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Author	Martin, Morag Corbett
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MOLECULAR MECHANISMS INVOLVED IN CYCLIC AMP AND GLUCOCORTICOID REGULATION OF GRANULOCYTE APOPTOSIS

MORAG CORBETT MARTIN

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ABSTRACT

Inflammation is a normally beneficial, complex interplay of events that occur in response to tissue injury or infection. Granulocytes are rapidly recruited to the inflammatory site, however, the activity of these cells must be carefully regulated as uncontrolled release of cytotoxic cell contents into the surrounding milieu may result in excessive tissue damage. In order to avoid this undesirable and inappropriate response, granulocytes undergo a regulated process of programmed cell death or apoptosis, allowing shutdown of secretory capacity and phagocytic removal of intact effete cells by a mechanism that does not incite an inflammatory response.

Although the apoptotic programme in granulocytes is an intrinsic cell process, the rate of apoptosis can be altered dramatically by a number of agents. We have shown that elevation of the second messenger cyclic AMP and glucocorticoids, profoundly delay constitutive neutrophil apoptosis. Further investigations demonstrated that cyclic AMP inhibits loss of mitochondrial membrane potential occurring during constitutive neutrophil apoptosis. Moreover, cyclic AMP was found to delay caspase activation in these inflammatory cells. Investigations were undertaken to examine the cyclic AMP signal transduction pathway responsible for delay of neutrophil apoptosis. Despite increasing protein kinase A (PKA) activity, this kinase is unlikely to mediate the effects of cyclic AMP in apoptosis since blockade of PKA activation did not influence the survival effects of cyclic AMP. Furthermore, cyclic AMP mediated delay of neutrophil apoptosis is independent of PI-3 kinase and MAP kinase activation. Our results suggest cyclic AMP delays neutrophil apoptosis via a novel, reversible and transcriptionally-independent mechanism. We show that proteasome activity in the neutrophil is vitally involved in this process and suggest that a balance of pro-apoptotic and anti-apoptotic proteins plays a key role in the powerful ability of cyclic AMP to delay neutrophil death.

Additional studies were aimed at elucidating the underlying mechanisms of glucocorticoid regulation of granulocyte apoptosis. Glucocorticoids were found to

exert diametrically opposed effects on eosinophils and neutrophils, causing induction of apoptosis in eosinophils while delaying neutrophil cell death. Granulocytes were found to express the glucocorticoid receptor (GR), however the nature of isoforms expressed remains undefined. Examination of the glucocorticoid signal transduction cascade suggested the requirement for hsp90 in glucocorticoid regulation of granulocyte apoptosis. Further studies were undertaken to establish the involvement of transcriptional transactivation or repression in glucocorticoid signalling pathway regulating granulocyte cell death.

In summary, there is good evidence implicating glucocorticoids and cyclic AMP in the regulation of granulocyte apoptosis. A greater understanding of the signalling mechanisms by which these mediators regulate granulocyte death could potentially lead to the development of novel strategies to therapeutically induce apoptosis for the resolution of inflammation.

DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

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CONTENTS

ABBREVIATIONS

-Ab	Antibody
-AP-1	Activator Protein-1
-Apaf-1	Apoptotic Protease Activating Factor-1
-ARDS	Acute Respiratory Distress Syndrome
-ATP	Adenosine Triphosphate
-Ca^{2+}	Calcium ion
-cAMP	Cyclic AMP
-CARD	Caspase Recruitment Domain
-CBP	CREB Binding Protein
-CGD	Chronic Granulomatous Disease
-CRE	Cyclic AMP Responsive Element
-CREB	Cyclic AMP Reponsive Element Binding Protein
-DD	Death Domain
-DED	Death Effector Domain
-ELISA	Enzyme Linked ImmunoSorbant Assay
-ERK	Extracellular Signal Regulated Kinases
-fMLP	f-Met-Leu Phe
-GEF	Guanine Exchange Factor
-G-CSF	Granulocyte Colony Stimulating Factor
-GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
-GR	Glucocorticoid Receptor
-GRE	Glucocorticoid Reponsive Element
-HAT	Histone Acetyltransferase Activity
-HDAC	Histone Deacetylation
-Hsp	Heat shock protein
-HRP	HorseRadish Peroxidase
-ICAM-1	Intercellular Adhesion Molecule-1
-IFN	Interferon
-IL-	Interleukin
-JNK	c-Jun N-terminal Kinase
-KO	Knock Out (gene deficient)
-LPS	Lipopolysaccharide
-LTB_4	Leukotriene B_4
-LTC_4	Leukotriene C_4
-mAb	Monoclonal Antibody
-MAPK	Mitogen Activated Protein Kinase
-MPO	Myeloperoxidase
-NFκB	Nuclear Factor -κB
-PAF	Platelet Activating Factor
-PARP	poly(ADP ribose) polymerase
-PBMCs	Peripheral Blood Mononuclear Cells
-PBS	Phosphate Buffered Saline
-PDE	Phosphodiesterase
-PGE_2	Prostaglandin E_2

-PI-3K	Phosphoinositide –3 Kinase
-PKA	Protein Kinase A
-PKC	Protein Kinase C
-PMA	Phorbol 12-Myristate 13-Acetate
-PS	Phosphatidylserine
-PTP	Permeability Transition Pore
-RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
-ROI	Reactive Oxygen Intermediates
-SLPI	Secretory Leukocyte Proteinase Inhibitor
-STAT	Signal Transducer and Activator of Transcription
-TBS	Tris-Buffered Saline
-TGF	Transforming Growth Factor
-Th	T helper
-Th_1	T helper 1
-Th_2	T helper 2
-TNF	Tumour Necrosis Factor
-TNFR	Tumour Necrosis Factor Receptor
-TSA	Trichostatin A
-VCAM	Vascular Cell Adhesion Molecule
-VDAC	Voltage Dependent Anion Channel
-VLA	Very Late Antigen

1 INTRODUCTION

1.1 INTRODUCTION LAYOUT

The introduction summarises the knowledge to date on the generation, structure and function of granulocytes and describes the surface phenotype and mediator release involved in the phagocytic capacity of these cells, following migration into tissues.

The following section describes the role of neutrophils and eosinophils in disease highlighting the differences in the structure and phenotype of granulocytes, which may lead to the differential involvement of these cells in many cellular processes.

The process of apoptosis is then described and the mechanisms involved in molecular and genetic control of apoptosis are discussed.

Finally the introduction ends with a summary of the current knowledge of the mechanisms involved in granulocyte apoptosis and the potential implications of manipulation of granulocyte apoptosis for therapeutic treatment of inflammatory disease.

1.2 THE ROLE OF NEUTROPHILS IN INFLAMMATION

1.2.1 GENERATION OF NEUTROPHILS

Neutrophils are the most abundant leukocytes in the blood and are derived from pluripotent stem cells located in the bone marrow. During their development pluripotent stem cells undergo proliferation and differentiation under the influence of three major haematopoietic cytokines G-CSF, GM-CSF and IL-3 (Metcalf and Nicola, 1983; Metcalf *et al.*, 1986; Bot *et al.*, 1988) allowing them to differentiate and mature into fully functional neutrophils. There appear to be several recognisable morphological stages including myeloblast, primary myeloblast, myelocyte, and metamyelocyte stages with subsequent formation of non-segmented (band) neutrophils before fully functional segmented neutrophils are formed (Zajicek *et al.*, 1984). During the developmental process neutrophils acquire their characteristic primary (azurophil), secondary (specific), and tertiary granules. The mature neutrophil is distinguished also by a lobulated chromatin dense nucleus.

Neutrophils are released from the bone marrow into the circulation in the healthy adult, at a rate of 10^{11} neutrophils per day (Cannistra and Griffin, 1988). During acute inflammation, release of neutrophils from the bone marrow is thought to increase to more than 10^{12} per day (Cannistra and Griffin, 1988). Neutrophils are short lived cells and in the circulation have an estimated half life of only 4 - 6 h. Neutrophils may also exist in marginated pools in the liver, spleen and lung, where they can live for 1 to 2 days (Bicknell *et al.*, 1994; Peters, 1998). A dynamic equilibrium exists between the circulating pools and marginated pools, allowing exchange with each other (Berkow and Dodson, 1987).

1.2.2 NEUTROPHIL MORPHOLOGY

Neutrophils have a mean diameter of ~7 μm and are characterised by a multilobed chromatin-dense nucleus and a large number of intracellular granules. Initially neutrophil granules were simply divided into two categories namely peroxidase

positive (azurophil or primary) and peroxidase negative (specific or secondary). However it appears that there are in addition two further granule subgroups namely tertiary/gelatinase granules and secretory vesicles (Borregaard *et al.*, 1993). Each category of granule can be distinguished by differences in morphology and biochemical constituents (For a recent review see Borregaard and Cowland, 1997). The importance of neutrophil granules is demonstrated in Chediak-Higashi syndrome in which an apparent inability to mount a proper inflammatory response leads to a congenital abnormality in neutrophil granule formation (Davis and Douglas, 1972).

The azurophil or primary granules vary in size and have an oval or round morphology (Bainton, 1975). They contain a wide variety of agents believed to be involved in bacterial killing and are primarily released into the phagolysosomal compartment during phagocytosis. Regulated secretion of granule contents is required to avoid detrimental pathology to host tissue. Among the many antimicrobial constituents of azurophil granules, one of the most important for effective bactericidal killing is myeloperoxidase (MPO) (For a recent review see Klebanoff, 1999). The enzymatic activity of MPO is responsible for the conversion of hydrogen peroxide to hypochlorous acid, providing the neutrophil with a highly effective microbicidal capacity. Azurophil granules also contain antimicrobial peptides called defensins, which form a large proportion of the proteins contained in azurophil granules (Chertov *et al.*, 2000). These small antimicrobicidal peptides are highly toxic to a wide variety of bacteria, fungi and some viruses. Other important constituents include serine proteases such as elastase and cathepsin G which are involved in hydrolytic degradation of many substrates including bacterial cell walls (Chertov *et al.*, 2000).

The second category of granules are specific granules which are round, oval or elongated in shape and are slightly larger than gelatinase granules. In contrast to azurophil granules, it is thought that specific granules release their contents extracellularly. They characteristically contain the iron sequestering protein lactoferrin, important for preventing the growth of ingested bacteria by depriving them of this vital growth nutrient (Spitznagel *et al.*, 1974). Specific granules are also

a source of cytoplasmic receptors for the small complement fragment iC3b (Berger *et al.*, 1984), laminin (Yoon *et al.*, 1987) and fMLP (Sengelov *et al.*, 1994). It is not clear if these receptors are fully functional when translocated to the plasma membrane. A further important constituent of specific granules is cytochrome b558, a component of the NADPH oxidase, responsible for the generation of superoxide anion (Borregaard, 1985).

Gelatinase (or tertiary) granules are smaller than specific granules and are principally composed of the protease gelatinase, which may be involved in digestion of the vascular basement membrane, allowing the extravasation of neutrophils (Murphy *et al.*, 1980; Dewald *et al.*, 1982). Similarly to specific granules, gelatinase granules contain cytochrome b558 and several acid hydrolases but do not contain lactoferrin.

The fourth category of granules namely secretory vesicles contain alkaline phosphatase and are characterised by their ability to be rapidly mobilised to the plasma membrane of neutrophils (Borregaard *et al.*, 1990). The possible importance of this ability to translocate is suggested by the presence of several receptors, which may be important for neutrophil adhesion and chemotaxis such as CD11b/CD18 (Mac-1) (Calafat *et al.*, 1993), and the fMLP receptor (Sengelov *et al.*, 1994). Furthermore, secretory granules may also contain CD16 (Fcγ receptor III) (Tosi and Zakem, 1992), CD14 (Detmers *et al.*, 1995) and cytochrome b558 (Calafat *et al.*, 1993).

1.2.3 NEUTROPHIL RECRUITMENT AT SITES OF INFLAMMATION

The neutrophil is key in first line defence against invading micro-organisms and is the first type of leukocyte summoned from the blood to sites of infection or injury. The rapid mobilisation of neutrophils relies on the ability of these cells to respond to chemotactic agents and adhere to endothelial cells. Initial interactions between neutrophils and the endothelium involves selectins, which allow the neutrophil to roll along the endothelium via transient contacts (Picker *et al.*, 1991; Lawrence and Springer, 1991; Lawrence and Springer, 1993). Involvement of each of the main

selectins (L-, P-, and E-selectin) has now been described (For a recent review see Gonzalez-Amaro and Sanchez-Madrid, 1999). Following activation by chemoattractant agents such as IL-8 or PAF, the neutrophil upregulates adhesion receptors such as CD11b/CD18. Consequently, firm adhesion to the endothelium is secured via binding through CD11/CD18 with endothelial cell ligands such as intercellular adhesion molecule-1 (ICAM-1) (Carlos and Harlan, 1994; Springer, 1994; Ley, 1992; Smith *et al.*, 1989). Transmigration of neutrophils across the endothelial barrier involves an interaction between leukocyte integrins and endothelial ICAM, and between glycosylated aminoglycans on the neutrophil plasma membrane and CD31, localised in the intercellular junctions of endothelial cells (Muller *et al.*, 1993). Diapedesis of neutrophils through the endothelium is then followed by migration to the site of infection along a chemotactic gradient (Figure 1.1). The invading microorganism may produce chemoattractants such as fMLP, involved in recruiting neutrophils to the site of infection. Other chemotactic agents such as IL-8 may be released from the phagocytes themselves following initial interactions with infectious agents (Hachicha *et al.*, 1998). This allows an amplification of the preliminary inflammatory response, initiating a second wave of neutrophil recruitment.

1.2.4 REGULATION OF NEUTROPHIL FUNCTION

It is well recognised that there are complex regulatory mechanisms in place to allow neutrophils to respond rapidly to infection when required. The magnitude of neutrophil responsiveness to infection or tissue injury may be influenced by several agents which may allow dormant neutrophils to acquire a state of preactivation, allowing enhanced responsiveness to microbial infection. This regulatory mechanism of *priming* is vitally important for generating a more rapid and powerful neutrophil response, following the initial microbial insult (For a recent review see Condliffe *et al.*, 1998). Priming agents include cytokines such as GM-CSF, G-CSF and TNFα and bacterial products such as LPS (Guthrie *et al.*, 1984). As a consequence, neutrophil functions such as agonist (e.g fMLP, C5a) induced degranulation (Fittschen *et al.*, 1988), the respiratory burst (Guthrie *et al.*, 1984) and production

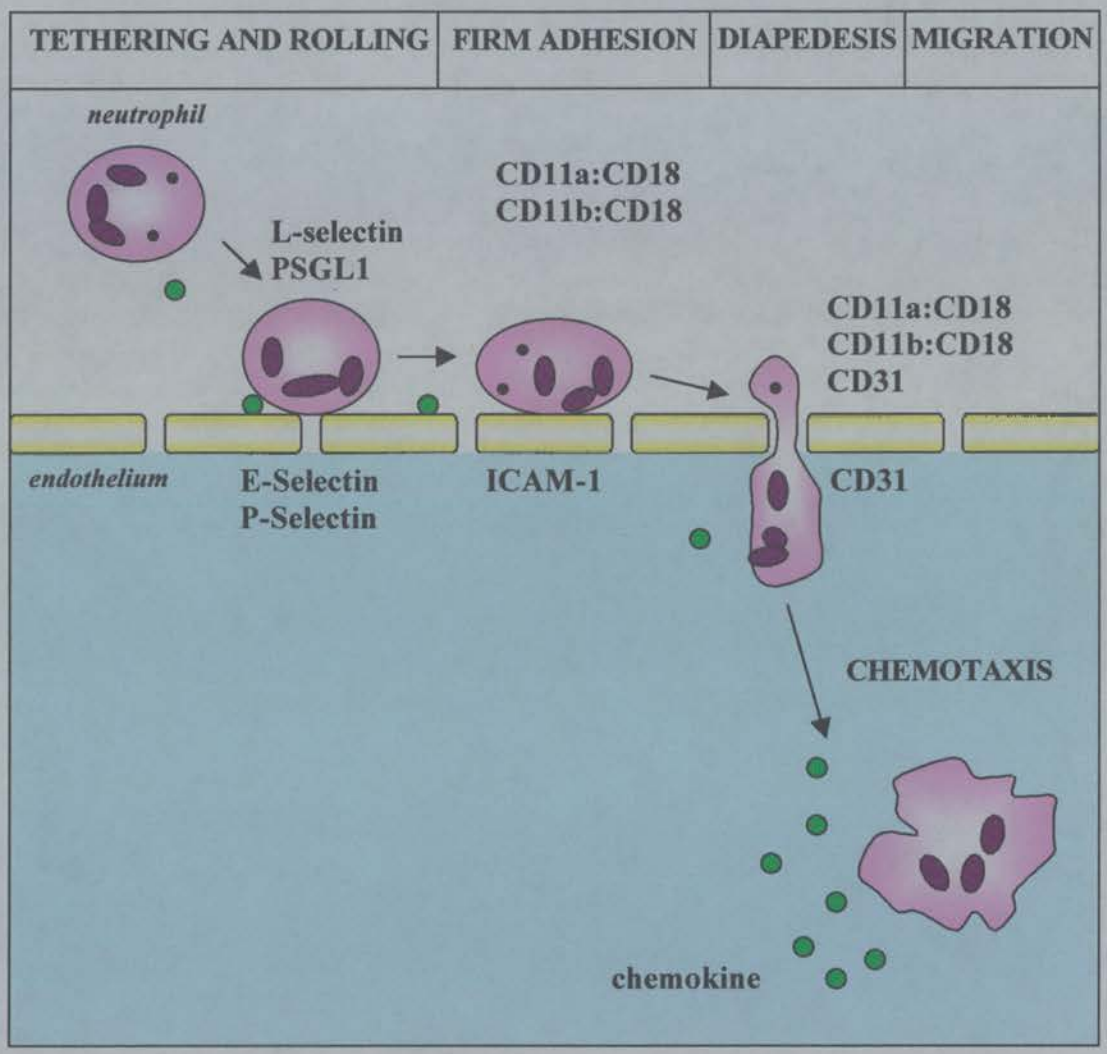


Figure 1.1 Schematic representation of the mechanisms involved in neutrophil recruitment. Initial interactions involve reversible binding to the endothelium through selectins on the endothelium and their carbohydrate ligands on the PMNs, which allows rolling of the leukocyte along the endothelial cell surface. Stronger binding is mediated through CD11a:CD18, CD11b:CD18 and ICAM-1. Tight binding arrests the rolling and allows the neutrophil to transmigrate across the endothelium and enter the site of infection. Finally the neutrophil migrates along a concentration gradient of chemokines secreted by the cells at the site of infection.

of lipid mediators (Doerfler *et al.*, 1989; Doerfler *et al.*, 1994) is greatly enhanced. It has become increasingly apparent that regulation of neutrophil function is also determined by the ability of neutrophils to produce a variety of mediators, which can influence neutrophil activity in an autocrine manner. This is in contrast to previously held beliefs that neutrophils already possess all mediators necessary to participate in the inflammatory response and have little synthetic capacity. However neutrophils are now known to be capable of producing a large variety of inflammatory mediators such as bioactive lipids and cytokines which can participate and influence the inflammatory response.

Through metabolism of arachadonic acid, neutrophils produce leukotrienes and prostaglandins such as LTB_4 (McColl *et al.*, 1991) and PGE_2 (Tolone *et al.*, 1977). Furthermore they can secrete a wide variety of cytokines such as IL-8 (Cassatella *et al.*, 1992; Bazzoni *et al.*, 1991), IL-6 (Cicco *et al.*, 1990), TNFα (Dubravec *et al.*, 1990; Djeu *et al.*, 1990) and IL-1 (Tiku *et al.*, 1986; Lindemann *et al.*, 1988, for a recent review see Scapini *et al.*, 2000). Thus the role of the neutrophil is not only determined by their ability to respond to agents in the extracellular milieu, but also by their capacity to direct and influence the inflammatory response.

1.2.5 NEUTROPHIL FUNCTIONS AND ROLE IN DISEASE

Once a neutrophil has migrated into the tissue, its primary purpose is to recognise and destroy pathogens. Phagocytosis is a process utilised by neutrophils to ingest and clear large particles (> 0.5 μM), including infectious agents and cellular debris. Neutrophils may phagocytose microbes through direct binding of lectins (Ofek *et al.*, 1995). However, in many instances effective phagocytosis and clearance of infectious agents additionally requires the availability of opsonins, to facilitate the adherence of the bacteria and other microbes to opsonin receptors on the surface of the neutrophil. Opsonisation prior to phagocytosis may be mediated for example through Fc receptor binding to Fc portion of antibodies deposited on the bacterial or

viral cell surface or via complement receptors binding to C3b (Scribner and Fahrney, 1976) (Figure 1.2).

Following recognition and pathogen binding, a phagosome forms containing engulfed micro-organisms. This then fuses with the intracellular granules, allowing the neutrophil to release a variety of antimicrobial agents into a contained microenvironment. It appears that both specific and azurophil granule products are released into the phagosome, while some specific granule products, together with products from secretory vesicles, are released extracellularly. During phagocytosis a substantial increase in oxygen consumption is observed, called the respiratory burst (For a recent review see Wientjes and Segal, 1995). Activation of respiratory burst activity occurs upon translocation and assembly of the cytosolic components of the NADPH oxidase enzyme system ($p45^{phox}$, $p67^{phox}$, $p21^{rac}$) with membrane-bound flavocytochrome, cytochrome b_{558}. This process, through the reduction of oxygen by NADPH oxidase, allows the generation of toxic reactive oxygen intermediates (ROI) such as superoxide anions and hydrogen peroxide. Further reduction leads to the production of more toxic oxygen radicals such hydroxyl radical ($OH^{\bullet}$) from H_2O_2, in a reaction catalysed by Fe^{2+} or the production of hypochlorous acid, through the action of MPO. These substances have highly powerful antimicrobial activities; the importance of which is illustrated in chronic granulomatous disease (CGD). In CGD, absence of a proper respiratory burst due to an abnormality in the NADPH oxidase system, manifests in children as an inability to fight bacterial infection (Hohn and Lehrer, 1975; Klebanoff, 1975).

The neutrophil plays an essential role in host defence however there is accumulating evidence that dysregulation of neutrophil function can result in injury to healthy tissue and is associated with the pathogenesis of a large number of inflammatory conditions. In many diseases such as rheumatoid arthritis, vasculitis, and glomerulonephritis, the neutrophil is thought to contribute to disease progression (Weissmann and Korchak, 1984; Weissmann, 1989; Heinzelmann *et al.*, 1999). In

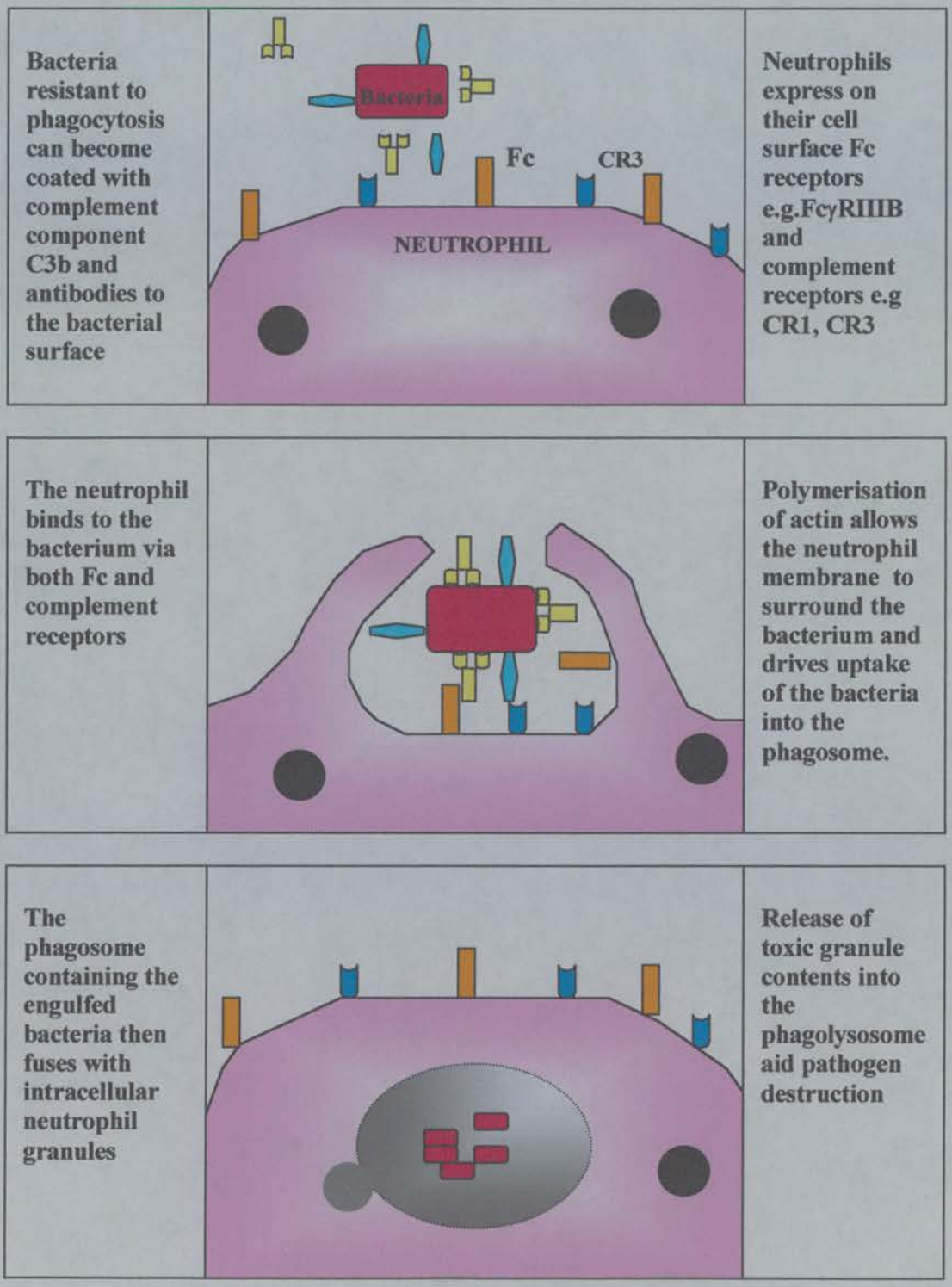


Figure 1.2. Fc and complement receptor mediated neutrophil phagocytosis.

acute respiratory distress syndrome (ARDS), neutrophils may cause injury through enhanced production of reactive oxygen species (McGuire *et al.*, 1982). Influx of neutrophils and subsequent activation is also thought to be involved in ischemia-reperfusion injury (Jordan *et al.*, 1999).

1.3 ROLE OF EOSINOPHILS IN INFLAMMATION

1.3.1 GENERATION OF EOSINOPHILS

The eosinophil, like the neutrophil, is a terminally differentiated cell derived by differentiation from pluripotent stem cells in the bone marrow. In contrast to neutrophils, the development of eosinophils is specifically promoted by GM-CSF, IL-3 and IL-5 (Bot *et al.*, 1988; Metcalf *et al.*, 1986; Campbell *et al.*, 1987), allowing the pluripotent stem cells to differentiate and mature into fully functional eosinophils over a period of 2-6 days. The eosinophil resides predominantly in the tissues and is mainly found in the epithelium of the respiratory and gastrointestinal tract. The eosinophil appears to be long-lived compared to the neutrophil. In the presence of growth factors such as IL-5, GM-CSF and IL-3, the eosinophil can survive *in vitro* culture for more than 7 days (Tai *et al.*, 1991). The half life of the eosinophil in the circulation is 18 h. However, as large numbers can be found in tissues even when the blood count is low, it is presumed they are able to reside in the tissues for several days (Weller, 1991).

1.3.2 EOSINOPHIL MORPHOLOGY

Eosinophils have a mean diameter of 8 μm, a bilobed chromatin-dense nucleus and a large number of distinctive intracellular granules (Sokol *et al.*, 1987). The characteristic identifying feature of eosinophils is the presence of large specific ellipsoid granules containing crystalline cores. These contain a wide variety of agents that are believed to be involved in host defence such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO) (Gleich *et al.*, 1976; Egesten *et al.*, 1986; Peters *et al.*,

1986). MBP is contained in the crystalline core whereas ECP, EDN and EPO are found in the surrounding matrix. These distinct cationic proteins contribute to varying degrees in defence against helminth parasites but have the potential to be involved in host tissue damage. Large specific granules also contain hydrolytic lysosomal enzymes and histaminase. In addition, the eosinophil possesses other so-called small granules which in contrast lack a crystalline core and contain aryl sulphatase and acid phosphatase. Although both eosinophil and neutrophil granules contain potent antimicrobial agents and toxins, granule contents may be discharged in very different ways by each cell. Whereas the neutrophil releases granule contents primarily into contained phagolysosome, eosinophil granules may be released onto extracellular parasites.

1.3.3 EOSINOPHIL RECRUITMENT AT SITES OF INFLAMMATION

Similarly to neutrophils, eosinophils will respond to chemotactic stimuli following tissue injury and will marginate and diapedese into the tissues, travelling towards the inflammatory focus along a chemotactic gradient (Resnick and Weller, 1993). Although the process of neutrophil and eosinophil recruitment are broadly similar at a molecular level, selectivity in recruitment of granulocytes is achieved through several regulatory mechanisms. For example differential expression of adhesion molecules may determine the type of granulocyte selectively recruited to the site of injury. In this regard, expression of β_1 integrin VLA-4 and β_7 integrin $\alpha_4\beta_7$ by eosinophils but not neutrophils may allow the specific accumulation of eosinophils at an inflammatory site (Weller *et al.*, 1991). The chemotactic agents present during the inflammatory response may determine further selectivity in recruitment. For example the presence of IL-8 may favour selective neutrophil accumulation whereas the presence of eotaxin, MCP-3 and RANTES may result in preferential recruitment of eosinophils (Leonard *et al.*, 1990; Griffiths-Johnson *et al.*, 1993; Ponath *et al.*, 1996; Dahinden *et al.*, 1994; Kameyoshi *et al.*, 1992; Rot *et al.*, 1992).

1.3.4 REGULATION OF EOSINOPHIL FUNCTION

Eosinophil function is also regulated by the ability of eosinophils to respond to local mediators during the inflammatory response and also by the capacity of eosinophils to synthesise and secrete mediators, which may in turn, govern and refine the subsequent immune response.

Eosinophils are capable of producing a number of cytokines that may be involved in various aspects of the inflammatory response such as IL-1α (Weller *et al.*, 1993), IL-4 (Moqbel *et al.*, 1995; Nonaka *et al.*, 1995), IL-6 (Hamid *et al.*, 1992; Melani *et al.*, 1993), IL-8 (Braun *et al.*, 1993; Kita *et al.*, 1995), TNFα (Costa *et al.*, 1993), RANTES (Lim *et al.*, 1995) and TGFβ (Wong *et al.*, 1991; Ohno *et al.*, 1992). Furthermore they also produce GM-CSF (Weller, 1992), IL-3 (Kita *et al.*, 1991a; Fujisawa *et al.*, 1994) and IL-5 (Broide *et al.*, 1992; Dubucquoi *et al.*, 1994) which may serve as autocrine growth factors. In addition, eosinophils secrete lipid mediators such as PAF (Lee *et al.*, 1984) and LTC_4 (Verhagen *et al.*, 1984), which alter permeability of the microvasculature and also cause bronchoconstriction and enhance the secretion of mucus (Henderson, Jr., 1991).

1.3.5 EOSINOPHIL FUNCTIONS AND ROLE IN DISEASE

The eosinophil is thought to play an important and specialised role in the adaptive immune response most notably against helminthic parasite worms (Butterworth *et al.*, 1975; Gleich and Adolphson, 1986). These large multicellular organisms cannot be phagocytosed and eosinophils instead defend against such parasites through release of helminthotoxins such as MBP and ECP, by extracellular release of eosinophil granule contents.

Although the eosinophil may potentially have an important role in host defence against helminthic parasites, there is a wide consensus that the eosinophil and its derived products may be important contributors in the pathogenesis of many allergic and asthmatic diseases. Extracellular release of granule products in an uncontrolled

manner may have detrimental consequences to host tissue. Many eosinophil products such as MBP are known to be toxic to mammalian cells and may produce pathological changes through alteration of lipid membranes (Kroegel *et al.*, 1987).

Airway inflammation is a distinctive feature of asthma and is characterised by a large inflammatory cell infiltrate with a notable abundance of eosinophils (Bousquet *et al.*, 1990; Djukanovic *et al.*, 1990). There is significant evidence that eosinophils and their derived mediators play a central role in the pathogenesis of this disease (Bousquet *et al.*, 1990; Martin *et al.*, 1996; Smith, 1992; Kay, 1991a; Wardlaw *et al.*, 1995). There appears to be a positive correlation between the severity of airway hyperreactivity and numbers of eosinophils found in the BAL fluid and peripheral blood of patients with asthma (Bousquet *et al.*, 1990; Bradley *et al.*, 1991). Persistent accumulation of eosinophils and the release of cytokines such as IL-4 and IL-5 from Th2 lymphocytes, may be in part be responsible for tissue accumulation of eosinophils in this disease (Kay, 1991b). It has been shown that IL-5 administration to the airways of asthmatic subjects is associated with peripheral blood eosinophilia, airway eosinophilia, and brochial hyperresponsiveness (Shi *et al.*, 1998; Shi *et al.*, 1999). It is believed that activation of eosinophils in the bronchial mucosa with subsequent release of cytokines and eosinophil granule proteins may not only be cytotoxic to the bronchial epithelium but may also increase bronchial responsiveness and amplify the asthmatic hyperreactivity.

The role of the eosinophil in the pathogenesis of asthma has however recently come into question in light of clinical reports that suggest reduction of eosinophil numbers has limited effects on airway hyperreponsiveness and the late asthmatic reaction. Leckie and colleagues report that monoclonal anti-IL-5 blocking antibodies, which substantially decrease allergen induced blood and sputum eosinophilia, fail to influence the late asthmatic reponse or alleviate airway hyperresponsiveness to histamine (Leckie *et al.*, 2000). From this particular report, it may be difficult to refute the idea of an important role for eosinophils, due to the very small sample size of this study. However, a recent study by van Rensen *et al.* suggests aerosol administration of IL-5 to mild asthmatics does not induce eosinophilic inflammation

or bronchial hyperresponsiveness, although there may be potential systemic effects of IL-5 on the bone marrow (van Rensen *et al.*, 2001). In a further study, the cytokine IL-12 was tested for its ability to suppress allergic and eosinophilic inflammation (Bryan *et al.*, 2000). IL-12 is key in regulating the balance between Th1 and Th2 cells. It is believed that allergic inflammation involves Th2 responses, which may favour eosinophil accumulation and enhance IgE synthesis. Animal studies have suggested treatment with IL-12 reduces airway hyperresponsiveness following antigen challenge (Kips *et al.*, 1996; Schwarze *et al.*, 1998). However, human studies by Bryan *et al* demonstrate that while IL-12 administration causes significant reduction of blood and sputum eosinophils, the late asthmatic response and airway hyperresponsiveness are unaffected (Bryan *et al.*, 2000). It is conceivable that approaches to suppress eosinophilic mediated inflammation by blocking a single mediator of eosinophil accumulation, may be ineffective, as only a small number of residual eosinophils capable of eliciting cytotoxic mediators, could potentially still orchestrate a detrimental inflammatory response. Furthermore, numbers of tissue dwelling eosinophils, which may be fundamentally involved in the asthmatic response, may not be effectively lowered in these studies. It will be interesting to observe if future studies demonstrate changes in the numbers of eosinophils in tissues of the airways following treatment with blocking antibodies to IL-5. Furthermore as many other mediators, in addition to IL-5, are involved in accumulation of eosinophils, a combinatorial approach to blocking eosinophil inflammation may be required for effective reduction in eosinophil accumulation and responsiveness. Verification or confirmation of the central role of the eosinophil in allergic inflammation, will therefore await future studies.

The eosinophil may also be involved in the pathogenesis of many other diseases including the development of fibrosis. In Hodgkin's disease, as in certain other malignancies, there appears to be elevated numbers of eosinophils in the circulation and tissues, which may contribute to the development of fibrosis through production of TGF-β (Kadin *et al.*, 1993).

It appears that in many chronic inflammatory diseases and distinctive cancers outlined above, the normal control mechanisms regulating eosinophil function are perturbed leading to detrimental sequelae for the host. The reasons for eosinophil dysregulation in these conditions are not fully understood however insight can be gained by examining divergence from the normal regulatory control mechanisms, which under normal physiological conditions, limit the potentially deleterious capacity of these inflammatory cells to damage host tissue. Increased production of anti-inflammatory mediators, a reduction in the effective concentrations of pro-inflammatory mediators and cellular and tissue desensitisation may all be involved. Perhaps most importantly, controlled death or apoptosis of cells, concomitant with their safe and effective removal may be essential for successful resolution of inflammation.

1.4 APOPTOSIS

1.4.1 MORPHOLOGY OF APOPTOSIS

Cell death is known to occur by two distinct mechanisms namely necrosis or apoptosis (programmed cell death). For many years it was assumed that all cells died by a process of necrosis, in which disintegration of the cell membrane ultimately leads to the uncontrolled release of cell contents (Trump *et al.*, 1981a; 1981b). A distinctive feature of necrosis is dysregulation of osmotic pressure as a consequence of plasma membrane damage by environmental insults such as physical or chemical trauma. This induces the swelling and rupturing of the plasma membrane, leading to dysregulated release of intracellular contents. As a consequence of increased cell membrane permeability, necrosis can often be measured by the ability of cells to take up dyes such as trypan blue. One could envisage that disintegration of a cell by necrosis, with particular regard to the granulocyte, could provoke undesirable inflammation that may prove deleterious to the host.

Apoptosis, in contrast to necrosis, represents a "physiological" form of cell death in which the cell dies in a highly regulated manner that importantly does not incite an

inflammatory response. There is wide consensus that apoptosis is involved in many physiological and homeostatic processes such as regulating cell numbers during embryonic development (Granerus *et al.*, 1995). Apoptosis may also allow the controlled removal of cells that could represent a threat to the integrity of the host i.e. those with DNA damage (Williams, 1991) or those infected with viruses (Vaux *et al.*, 1994).

Apoptosis was initially described with respect to a succession of morphological changes that contrasted to molecular events observed in necrosis (Wyllie *et al.*, 1980). Although apoptosis may be differentially regulated in different cell types, there are many common morphological and biochemical changes during apoptosis. Major cell surface changes such as plasma membrane ruffling, together with loss of microvilli is often evident. Furthermore, there may be exposure of phosphatidylserine on the outer leaflet of the plasmalemma, often used as an indicator of apoptotic onset. Commonly, apoptotic cells display cell shrinkage due to cytoplasmic condensation through fluid loss. The most striking changes, however, are observed in the nucleus. Condensation of chromatin forms crescent shaped aggregates around the nuclear envelope, which may coalesce into one or two dense 'pyknotic' spheres when the nucleus is multilobular. This is accompanied by activation of endogenous endonucleases resulting in internucleosomal cleavage of chromatin and the characteristic 'ladder' pattern of fragmented DNA subunits (Wyllie, 1980). Throughout this process the plasma membrane integrity is preserved and cells maintain their osmotic gradients. Cells may remain as intact apoptotic cells or may eventually form small membrane-bound apoptotic bodies that enclose the intracellular contents of the cells (Arends and Wyllie, 1991; Cohen, 1993). In either case, the apoptotic cells or bodies are rapidly phagocytosed and degraded by macrophages preventing the release of cellular contents into the extracellular milieu and limiting the inflammatory response.

1.4.2 CASPASES

A common feature in the series of events underlying apoptosis regulation is the activation of a group of cysteine proteases termed the caspases (cytosolic aspartate-specific cysteine proteases). These death proteases, which are highly conserved through evolution, are thought to be responsible for most of the morphological changes observed in apoptosis and are thus viewed as the central executioners of cell death (Budihardjo *et al.*, 1999; Cikala *et al.*, 1999; Thornberry, 1997; Cohen, 1997; Cohen, 1997; Martin and Green, 1995; Kothakota *et al.*, 1997; Rao *et al.*, 1996; Enari *et al.*, 1998).

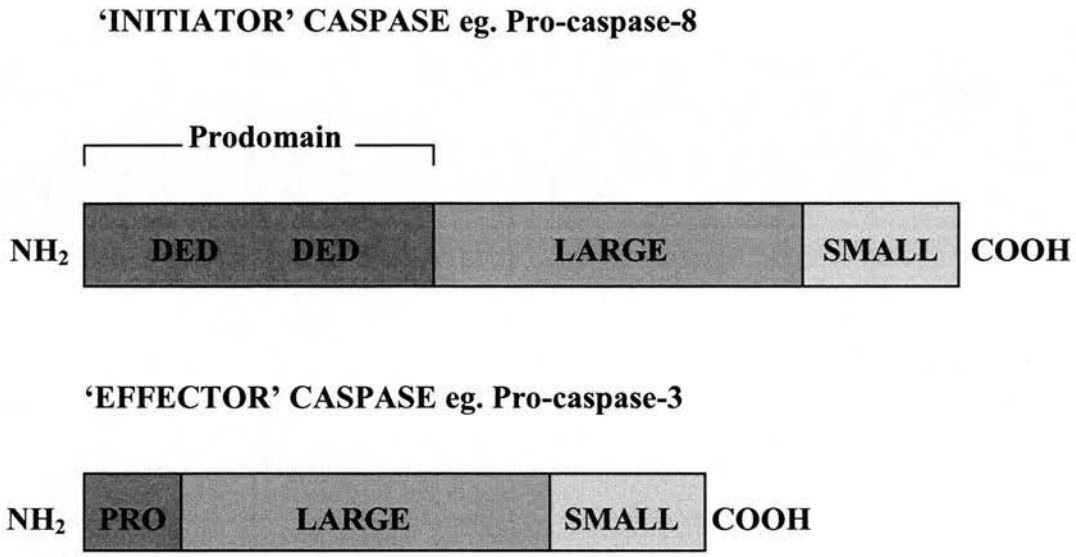
Understanding the mechanisms of apoptosis and the involvement of caspase activation has benefited from insight gained from genetic studies in nematode *Caenorhabditis elegans (C.elegans)* (Ellis and Horvitz, 1991; Hengartner and Horvitz, 1994). The *C.elegans* gene, ced-3 is required for apoptosis in the nematode and was found to have similarity to the interleukin-1β (IL-1β)-converting enzyme (ICE)-like protease (Miura *et al.*, 1993; Thornberry *et al.*, 1992; Yuan *et al.*, 1993). Since this discovery, enormous progress has been made in the identification and elucidation of the role of caspases in progression of cell death.

Caspases are synthesised as inactive pro-enzymes, which are activated by cleavage after aspartic acid residues, a process that initiates the subsequent cleavage of the next caspase in the pathway, thus stimulating a cascade of caspase activation (Harvey and Kumar, 1998; Thornberry, 1997). It is this caspase cascade which is thought to be responsible for the cleavage of cellular proteins which bring about the visible changes characteristic of apoptotic cell death (Martin and Green, 1995). The mature caspase zymogens are composed of an N-terminal prodomain and the p20 and p10 domains (Cohen, 1997). Although many caspase substrates have been identified, it is widely believed that caspases recognise and cleave a limited set of target proteins (Nicholson, 1999). Substrates include enzymes involved in DNA repair such as DNA-dependent protein kinase (DNA-PK) (Casciola-Rosen *et al.*, 1995) and poly(ADP-ribose) polymerase (PARP) (Lazebnik *et al.*, 1994), structural proteins of

the nuclear envelope such as lamins A and B (Lazebnik *et al.*, 1995; Neamati *et al.*, 1995; Orth *et al.*, 1996; Rao *et al.*, 1996) and cytoskeletal proteins such as gelsolin and fodrin (Martin and Green, 1995; Kothakota *et al.*, 1997 Buendia *et al.*, 1999).

Caspases can be separated into two distinct groups according to structural and functional differences (Thornberry and Lazebnik, 1998). The first group such as caspase 8, -9 and –10 are distinguished by a long prodomain and are involved in the initiation of the caspase cascade and are appropriately termed 'initiator' caspases. A further group known as 'effector' caspases which includes caspases 3, -6 and-7, are distinguished by a relatively short prodomain and are activated upon cleavage by initiator caspases (Figure 1.3). In general it is assumed that effector caspases become active when proteolytically cleaved by upstream caspases, usually between the p20 and p10 domains. As cleavage occurs after aspartic acid residues, it suggests the possibility of autocatalysis (Thornberry, 1997). However this mechanism of activation cannot account for activation of initiator caspases, as being the apical proteases in the pathway, they are unable to be enzymatically cleaved by upstream caspases. In view of this, two alternative mechanisms have been proposed to explain activation of initiator caspases. In the first model, initiator caspases are recruited in response to death stimuli via adaptor proteins to the ligand-receptor complex, increasing their local concentration. It is postulated that low intrinsic protease activity of the zymogen is sufficient to allow transcatalysis and autoproteolytic processing, thereby fully activating the caspase (Muzio *et al.*, 1998). This simplistic model of caspase activation by induced proximity has been implicated in the activation of caspase-8 following ligation of TNFR1 and Fas by their respective ligands (Muzio *et al.*, 1998). Caspase-2 and –10 may also be activated in this manner (Salvesen and Dixit, 1999).

A further model of caspase activation has been proposed in which enzymatic maturity of the caspase is dependent on co-factor association. This mechanism of activation is used by caspase-9 and is dependent on an essential association with the cytosolic protein Apaf-1 (Li *et al.*, 1997; Zou *et al.*, 1997). The release of cytochrome c from mitochondria is believed to induce a conformational change in



INITIATOR	EFFECTOR	UNKNOWN
Caspase-8	Caspase-3	Caspase-1
Caspase-9	Caspase-6	Caspase-2
Caspase-10	Caspase-7	Caspase-4
		Caspase-5

Figure 1.3 Proenzyme organisation of the caspases. Caspases are synthesised as proenzymes with an N-terminal prodomain (PRO) and a large and small subunit often separated by a linker peptide. Caspases are subdivided into two structurally and functionally distinct groups, 'initiator' or 'effector', characterised by long and short prodomains respectively. Caspase-8 and –10 contain N-terminal death effector domains (DED) allowing interaction with adaptor molecules such as FADD. Other caspase such as caspase-9 contain caspase recruitment domains (CARD), which permits interaction with other caspases or adaptor proteins such as IAPs.

Apaf-1, allowing it to form an active complex with caspase-9, which is believed to be the enzymatically active form of caspase-9 (Rodriguez and Lazebnik, 1999). The hierarchy of caspase activation, which ultimately may lead to the disassembly and dismantling of the cell can be separated into two distinct signalling pathways. Which of these cascades of caspase activation is induced is dependent on the whether apoptosis is initiated by cell surface 'death' receptor (extrinsic) signalling or by intracellular stress (intrinsic) signals (Figure 1.4) (For a recent review see Strasser *et al.*, 2000).

