was washed 4x with TBST for 5 min, incubated for 30 min at room temperature with the secondary antibody in the blocking solution and washed 4x with TBST for 5 min. Bound antibody was detected by using SuperSignal West Dura chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging

system (Alpha Innotech Corporation, San Leandro, CA, USA). The chemiluminescent signal was quantified by using the FluorChem software version 3.1.

Materials

L-JNKI1 (c-Jun N-terminal kinase peptide inhibitor 1, L-stereoisomer) and L-TAT control peptide were purchased from Alexis Corp. (Läufelfingen, Switzerland). JNK1 antibodies and goat anti-rabbit polyclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and phospho-JNK (Thr183/Tyr185, Thr221/Tyr223) antibody was from Upstate (Lake Placid, NY, USA). Other reagents were obtained as follows: human recombinant IL-5 (R&D system Europe, Abingdon, UK), propidium iodide (Tocris, Bristol, UK), anti-CD16 microbeads and the magnetic cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany), Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), antibiotics and RPMI 1640 (Dutch modification) (Gibco BRL, Paisley, UK), fetal calf serum, Hank's balanced salt solution (HBSS) and RPMI 1640 (BioWhittaker, Verviers, Belgium), May-Grünwald (Merck, Darmstadt, Germany) and Giemsa (J.T.Baker, Deventer, Holland). Diphenhydramine hydrochloride, chlorpheniramine maleate and all other reagents were purchased from Sigma Chemical Company (Poole, Dorset, UK) unless otherwise stated. Diphenhydramine hydrochloride and chlorpheniramine maleate were dissolved in RPMI 1640 medium (Dutch modification). The peptides L-JNKI1 and L-TAT were dissolved in HBSS.

Statistics

The results are expressed as the mean ± SEM. Apoptosis is expressed as an apoptotic index (number of apoptotic cells/total number of cells, ie. apoptotic index 0.1 means 10% of the cells are apoptotic). Statistical significance was calculated by analysis of variance for repeated measures supported by the Dunnett test or by paired t-tests. Differences were regarded significant when $p<0.05$.

Results

Effect of Diphenhydramine and Chlorpheniramine on IL-5-afforded Eosinophil Survival

Eosinophils cultured for 40 h without survival-prolonging cytokines underwent apoptosis (apoptotic index = 0.59 ± 0.06, n=6) as assessed by measuring fragmented DNA of PI-stained cells. In contrast, IL-5 promoted eosinophil survival in a concentration-dependent manner. Thus, at 1 pM and 10 pM IL-5, the apoptotic index was 0.14 ± 0.03 (n=6, $p<0.01$) and 0.10 ± 0.03 (n=6, $p<0.01$) respectively. In addition, diphenhydramine (1 mM) reversed the eosinophil survival enhancing activity of both concentrations of IL-5 studied by inducing apoptosis (n=6, $p<0.01$) (Fig. 1A) as measured by the relative DNA fragmentation assay in PI-staining. When eosinophil morphology was used as an indicator of apoptosis, the effects of 1 mM diphenhydramine were similar as in flow cytometric analyses (n=6, $p<0.01$) (Table 1A). Another H_1-receptor antagonist, chlorpheniramine (100 µM) also increased apoptosis (n=6, $p<0.05$) when eosinophils were cultured with 1 pM IL-5 as assessed by measuring fragmented DNA of PI-stained cells. However, this effect of chlorpheniramine was suppressed when the concentration of IL-5 was increased to 10 pM (Fig.1B). In contrast to the data obtained by measuring DNA fragmentation, chlorpheniramine did not increase the number of cells showing the typical morphological features of apoptosis in the presence of IL-5 (Table 1B).

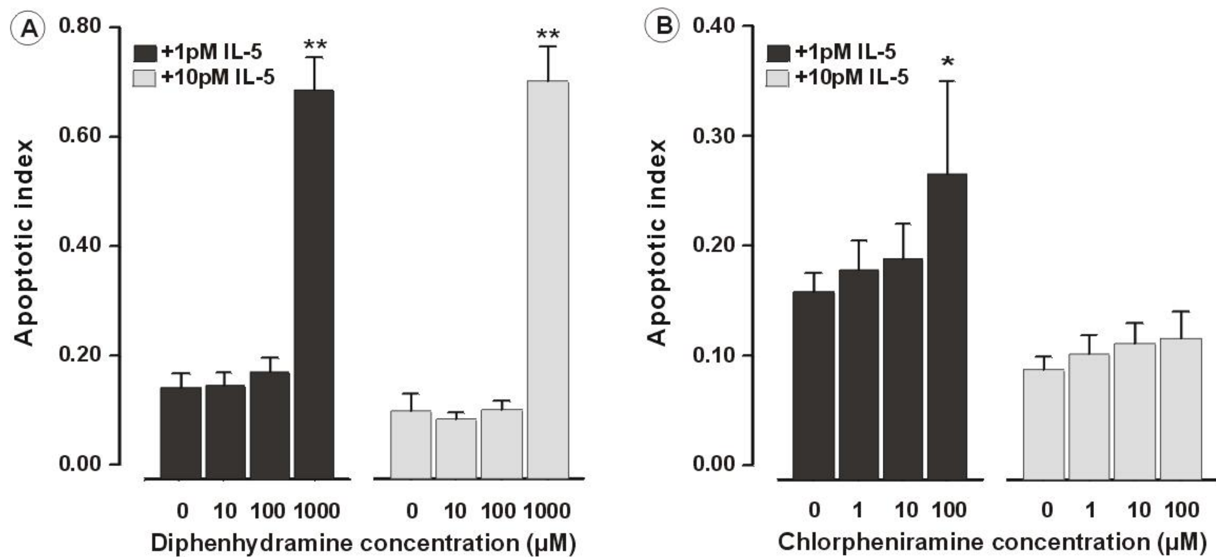

Figure 1. Antihistamines reverse IL-5-afforded eosinophil survival. The effects of A) diphenhydramine (10-1000 µM) and B) chlorpheniramine (1-100 µM) on eosinophil apoptosis in cells cultured for 40 h with 1 pM and 10 pM IL-5. Apoptosis was assessed by the relative DNA fragmentation assay of propidium iodide –stained cells. * indicates $p<0.05$ and ** indicates $p<0.01$ as compared with the respective control. Each data point represents the mean ± SEM of n=6 independent measurements using eosinophils from different donors.

Table 1. The effects of antihistamines on eosinophil apoptosis in the absence and presence of IL-5.

		Apoptotic indices		
		No IL-5	1 pM IL-5	10 pM IL-5
A	No diphenhydramine	0.41 ± 0.06	0.08 ± 0.02	0.03 ± 0.00
	1 mM diphenhydramine	0.83 ± 0.04 **	0.84 ± 0.04 **	0.85 ± 0.04 **
B	No chlorpheniramine	0.41 ± 0.06	0.08 ± 0.02	0.03 ± 0.00
	100 µM chlorpheniramine	0.31 ± 0.05	0.07 ± 0.01	0.05 ± 0.01

*The apoptotic indices of morphologically analyzed eosinophils after 40 h incubation. Values represent the mean ± SEM of six experiments with cells from different donors. ** indicates p<0.01 as compared with the respective control.*

JNK Activation in Diphenhydramine-Induced Eosinophil Apoptosis

The activity of JNK was assessed by calculating the phospho-JNK/total JNK ratio measured by Western blotting by using antibodies that recognize the 46 and 55 kDa molecular weights of JNK. In freshly isolated human eosinophils with IL-5 (10 pM), JNK was spontaneously active (Fig. 2). After incubation for the indicated time with diphenhydramine (1 mM) and IL-5 (10 pM), a slow activation of the 55 kDa JNK isoform could be observed in eosinophils (Fig. 2). This suggests that the c-Jun N-terminal kinase is slowly activated during antihistamine-induced reversal of cytokine-afforded human eosinophil survival and that the increased JNK activity may mediate the enhanced apoptosis by antihistamines.

Effect of JNK Inhibition on Eosinophil Apoptosis

When eosinophils were cultured for 40 h with 10 pM IL-5, the apoptotic index was 0.10 ± 0.01 (n=6) as analyzed by flow cytometry of propidium iodide-stained cells. Diphenhydramine (1 mM) increased the apoptotic index to 0.45 ± 0.02 (n=6, p<0.001). In the presence of L-TAT, the control for the JNK inhibitor peptide L-JNKI1, the apoptotic indices were 0.39 ± 0.03 and 0.08 ± 0.01 with and without 1 mM diphenhydramine, respectively (n=6, p<0.05). The JNK inhibitor L-JNKI1 markedly inhibited eosinophil apoptosis in the absence (n=6, p<0.05) and presence of diphenhydramine (1 mM, n=6, p<0.01) (Fig. 3). The apoptotic indices were 0.06 ± 0.01 and 0.20 ± 0.04, respectively. When the morphological features of apoptosis were analyzed, L-JNKI1 decreased the number of cells showing the typical apoptotic features in the presence of diphenhydramine (1 mM) (Table 2). Our results suggest that since L-JNKI1 inhibits apoptosis, antihistamine-induced reversal of IL-5-afforded eosinophil survival and enhancement of eosinophil apoptosis are at least partly mediated through the JNK pathway.

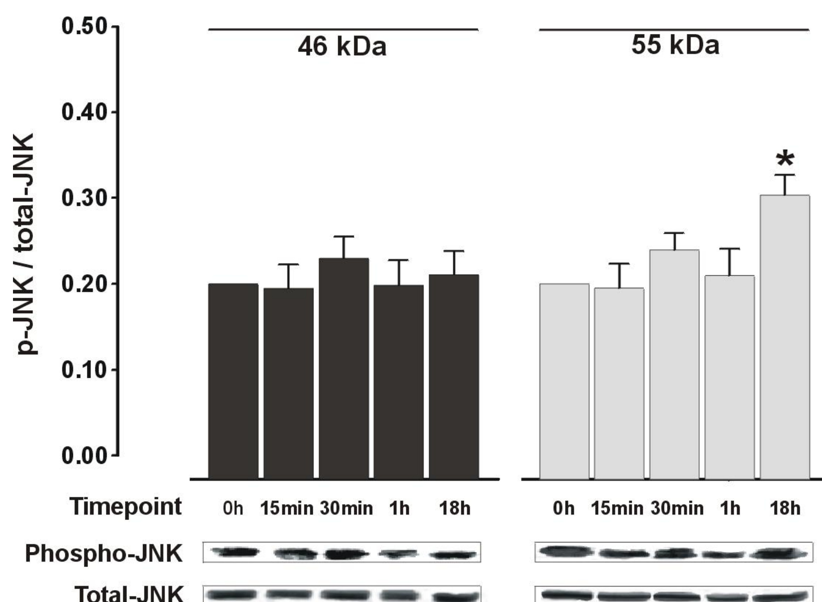

Figure 2. The activation of JNK (46 and 55 kDa) during diphenhydramine-induced eosinophil apoptosis in the presence of IL-5 (10 pM). The bars represent the mean ± SEM of the phosphorylated JNK/total JNK –ratio of n=4-6 independent Western blot analyses using eosinophils from different donors. The p-JNK/total JNK –ratio at time point 0 h is set as 0.20. * indicates $p<0.05$ as compared with the 0 h time point. The immunoblots are representative of n=4-6 essentially identical experiments.

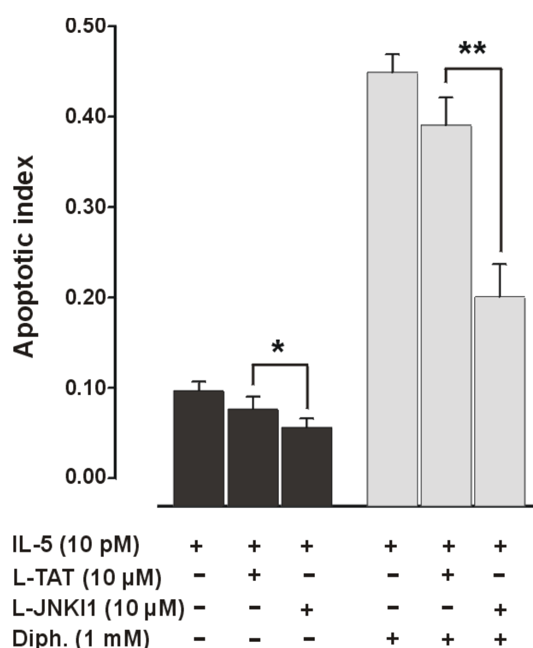

Figure 3. L-JNKI1 inhibits eosinophil apoptosis. The effects of L-JNKI1 on eosinophil apoptosis in the absence and presence of diphenhydramine (1 mM) and in the presence of IL-5 (10 pM). Apoptosis was assessed by the relative DNA fragmentation assay of propidium iodide –stained cells. L-TAT is the control peptide for L-JNKI1. HBSS was used as the negative control without peptides. * indicates $p<0.05$ and ** indicates $p<0.01$ as compared with the respective control. The values represent the mean ± SEM of six experiments with eosinophils from different donors.

Table 2. The effects of L-JNKI1 on eosinophil apoptosis.

	Apoptotic indices		
	HBSS	L-TAT	L-JNKI1
No diphenhydramine	0.10 ± 0.01	0.10 ± 0.01	0.05 ± 0.01***
1 mM diphenhydramine	0.75 ± 0.03	0.74 ± 0.04	0.56 ± 0.01**

*The apoptotic indices of eosinophils after 40 h incubation in the presence of IL-5 (10 pM) as analyzed by measuring the morphological features of apoptosis by bright field microscopy. L-TAT is the control peptide for L-JNKI1. HBSS was used as the negative control without peptides to exclude the effect of L-TAT. Each value represents the mean ± SEM of six independent experiments with eosinophils from different donors. ** indicates $p<0.01$ and *** indicates $p<0.001$ as compared with the respective control in the presence of L-TAT.*

Effect of Diphenhydramine and Chlorpheniramine on Constitutive Eosinophil Apoptosis

Eosinophils cultured for 40 h in the absence of IL-5 underwent spontaneous apoptosis with an apoptotic index of 0.59 ± 0.06 (n=6). Diphenhydramine (1 mM) tended to increase spontaneous eosinophil apoptosis but the increase was not quite statistically significant (n=6, $p>0.05$) (Table 3A). When the morphological features of apoptosis were analyzed, the enhancement of apoptosis by diphenhydramine (1 mM) was significant (n=6, $p<0.01$) (Table 1A). Chlorpheniramine (100 µM) decreased constitutive apoptosis (n=6, $p<0.01$) (Table 3B). When morphological analysis of apoptosis was performed, a similar tendency was found (n=6, $p>0.05$) (Table 1B).