1.5 INITIATION OF APOPTOSIS

1.5.1 APOPTOSIS INITIATED BY EXTRINSIC SIGNALLING

There are two principal pathways by which apoptosis may be initiated. In the first mechanism, activation of cell death is initiated through the engagement of cell surface death receptors belonging to the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. Members of this family include TNFR1, Fas/APO-1/CD95, DR-3/Apo-3/TRAMP, DR4/TRAIL-R1 and DR5/TRAIL-R2/TRICK2 (Schmitz *et al.*, 2000; Ashkenazi and Dixit, 1998). The extracellular domains of these receptors share characteristic cysteine-rich motifs, however, a subset of this superfamily, including Fas and TNFR1, contain an important 68 amino acid region of homology in their cytoplasmic tail called the 'death domain' (DD), which as the name implies is essential for apoptotic signalling (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993; Boldin *et al.*, 1995). Death receptor signalling involves a well characterised sequence of events commencing with ligand-induced receptor trimerisation, followed by the recruitment of receptor-associated proteins and finally initiation of caspase activation. Upon receptor activation, the death domain of the cell surface death receptor undergoes homotypic interaction with death domain containing proteins such as TRADD and FADD, recruiting them to the so-called death inducing signalling complex (DISC) (Chinnaiyan *et al.*, 1995). Death receptor signalling through Fas involves direct recruitment of FADD to the DISC whereas

TNFR1 binds FADD indirectly via TRADD. The adaptor protein FADD is essential for cell death signalling from both receptors and through an N-terminal 'death effector domain' (DED), allows recruitment of DED containing procaspase-8 and/or procaspase-10 to the DISC. As a result, procaspase-8 is proteolytically cleaved into its active form and in turn initiates cleavage of downstream caspases (Muzio *et al.*, 1996; Srinivasula *et al.*, 1996; Muzio *et al.*, 1998). Caspase-8 may also cleave the BH3 only protein Bid, allowing its recruitment to the mitochondria, inducing the release of cytochrome c (Luo *et al.*, 1998). As will be discussed in the next section, cytochrome c release forms an essential part of cell death mediated by stress-induced stimuli. There is controversy regarding the importance of mitochondrial perturbation and cytochrome c release in death receptor signalling. It is likely that death receptors may initiate differential signalling pathways and depending on cell type may or may not require mitochondrial release of cytochrome c (Scaffidi *et al.*, 1998). Receptor activation through TNFR1 complex may also lead to recruitment of the serine-threonine kinase RIP (Receptor interacting protein) and TRAF2 (TNFR associated factor 2) through TRADD. RIP and TRAF2 both seem to be involved in NFκB activation (Liu *et al.*, 1996; Ting *et al.*, 1996). Additionally TRAF2 allows activation of JNK (Natoli *et al.*, 1997; Reinhard *et al.*, 1997).

It is perhaps not surprising that there are regulatory mechanisms in place to control and inhibit death receptor signalling when required. There are multiple levels of regulation, including inhibitory proteins and decoy receptors. The latter bind ligands such as TRAIL, and Fas ligand but are unable to transduce normal death signals due to truncated death domains (Marsters *et al.*, 1997; Pitti *et al.*, 1998). Thus, decoy receptors may prevent normal death stimuli from binding their appropriate receptor, preventing apoptosis (Pan *et al.*, 1997; Sheridan *et al.*, 1997; Degli-Esposti *et al.*, 1997). Apoptosis induced by death receptor activation may also be inhibited by FLICE-like inhibitory proteins (FLIPs) which possess DED domains allowing interaction with FADD, procaspase-8 and –10, thereby by preventing normal recruitment of procaspases to the DISC by competitive inhibition (Cryns and Yuan, 1998; Hu *et al.*, 1997a; Thome *et al.*, 1997; Irmler *et al.*, 1997; Tschopp *et al.*, 1998).

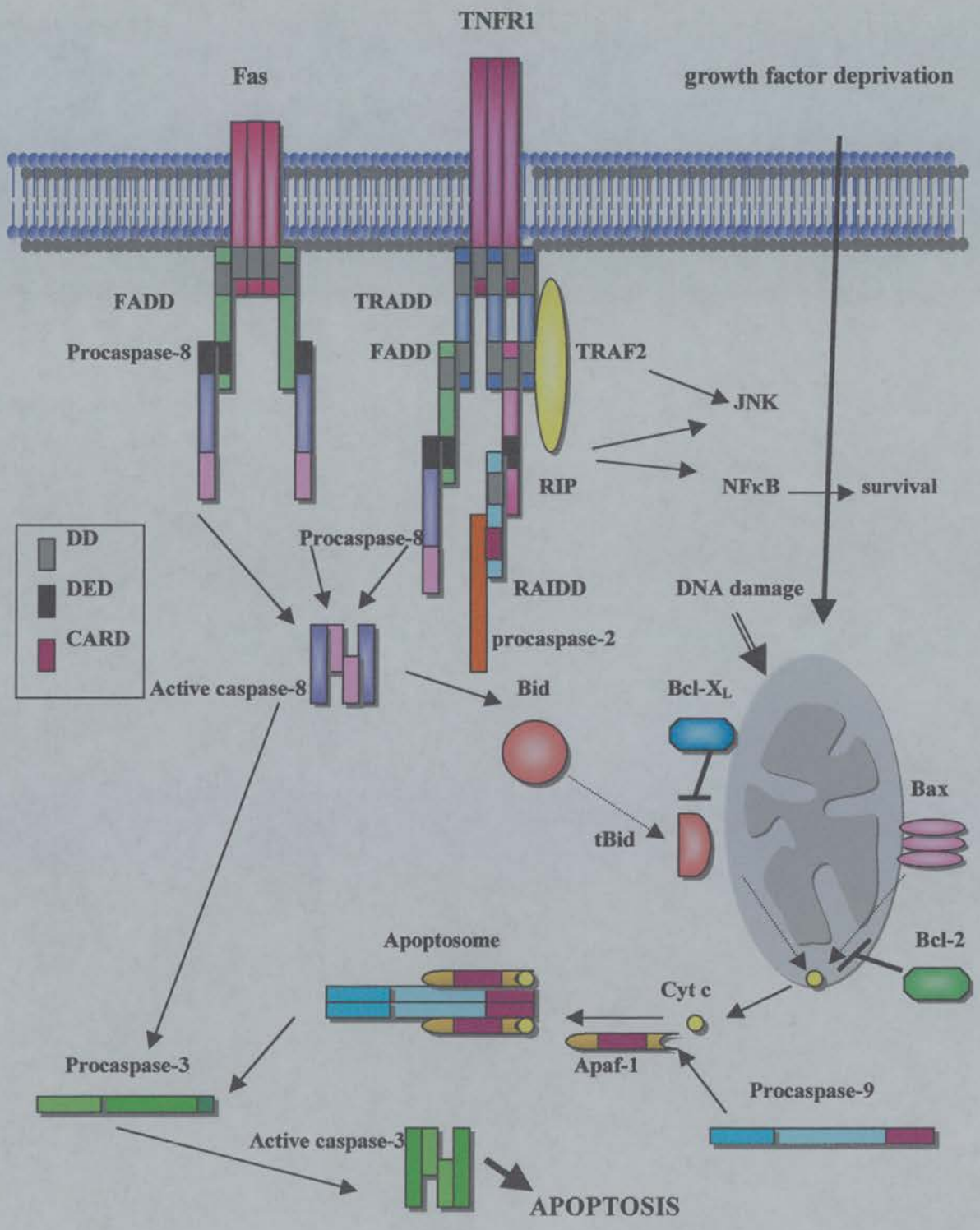


Figure 1.4 Death and non death receptor apoptotic signalling pathways Ligation of death receptors induces processing of caspase-8 and subsequent activation of effector caspases. Caspase-8 cleavage of Bid induces release of cytochrome c from the mitochondria. Stress/chemical induced apoptosis also results in release of cytochrome c causing processing of caspase-9 within the apoptosome complex.

It is interesting to note that immune evasion strategies may involve manipulation of death receptor signalling mechanisms. For example viral proteins such as crmA that can directly inhibit autocatalysis of procaspase-8 and –10 preventing their activation and consequently preventing death receptor signals from being transduced (Ray *et al.*, 1992; Komiyama *et al.*, 1994). Prevention of cell death by inhibiting activation of the caspase cascade may constitute an important viral counter-measure against host defence during infection.

1.5.2 APOPTOSIS INITIATED BY INTRINSIC SIGNALLING

An alternative mechanism for apoptosis to that triggered by cell surface death receptors, may result as a consequence of growth factor withdrawal, chemical treatment (e.g. staurosporine) or DNA damage to the cell. Activation of this death pathway in response to internal insults or extracellular cues, is thought to converge on the mitochondria, which may be the central control point of this pathway. The first suggestion of mitochondrial involvement in apoptosis, resulted from studies which demonstrated mitochondria to be required for cytosolic extracts to induce apoptotic changes in isolated nuclei from *Xenopus* eggs (Newmeyer *et al.*, 1994). Further fractionation of cytosolic extracts revealed that cytochrome c, a resident protein of the mitochondrial intermembrane space, was necessary to activate the apoptotic effector caspases (Kluck *et al.*, 1997). Since these initial findings there have been many advances in understanding the extent of mitochondrial involvement in apoptosis and the molecular events which may converge on the mitochondria, leading to commitment to cell death.

Cytochrome c forms an important part of a high molecular weight complex termed the 'apoptosome', which is composed of cytochrome c, Apaf-1 and procaspase-9 (Zou *et al.*, 1999; Cain *et al.*, 1999). Once released from mitochondria, cytochrome c binds to Apaf-1, which recruits and activates procaspase-9 in the presence of ATP or dATP (Li *et al.*, 1997; Hu *et al.*, 1999; Zou *et al.*, 1999). Activation of procaspase-9 is mediated by means of conformational change, not proteolysis. As a result, activation of caspase–9 then processes and activates other caspases to orchestrate the

biochemical execution of cells (Slee *et al.*, 1999). Apaf-1 shares an 85 amino acid region of homology to the prodomains of several caspases and functions as a caspase recruitment domain (CARD) (Hofmann *et al.*, 1997). The central region of Apaf-1 contains a nucleotide-binding domain, which is essential for function (Zou *et al.*, 1997). This region shows structural and functional homology to the *C. elegans* death promoting protein Ced-4 (Zou *et al.*, 1997). The C-terminal region is made up of WD-40 repeats which mediate protein-protein interactions. As mitochondrial cytochrome c is located in the intermembrane space, passage of cytochrome c through the outer mitochondrial membrane is critical for activation of the caspase cascade.

In addition to cytochrome c, other mediators involved in regulation of caspase activation and apoptosis are also released from mitochondria. In some cells, mitochondria contain a pool of procaspase-3 that is liberated into the cytosol during induction of apoptosis (Mancini *et al.*, 1998). Another caspase activating protein, namely 'apoptosis inducing factor' (AIF), can also be released from the intermembrane space of mitochondria (Susin *et al.*, 1996; Susin *et al.*, 1997).

The integrity of the outer mitochondrial membrane, appears to be an important regulator of the release of apoptotic mediators and consequently the control of induction of apoptosis. In light of this, it is perhaps not surprising there are several control mechanisms in place to regulate this process, which involve several members of the Bcl-2 family of proteins.

1.6 REGULATION OF MITOCHONDRIA BY BCL-2 FAMILY MEMBERS

Members of the expanding family of Bcl-2 like proteins have emerged as important regulators of programmed cell death. However, until recently the mechanisms by which Bcl-2 related proteins regulate apoptosis have remained unknown. There are currently 15 Bcl-2-like proteins identified in mammals (Gross *et al.*, 1999)

characterised by the presence of α-helical conserved regions termed Bcl-2 homology domains (BH1-BH4) which allow homo- and heterotypic protein interactions (Kelekar and Thompson, 1998; Oltvai *et al.*, 1993; Reed *et al.*, 1996). Bcl-2 like proteins are either anti-apoptotic (e.g. Bcl-2, Bcl-X_L) and contain at least three BH domains or are pro-apoptotic (e.g. Bax, Bak) and contain two or more BH domains (Chao and Korsmeyer, 1998; Reed, 1998). Some pro-apoptotic members of this family are more distantly related to Bcl-2 and possess only the BH3 domain (e.g. Bid and Bad) which is required for both their ability to transduce apoptotic signals and for their interactions with other family members (Kelekar and Thompson, 1998; Chao and Korsmeyer, 1998; Reed, 1998). As Bcl-2 family members have the ability to localise constitutively or inducibly to the outer mitochondrial membrane, it has been proposed that they regulate apoptosis through an ability to control mitochondrial compartmentalisation of cytochrome c (Goping *et al.*, 1998; Antonsson *et al.*, 2000; Antonsson *et al.*, 2001; Gross *et al.*, 1999). It has been shown that the anti-apoptotic proteins Bcl-2 and Bcl-X_L, prevent release of mitochondrial cytochrome c in response to death stimuli (Yang *et al.*, 1997; Vander Heiden *et al.*, 1997). In contrast, the pro-apoptotic Bcl-2 family member Bax has been demonstrated to accelerate programmed cell death (Oltvai *et al.*, 1993) and can induce release of cytochrome c when added directly to isolated mitochondria (Jurgensmeier *et al.*, 1998; Eskes *et al.*, 1998). Although members of the Bcl-2 family have been implicated in regulating mitochondrial release of cytochrome c, the precise biochemical events by which this occurs has not been fully elucidated. Currently there are three basic models by which Bcl-2 like proteins have been proposed to regulate cytochrome c release from mitochondria. These are described below:

1.6.1 FORMATION OF ION CHANNELS BY BCL-2 FAMILY MEMBERS

The structural similarity of Bcl-X_L to diphtherial bacterial toxin, which is known to allow the transport of ions by forming pores in lipid bilayers, suggested Bcl-2 proteins may have similar properties (Muchmore *et al.*, 1996). Subsequently, it has been demonstrated that Bcl-X_L, tBid and Bax, can form functional ion channels in

synthetic lipid vesicles and planar bilayers (Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997; Antonsson *et al.*, 1997). However, there is no evidence that Bcl-2 family proteins form channels *in vivo*. Furthermore, it is uncertain whether the diameter of such channels would be sufficient for proteins such as cytochrome c to pass through.

1.6.2 FORMATION OF LARGE PROTEIN CHANNELS BY BCL-2 FAMILY MEMBERS

Bcl-2 family members may also be able to facilitate release of apoptosis-inducing factors by interacting with other proteins in the outer mitochondrial membrane to form a large pore. Pro-apoptotic Bcl-2 family members may co-operate with the voltage dependent anion channel (VDAC) and form a large cytochrome c conducting channel (Shimizu *et al.*, 1999). It could be envisaged that pro-apoptotic proteins such as Bax and Bak, may allow opening of the channel, whereas anti-apoptotic proteins such as Bcl-X_L, may facilitate its closure (Shimizu *et al.*, 1999). It is predicted that VDAC on its own would be unable to form channels big enough to allow cytochrome c to pass through. Instead, it is proposed that a conformational change in VDAC would be required, together with co-operation Bcl-2 family proteins, to allow formation of a megapore of the appropritate size (Shimizu *et al.*, 2000).

Other studies have suggested that some BH3-only proteins such as Bid alone can act through a separate undefined pathway, which is sufficient to cause release of cytochrome c from mitochondria. It remains elusive how BH3-only proteins can mediate release of cytochrome c, although it is possible that they may associate with novel proteins in the outer mitochondrial membrane to allow redistribution of cytochrome c (Wang *et al.*, 1996a; Luo *et al.*, 1998; Li *et al.*, 1998).

1.6.3 REGULATION OF OUTER MITOCHONDRIAL MEMBRANE RUPTURE BY BCL-2 FAMILY MEMBERS

A further proposed mechanism to account for cytochrome c redistribution during apoptosis, may involve the non-specific disruption of the outer mitochondrial membrane. This disruption could occur following alterations in mitochondrial physiology as a consequence of apoptotic signalling, allowing the diffusion of proteins through the lipid bilayer. Non-specific rupture of the outer mitochondrial membrane may involve the opening of a large conductance channel called the mitochondrial permeability transition pore (PTP) (Zamzami *et al.*, 1996a; Qian *et al.*, 1997; Crompton, 1999). Opening of the PTP results in collapse of the mitochondrial inner transmembrane potential ($\Delta\Psi m$) and matrix swelling (Zamzami *et al.*, 1996b). As a result of organellar swelling, the outer mitochondrial membrane may rupture, causing the release of apoptosis inducing factors localised within the intermembrane space (Kroemer *et al.*, 1997). It is possible that Bcl-2 family members could directly regulate PTP activity (Marzo *et al.*, 1998; Narita *et al.*, 1998). Evidence to support this model of mitochondrial disruption is implied from the ability of inhibitors of PT pore opening such as bongkrekic acid and cyclosporin to block apoptosis in some (Zamzami *et al.*, 1996a), but not all systems (Eskes *et al.*, 1998). At present it is controversial whether mitochondrial depolarisation as a result of PT pore opening initiates the induction of apoptosis or if PTP opening is a late event which occurs as a consequence of apoptosis.

In summary, Bcl-2 family members are important regulators of apoptosis and this may depend in part on their ability to control mitochondrial release of cytochrome c. It could be envisaged that regulation of apoptosis by Bcl-2 like proteins involves a balance of pro-apoptotic and anti-apoptotic signals possibly at the level of the mitochondria, which may determine cellular fate in many systems.

1.7 GRANULOCYTE APOPTOSIS

As described in section 1.2.3, granulocytes are recruited to inflammatory sites in response to infection or tissue injury. In acute inflammation, neutrophils accumulate in tissues due to targeted influx from the circulation in response to inflammatory stimuli. However the recruitment of large numbers of inflammatory cells also increases the potential for tissue damage through liberation of destructive enzymes or toxic oxygen metabolites. This is exemplified in asthmatic and allergic inflammation where accumulation of eosinophils in the bronchial mucosa, and subsequent release of eosinophil granule products may lead to bronchial epithelial cell damage and contribute to the pathogenesis of asthma (Walsh, 2001). The beneficial contribution of inflammatory cell recruitment must be balanced by the safe removal and cessation of cell accumulation when these cells are no longer required.

The majority of circulating granulocytes are eliminated by the reticuloendothelial system of the liver and spleen. Extravasated granulocytes are not thought to return to the blood stream (Haslett and Henson, 1988) and instead meet their fate at the inflammatory site. It was originally assumed granulocytes would die *in situ* by cellular necrosis once their contribution to the inflammatory response was complete (Hurley, 1983). However, death by necrosis results in the release of toxic granule contents into the extracellular milieu, thereby inciting an exaggerated and potentially deleterious inflammatory response. Since host tissue damage does not normally arise following an acute inflammatory response, it is likely that resolution of inflammation must involve a clearance mechanism in which the cells are removed in a manner that limits the release of inflammatory mediators and granule contents. It is believed that termination of the inflammatory role of granulocytes occurs by death by apoptosis and subsequent clearance of intact cells by tissue macrophages (Savill *et al.*, 1989; Savill *et al.*, 1990; Savill, 1992).

1.7.1 MOLECULAR CHANGES IN GRANULOCYTES UNDERGOING APOPTOSIS AND CLEARANCE

Granulocytes will undergo constitutive apoptosis during *in vitro* culture and exhibit classic changes associated with apoptosis including cytoplasmic condensation and internucleosomal cleavage of DNA by endogenous endonucleases (Savill *et al.*, 1989). There are several changes to expression of cell surface molecules during granulocyte apoptosis, some of which may be unique to these cells, such as loss of CD16 (FcγRIII) expression in neutrophils (Dransfield *et al.*, 1994). Other cell surface changes such as exposure of phosphatidylserine on the outer leaflet of the plasmalemma, may be important for phagocytic recognition of apoptotic granulocytes (Fadok *et al.*, 1992). There is also downregulation of the functional capacity of granulocytes during apoptosis, with cells having a reduced ability to generate a respiratory burst, degranulate or undergo chemotaxis in response to external stimuli (Whyte *et al.*, 1993). This decreased responsiveness and reduced adhesive capacity, together with exposure of ligands facilitating apoptotic cell recognition, may serve to limit tissue injury and promote resolution of inflammation.

A variety of studies, including experimental models of glomerulonephritis (Savill *et al.*, 1992a) and endotoxin induced lung injury (Cox *et al.*, 1995), have given evidence to support a role for apoptosis in the clearance of apoptotic neutrophils *in vivo.* Furthermore, it has been demonstrated *in vitro* that apoptotic granulocytes are rapidly recognised and ingested by macrophages (Haslett *et al.*, 1989; Stern *et al.*, 1992; Savill *et al.*, 1989; Savill, 1992; Meagher *et al.*, 1992), without elicitation of proinflammatory mediators but importantly with release of anti-inflammatory mediators such as IL-10 (Voll et al., 1997) and TGF-β (Fadok *et al.*, 1998). Thus removal of apoptotic granulocytes by this manner could be crucial in resolution of the inflammatory process and in the disposal of effete granulocytes from the sites of inflammation. Macrophages are the principal cells involved in clearance of apoptotic granulocytes although semi-professional phagocytes such as fibroblasts and glomerular mesangial cells may also recognise and ingest apoptotic neutrophils (Savill *et al.*, 1992a; Hall *et al.*, 1994; Hughes *et al.*, 1997).

Several molecules have been implicated in apoptotic cell recognition including the phosphatidylserine receptor (Fadok *et al.*, 2000), the vitronectin receptor $\alpha_v\beta_3$ integrin (Savill *et al.*, 1990), CD36 (Savill *et al.*, 1992b), the ATP binding cassette transporter (ABC1) (Luciani and Chimini, 1996), class A scavenger receptor (Platt *et al.*, 1996) and the CD14 receptor (Devitt *et al.*, 1998). Considering the number of receptors that have been proposed to be involved, there may be functional redundancy in the molecular pathways that are used for phagocytic cell clearance. There may be complex subtleties in the involvement of each of these molecules in apoptotic cell recognition, perhaps with some molecules engaged in initial recognition of the apoptotic cell, while others may be preferentially used in engulfment. It is probable that many phagocyte surface receptors implicated in apoptotic cell recognition, may act in concert. However, research is still required to resolve apparent differences in receptor usage between different phagocyte and apoptotic cell populations and to understand the precise regulatory mechanisms underlying apoptotic cell clearance.

1.7.2 REGULATION OF GRANULOCYTE APOPTOSIS BY BCL-2 FAMILY MEMBERS

As previously discussed, members of the Bcl-2 family have been well characterised as important regulators of apoptosis. It is thought that the relative levels of these pro- and anti-apoptotic proteins and their molecular interactions, are crucial in determining whether a cell will survive or become apoptotic. Mature neutrophils do not express Bcl-2 (Delia *et al.*, 1992; Hannah *et al.*, 1994) but do express the pro-apoptotic proteins Bax (Moulding *et al.*, 1998), Bik (Moulding *et al.*, 2001) and Bak (Bazzoni *et al.*, 1999) and the anti-apoptotic proteins Mcl-1 and A1 (Moulding *et al.*, 1998; Moulding *et al.*, 2001). Eosinophils on the other hand are thought to express Bcl-X_L (Dibbert *et al.*, 1998) Bax (Druilhe *et al.*, 1998; Dibbert *et al.*, 1998; Dewson *et al.*, 2001) and in some reports, low levels of Bcl-2 (Druilhe *et al.*, 1998). There have been some discrepancies in the literature, due to different methods of detection and variability in levels of expression, perhaps reflecting the maturation and activation status of the cells being examined. Thus, caution must be taken when

trying to correlate expression of these family members with granulocyte survival. Expression of Mcl-1, another Bcl-2 family member, is reportedly decreased prior to onset of apoptosis in neutrophils but is increased in response to the apoptosis delaying agents, LPS and GM-CSF (Moulding *et al.*, 1998). Neutrophils also express mRNA for A1, a further anti-apoptotic member of the Bcl-2 family however reliable expression of A1 at the protein level is yet to be obtained (Chuang *et al.*, 1998; Moulding *et al.*, 2001). Both Mcl-1 and A1 are reported to be expressed in eosinophils, however, convincing evidence of a correlation between expression of these proteins and eosinophil apoptosis is yet to be produced (Druilhe *et al.*, 1998; Dibbert *et al.*, 1998).

1.7.2 REGULATION OF GRANULOCYTE APOPTOSIS BY EXTRACELLULAR STIMULI

Although granulocytes undergo constitutive apoptosis, it is apparent that this process is not immutable and it is well established that many inflammatory mediators can prolong or suppress granulocyte survival by altering the rate of apoptosis (Haslett *et al.*, 1991) (Figure 1.5). Considering extravasated granulocytes die by apoptosis *in situ* at inflammatory foci, the ability of exogenous inflammatory mediators and cytokines to differentially modulate granulocyte survival, has important consequences for resolution of the inflammatory response. Thus, the longevity and resolution of an acute inflammatory response may depend on exogenous stimuli present at an inflammatory focus. Pro-inflammatory mediators including cytokines such as GM-CSF, G-CSF, IFN-γ, bacterial products such as LPS and chemotactic peptides such as C5a, delay granulocyte apoptosis (Brach *et al.*, 1992; Colotta *et al.*, 1992; Stern *et al.*, 1992; Lee *et al.*, 1993). It is important to note that granulocytes show differential responsiveness to apoptotic stimuli compared with other leukocytes. Indeed, there appears to be differences in the regulatory mechanisms controlling apoptosis in neutrophils and eosinophils (Figure 1.5). This may be due to the presence of distinct receptors on the surface of the eosinophil compared to the neutrophil. For example IL-5 can profoundly enhance eosinophil survival yet does not modulate neutrophil apoptosis (Tai *et al.*, 1991; Yamaguchi *et al.*, 1991).

Similarly IL-8 can suppress neutrophil apoptosis but has no effect on eosinophil longevity (Leuenroth *et al.*, 1998; Kettritz *et al.*, 1998). The situation is further complicated by the ability of certain cytokines such as TNFα to have bi-phasic effects; *in vitro* TNFα will induce neutrophil apoptosis at early time points while at later time points apoptosis is inhibited by this cytokine (Murray *et al.*, 1997).

Granulocyte apoptosis may also be modulated by stimulation of the Fas signalling pathway, with reports that both neutrophils and eosinophils are susceptible to induction of apoptosis by Fas mediated signals (Liles *et al.*, 1996; Renshaw *et al.*, 2000; Iwai *et al.*, 1994; Brown *et al.*, 1997). Initial findings that neutrophils could produce and secrete FasL, led to the proposal that Fas/FasL could regulate spontaneous granulocyte apoptosis (Liles *et al.*, 1996). However this is somewhat controversial as induction of granulocyte death has been achieved using Fas activating antibodies but not reproducibly with soluble FasL (Brown *et al.*, 1997; Renshaw *et al.*, 2000). Moreover, constitutive apoptosis in neutrophils isolated from FasL (gld)- and Fas (lpr)-deficient mice appears to proceed at a rate comparable to neutrophils obtained from control mice, arguing against a role for Fas–FasL in the regulation of constitutive neutrophil death (Fecho and Cohen, 1998; Villunger *et al.*, 2000).

Other extracellular modulators of granulocyte apoptosis include nitric oxide (NO), which can promote neutrophil apoptosis (Fortenberry *et al.*, 1998; Fortenberry *et al.*, 1999; Ward *et al.*, 2000) but delay cell death in eosinophils (Beauvais *et al.*, 1995). Moreover hypoxia, in contrast to many other cell types, delays neutrophil apoptosis (Hannah *et al.*, 1995). This may have important implications as neutrophil lifespan may be increased in the hypoxic environment of the tissues following extravasation from the circulation during the inflammatory response. As yet, little is known of the regulatory mechanisms which underlie regulation of apoptosis by these conditions.

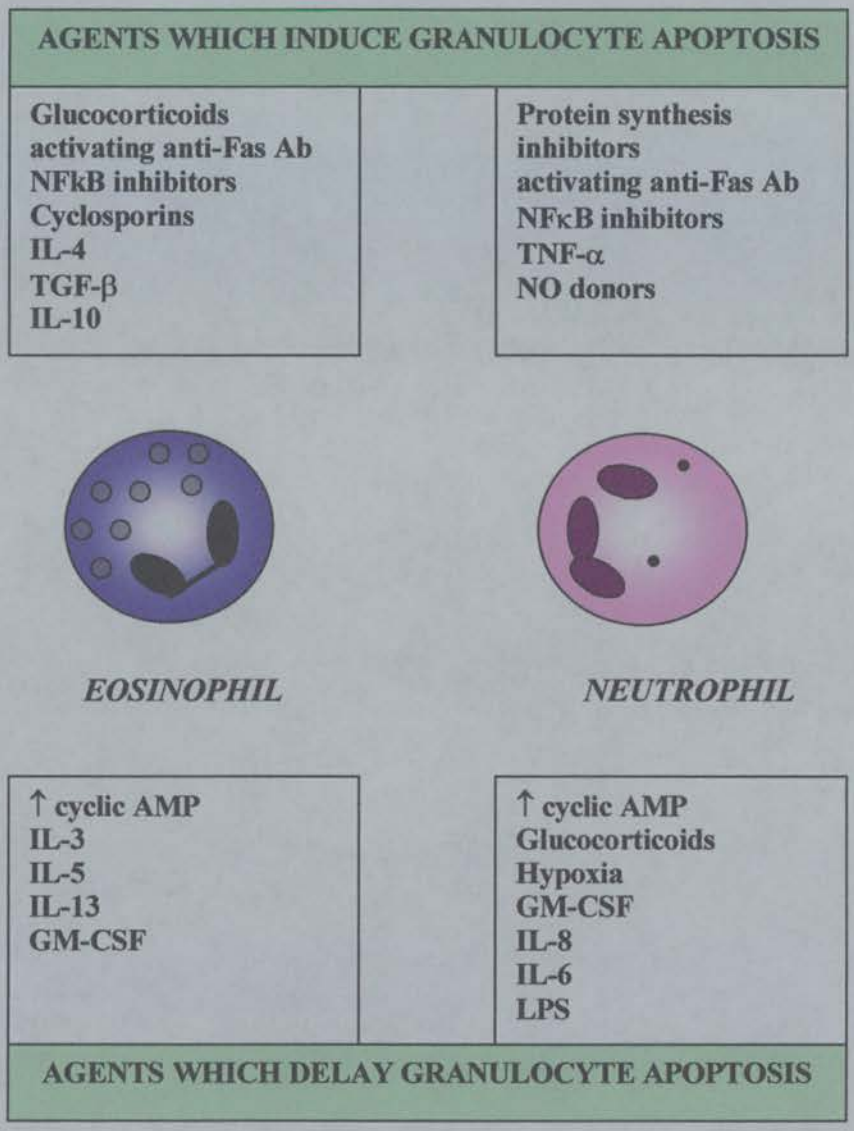


Figure 1.5 Factors which influence the acceleration or delay of constitutive apoptosis in granulocytes

Granulocyte apoptosis *in vitro* is accelerated by inhibitors of protein synthesis such as cycloheximide and actinomycin D suggesting that granulocyte survival is prolonged by the expression of one or more survival proteins (Whyte *et al.*, 1997). It has also been reported that many agents which delay granulocyte apoptosis require new protein synthesis for their survival enhancing effects (Brach *et al.*, 1992; Hachiya *et al.*, 1995; Kato *et al.*, 1995; Cox and Austin, 1997). It is possible that extracellular modulators of neutrophil survival may achieve their effects through upregulation of transcription of genes important for survival or alternatively by postranslational modification of pre-existing proteins, which may alter their cellular activity.

1.7.3 SIGNALLING PATHWAYS REGULATING GRANULOCYTE APOPTOSIS

Despite the wide numbers of agents implicated in modulation of granulocyte apoptosis, surprisingly little has been described of the intracellular signalling pathways used by these agents to regulate cell death. Dissection of the signalling pathways leading to granulocyte death have been hindered by the inability of these cells to be transfected by conventional methods. Elucidation of the signalling pathways involved has relied principally on pharmacological approaches.

There is evidence that granulocyte apoptosis is regulated by activation of phosphorylation cascades by extracellular agents through cell surface receptors. Many studies have implicated a role for the mitogen-activated protein kinase (MAPK) signal transduction cascade in the regulation of granulocyte apoptosis. There are three different types of MAP kinases: the p42/p44 extracellular signal-related protein kinases (ERKs), the c-Jun N-Terminal kinase/stress activated MAPKs (JNKS/SAPKs) and p38 MAPKs. The role of p38 MAPK in regulation of granulocyte survival is somewhat controversial. Some reports suggest a role for this signalling pathway in activation of constitutive neutrophil apoptosis (Aoshiba *et al.*, 1999) although further studies have not supported these findings (Villunger *et al.*, 2000; Frasch *et al.*, 1998). Paradoxically, it appears that p38 MAPK is required for

irradation-induced apoptosis and also in the survival effects of hypoxia (Frasch *et al.*, 1998; Leuenroth *et al.*, 2000). Thus, at present the exact function of p38 MAPK in the signalling pathways controlling survival and death of granulocytes is still uncertain.

Activation of classical p42/p44 ERKs appear important in the regulation of constitutive granulocyte apoptosis. For example, LPS, IL-8 and GM-CSF stimulate survival pathways in neutrophils, which are dependent on the activation of MEK and presumably therefore ERKs (Nolan *et al.*, 1999; Klein *et al.*, 2000). Additionally, survival signals stimulated by IL-8 and GM-CSF appear to involve activation of the phosphoinositide 3-kinase (PI-3K) signalling pathway (Klein *et al.*, 2000). PI-3 kinase has been implicated as an important pathway in apoptotic control through the ability of PI-3 kinase to activate Akt, which in turn phosphorylates the Bcl-2 family member, Bad (del Peso *et al.*, 1997; Franke and Cantley, 1997). Phosphorylation of Bad sequesters Bad from Bcl-X_L resulting in binding to 14-3-3 in the cytosol, reducing its pro-death properties (Zha *et al.*, 1996). Whether Akt and Bad phosphorylation are required for the survival pathways stimulated by GM-CSF and IL-8 has yet to be determined.

The downstream targets of both ERK and PI-3K in neutrophils, are not yet characterised. However, from other cell systems it is likely that NFκB and other transcription factors may be involved. Indeed, NFκB has been shown to be an important survival signal in neutrophils and is involved in the anti-apoptotic effects of TNFα in these cells (Ward *et al.*, 1999a). Blockade of NFκB activation will rapidly induce apoptosis in neutrophils (Ward *et al.*, 1999a) which is in contrast to thymocytes and HL60 cell lines where NFκB activation is required for cell death (Slater *et al.*, 1995; Hettmann *et al.*, 1999; Boland *et al.*, 2000). However, not all agents that delay granulocyte apoptosis activate NFκB and it is likely that other transcriptional regulators may be involved in regulating cell survival (McDonald *et al.*, 1997).

Constitutive granulocyte apoptosis may also be regulated through the activity of the serine/threonine kinase PKC and, in particular, the PKC isoform PKCδ may be involved in control of neutrophil apoptosis (Pongracz *et al.*, 1999; Khwaja and Tatton, 1999). Blockade of PKC activity pharmacologically with Ro318220 or using the non-selective PKC inhibitor staurosporine, induce granulocyte apoptosis although the downstream substrates of PKC activated by this signalling pathway have not been fully identified (Cousin *et al.*, 1997).

Second messengers appear to be important regulators of apoptosis in diverse cell types and it is therefore perhaps unsurprising that these molecules are potent regulators of apoptosis in granulocytes. In contrast to the induction of apoptosis in thymocytes (McConkey *et al.*, 1990; Suzuki *et al.*, 1991) and leukaemic cell lines (Lanotte *et al.*, 1991), elevation of the second messenger cyclic AMP can profoundly delay cell death in both neutrophils and eosinophils (Rossi *et al.*, 1995; Ottonello *et al.*, 1998). The intracellular signalling pathway of cyclic AMP is well characterised but remarkably little is known of the underlying regulatory signal transduction cascades involved in the ability of cyclic AMP to modulate apoptosis. Manipulation of cyclic AMP levels, through phosphodiesterase (PDE) inhibition, has been a target for treatment of chronic airways disease, due to the ability of cyclic AMP to mediate relaxation of airways smooth muscle and suppress inflammatory cell responsiveness to secretory agonists (Underwood *et al.*, 1993; Dent and Giembycz, 1996; Lad *et al.*, 1985; Wada, 1989; Dent *et al.*, 1994). However, the observations that cyclic AMP may delay granulocyte apoptosis together with reports that cyclic AMP can inhibit macrophage phagocytosis of apoptotic cells (Rossi *et al.*, 1998) may have important implication for the potential usefulness of PDE inhibitors in treatment of asthma.

Also applicable to chronic airways disease is the observation that glucocorticoids exert differential effects on granulocyte apoptosis (Meagher *et al.*, 1996). Glucocorticoids are known to be potent anti-inflammatory agents and have been most widely used in the treatment of chronic inflammatory diseases such as rheumatoid arthritis and asthma (Barnes, 1998). Although glucocorticoids exert divergent effects on many cell types, the efficacy of steroid treatment in asthma may

relate in part to their ability to induce eosinophil death. Further, it has been demonstrated that glucocorticoids *in vivo* reduce the number of eosinophils in airway secretions (Woolley *et al.*, 1996). Considering the ability of glucocorticoids to increase macrophage phagocytosis of apoptotic cells (Liu *et al.*, 1999), the anti-inflammatory capacity of glucocorticoids may partially rest in their ability to both induce apoptosis concomitant with upregulation of normal clearance mechanisms. Surprisingly very little has been elucidated of the signalling pathways and regulatory mechanisms involved in the divergent effect on granulocyte apoptosis.

In summary, there is good evidence implicating glucocorticoids and second messengers such as cyclic AMP in the regulation of apoptosis in granulocytes, however their mechanisms of action are still obscure. The mechanism by which these agents may regulate the activation of the members of the caspase family and how this in turn modulates endonuclease activation and cell death, is not well understood. Although granulocytes express several members of the Bcl-2 family, it is unclear how agents which modulate the rate of granulocyte apoptosis, such as cyclic AMP and glucocorticoids, integrate with the regulatory control mechanisms of Bcl-2 family proteins. Moreover, little progress has been made to understanding why one signal may induce apoptosis in eosinophils yet inhibit or delay neutrophil cell death. Understanding these complex interactions in the apoptotic cascades regulating granulocyte death could lead to development of novel strategies to therapeutically induce apoptosis for the resolution of inflammation. Furthermore, as there appear to be significant differences in apoptotic regulation between granulocytes and other immune cells and additionally between neutrophils and eosinophils, there may be the opportunity to induce apoptosis in inflammatory cell types selectively, for therapeutic gain.

1.8 AIMS OF THESIS

The principal aims of this thesis were to determine the signalling mechanisms involved in cyclic AMP and glucocorticoid regulation of human granulocyte apoptosis.

- Preliminary studies established that elevation of cyclic AMP caused a marked enhancement of neutrophil and eosinophil longevity by inhibiting apoptosis. Studies were undertaken to investigate the influence of cyclic AMP on key cellular events such as caspase activation and dissipation of mitochondrial transmembrane potential, which may regulate constitutive granulocyte apoptosis

- Subsequent experiments, designed to further assess the involvement of this second messenger in the regulation of granulocyte apoptosis, investigated the cyclic AMP signalling transduction pathway directly and examined the involvement of transcriptional regulation in cyclic AMP mediated control of granulocyte cell death. As cyclic AMP delays apoptosis in both neutrophils and eosinophils, studies of the signalling pathways involved were performed primarily in neutrophils.

- It was also established that glucocorticoids differentially regulate granulocyte apoptosis; promoting eosinophil apoptosis while delaying neutrophil cell death. Studies were undertaken to elucidate the glucocorticoid signal transduction pathways involved in regulation of apoptosis in both cell types. The involvement of transactivation and transrepression of gene transcription in both glucocorticoid mediated enhancement of neutrophil longevity, and glucocorticoid promotion of eosinophil cell death, were also examined.

2 MATERIALS AND METHODS

2.1 MATERIALS AND BUFFERS

All materials were purchased from Sigma (Poole, Dorset) unless otherwise indicated:

Blocking Buffer

TBS with 0.1% Tween-20 and 5% Milk powder (Marvel). Made fresh.

ELISA Blocking Buffer

TBS containing 0.1% bovine serum albumin (BSA). Made fresh.

ELISA Wash Buffer

TBS containing 0.1% Tween-20. Kept at room temperature.

ELISA Substrate Buffer

Sodium acetate-citrate 100 mM pH 4.9 (80 ml 0.1M sodium acetate with 17 ml 0.1M citric acid, dH_2O up to 100 ml). Made fresh.

ELISA Substrate solution

100 µl TMB ((3,3'.5,5'-Tetramethylbenzidine) dissolved at 10 mg/ml in DMSO - stored in dark for up to 2 weeks) added to 10 ml ELISA substrate buffer with 5 µl H_2O_2 (30%) added just prior to use. Made up fresh.

FACS Annexin V binding buffer

500 ml Hanks Balanced Salt Solution (Gibco, Paisley, UK, H9394) with 5 µM $CaCl_2$ (final). Kept at 4°C.

Methanol Based Transfer Buffer

14.6 g Glycine, 2.9 g Tris-HCl, 200 ml methanol, dH_2O up to 1000 ml. Made fresh.

NP-40 Cytoplasmic Lysis Buffer for protease sensitive proteins (cLBPSP)

10 mM HEPES pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM ABSEF, 10 µg/ml aprotinin, 2 mM levamisole, 10 µg/ml leupeptin, 1mM sodium orthovanadate, 0.5 mM benzamidine, 10 mM β-glycerophosphate, 10 µg/ml pepstatin A, 1 mM phenanthroline, 1 mM PMSF. Made up fresh.

NP-40 Nuclear Lysis Buffer for protease sensitive proteins (nLBPSP)

50 mM HEPES pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10 % glycerol, 0.5 mM ABSEF, 10 µg/ml aprotinin, 2 mM levamisole, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, 0.5 mM benzamidine, 10 mM β-glycerophosphate, 10 µg/ml pepstatinA, 1 mM phenanthroline, 1 mM PMSF. Made up fresh

PKA Extraction Buffer

25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM beta-mercaptoethanol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM PMSF and 1% Triton X-100. Made up fresh.

Sample buffer (x3)

1 ml stacking gel buffer, 1 ml 20 % SDS, 500 µl β-mercaptoethanol, 1 ml glycerol, bromophenol blue. Store at 4°C.

SDS-Tris Glycine Electrophoresis Buffer (x10)

250 mM Tris-HCl, 1.92 M Glycine, 1% SDS. Kept at room temperature.

Separating Gel Buffer

36.30 g Tris-HCl, 0.8 g SDS, dH_2O up to 100 ml pH 8.9

Stacking Gel Buffer

5.1 g Tris-HCl , 0.4 g SDS, dH_2O up to 100 ml pH 6.7

Standard NP40 cytoplasmic lysis buffer

50 mM sodium fluoride, 5 mM tetra sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.5% NP40, 2 mM EDTA, 20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 1 protease inhibitor tablet. Stored at –20°C.

Standard NP40 nuclear lysis buffer

50 mM sodium fluoride, 5 mM tetra sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.5% NP40, 2 mM EDTA, 20 mM Na_2HPO_4, 20 mM NaH_2PO_4 300 mM NaCl, 1 protease inhibitor tablet. Stored at –20°C.

Tris Buffered Saline (TBS)

Tris-HCl (20 mM) pH 7.4, NaCl (15 mM)

Western Wash Buffer

TBS with 0.1% Tween-20. Kept at room temperature.

Triton X-100 Lysis Buffer

10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.4. and 1 protease inhibitor tablet (1 per 20 ml lysis buffer). Stored at –20°C.

Further materials were purchased from the following companies:

Affiniti Research Products Ltd, Mamhead, Exeter, UK: z-VAD-fmk methyl ester, lactacystin, epoxomicin.

Affinity Bioreagents Inc, Golden, CO, USA: polyclonal rabbit anti-human glucocorticoid receptor antibody (PA1-511), polyclonal rabbit anti-human glucocorticoid receptor beta antibody (PA3-514).

Amersham Pharmacia Biotech, UK Ltd, Buckinghamshire, UK: Hybond C nitrocellulose membrane, horse radish peroxidase-conjugated donkey anti-rabbit secondary antibody, Phosphorus-33 (370MBq/ml, 10 μci/ml).

Baxter Healthcare Ltd, Baillieston, Glasgow, Scotland, UK: Diff Quik™ stain. Solution I (Eosin G in phosphate buffer, pH 6.0), Solution II (Thiazine blue in phosphate buffer; pH 6.0), saline solution 0.9% (sterile).

Boehringer Mannheim, Germany: Annexin-V-FLUOS

Calbiochem-Novabiochem UK, Nottingham, UK: Rp-8-Br-cAMPS sodium, trichostatin A Streptomyces sp, dibutyryl-cyclic AMP sodium, H-89 Dihydrochoride, PD 98059, LY 294002, GGTI-286, geldanamycin

Cell Signalling Technology, Beverly, MA, UK: polyclonal rabbit anti-IκBα antibody

DAKO Ltd Cambridgeshire, UK: Horse radish peroxidase-conjugated, goat anti-mouse, secondary antibody and rabbit immunoglobulin fraction (normal).

Dynal UK, Wirral, UK: Dynabeads M-450 sheep anti-mouse IgG. Supplied as 4 x 10^8 beads/ml in PBS pH 7.4 with 0.1% human serum albumin and 0.2% sodium azide.

Genzyme Diagnostics, Kent, UK: GM-CSF (1000 U/ml in PBS) was stored at -70°C.

Gibco Life Technologies, Paisley, Scotland, UK: Iscove's Dulbecco's modified Eagles medium, without supplements with L-glutamine (Iscove's MDM); Hanks Balanced Salt Solution (HBSS); culture supplements penicillin (50 U/ml)/streptomycin (50 U/ml); L-glutamine (200 mM); 10 % SDS; and 30 % (w/v) acrylamide/bis solution.

Martindale Pharmaceuticals Ltd, Romford, UK: calcium chloride.

Millipore, Bedford, Mass, USA.: Immobilon-P PVDF

Molecular Probes Inc, Eugene, OR, USA: TO-PRO-3, iodide (642/661), JC-1 [5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazocarbocyaniniodide].

Organon Laboratories Ltd, Cambridge, UK: Dexamethasone.

Phoenix Pharmaceuticals Ltd, Gloucestershire, UK: Sodium citrate solution (3.8%).

Promega Corporation, Southampton, UK: SignaTECT^{TM} cAMP-Dependent Protein Kinase (PKA) Assay System

R&D Systems Europe Ltd, Oxon, UK: TNFα (stock solution 10 μg/ml); mouse IgG_1 anti-human IL-8 monoclonal antibody (stock solution 500 μg/ml), biotinylated

anti human IL-8 polyclonal antibody (stock solution 50 μg/ml), human IL-8 (stock solution.

Shandon, Pittsburgh, PA, USA: Shandon Filter Cards

Spectrum Companies, Gardena, CA, USA: Spectra/Por Molecular porous membrane tubing MWCO 3,500

Transduction Laboratories, San Diego, California, USA: Rabbit anti-human caspase-3 polyclonal antibody, rabbit anti-human Bax polyclonal antibody, mouse IgG_{2b} PKA R_I antibody, mouse IgG_{2b} PKAc antibody.

TCS Biologicals, Botolph Clayton, Bucks, UK: anti-Fas human clone CH-11

The following reagents were kindly donated as gifts: RU24858 and RU27842 were obtained from Roussel UCLAF, Romainville, Cedex, France; ZK77945 and ZK55740 from Schering AG, Berlin, Germany; *Clostridium sordelli* lethal toxin from M.R Popoff, Centre National de Reference des Bacteries Anaerobies, Institut Pasteur, Paris; polyclonal rabbit anti-SLPI antibody from J.M Sallenave, CIR, University of Edinburgh; NH3 mouse monoclonal antibody from I.Dransfield, CIR, University of Edinburgh and murine anti-neutrophil antibody 3G8 from Dr J. Unkeless, Mount Sinai Medical School, New York.