Table 3. The effects of antihistamines on constitutive eosinophil apoptosis.

	Apoptotic indices				
Concentration	0	1 µM	10 µM	100 µM	1000 µM
A Diphenhydramine	0.59 ± 0.06		0.63 ± 0.05	0.56 ± 0.06	0.69 ± 0.06
B Chlorpheniramine	0.65 ± 0.06	0.63 ± 0.06	0.63 ± 0.07	0.54 ± 0.07**	

*The apoptotic indices of eosinophils after 40 h incubation. Apoptosis was assessed by the relative DNA fragmentation assay. Values represent the mean ± SEM of six experiments with cells from different donors. ** indicates $p<0.01$ as compared with the respective control.*

Discussion

In the present study, we have shown that first-generation antihistamines diphenhydramine and chlorpheniramine reverse IL-5-afforded human eosinophil survival by enhancing apoptosis *in vitro*. This is likely to be of clinical importance due to the critical pathogenetic role of eosinophils in allergic conditions and the common use of antihistamines in their treatment. Asthma is a chronic eosinophilic disease and in asthmatic patients, eosinophil apoptosis has been shown to be delayed.[2,4,5] Allergic rhinitis is characterized by recruitment and accumulation of nasal mucosal and luminal eosinophils.[3] IL-5 is a key cytokine involved in the recruitment of tissue eosinophils, acting on both bone marrow stimulation and tissue recruitment of circulating progenitor cells together with different chemokines and inflammatory mediators.[3] In a recent study evaluating the relationships among upper and lower airway function and nasal inflammation in subjects with asthma and seasonal allergic rhinitis by Ciprandi et al., nasal eosinophils were found to be correlated with nasal symptoms and airflow as well as with forced expiratory volume in 1 s (FEV_1).[23] At present, the united airways disease concept is well acknowledged and supports the link between asthma and rhinitis.[24-26] Therefore, it might be hypothesized that the reversal IL-5-induced survival of human eosinophils through antihistamine-enhanced apoptosis observed in this study would have clinical implications in the treatment of asthma and other allergic disorders.

A plethora of evidence from multiple *in vitro* and *in vivo* studies demonstrates that antihistamines possess anti-inflammatory properties unrelated to their ability to antagonize the effect of histamine at H_1-receptors. For example, cetirizine, levocetirizine and desloratadine have been shown to inhibit the expression or release of pro-inflammatory mediators or to decrease inflammatory cell chemotaxis or adherence to endothelial cells.[13,27] *In vivo*, cetirizine has been demonstrated to inhibit allergen-induced eosinophil recruitment in the airways and to attenuate the synthesis or release of pro-inflammatory mediators.[13] Desloratadine was recently observed to inhibit eosinophil and mast cell activation in nasal polyps.[28] Desloratadine has also been suggested to decrease eosinophil influx into the nasal mucosa during allergy season.[27] In addition, cetirizine has been shown to prevent sensitization to new allergens and to delay or even to prevent the development of asthma in certain groups of allergic children.[13]

With respect to the amount of eosinophils, levocetirizine has been observed to decrease the numbers of nasal eosinophils and neutrophils and the levels of IL-4 and IL-8 in addition to symptom relief and improved nasal airflow in patients with seasonal allergic rhinitis.[29] Desloratadine has recently been shown to decrease blood eosinophils and to prevent the increase in circulating eosinophil numbers after nasal allergen challenge although the numbers of eosinophils in the nasal or bronchial mucosa were not altered by desloratadine.[30] However, only limited data exists on the specific effects of antihistamines on eosinophil apoptosis. An anti-allergic compound oxatomide has been observed to suppress IL-5-induced survival and to promote apoptosis of human eosinophils.[14] Fexofenadine has also been shown to induce eosinophil apoptosis in the presence of IL-5 at a similar concentration range as used in this study.[15] In addition, it has been shown that cetirizine[31] and desloratadine[32] inhibit eosinophil survival although apoptosis was not measured. Recently, we have demonstrated that ketotifen reverses IL-5-mediated human eosinophil survival through enhancement of primary eosinophil necrosis and not apoptosis at a similar concentration range as used in the present study.[16] It therefore seems that not all antihistamines possess similar anti-inflammatory effects and that not all antihistamines necessarily affect human eosinophil apoptosis. The enhancement of eosinophil apoptosis by certain H_1-antagonists may provide

additional benefit in the treatment of allergic diseases and support the use of these more anti-inflammatory antihistamines. This study was limited only to first-generation antihistamines diphenhydramine and chlorpheniramine since no second or third generation antihistamines were available for us during the course of the study. Thus, studies investigating the effects of other antihistamines on human eosinophil apoptosis are highly warranted.

Frequently, it has been suggested that the additional anti-inflammatory effects of antihistamines may not be mediated through H_1-antagonism.[12,13,27] Recently, while demonstrating the pro-necrotic role of ketotifen in human eosinophils we also suggested that the reversal of IL-5-induced eosinophil survival by ketotifen was not mediated through the H_1-receptor since mepyramine, a highly selective H_1-receptor antagonist, did not exert any effects on eosinophil viability.[16] The observation that not all H_1-receptor antagonists affect eosinophil survival suggests that the pro-apoptotic effects of diphenhydramine and chlorpheniramine demonstrated in this study are not a common feature for the drug-group of antihistamines and may not be mediated through the H_1-receptor. This hypothesis is also supported by the finding that the concentrations needed to achieve the pro-apoptotic effects were found to be higher than those needed for H_1-receptor antagonism. The concentrations found to induce apoptosis in IL-5-treated eosinophils in the present study are similar to those used in the other studies with fexofenadine[15] and ketotifen[16] and could be achieved in topical preparations such as nasal sprays or eye drops. Therefore, it could be speculated that in allergic rhinitis, topically administered antihistamines would relieve the accumulation of nasal mucosa eosinophils through induction of apoptosis.

After suggesting that the H_1-receptor may not be responsible for the induction of apoptosis by antihistamines, we further studied the role of JNK to elucidate the mechanism behind the pro-apoptotic effects. JNK was found to be constitutively active in eosinophils in the presence of IL-5. In addition, a slow activation of JNK was observed in the presence of diphenhydramine. However, only the 55 kDa JNK isoform but not the 46 kDa JNK was activated after incubation of 18 h with diphenhydramine and IL-5. This has not previously been described in eosinophils. Similar phenomenon has, however, been found in primary glial tumours in which the activation of only the 55 kDa JNK isoform was demonstrated to be the result of autophosphorylation of isoforms of the JNK2 gene family.[33,34] However, it remains unresolved whether the activation of 55 kDa JNK in eosinophils stimulated with diphenhydramine and IL-5 observed in this study is due to similar autophosphorylation activity. In addition, in the present study we found that inhibition of JNK by a peptide inhibitor L-JNKI1 partly reversed the pro-apoptotic effects of diphenhydramine suggesting that the c-Jun N-terminal kinase is involved in antihistamine-induced human eosinophil apoptosis. JNK has previously been proposed to be activated in NO- and dexamethasone-induced eosinophil apoptosis.[19,20] These results support the significance of JNK in mediating the apoptotic response in eosinophils. However, it can be speculated that JNK activation may not be a specific mechanism selective to NO, glucocorticoids or antihistamines but may rather be a more generalized feature in eosinophil apoptosis.

In conclusion, in the present study we have shown that antihistamines diphenhydramine and chlorpheniramine reverse IL-5-afforded human eosinophil survival through induction of apoptosis. The enhanced apoptosis is at least in part mediated through increased activity of the c-Jun N-terminal kinase.

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c-Jun N-terminal kinase mediates constitutive human eosinophil apoptosis

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Abstract

Eosinophils are considered to play an important role in the pathogenesis of asthma. Glucocorticoids are potent anti-inflammatory agents for the treatment of chronic inflammatory diseases and they have been shown to increase the rate of eosinophil apoptosis. c-Jun N-terminal kinase (JNK) has been suggested to participate in the signaling pathways of apoptosis. The aims of the present study were to examine whether JNK is involved in the regulation of constitutive eosinophil apoptosis and whether it mediates dexamethasone-induced apoptosis of human eosinophils.

Isolated human eosinophils were cultured with and without dexamethasone and the JNK inhibitor L-JNKI-1. Apoptosis was assessed by measuring the relative DNA content of propidium iodide-stained cells and confirmed by Annexin V-binding and morphological analysis with bright field microscopy. The phosphorylation of both JNK and c-Jun were measured by Western blotting.

During a 40 h culture, dexamethasone (1 μM) enhanced human eosinophil apoptosis by 10–30%. Culture with L-JNKI1 (10 μM) inhibited apoptosis in dexamethasone-treated cells by 53%. Furthermore, L-JNKI1 decreased the rate of constitutive eosinophil apoptosis by 64%. However, the enhancement of eosinophil apoptosis by dexamethasone was not reversed by L-JNKI1. Slow activation of JNK in constitutive apoptosis as well as a similar tendency in dexamethasone-induced eosinophil apoptosis could be observed by Western blot analyses. c-Jun was found to be active both in the presence and absence of dexamethasone. However, no further phosphorylation of the serine residue 63 of c-Jun could be seen.

Taken together, our present results suggest that JNK is active during apoptosis of human eosinophils both in the presence and absence of glucocorticoids. JNK seems to mediate constitutive human eosinophil apoptosis. However, the activity of JNK is not enhanced by glucocorticoids and the effects of glucocorticoids cannot be reversed by JNK inhibition. JNK therefore seems not to mediate glucocorticoid-induced human eosinophil apoptosis.
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Keywords: Eosinophils; Apoptosis; JNK; c-Jun; Glucocorticoids

1. Introduction

Eosinophils have been implicated in the pathogenesis of allergic inflammatory diseases such as bronchial asthma and allergic rhinitis [1,2]. Activation and degranulation of eosinophils in the airways is considered to cause epithelial tissue injury and airway remodelling [3–5]. Eosinophil apoptosis or programmed cell death has been suggested as a major mechanism in the resolution of eosinophilic inflammation for example in asthma [6–8]. Moreover, it

Abbreviations: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PI, propidium iodide; IL; interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF-α, tumour necrosis factor α; Ser, serine; L-JNKI1, c-Jun N-terminal kinase inhibitor, L-stereoisomer

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has been demonstrated that eosinophil apoptosis is delayed in asthmatic patients [9]. Apoptosis is characterized by specific biochemical and morphological changes such as cell shrinkage, nuclear coalescence, chromatin condensation and endonuclease-catalyzed DNA breakdown followed by fragmentation of the cell into discrete apoptotic bodies, which are phagocytozed intact without releasing their granule contents to the surrounding tissues, which might induce inflammation [7,10,11]. In the absence of cytokines, in vitro cultured human eosinophils undergo apoptosis that can be inhibited by different cytokines, principally interleukin (IL-) 3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) [1,8,11,12].

Glucocorticoids are commonly used anti-inflammatory agents for the treatment of asthma and several other chronic inflammatory diseases [13]. Glucocorticoids decrease the transcription of many inflammatory cytokines, such as IL-5, tumour necrosis factor α (TNF-α) and GM-CSF, and reduce the numbers and activation of airway inflammatory cells [14]. The target cells include eosinophils, neutrophils, T-lymphocytes, macrophages, mast cells, dendritic cells, epithelial cells and endothelial cells [14,15]. Inhaled glucocorticoids have been reported to reduce the numbers of tissue and blood eosinophils [15]. Consistently, clinically relevant concentrations of glucocorticoids have been shown to enhance eosinophil apoptosis in vitro [16,17]. The induction of eosinophil apoptosis may therefore constitute one of the anti-inflammatory actions of glucocorticoids in allergy and asthma. The mechanisms by which glucocorticoids promote eosinophil apoptosis are currently, however, poorly understood.

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that are characteristically activated by threonine and tyrosine phosphorylation. In mammals, three major MAPK subfamilies have been identified, the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAP kinase (p38 MAPK) [18]. Both p38 MAPK and JNK have been proposed to play a role in the signaling pathways of apoptosis [18]. It has previously been shown that p38 MAPK activity is involved in the inhibition of human eosinophil apoptosis [19]. JNK is activated by dual phosphorylation induced by cytokines or environmental stress (e.g. oxidative stress) [18,20]. The signaling cascades that lead to the regulation of apoptosis after JNK activation are not fully understood but a critical role for JNK has been suggested in the regulation of activator protein (AP) -1 transcriptional activity, and phosphorylation of c-Jun on serine 63 and 73 residues by JNK has been found to increase its transcriptional activity [18]. Recent evidence suggests that nitric oxide and glucocorticoids may activate MAP kinases in human eosinophils [21–23]. Activation of JNK by dexamethasone was associated with induction of eosinophil apoptosis, a process that could be inhibited by GM-CSF and by blocking JNK activation by a JNK inhibitor (SP600125) [21]. However, the results concerning the importance of JNK activation in glucocorticoid-induced eosinophil apoptosis remain controversial as JNK1/2 antisense phosphorothioate oligodeoxynucleotides did not exert any significant effect on dexamethasone-induced eosinophil apoptosis [22].

Given the possible role of JNK in regulating apoptosis of eosinophils and other cell types, the aim of the present study was to elucidate the role of JNK in spontaneous and dexamethasone-enhanced apoptosis of human eosinophils.

2. Materials and methods

2.1. Eosinophil isolation

One hundred millilitres of peripheral venous blood was obtained from healthy or atopic volunteers. Before donating blood, the volunteers gave written informed consent to a study protocol approved by the Ethics committee of Tampere University Hospital (Tampere, Finland). Eosinophils were isolated under sterile conditions to >99% purity as previously described [9,19,23,24]. Eosinophils were cultured (+37 °C, 5% CO_2) for 40 h in the absence and presence of dexamethasone and a JNK inhibitor in RPMI 1640 medium (Dutch modification) with 10% fetal calf serum and antibiotics.

2.2. Determination of apoptosis by the relative DNA content assay

The percentage of apoptotic cells was assessed by measuring the relative DNA content by flow cytometry (FACScan, Becton Dickinson, San Hose, CA) of propidium iodide (PI)-stained cells as previously described [9,17,24]. Endonuclease-catalysed DNA fragmentation is considered to be a specific feature of apoptosis [24]. Therefore, the cells showing decreased relative DNA content were considered as apoptotic. Eosinophils were suspended in 300 µl of hypotonic propidium iodide solution (25 µg ml^{-1} in 0.1% sodium citrate and 0.1% Triton X-100), protected from light and incubated at +4 °C for 1 h before flow cytometric analysis.