2.2 CELL ISOLATIONS AND PURIFICATIONS

2.2.1 ISOLATION OF HUMAN GRANULOCYTES FROM PERIPHERAL BLOOD

Human neutrophils were purified from the peripheral blood of healthy human volunteers by modification of previously described methods (Haslett *et al.*, 1985; Dransfield *et al.*, 1994). Neutrophil isolation was performed at room temperature, under sterile conditions and using endotoxin-free reagents and plasticware (Falcon, Oxford, UK). Venous blood was collected into 50 ml polypropylene tubes, anticoagulated (4 ml 3.8% sodium citrate/36 ml blood) and centrifuged (350g, 20 min., room temperature). This gives two layers, an upper layer containing plasma and platelets, and a lower layer containing a mixture of erthyrocytes and leukocytes. The platelet-rich plasma (PRP) was aspirated and used to prepare autologous serum in glass tubes by the addition of $CaCl_2$ (220 µl of 1 M $CaCl_2$ added to 10 ml PRP) at 37°C. To sediment the erythrocytes, 5 ml of 6% dextran (T500 pre-warmed to 37°C) was added to the pelleted cells and the volume made up to 50 ml with 0.9 % saline (pre-warmed to 37°C). The tubes were mixed gently and the cells allowed to sediment for ~30 min at room temperature resulting in formation of two distinguishable layers; a bottom layer containing mainly sedimented erythrocytes and an upper leukocyte-rich layer. The leukocyte-rich layer was aspirated, centrifuged (350g, 6 min) and the supernatant discarded. The resulting leukocyte pellet was resuspended in 2.5ml of 55% isotonic Percoll (9:1 v/v Percoll: 10 x PBS) in 1 x PBS without divalent cations. Discontinous Percoll gradients were prepared by overlaying 2.5ml of 68% Percoll onto 2.5ml of 79% isotonic Percoll in a 15 ml Falcon tube. Leukocytes were then resuspended in 55% Percoll and overlayed to form the final layer of the gradient. The gradients were centrifuged (720g, 20 min) and polymorphonuclear cells harvested from the 68%/79% Percoll interface. Mononuclear cells sedimented at the 55%/68% Percoll interface. Purified cells were washed sequentially in PBS twice and cell yield assessed using a haemocytometer. Although the above density gradient centrifugation method does not separate neutrophilic from eosinophilic or basophilic granulocytes, however harvested

polymorphonuclear cells generally consisted of <3% eosinophils, and basophils were rarely seen. Preparations of granulocytes containing >5% eosinophils were used for preparation and study of eosinophils as described in section 2.2.2. Cell viability was assessed by trypan blue exclusion and was routinely >99%. The typical yield for this isolation method was 100 x 10^6 polymorphonuclear cells/40 ml whole blood. Cells were minimally activated by the methods used for cell preparation as outlined above.

2.2.2 PREPARATION OF HUMAN EOSINOPHILS FROM PERIPHERAL BLOOD

Eosinophils were prepared from polymorphonuclear cells, isolated as described above (Section 2.2.1). Separation of eosinophils from neutrophils was achieved through negative selection by immunomagnetic separation using the murine anti-neutrophil antibody 3G8 (anti-CD16)-coated sheep anti-mouse IgG-Dynabeads. CD16-Dynabeads were prepared under sterile conditions by combining 3G8 supernatants with Dynal M450 sheep anti-mouse dynabeads (10 ml supernatant: 500µl beads), in a 15ml falcon tube. The antibody/bead mixture was rotated at 4°C for at least 20 mins to allow antibody binding. The coated beads were then sequentially washed 4 times in PBS without divalent cations (4°C) and the beads retrieved using a stationary contact (3 min) with a magnet (Dynal Magnetic Particle concentrator, MPC-1).

Granulocytes, isolated as in Section 2.2.1 were incubated with washed 3G8-Dynabeads at a bead : granulocyte ratio of 3:2 on a rotary mix at 4°C for 10 min and the beads with attached neutrophils were magnetically separated by stationary contact (3 min) with a magnet. This procedure was repeated once. Purity was assessed by light microscopy of cyto-centrifugated cells, stained with Diff-Quik™. Purified eosinophils (> 98%) were washed and centrifuged (220g, 5 min) twice in PBS without divalent cations, before cell yield was assessed by haemacytometer counts.

2.2.3 SEPARATION OF APOPTOTIC NEUTROPHILS FROM NON APOPTOTIC NEUTROPHILS

Purification of apoptotic neutrophils from a mixed population of aged neutrophils was performed according to the method of Dransfield *et al.*, (1994). Non-apoptotic neutrophils were removed using immunomagnetic separation with sheep anti mouse IgG-Dynabeads (Dynabeads M-450, Dynal, Merseyside, United Kingdom) coated with the murine anti-neutrophil antibody 3G8 (anti-CD16; a gift from Dr J. Unkeless, Mount Sinai Medical School, New York). Cells were mixed with washed antibody-coated magnetic beads on a rotary mixer at 4 °C for 20 minutes, and the beads removed magnetically by two 3 minute stationary magnetic contacts (Dynal Magnetic Particle Concentrator, MPC-1) to yield an apoptotic neutrophil preparation (> 99%).

2.3 CELL CULTURE

2.3.1 GRANULOCYTE CULTURE

Unless otherwise stated, freshly isolated neutrophils and eosinophils were routinely suspended at a density of 5 x 10^6/ml or 2.5 x 10^6/ml respectively in Iscove's DMEM supplemented with 10% autologous serum, 50 U/ml penicillin, and 50 U/ml streptomycin. Cells were cultured in a final volume of 150 µl in flat-bottomed 96-well Falcon flexiwell plates (Becton-Dickinson, UK) at 37°C in a humidified, 5% CO_2 atmosphere for the time periods indicated. Cells showed minimal activation and remained non-adherant during the culture period, making this *in vitro* culture system a good model for examining constitutive granulocyte apoptosis. Reagents to be examined in this assay system were diluted to 10 x the final concentration required in Iscove's DMEM before addition of 15 µl of each reagent to be investigated.

2.4 ASSESSMENT OF APOPTOSIS

2.4.1 ASSESSMENT OF APOPTOSIS BY MORPHOLOGICAL CRITERIA

Neutrophil apoptosis was assessed morphologically according to the method of Savill *et al* (Savill *et al.*, 1989). Cells were gently resuspended and 100 µl of cell suspension (approximately 5 x 10^5 neutrophils) harvested from each well, cytocentrifuged (300 rpm, 3 min) and the resulting slide preparations air dried, fixed in methanol and stained with Diff-Quik™ and cells were counted using oil immersion light microscopy. Cell recovery was measured in parallel using a haemocytometer and cell viability assessed by trypan blue exclusion. Apoptotic cells were defined as those containing one or more darkly stained pyknotic nuclei (see Sections 3.1 and 4.1). At least 500 cells were counted over five fields of view, with slides prepared in triplicate per well and the observer blinded to the experimental conditions.

2.4.2 ASSESSMENT OF APOPTOSIS BY ANNEXIN V BINDING

In addition, apoptosis was assessed by flow cytometry using FITC-labelled recombinant human Annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells. A working solution of Annexin V was made from stock Annexin V (Annexin-V-FLUOS, Boeringer), diluted 1:3000 with Annexin V binding buffer (see buffers). Neutrophils (20 µl of 5 × 10^6/ml) were added to 200 µl of the working solution of Annexin V-FLUOS before being assessed by flow cytometry on a FACSCalibur (Becton Dickinson, Oxford, UK) and analysed on associated CellQuest (Becton Dickinson) software. All experiments were performed at least three times unless otherwise indicated.

2.5 MEASUREMENT OF MITOCHONDRIAL DISSIPATION

Changes in mitochondrial potential were measured in neutrophils following stimulation using JC-1 [5,5',6,6'-tetrachloro-1,1',3,3'-tetra-ethyl-benzimidazocarbo-cyaniniodide] (Molecular Probes), a cationic dye which exhibits potential dependent accumulation in mitochondria indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). Mitochondrial depolarisation is indicated therefore by a decrease in the red/green fluorescence intensity ratio. JC-1 (10 μg/ml) was diluted in PBS from stock JC1 (5 mg/ml in DMSO) and added to neutrophils (1×10^6/ml) for 10 min at 37°C. Neutrophil mitochondria labelled with JC-1, were examined by confocal fluorescent microscopy together with TO-PRO-3 (1 μM) (Molecular Probes) to assess neutrophils with necrotic morphology. Alternatively neutrophils labelled with JC-1 were assessed by flow cytometric analysis using FACSCalibur (Becton Dickinson, Oxford, UK) and analysed on associated CellQuest (Becton Dickinson) software.

2.6 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

Interleukin-8 (IL-8) secretion was analysed by detecting soluble protein in aliquots of supernatants collected from granulocyte cultures at 24 h. Reagents for IL-8 detection and standards were purchased from R&D systems (Oxon, UK). Briefly, anti-cytokine capture antibodies were diluted to 4 μg/ml in PBS. 100 μl of diluted capture antibody was added to the wells of enhanced protein binding ELISA plates (Corning, NY, USA). The plates were sealed and incubated overnight at 37°C. Capture antibody was removed, and wells were washed 3 times with ELISA wash buffer, 200 μl/well. 100 μl of ELISA blocking buffer was added to each well and the plates incubated at 37°C for 1 h. Wells were washed 3 times with ELISA wash buffer (200 μl/well) and 100 μl of standards and samples were added to the wells (appropriately diluted in ELISA wash buffer). Plates were sealed and incubated for 1 h at 37°C. Wells were washed 3 times with 200 μl ELISA wash buffer and 100 μl of 20 ng/ml biotinylated detection antibody diluted in ELISA blocking buffer was

added to each well. Plates were incubated for 1 h at room temperature after which wells were washed 3 times with 200µl ELISA wash buffer and 100µl of 1 in 2000 diluted Streptavidin-HRP (Amersham Life Sciences, Amersham, U.K.) was added to the wells and incubated for 20 mins at room temperature. Wells were washed 3 times with ELISA wash buffer and 100 µl of substrate solution was then added. Plates were placed in the dark for development before the reaction was stopped by the addition of 50 µl H_2SO_4 to each well and read on a Microplate Reader 450 (BioRad Laboratories, Hemel Hempstead, U.K.) and associated software.

2.7 SIGNALLING

2.7.1 MEASUREMENT OF PKA ACTIVITY

PKA activity was measured using Promega's SignaTECT^{TM} cAMP-Dependent Protein Kinase (PKA) Assay System which utilises biotinylated Kemptide (LRRASLG), a peptide substrate derived from the *in vivo* substrate pyruvate kinase. Unless otherwise stated, neutrophils (5 x 10^6 cells) were pre-incubated with control buffer or 10 µM H89 (Calbiochem, Nottingham, UK) for 1 h in PBS with Ca^{2+}/Mg^{2+} at 37 °C before being stimulated with 0.2 mM dbcAMP or 1 µM PGE_2, for 30 min at 37 °C. Following one wash in ice cold PBS, neutrophils were resuspended in 0.5 ml of cold PKA extraction buffer. The lysates were centrifuged (5 min; 4 °C; 14,000 g) and the supernatants retained. The PKA reaction mix consisting of 5 µl of 5 × PKA Assay Buffer, 5 µl of cyclic AMP (0.025 mM), 5 µl of PKA Biotinylated Peptide Substrate (0.5 mM), 5 µl [gamma-^{33}P]ATP mix (5 µl 0.5 mM ATP and 0.05 µl [gamma-^{33}P]ATP (3,000Ci/mmol) 10µCi/µl) was mixed gently and pre-incubated at 30 °C for 5 min. A control reaction without substrate was performed to determine background counts. The PKA activity reaction was initiated by adding 5 µl of the lysates to the reactants and incubated at 30°C for 5 min. The reaction was terminated by adding 12.5 µl of Termination buffer to each sample (Promega, Southampton, UK). Aliquots (10 µl) from each terminated reaction sample were spotted onto prenumbered SAM^{2TM}

Membrane squares (Promega, Southampton, UK). The SAM^{2TM} Membrane squares containing the spotted samples were then washed 1 × 30 seconds with 200 ml 2 M NaCl (Sigma, Poole, Dorset, UK) followed by 3 × washes for 2 min of 200 ml 2 M NaCl then 4 × washes for 2 min of 200 ml 2 M NaCl in 1% H_3PO_4. Finally the Membrane squares were quickly washed in deionized water before being allowed to dry. PKA activity was measured by scintillation counting.

2.7.2 WESTERN BLOTTING

A number of lysis methods were employed to extract neutrophil proteins as inconsistent results and glucocorticoid receptor (GR) proteolysis were found when standard lysis methods were utilised (Figures 2.1 and 2.2). These problems occurred despite inclusion of a broad spectrum of protease inhibitors and may reflect the major protease content of neutrophils.

2.7.2.1 Extraction of proteins from neutrophils using Triton X-100 lysis buffer

200 µl of Triton X-100 lysis buffer (see buffers) was added to the PBS washed, pelleted (10 x 10^6) neutrophils and the cells thoroughly resuspended by repeat pipetting. The samples were then incubated on ice for 10 min before being centrifuged (20000g, 4°C, 10 min). The resulting supernatants were stored at -80°C prior to analysis. Prior to electrophoresis, 15 µl of 3 x sample buffer was added to 30 µl of lysate and samples boiled for 2 min, 95°C.

2.7.2.2 Extraction of proteins from neutrophils using boiling sample buffer

Washed cell pellets were resuspended in 40 µl of 1x sample buffer, preheated to 95°C. Samples were then vortexed and sonicated before being stored at 4°C.

2.7.2.3 Extraction of proteins from neutrophils using NP40 lysis buffer

100 µl of cytoplasmic NP40 lysis buffer (see buffers) was added to the washed, pelleted neutrophils and the cells thoroughly resuspended by repeat pipetting. The samples were then incubated on ice for 10 min before being centrifuged (4300g, 4°C, 10 min). Supernatants were harvested and stored at -80°C prior to analysis. To the remaining pellet, 50 µl of nuclear NP40 lysis buffer was added and the samples incubated on ice for 10 min before being centrifuged (23100g, 4°C 10 min). Supernatants were saved and stored at –80°C prior to analysis

2.7.2.4 Extraction of proteins from neutrophils using NP40 lysis buffer for proteins sensitive to proteases (PSP)

100 µl of cytoplasmic NP-40 lysis buffer (cLBPSP) (see buffers) was added to the washed, pelleted neutrophils and the cells thoroughly resuspended by repeat pipetting. The samples were incubated on ice for 10 min to help aid neutralisation of surfaces proteases. Following the 10 min incubation time, 10 µl of NP-40 was added, the sample flick-mixed, before being centrifuged (4300g, 10 min). Supernatants were harvested and immediately added to 50µl of 3 x sample buffer, before being boiled for 2 min, 95°C. The remaining cell pellet was resuspended in 50 µl of nuclear NP-40 lysis buffer (nLBPSP) (see buffers) and the samples placed at 4°C with constant shaking, to solubilise nuclear proteins. After 20 min incubation the samples were centrifuged (23100g, 10 min) and the supernatants added to 25µl of 3 x sample buffer before being boiled for 2 min, 95°C.

2.7.3 ASSESSMENT OF PROTEIN CONCENTRATIONS

Protein concentrations were quantified using a BCA protein assay (Pierce, IL, USA). This assay is based on the ability of protein present in the test samples to cause a reduction of Cu^{2+} to Cu^{+} and bicinchoninic acid (BCA) to chelate Cu^{+} forming a purple compound which can be measured using spectrophotometry (562 nm) (Smith

et al., 1985). Samples were diluted 1 in 10 in dH_2O and 10 µl incubated with 200 µl of test solution (30 min, 37°C) in 96 well plates prior to analysis using an automated plate reader (MR5000, Dynatech, UK). Samples were assayed in triplicate and standard curves formed using pre-made BSA standards.

2.7.4 SEPARATION OF PROTEIN EXTRACTS BY POLYACRYLAMIDE GEL ELECROPHORESIS (PAGE)

Protein extracts (50 µg protein) were subject to SDS-PAGE on polyacrylamide gels. Samples were electrophoresed at 150 Volts using SDS-Tris-Glycine electrophoresis buffer (Mini Protean II apparatus, Biorad, CA) for one hour beside pre-stained molecular weight markers (Gibco BRL, Paisley, U.K.). Unless otherwise stated, proteins were transferred to nitrocellulose (Hybond C, Amersham Pharmacia Biotech, Amersham, U.K.) using methanol based transfer buffer at 60 Volts for 1 hr. Non-specific protein binding sites on the nitrocellulose membrane were blocked by incubation of the membranes in 50 ml of blocking buffer for one hour at 37°C. Membranes were then incubated at 4°C overnight in 3 ml blocking buffer containing the appropriate primary antibodies with constant shaking. This was followed by 3 sequential washes (5 min, 25°C) in Western wash buffer. Membranes were then incubated with the appropriate horse-radish peroxidase (HRP)-conjugated secondary antibodies either (a) HRP-Goat anti mouse Ig (Dako Corporation, Cambridege, UK) or (b) HRP-donkey anti rabbit Ig (Amersham) diluted 1 in 2000 in blocking buffer for one hour at room temperature with gentle shaking. Membranes were washed a further 3 times with 50 ml wash buffer before 1 min incubation with ECL reagent (Amersham). Excess ECL reagent was removed and the membrane placed under BioMax MS-1 X-ray sensitive film. Films were processed through an X-ray developer (X-Ograph Imaging Systems, Wilts, U.K.) at various exposure time points (1min, 3min, 5 min and 10 mins).

2.7.5 WESTERN BLOTTING FOR THE GLUCOCORTICOID RECEPTOR (GR)

When neutrophil lysates, prepared using a standard Triton X-100 based lysis buffer (section 2.7.2.1), were separated with SDS-PAGE and the Western blots developed using an anti-GR antibody, several bands were observed (Figure 2.1A), suggesting non-specific binding of the rabbit polyclonal antibody or severe protein degradation during the neutrophil lysis. A similar result was observed when neutrophil lysates were prepared by direct lysis of neutrophils using boiling Laemelli buffer (Section 2.7.2.2, Figure 2.1B). Furthermore neutrophil lysates prepared using a standard NP-40 lysis buffer, failed to produce a band of the appropriate molecular weight for GR (Section 2.7.2.3, Figure 2.2A). To investigate if problems in western blotting for GR were due to poor antibody binding, anti-GR antibody was tested against pre-prepared human epidermal A431 cell line lysates from Santa Cruz and A431 and neutrophil cytoplasmic extracts prepared by a standard NP40 lysis buffer (Section 2.7.2.3, Figure 2.2Aand 2.2B). Figure 2.2A shows a Western blot of neutrophil proteins prepared using by a standard NP-40 lysis buffer in which neutrophils do not appear to express GR. Expression of GR was found in A431 cytoplasmic lysates prepared by the same lysis method or in pre-prepared A431 lysates from Santa Cruz (Figure 2.3). Neutrophil cytoplasmic extracts could be successfully immunoblotted for actin (NH3) indicating that extraction method was sufficient for detecting high abundance proteins in neutrophils (Figure 2.2B). From this result it would appear that anti-GR antibody does recognise GR in A431 cells yet cannot detect GR expression in neutrophils. This suggests that difficulty in detection of GR in neutrophils is not due to poor antibody binding but may instead reflect either a low expression level of GR in neutrophils or degradation of GR in these cells, despite inclusion of a broad range of protease inhibitors in the lysis buffer. To investigate if difficulty in detection of GR in granulocytes was due to proteolytic degradation of GR in these cells, we further optimised a standard NP40 lysis protocol (Section 2.7.2.4) to include a high protease inhibitor content lysis buffer and incubation of granulocytes with protease inhibitors before lysis to neutralise surface proteolytic activity. Using this lysis method, a band of the appropriate molecular weight to GR could be consistently

detected in neutrophils (See Figures 4.4.1A and 4.4.1B). This suggests that protease activity of granulocytes is a major problem for successful immunoblotting and consequently, this optimised NP40 lysis protocol was used for all other immunoblotting.

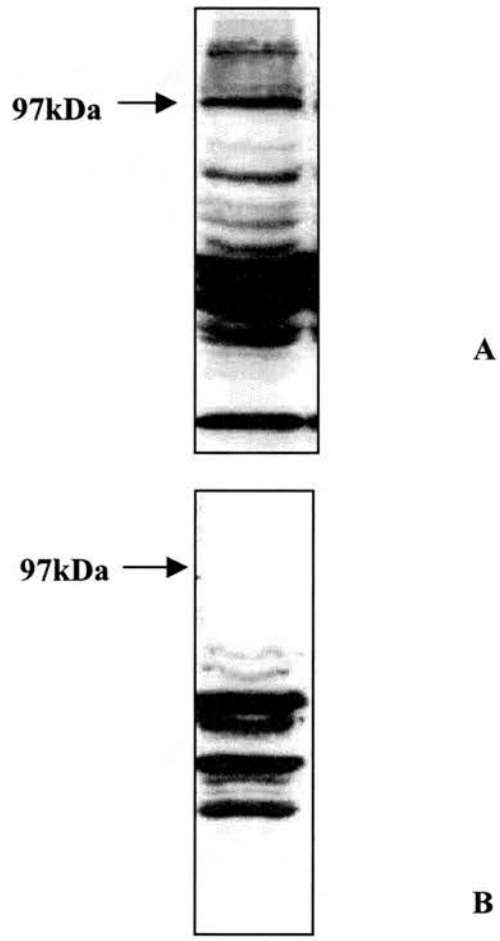


Figure 2.1 Immunoblotting for GR in neutrophil lysates. Neutrophil lysates were prepared using a standard Triton X-100 lysis buffer (A) or by direct addition of boiling Laemelli buffer (B). Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using a 8.5 % polyacrylamide gel and membranes probed with a rabbit anti-human GR antibody (PA1-511). The gels are representative of 3 experiments.

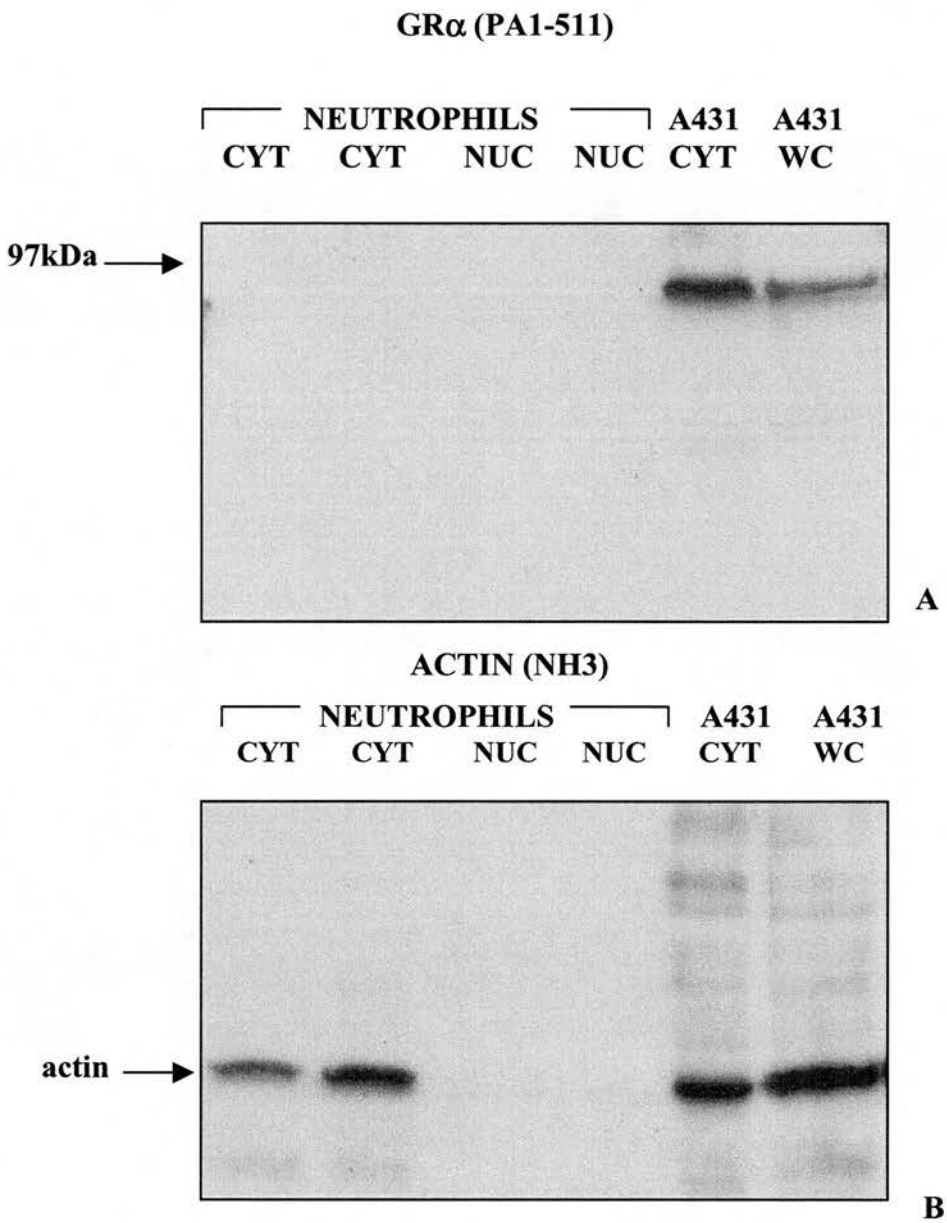


Figure 2.2. Western blot analysis for GRα in neutrophils. Neutrophil and A431 cytoplasmic (CYT) and nuclear (NUC) lysates were prepared using standard NP40 lysis buffer and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using antibodies against either GRα (PA1-511) (A) or actin (NH3) (B). As a positive control the last lane contains a whole cell lysate (WC) supplied by Santa Cruz made from A431 cell lines.

2.7.6 WESTERN BLOTTING FOR SECRETORY LEUKOCYTE PROTEINASE INHIBITOR (SLPI)

Neutrophils and eosinophils were cultured at 5 x 10^6/ml or 2.5 x 10^6/ml respectively in Iscove's DMEM supplemented with 10% autologous serum, 50 U/ml penicillin, and 50 U/ml streptomycin. Cells were cultured for 20 h in the presence or absence of appropriate concentrations of dexamethasone. The supernatants were then harvested and dialysed overnight using dialysis tubing (MWCO 3,500) to remove excess salts from the medium. Supernatants were run on a 15 % polyacrylaminde gel and Western blotting was performed as in Section 2.7.4 using a rabbit anti-human SLPI antibody (gift from J.Sallenave).

2.8 STATISTICAL ANALYSIS

Results are reported either as pooled data from a series of n separate experiments (mean ± S.E.) or as individual representative experiments (mean ± SD, 3 replicates/condition). Statistical significance was assessed by the students t-test or, by one way analysis of variance with comparisons between groups made using the Newman-Keuls procedure.

3 CYCLIC AMP REGULATION OF GRANULOCYTE APOPTOSIS

Cyclic AMP is a key second messenger that plays a central role in regulating a multitude of cellular processes. It is widely recognised that cyclic AMP is involved in the control of many cellular events occurring in the immune system such as proliferation, differentiation and chemotaxis. For example, cyclic AMP induces eosinophilic differentiation of human leukaemia cell line Eol-1 (Jung *et al.*, 1994) and potentiates granulocytic differentiation of retinoid induced maturation of human myeloid leukaemia cells (Olsson and Breitman, 1982). Cyclic AMP is also able to influence development of the immune system through its ability to affect T cell effector function. Elevation of cyclic AMP has been shown to both inhibit lymphocyte proliferation (Estes *et al.*, 1971; Skalhegg *et al.*, 1992; Bauman *et al.*, 1994; Bryce *et al.*, 1999) and regulate mononuclear cell production of cytokines influencing T cell polarisation. For example, elevation of cyclic AMP has been shown to inhibit secretion of IFN γ and IL-2 (Betz and Fox, 1991; Snijdewint *et al.*, 1993) but enhance production of IL-5 (Lacour *et al.*, 1994), IL-10 (Platzer *et al.*, 1995) and IL-1-β (Lorenz *et al.*, 1995). The ability of cyclic AMP to inhibit Th1 type cytokine production lead to the proposal that cyclic AMP favoured development of a Th2 phenotype (Gajewski *et al.*, 1990; Betz and Fox, 1991) however the finding that cyclic AMP also inhibited IL-4 secretion (Borger *et al.*, 1996; Sottile *et al.*, 1996) has put this into question.

In addition to influencing adaptive immune responses, cyclic AMP plays an important role in regulating innate immune cell function. β2-adrenoceptor agonists have been found to be very effective in the treatment of inflammatory diseases such as asthma (Barnes, 1999). It is postulated that this relates partly to the ability of β2-adrenoceptor agonists to stimulate increases in cyclic AMP, leading to suppression of inflammatory cell function (Hallsworth *et al.*, 2001). For example, elevation of cyclic AMP is known to inhibit directly many granulocyte functions such as superoxide anion release (Schudt *et al.*, 1991; Lad *et al.*, 1985), degranulation (Kita *et al.*,

1991b), enzyme secretion (Wada, 1989) and the induction of the respiratory burst (Dent *et al.*, 1994). Cyclic AMP will also inhibit indirectly inflammatory cell activity by suppressing release of many proinflammatory mediators such as eicosanoids (Ham *et al.*, 1983), LTC_4 (Tenor *et al.*, 1996), thromboxane A_2 (Zheng *et al.*, 1991) and eosinophil activating cytokines such as GM-CSF, RANTES and eotaxin (Hallsworth *et al.*, 2001). The effect of cyclic AMP on neutrophil chemotaxis however is somewhat more controversial with reports that β2-adrenoceptor agonist suppression of LTB_4 induced chemotaxis does not relate to its ability to elevate cyclic AMP (Harvath *et al.*, 1991). Forskolin, a more effective elevator of cAMP, also was found to be ineffectual at inhibiting LTB_4 induced chemotaxis in this cell type (Harvath *et al.*, 1991) suggesting that intracellular concentration of cyclic AMP is not the key determinant in suppression of neutrophil chemotaxis. In light of these findings it appears cyclic AMP suppresses certain responses in neutrophils such as release of PAF and superoxide anions yet fails to affect other neutrophil responses such as priming, chemotaxis and transmigration (Daniels *et al.*, 1993; Armstrong, 1995)

The mechanism by which cyclic AMP regulates control of various cellular processes involves a well-characterised signalling pathway initiated by the specific ligation of appropriate G protein coupled receptors (Figure 3). Following conformational changes induced by ligand binding, Gs composed of α_s and $\beta\gamma$ subunits, becomes activated causing the α_s subunit to dissociate from the Gs complex, exposing a binding site for adenylate cyclase. Binding of the α_s subunit to adenylate cyclase, of which there are at least nine membrane isoforms, leads to the formation of cyclic AMP from ATP (Hanoune and Defer, 2001). Cyclic AMP signalling is typically transient, with formation of cyclic AMP in the cell being balanced by progressive degradation by cyclic nucleotide phosphodiesterases (PDEs). There have been at least 10 different isoenzyme classes of PDEs described in mammalian cells (PDE1-PDE10) with this superfamily being further subdivided due to the presence of multiple splice variants (Houslay and Milligan, 1997). In inflammatory cells, the predominant PDEs expressed are type III and IV, with granulocytes reported to

contain only PDE IV (Dent *et al.*, 1991). The formation of cyclic AMP by adenylate cyclase initiates cyclic AMP binding to cytoplasmic protein kinase A; a tetrameric structure composed of two regulatory (R) and two catalytic (C) subunits which exists as an inactive holoenzyme complex in the absence of cyclic AMP (Daniel *et al.*, 1998). There are two isoforms of PKA identified, namely PKA-I and PKA-II, which are differentiated on the basis of their regulatory subunits (RI and RII) (Rubin, 1994; Beebe, 1994) Expression of a particular isoform of PKA regulatory subunits has important consequences for the subcellular localisation of PKA. For example, it has been found that a family of proteins called A-kinase anchoring proteins (AKAPs) will interact with RII subunits of PKA when inactive, sequestering PKA at specific locations within the cell (Rubin, 1994). AKAPs do not appear to bind RI subunits of PKA, thus cyclic AMP dependent signalling may vary as a consequence of which PKA isoforms are present in a particular cell (Rubin, 1994; Scott and McCartney, 1994). There is also some evidence that cyclic AMP signalling may be further compartmentalised. Caveolae membrane microdomains have been found to contain many components involved in cyclic AMP signalling. Furthermore, caveolin1 has been shown to be capable of abrogating cyclic AMP signalling (Razani *et al.*, 1999). The consequences of compartmentalisation on downstream signalling events of cyclic AMP have still to be elucidated.

Activation of PKA by cyclic AMP results in the translocation of the catalytic subunits to the nucleus, allowing phosphorylation of specific cellular substrates such as transcription factors. These include cyclic AMP responsive element binding protein (CREB), cyclic AMP responsive element modulator (CREM) and activating transcription factor 1 (ATF1) (Sassone-Corsi, 1995). These transcription factors bind as dimers to the cyclic AMP consensus sequence TGACGTXA, known as the cyclic AMP responsive element (CRE) (Sassone-Corsi, 1995). Binding of transcription factors to CRE is thought to allow cyclic AMP stimulated signals to subsequently affect gene transcription (Sassone-Corsi, 1995). Transcription of CRE regulated genes may be augmented by interaction with CBP (CREB binding protein) which bridges CREB with the basal transcriptional machinery (Goldman *et al.*, 1997). CBP

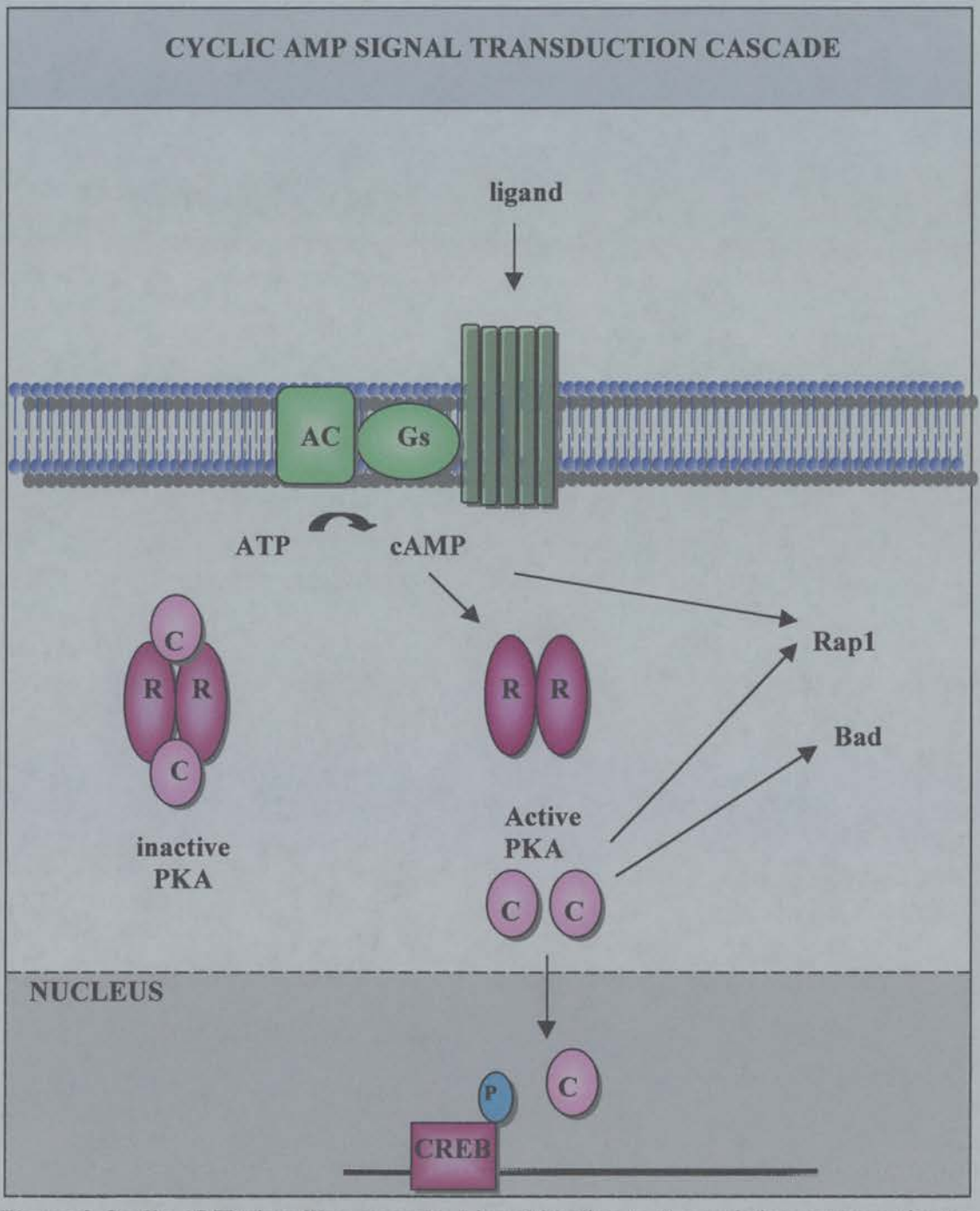


Figure 3 Cyclic AMP signalling cascade. Ligand binding to G-coupled receptors activates a stimulatory G-protein (Gs). This activates adenylate cyclase (AC) leading to formation of cyclic AMP from ATP. Active PKA catalytic subunits are released following cyclic AMP binding to PKA regulatory subunits. The C subunits phosphorylate targets in the cytoplasm (e.g. Bad, Rap1) and in the nucleus (e.g. cyclic AMP response element binding protein (CREB)).

is believed to have a more universal role and may also allow integration of signals from other transcription factors such as AP-1 (Kamei *et al.*, 1996). Other transcription factors regulated by and responsive to the activation of the cyclic AMP signalling pathway include NFκB (Chen and Rothenberg, 1994; Satriano and Schlondorff, 1994) and nuclear receptors (Darwish *et al.*, 1993).

It is important to note that PKA has been reported to act independently of cyclic AMP in some circumstances. It has been demonstrated that PKA is capable of regulating NFκB transcription by phosphorylating p65 and initiating the degradation of IκB (Zhong *et al.*, 1997), in a ligand independent manner. Furthermore it has been reported that PKA can form part of an inactive complex with NFκB/IκB (Zhong *et al.*, 1997) and is thought to be responsible for regulating glucocorticoid-mediated suppression of NFκB independently of cyclic AMP (Doucas *et al.*, 2000).

Until recently it was assumed that cyclic AMP exerted its physiological functions almost solely through activation of PKA. However this appears to not be the case with the discovery of a new family of cyclic AMP binding proteins which have close sequence similarity to PKA (Kawasaki *et al.*, 1998; de Rooij *et al.*, 2000). These cyclic AMP binding proteins termed cyclic AMP specific guanine nucleotide exchange factors (cAMP-GEFs) contain a guanine nucleotide exchange factor (GEF) domain and a cyclic AMP binding domain which closely resemble the cyclic AMP binding sites of PKA (de Rooij *et al.*, 2000; Kawasaki *et al.*, 1998). There have been two cAMP-GEFs identified, namely cAMP-GEFI (or Epac) and cAMP-GEFII (de Rooij *et al.*, 2000; Kawasaki *et al.*, 1998). Binding of cyclic AMP to cAMP-GEFs results in the activation of the small Ras like GTPase, Rap-1 (de Rooij *et al.*, 2000). The functional consequences of activation of Rap1 have not been fully established, however Rap-1 is postulated to have a role in platelet aggregation (Bos, 1998), cell differentiation (York *et al.*, 1998), cell proliferation (Altschuler and Ribeiro-Neto, 1998) and T cell anergy (Boussiotis *et al.*, 1997). The downstream signalling cascade of Rap1 is poorly defined, however there have been suggestions that Rap1 may antagonise Ras signalling (Cook *et al.*, 1993; Hu *et al.*, 1997b). This has recently

been disputed with the finding that ERK activation of Rap1 fails to interfere with Ras effector signalling (Zwartkruis *et al.*, 1998). The localisation of Rap1 may however give clues as to its function as it has been shown to be present in the mid Golgi, early and late endocytic vesicles and lysosomes whereas Ras is mainly localised in the plasma membrane (Pizon *et al.*, 1994).

Many of the molecular mechanisms underlying cyclic AMP regulation of cellular events are well characterised. However, the ability of this ubiquitous second messenger to regulate one aspect of cellular function remains poorly defined. Cyclic AMP can powerfully and differentially modulate apoptosis in a wide variety of cell types yet little is known of the molecular mechanisms controlling this process. It has recently come to light that cyclic AMP is involved in the regulation of the pro-apoptotic Bcl-2 family member Bad (Harada *et al.*, 1999). Bad is found to be associated with Bcl-X_L at the mitochondrial outer membrane (Zha *et al.*, 1997). It has been proposed that following cyclic AMP mediated activation of PKA there is phosphorylation of key serine residues on Bad, allows the dissociation of Bad from Bcl-X_L(Harada *et al.*, 1999). Bad is then thought to be sequestered in the cytoplasm by 14-3-3 resulting in abrogation of its pro-apoptotic properties, allowing cell survival (Zha *et al.*, 1996). The physiological importance of this pathway has yet to be determined however it is unlikely that this is the universal mechanism controlling cyclic AMP regulated apoptosis, considering the divergent effects cyclic AMP has on cell death in different cell types.

The importance of understanding the signalling pathways mediated by cyclic AMP, in the control of apoptosis, is exemplified by the wide variety of cells in which cyclic AMP determines cell fate. For example, elevation of cyclic AMP induces apoptosis in thymocytes (McConkey *et al.*, 1990; Suzuki *et al.*, 1991), primary granulosa cells (Aharoni *et al.*, 1995), myeloid cell lines (Lanotte *et al.*, 1991) and WEHI7.2 murine lymphocytes (Dowd *et al.*, 1992). The signalling mechanisms used by cyclic AMP to control these events are likely to be complex and cell type specific. For example, in contrast to the profound induction of apoptosis in the cell types above, evidence has emerged demonstrating that cyclic AMP can provide protection from cell death in

many cell types. Cyclic AMP inhibits apoptosis in pancreatic cancer cells (Boucher *et al.*, 2001), protects human osteoblasts from NO induced apoptosis (Chae *et al.*, 2001) and promotes neuronal survival (Deckwerth and Johnson, Jr., 1993; D'Mello *et al.*, 1993; De *et al.*, 1994; Li *et al.*, 2000a). There has been recent interest in the finding that cyclic AMP can also regulate granulocyte apoptosis. Elevation of cyclic AMP using either analogues of cyclic AMP (Rossi *et al.*, 1995) or receptor directed prostaglandins (Rossi *et al.*, 1995; Ottonello *et al.*, 1998) has been reported to profoundly delay neutrophil apoptosis. Various studies have described similar findings in eosinophils yet little is known of the signalling mechanism by which cyclic AMP appears to regulate this cell function (Chang *et al.*, 2000; Peacock *et al.*, 1999; Hallsworth *et al.*, 1996). We have previously highlighted the importance of regulated granulocyte apoptosis, as a vital process for ensuring the successful resolution of an inflammatory response (Savill, 1992; Stern *et al.*, 1992). The obvious consequence of the knowledge that cyclic AMP can protect against granulocyte apoptosis, is the chance to promote apoptosis indirectly by blocking the influence of this survival factor and augment phagocytic removal of these cells. Since the mechanisms by which cyclic AMP influences granulocyte apoptosis and the signalling pathways involved in regulating granulocyte cell death remain ill defined, our aim was to investigate the underlying molecular mechanism and establish a greater understanding of the control of neutrophil cell death.

3.1 ELEVATION OF CYCLIC AMP DELAYS CONSTITUTIVE NEUTROPHIL APOPTOSIS

To examine the effects of cyclic AMP on constitutive neutrophil apoptosis, neutrophils (5 x 10^6/ml) cultured in serum supplemented Iscove's DMEM, were exposed to dibutyryl cyclic AMP (dbcAMP), a membrane-permeant cyclic AMP analogue and the receptor directed stimulus prostaglandin E_2 (PGE_2), for 20 h. Both dbcAMP and PGE_2 delay morphological changes characteristic of constitutive neutrophil apoptosis, such as cytoplasmic shrinkage and nuclear condensation (Figure 3.1.1). DbcAMP and PGE_2 also delay cell membrane changes associated with apoptosis such as the exposure of phosphatidylserine measured by Annexin V binding (Figures 3.1.2 and 3.1.3). Figure 3.1.2 illustrates the percentage of cells within the Annexin V "high" gate was reduced by dbcAMP treatment from 63% (B; control) to 34% (C). It is interesting to note that maximal concentrations of dbcAMP, established previously as 0.2 mM (Rossi *et al.*, 1995), are more effective at delaying neutrophil apoptosis compared to maximal concentrations of PGE_2 (10 μM) (Figure 3.1.3). Assessment of cell viability by trypan blue exclusion demonstrated that dbcAMP or PGE_2 did not alter this parameter (data not shown).

Cyclic AMP mediated delay of neutrophil apoptosis was also measured by loss of cell surface CD16 (FcγRIII), a process that has been characterised as a further marker of spontaneous neutrophil death (Dransfield *et al.*, 1994). The marked inhibition of apoptosis induced by dbcAMP (as determined by CD16 shedding) is exemplified by the representative flow cytometric analysis depicted in Figure 3.1.4. The CD16 " low peak" in the control 24 h cells represents apoptotic cells that have shed their cell surface CD16; the percentage cells with "low" CD16 was markedly reduced when compared to cells that have been treated with dbcAMP whereas the corresponding CD16 "high" peak was increased. Together these results demonstrate morphological and surface alterations that accompany apoptosis are inhibited by elevators of cyclic AMP.

SUMMARY

- Elevation of cyclic AMP by dbcAMP and PGE_2 delays morphological changes that occur during neutrophil apoptosis.
- Elevation of cyclic AMP delays expression of phosphatidylserine and inhibits cell surface loss of CD16 (FCRγIII) that occur during neutrophil apoptosis.