2.3. Annexin V-FITC

Annexin-V binding and analysis by flow cytometry were performed as previously reported [17]. Briefly, after 18 h incubation, the cells were washed in PBS solution and suspended in binding solution (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The mixture of 5 µl annexin V-FITC (solution containing 50 mM Tris, 100 mM NaCl, 1% BSA, sodium azide, pH 7.4) and 195 µl of the 5×10^5 cells/ml cell suspension was incubated at room temperature for 10 min. The cells were washed and resuspended in 190 µl of binding buffer, and 10 µl of 20 µg ml^{-1} propidium iodide solution was added. Annexin-positive cells were considered to be apoptotic.

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2.4. Morphological analysis

For morphological analysis, eosinophils were spun onto cytospin slides (500 rev min^{-1}, 5 min) and stained with May-Grünwald-Giemsa after fixation in methanol. Cells showing the typical features of apoptosis, such as cell shrinkage, nuclear coalescence and chromatin condensation, were considered as apoptotic [24].

2.5. Western blotting

Eosinophils were suspended at 10^6 cells/ml and cultured at +37 °C. At indicated time points, the samples were centrifuged at 12,000g for 15 s. The cell pellet was lysed by boiling for 5 min in 30 µl of Laemmli sample buffer. The sample was centrifuged at 12,000g for 10 min and the debris was carefully removed. Samples were stored at −20 °C until the Western blot analysis. The protein sample (30 µl) was loaded onto 10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 2 h at 100 V. The separated proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) with a semidry blotter at 2.5 mA cm^{-2} for 60 min. After transfer, the membranes were blocked by 5% bovine serum albumin (BSA) in TBST (20 mM Tris base pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature and incubated with the specific primary antibody overnight at +4 °C in the blocking solution. The membrane was thereafter washed 4× with TBST for 5 min, incubated for 30 min at room temperature with the secondary antibody in the blocking solution and washed 4× with TBST for 5 min. Bound antibody was detected by using SuperSignal West Dura chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). The chemiluminescent signal was quantified by using the FluorChem software version 3.1.

2.6. Materials

L-JNKI1 (c-Jun N-terminal kinase peptide inhibitor 1, L-stereoisomer) and L-TAT control peptide were purchased from Alexis Corp. (Läufelfingen, Switzerland). c-Jun and JNK1 antibodies and goat anti-rabbit polyclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), phospho-c-Jun (Ser63) antibody was from Cell Signaling Technology, Inc. (Beverly, MA, USA) and phospho-JNK (Thr183/Tyr185, Thr221/Tyr223) antibody was from Upstate (Lake Placid, NY, USA). Other reagents were obtained as follows: propidium iodide (Tocris, Bristol, UK), anti-CD16 microbeads and the magnetic cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany), Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), antibiotics and RPMI 1640 (Dutch modification) (Gibco BRL, Paisley, UK), fetal calf serum, Hank's balanced salt solution (HBSS) and RPMI 1640 (BioWhittaker, Verviers, Belgium), Annexin V-FITC kit (Bender medSystems, Vienna, Austria), May-Grünwald (Merck, Darmstadt, Germany) and Giemsa (J.T.Baker, Deventer, Holland). Dexamethasone and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Dexamethasone, L-JNKI1 and L-TAT were dissolved in HBSS.

2.7. Statistics

All results are expressed as the mean ± SEM. Apoptosis is expressed as an apoptotic index (number of apoptotic cells/total number of cells, i.e. apoptotic index 0.1 means 10% of the cells are apoptotic). Statistical significance was calculated by analysis of variance for repeated measures supported by the Dunnett test or by paired t-tests. Differences were regarded as significant when $p<0.05$.

3. Results

3.1. Effect of JNK-inhibition on constitutive eosinophil apoptosis

Human eosinophils cultured for 40 h in cytokine-deprived conditions underwent spontaneous apoptosis. The constitutive apoptotic index was 0.36 ± 0.07 ($n = 6$) as defined by the relative DNA fragmentation assay in propidium iodide-staining. The novel cell-permeable JNK inhibitor peptide L-JNKI1 [25,26] (10 µM) was found to decrease constitutive eosinophil apoptosis by 64% when apoptosis was assessed by using the relative DNA fragmentation assay (Fig. 1). The negative control peptide L-TAT did not affect apoptosis as compared with medium control ($n = 6$, data not shown). The flow cytometry histograms of eosinophils treated with L-JNKI1 clearly demonstrate the reduction in the numbers of cells with decreased relative DNA content i.e. reduced apoptosis by L-JNKI1 as compared with the negative control L-TAT (Fig. 2A and B). When apoptosis was assessed by measuring phosphatidylserine expression (i.e. Annexin V-binding), the effect was similar as L-JNKI1 decreased apoptosis by 40% (Fig. 2C and D). The apoptotic indices were 0.25 ± 0.02 and 0.15 ± 0.01 ($p<0.001$, $n = 8$) for L-TAT and L-JNKI1, respectively. Consistently, L-JNKI1 also reduced the number of eosinophils showing the typical characteristics of apoptosis such as cell shrinkage, chromatin condensation and nuclear coalescence. The apoptotic indices were 0.61 ± 0.07 and 0.46 ± 0.05 in the presence of L-TAT and L-JNKI1, respectively ($p<0.01$, $n = 6$, Fig. 2E and F).

3.2. Effect of JNK-inhibition on dexamethasone-enhanced eosinophil apoptosis

Human eosinophils cultured for 40 h in cytokine-deprived conditions underwent spontaneous apoptosis (apoptotic index 0.36 ± 0.07). In the presence of

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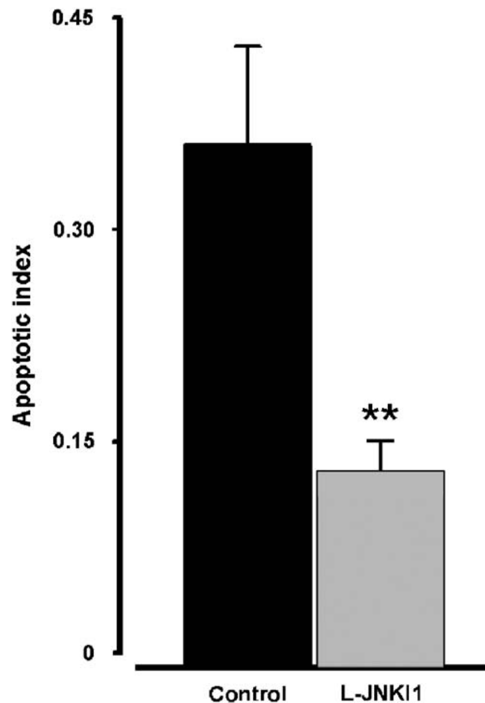

Fig. 1. The effect of the JNK inhibitor L-JNKI1 (10 μM) on constitutive eosinophil apoptosis. Apoptosis was analyzed by the relative DNA fragmentation assay (see methods). Each data point represents the mean ± SEM of six independent determinations using eosinophils from different donors. **indicates $p<0.01$ as compared with the respective control (10 μM L-TAT).

dexamethasone (1 μM), the apoptotic index was enhanced up to 0.43 ± 0.06 ($n=6$) when analyzed by using the relative DNA fragmentation assay. L-JNKI1 (10 μM), but not the negative control peptide L-TAT, was found to decrease dexamethasone-induced eosinophil apoptosis by 53% (Fig. 3). The flow cytometry histograms of dexamethasone-enhanced eosinophil apoptosis in the presence of L-JNKI1 show the decrease in the number of cells showing hypodiploid DNA content suggesting reduced apoptosis by L-JNKI1 as compared with L-TAT (Fig. 2G and H). When Annexin V-binding was used as an indicator of apoptosis, the results were similar as L-JNKI1 decreased eosinophil apoptosis in the presence of dexamethasone by 37% (Fig. 2I and J). The apoptotic indices were 0.29 ± 0.02 and 0.18 ± 0.02 ($p<0.001$, $n=8$) in the presence of L-TAT and L-JNKI1, respectively. Consistently, L-JNKI1 reduced eosinophil apoptosis in the presence of dexamethasone as assessed by morphological analysis by bright field microscopy. The apoptotic indices were 0.74 ± 0.06 and 0.65 ± 0.05 with L-TAT and L-JNKI1, respectively ($p<0.05$, $n=6$, Fig. 2K and L).

3.3. Fold-increase in eosinophil apoptosis by dexamethasone

The JNK inhibitor L-JNKI1 delays human eosinophil apoptosis both in the presence and absence of dexamethasone by 53% and 64%, respectively. As the JNK inhibitor seems to reduce apoptosis to a similar extent in the presence and absence of dexamethasone, we calculated how many fold does dexamethasone increase apoptosis in the absence and presence of the JNK inhibitor. These fold-increased rates of eosinophil apoptosis (apoptotic index in the presence of dexamethasone/apoptotic index in the absence of dexamethasone) showed that L-JNKI1 did not reverse the steroid-effect (Fig. 4), suggesting that the enhancement of eosinophil apoptosis by dexamethasone may not be mediated through activation of JNK.

3.4. JNK activation during constitutive and dexamethasone-enhanced eosinophil apoptosis

Dexamethasone has been proposed to activate JNK in human eosinophils [21,22]. To further evaluate the possible role of JNK in spontaneous and dexamethasone-induced eosinophil apoptosis, the activation of JNK was studied. The activity of JNK was assessed by calculating the ratio of phosphorylated/total JNK by using antibodies directed against Thr183/Tyr185 and Thr221/Tyr223 phosphorylated (activated) JNK and total JNK, which recognize JNK at molecular weights of 46 and 55 kDa. In freshly isolated human eosinophils, a spontaneously active JNK could be seen (Fig. 5A). During spontaneously occurring eosinophil apoptosis, a slow activation of 55 kDa JNK could be observed (Fig. 5A). In the presence of dexamethasone, a non-significant tendency towards slow activation of JNK could be seen (Fig. 5B). This suggests that the JNK is slowly activated during human eosinophil apoptosis but steroids do not increase JNK activity. In addition, an analysis of a downstream effector of JNK was conducted. c-Jun is an inducible transcription factor and can be activated by phosphorylation at specific serine 63 and 73 residues only by JNK [27]. The activity of c-Jun was analyzed by Western blotting by using antibodies directed against Ser-63 phosphorylated c-Jun and total c-Jun. Similarly to the active JNK seen in freshly isolated human eosinophils, a phosphorylated c-Jun could be detected in these cells. However, during constitutive apoptosis of eosinophils, c-Jun phosphorylation at the serine 63 residue was not increased (Fig. 6A). Dexamethasone (1 μM) did not increase c-Jun phosphorylation (Fig. 6B), which further suggests that dexamethasone does not induce further activation of JNK.

4. Discussion

We have shown here that JNK mediates spontaneous eosinophil apoptosis and that inhibition of the JNK pathway leads to increased survival of human eosinophils. The JNK inhibitor L-JNKI1 markedly reduced the rate of human eosinophil apoptosis. These effects could be observed with each method used to measure apoptosis, the relative DNA fragmentation assay, Annexin V-binding and morphological analysis by bright field microscopy. Moreover, the presence of active JNK as evidenced by the presence of phosphorylated JNK as well as phosphorylated

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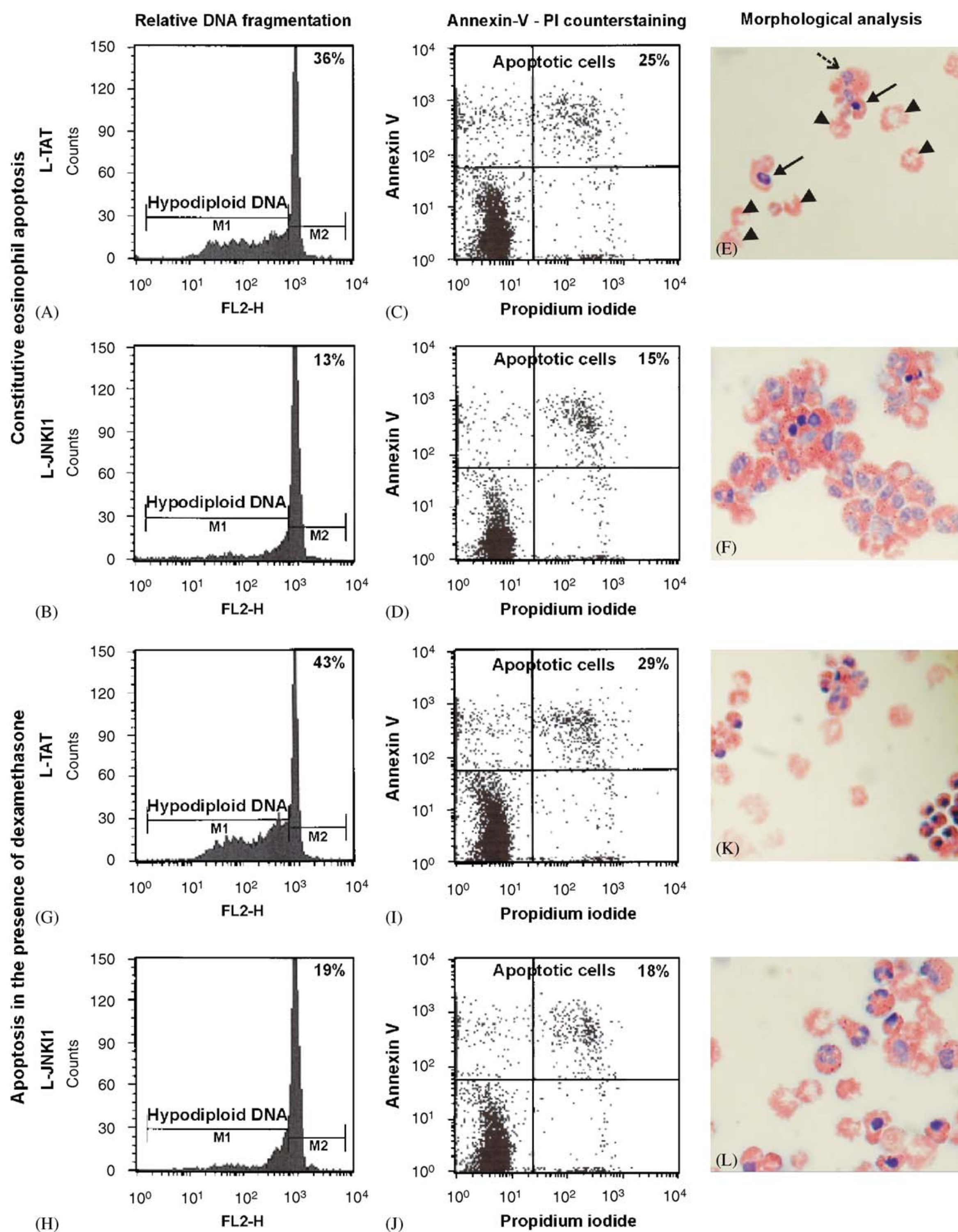