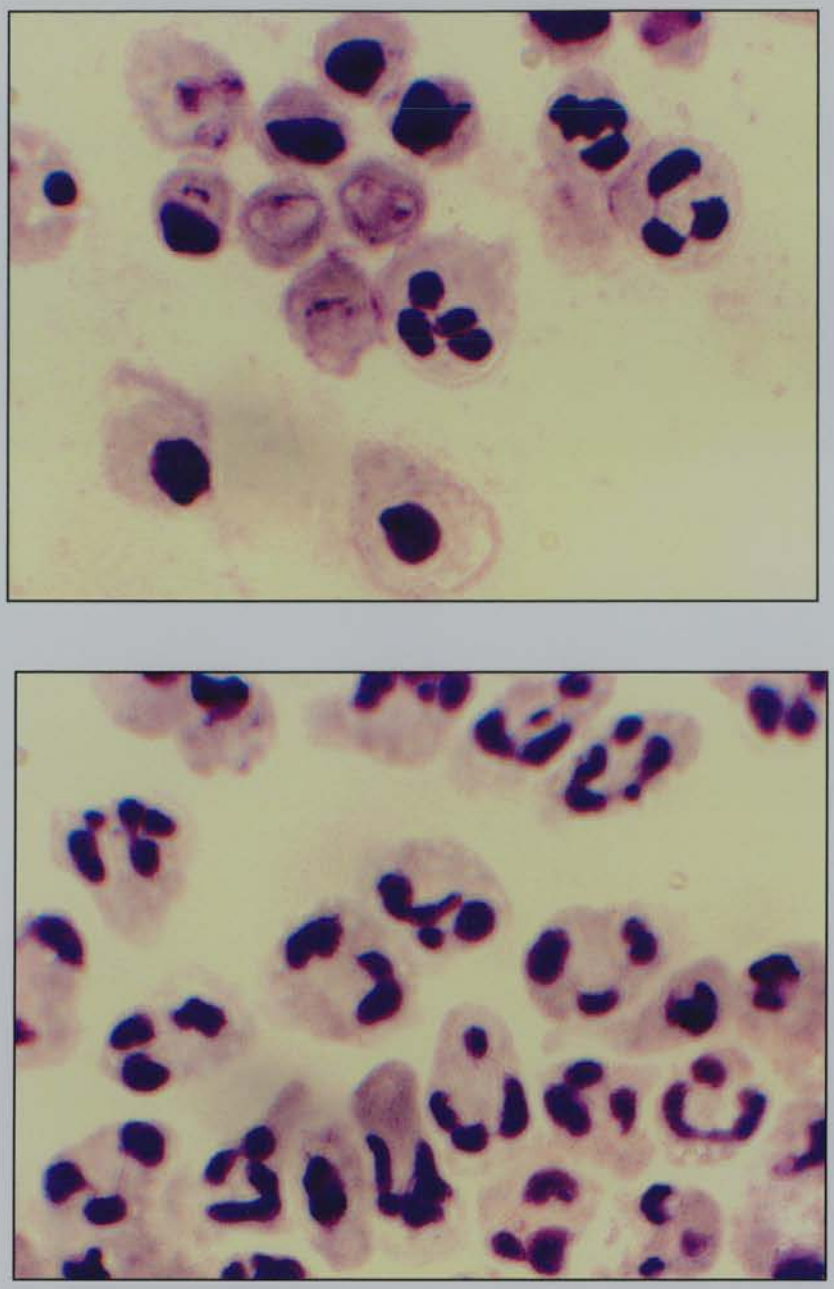


Figure 3.1.1 Neutrophil apoptosis is delayed by elevation of cyclic AMP. Human neutrophils (5 x 10^6/ml) were cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, with or without dbcAMP (0.2mM). After 20 h, cells were harvested and assessed morphologically for apoptosis. The upper panel indicates control neutrophils after 20 h in culture. The bottom panel indicates neutrophils treated with dbcAMP for 20 h.

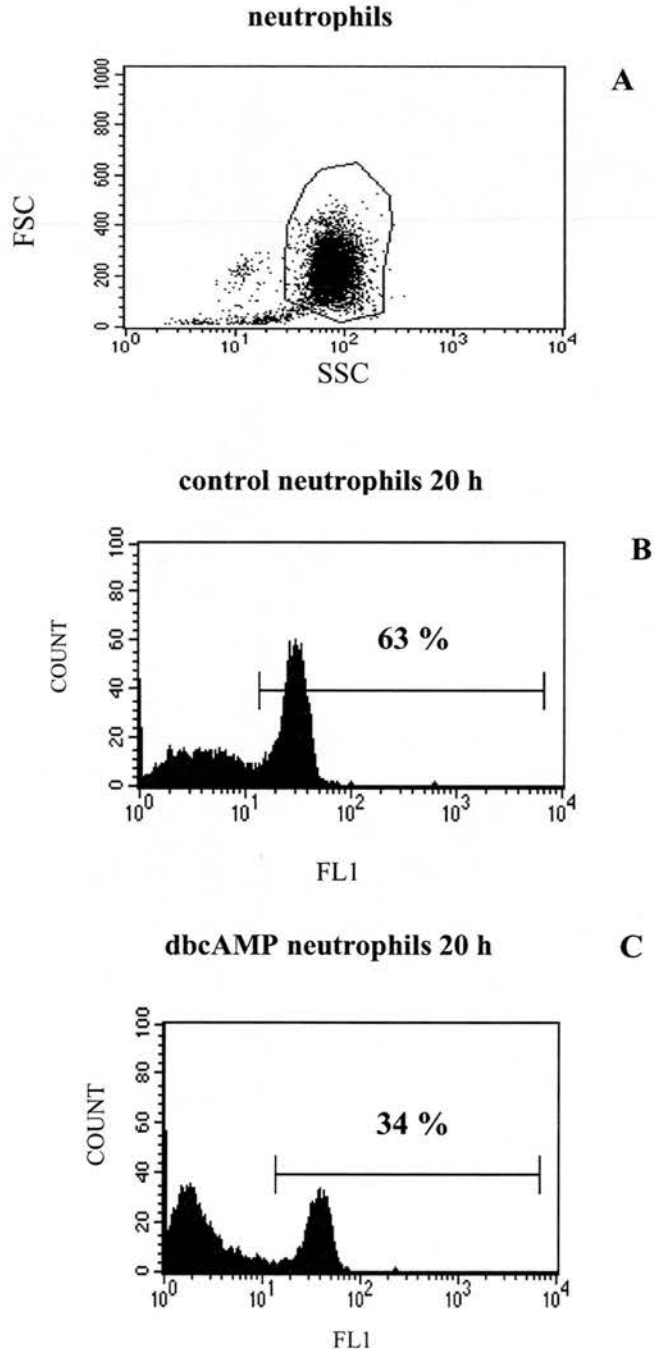


Figure 3.1.2 Cyclic AMP elevation delays neutrophil apoptosis. (A) represents a typical flow cytometric scatter plot for neutrophils. (B) represents control neutrophils after 20h in culture incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. Similarly (C) represents neutrophils stimulated with dbcAMP for 20 h before incubation with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine exposure.

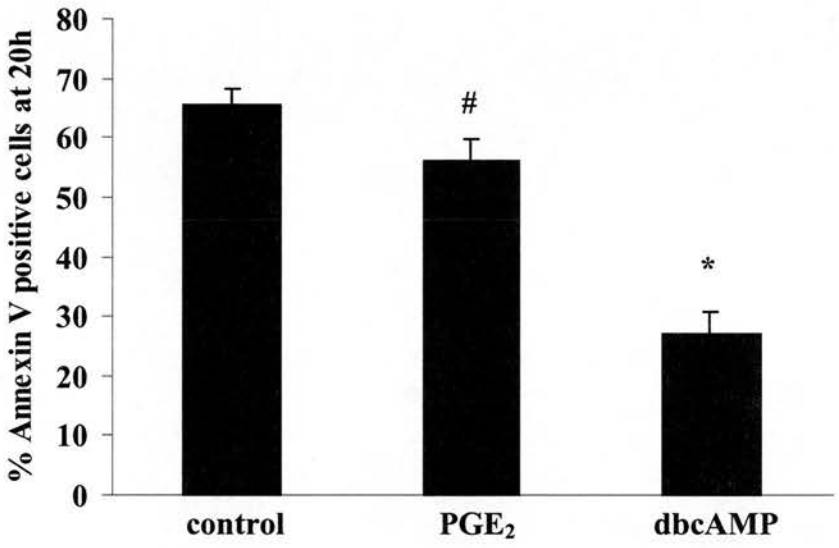


Figure 3.1.3 Cyclic AMP elevation delays neutrophil apoptosis as assessed by Annexin V binding. Human neutrophils (5 x 10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum and treated with dbcAMP (0.2 mM) or PGE_2 (10 μM). After 20 h, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine exposure. All values represent mean ± S. E. of n = 5 - 8 experiments, each performed in duplicate where significant difference from control is represented by * $P<0.001$ and $^{\#}P<0.05$. Similar results were found by morphological assessment of apoptosis (data not shown).

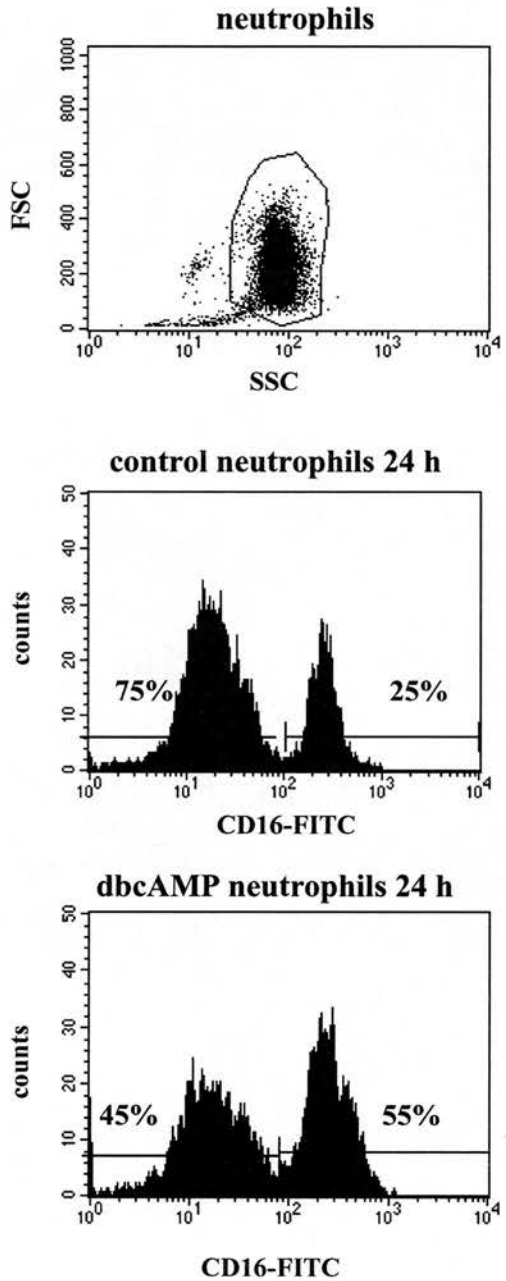


Figure 3.1.4 Cyclic AMP elevation delays neutrophil apoptosis as assessed by CD16 shedding. Human neutrophils (5 x 10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum and treated with or without dbcAMP (0.2 mM). After 24 h, the cells were measured for CD16 expression as outlined in the "Methods" section before being analysed on FACS Calibur and the CellQuest associated software. Data presented is of one representative experiment.

3.2 CYCLIC AMP ELEVATION INHIBITS CASPASE-3 EXPRESSION DURING CONSTITUTIVE NEUTROPHIL APOPTOSIS

In light of the findings that cyclic AMP elevating agents delay neutrophil apoptosis, we were interested in investigating the point at which cyclic AMP influenced the apoptotic signalling cascade. It is widely believed that caspases act as the main executioners of apoptosis, with their activation resulting in chromatin condensation and DNA fragmentation. Whether cyclic AMP delays constitutive neutrophil apoptosis by directly suppressing caspase activation in these cells has not been examined. We therefore investigated the effect of cyclic AMP on activation of caspase-3 during constitutive neutrophil apoptosis. Cytoplasmic extracts were obtained from neutrophils which had been treated with control buffer or dbcAMP (0.2 mM) over a time course of 20 h. Lysates were prepared from equivalent numbers of cells and were subject to SDS-PAGE/immunoblot analysis using a rabbit polyclonal antibody specific for caspase-3. The caspase-3 antibody recognizes both the 32 kD pro-caspase-3 and the 17 kD subunit of active caspase-3. Neutrophils began to express active caspase-3 (17kD) at 8 h and by 20 h there was significant caspase-3 activity (17kD) which could be inhibited by dbcAMP (Figure 3.2.1.). We also found that expression of caspase-3 correlated with the phosphatidylserine exposure that occurs during constitutive neutrophil apoptosis (Figure 3.2.2). The appearance of some caspase-3 activity in the presence of dbcAMP at 20 h probably reflects the presence of some apoptotic cells in the population (Figure 3.2.2). The intriguing nature of the results illustrated above raised the possibility that cyclic AMP mediated delay of neutrophil apoptosis is due to suppression of caspase activation. We therefore studied the effect of co-culturing dbcAMP and the pan caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) on the rate of neutrophil apoptosis. Figure 3.2.3 shows that when neutrophils are co-cultured in the presence of both dbcAMP and zVAD-fmk their individual effects are less than additive suggesting that these reagents delay neutrophil apoptosis by a common

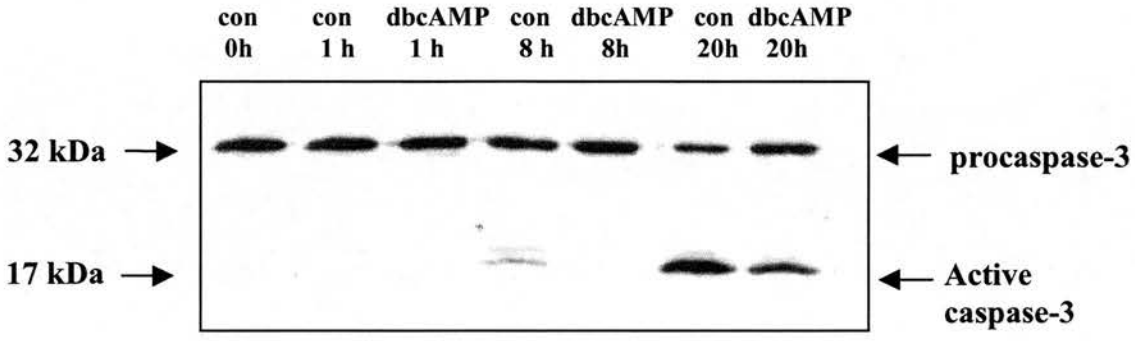


Figure 3.2.1 Time course for the effect of dbcAMP on caspase-3 expression during human neutrophil apoptosis Western blot of cytoplasmic extracts from neutrophils treated with control buffer or dbcAMP (0.2 mM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis. A 12.5% gel was used. The rabbit polyclonal antibody to caspase-3 antibody recognizes both the 32 kDa pro-caspase-3 and the 17 kDa subunit of active caspase-3. The 17 kD caspase-3 cleavage product is faintly visible in control lysates at 8 h becoming more apparent by 20 h. The gel is representative of 3 experiments.

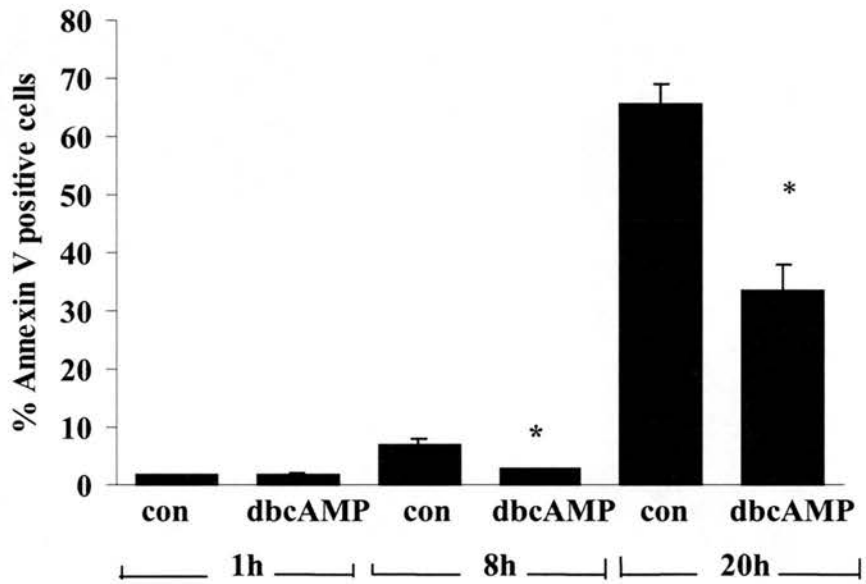


Figure 3.2.2 Time course for phosphatidylserine exposure during overnight neutrophil culture. Human neutrophils were treated with or without dbcAMP (0.2 mM) for the time points indicated under equivalent culture conditions as the cells used for caspase-3 expression assessment above. Cells were assessed for apoptosis by measurement of phosphatidylserine expression using Annexin-V FITC. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by * $P<0.001$.

inhibitory mechanism. However further experiments involving much wider concentration ranges of both reagents are necessary to make any firm conclusions regarding this data. It is intriguing that elevation of cyclic AMP delayed neutrophil apoptosis more effectively than blockade of caspase activation by zVAD-fmk. This suggests either that cyclic AMP elevation more effectively inhibits caspase activation than zVAD-fmk or alternatively cyclic AMP may act partially through a mechanism not involving inhibition of caspase activation, in order to suppress neutrophil apoptosis.

It is interesting that blockade of caspase activation by zVAD-fmk does not fully suppress neutrophil apoptosis. This is unlikely to be due to poor cell permeability, as zVAD-fmk will effectively block TNFα induced apoptosis in neutrophils (Ward *et al.*, 1999a). It is however possible that zVAD-fmk may be degraded during overnight culture and therefore caspase activity may not be effectively blocked during the 20 h culture period. Although this may be possible, a further and more interesting interpretation of the data would be that constitutive neutrophil apoptosis occurs partially independently of caspase activation.

SUMMARY

- Constitutive neutrophil apoptosis is accompanied by expression of active caspase-3.
- Elevation of cyclic AMP delays expression of caspase-3 during constitutive neutrophil apoptosis.
- Studies with zVAD-fmk suggest constitutive neutrophil apoptosis may occur partially through a caspase-independent mechanism.

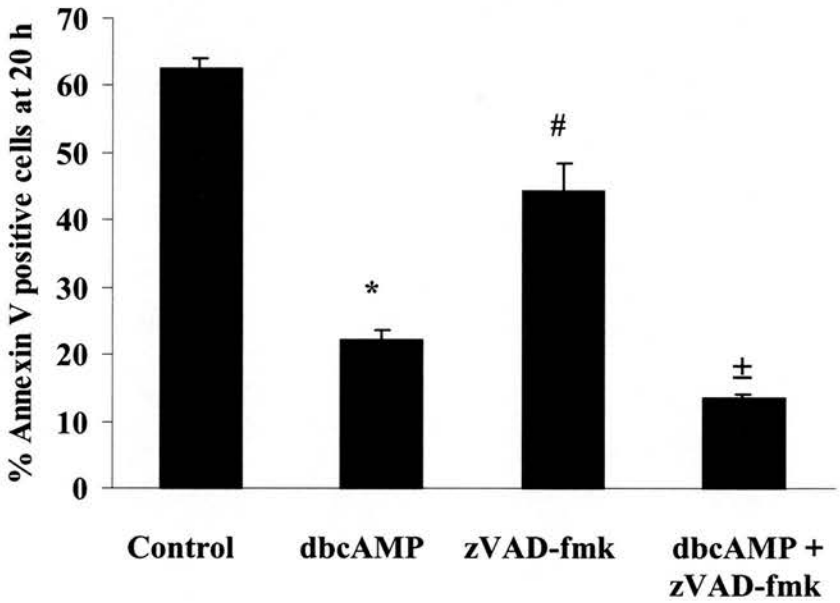


Figure 3.2.3 Comparison of the effects of dbcAMP and zVAD-fmk on neutrophil apoptosis. Human neutrophils (5 x 10 ^{6}/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum and treated with dbcAMP (0.2 mM) or zVAD-fmk (100 μM). After 20 h, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S. E. of n = 3 experiments, each performed in duplicate where significance from control is represented by *P<0.001 or #P<0.01 and significance from dbcAMP alone is represented by ±P<0.05.

3.3 CYCLIC AMP INHIBITS LOSS OF MITOCHONDRIAL POTENTIAL OCCURRING DURING CONSTITUTIVE NEUTROPHIL APOPTOSIS

3.3.1 DETERMINATION OF WHETHER NEUTROPHILS CONTAIN MITOCHONDRIA

In many cell models, apoptosis is accompanied by an early dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$) (Zamzami *et al.*, 1995). Previous data have indicated that neutrophils do not respire and it was thought unlikely that they contained many, if any mitochondria (Simon, 2001). To investigate whether cyclic AMP mediated delay of neutrophil apoptosis involves regulating changes in mitochondrial membrane potential we firstly examined if changes in mitochondrial membrane potential occur during constitutive neutrophil apoptosis. Using confocal microscopy and flow cytometry we have been able to demonstrate that neutrophils do contain mitochondria (orange), which during overnight culture exhibit loss of mitochondrial potential, as indicated by an increase in green fluorescence using the mitochondrial specific dye JC-1 (Reers *et al.*, 1991) (Figure 3.3.1). Cells displaying necrotic morphology were distinguished from the rest of the cell population by using the impermeant nucleic acid dead-cell stain TOPRO-3 (van Hooijdonk *et al.*, 1994). We found that >99% of neutrophils were viable as assessed by exclusion of TOPRO-3.

3.3.2 EFFECT OF CYCLIC AMP ON DISSIPATION OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL DURING NEUTROPHIL APOPTOSIS

To investigate if cyclic AMP delays neutrophil apoptosis by affecting mitochondrial membrane potential, cells were cultured in the presence or absence of dbcAMP for 20 h, before measurement of mitochondrial membrane potential by JC-1. Our studies reveal that dbcAMP inhibits changes in mitochondrial potential associated with constitutive neutrophil apoptosis (Figure 3.3.2 A & B). As the number of cells

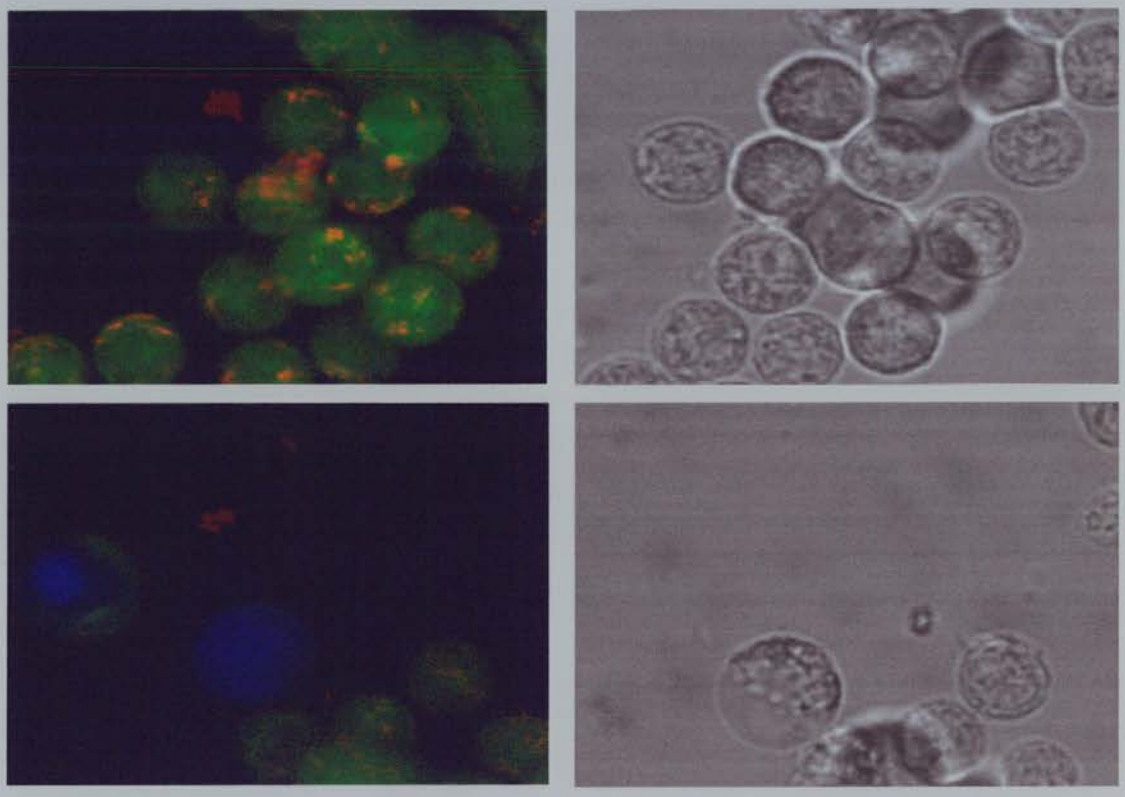


Figure 3.3.1 Demonstration of mitochondria within neutrophils. Human neutrophils (1 x 10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum. Neutrophils were labelled with JC-1, a mitochondrial specific dye, and examined by confocal fluorescent microscopy as described under "Materials and Methods". Bottom panels show TO-PRO-3 staining for neutrophils with necrotic morphology (blue).

showing loss of mitochondrial potential appeared to correlate with the number of apoptotic cells measured by Annexin-V positivity (Figure 3.3.2 C), we wanted to examine directly if loss in mitochondrial potential occurs in those neutrophils undergoing apoptosis. By using conjugated anti-CD16 magnetic beads we were able to perform immunodepletion of non-apoptotic neutrophils. We confirmed that the remaining cells following immunodepletion were apoptotic by Annexin-V binding (Figure 3.3.2 E). Furthermore, we found that the remaining apoptotic neutrophils were indeed positive for loss of mitochondrial potential, indicating that dissipation of mitochondrial membrane potential occurs in neutrophils undergoing programmed cell death (Figure 3.3.2 D). It has been previously shown that inhibitors of the mitochondrial respiratory chain do not affect constitutive neutrophil apoptosis, raising the question of the source of their $\Delta\Psi m$ (Mecklenburgh, 1999). It may be the case that the neutrophil maintains a transmembrane gradient by a functional F1 Fo ATPase, however this has to be investigated in more detail. We have demonstrated that cyclic AMP mediated delay of neutrophil apoptosis appears to suppress both caspase activation and changes in mitochondrial potential. We next investigated the cyclic AMP signal transduction pathway responsible for this suppression of neutrophil apoptosis and attempted to establish how it is coupled to controlling components of the molecular machinery for cell death as described above.

SUMMARY

- Neutrophils contain a small number of mitochondria.
- There is dissipation of mitochondria transmembrane potential in neutrophils undergoing apoptosis.
- Elevation of cyclic AMP inhibits loss of mitochondrial membrane potential, during constitutive neutrophil apoptosis.

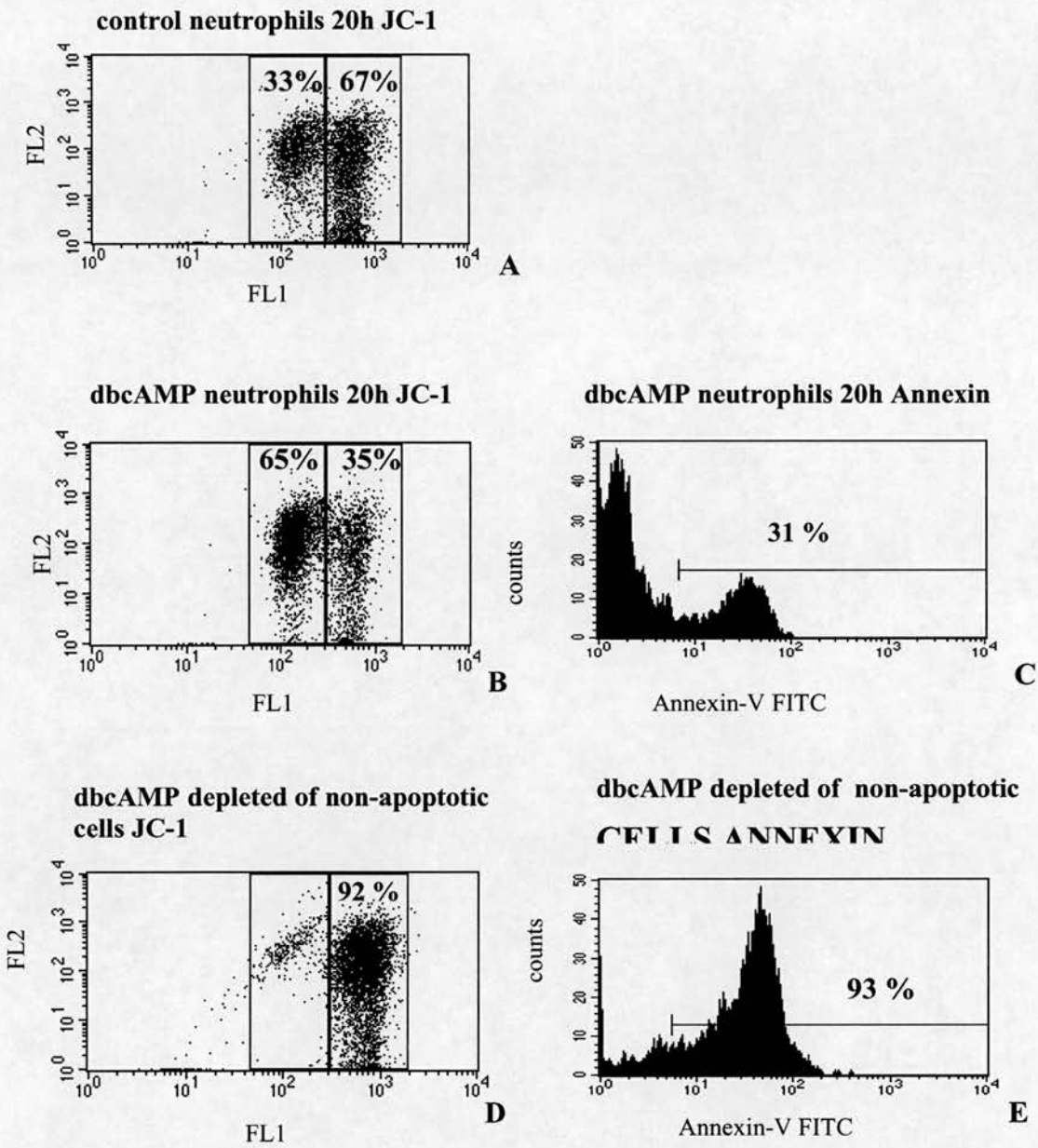


Figure 3.3.2 Effect of cyclic AMP on dissipation of mitochondrial transmembrane potential during human neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) were cultured for 20 h at 37°C in Iscoves's DMEM containing 10% autologous serum with or without dbcAMP (0.2 mM). Cells were then labelled with the mitochondrial specific dye JC-1 as described under 'Materials and Methods' before flow cytometric analysis of mitochondrial membrane potential (A, B & D). Apoptosis was assessed by incubation of cells with FITC labelled recombinant human Annexin-V to determine the phosphatidylserine exposure (C & E). Non-apoptotic neutrophils were removed by anti-CD16 immunodepletion before the remaining cells were labelled with either Annexin-V FITC or JC-1. Shown as one representative experiment.

3.4 CYCLIC AMP REGULATION OF NEUTROPHIL APOPTOSIS OCCURS INDEPENDENTLY OF PKA

3.4.1 CYCLIC AMP ELEVATION STIMULATES PKA ACTIVATION IN NEUTROPHILS, AN EFFECT THAT IS BLOCKED BY PHARMACOLOGICAL INHIBITORS

The major intracellular receptor for cyclic AMP is the regulatory (R) subunits of protein kinase A (PKA) (Daniel *et al.*, 1998). Binding of cyclic AMP to the R subunits releases catalytic C subunits, which consequently phosphorylate target proteins such as cyclic AMP response element binding protein (CREB) (Daniel *et al.*, 1998). It has been proposed that the increase of intracellular cyclic AMP resulting in PKA activation is essential for neutrophil survival (Rossi *et al.*, 1995; Parvathenani *et al.*, 1998; Tortorella *et al.*, 1998a). To further investigate this we examined the effects of cyclic AMP elevation on endogenous PKA activation (Figure 3.4.1). PKA activity was assessed in neutrophils by measuring the phosphorylation of a biotinylated peptide substrate kemptide, which is highly specific for PKA (See Materials & Methods). The transfer of a labelled phosphate to kemptide in neutrophil lysates containing PKA activity, was measured by capture of the labelled substrate on a streptavadin matrix, followed by scintillation counting. We found rapid activation of PKA when cells were stimulated with both dbcAMP and PGE_2 (Figure 3.4.1). Furthermore preincubating for 30 minutes with the pharmacological PKA inhibitor H-89 (Chijiwa *et al.*, 1990) blocked activation of PKA upon stimulation of neutrophils with cyclic AMP elevators (Figure 3.4.1).

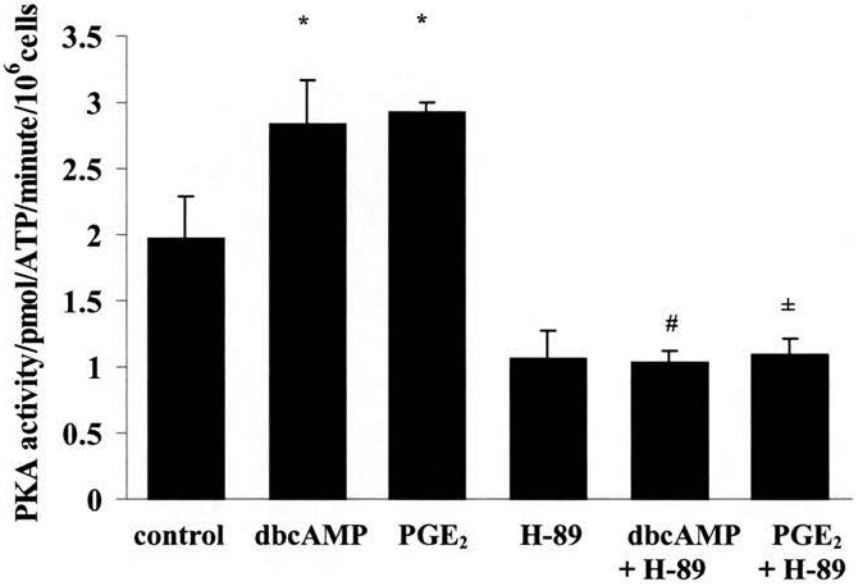


Figure 3.4.1. Measurement of PKA activation by elevators of cyclic AMP in human neutrophils. Human neutrophils (5 x 10^6/ml) were pre-incubated with 10 μM H-89 for 1 h before being stimulated with dbcAMP (0.2 mM) or PGE_2 (1 μM) for 30 minutes at 37 °C. PKA activity was measured as described under "Materials and Methods". All values represent mean ± S. E. of n = 3 experiments where significant difference from control values is represented by $^{*}P<0.05$. Significant difference from dbcAMP alone is represented by $^{\#}P<0.01$ and significant difference from PGE_2 alone is represented by $^{\pm}P<0.01$.

3.4.2 ACTIVATION OF THE PKA PATHWAY DOES NOT ACCOUNT FOR CYCLIC AMP MEDIATED PROTECTION AGAINST APOPTOSIS.

To investigate if PKA activation by cyclic AMP was necessary for cyclic AMP mediated delay of apoptosis, neutrophils were incubated with the PKA inhibitor H-89 before being stimulated with dbcAMP and assessed for apoptosis. To our surprise although pre-treatment with H-89 prevented activation of PKA, it did not prevent inhibition of apoptosis by dbcAMP (Figure 3.4.2). Additionally other pharmacological inhibitors of PKA such as the highly specific inhibitor Rp-8-BrcAMPS (Gjertsen *et al.*, 1995) also failed to block dbcAMP inhibition of neutrophil apoptosis (Figure 3.4.3). This demonstrates that cyclic AMP elevation stimulates PKA activity but PKA does not play a major role in the anti-apoptotic effect of cyclic AMP elevation in neutrophils.

Moreover it is interesting that inhibition of PKA activity by H-89 suppresses basal activation of PKA compared to control yet H-89 does not alter the rate of apoptosis in neutrophils (Figure 3.4.1 and Figure 3.4.2). This suggests that basal turnover of cyclic AMP and subsequent activation of PKA may not be an important factor in regulating constitutive neutrophil apoptosis. The ability of H-89 to block PKA activity, as shown by direct measurement of kinase activity, suggests a lack of involvement of PKA in the anti-apoptotic effect of cyclic AMP in neutrophils. It was therefore important to determine whether H-89 could block PKA activity for the full overnight culture period and under identical culture conditions that we use for our apoptosis assay. Neutrophils were cultured in serum supplemented Iscove's DMEM for 19 h in the presence or absence of H-89 before stimulation with PGE_2 for 1 h. PKA activity was then measured as described in the Materials and Methods. We found that H-89 could still block PGE_2 stimulated PKA activity at 20 h (Figure 3.4.4). This is very important as it demonstrates that the inability of H-89 to reverse cyclic AMP mediated delay of neutrophil apoptosis is not due to degradation of H-89 during the overnight culture period. Furthermore it also demonstrates that H-89 is not inactivated by autologous serum which is used in our apoptosis assay.

SUMMARY

- Elevation of cyclic AMP by dbcAMP and PGE_2 stimulate PKA activity in neutrophils.
- PKA stimulated activity is blocked by the pharmacological inhibitor H-89.
- Cyclic AMP mediated delay of neutrophil apoptosis is not reversed by the PKA inhibitors H-89 and Rp-8-Br-cAMPS.
- Failure of H-89 and Rp-8-Br-cAMPS to reverse cyclic AMP mediated delay of neutrophil apoptosis is not due to inactivation by autologous serum or degradation of the inhibitors during overnight culture.
- Cyclic AMP mediated delay of neutrophil apoptosis likely occurs via a PKA independent mechanism.

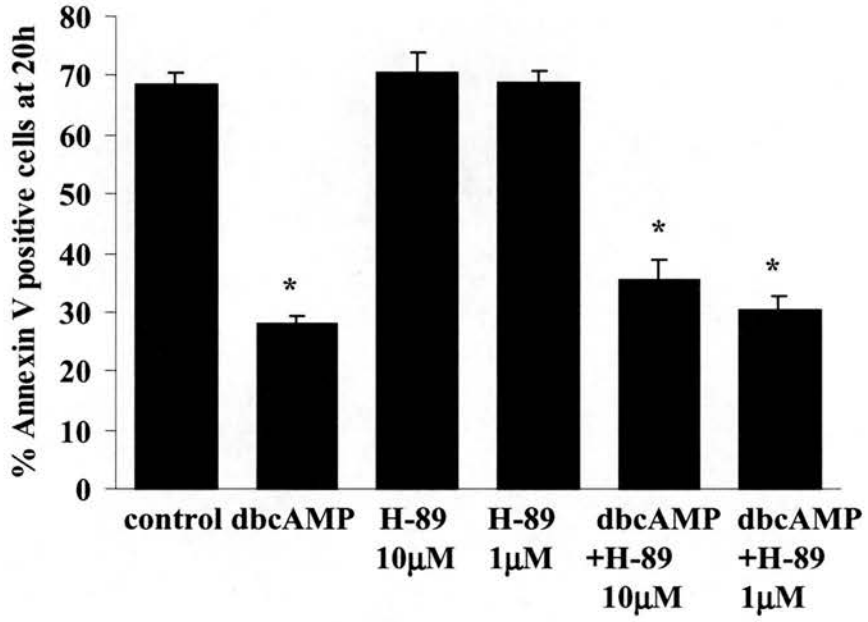


Figure 3.4.2. The effect of pharmacological blockade of PKA activity on dbcAMP mediated delay of neutrophil apoptosis. Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, were pre-incubated for 30 min with H-89 (1 or 10 μM) before stimulation with dbcAMP (0.2 mM). After a further 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by *P<0.001. Similar results were found when cells were assessed for apoptosis by morphological examination (data not shown).

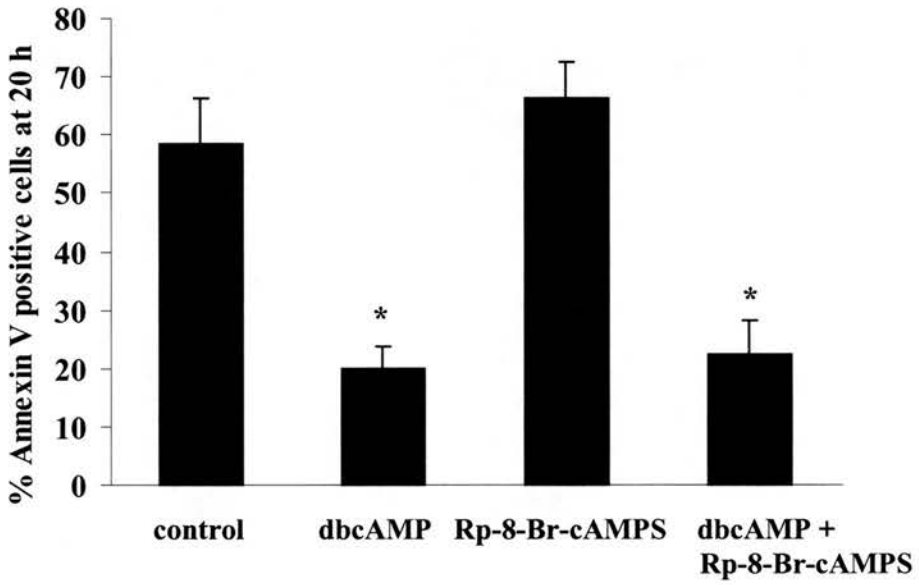


Figure 3.4.3 The effect of pharmacological blockade of PKA activity on dbcAMP mediated delay of neutrophil apoptosis. Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, were pre-incubated for 30 min with Rp-8-Br-cAMPS (100 μM) before stimulation with dbcAMP (0.2 mM). After a further 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S. E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by *P<0.001. Similar results were found when cells were assessed for apoptosis by morphological examination (data not shown).

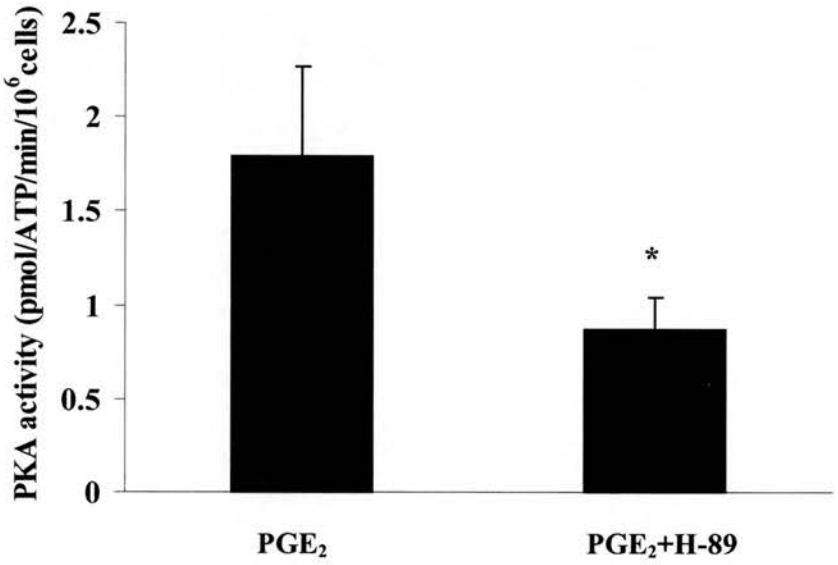


Figure 3.4.4. Measurement of PKA activation by elevators of cyclic AMP in human neutrophils. Human neutrophils (5 x 10^6 /ml) were cultured in serum supplemented Iscove's DMEM and were pre-incubated with 10 μM H-89 for 19 h before being stimulated with PGE_2 (1 μM) for 1 h at 37 °C. PKA activity was measured as described under "Materials and Methods". Significant difference from PGE_2 alone is represented by $^*P<0.01$.

3.5 ACTIVATION OF AKT/PI-3 KINASE PATHWAY DOES NOT ACCOUNT FOR CYCLIC AMP MEDIATED DELAY OF NEUTROPHIL APOPTOSIS

In light of the surprising finding that cyclic AMP mediated delay of neutrophil apoptosis was independent of PKA, we sought to investigate the involvement of other signalling molecules which may act downstream of cyclic AMP to suppress neutrophil apoptosis. The phosphoinositide-3 kinase/Akt pathway plays an essential role in cell survival in various cell types (Datta *et al.*, 1997). Activation of PI 3-kinase leads to activation of downstream signalling molecules such as Akt/PKB (Franke *et al.*, 1995; Burgering and Coffer, 1995). It has been proposed that PI-3 kinase may regulate apoptosis through the serine phosphorylation of Bad, a pro-apoptotic protein of Bcl-2 family (Datta *et al.*, 1997). Furthermore, PI-3 kinase may be involved in cyclic AMP signalling cascade in a variety of cells. For example, it has been reported that cyclic AMP requires PI-3 kinase activation for DNA synthesis induced by IGF-I in FRTL-5 cells (Nedachi *et al.*, 2000), and is involved in the ability of cyclic AMP to attenuate chemoattractant induced respiratory burst in neutrophils (Ahmed *et al.*, 1995). Therefore we examined if PI-3 kinase was involved in the signalling pathway mediating the protective effect of cyclic AMP on neutrophil survival. Cells were pre-incubated with the specific PI-3 kinase inhibitor LY294002 (Vlahos *et al.*, 1994) prior to exposing them to dbcAMP or GM-CSF. We found that the PI-3 kinase inhibitor suppressed GM-CSF mediated delay of neutrophil apoptosis, which has been previously reported (Klein *et al.*, 2000), yet had no effect on suppression of apoptosis by dbcAMP (Figure 3.5.1). This suggests cyclic AMP mediated delay of neutrophil apoptosis does not require PI-3 kinase activation.

SUMMARY

- The PI-3 kinase inhibitor LY294002 suppresses GM-CSF but not dbcAMP mediated delay of neutrophil apoptosis
- Cyclic AMP mediated delay of neutrophil apoptosis occurs independently of PI-3 kinase activity.

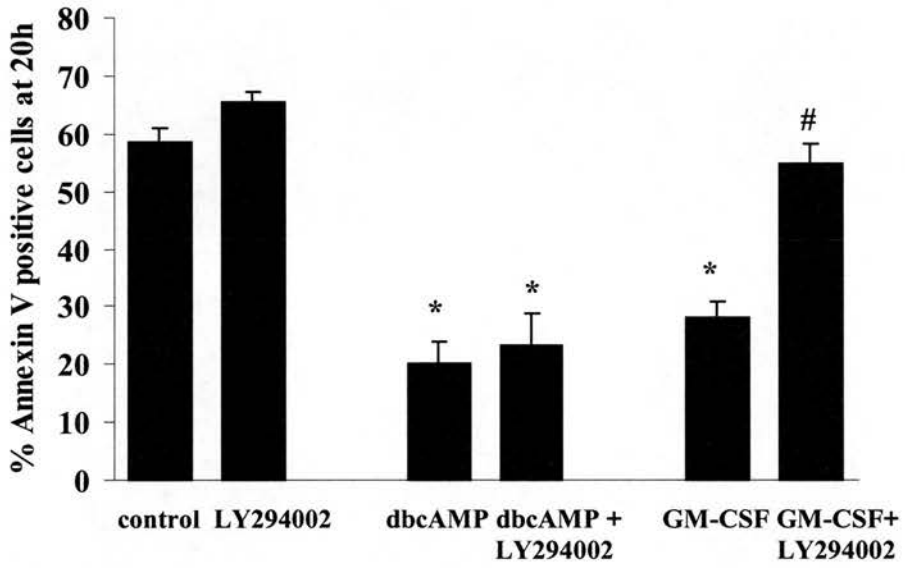


Figure 3.5.1. Effect of PI-3 kinase inhibition on cyclic AMP and GM-CSF mediated delay of neutrophil apoptosis. Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C were treated with LY294002 (10 μM) for 30 minutes prior to stimulation by dbcAMP (0.2 mM) or GM-CSF (50 U/ml). After a further 20 h in culture, cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S. E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by $^*P<0.001$ and significant difference from GM-CSF alone is represented by $^{\#}P<0.01$.

3.6 CYCLIC AMP MEDIATED DELAY OF NEUTROPHIL APOPTOSIS DOES NOT INVOLVE ACTIVATION OF EXTRACELLULAR SIGNAL REGULATED KINASES

We also investigated if cyclic AMP could act through the extracellular signal regulated kinases (ERK) signalling pathway to inhibit neutrophil apoptosis. Activation of ERK has been implicated in a number of systems to contribute as a negative regulator of apoptosis (Tran *et al.*, 2001; Xia *et al.*, 1995). Increasing cyclic AMP levels are also known to either inhibit or activate ERK in a cell type- and stimulus-specific manner. The mechanism by which cyclic AMP activates ERK is unclear at present, however it has been proposed that cyclic AMP may activate ERK through small GTPases such as Ras and Rap-1 (Busca *et al.*, 2000; Altschuler *et al.*, 1995). The physiological role of Rap-1 has yet to be elucidated but has been postulated to antagonise Ras dependent signalling (Kitayama *et al.*, 1989; Cook *et al.*, 1993; Boussiotis *et al.*, 1997) although this has recently been disputed (Zwartkruis *et al.*, 1998). We decided to investigate if activation of Rap-1 or Ras and subsequently ERK signalling by cyclic AMP, was important for cyclic AMP mediated suppression of apoptosis.

As an approach to investigating the role of small GTP-binding proteins of the Ras family, we used *Clostridium sordellii* lethal toxin (LT), which has been reported to specifically inhibit the small GTPases Ras, Rap-1 and Rac (Popoff *et al.*, 1996). We found that blockade of the activity of these small GTPases by LT did not reverse cyclic AMP mediated delay of neutrophil apoptosis (Figure 3.6.1A). Replacing autologous serum with 0.1% BSA in our culture system resulted in the induction of neutrophil apoptosis by LT (Figure 3.6.1.B). This induction of apoptosis was partially inhibited by cyclic AMP. Thus it appears that a component of autologous serum suppresses or inactivates the activity of LT. From this data it is difficult to establish whether blockade of Rap-1 and Ras by *Clostridium sordellii* lethal toxin can reverse cyclic AMP mediated delay of apoptosis. At concentrations of LT which do not cause induction of apoptosis, which have been reported to cause effective

blockade of small GTPases (Busca *et al.*, 2000), there was no reversal of cyclic AMP mediated delay of neutrophil apoptosis. However at higher concentrations of LT which cause induction of neutrophil apoptosis, cyclic AMP mediated suppression of apoptosis was lost. From this data, it is unlikely that Rap-1 and other small GTPases such as Ras play a vital role in cyclic AMP mediated delay of apoptosis. However, activity of small GTPases may be important in regulating constitutive neutrophil apoptosis.

Further evidence that Rap-1 is not involved in the anti-apoptotic effect of cyclic AMP was suggested by studies in which Rap-1 processing was inhibited by the geranylgeranyltransferase inhibitor GGTI-286. Activation of Rap-1 requires post-translational modifications that facilitate its attachment to the inner surface of the plasma membrane. Geranylgeranylation is thought to be required for Rap-1 to mature into its biologically active form (Lerner *et al.*, 1995). Neutrophils were cultured overnight in serum supplemented Iscove's DMEM in the presence or absence of GGTI-286. We found that blockade of Rap-1 processing by GGTI-286 did not reverse cyclic AMP mediated suppression of neutrophil apoptosis (Figure 3.6.2). Similar results were found when autologous serum was replaced by 0.1% BSA in the culture system (data not shown). This result further suggests that activation of Rap-1 is not important for cyclic AMP suppression of apoptosis.

Lastly we investigated whether ERK activation was required for cyclic AMP mediated delay of neutrophil apoptosis. The protective effect of cAMP-elevating agents does not appear to act through the ERK pathway in our system. This is suggested by the finding that the p42/p44 MAPK kinase inhibitor PD98059 (Dudley *et al.*, 1995) had no effect on the anti-apoptotic functions of cyclic AMP in neutrophils yet can reverse the anti-apoptotic functions of GM-CSF treatment in neutrophils which has been also shown recently by Klein *et al* (Figure 3.6.3). Similarly the p38 MAPK inhibitor SB203580 could not reverse dbcAMP mediated delay of neutrophil apoptosis (data not shown).

SUMMARY

- Cyclic AMP mediated delay of neutrophil apoptosis is *not* reversed by :

  Clostridium sordellii lethal toxin

  Geranylgeranyltransferase inhibitor GGTI286

  p42/p44 MAP kinase inhibitor PD 98059.

  p38 MAP kinase inhibitor SB 203580

- It is unlikely that cyclic AMP mediated delay of neutrophil apoptosis involves Rap1 signalling or MAP kinase activation.

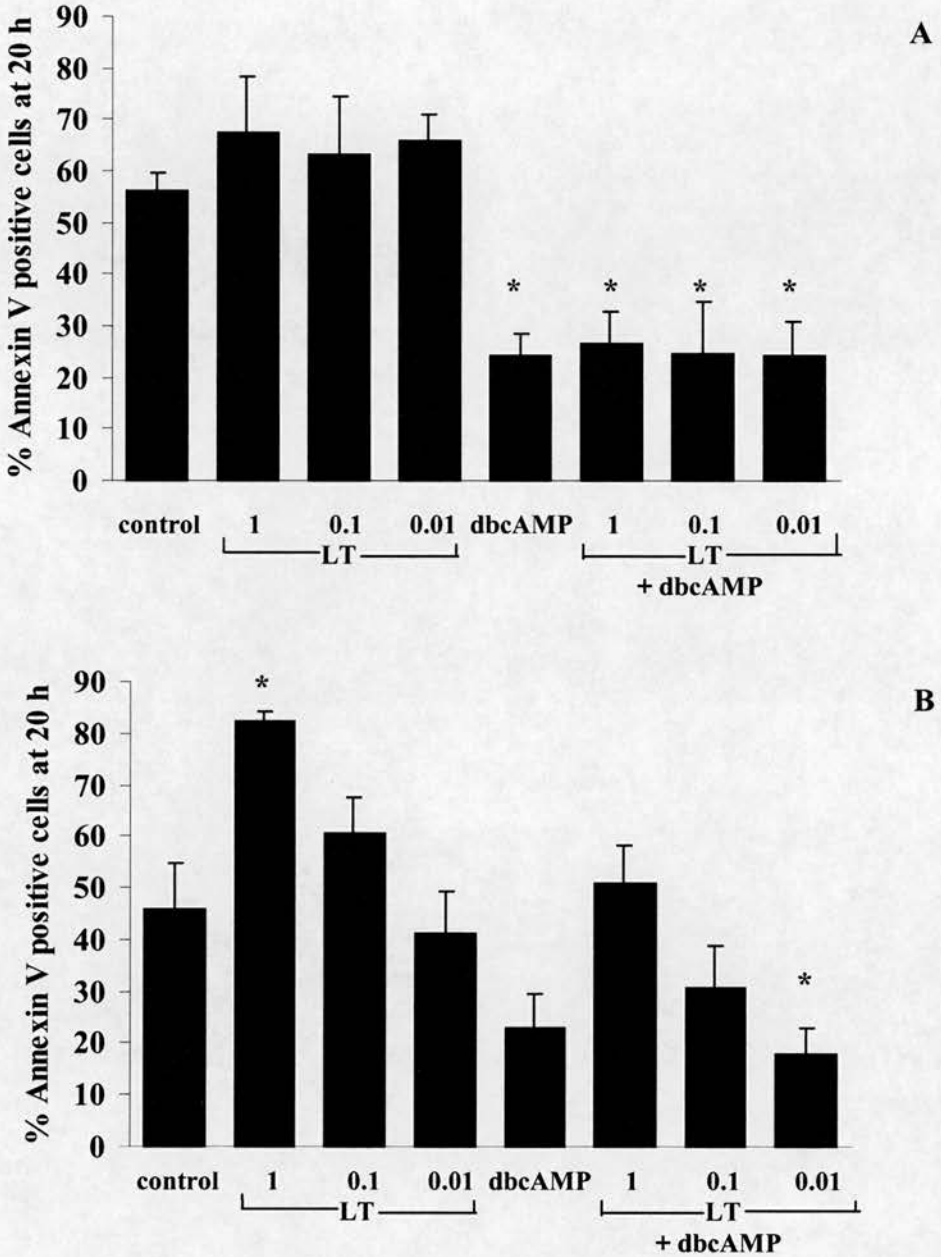


Figure 3.6.1. Effect of *Clostridium sordellii* lethal toxin on dbcAMP mediated delay of neutrophil apoptosis. Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum (A) or 0.1% BSA (B) at 37 °C were treated with the indicated concentrations of *Clostridium sordellii* lethal toxin (LT) alone (μg/ml) and LT plus dbcAMP (0.2 mM). After 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference control is represented by *P<0.01

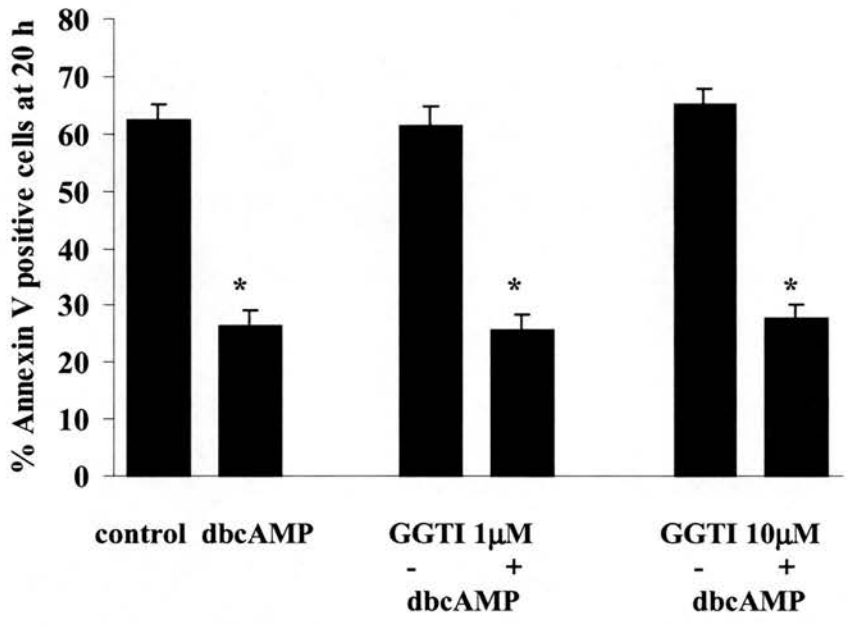


Figure 3.6.2. Blockade of geranylgeranyltransferase activity does not affect cyclic AMP mediated delay of neutrophil apoptosis Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C were treated with GGTI-286 for 10 minutes prior to stimulation by dbcAMP (0.2 mM). After a further 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 4 - 6 experiments, each performed in duplicate where significant difference from control is represented by *P<0.001.

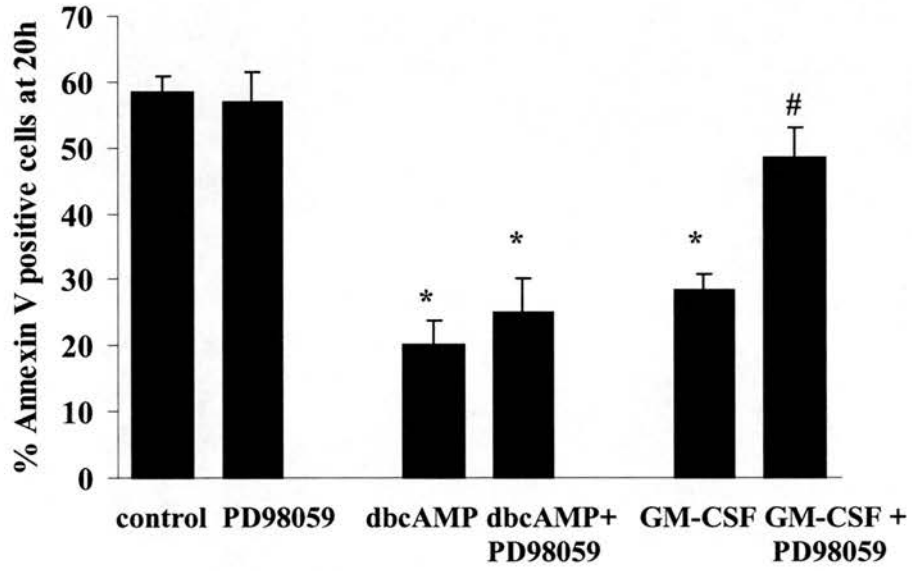


Figure 3.6.3. Effect of MAP kinase inhibition on dbcAMP and GM-CSF mediated delay of neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C were treated with PD98059 (10 μM) for 30 minutes prior to stimulation by dbcAMP (0.2 mM) or GM-CSF (50 U/ml). After a further 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S. E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by $^*P<0.001$ and significant difference from GM-CSF alone is represented by $^{\#}P<0.01$.

3.7 CYCLIC AMP MEDIATED DELAY OF NEUTROPHIL APOPTOSIS OCCURS VIA A TRANSCRIPTIONALLY INDEPENDENT AND REVERSIBLE SIGNALLING PATHWAY

3.7.1 CYCLIC AMP MEDIATED SUPPRESSION OF NEUTROPHIL APOPTOSIS DOES NOT REQUIRE NEW PROTEIN SYNTHESIS

Our results suggest cyclic AMP elevation suppresses neutrophil apoptosis via a previously uncharacterised signalling mechanism. In order to investigate the fundamental nature of this signalling mechanism, it was important to establish if cyclic AMP stimulated a novel signalling pathway which would require transcriptional activation to suppress neutrophil apoptosis.

Cycloheximide, used to block protein synthesis, was titrated to low concentrations to minimise the induction of neutrophil apoptosis by this compound on its own (Whyte *et al.*, 1997). Following overnight culture of neutrophils with dbcAMP and cycloheximide, apoptosis was assessed by standard morphological criteria and exposure of phosphatidylserine by Annexin-V binding. It was found that cycloheximide was unable to reverse the suppression of apoptosis by dbcAMP (Figure 3.7.1). Cycloheximide however blocked glucocorticoid-mediated suppression of neutrophil apoptosis at these concentrations (see Figure 4.3.1). These data suggest that gene transcription is not necessary for suppression of neutrophil apoptosis by cyclic AMP.

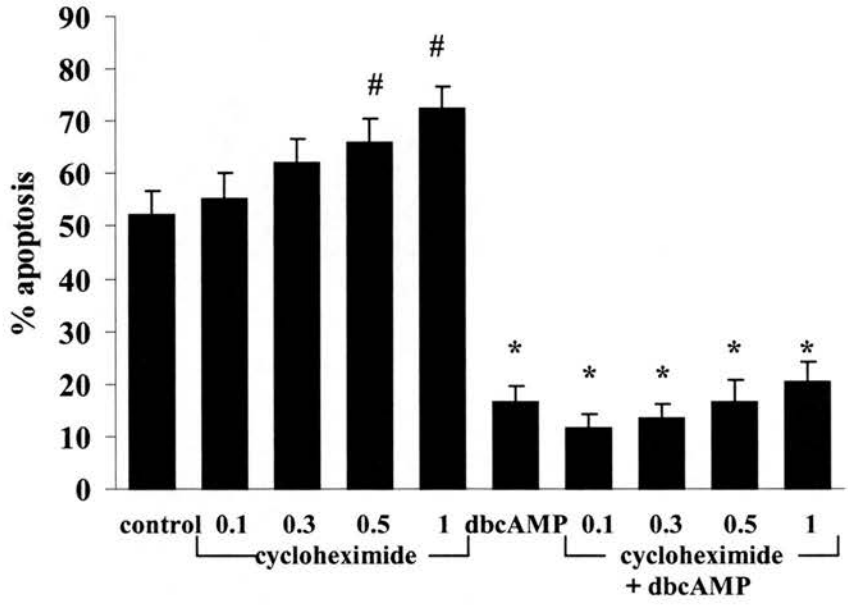


Figure 3.7.1 Effect of protein synthesis inhibition by cycloheximide on cyclic AMP mediated delay of neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with the indicated concentrations of cycloheximide (μg /ml) with or without dbcAMP (0.2mM). After 20 h, cells were harvested and assessed morphologically for apoptosis. All values represent mean ± S.E. of n = 10 experiments, each performed in triplicate. Similar results were found when cells were assessed for apoptosis by Annexin-V binding (data not shown). Significance from control is represented by $^{*}P<0.001$ or $^{\#}P<0.01$

3.7.2 CYCLIC AMP MEDIATED DELAY OF NEUTROPHIL APOPTOSIS IS DEPENDENT ON CONTINUOUS STIMULATION

As we had eliminated the requirement for gene transcription for the ability of cyclic AMP to delay neutrophil apoptosis, we examined if cyclic AMP could activate a rapid and direct signalling pathway instead of stimulating new protein synthesis, which would occur over several hours. Evidence that this was indeed the case was implied from experiments in which neutrophils were cultured in the presence of dbcAMP for the time points indicated (i.e. 0.5, 1, 2, 4 h) before dbcAMP was removed from culture by gently washing in PBS and the cells returned to normal culture conditions (Figure 3.7.2). DbcAMP was required to be continually present in the culture medium in order to suppress neutrophil apoptosis suggesting again that cyclic AMP does not stimulate production of a survival protein in order to enhance neutrophil survival (Figure 3.7.2).

3.7.3 CYCLIC AMP ELEVATION CAN RESCUE NEUTROPHILS FROM APOPTOSIS WHEN ADDED AFTER ONSET OF CULTURE

The results above suggest cyclic AMP activates a rapid signalling pathway in neutrophils to delay neutrophil apoptosis. We wished to examine if cyclic AMP was capable of rescuing neutrophils from constitutive cell death by adding cyclic AMP late after onset of culture. We found that dbcAMP could rescue neutrophils from apoptosis when added at time points after onset of culture (Figure 3.7.3). It was striking that cyclic AMP was still able to suppress neutrophil apoptosis even after 8 hours in culture to levels comparable with a full 24 h incubation with cyclic AMP. It has previously been demonstrated that early measurable signs of neutrophil apoptosis can occur by 8 hours (Ward *et al.*, 1999a) and so one could assume that the apoptotic signalling machinery would presumably already be activated in these cells at this time point. Together these results suggest that cyclic AMP is capable of a powerful direct signalling mechanism independent of new protein synthesis in order

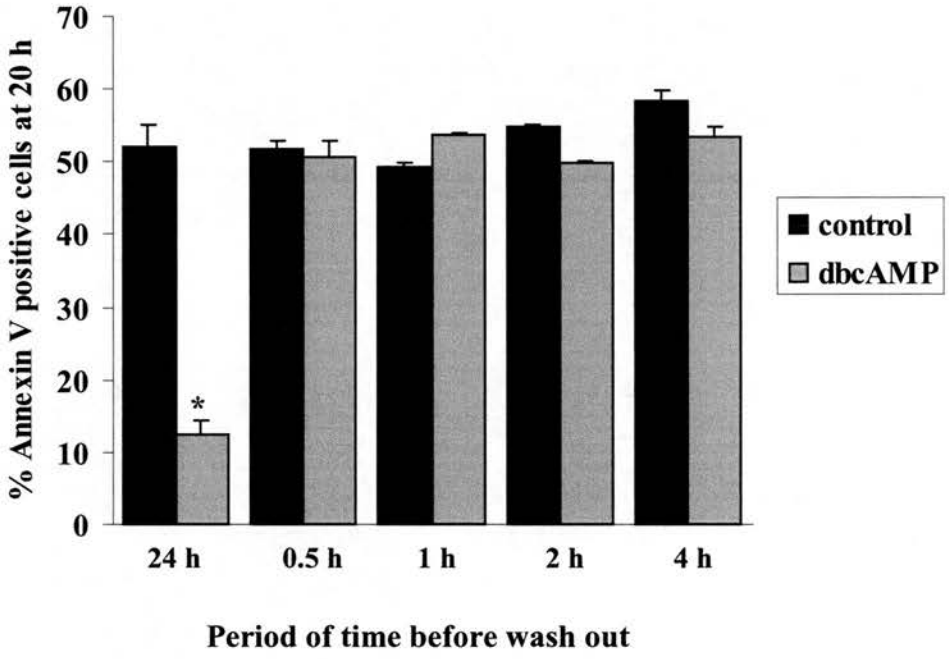


Figure 3.7.2. Loss of cyclic AMP mediated delay of neutrophil apoptosis by washing. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C were treated with or without dbcAMP (0.2 mM) for the time points indicated before the cells were washed x 2 in PBS to remove dbcAMP and returned to culture. Cells were cultured in Iscove's DMEM containing 10 % autologous serum until 20 h when the cells were resuspended and incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by *P<0.001.

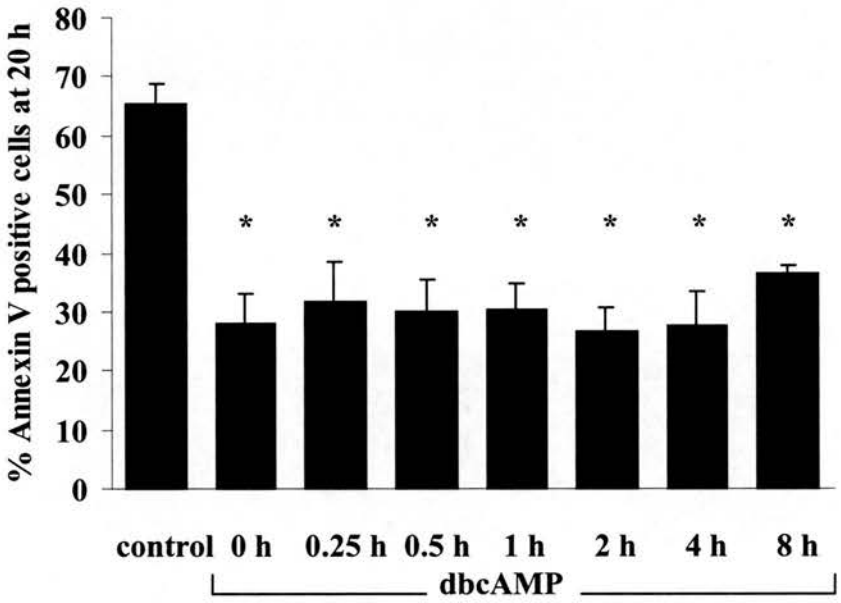


Figure 3.7.3 Rescue of cultured neutrophils from apoptosis by delayed addition of dbcAMP. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37°C for the time points indicated before addition of dbcAMP (0.2 mM). At 20 h, cells were resuspended and incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S. E. of n = 3 experiments, each performed in duplicate and were significantly different from control *P<0.01

to suppress neutrophil apoptosis and that this suppression is rapidly lost when cyclic AMP is removed from culture. It is likely therefore that cyclic AMP is exerting its effects via post-translational modifications within the neutrophil.

SUMMARY

- Cyclic AMP mediated delay of neutrophil apoptosis is not reversed by cycloheximide.
- Suppression of neutrophil apoptosis by cyclic AMP is rapidly lost in "wash-out" experiments.
- Cyclic AMP will rescue neutrophils from apoptosis up to at least 8 hours after onset of culture.
- Cyclic AMP may be acting independently of protein synthesis, via a direct and rapid signalling pathway to delay neutrophil apoptosis.

3.8 PROTEASOME INHIBITORS ARE ABLE TO REVERSE SUPPRESSION OF NEUTROPHIL APOPTOSIS BY CYCLIC AMP

It has been postulated that cell fate may depend on the balance in the cell between pro-apoptotic and anti-apoptotic proteins (Ward *et al.*, 1999b; Akgul *et al.*, 2001). It has been shown that neutrophils contain death regulator proteins such as Bax and Bad and also express some anti-apoptotic Bcl-2 family proteins such as Mcl-1 and Bcl-X_L but not Bcl-2 (Ward *et al.*, 1999b; Akgul *et al.*, 2001). It has been proposed that neutrophils may prolong their longevity by a mechanism whereby they synthesise anti-apoptotic proteins such as Mcl-1 (Ward *et al.*, 1999b; Akgul *et al.*, 2001). It is unlikely however that cyclic AMP mediated delay of neutrophil apoptosis involves such a mechanism as we have demonstrated that cyclic AMP mediated survival does not require gene transcription. We decided to examine if cyclic AMP could be accelerating the degradation or modification of pro-death proteins within the neutrophil to promote survival. Proteasomes are thought to have a major role in the degradation and disposal of intracellular proteins (Rock *et al.*, 1994) however it is becoming apparent that these organelles play a vital role in the regulation of many other cellular processes. Proteasomes are now known to selectively degrade many proteins involved in cell regulation and thus are directly or indirectly involved in many cell functions including apoptosis (Orlowski, 1999). Recent findings suggest that proteasomes can modulate the balance between Bcl-2 family members and thus activity of the proteasome may determine cellular fate (Breitschopf *et al.*, 2000; Marshansky *et al.*, 2001). For example, it has been shown that blockade of proteasome activity in Jurkat T cells allows accumulation of Bax and subsequently induced cytochome c dependent apoptosis (Li and Dou, 2000). To investigate if cyclic AMP causes targeted degradation of apoptotic proteins in the neutrophil to promote survival, we blocked proteasome activity with the irreversible proteasome inhibitors lactacystin and epoxomicin for 20 h. Lactacystin, a *Streptomyces* metabolite, is a specific inhibitor of the proteasome, which modifies irreversibly the amino terminal threonine residue of the mammalian 20S proteasome subunit X,

which is required for proteolysis (Fenteany *et al.*, 1995). Epoxomicin covalently modifies 4 catalytic subunits of the 20S proteasome and is thought to have greater specificity than all other available proteasome inhibitors (Meng *et al.*, 1999). We found that both lactacystin and epoxomicin could eliminate suppression of neutrophil apoptosis by dbcAMP thus suggesting cyclic AMP mediated suppression of neutrophil apoptosis requires the degradation of unknown protein(s) to confer a pro-survival signal on the cell (Figure 3.8.1 & 3.8.2).

SUMMARY

- Proteasome inhibitors lactacystin and epoxomicin reverse cyclic AMP mediated delay of neutrophil apoptosis.

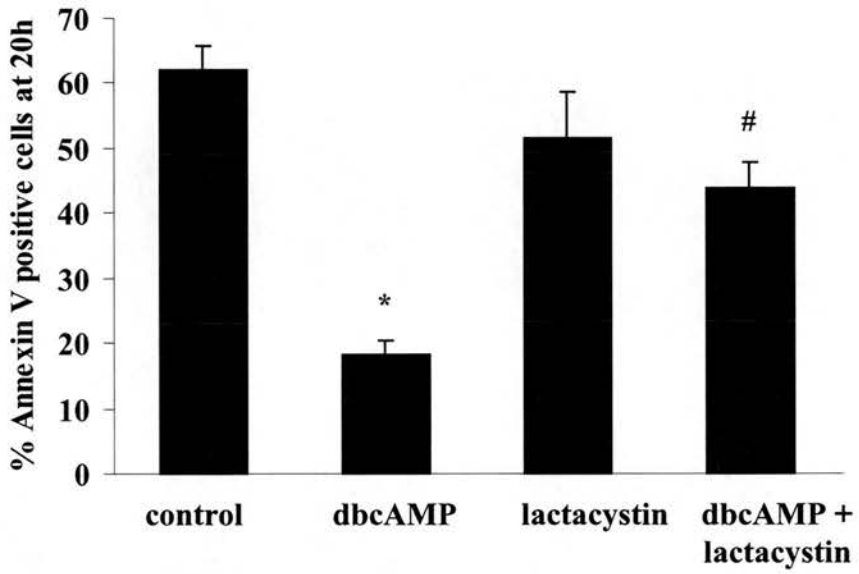


Figure 3.8.1 Effects of lactacystin on dbcAMP mediated delay of neutrophil apoptosis. Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with lactacystin (10 μM) with or without dbcAMP (0.2 mM). After 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by $^*P<0.001$. Significant difference from dbcAMP alone represented by $^{\#}P<0.001$.

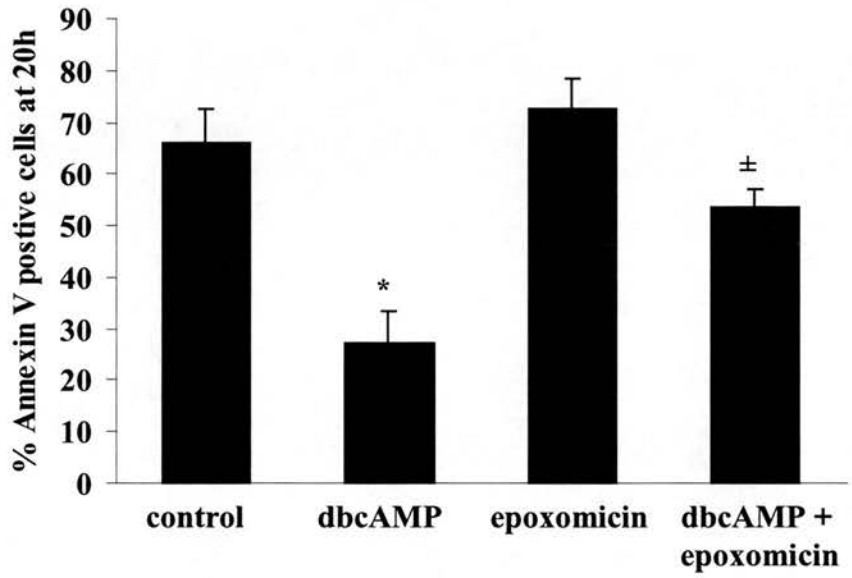


Figure 3.8.2 Effects of epoxomicin on dbcAMP mediated delay of neutrophil apoptosis. Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with epoxomicin (10 μM) with or without dbcAMP (0.2 mM). After 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n=3 experiments, each performed in duplicate where significant difference from control is represented by $*P<0.001$. Significant difference from dbcAMP alone represented by $^{\pm}P<0.01$.

3.9 EFFECT OF CYCLIC AMP ON "DEATH PROTEIN" EFFECTOR EXPRESSION IN NEUTROPHILS

In light of the previous findings, we decided to examine the effect of cyclic AMP on expression of possible "death" proteins in neutrophils by Western blotting. Neutrophils were incubated with dbcAMP for various time points over a 20 hour period from which whole cell lysates were prepared. Neutrophils have been demonstrated to contain the pro-apoptotic protein Bax. As shown in Figure 3.9.1, we found that levels of Bax remain unchanged over the 20 hour time course and are not altered by dbcAMP treatment. Moreover, neutrophils have also been reported to contain the pro-apoptotic protein Bad, however difficulty in achieving reproducible blots for Bad in neutrophils has meant that a role for Bad in cyclic AMP-mediated delay of neutrophil apoptosis has yet to be fully elucidated (data not shown).

It has been demonstrated in neuronal cell lines that exposure to forskolin, a direct activator of adenylyl cyclase, results in a decrease in the levels of the catalytic and regulatory subunits of PKA (Boundy *et al.*, 1998). Further, this process was shown to be independent of gene transcription and involves proteasome mediated degradation (Boundy *et al.*, 1998). We therefore investigated the possibility that cyclic AMP could be targeting the degradation of PKA, in order to suppress neutrophil apoptosis. Immunoblotting revealed that in neutrophils, expression of both PKA regulatory and catalytic subunits remain unchanged during neutrophil apoptosis (Figure 3.9.2). Furthermore, treatment of neutrophils with dbcAMP does not alter protein levels of the PKA subunits tested. We also investigated if cyclic AMP could alter the expression of IκBα, an inhibitor of NFκB in order to delay neutrophil apoptosis. NFκB has been shown to be an important survival factor in neutrophils (Ward *et al.*, 1999a) and activation of NFκB is dependent on the degradation of IκBα, which as a consequence allows NFκB translocation to the nucleus (Chen *et al.*, 1995). As degradation of IκBα is mediated by proteasomes (Chen *et al.*, 1995) it was pertinent to examine the possibility that cyclic AMP may cause

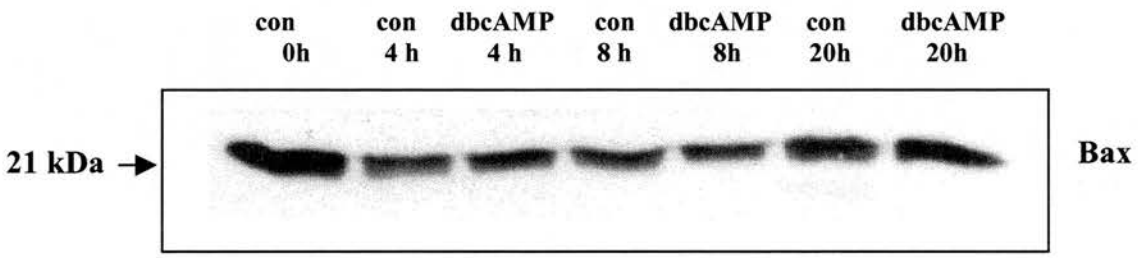


Figure 3.9.1 Time course for the effect of dbcAMP on Bax expression during human neutrophil apoptosis Western blot of cytoplasmic extracts from neutrophils treated with control buffer or dbcAMP (0.2 mM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using a rabbit polyclonal antibody specific for Bax. The gel is representative of 3 experiments.

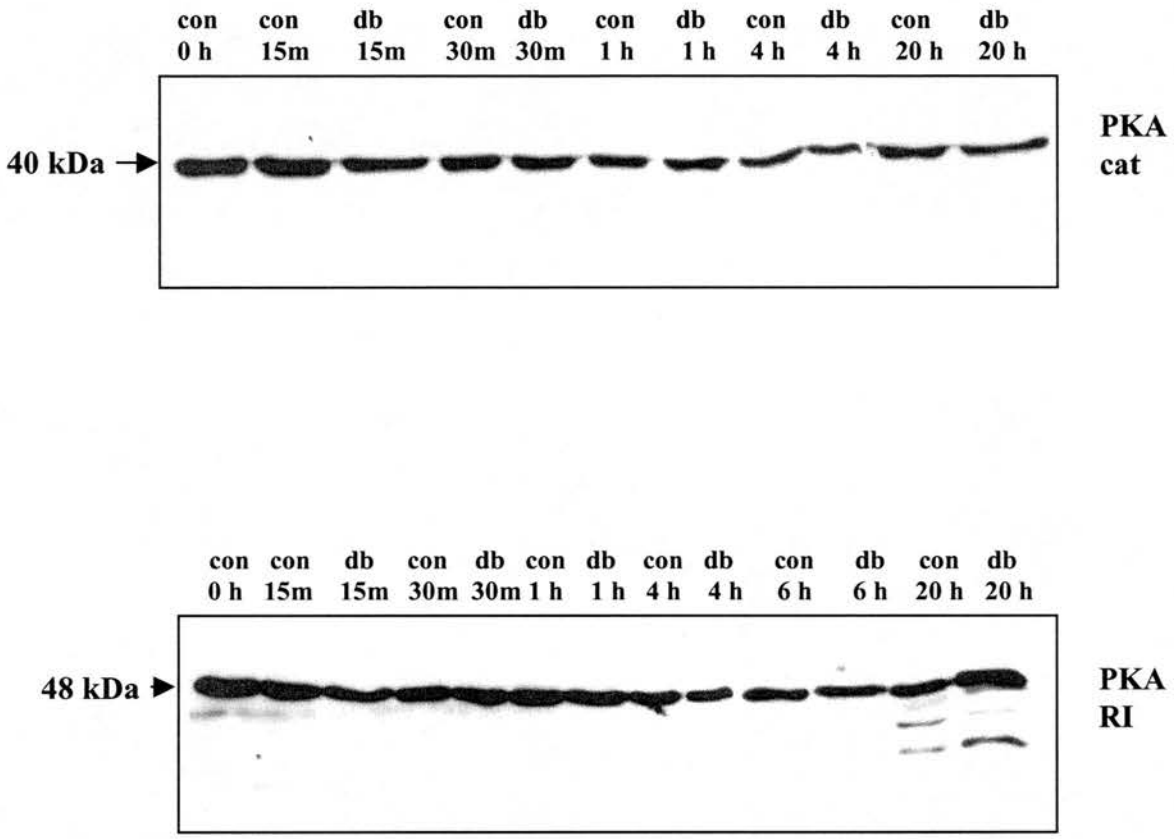


Figure 3.9.2 Time course for the effect of dbcAMP on PKA catalytic (C) and regulatory (RI) subunit expression during human neutrophil apoptosis Western blot of cytoplasmic extracts from neutrophils treated with control buffer or dbcAMP (0.2 mM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using mouse IgG2b monoclonal antibodies specific for PKA catalytic (C) and PKA regulatory (RI) subunit. The gels are representative of 3 experiments.

degradation of IκBα, allowing activation of NFκB which has been demonstrated to favour neutrophil survival. This appeared unlikely to be the case, as cyclic AMP does not alter IκBα expression (Figure 3.9.3). As a positive control we were able to demonstrate that unlike cyclic AMP, TNFα does cause degradation of IκBα. Furthermore cyclic AMP stimulation did not alter TNFα mediated degradation of IκBα.

SUMMARY

- Cyclic AMP does not appear to alter protein expression of Bax, PKA regulatory and catalytic subunits and IκB-α

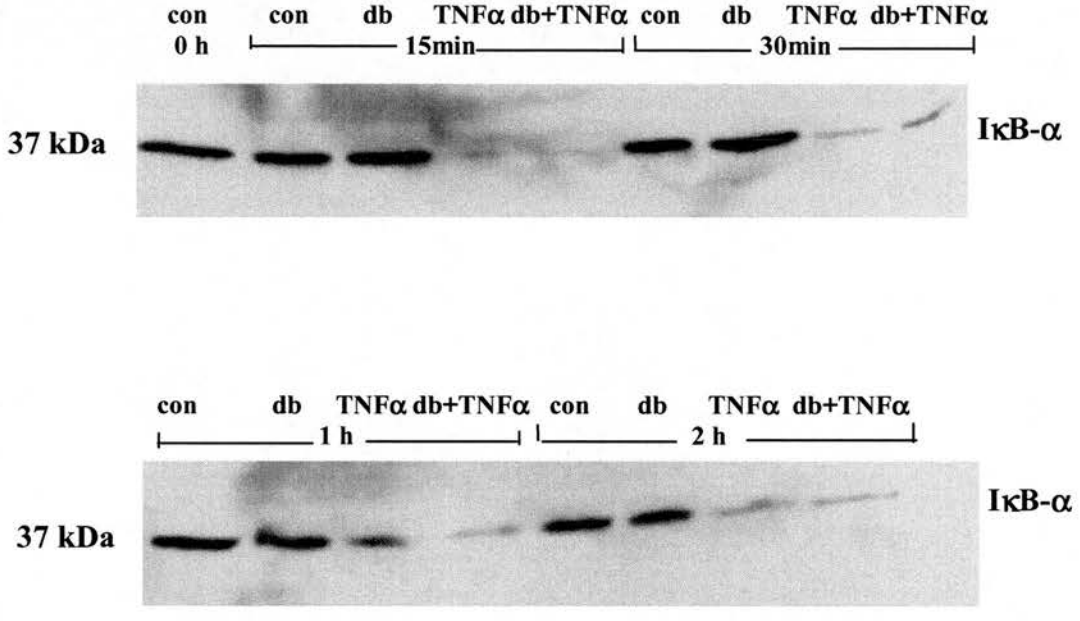


Figure 3.9.3 Time course for the effect of dbcAMP on IκB-α expression during human neutrophil culture. Western blot of cytoplasmic extracts from neutrophils treated with control buffer, dbcAMP (0.2 mM) or TNFα (12.5 ng/ml) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using a rabbit polyclonal antibody to IκB-α. The gels are representative of 3 experiments.

3.10 REGULATION OF DEATH RECEPTOR SIGNALLING IN GRANULOCYTES BY CYCLIC AMP

We have demonstrated that cyclic AMP plays an important role in the regulation of constitutive neutrophil apoptosis. We wished to further our studies to investigate the role of cyclic AMP in regulating death receptor signalling pathways in the neutrophil. The pathways regulating constitutive neutrophil apoptosis may be very different from the death receptor signalling mechanisms mediated by the tumour necrosis factor receptor (TNFR)/nerve growth factor family. Curiously TNFα has been found to exert both pro-and antiapoptotic effects on the neutrophil (Murray *et al.*, 1997). A second member of the TNF receptor family, Fas can also transduce death signals in the granulocyte (Liles *et al.*, 1996). The precise role of Fas and the natural ligand for Fas, FasL in controlling neutrophil apoptosis is however not yet clear (Liles *et al.*, 1996; Fecho and Cohen, 1998; Tortorella *et al.*, 1998b). We decided to establish whether elevation of cyclic AMP could inhibit death receptor mediated apoptosis and the mechanisms by which this may occur.

3.10.1 SUPPRESSION OF TNFα INDUCED DEATH IN NEUTROPHILS BY CYCLIC AMP

To examine the effects of cyclic AMP on death receptor mediated apoptosis, we firstly assessed the ability of cyclic AMP to modulate TNFα induced apoptosis in neutrophils. TNFα has been shown to induce apoptosis at early time points during neutrophil culture but inhibits neutrophil apoptosis at later time points (Murray *et al.*, 1997). To assess if cyclic AMP can modulate TNFα induced apoptosis in neutrophils, cells were pre-incubated for 10 minutes with dbcAMP prior to stimulation with TNFα for 6 hours. As previously described, we found that TNFα, will cause a modest induction of apoptosis at 6 hours (Figure 3.10.1). Furthermore it appears that elevation of cyclic AMP will block the pro-apoptotic effect of TNFα (Figure 3.10.1).

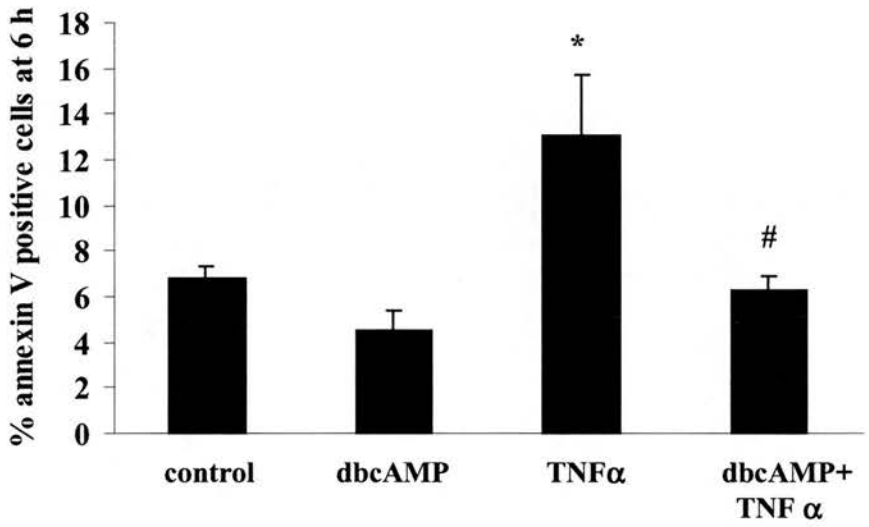


Figure 3.10.1 Suppression of TNFα induced apoptosis by cyclic AMP elevation. Human neutrophils (5 x10^6/ml) were cultured in phosphate buffered saline and pre-incubated at 37 °C, with dbcAMP (0.2 mM) for 10 min before addition of TNFα (12.5 ng/ml). After 6 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by $^*P<0.05$. Significant difference from TNFα alone represented by $^{\#}P<0.05$.

3.10.2 SUPPRESSION OF FAS INDUCED DEATH IN NEUTROPHILS BY CYCLIC AMP

As cyclic AMP has the ability to inhibit TNFα induced apoptosis in neutrophils it was important to ascertain if cyclic AMP could also inhibit other death receptor signalling pathways. Thus we examined the ability of cyclic AMP to modulate Fas induced apoptosis in neutrophils. As mentioned previously there is some controversy as regards the role of the Fas signalling pathway in the regulation of neutrophil apoptosis. Nevertheless it was important to establish if cyclic AMP could modulate Fas induction of apoptosis using the current tools available for investigating this signalling pathway.

Neutrophils were incubated with CH-11, an anti Fas activating antibody (Alderson *et al.*, 1994), in the presence or absence of dbcAMP for the time points indicated. At 4 hours, CH-11 significantly induced apoptosis compared to control (Figure 3.10.2.A). Furthermore it appeared that CH-11 induced apoptosis was inhibited by elevation of cyclic AMP (Figure 3.10.2.A). Additionally, an even greater induction of apoptosis by CH-11 at 6 hours (Figure 3.10.2.B) which was also inhibited by elevation of cyclic AMP. It is interesting to note however that dbcAMP does not completely block CH-11 induced killing in neutrophils observed at 6 hours whereas at 4 hours it appeared that dbcAMP inhibited CH-11 induced apoptosis to levels comparable with the control rate of apoptosis. One possibility is that cyclic AMP is not be able to prevent death receptor induced apoptosis when a greater number of cells are induced to die. Unfortunately we were unable to test whether the same is true of cyclic AMP mediated inhibition of TNFα induced apoptosis due to the biphasic effect of TNFα on neutrophil apoptosis.

SUMMARY

- Cyclic AMP inhibits TNFα induction of neutrophil apoptosis
- Cyclic AMP partially inhibits CH-11 induction of neutrophil apoptosis
- Cyclic AMP may inhibit death receptor mediated neutrophil apoptosis however induction of cell death by a powerful death stimulus may overwhelm the capacity of cyclic AMP to offer protection against apoptosis.

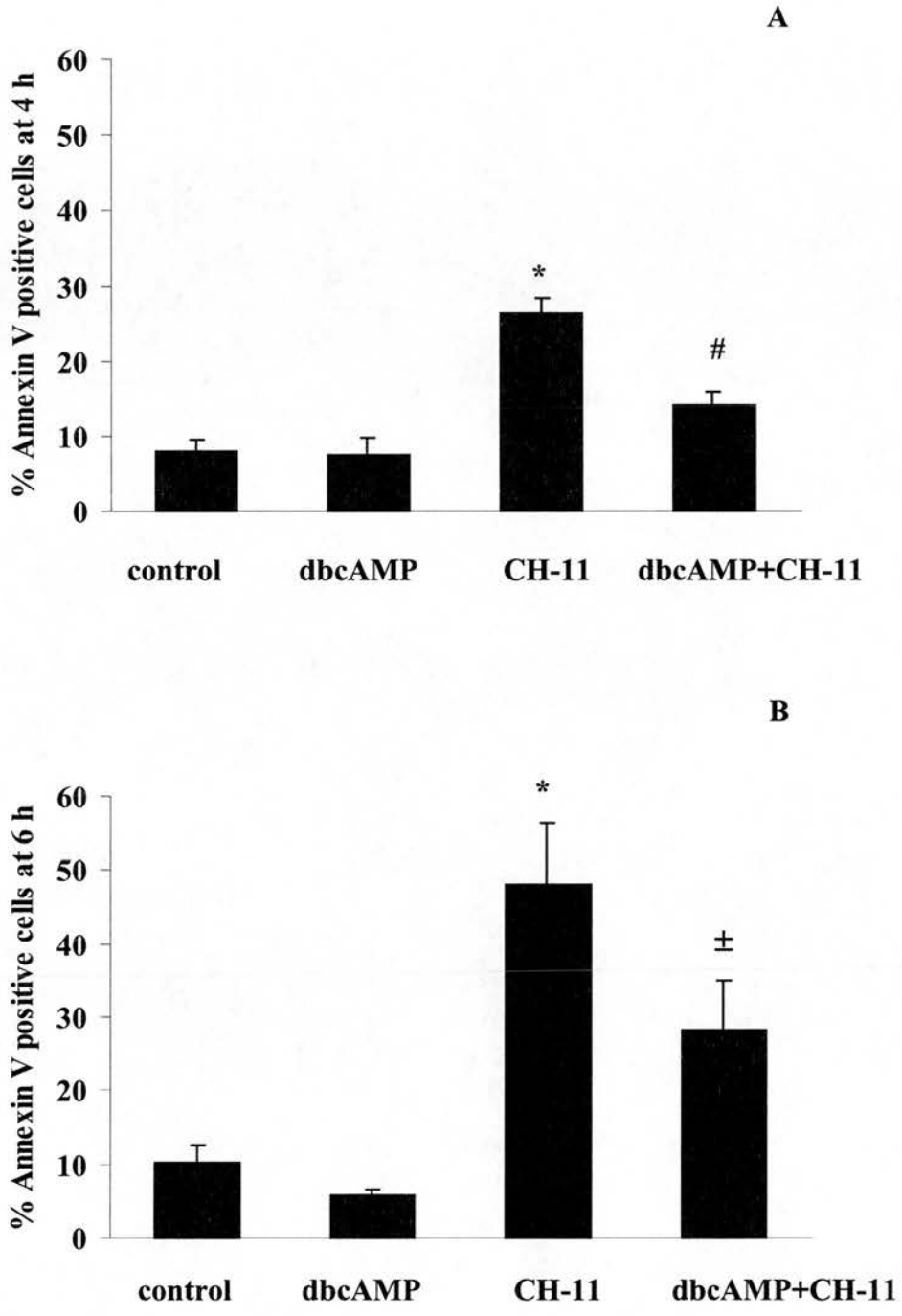


Figure 3.10.2 Suppression of CH-11 induced apoptosis by cyclic AMP elevation. Human neutrophils (5 x10^6/ml) were cultured in phosphate buffered saline with divalent cations and pre-incubated at 37 °C, with dbcAMP (0.2 mM) for 10 min before addition of CH-11 (500ng/ml). After the indicated time points (4 h A and 6 h B), the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 4 experiments, each performed in duplicate where significant difference from control is represented by $^*P<0.001$. Significant difference from CH-11 alone is represented by $^{\#}P<0.01$ or $^{\pm}P<0.05$.