Fig. 2. The effect of L-JNKI1 on constitutive and dexamethasone (1 μM)-induced human eosinophil apoptosis. Shown are the results of one independent experiment representative of six essentially identical experiments of constitutive eosinophil apoptosis (A–F) and dexamethasone (1 μM)-induced apoptosis (G–L). The results were analyzed by the relative DNA fragmentation assay (A, B, G, H), in which the cells showing hypodiploid DNA concentration were considered as apoptotic, the Annexin-V assay (C, D, I, J), in which cells with increased Annexin-V-binding (FL1-H) or both increased Annexin-V- and PI (FL2-H)-binding were considered as apoptotic, and by morphological analysis (E, F, K, L), where the typical features of normal cells (dotted arrows), apoptotic (solid arrows) and late apoptotic (arrowheads) cells were assessed. The figure in the top right corner represents the percentage of apoptotic cells.

c-Jun in freshly isolated cells and the slight activation of JNK during spontaneously occurring apoptosis support the hypothesis that JNK mediates constitutive apoptosis of human eosinophils. Furthermore, in line with our results, Gardai and co-workers have reported that the JNK inhibitor SP600125 prevents dexamethasone-induced

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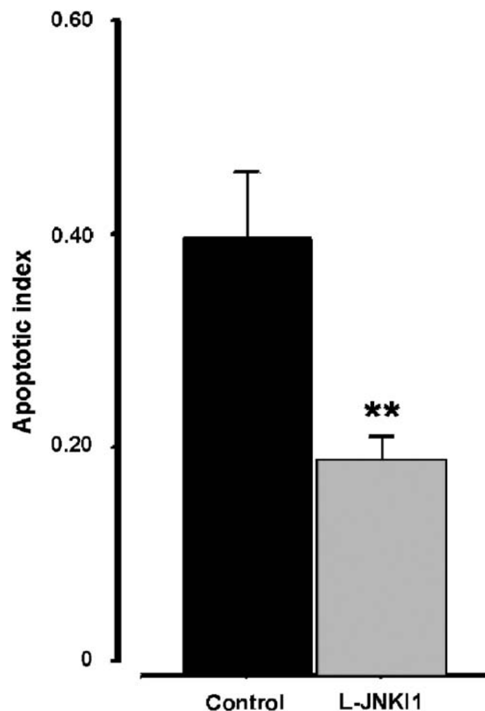

Fig. 3. The effect of the JNK inhibitor L-JNKI1 (10 μM) on dexamethasone (1 μM)-induced human eosinophil apoptosis. Apoptosis was assessed by the relative DNA fragmentation assay. Each data point represents the mean ± SEM of six independent determinations using eosinophils from different donors. **indicates $p<0.01$ as compared with the respective control (10 μM L-TAT).

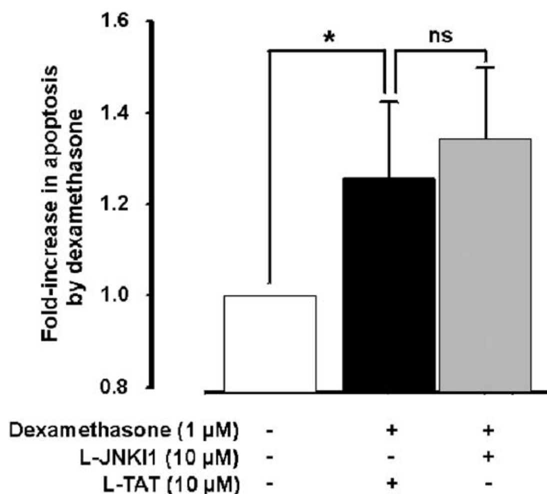

Fig. 4. The effect of JNK inhibition on the increase in eosinophil apoptosis by dexamethasone (1 μM). The bars represent how many fold does dexamethasone increase apoptosis without and with JNK inhibition. Apoptosis in the absence of dexamethasone and the JNK-inhibitor is set as 1.0 (white column). Dexamethasone (1 μM) was found to significantly enhance human eosinophil apoptosis. The JNK inhibitor, L-JNKI1 (10 μM), did not reverse the apoptosis-promoting effect of dexamethasone (i.e. did not return the fold-increase rate of apoptosis back to 1.0) suggesting that JNK does not mediate glucocorticoid-induced apoptosis in human eosinophils. Apoptosis was measured by the relative DNA fragmentation assay in PI-stained cells, $n=4$, ns (non-significant) indicates $p>0.05$ and *indicates $p<0.05$.

eosinophil apoptosis while a similar although non-significant anti-apoptotic tendency could be observed in the absence of dexamethasone ($n=3$) [21].

In addition to decreasing constitutive apoptosis, inhibition of the JNK pathway was in the present study found to diminish eosinophil apoptosis in the presence of dexamethasone. By calculating the ratios of how many fold does dexamethasone increase apoptosis in the absence and presence of the JNK inhibitor L-JNKI1, we concluded that JNK inhibition did not, however, reverse the steroid effect indicating that the JNK may not mediate glucocorticoid-induced apoptosis in human eosinophils as recently suggested by Gardai et al. [21]. In conjunction with our present results, Zhang et al. [22] reported that inhibition of JNK by JNK1/2 antisense oligodeoxynucleotides did not exert any significant effects on dexamethasone-induced eosinophil apoptosis. Dexamethasone has previously been shown to activate JNK in human eosinophils [21,22]. In this study we showed that JNK is slowly activated during constitutive human eosinophil apoptosis and the same tendency can be seen in dexamethasone-induced apoptosis. However, we did not observe any increased activity of JNK in dexamethasone-treated eosinophils as compared with untreated cells. This is in line with our results showing that JNK inhibitors do not reverse the effect of steroids. In this study we also showed that serine 63-phosphorylated c-Jun can be detected in freshly isolated human eosinophils, which reflects the activation of JNK [27]. However, no further activation (i.e. phosphorylation of serine 63 residues) of c-Jun could be observed although JNK was found to be slightly activated. This can be explained by the fact that activities of c-Jun and JNK can be separately regulated apart from phosphorylation of serine 63 and 73 residues of c-Jun that is strictly dependent of nuclear JNK activity [27]. JNK activation and even nuclear translocation does not inevitably result in phosphorylation of c-Jun since JNK also catalyzes the phosphorylation of other nuclear substrates [27]. In addition, JNK can associate with c-Jun through multiple contacts which do not require the N-terminal phosphorylation of the serine residues 63 and 73 [27].

Addressing the question of the specificity of the JNK inhibitors, which is essential when drawing conclusions from studies made with protein kinase inhibitors, Bain and co-workers [28] have recently shown that the most commonly used JNK inhibitor SP600125 is a relatively weak and non-specific inhibitor of JNK. To further complicate the studying of signalling cascades associated with protein kinases, the use of transfection techniques to either block or enhance certain signalling pathways in non-dividing and rapidly dying cells like eosinophils is by large impossible. To overcome these problems, we used another pharmacological approach to inhibit JNK. L-JNKI1 is novel cell-permeable inhibitor of JNK that is coupled to the HIV transcription factor TAT, which acts as a protein transduction domain (PDT) and rapidly delivers the peptide into the cell [25,26,29]. Even though there exist

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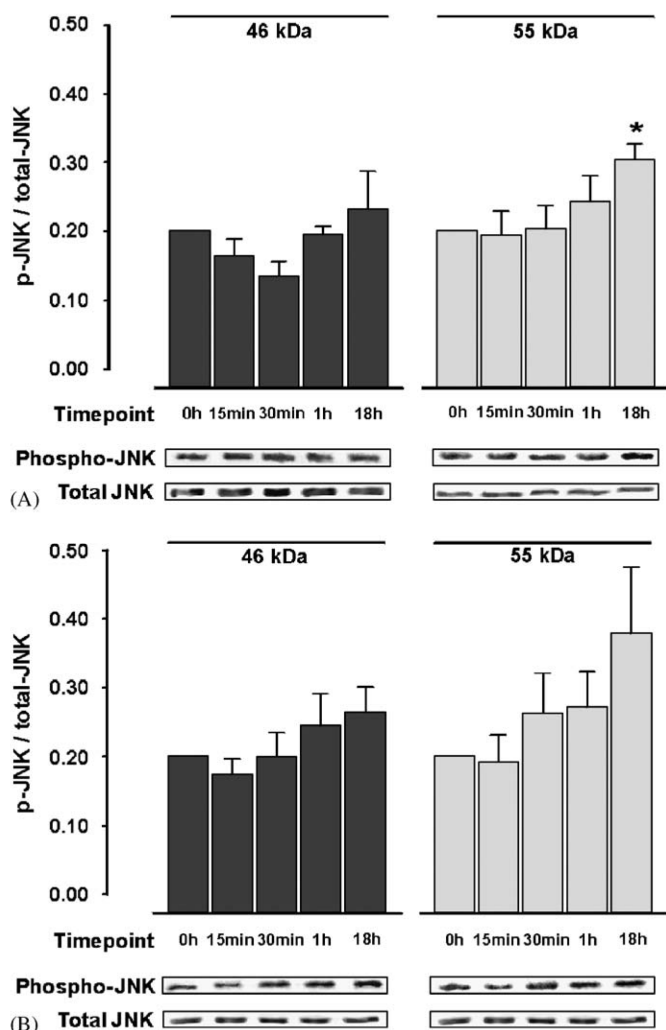

Fig. 5. The activation of JNK (46 and 55 kDa) during constitutive (A) and dexamethasone-enhanced (B) eosinophil apoptosis. The bars represent the ratio of phosphorylated JNK/total JNK from 4 to 6 independent Western blot analyses using eosinophils from different donors (mean ± SEM). The p-JNK/total JNK-ratio at time point 0 h is set as 0.20. *indicates $p<0.05$ as compared with the 0 h time point. The immunoblots are representative of $n = 4-6$ essentially identical experiments.

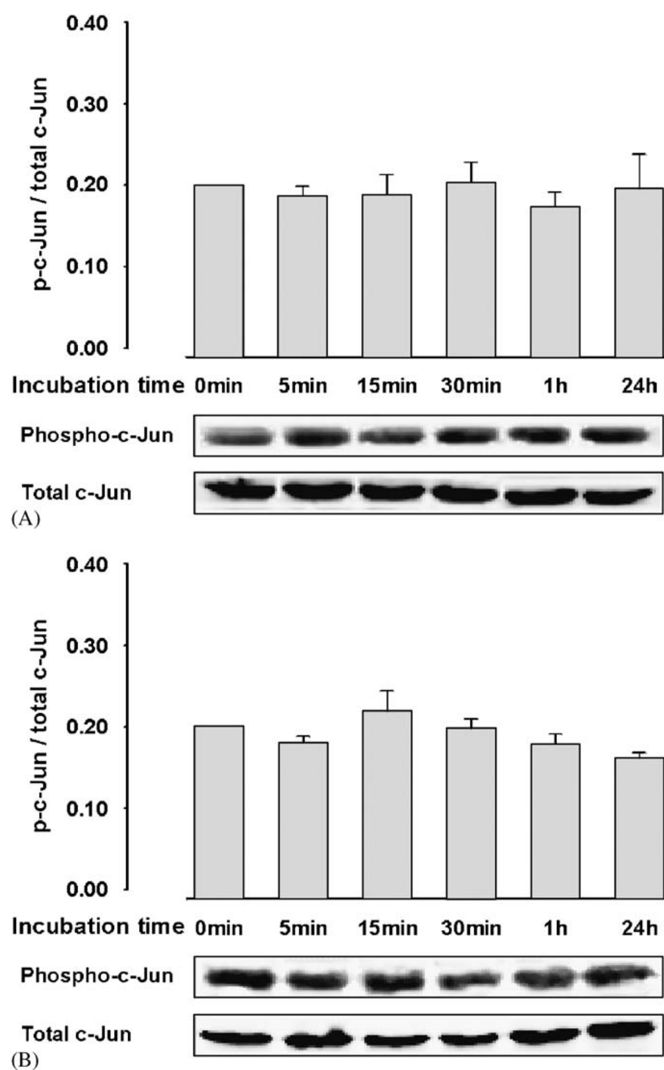

Fig. 6. The activation of c-Jun (phosphorylation at Ser-63) during constitutive (A) and dexamethasone (1 μM)-enhanced (B) eosinophil apoptosis. The bars represent the ratio of phosphorylated c-Jun/total c-Jun from 3 to 5 independent Western blot analyses using eosinophils from different donors (mean ± SEM). The p-c-Jun/total c-Jun-ratio at time point 0 h is set as 0.20. The immunoblots are representative of $n = 3-5$ essentially identical experiments.

some limitations to the use of protein transduction domains, their low toxicity and ability to deliver to at least some primary non-dividing cells make PDTs an attractive tool for both in vitro and in vivo studies [29]. Based on our observations, L-JNKI1 can be applied to studies in eosinophils. For example, we have recently shown that nitric oxide induces activation of JNK and reverses IL-5-mediated human eosinophil survival by inducing apoptosis, which can be inhibited by L-JNKI1 [23].

Recently, based on studies with sensitized animals, a role for the JNK in chronic allergic airway inflammation and remodeling associated with bronchial hyperresponsiveness has been proposed [30]. Pretreatment with the JNK inhibitor SP600125 has been shown to reduce the numbers of inflammatory cells, including eosinophils in the bronchoalveolar lavage fluid after allergen challenge in mice and rats [30–32]. Interestingly, the numbers of eosinophils in the bronchial tissue were not reduced after pre-treatment with SP600125 [32]. Our present result that inhibition of JNK activity does not increase but rather decreases apoptosis of eosinophils is in line with the inability of SP600125 to reduce tissue eosinophilia. However, SP600125 reduces the production of several cytokines and chemokines [30–32] as well as inhibits several other kinases [28] and thus the net effect of SP600125 on pulmonary inflammatory indices is a result of a combination of all its effects. However, based on our present results and those reported from animal studies [32], inhibition of JNK activity may not be an optimal target to reduce lung tissue eosinophilia.

In conclusion, we have in the present study demonstrated that JNK mediates spontaneous apoptosis of

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human eosinophils. In contrast, our results suggest that JNK may not be responsible for the enhancement of eosinophil apoptosis by glucocorticoids.

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