3 DISCUSSION

Human neutrophils undergo apoptosis, a process that is centrally important in the resolution of inflammation. It has been shown previously that cyclic AMP is an important regulator of neutrophil apoptosis (Rossi *et al.*, 1995; Parvathenani *et al.*, 1998; Ottonello *et al.*, 1998; Tortorella *et al.*, 1998a; Tortorella *et al.*, 1998a) yet little is known of the signalling mechanism by which by cyclic AMP controls neutrophil cell death. The studies herein have established that cyclic AMP acts upstream of caspase-3 activation to inhibit the apoptotic pathway in neutrophils. This finding suggests that cyclic AMP delays neutrophil apoptosis before the main phase of apoptotic execution. In mammalian cells, execution of apoptosis is thought to involve either direct activation of procaspase-3 by caspase-8 (Stennicke *et al.*, 1998) or indirect activation of procaspase-3 through release of apoptosis inducing agents such as cytochrome c and apoptosis inducing factor (AIF) from the mitochondria (Kuwana *et al.*, 1998). As cyclic AMP is able to delay the appearance of active caspase-3 in neutrophils, it suggests that cyclic AMP is acting upstream of at least the execution step of caspase activation. We have not been able to establish if cyclic AMP is able to prevent the activation of procaspase-9, regarded as the initial caspase in the intrinsic (stress and genomic) pathway (Li *et al.*, 1997; Slee *et al.*, 1999). Immunoblotting for caspase-9 was technically difficult, perhaps due to low levels of this protein in neutrophils. It would be interesting to establish if cyclic AMP delayed neutrophil apoptosis by a mechanism which prevented the activity of this apical caspase perhaps through use of specific caspase inhibitors.

It is unclear at present if cyclic AMP is also able to prevent expression of caspase-8, considered to be the apical caspase of the extrinsic (death receptor) pathway (Muzio *et al.*, 1996; Peter and Krammer, 1998). This would be of particular relevance in understanding the nature of cyclic AMP mediated regulation of death receptor pathways. We have found that cyclic AMP can inhibit but not prevent death receptor induction of apoptosis in neutrophils. It would be pertinent therefore to establish whether cyclic AMP is able to delay expression of active caspase-8 or indeed assess

the ability of cyclic AMP to delay death receptor induction of apoptosis if caspase-8 has already been activated. This would help ascertain the "point of no return" at which cyclic AMP is no longer able to delay apoptosis and establish a better picture of the hierarchy of signalling involving cyclic AMP which determines cellular fate.

Novel interactions between caspases and the cyclic AMP signalling pathway have been demonstrated in studies by Huston *et al* who report that cyclic AMP specific phosphodiesterase PDE4A5 is cleaved by caspase-3 during apoptosis in Rat-1 cells (Huston *et al.*, 2000). PDE4A5 was found to be cleaved downstream of its SH3 interaction domain, causing a decrease in its catalytic activity. It is suggested therefore that cleavage by caspase-3 may influence the intracellular targeting of PDE45 and thus affect its activity and ability to regulate levels of cyclic AMP within cells. As elevation of cyclic AMP in Rat-1 cells by forskolin and the PDE4 inhibitor rolipram induces apoptosis, cleavage of PDE4A5 which decreases its ability to breakdown cyclic AMP may consequently contribute to the regulation of apoptosis in Rat-1 cells. It would be interesting to investigate if PDE4 is cleaved during neutrophil apoptosis. Potentially such a mechanism could either upregulate or downregulate the activity of PDE4A5, changing the levels of cyclic AMP, and possibly act as a feedback mechanism influencing the rate of neutrophil apoptosis.

For the first time, we have demonstrated that neutrophils contain a small but significant number of mitochondria, which exhibit loss of membrane potential during constitutive apoptosis. We are currently investigating if this process occurs before other indices of apoptosis in neutrophils, such as phosphatidylserine exposure and nuclear condensation. This would help ascertain whether loss of mitochondrial potential, shown to trigger apoptosis in other cell types, has a similar function in neutrophils. We have also demonstrated that dissipation of mitochondrial membrane potential can be inhibited by elevation of cyclic AMP. It will be important to establish if cyclic AMP can directly affect changes in mitochondrial membrane potential and if this is key to cyclic AMP mediated delay of neutrophil apoptosis.

A very recent report has highlighted the involvement of mitochondria in eosinophil apoptosis (Peachman *et al.*, 2001). Similarly to neutrophils, it appears that eosinophils contain a small number of mitochondria which appear to provide insignificant respiration yet play a vital role in initiation of apoptosis. There is evidence to suggest that in neutrophils mitochondria respiratory chain inhibitors do not cause induction of apoptosis (Mecklenburgh, 1999) indicating that progression of neutrophil apoptosis does not requires an intact respiratory chain. It will be important to examine if neutrophils generate changes in mitochondrial membrane potential from hydrolysis of ATP rather than from respiration and whether blockade of this activity will cause induction of neutrophil apoptosis. This will help determine whether mitochondria directly play a functional role in the regulation and initiation of apoptosis in neutrophils. This also holds importance for the elucidation of the role of Bcl-2 family members in regulating neutrophil apoptosis. Many of these proteins are known to associate with the mitochondria and some such as Bax, undergo conformational changes when associated with these organelles, leading to release of apoptosis inducing factors such as cytochrome c (Wolter *et al.*, 1997; Zamzami *et al.*, 1998; Jurgensmeier *et al.*, 1998). Although there is indirect evidence that Bcl-2 family members play a role in neutrophil apoptosis (Chuang *et al.*, 1998; Moulding *et al.*, 1998) it is unclear whether they require association with mitochondria to mediate their effects. Indeed it has not yet been established if release of cytochrome c from mitochondria is an absolute requirement for induction of apoptosis in neutrophils due to difficulties in detection of this molecule in this cell type. Establishing the subcellular location and possible association of various Bcl-2 family members proteins with these organelles in neutrophils, will give a clearer indication if mitochondria play a key role in regulating neutrophil apoptosis, as has been shown in other cell types.

It has been suggested that PKA plays an important role in cyclic AMP-mediated delay of neutrophil apoptosis (Rossi *et al.*, 1995; Parvathenani *et al.*, 1998; Ottonello *et al.*, 1998). It has been demonstrated that cyclic AMP analogues, which selectively activate type I PKA, attenuate neutrophil apoptosis, compared to analogues that preferentially activate type II PKA suggesting that type I PKA is necessary and

sufficient to mediate the cyclic AMP induced delay of neutrophil apoptosis (Parvathenani *et al.*, 1998). We suggest, alternatively, that PKA activation by cyclic AMP is not responsible for the major apoptosis-retarding influences of cyclic AMP in neutrophils. Indeed, we have demonstrated directly that cyclic AMP elevation in neutrophils stimulates an increase in PKA activity, which is blocked by pharmacological inhibitors. Importantly however, blockade of PKA was not sufficient to reverse the anti-apoptotic effect of cyclic AMP, implying that this molecule has little or no role in the cyclic AMP signalling pathway responsible for the delay of neutrophil apoptosis.

Previous publications have implicated a role for PKA in cyclic AMP regulation of neutrophil apoptosis using concentrations of H-89 greater than 10 μM (Rossi *et al.*, 1995; Parvathenani *et al.*, 1998). The specificity of H-89 at these concentrations is questionable and it has been published that H-89 may inhibit several other kinases, some with potency similar to, or greater than that for PKA (Davies *et al.*, 2000). We propose that failure to directly measure PKA activity together with the use of high and possibly non-specific concentrations of H-89, could have led to misinterpretation of previous data. We have demonstrated that 10 μM H-89 is sufficient to block PKA activity for extended culture periods and is active in the presence of autologous serum. The failure therefore of both H-89 *and* Rp-8-Br-cAMPS, a highly specific inhibitor of PKA, to reverse cyclic AMP mediated delay of neutrophil apoptosis, point to a novel signaling pathway used by cyclic AMP to inhibit neutrophil apoptosis, which is independent of PKA activation.

There have been a few studies reporting PKA independent effects of cyclic AMP. For example in contradiction to previous findings (Skalhegg *et al.*, 1992; Bauman *et al.*, 1994), it has been reported recently that cyclic AMP suppression of T cell proliferation and inhibition of release of T cell cytokines proceeds in a PKA independent manner (Bryce *et al.*, 1999). Furthermore cyclic AMP mediated suppression of TNFα mediated apoptosis in primary hepatocytes has been found to be only partially reversed by the PKA inhibitor KT5720 (Li *et al.*, 2000b). Other

PKA independent effects include cyclic AMP stimulated Akt phosphorylation and membrane ruffling in thyroid cells (Cass *et al.*, 1999).

Although these recent studies have suggested cyclic AMP is capable of mediating effects independently of PKA, there has been little elucidated of the alternative signalling pathways involved. Pharmacological blockade of the MAP kinase and PI-3 kinase signalling cascades in this study suggest that neither of these signalling pathways are likely to be important in the cyclic AMP mediated delay of neutrophil apoptosis. There has been interest in the discovery that cyclic AMP can bind specifically to and activate small guanine nucleotide exchange factors (GEFs) which will activate the small Ras like GTPase, Rap1 (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). The biological function of Rap1 is still unclear but it has been proposed that activation of this small GTPase may feed into MAPK signaling pathways (Bos, 1998). It was important to investigate if Rap1 was involved in cyclic AMP mediated protection of neutrophil cell death, as Rap1 has been found to be highly expressed in neutrophils (Quilliam *et al.*, 1991; M'Rabet *et al.*, 1998). Furthermore, activation of Rap1 has been shown to occur in neutrophils in response to fMLP, PAF, GM-CSF and IgG coated particles (M'Rabet *et al.*, 1998). As an approach to establishing if Rap1 has a role in cyclic AMP mediated delay of neutrophil apoptosis, we blocked Rap1 activity using the *Clostridium sordellii* lethal toxin (LT), which has been reported to specifically inhibit the small GTPases Rap1, Ras and Rac (Popoff *et al.*, 1996). Furthermore we tested GGTI-286, a geranylgeranyltransferase inhibitor, which blocks geranylgeranylation required by Rap1 to achieve its mature, biologically active form (Lerner *et al.*, 1995). Our preliminary experiments have suggested that Rap1 is not involved in cyclic AMP mediated delay of neutrophil apoptosis. However due to the lack of specificity of both *Clostridium sordellii* lethal toxin and GGTI-286 as inhibitors of Rap1, it is difficult to rule out completely a role for this small GTPase in cyclic AMP mediated neutrophil survival. It would be interesting to measure Rap1 activity directly to ascertain if treatment of neutrophils with elevators of cyclic AMP results in activation of Rap1. Without the ability to "knock-out" the activity of Rap1 in neutrophils either with a specific

pharmacological inhibitor or by other means, it is difficult to directly prove the involvement of Rap1 in cyclic AMP mediated effects on apoptosis at this time.

Our studies are in accord with very recent publications, which demonstrate that cyclic AMP can mediate effects independently of all currently known downstream substrates. For example, cyclic AMP-dependent inhibition of IL-5 from human T lymphocytes was found not to be mediated by PKA or by the Rap1 signalling pathways (Staples *et al.*, 2001) and in melanocytes, cyclic AMP was found to activate Ras and B-Raf independently of PKA, the Ras exchange factor, Son of sevenless (SOS) and Epac (Busca *et al.*, 2000). This reveals the possibility that there may be an as yet undiscovered downstream substrate(s) for cyclic AMP, which may mediate the effects of this second messenger in not only neutrophil apoptosis but also a whole host of other cellular responses. The challenge will be to ascertain the identity of novel signalling molecules involved in cyclic AMP regulation and contest the current dogma that cyclic AMP exerts is physiological functions almost entirely through activation of PKA.

Regulation of neutrophil apoptosis is thought to depend on the balance between pro-apoptotic and anti-apoptotic death factors expressed in the cell (Ward *et al.*, 1999b; Akgul *et al.*, 2001). Neutrophils contain death regulator proteins, including Bax and Bad, and also express some anti-apoptotic Bcl-2 family proteins such as Mcl-1 and Bcl-xL but not Bcl-2 (Ward *et al.*, 1999b; Akgul *et al.*, 2001). It has been proposed that neutrophil longevity may be prolonged by the synthesis of anti-apoptotic proteins such as Mcl-1 (Moulding *et al.*, 1998). However, it is unlikely that cyclic AMP effects are mediated by such a mechanism since we have demonstrated that cyclic AMP-mediated delay of neutrophil apoptosis does not require gene transcription. Furthermore, "wash out" experiments have revealed that retardation of neutrophil apoptosis is rapidly lost when dbcAMP is removed from culture, even after incubation periods that should permit new protein synthesis.

Together, these data suggest a mechanism whereby cyclic AMP does not stimulate production of a survival protein but may alternatively induce post-translational

modifications in the neutrophil to promote survival. One potential mechanism for cyclic AMP-mediated retardation of neutrophil apoptosis may involve cyclic AMP specifically targeting a death protein(s) to the proteasome for degradation. We have demonstrated that blockade of proteasome activity results in a dramatic loss of the pro-survival effect of cyclic AMP. We speculate that cyclic AMP may be involved in the post-translational modification of a death protein, which it may specifically target to the proteasome. If cyclic AMP stimulation is removed or proteasome activity is blocked then the accumulation of a death protein(s) would be predicted to permit the rapid onset of cell death.

There is evidence that in at least other cell types, cyclic AMP is capable of stimulating proteasomal activity. For example in human embryonic kidney cells (HK293) elevation of cyclic AMP results in increased proteasomal activity allowing secretion of a C terminally truncated fragment of the β amyloid precursor protein (Marambaud *et al.*, 1996). Furthermore, inducible cAMP early repressor (ICER), a member of the CREB/ATF family of transcription factors, has been shown to be degraded by the ubiquitin-proteasome pathway (Folco and Koren, 1997), allowing regulation of its transcriptional activity. There is also evidence that treatment of cells with proteasomal inhibitors can result in the accumulation of apoptotic regulatory proteins such as the accumulation of Bax and Bik in Jurkat T cells (Li and Dou, 2000; Marshansky *et al.*, 2001).

To investigate whether cyclic AMP elevation targets the specific degradation of death protein(s) in neutrophils, we have tried to examine individually possible "death" proteins that may be degraded by cyclic AMP. We were not able to detect any changes in the levels of protein expression of Bax, PKA regulatory and catalytic subunits and IκBα. We did however observe alterations in proteins levels of caspase-3 with cyclic AMP treatment (Figure 3.2.1). This, we assumed, reflected the ability of cyclic AMP to delay neutrophil apoptosis and thus decreased expression of active caspase-3 was likely to be due to a decreased number of apoptotic cells in the population compared to control. However, it has recently been published that X-

linked inhibitor of apoptosis (XIAP) promotes degradation of caspase-3 due to its ubiquitin-ligase activity (Suzuki *et al.*, 2001). The anti-apoptotic activity of XIAP is thought to be due to the ability of XIAP to promote degradation of caspase-3. It is possible that a more exciting inference for the ability of cyclic AMP to inhibit caspase-3 expression is that cyclic AMP is actively causing the specific degradation of active caspase-3 thus decreasing its expression, delaying apoptosis. In future experiments it would be interesting to establish if XIAP is present in human neutrophils and if it has any role to play in cyclic AMP mediated delay of neutrophil apoptosis.

There is of course the possibility that cyclic AMP is targeting the degradation of a protein which, is as yet, unidentified. We have also tried to examine the effects of cyclic AMP in neutrophils by investigating global changes in protein expression by two-dimensional electrophoresis. However, due to time constraints and difficulty in identifying new proteins, particularly those expressed at low levels, has meant that identification of novel targets of cyclic AMP by this method, will await future studies. It would also be interesting to measure proteasome activity directly and so further characterise the interplay between the activity of the proteasome and signalling stimulated by cyclic AMP, in the regulation of neutrophil apoptosis.

We have demonstrated that cyclic AMP can dramatically modulate the rate of constitutive neutrophil apoptosis via a novel signalling pathway involving proteasomal regulation. We have also shown in preliminary experiments that cyclic AMP may regulate apoptosis induced in neutrophils by a variety of death receptor stimuli. We have found that cyclic AMP inhibits the pro-apoptotic effect of TNFα in neutrophils. Cyclic AMP is also able to inhibit apoptosis induced by the Fas activating antibody CH-11, at time points of 4 and 6 hours. It is important to note however that cyclic AMP does not appear to be as proficient at inhibiting CH-11 induced apoptosis at 6 hours compared to 4 hours when the percentage of cells induced to die is more modest. It is possible that cyclic AMP is more effective at inhibiting death receptor mediated apoptosis when induction of cell death is moderate. Cyclic AMP may not be able confer pro-survival effects in the presence of

a more powerful death stimulus. Our results are similar to findings made by Parvathenani *et al.* in which elevation of cyclic AMP inhibited CH-11 induced apoptosis better at the early time point of 2 h compared to 8 h where inhibition was minimal (Parvathenani *et al.*, 1998). Consistent with this suggestion, Tortorella *et al.* have reported that dbcAMP could not inhibit CH-11 induced death in neutrophils at 12 h, when the death stimulus is more potent (Tortorella *et al.*, 1998b).

Further support for this hypothesis, is our observation that cyclic AMP is not able to inhibit induction of cell death in neutrophils by the NFκB inhibitor, gliotoxin or in the synchronous death during temperature re-warming of neutrophils from 15°C to 37°C (data not shown). In both gliotoxin and temperature shift mediated neutrophil death, 80-90% of cells are apoptotic after 2 h (Ward *et al.*, 1999a; Pryde *et al.*, 2000). In both systems, there is a rapid onset of neutrophil apoptosis yet pre-incubation with elevators of cyclic AMP fails to inhibit induction of neutrophil apoptosis.

We have demonstrated that during constitutive neutrophil apoptosis, cyclic AMP is able to rescue neutrophils from cell death up to 8 h after the onset of culture. However we have yet to establish if cyclic AMP is able to rescue cells at time points after 8 h when we know there is an exponential increase in the rate of cell death. It would be very interesting to investigate if cyclic AMP continues to delay neutrophil apoptosis at these later time points or if during this phase of culture, there is a point at which cyclic AMP is no longer effective. Interpretation of data from these types of experiments is difficult, as the neutrophils are not dying in a synchronous fashion. However in experimental systems such as death receptor mediated death or temperature re-warming, apoptosis is more synchronous and it is in these instances that we have found cyclic AMP to be unable to effectively mediate protection.

Collectively it appears possible that cyclic AMP powerfully delays neutrophil apoptosis in circumstances when the cells are constantly stimulated by cyclic AMP before onset of apoptosis. If cyclic AMP is removed, normal onset of apoptosis is resumed. Furthermore, if neutrophils are induced to die by a very powerful death

stimulus, cyclic AMP may no longer be effective at mediating its pro-survival effects.

Our hypothesis is supported by finding of Niwa *et al* who show that elevation of cyclic AMP inhibits neutrophil apoptosis induced by a combination of TNFα and cycloheximide (Niwa *et al.*, 1999). In this paper it is suggested that cyclic AMP is only able to suppress apoptosis if elevators of cyclic AMP were added to the neutrophils before treatment with TNFα. It appears that once the apoptotic signalling cascade is activated by TNFα, cyclic AMP can no longer inhibit cell death. It is possible that cyclic AMP may no longer prevent apoptosis once a particular component of the death pathway becomes engaged and it is tempting to speculate the identity of such a protein. During neutrophil temperature rewarming, there is an exponential increase in cell death associated with Bax insertion into the mitochondrial membrane (Pryde *et al.*, 2000). Failure of cyclic AMP to prevent apoptosis induced by temperature rewarming may therefore be due to the inability of this second messenger to inhibit cell death once this death protein is inserted into the mitochondrial membrane. However, as we have not directly measured the effect of cyclic AMP on insertion of Bax during temperature rewarming, we can only hypothesise that this may be the point at which cyclic AMP may no longer be able to inhibit apoptosis. Furthermore, due to the difficulty at present to 'knock-out' the effects of Bax and other proteins in neutrophils, one cannot conclude whether involvement of Bax or other Bcl-2 family proteins in induction of granulocyte death is associative or causative.

Further research into the mechansims behind the ability of cyclic AMP to influence neutrophil apoptosis is vital as these findings have potentially important implications for the role of cyclic AMP in controlling inflammatory cell function *in vivo*. In particular, in situations where cyclic AMP may be artificially elevated, such as the treatment of inflammatory diseases by β2 agonists or selective phosphodiesterase (PDE) inhibitors. There have been widespread interest in the potential usefulness of PDE inhibitors for the treatment of inflammatory diseases (Torphy *et al.*, 1992;

Giembycz and Dent, 1992; Nicholson and Shahid, 1994). Studies have shown that selective PDE inhibitors through elevation of cyclic AMP, suppress a variety of inflammatory cell functions such as degranulation and secretion of granule proteins (Nourshargh and Hoult, 1986; Dent *et al.*, 1991). Rolipram, a PDEIV inhibitor, has been shown to inhibit allergen induced eosinophil and neutrophil accumulation into the lungs of ovalbumin sensitised Brown-Norway rats (Elwood *et al.*, 1995). Furthermore β2 agonists are used as first line therapy in the treatment of asthma and this is may reflect in part their ability to suppress inflammatory cell functions such as superoxide anion release through elevation of cyclic AMP. However, our results point to enhanced neutrophil longevity in the presence of elevated cyclic AMP which may be deleterious in chronic inflammatory conditions. Given that elevated cyclic AMP has also been shown to inhibit macrophage phagocytosis of apoptotic cells (Rossi *et al.*, 1998) it could be predicted that prolonged elevation of cyclic AMP may lead to both enhanced granulocyte survival and ineffective clearance, exacerbating rather than alleviating chronic inflammatory conditions. It is possible that PDE inhibitors although possessing the ability to suppress the inflammatory potential of granulocytes (Nourshargh and Hoult, 1986; Dent *et al.*, 1991), may also antagonise the successful clearance and resolution of inflammation by enhancing granulocyte survival.

There have been intriguing results regarding the influence of cyclic AMP in the presence of pro-inflammatory cytokines on granulocyte survival. It has been reported that elevation of cyclic AMP will favour survival of neutrophils but will reduce the anti-apoptotic effects of GMCSF (Tortorella *et al.*, 1998a). Similarly in eosinophils it has been demonstrated that elevation of cyclic AMP enhances survival but reduces the anti-apoptotic effects of GMCSF (Hallsworth *et al.*, 1996) and IL-5 (Chang *et al.*, 2000). Thus there is the possibility that during inflammation, when cytokines such as GM-CSF may be in abundance, cyclic AMP suppresses inflammation by both dampening down inflammatory cell function and reducing the survival influencing effects of GM-CSF. One could envisage a scenario in which once the inflammation had subsided and activation of various cytokines had decreased, elevation of cyclic AMP by PDE inhibition for example could be deleterious, as elevation of cyclic

AMP alone would likely prevent apoptosis and decrease phagocytic clearance by macrophages.

In summary, our findings have suggested that elevation of cyclic AMP delays constitutive neutrophil apoptosis via a novel, rapid and transcriptionally independent signalling pathway. We believe that protection from apoptosis afforded by cyclic AMP appears to be upstream of changes in mitochondrial potential and executioner caspases. Although elevation of cyclic AMP is very powerful at delaying constitutive neutrophil apoptosis, preliminary experiments indicate that the pro-survival effects of this second messenger may not be as effective when neutrophils are induced to die by powerful death stimuli. It is yet to be established *in vivo*, the relative importance of the ability of cyclic AMP to influence granulocyte apoptosis in the multitude of cellular processes influenced by this powerful second messenger.

4 GLUCOCORTICOID REGULATION OF GRANULOCYTE APOPTOSIS

Glucocorticoids are highly effective in the control of many inflammatory and immune diseases. Glucocorticoids have been employed therapeutically to treat diseases such as asthma and rheumatoid arthritis, however, their use is often limited by systemic side effects. Considering glucocorticoids are widely used in controlling many inflammatory conditions, it is perhaps surprising how little is known of their mechanism of action. Elucidating the underlying signalling pathways of glucocorticoids could be key in the development of more selective anti-inflammatory steroids with fewer side effects.

Glucocorticoids exert their action by diffusing passively through the cell membrane where they bind to glucocorticoid receptors (GRs) located in the cytoplasm of target cells (Levinson *et al.*, 1972; Giannopoulos, 1975). The glucocorticoid receptor belongs to the steroid hormone receptor superfamily whose members include cytosolic receptors for other steroid hormones such as progesterone and oestrogen (Carson-Jurica *et al.*, 1990). Unoccupied glucocorticoid receptors form part of a large heteromeric complex in the cytoplasm that includes proteins such as heat shock protein 90 (hsp90). These act as molecular chaperones, preventing the nuclear translocation of glucocorticoid receptors in the absence of steroid (Bresnick *et al.*, 1989; Bresnick *et al.*, 1988; Pratt *et al.*, 1989). Hsp90, hsp70 and hsp40 together with co-chaperones Hop and p23 are believed to be involved in the assembly of glucocorticoid receptors in a conformation receptive for binding (Rajapandi *et al.*, 2000; Morishima *et al.*, 2000a; Morishima *et al.*, 2000b). It is believed that a conformational change ensues following glucocorticoid binding to GR, allowing GR to dissociate from hsp90 and translocate to the nucleus (Pratt *et al.*, 1989; Stancato *et al.*, 1996).

As the glucocorticoid receptor was one of the first transcription factors to be isolated, there have been many studies elucidating the structure and function of this protein in

glucocorticoid mediated effects. Mutagenesis studies have revealed that the steroid binding domain of GR is located at the carboxy terminus of the molecule (Giguere *et al.*, 1986; Danielsen *et al.*, 1987). Additional functions of the C-terminal domain are thought to include interaction with hsp90, nuclear translocation and transcriptional transactivation (Picard and Yamamoto, 1987; Dalman *et al.*, 1991; Hollenberg and Evans, 1988; Webster *et al.*, 1988; Danielian *et al.*, 1992; Webster *et al.*, 1988)

Following glucocorticoid binding to GR, the subsequent step in the glucocorticoid signal transduction pathway is the rapid translocation of the activated receptor to the nucleus, where GR binds DNA. The DNA binding domain is situated in a central region of the receptor comprising of two zinc motifs and two α helical regions that contact DNA (Luisi *et al.*, 1991; Giguere *et al.*, 1986; Dahlman-Wright *et al.*, 1994; Danielsen *et al.*, 1986; Green *et al.*, 1988). Formation of each zinc finger is thought to require four cysteine residues bound to one zinc molecule (Freedman *et al.*, 1988; Zilliacus *et al.*, 1992). GR is thought to bind to DNA at consensus sites known at glucocorticoid response elements (GREs) in the 5' upstream promoter region of glucocorticoid responsive genes (Tsai *et al.*, 1988; Hard *et al.*, 1990; Truss and Beato, 1993). Transactivation is believed to involve the N-terminal region, which is the largest of the three major GR protein domains. Situated in this N-terminal region is a domain called tau1 (τ_1) which contains a 41 amino acid core sequence which is critical for transactivation. τ_1 may also be involved in binding other transcription factors (Giguere *et al.*, 1986; Dahlman-Wright *et al.*, 1995). A further transactivation domain (τ_2) is also found in human GR and may be important for localisation of GR to the nucleus (Hollenberg and Evans, 1988). GR is highly phosphorylated predominantly on serine residues at the N-terminal domain however the definitive role and consequences of phosphorylation on steroid action remains largely undetermined (Muller *et al.*, 1991).

Glucocorticoids mediate their effects by activating a signalling pathway in which GR directly or indirectly regulates transcription of target genes (Beato *et al.*, 1989; Gronemeyer, 1992) (Figure 4.1).

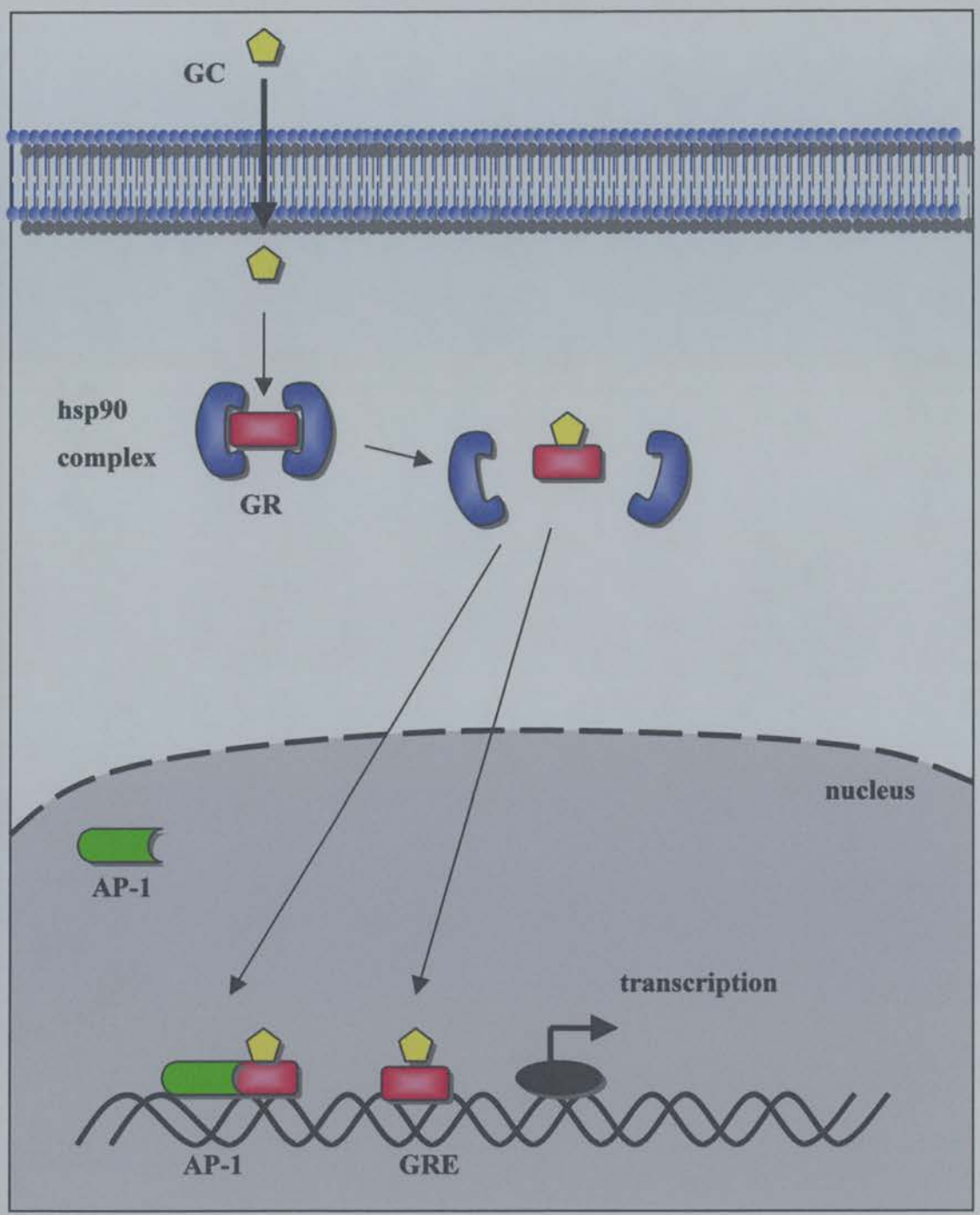


Figure 4.1 Schematic representation of the glucocorticoid signal transduction pathway. Glucocorticoids (GCs) penetrate the cell membrane and bind directly to cytosolic glucocorticoid receptors (GRs). GRs are held in a large multiprotein complex including hsp90. Binding of ligand allows GR to dissociate from this complex and translocate to the nucleus where GR may bind a glucocorticoid recognition sequence (GRE) on the 5' upstream promoter of glucocorticoid responsive genes, allowing modulation of transcription. Alternatively activated GR may indirectly modulate transcription by associating with other transcription factors e.g. AP-1 which may result in mutual repression.

This is thought to involve transcriptional transactivation by direct DNA binding of GR followed by transactivation through τ_1 domain (McEwan *et al.*, 1995). Alternatively GR is thought to control gene expression indirectly through protein-protein interactions in the nucleus with other transcription factors. The first mechanism involves the binding of GR to GRE consensus sites on responsive genes, altering the rate of transcription, resulting in induction or repression of the gene (Tsai and O'Malley, 1994). The rate of transcription may be influenced by a number of factors including the number of GRE consensus sites present and the binding of other transcription factors in the vicinity of GRE, which may alter steroid inducibility (Tsai and O'Malley, 1994; Bastian and Nordeen, 1991; McEwan *et al.*, 1994). There are several examples of DNA-dependent GR mediated repression such as GR mediated repression of prolactin and c-fos genes (Sakai *et al.*, 1988; Mittal *et al.*, 1994). GR has also been proposed to regulate activity of NFκB via GR-GRE induction of transcription of the gene encoding IκB-α (Auphan *et al.*, 1995; Scheinman *et al.*, 1995a). However later studies have shown this mechanism of NFκB regulation by glucocorticoids to be cell type restricted and is not universally applicable to all cell types (Brostjan *et al.*, 1996). It has therefore been suggested that GR mediated transactivation may not represent the principal mechanism of GR mediated transcriptional regulation. There is now abundant evidence that GR can also function in a DNA-independent manner, through a transrepression mechanism involving protein-protein interactions (Karin *et al.*, 1993; Heck *et al.*, 1994; Helmberg *et al.*, 1995). For example GR mediated repression of collagenase gene induction is thought to occur independently of GR-DNA binding and instead involves GR forming a protein–protein complex with AP-1, preventing this transcription factor from stimulating collagenase activity. GR may also interact directly with other important transcription factors involved in signal transduction such as NFκB, signal transducers and activators of transcription (STATs) and cyclic AMP responsive element binding protein (CREB) (Ray and Prefontaine, 1994; Caldenhoven *et al.*, 1995; Scheinman *et al.*, 1995b; Stocklin *et al.*, 1996). It has been proposed that GR transcriptional transrepression mechanisms are important for the anti-inflammatory action of glucocorticoids. Many of the transcription factors demonstrated to be subject to GR

transrepression mechanisms such as NFκB and AP-1, are involved in the regulation of genes centrally involved in inflammation (Barnes and Adcock, 1993).

Glucocorticoids are thought to also regulate gene transcription through effects on chromatin structure (Figure 4.2). DNA is normally tightly coiled around histone proteins, which form a repeating array of DNA-protein particles called nucleosomes. During transcription, histone residues become acetylated, resulting in uncoiling of DNA, which is wrapped around the histone proteins. This allows increased access of transcription factors, resulting in enhanced transcriptional activity. Several transcription factors such as AP-1, STATs and NFκB are thought to bind to co-activator molecules such as CREB binding protein (CBP) and the related p300 protein (Arias *et al.*, 1994; Kamei *et al.*, 1996; Zhang *et al.*, 1996; Gerritsen *et al.*, 1997). CBP is thought to regulate transcription through its ability to both associate with transcription factors and through is possession of intrinsic histone acetyltransferase (HAT) activity, allowing it to acetylate histone residues and regulate transcription (Ogryzko *et al.*, 1996). CBP has been shown to associate with GR via various co-activator proteins such as glucocorticoid receptor interacting protein-1 (GRIP-1) (Hong *et al.*, 1997) and glucocorticoid receptor coactivator-1 (SRC-1) (Smith *et al.*, 1996) thus linking GR to the basal transcriptional machinery. It has therefore been suggested that glucocorticoid repression of transcription factors such as NFκB may be due to competition between GR and NFκB for limiting amounts of CBP or p300 (Kamei *et al.*, 1996; Sheppard *et al.*, 1998). However, other studies have suggested that glucocorticoids are capable for repressing NFκB and other transcription factors independently of the amount of co-activator present in the cell (De Bosscher *et al.*, 2000; De Bosscher *et al.*, 2001).

During transcriptional repression, deacetylation of histones is thought to occur resulting in tighter coiling and reduced access of transcription factors to their binding sites. It has been recently proposed that glucocorticoids in association with co-repressors and proteins with intrinsic histone deacetylation activity may cause deacetylation of histones, leading to gene repression (Wolffe, 1997).

Glucocorticoids are known to be potent anti-inflammatory agents and have been widely used in the treatment of chronic airways diseases such as asthma (Barnes and Adcock, 1993; Barnes, 1997). The anti-inflammatory effects of glucocorticoids may relate, in part, to their ability to downregulate adhesion molecules that may be required in the recruitment of inflammatory cells to the sites of inflammation. Glucocorticoids are known to downregulate ICAM-1 and E-selectin via a direct signalling mechanism (Cronstein *et al.*, 1992; van de *et al.*, 1993). Glucocorticoids have also been shown to suppress PAF induced upregulation of CD11/CD18 and downregulation of L selectin, which may have important implications for leukocyte accumulation (Filep *et al.*, 1997). Glucocorticoids may also suppress inflammation through enhanced synthesis of anti-inflammatory mediators such as IL-1 receptor antagonist (IL-1ra) (Levine *et al.*, 1996), secretory leukocyte protease inhibitor (SLPI) (Abbinante-Nissen *et al.*, 1995) and lipocortin-1 (Browning *et al.*, 1990; Errasfa *et al.*, 1985). Production of lipocortin–1 would presumably decrease the activity of lipid mediators such as prostaglandins (Fradin *et al.*, 1988) and SLPI may be involved in anti-inflammatory activity in the airways, through neutralisation of destructive proteases such as neutrophil elastase (Hochstrasser *et al.*, 1981). The mechanism by which glucocorticoids upregulate these anti-inflammatory proteins is thought to be through transcriptional transactivation. Glucocorticoids also suppress the synthesis of many proinflammatory cytokines from a variety of cells including macrophages (Martinet *et al.*, 1992; Linden and Brattsand, 1994), T lymphocytes (Kelso and Munck, 1984), epithelial (Kwon *et al.*, 1994; Kwon *et al.*, 1995; Wang *et al.*, 1996b) and endothelial cells (Waage *et al.*, 1990; Kerner *et al.*, 1992). Further, glucocorticoids also suppress the synthesis and effects of many chemokines such as IL-8 (Kwon *et al.*, 1994), RANTES (Kwon *et al.*, 1995), and eotaxin (Lilly *et al.*, 1997) potentially abrogating the ability of inflammatory cells to be recruited to the site of inflammation. As several of these genes do not contain the appropriate GRE consensus sites in the 5'end region of their promoter region, it is believed glucocorticoids suppress their production by indirect transrepression of the transcription factors that regulate their expression.

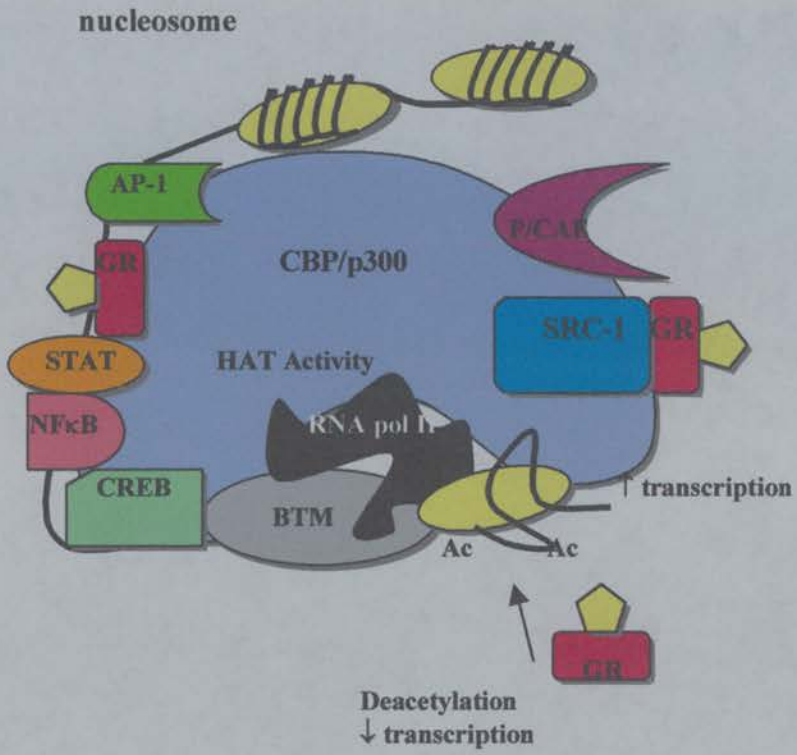


Figure 4.2 Effects of glucocorticoids on chromatin structure. Several transcription factors such as NFκB and CREB bind to the cointegrator molecule CBP/p300 which possesses intrinsic histone acetyltransferase (HAT) activity. During transcription, acetylation of histone proteins by CBP, allows DNA that is wrapped around histone proteins to uncoil. This allows increased accessibility of transcription factors resulting in enhanced transcription. Glucocorticoids through GR may modulate transcription through association with CBP via glucocorticoid receptor coactivator-1 (SRC-1). GR may cause gene repression by competing with other transcription factors for binding to limited amounts of CBP or instead may disturb other transcription factors from binding to the basal transcriptional machinery. Alternatively GR may indirectly deacetylate histones resulting in increased coiling of DNA around histone thereby preventing transcription factor binding. BTM, basal transcription machinery, Ac, acetylated nucleosome.

The beneficial anti-inflammatory properties of glucocorticoids may also occur through direct effects on cell function and may relate to the ability of glucocorticoids to modulate granulocyte function. Glucocorticoids may suppress eosinophilic inflammation by downregulating the release of eosinophil cytotoxic cell contents. However, this may not be the universal mechanism by which glucocorticoids suppress eosinophil activity, as glucocorticoids have been shown to only weakly suppress eosinophil degranulation and secretion of eosinophil basic proteins (Kita *et al.*, 1991c). One of the best-described actions attributed to steroids in asthma is a reduction in the number of circulating eosinophils following glucocorticoid therapy (Schleimer, 1990). Diminished numbers of eosinophils may be the result of suppression of eosinophil recruitment through glucocorticoid inhibition of eosinophil adherence and chemotaxis (Clark *et al.*, 1979; Altman *et al.*, 1981). Furthermore, glucocorticoids may suppress production of inflammatory mediators and cytokines involved in recruiting these cells to inflammatory sites. In addition to suppressing eosinophil influx, a reduction in eosinophil numbers in the airways may be caused by redistribution of eosinophils to other compartments. However studies by Kawabori *et al* have demonstrated that glucocorticoid induced depletion of intestinal eosinophils does not result in redistribution of eosinophils to the spleen, lymph nodes or peripheral blood, suggesting that this may not be a major mechanism for glucocorticoid reduction of eosinophil numbers (Kawabori *et al.*, 1991). There is accumulating evidence however that glucocorticoid depletion of eosinophils may instead occur by an important regulatory mechanism, in which glucocorticoids accelerate eosinophil cell death, allowing engulfment and clearance by macrophages. To this end, Woolley *et al.* have reported increased eosinophil apoptosis in the airways of asthmatics following glucocorticoid treatment. Furthermore, increased apoptosis has been correlated with clinical improvement and resolution of eosinophilic inflammation. As it has been demonstrated that activation of inflammatory cells is reduced when they undergo apoptosis (Whyte *et al.*, 1993), apoptosis could contribute to the resolution of inflammation by reducing eosinophil activity and facilitating their safe removal. Furthermore, apoptosis may allow the shutdown of secretory capacity while maintaining an intact cell membrane thus preventing leakage of cytotoxic cell contents thereby suppressing any further

incitement of inflammation. Furthermore, as glucocorticoids have also been demonstrated to increase the clearance of apoptotic granulocytes (Liu *et al.*, 1999), the effectiveness of glucocorticoids in treatment of inflammatory diseases such as asthma may relate in part to their ability to promote apoptosis and clearance of inflitrating eosinophils.

In contrast to eosinophils, where it has been demonstrated that glucocorticoids induce apoptosis (Meagher *et al.*, 1996), glucocorticoids have been shown *in vitro* to increase the lifespan of the closely related neutrophil (Cox, 1995; Kato *et al.*, 1995), (Liles *et al.*, 1995; Meagher *et al.*, 1996). Indeed, in comparison to the reduction in eosinophil numbers observed following systemic treatment with glucocorticoids, neutrophil numbers in fact show an increase (Schleimer, 1990). Although neutrophil lifespan appears to be influenced by glucocorticoids, it has generally been assumed that neutrophils are less sensitive to the influence of glucocorticoids compared to other white blood cells. Early reports suggested that high concentrations of glucocorticoids could modulate a variety of neutrophil responses (Levine *et al.*, 1981; Umeki and Soejima, 1990; Shea and Morse, 1978) however later studies have reported that physiological concentrations of steroids are unable to suppress a wide variety of neutrophil functions such as chemotaxis, adhesion, degranulation and secretion (Schleimer *et al.*, 1989). The inability of glucocorticoids to suppress neutrophil activity has been proposed to explain the lower efficacy of these drugs in neutrophilic inflammatory disease. For example the beneficial effect of glucocorticoids on 'eosinophilic' inflammatory diseases such as asthma and allergy has not been observed in neutrophil-associated inflammatory diseases such as chronic obstructive airways disease (Zainudin, 1997). Although ineffectiveness of glucocorticoids may be partly due to the inability of these drugs to influence neutrophil responses such as degranulation, it is reasonable to predict that glucocorticoid enhancement of neutrophil longevity may also play a part.

The mechanisms by which eosinophil and neutrophil apoptotic responses to glucocorticoids differ are unknown. It has been proposed that glucocorticoids are likely to exert their effects on granulocyte apoptosis by regulating the cytokine

environment that influences the longevity of these cells. Several groups have reported that glucocorticoids inhibit the effect of cytokines such as GM-CSF in prolonging eosinophil survival (Her *et al.*, 1991; Lamas *et al.*, 1991; Wallen *et al.*, 1991; Hallsworth *et al.*, 1992). Furthermore it has also been reported that glucocorticoids enhance the neutrophil survival properties of GM-CSF (Cox, 1995; Meagher *et al.*, 1996). As eosinophils and neutrophils when appropriately activated, are capable of producing GM-CSF (Kita *et al.*, 1991a), it has been proposed that glucocorticoids exert their influence on granulocyte apoptosis through differential regulation of the elaboration of GM-CSF. However GM-CSF is undetectable in ageing granulocyte cultures and excess of blocking anti-GM-CSF Ab fails to affect modulation of apoptosis caused by glucocorticoids (Meagher *et al.*, 1996). Thus the direct influence of glucocorticoids on granulocyte apoptosis does not appear to be mediated by GM-CSF. It is important to note that *in vivo,* glucocorticoids may influence secretion of cytokines such as GM-CSF from a variety of cell types, which may in turn influence granulocyte apoptosis. For example, glucocorticoids have been reported to suppress GM-CSF production from human bronchial epithelial cells, which consequently abrogates the survival influence of GM-CSF on eosinophils (Cox *et al.*, 1991). These findings may have importance for the use of glucocorticoids in inflammatory conditions, at times where GM-CSF may also be present. However of equal importance is the direct influence of glucocorticoids on granulocytes, which we propose may be a major influence in the long observed divergent response among granulocyte types following glucocorticoid administration *in vivo*. Our aim was to try to elucidate the mechanisms by which glucocorticoids can exert their effects on granulocytes, examining the intracellular signalling pathways initiated by the binding of glucocorticoids to these cells and investigating the differential responsiveness of granulocytes that suggest distinct apoptotic control mechanisms are present in each cell type. Elucidation of the signalling pathways by which glucocorticoids directly influence granulocyte apoptosis, may contribute to a greater understanding of the anti-inflammatory action of glucocorticoids in inflammation.

4.1 DIFFERENTIAL EFFECT OF GLUCOCORTICOIDS ON NEUTROPHIL AND EOSINOPHIL APOPTOSIS

Glucocorticoids have been previously reported to exert diametrically opposed effects upon the rate of granulocyte apoptosis *in vitro*; promoting eosinophil apoptosis while inhibiting neutrophil apoptosis (Cox, 1995; Kato *et al.*, 1995; Liles *et al.*, 1995; Meagher *et al.*, 1996). To confirm this is indeed the case, neutrophils (5 x 10^6/ml) and eosinophils (2.5 x 10^6/ml) were cultured in serum supplemented Iscove's DMEM and exposed to dexamethasone (1 μM) for 20 h and 40 h respectively. We found that dexamethasone delayed morphological changes characteristic of constitutive neutrophil apoptosis, such as cytoplasmic shrinkage and nuclear condensation (Figure 4.1.1). Dexamethasone also delayed cell membrane changes associated with apoptosis such as the exposure of phosphatidylserine measured by Annexin V binding (Figures 4.1.2 and 4.1.3). Figure 4.1.2 illustrates the percentage of cells within the Annexin V "high" gate was reduced by dexamethasone treatment of neutrophils from 57% (B; control) to 23% (C; dexamethasone).

In contrast to the effects observed on neutrophils, eosinophils (2.5 x 10^6/ml) cultured in serum supplemented Iscove's DMEM showed increased apoptosis in the presence of 1 μM dexamethasone at 40 h as measured by standard morphological assessment (Figure 4.1.4). The morphological changes in apoptosis associated with dexamethasone treatment of eosinophils include nuclear pyknosis and chromatin condensation together with decreased cell size and cytoplasmic vacuolation (Bottom panel Figure 4.1.4). Numerous anucleate eosinophils or "ghosts" habitually appeared on the cytocentrifuge preparations, indicative of nuclear extrusion. The effect of dexamethasone on eosinophil apoptosis was also examined by Annexin V binding (Figure 4.1.5 and 4.1.6). Measurement of Annexin-V binding during eosinophil apoptosis revealed an increase in the percentage of cells showing Annexin V "high positivity in dexamethasone treated eosinophils (83%) compared to untreated controls (45%) (Figure 4.1.6).

Assessment of cell viability demonstrated that dexamethasone did not significantly alter this parameter (data not shown). Thus we have confirmed the previous observation that glucocortiocoids exert differential effects on neutrophils and eosinophils.

SUMMARY

- Dexamethasone delays neutrophil apoptosis as assessed by morphological and cell membrane changes.
- Dexamethasone induces eosinophil apoptosis as assessed by morphological and cell membrane changes.

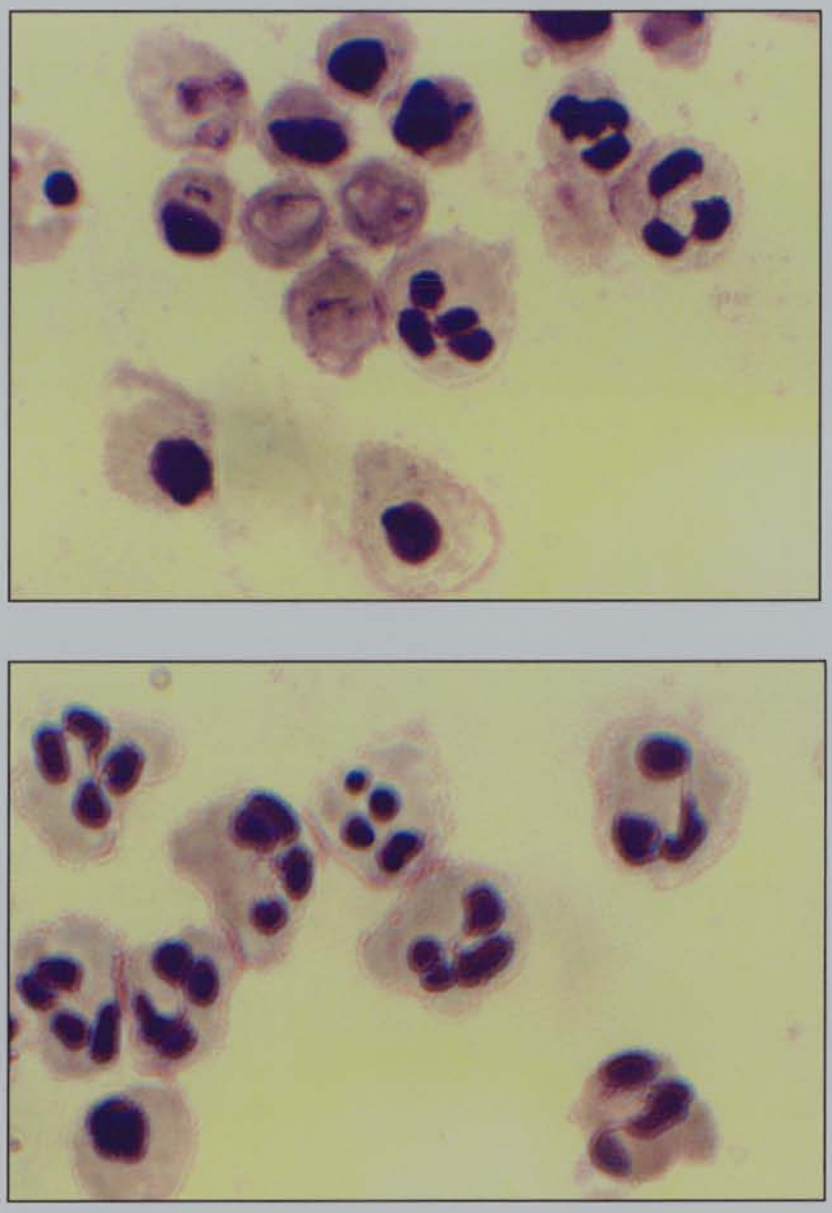


Figure 4.1.1 Neutrophil apoptosis is delayed by dexamethasone. Human neutrophils (5 x10^6/ml) were cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, with or without dexamethasone (1μM). After 20 h, cells were harvested and assessed morphologically for apoptosis. The upper panel indicates control neutrophils after 20 h in culture. The bottom panel indicates neutrophils treated with dexamethasone for 20 h. Note that the number of cells exhibiting classical apoptotic morphology (i.e. condensed nuclei) is much less in the dexamethasone treated cells.

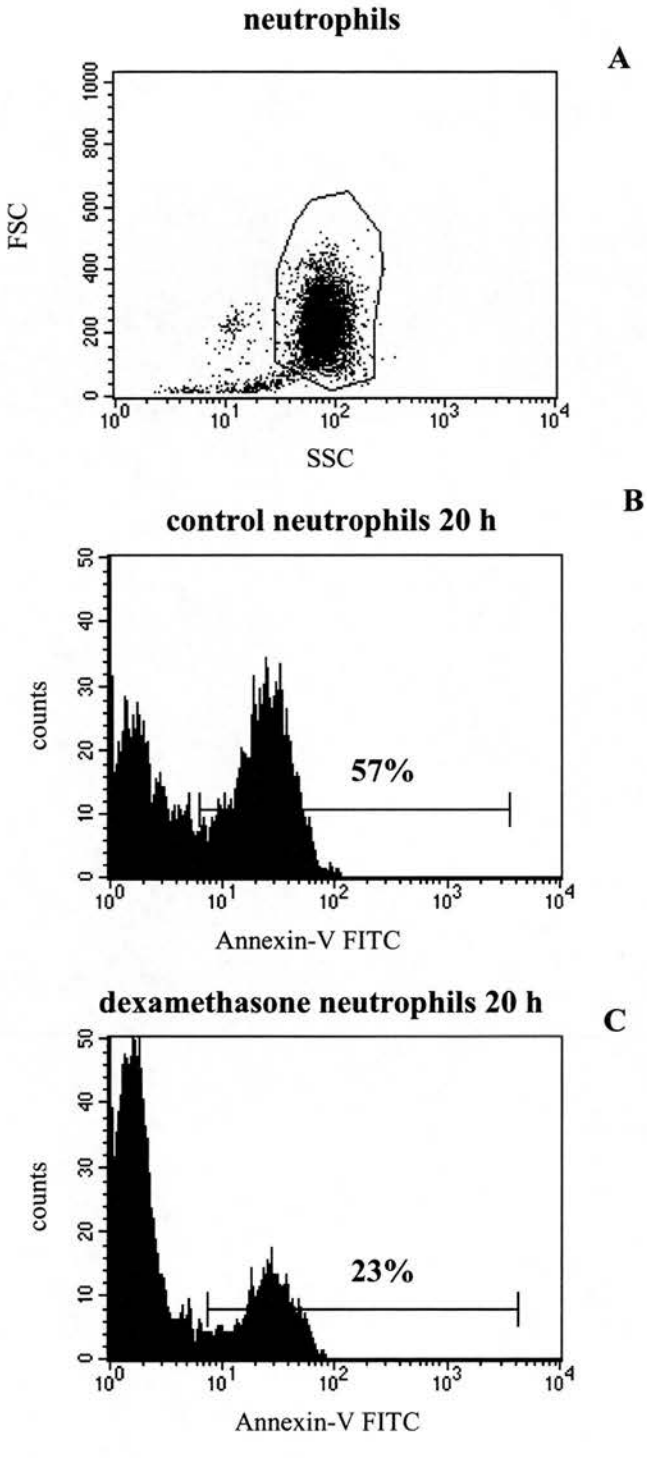


Figure 4.1.2 Dexamethasone mediated delay of neutrophil apoptosis. (A) represents a typical flow cytometric plot for neutrophils. (B) represents control neutrophils after 20 h in culture incubated with FITC labelled recombinant human Annexin-V to measure phosphatidylserine expression. (C) represents neutrophils stimulates with dexamethasone (1 μM) for 20 h before being incubated with FITC labelled recombinant human Annexin-V to measure phosphatidylserine expression. The data is of one representative experiment.

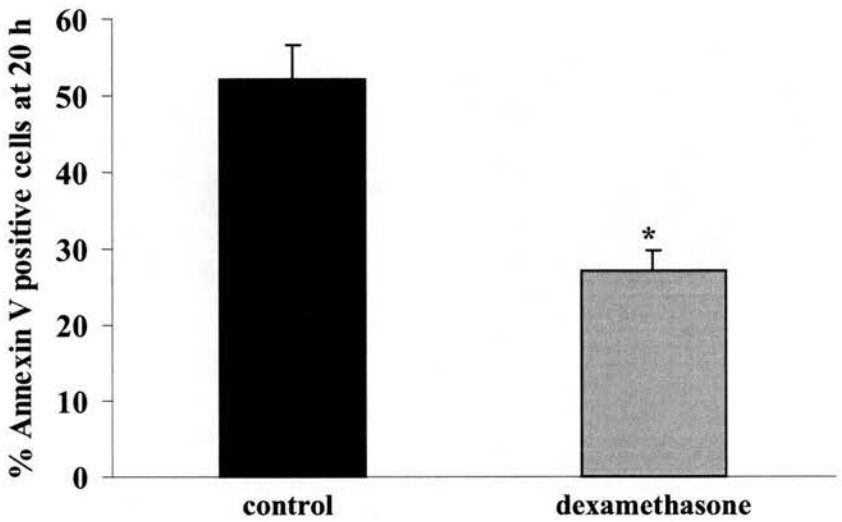


Figure 4.1.3 Dexamethasone delays neutrophil apoptosis. Human neutrophils (5 x10^6/ml) were cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with dexamethasone (1 μM). After 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 6 experiments, each performed in duplicate where significance from control is represented by *P<0.001.

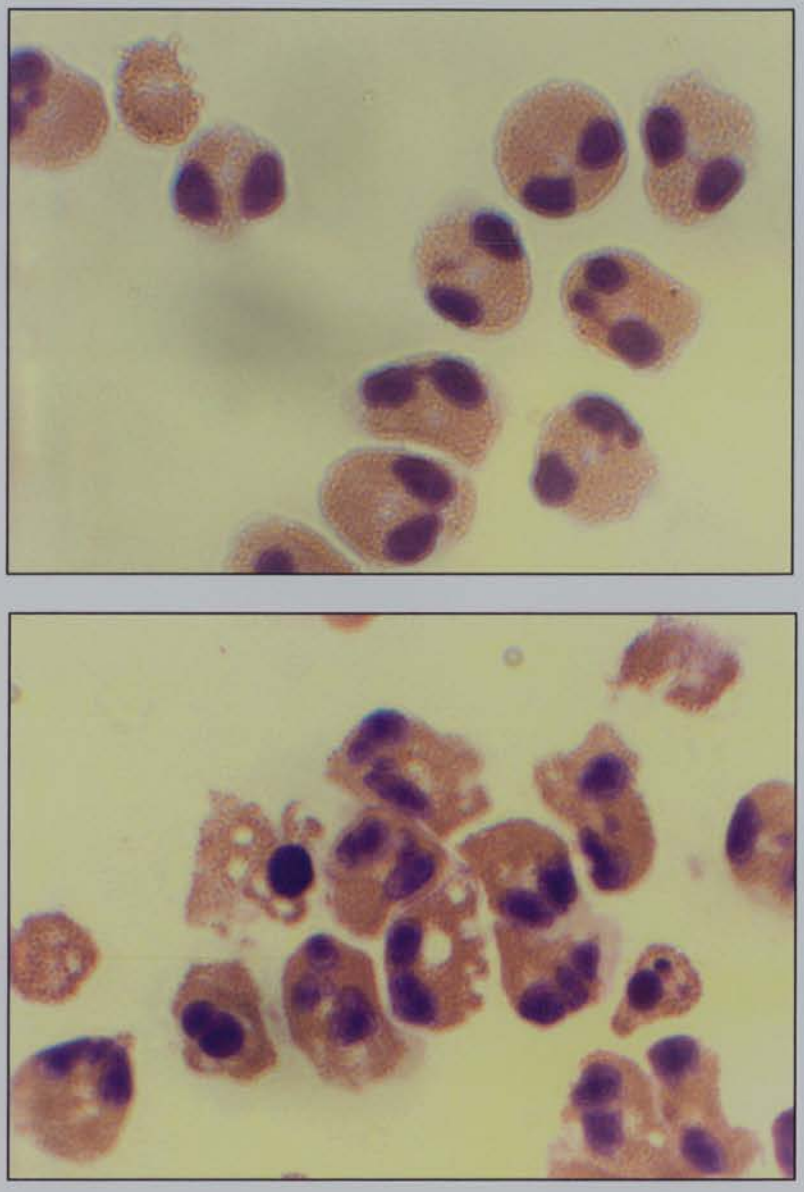


Figure 4.1.4 Dexamethasone induces eosinophil apoptosis. Human eosinophils (2.5 x10^6/ml) were cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, with or without dexamethasone (1μM). After 40 h, cells were harvested and assessed morphologically for apoptosis. The upper panel indicates control eosinophils after 40 h in culture. The bottom panel indicates eosinophils treated with dexamethasone for 40 h. Note that the number of cells exhibiting classical apoptotic morphology (i.e. condensed nuclei) is much higher in the dexamethasone treated cells.

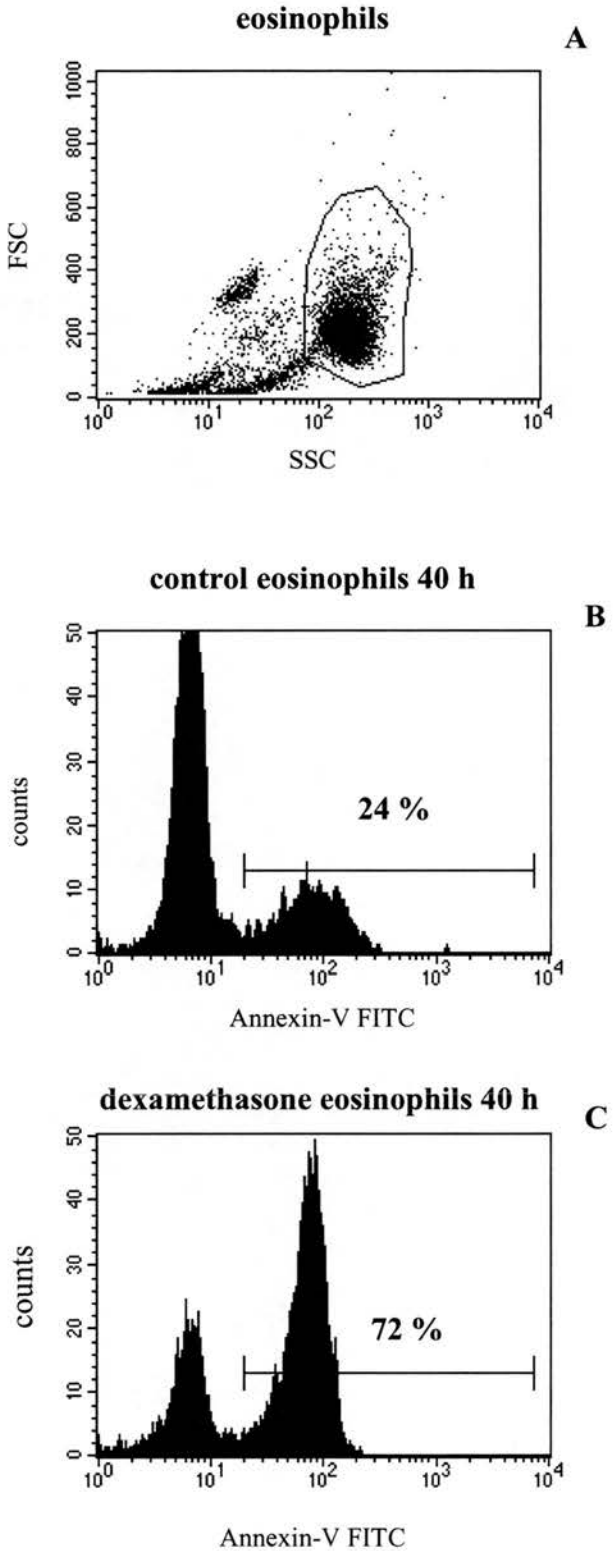


Figure 4.1.5 Dexamethasone induction of eosinophil apoptosis. (A) represents a typical flow cytometric scatter plot for eosinophils. (B) represents control eosinophils after 40 h in culture incubated with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. Similarly (C) represents eosinophils stimulated with dexamethasone (1 μM) for 40 h before incubation with FITC-labelled recombinant human Annexin-V to determine phosphatidylserine expression. The data above is one representative experiment.

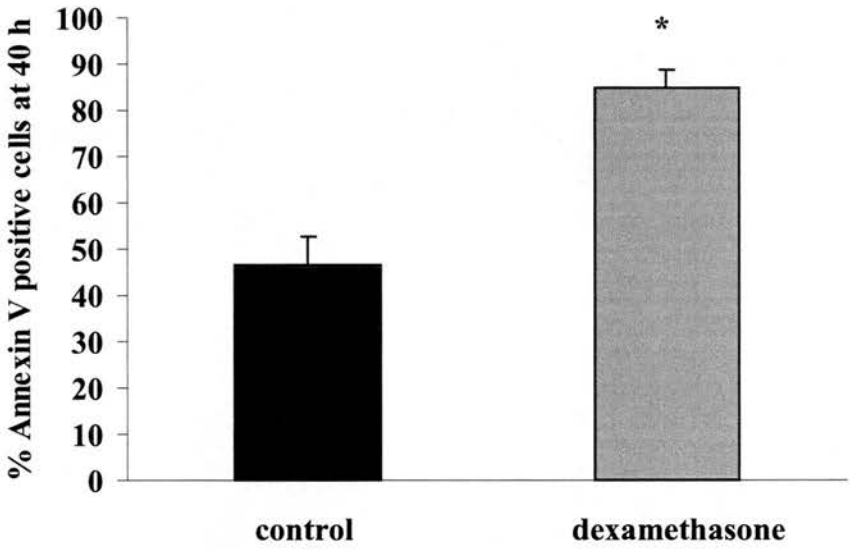


Figure 4.1.6 Dexamethasone induces eosinophil apoptosis. Human eosinophils (2.5 x10^6/ml) cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with dexamethasone (1 μM). After 40 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significance from control is represented by *P<0.001

4.2 CONCENTRATION DEPENDENCY OF THE EFFECT OF DEXAMETHASONE ON NEUTROPHIL AND EOSINOPHIL APOPTOSIS

To determine whether the differential effect of dexamethasone on neutrophil and eosinophil apoptosis is concentration dependent, we performed the following experiments. Neutrophils (5 x 10^6/ml) cultured in serum supplemented Iscove's DMEM showed decreasing levels of neutrophil apoptosis in the presence of increasing concentrations of dexamethasone (0.001 - 10 μM). Furthermore, eosinophils (2.5 x 10^6/ml) cultured in serum supplemented Iscove's DMEM showed increasing levels of apoptosis in the presence of increasing concentrations of dexamethasone (0.01-10 μM). It is important to note that neutrophils appear sensitive to the survival effects of dexamethasone at low concentrations of this glucocorticoid (10 nM). In comparison, it appears that higher concentrations of dexamethasone are needed to induce eosinophil apoptosis (0.1-10 μM).

It has been widely suggested that neutrophils are less sensitive to the effects of glucocorticoids compared to other cell types (Schleimer *et al.*, 1989). The inability of glucocorticoids to modulate key neutrophil functions such as chemotaxis and degranulation led to the prevailing notion that these cells are unresponsive to the effects of glucocorticoids. The data above clearly demonstrates that this is not the case with regards to the ability of nanomolar concentrations of dexamethasone to inhibit neutrophil apoptosis. Furthermore, it is interesting that glucocorticoid induction of eosinophil apoptosis requires higher concentrations of dexamethasone compared with that required to modulate neutrophil apoptosis.

SUMMARY

- Dexamethasone delays neutrophil apoptosis in a concentration dependent manner
- Dexamethasone induces eosinophil apoptosis in a concentration dependent manner
- Neutrophils appear more sensitive to the apoptotic regulatory effects of glucocorticoids compared to eosinophils

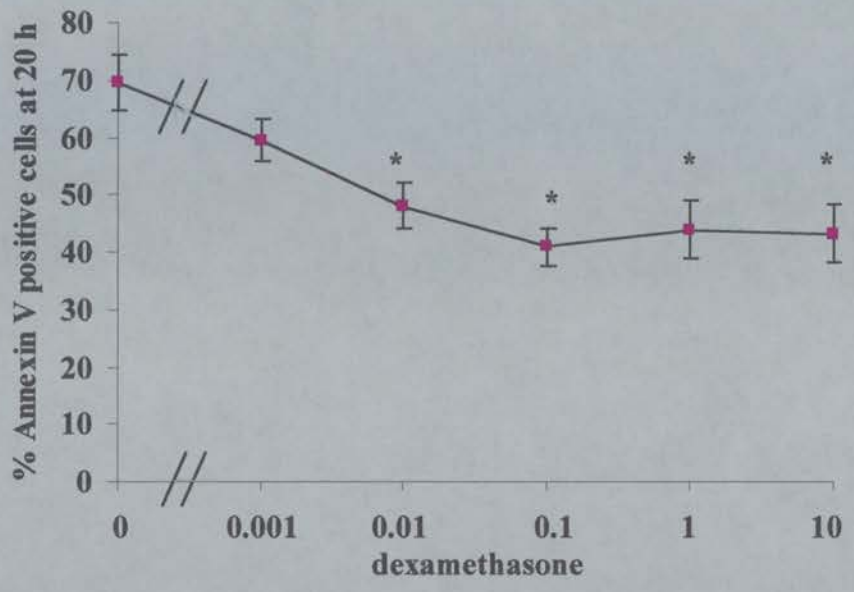


Figure 4.2.1 Dexamethasone delays neutrophil apoptosis in a concentration dependent manner. Human neutrophils (5 x10^6/ml) were cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C with the indicated concentrations of dexamethasone (μM). After 20 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3-7 experiments, each performed in duplicate where significance from control is represented by *P<0.01

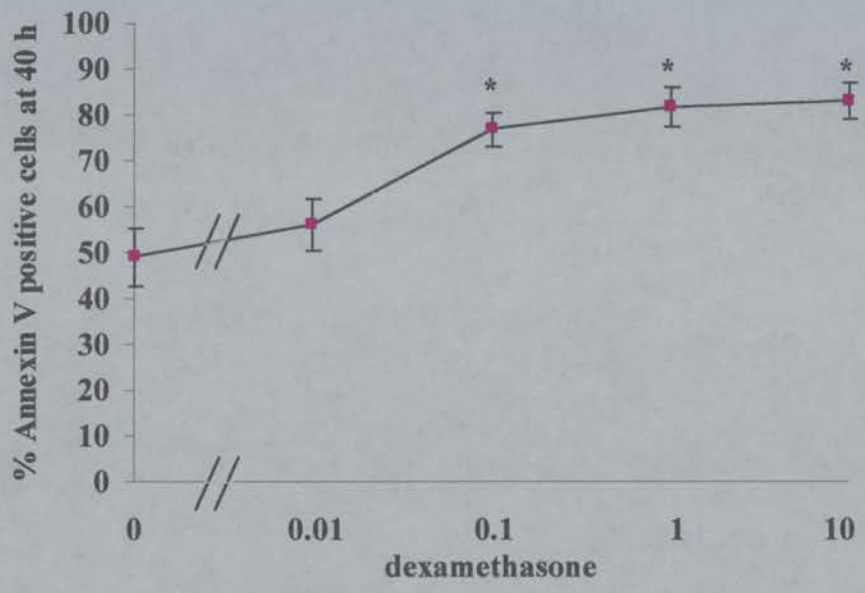


Figure 4.2.2 Dexamethasone induces eosinophil apoptosis in a concentration dependent manner. Human eosinophils (2.5 x10^6/ml) were cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C with the indicated concentrations of dexamethasone (μM). After 40 h in culture, the cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significance from control is represented by *P<0.01

4.3 GLUCOCORTICOID REGULATION OF GRANULOCYTE APOPTOSIS REQUIRES HSP90

Glucocorticoids exert their effects by diffusing passively through the cell membrane where they bind to glucocorticoid receptors (GRs) located in the cytoplasm of target cells. Glucocorticoid receptors form part of a large heterocomplex in the cytoplasm with specific molecular chaperones and other proteins, which prevent unoccupied glucocorticoid receptors from translocating to the nucleus, in the absence of ligand. The large protein complex which binds the carboxy terminal of the glucocorticoid receptor includes two subunits of the heat shock protein hsp90, hsp70 and several other co-chaperones (Bresnick *et al.*, 1989; Pratt *et al.*, 1989; Morishima *et al.*, 2000b; Rajapandi *et al.*, 2000). The role of the hsp 90 as a molecular chaperone of the glucocorticoid receptor is well characterised in many cell systems however involvement of hsp90 and other molecular chaperones in glucocorticoid regulated processes in granulocytes has not been described.

To establish if hsp90 in involved in the glucocorticoid signalling mechanisms controlling cell death in granulocytes we have made use of the hsp90 binding benzoquinoid ansamycin, geldanamycin (GA). Geldanamycin occupies the nucleotide-binding site on hsp90 and prevents the switch to its ATP-bound conformation, which is required for assembly of GR-hsp90 heterocomplexes receptive to ligand binding (Prodromou *et al.*, 1997; Roe *et al.*, 1999). Neutrophils were incubated in serum supplemented Iscove's DMEM in the presence of dexamethasone (1 μM) with or without geldanamycin (10 μM) for 20 h, before assessment of apoptosis (Figure 4.3.1). We found that dexamethasone mediated delay of neutrophil apoptosis is abrogated in the presence of geldanamycin indicating that hsp90 is required for glucocorticoid inhibition of neutrophil apoptosis.

In parallel studies, eosinophils were incubated for 40 h in the presence of dexamethasone (1 μM) with or without geldanamycin (10 μM) before assessment of apoptosis. Dexamethasone treatment of eosinophils induces apoptosis, which we

found could be significantly inhibited by geldanamycin (Figure 4.3.2), suggesting a role for hsp90 in glucocorticoid regulation of eosinophil cell death. It is interesting that geldanamycin causes a small induction of apoptosis on its own in eosinophils suggesting that hsp90 may be involved in regulation of constitutive eosinophil apoptosis.

Our results suggest that both glucocorticoid inhibition of neutrophil apoptosis and glucocorticoid induction of eosinophil cell death require the involvement of hsp90. As geldanamycin is known to block the formation of steroid binding competent aporeceptor complexes, our results may suggest that correctly assembled GR-hsp90 complexes are vital for glucocorticoid regulation of granulocyte apoptosis. However there are as yet no studies demonstrating that GR heterocomplexes form in granulocytes or are required for glucocorticoid signalling in these cells. Thus abrogation of glucocorticoid mediated effects in granulocyte apoptosis by geldanamycin only gives indirect evidence of hsp90 involvement in generating glucocorticoid receptors of the correct conformation for ligand binding. To further elucidate the signalling mechanism by which glucocorticoids differentially regulate granulocyte apoptosis we decided to try to examine the role of GR and its downstream effector mechanisms which may be participating in this process.

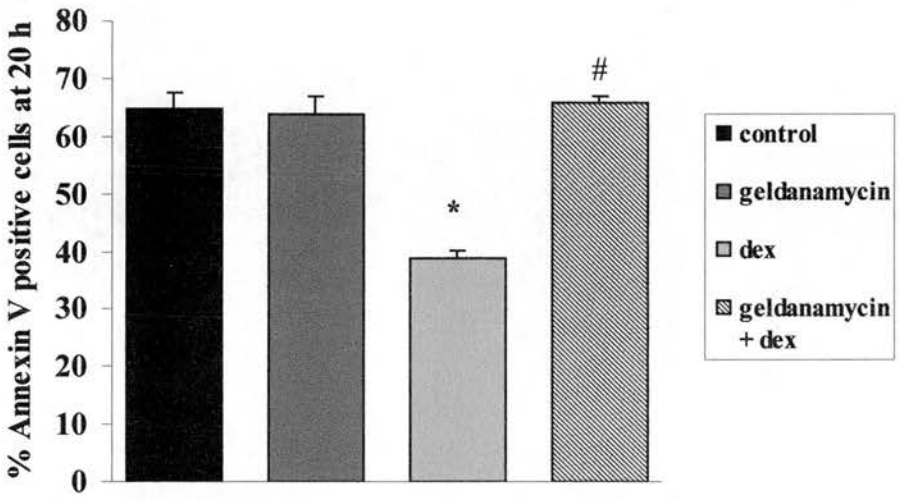


Figure 4.3.1 Blockade of hsp90 with geldanamycin abrogates dexamethasone mediated delay of neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with geldanamycin (10 μM) with or without dexamethasone (1 μM). After 20 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by *P<0.001 and difference from dexamethasone alone is represented by #*P*<0.001.

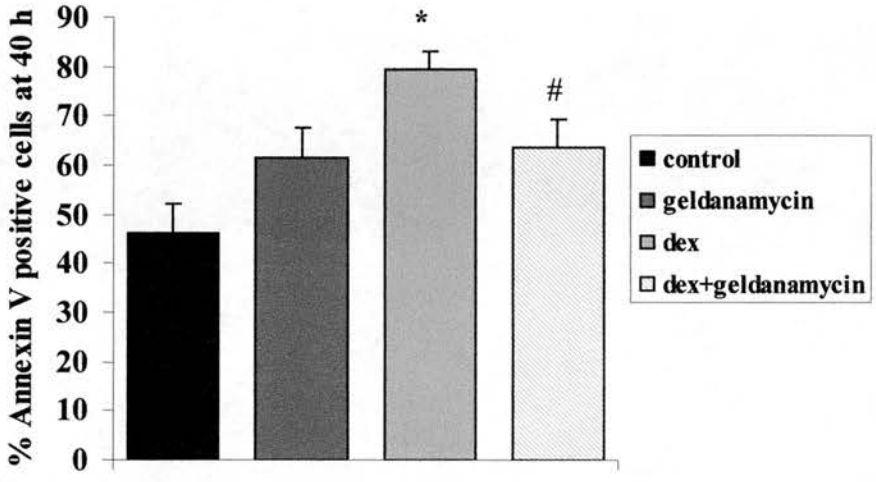


Figure 4.3.2 Blockade of hsp90 with geldanamycin abrogates dexamethasone mediated induction of eosinophil apoptosis. Human eosinophils (2.5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with 10 μM geldanamycin with or without dexamethasone. After 40 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by *P<0.001 and significant difference from dexamethasone alone is represented by #*P*<0.05.

SUMMARY

- Inhibition of hsp90 by geldanamycin abrogates glucocorticoid mediated delay of neutrophil apoptosis
- Inhibition of hsp90 by geldanamycin abrogates glucocorticoid mediated induction of eosinophil apoptosis
- Inhibition of hsp90 by geldanamycin causes a small induction of constitutive eosinophil apoptosis

4.4 ROLE OF THE GLUCOCORTICOID RECEPTOR IN GLUCOCORTICOID REGULATION OF GRANULOCYTE APOPTOSIS

4.4.1 EXPRESSION OF GLUCOCORTICOID RECEPTOR ISOFORMS IN GRANULOCYTES

In light of the fact that neutrophils, compared to eosinophils, appear to have both a differential response and enhanced sensitivity to the apoptotic regulatory influence of glucocorticoids, we investigated if this was due to differences in the glucocorticoid signalling pathway at the level of the GR. The human glucocorticoid receptor exists as two different isoforms, GRα and GRβ, which arise from the same gene by alternative splicing (Hollenberg *et al.*, 1985; Encio and Detera-Wadleigh, 1991). The two isoforms differ only at their carboxy terminus with the last 50 amino acids of GRα being replaced by a 15 AA sequence in GRβ, which is thought to lack a steroid binding domain. As a result, it is believed that GRβ is incapable of binding glucocorticoids and is unable to stimulate gene transcription. Moreover it has been suggested that GRβ can antagonise the effects of GRα possibly through formation of antagonistic GRα/GRβ heterodimers (Bamberger *et al.*, 1995; de Castro *et al.*, 1996; Oakley *et al.*, 1996; Oakley *et al.*, 1999).

It has previously been shown that neutrophils and eosinophils exhibit similar numbers of glucocorticoid receptors (Peterson *et al.*, 1981). The saturable glucocorticoid binding in human neutrophils is similar to that observed in eosinophils (Kd = 17.7 ± 0.8 nM in the neutrophil and Kd = 15.3 ± 0.6 nM in the eosinophil) (Peterson *et al.*, 1981). This would appear to suggest that differences in receptor number and affinity are not responsible for the differential effect of glucocorticoids on granulocyte apoptosis. It is currently unknown which isoforms of GR are expressed in neutrophils and eosinophils and indeed if there are any differences in the nature of the isoforms expressed in these cell types. Although binding studies using ^{3}H-labelled agonists have indicated neutrophils and eosinophils contain similar

numbers of glucocorticoid receptors, this technique would not be able to take into account effects mediated by the presence of GRβ, which cannot bind ligand and whose effects may be independent of this activity. Therefore we decided to examine if the differential responsiveness and effect of glucocorticoids in granulocytes is due to differential expression and signalling of GR isoforms.

Numerous studies have shown that GRα resides mainly in the cytoplasm of cells in the absence of hormone and translocates to the nucleus in a hormone dependent manner (Cidlowski *et al.*, 1990; Antakly *et al.*, 1989). In contrast, it has been shown that GRβ resides mainly in the nucleus although it is unclear if GRβ is able to change its subcellular localisation upon hormone treatment (Oakley *et al.*, 1996). As an initial approach to attempt to dissect the signalling mechanisms involved in glucocorticoid regulation of granulocyte apoptosis, we examined GR protein expression in neutrophils and eosinophils by Western blotting. Protein extracts were obtained from granulocytes which had been treated with control buffer or dexamethasone (1 μM) over a time course of 20 h. Lysates were prepared from equivalent numbers of cells and subject to SDS-PAGE/immunblot analysis. Numerous lysis methods and antibodies were tested as we found extreme difficulty in obtaining successful Western blots for GR using standard techniques. These problems occurred despite inclusion of a broad spectrum of protease inhibitors and may reflect the major protease content of these cells. The various lysis methods and antibodies that were tested are outlined in Chapter 2. Following lengthy optimisation, GR was detectable as a 97 kDa species upon Western blotting of neutrophil cytoplasmic extracts (Figure 4.4.1A). Levels of expression of GR did not alter during the 4 h time course. Furthermore there was surprisingly no detectable loss of GR from the cytoplasm upon dexamethasone treatment at all time points tested (Figure 4.4.1A). Moreover, we could not detect expression of GR in neutrophil nuclear extracts. Similarly to neutrophils, we found that expression of GR in eosinophils remains unchanged over a time course of 4 h (Figure 4.4.1B). Furthermore there is no detectable loss of GR from the cytoplasm of eosinophils upon dexamethasone treatment (Figure 4.4.1B) or expression of GR in nuclear extracts at all time points

tested (data not shown). An affinity purified rabbit IgG verified that the band appearing at 97kDa was not as a result of non-specific binding of the rabbit polyclonal antibody (data not shown).

Western blotting analysis of expression of GRβ specifically was performed using a commercially available rabbit anti-human polyclonal antibody which recognises specifically the carboxy terminus of GRβ (PA3-514). In granulocytes, immunoblotting using this antibody, resulted in the appearance of a ~60 kDa protein band in lysates from both neutrophils and eosinophils (Figure 4.4.1C & Figure 4.4.1D). There was no evidence of higher molecular weights proteins present in these immunoblots, even after a long exposure time. The disparity in the molecular weight of the protein observed and GRβ, which is ~94kDa, suggested the antibody was not recognising GRβ specifically. It was found that immunoblotting with an affinity purified rabbit IgG used at equivalent concentrations, produced a band of the same molecular weight, suggesting the protein detected is not GRβ and is due to non-specific binding of rabbit IgG.

SUMMARY

- Neutrophils and eosinophils express GR by Western blotting however the nature of GR isoforms present in these cells remains undefined.

- Translocation of GR upon dexamethasone treatment could not be detected in neutrophils and eosinophils

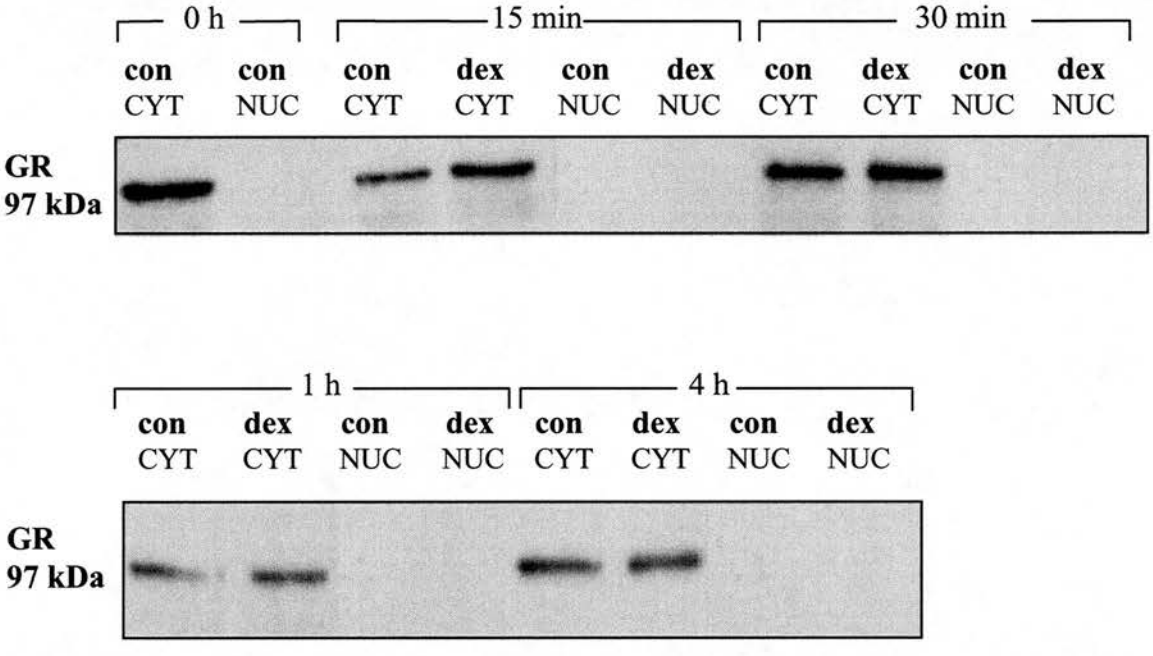


Figure 4.4.1A Time course for the effect of dexamethasone on GR expression during human neutrophil apoptosis Western blot of cytoplasmic (CYT) and nuclear extracts (NUC) from neutrophils treated with control buffer or dexamethasone (1 μM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using a rabbit anti-human polyclonal antibody (PA1-511) which recognises all forms of GR. The antibody recognises a 97 kDa GR protein. The gel is representative of 3 experiments.

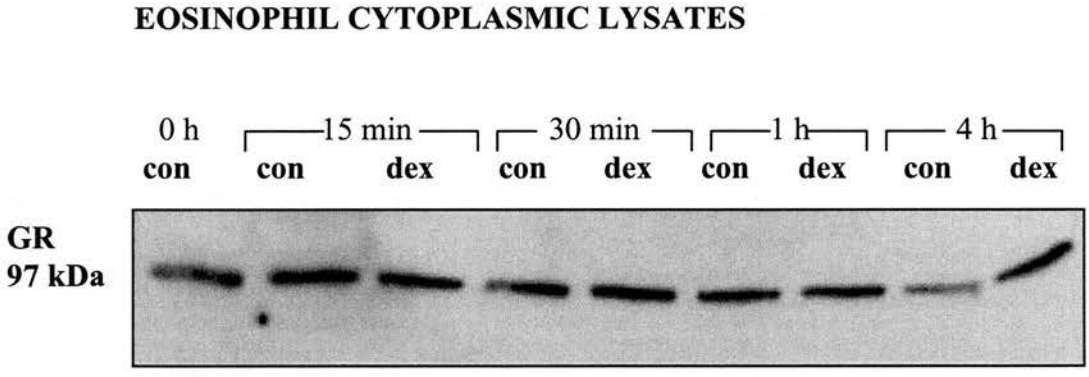


Figure 4.4.1B Time course for the effect of dexamethasone on GR expression in human eosinophils. Western blot of cytoplasmic extracts from eosinophils treated with control buffer or dexamethasone (1 μM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using a rabbit anti-human polyclonal antibody (PA1-511) which recognises all forms of GR. The antibody recognises a 97 kDa GR protein. The gel is representative of 3 experiments.

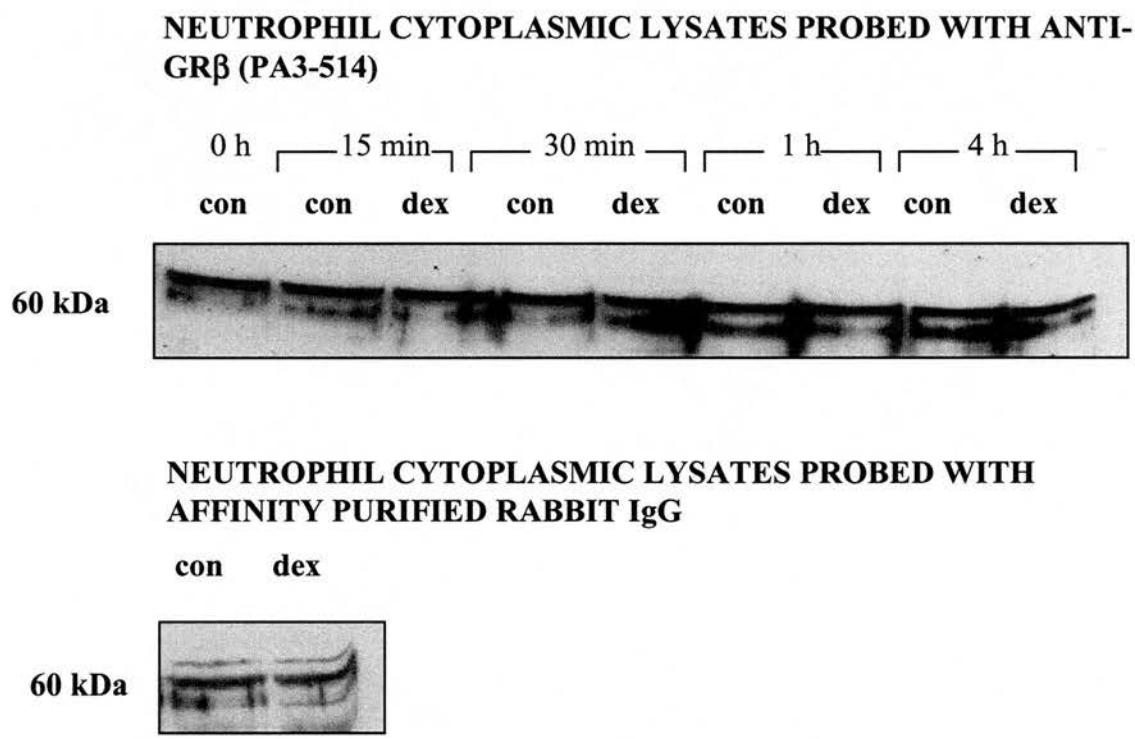


Figure 4.4.1C Time course for the effect of dexamethasone on GRβ expression in human neutrophils. Western blot of cytoplasmic extracts from neutrophils treated with control buffer or dexamethasone (1 μM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using a rabbit anti-human polyclonal antibody (PA3-514) which specifically recognises GRβ or as a negative control an affinity purified rabbit IgG. The gel is representative of 3 experiments

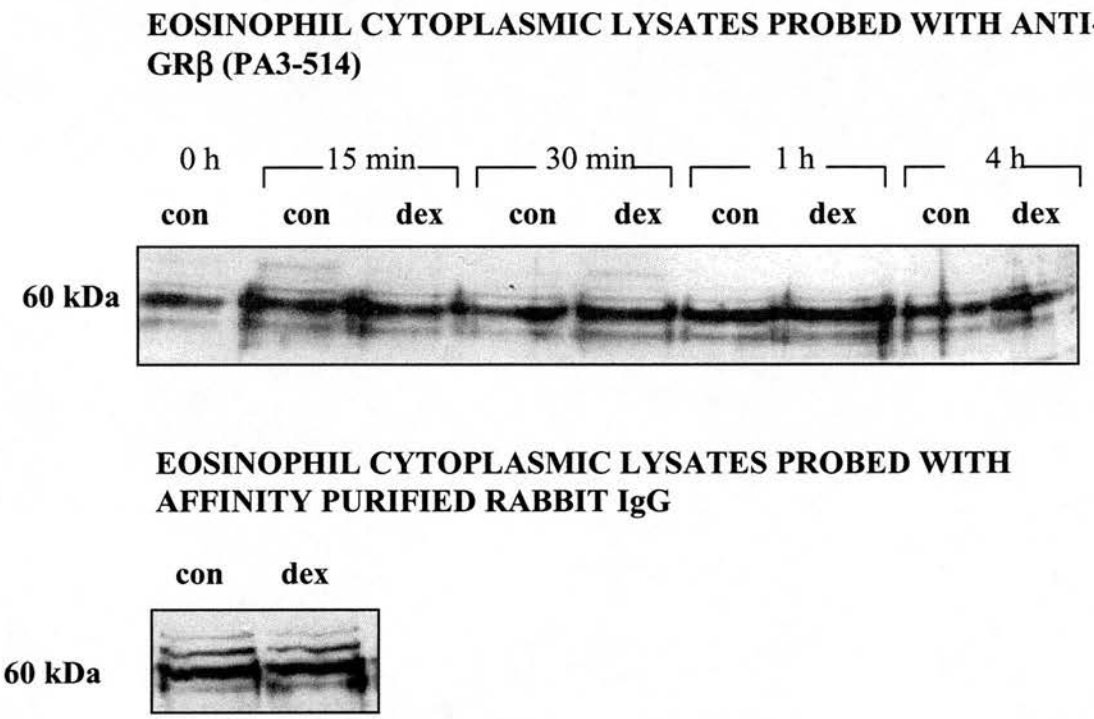


Figure 4.4.1D Time course for the effect of dexamethasone on GRβ expression in human eosinophils. Western blot of cytoplasmic extracts from eosinophils treated with control buffer or dexamethasone (1 μM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Materials and Methods". Lysates were prepared from equivalent numbers of cells and subjected to SDS-PAGE/immunoblot analysis using a rabbit anti-human polyclonal antibody (PA3-514) which recognises s GRβ specifically or as a negative control an affinity purified rabbit IgG. The gel is representative of 3 experiments

4.4.2 EFFECT OF GLUCOCORTICOID ANTAGONIST RU486 ON DEXAMETHASONE MEDIATED DELAY OF NEUTROPHIL APOPTOSIS

In the previous experiments we could not detect glucocorticoid mediated activation of the glucocorticoid receptor and did not observe dexamethasone-induced translocation of GR to the nucleus in either cell type. Although this may be due to limitations in the techniques used, it was necessary to try to elucidate whether glucocorticoid regulation of granulocyte apoptosis requires classical glucocorticoid signalling through GR. As an alternative approach, we therefore examined the effects of the glucocorticoid and progesterone receptor antagonist RU 486 on glucocorticoid regulation of granulocyte apoptosis. RU 486 is believed to act as a competitive antagonist, capable of binding to cytosolic glucocorticoid receptors but unable to stimulate GR dependent transcription (Moguilewsky and Philibert, 1984; Bourgeois *et al.*, 1984). Neutrophils were co-cultured with the indicated concentrations of dexamethasone and RU 486 for 20 h before assessment of apoptosis (Figure 4.4.2A). 10 μM RU 486 was found to fully reverse the delay of neutrophil apoptosis mediated by 0.01-0.1 μM dexamethasone however only a partial reversal was found in cells co-cultured in 1 μM dexamethasone and 10 μM RU 486.

It has been suggested that RU 486 has to be in at least 10 fold excess in order to fully abrogate dexamethasone binding to GR which would explain why only a partial reversal is found with 10 μM RU486 in the presence of 1 μM dexamethasone. It is striking however that there appears to be no reversal of dexamethasone inhibition of neutrophil apoptosis using 1 μM RU 486 when dexamethasone is titrated to concentrations as low at 0.01 μM.

To establish if glucocorticoid induction of eosinophil apoptosis required the involvement of the glucocorticoid receptor, eosinophils were co-cultured with the indicated concentrations of dexamethasone and RU 486 for 40 h, before assessment of apoptosis (Figure 4.4.2B). As previously shown in Figure 4.1.4, dexamethasone induces eosinophil apoptosis at concentrations of 0.1 μM and above. It appears that

RU 486 may act as a partial agonist with regards to the induction of eosinophil apoptosis. RU 486 at 10 μM will cause a small induction of apoptosis in eosinophils yet appears to also act as a competitive antagonist and will compete with dexamethasone for binding to GR resulting in partial abrogation of dexamethasone mediated eosinophil death. RU 486 was effective at reversing dexamethasone induction of eosinophil apoptosis when used at a concentration of 10 μM but again was ineffective at concentrations below this.

It is perhaps surprising that such high concentrations of RU 486 are needed to abrogate the apoptosis influencing effects of glucocorticoids in granulocytes. In particular it is remarkable that glucocorticoids can influence neutrophil apoptosis at nanomolar concentrations yet, 10 μM RU486 is required to abrogate this effect. These findings may suggest that glucocorticoids regulate neutrophil apoptosis via a mechanism other than classical signalling through GR. Although we have not been able to demonstrate the presence of GRβ in granulocytes due to poor antibody specificity it is possible that some of the effects of glucocorticoids could be mediated by GRβ which may not be antagonised be RU 486 (Oakley *et al.*, 1996).

SUMMARY

- Dexamethasone inhibition of neutrophil apoptosis is partially abrogated by RU 486 (≥10μM) but not at lower concentrations.
- Dexamethasone induction of eosinophil apoptosis is partially abrogated by RU486 (≥10μM) but not at lower concentrations.
- RU486 may also act as a partial agonist and induce apoptosis in eosinophils.

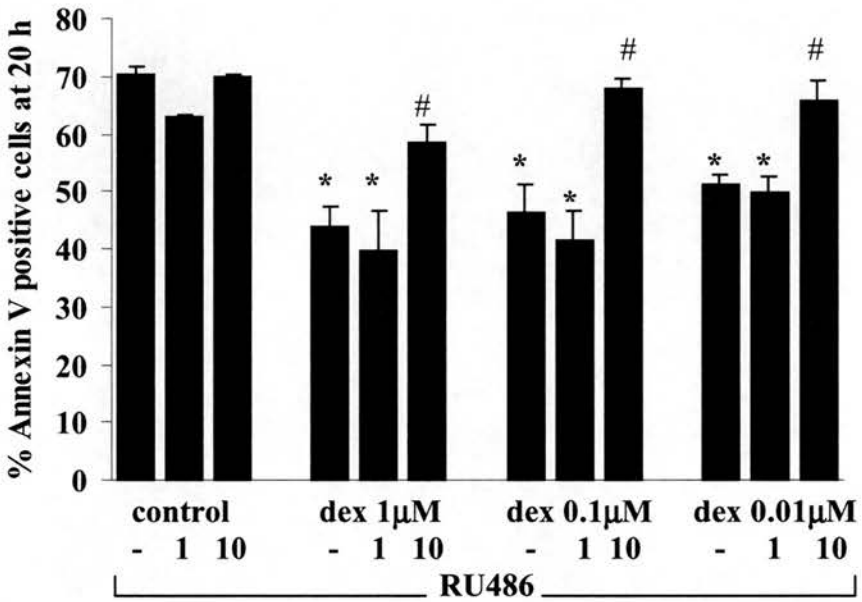


Figure 4.4.2A Effect of glucocorticoid antagonist RU486 on dexamethasone mediated delay of neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with the indicated concentrations of RU486 (μM) with or without dexamethasone. After 20 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 experiments, each performed in duplicate where significant difference from control is represented by *P<0.01 and significant difference from dexamethasone alone is represented by # P<0.01.

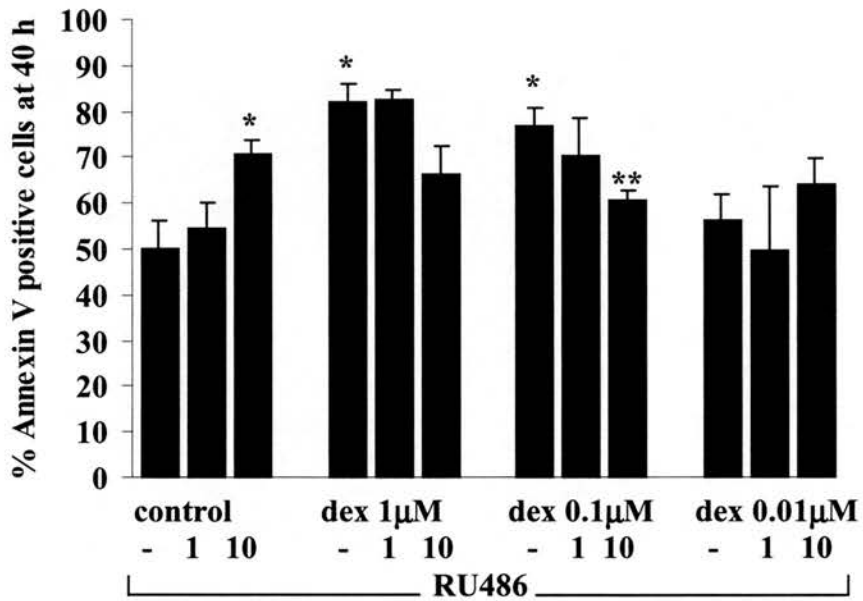


Figure 4.4.2B Effect of glucocorticoid antagonist RU486 on dexamethasone mediated induction of eosinophil apoptosis. Human eosinophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with the indicated concentrations of RU486 (μM) with or without dexamethasone. After 40 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3 - 4 experiments, each performed in duplicate where significant difference from control is represented by *P<0.05 and significant difference from dexamethasone alone (0.1 μM) by **P<0.01.

4.5 REQUIREMENT FOR PROTEIN SYNTHESIS IN GLUCOCORTICOID MEDIATED INHIBITION OF NEUTROPHIL APOPTOSIS

In light of the difficulties in establishing direct GR involvement in glucocorticoid regulation of granulocyte apoptosis, an alternative approach was sought to dissect the mechanism behind the differential effect of glucocorticoids on granulocytes. Glucocorticoids produce their effects on responsive cells by activating GR to directly or indirectly regulate the transcription of target genes. It has been highly controversial whether glucocorticoid regulation of apoptosis is a consequence of DNA-binding dependent transcriptional activation or transrepression. It has been previously shown that DNA binding of GR is a prerequisite for glucocorticoid mediated thymocyte apoptosis (Reichardt *et al.*, 1998). Similarly, Chapman *et al* have reported that transcription transactivation by GR is required for glucocorticoid induction of apoptosis in murine thymoma cells (Chapman *et al.*, 1996). In contrast, Helmberg *et al* have demonstrated that transrepression by GR is involved in glucocorticoid induction of apoptosis in human leukemic cells (Helmberg *et al.*, 1995). A key question we wished to address therefore was whether glucocorticoid regulation of granulocyte apoptosis required the direct effects of glucocorticoids through GR-DNA binding or instead involved transcriptional repression via GR association with other transcription factors. We also wished to ascertain if divergence in the mechanisms regulating glucocorticoid stimulated transcriptional activity, was responsible for the differential responsiveness of eosinophils and neutrophils to apoptotic regulation by glucocorticoids.

As an initial approach to verify the necessity of gene transcription for glucocorticoid modulation of apoptosis in granulocytes, cells were co-cultured with the protein synthesis inhibitor, cycloheximide. This compound was titrated to low concentrations to minimise the induction of apoptosis that has been reported to occur by this compound on its own. We first tested if glucocorticoid inhibition of neutrophil apoptosis required gene transcription. Neutrophils were cultured in serum-

supplemented Iscove's DMEM in the presence of dexamethasone (1 μM) together with the indictaed concentrations of cycloheximide. After 20 h, apoptosis was assessed by standard morphological criteria and exposure of phosphatidylserine by Annexin V binding. We found that cycloheximide blocked glucocorticoid-mediated suppression of neutrophil apoptosis at these concentrations (Figure 4.5.1) suggesting glucocorticoids are required to stimulate gene transcription in order to delay neutrophil apoptosis.

Similar experiments were performed to establish if glucocorticoid induction of eosinophil cell death required gene transcription. However interpretation was difficult as eosinophils are particularly sensitive to induction of apoptosis by cycloheximide thus masking any changes that may occur to glucocorticoid mediated induction of eosinophil apoptosis when gene transcription is blocked (data not shown). However this data does suggest that transcriptional events may be involved in prolonging eosinophil survival as cycloheximide will rapidly induce eosinophil apoptosis.

SUMMARY

- Cycloheximide reverses glucocorticoid mediated delay of neutrophil apoptosis
- Cycloheximide induces apoptosis in both neutrophils and eosinophils
- The requirement of gene transcription in glucocorticoid mediated induction of eosinophil apoptosis could not be determined due to the sensitivity of eosinophils to the apoptosis inducing properties of cycloheximide

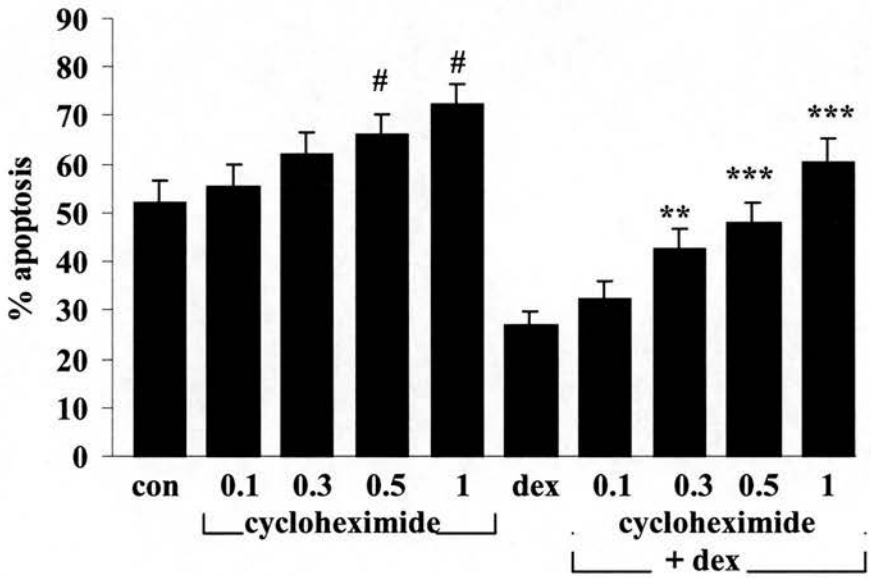


Figure 4.5.1 Effect of protein synthesis inhibition by cycloheximide on dexamethasone mediated delay of neutrophil apoptosis. Human neutrophils (5 x10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with the indicated concentrations of cycloheximide (μg/ml) with or without dexamethasone (1μM). After 20 h, cells were harvested and assessed morphologically for apoptosis. All values represent mean ± S.E. of n = 5 experiments, each performed in triplicate. Similar results were found when cells were assessed for apoptosis by Annexin-V binding (data not shown). Significant difference from control is represented by $^{\#}P<0.01$ and significant difference from dexamethasone alone is represented by $^{**}P<0.01$ or $^{***}P<0.001$

4.6 INVOLVEMENT OF GENE TRANSACTIVATION AND TRANSREPRESSION IN GLUCOCORTICOID REGULATION OF GRANULOCYTE APOPTOSIS.

As transcriptional regulation by glucocorticoids likely plays a role in glucocorticoid mediated modulation of granulocyte apoptosis, we sought to determine the mechanism by which glucocorticoids affect transcriptional activity in order to influence apoptosis and in particular, if different mechanisms of transcriptional control are involved in the ability of glucocorticoids to differentially affect apoptosis in these cells. Firstly, we investigated whether glucocorticoids stimulate transcriptional induction through GRE mediated stimulation of steroid responsive genes. As a measurement of GR-mediated transactivation we examined glucocorticoid stimulation of secretory leukocyte protease inhibitor (SLPI) production by Western blotting. Glucocorticoids have been shown to increase the transcription of SLPI through direct DNA binding of GR the promoter region of the SLPI gene (Abbinante-Nissen *et al.*, 1995). SLPI is an important antiprotease found in the upper airways during pulmonary inflammation and may serve to counteract the effects of inflammatory enzymes. Further SLPI is a major leukocyte elastase inhibitor and has been shown to be present in human neutrophils (Sallenave *et al.*, 1997).

Neutrophils (5 x 10^6/ml) and eosinophils (2.5 x 10^6 /ml) were cultured in serum supplemented Iscove's DMEM in the presence of dexamethasone (0.01-1 μM) for 20 h before supernatants were harvested. The supernatants were then dialysed to remove excess salts contained in culture medium before SDS-PAGE/immunblot analysis using a rabbit polyclonal antibody specific for human SLPI. Surprisingly we found that dexamethasone did not increase SLPI production in either neutrophils or eosinophils (Figure 4.6.1 & Figure 4.6.2). Moreover, in neutrophils, dexamethasone may even downregulate SLPI production compared to unstimulated cells (Figure 4.6.1) although this was not always reproducible. Phorbol myristate acetate (PMA) was used as our positive control and was found to upregulate SLPI production.

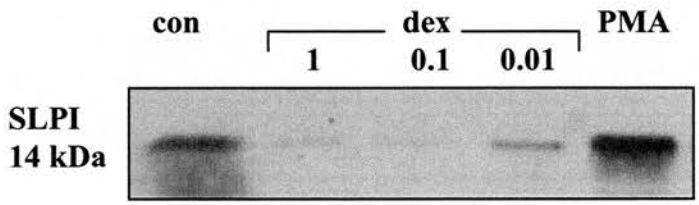


Figure 4.6.1 Effect of dexamethasone on SLPI expression in neutrophils. Western blot of supernatants from neutrophils treated with the indicated concentrations of dexamethasone (μM) or PMA (100nM) for 20 h. Supernatants were prepared and immunoblotted as described under "Materials and Methods" and subjected to SDS-PAGE/immunoblot analysis using a rabbit polyclonal antibody specific for SLPI. The SLPI antibody recognizes a 14kDa protein. The gel is of a representative experiment.

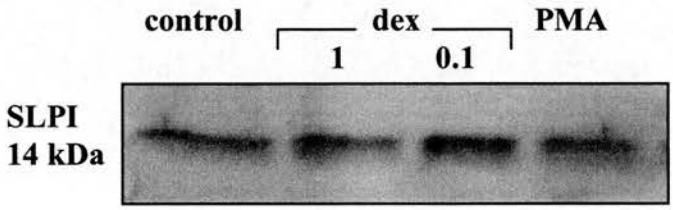


Figure 4.6.2 Effect of dexamethasone on SLPI expression in eosinophils. Western blot of supernatants from eosinophils treated with the indicated concentrations of dexamethasone (μM) or PMA (100nM) for 20 h. Supernatants were prepared and immunoblotted as described under "Materials and Methods" and subjected to SDS-PAGE/immunoblot analysis using a rabbit polyclonal antibody specific for SLPI. The SLPI antibody recognizes a 14kDa protein. The gel is of a representative experiment.

We also examined if glucocorticoids could also transrepress gene transcription in granulocytes. Many studies examining the anti-inflammatory action of glucocorticoids have focussed on glucocorticoid transrepression of NFκB, as NFκB binding sites are found in the promoter regions of many cytokines known to be downregulated by glucocorticoids. As NFκB has been demonstrated to be an important regulator of neutrophil apoptosis (Ward *et al.*, 1999a) we decided to study if glucocorticoids transrepress NFκB activation in neutrophils and eosinophils as a measurement of glucocorticoid transrepression in these cells. NFκB-mediated transcription was assessed by measuring the influence of glucocorticoids on LPS stimulated IL-8 production by ELISA. NFκB is believed to be essential for transcription of IL-8 although other transcription factors may also be important for the regulation of this gene such as AP-1 (Mukaida *et al.*, 1994a). Furthermore NFκB has been shown to be the target in glucocorticoid regulation of IL-8 secretion (Mukaida *et al.*, 1994b).

Neutrophils (5 x 10^6 cells/ml) and eosinophils (1 x 10^6 cells/ml) were cultured in serum supplemented Iscove's DMEM together with LPS (100 ng/ml) in the presence or absence of the indicated concentrations of dexamethasone. After 20 h the supernatants were saved and IL-8 was measured by IL-8 sandwich ELISA. Dexamethasone concentration dependently suppresses basal and LPS stimulated IL-8 production in neutrophils (Figure 4.6.3). As there is donor variation in basal and stimulated IL-8 production between experiments the data are represented as two typical experiments together with the averaged data of several experiments to show the similarity in individual trends, although there is variation in baseline IL-8 production. This data confirms that concentrations of dexamethasone which transrepress NFκB regulated IL-8 production in neutrophils are capable of delaying neutrophil apoptosis. In eosinophils there was greater donor variation however dexamethasone tended to suppress basal IL-8 production (Figure 4.6.4). Although dexamethasone (0.1 μM) could suppress LPS stimulated IL-8 production, high concentrations of dexamethasone (1 μM) did not significantly inhibit LPS stimulated IL-8 secretion.

SUMMARY

- Dexamethasone does not upregulate GRE-mediated SLPI production in neutrophils and eosinophils measured by Western blotting.
- Dexamethasone concentration dependently suppresses NFκB regulated IL-8 production in neutrophils
- Dexamethasone (0.1 μM) suppresses NFκB regulated IL-8 production in eosinophils but not at higher concentrations (1 μM).

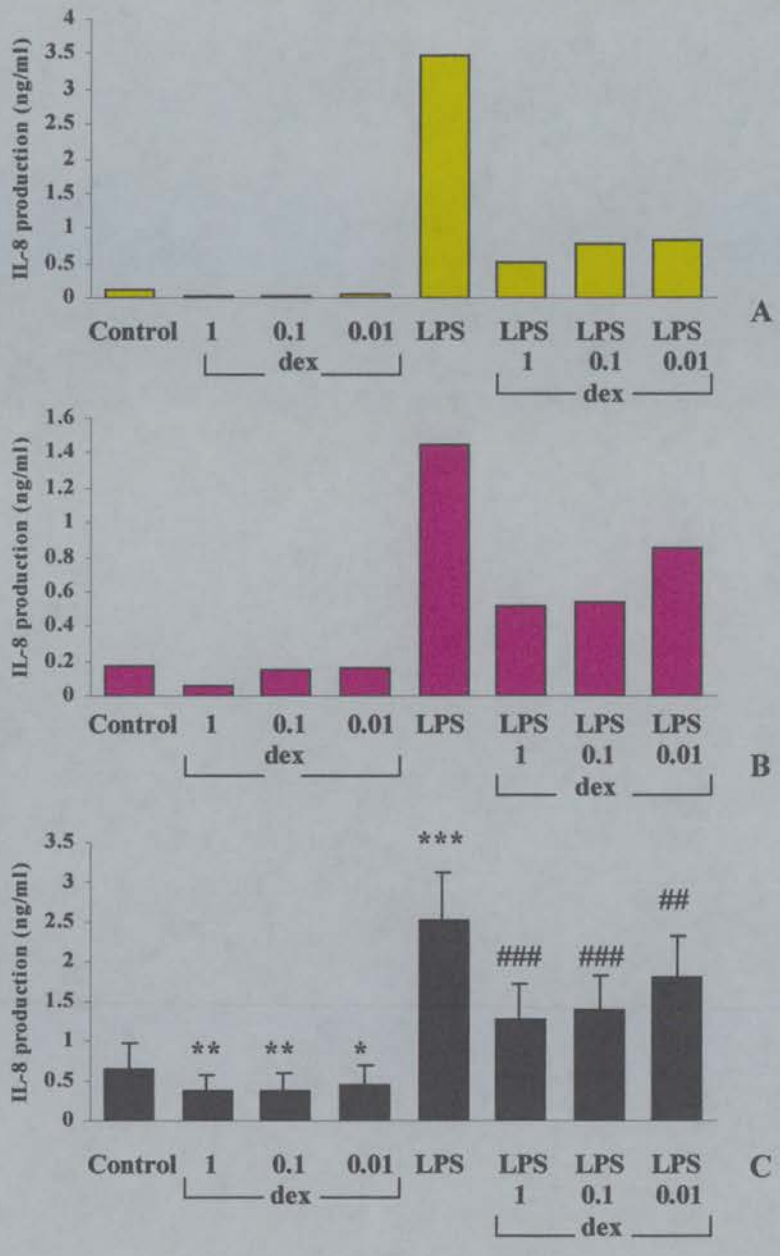


Figure 4.6.3 Effect of dexamethasone on LPS stimulated IL-8 production in neutrophils. Human neutrophils (5 x 10^6/ml) were cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with indicated concentrations of dexamethasone with or without LPS (100 ng/ml). After 20 h, supernatants were saved and IL-8 production was measured by ELISA. Graphs A and B represent results from two different individual donors. Graph C represents mean ± S.E. of n = 13 experiments, each performed in duplicate where significant difference from control is represented by $^{***}P<0.001$, $^{**}P<0.01$ or $^{*}P<0.05$ and significant difference from LPS alone is represented by $^{###}P<0.001$ or $^{##}P<0.01$.

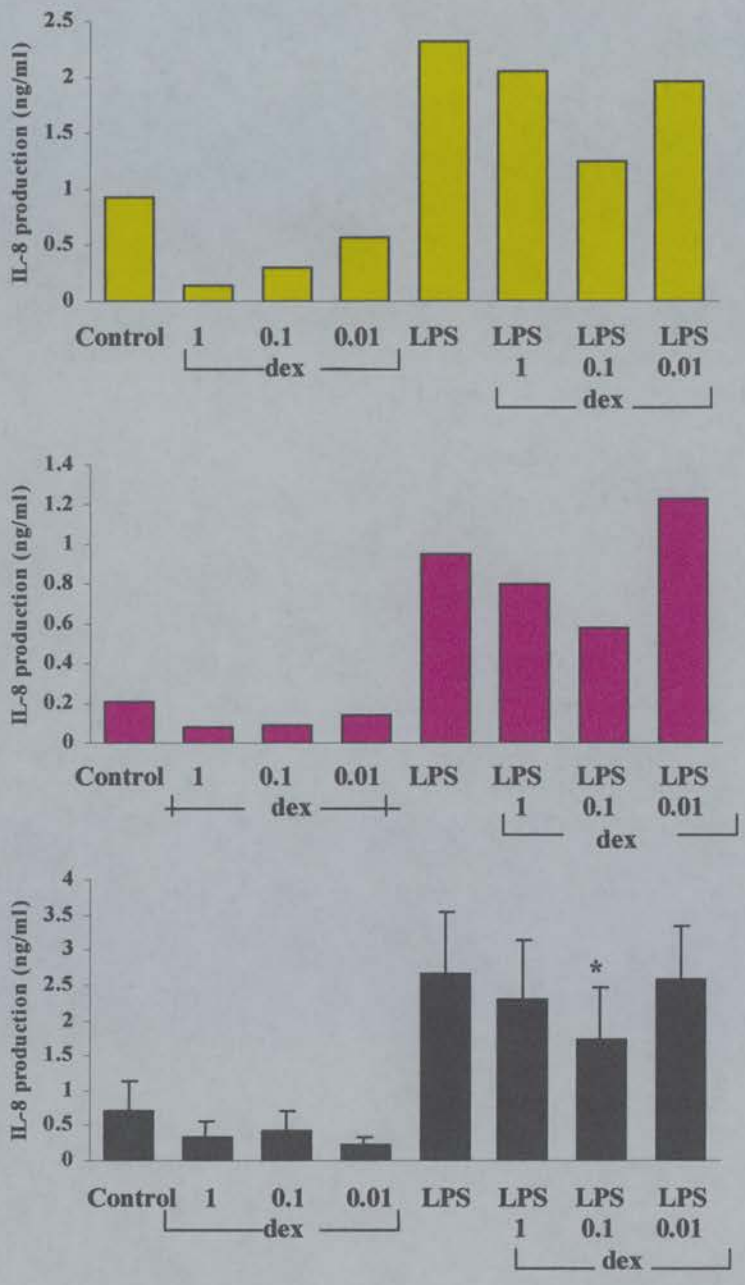


Figure 4.6.4 Effect of dexamethasone on LPS stimulated IL-8 production in eosinophils. Human eosinophils (1 x 10^6/ml) were cultured in Iscove's DMEM containing 10 % autologous serum at 37 °C, and treated with indicated concentrations of dexamethasone with or without LPS (100 ng/ml). After 20 h, supernatants were saved and IL-8 production was measured by ELISA. Graphs A and B represent results from two different individual donors. Graph C represents mean ± S.E. of n = 4-6 experiments, each performed in duplicate where significant difference from control is represented by *P<0.05.

4.7 EFFECT OF DISSOCIATED GLUCOCORTICOIDS ON GRANULOCYTE APOPTOSIS

In neutrophils it appears that glucocorticoids are capable of regulating gene expression by a mechanism of transrepression. On the other hand glucocorticoids appear incapable of transactivation through direct DNA binding to GRE consensus sites in these cells as measured by glucocorticoid effects on production of SLPI. We have also found that concentrations of dexamethasone, capable of transrepressing NFκB regulated IL-8 production, are also able to delay neutrophil apoptosis. This does not directly prove however that transrepression is required for the anti-apoptotic effect of glucocorticoids in neutrophils. In eosinophils, glucocorticoids may not act through transcriptional transactivation, as measured by SLPI production. Glucocorticoids were found to transrepress IL-8 secretion but not at the concentrations which most effectively induce eosinophil apoptosis.

In order to elucidate directly if glucocorticoid transactivation or transrepression are directly required for glucocorticoid regulation of granulocyte apoptosis, we took advantage of the availability of dissociated glucocorticoids which have been published to distinguish between transactivation and repression. We tested these compounds for their ability to affect apoptosis in neutrophils and eosinophils, in comparison to the classical glucocorticoid dexamethasone. The dissociated glucocorticoids used in these experiments differ from classic glucocorticoids, as they have been designed to be either defective in transactivation but retain the ability to transrepress or vice-versa, unlike classical glucocorticoids which are thought capable of acting via both mechanisms to a greater or lesser extent. The first set of compounds from Hoechst Marion Roussel Pharmaceutical Corp, RU24782 and RU24858, have been demonstrated to be only weakly capable of transactivation as measured by a GR reporter gene (Vayssiere *et al.*, 1997). These compounds have been shown to be capable of transrepressing AP-1 by measurement of the activation of c-Jun activated collagenase promoter-Cat reporter gene (Vayssiere *et al.*, 1997). A further set of steroids were obtained from Schering AG, namely ZK55740 and ZK77945. These compounds have been reported to transactivate transcription of

metallothionein Iia in HeLa cells, though they were not as efficient as dexamethasone (Heck *et al.*, 1997). Importantly, ZK55740 and ZK77945 were only weakly capable of transrepressing AP-1 regulated collagenase 1 activity unlike dexamethasone which efficiently suppressed collagenase 1 (Heck *et al.*, 1997).

4.7.1 EFFECT OF DISSOCIATED GLUCOCORTICOIDS ON NEUTROPHIL APOPTOSIS

To investigate if transactivation is required for glucocorticoid mediated delay of neutrophil apoptosis neutrophils were cultured in serum supplemented Iscove's DMEM in the presence of the indicated concentrations of dexamethasone, ZK55740 or ZK77945. After 20 h cells were assessed for apoptosis by measurement of Annexin V binding. We found that ZK77945 significantly inhibited neutrophil apoptosis (0.1 – 10 μM) as did ZK55740 albeit only at high concentrations (1 – 10 μM) (Figure 4.7.1A). In comparison to dexamethasone however, ZK77945 and ZK55740 did not inhibit neutrophil apoptosis as efficiently (Figure 4.7.1A).

To test whether glucocorticoids require the ability to transrepress gene expression in order to delay neutrophil apoptosis, neutrophils were incubated in serum supplemented Iscove's DMEM in the presence of the indicated concentrations of dexamethasone, RU24858 or RU24782. After 20 h apoptosis was assessed by measurement of Annexin V binding. We found that RU24858 and RU24782 both significantly inhibit neutrophil apoptosis (0.01-10 μM) with RU24858 being more effective at lower concentrations than RU24782 (Figure 4.7.1B). Moreover, dexamethasone appears to be more efficient at delaying neutrophil apoptosis compared to either compound (Figure 4.7.1B). It is important to note however that at low concentrations (0.01 μM), both RU24858 and RU24782 significantly delay

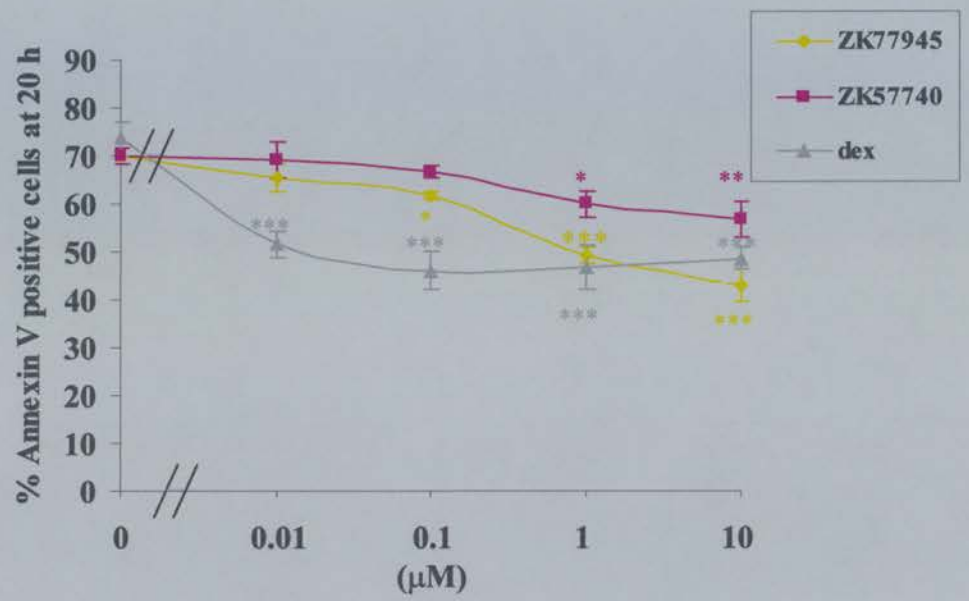


Figure 4.7.1A Effect of ZK transactivating compounds compared to dexamethasone on neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with the indicated concentrations of ZK55740, ZK77945 or dexamethasone. After 20 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 3-6 experiments, each performed in duplicate where significant difference from control is represented by ***P<0.001, **P<0.01 and *P<0.05.

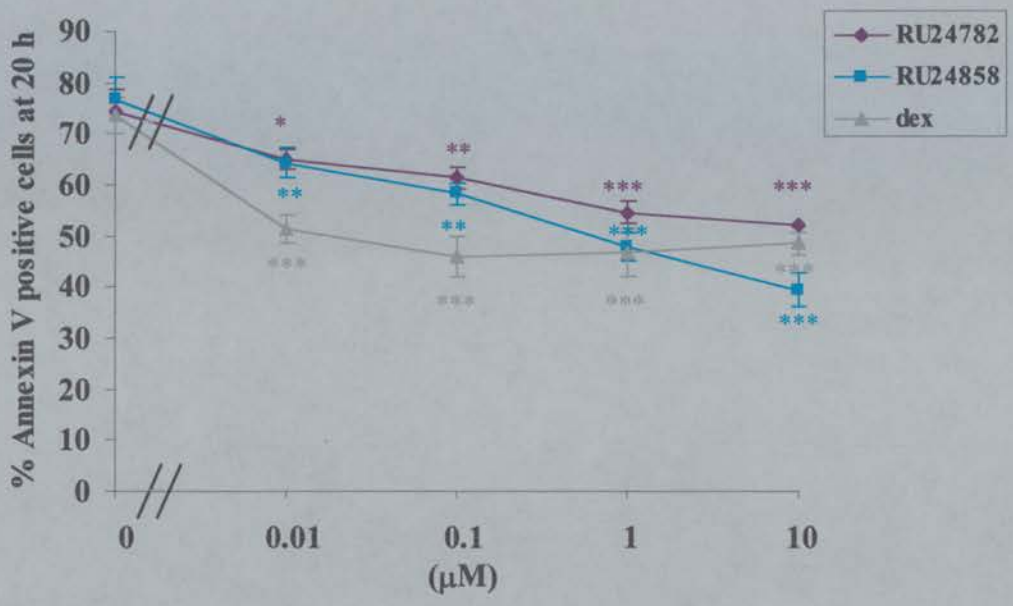


Figure 4.7.1B Effect of RU transrepressing compounds compared to dexamethasone on neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with the indicated concentrations of RU24782, RU24858 or dexamethasone. After 20 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 4-5 experiments, each performed in duplicate where significant difference from control is represented by ***P<0.001, **P<0.01 and *P<0.05.

neutrophil apoptosis whereas the transrepressing compounds ZK77945 and ZK55740 are ineffective (Figure 4.7.1B & Figure 4.7.1B). The ability of high concentrations of ZK transactivating compounds to delay neutrophil apoptosis, could be argued to be due to a residual albeit impaired ability to transrepress, which is more noticeable at these concentrations. However as dexamethasone is more efficient at delaying neutrophil apoptosis compared to either set of dissociated glucocorticoids and is known to retain the ability to both transactivate and transrepress, it is possible that both transactivation and transrepression to some degree are involved in glucocorticoid regulation of neutrophil apoptosis.

SUMMARY

- ZK55740 and ZK77945 significantly delay neutrophil apoptosis.
- ZK55740 will delay neutrophil apoptosis only at high concentrations (1-10 μM) whereas ZK77945 is slightly more effective delaying neutrophil apoptosis between (0.1-10 μM).
- ZK55740 and ZK77945 are less efficient at inhibiting neutrophil apoptosis compared to dexamethasone
- RU24858 and RU24782 significantly delay neutrophil apoptosis (0.01-10 μM)
- RU24858 and RU24782 are less efficient at inhibiting neutrophil apoptosis compared to dexamethasone
- RU transrepressing compounds appear more efficient at delaying neutrophil apoptosis compared to transactivating ZK compounds

4.7.2 EFFECT OF DISSOCIATED GLUCOCORTICOIDS ON EOSINOPHIL APOPTOSIS

To assess the involvement of transactivation by glucocorticoids in the induction of eosinophil apoptosis, eosinophils were cultured in serum supplemented Iscove's DMEM in the presence of dexamethasone (1 μM), ZK77945 (1 μM) or ZK55740 (1 μM). After 40 h apoptosis was assessed by Annexin V binding. We found that ZK77945 could significantly induce eosinophil apoptosis albeit less effectively than dexamethasone. ZK57740 was unable to induce eosinophil apoptosis.

To assess the requirement of transrepression in glucocorticoid induction of eosinophil apoptosis, cells were cultured for 40 h in the presence of RU24858 (1 μM), RU24782 (1 μM) or dexamethasone (1 μM) before assessment of apoptosis. We found that RU24858 could significantly induce eosinophil apoptosis unlike RU24782, which had little effect. Again dexamethasone was more efficient at inducing eosinophil cell death than either RU compounds.

Our results suggest that dissociated glucocorticoids, defective in either their ability to transactivate or transrepress transcriptional activity, are less efficient at inducing eosinophil apoptosis compared to dexamethasone, which is capable of performing both these functions. As we have used reasonably high concentrations of dissociated steroids in these experiments (1 μM) and a full concentration response curve has not yet been performed, it is difficult to conclude if either transactivation or transrepression are absolutely necessary for glucocorticoid mediated induction of apoptosis. At these concentrations, any residual ability to either transrepress in the case of the ZK transactivating compounds or the ability to transactivate in the RU transrepressing compounds, may have important effects. It would be useful to perform a full concentration response curve to further elucidate the mechanisms of glucocorticoid mediated transcriptional regulation during induction of eosinophil apoptosis.

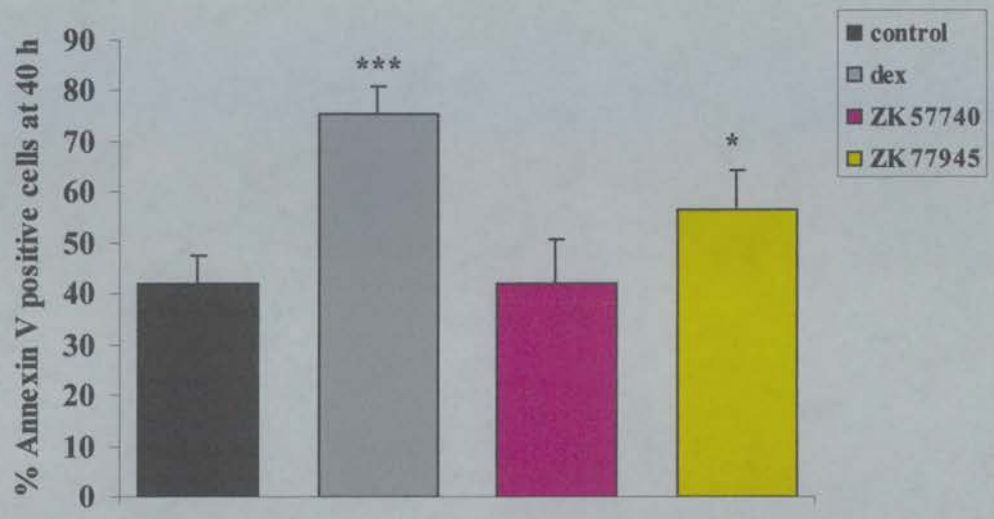


Figure 4.7.2A Effect of ZK transactivating compounds compared to dexamethasone on eosinophil apoptosis. Human neutrophils (2.5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37°C, and treated with ZK57740, ZK77945 or dexamethasone (1μM). After 40 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 4 experiments, each performed in duplicate where significant difference from control values is represented by ***P<0.001 and * P<0.05

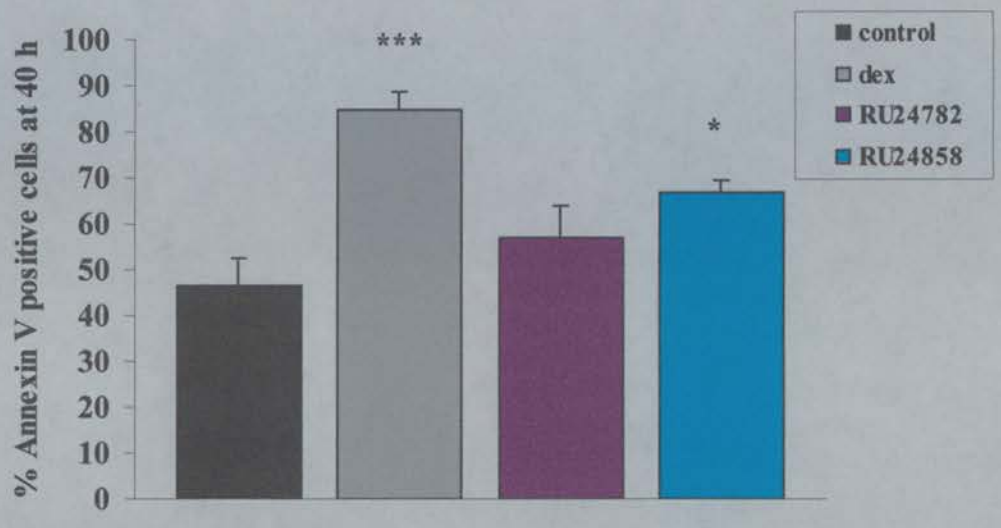


Figure 4.7.2B Effect of RU transrepressing compounds compared to dexamethasone on eosinophil apoptosis. Human neutrophils (2.5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with RU 24858, RU24782 or dexamethasone (1μM). After 40 h, cells were incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. All values represent mean ± S.E. of n = 4 experiments, each performed in duplicate where significant difference from control values is represented by ***P<0.001 and *P<0.05.

SUMMARY

- ZK77945 significantly induces eosinophil apoptosis however ZK55740 was found to be ineffective.
- Dexamethasone was more efficient at inducing eosinophil apoptosis compared to ZK77945
- RU24858 significantly induces eosinophil apoptosis however RU24782 was found to be ineffective
- Dexamethasone was more efficient at inducing eosinophil apoptosis compared to RU24858.

4.8 ROLE OF HISTONE DEACETYLATION IN GLUCOCORTICOID REGULATION OF GRANULOCYTE APOPTOSIS

We have attempted to explore the potential mechanisms used by glucocorticoids to govern transcriptional regulation in granulocytes and ascertain the importance of these mechanisms in glucocorticoid mediated control of granulocyte apoptosis. A further aspect of glucocorticoid transcriptional control is related to the recently suggested ability of glucocorticoids to affect chromatin structure.

During activation of transcription, access of transcription factors to DNA is thought to be increased by the unwinding of DNA wrapped around histone proteins following the acetylation of histone residues. During transcriptional repression, deacetylation of histones is thought to occur resulting in tighter coiling and reduced access of transcription factors to their binding sites. It has been recently proposed that glucocorticoids in association with co-repressors and proteins with intrinsic histone deacetylation activity may cause deacetylation of histone leading to gene repression (Wolffe, 1997). There is no evidence as yet that glucocorticoid-mediated regulation of apoptosis involves changes in chromatin structure.

To investigate if glucocorticoid regulation of granulocyte apoptosis involves chromatin remodelling, we made use of a pharmacological inhibitor called trichostatin A, a potent histone deacetylation inhibitor (Yoshida *et al.*, 1990). Neutrophils (5 x 10^6/ml) and eosinophils (2.5 x 10^6/ml) were cultured in serum supplemented Iscove's DMEM in the presence of dexamethasone (1 μM) with or without the indicated concentrations of trichostatin A (TSA) for 20h and 40h respectively. Apoptosis was then assessed by measurement of phosphatidylserine exposure by Annexin V binding and morphological examination. We found that blockade of histone deacetylation did not significantly reverse dexamethasone mediated delay of neutrophil apoptosis. Trichostatin A has been reported to block histone deacetylation at low concentrations (Ki 3.4 nM) (Yoshida *et al.*, 1990)

however we found that equivalent concentrations and above did not appear to modulate dexamethasone inhibition of neutrophil apoptosis.

In further studies we observed that concentrations of TSA greater than or equal to 0.1 μM, cause induction of constitutive eosinophil apoptosis. Although this was not statistically significant due to donor variation, it is possible that acetylation of histones may be important in regulating the progress of constitutive eosinophil cell death. Importantly, it appears that dexamethasone mediated induction of eosinophil apoptosis does not require histone deacetylation activity. At concentrations of TSA which do not modulate basal eosinophil apoptosis, there was no reversal of dexamethasone mediated acceleration of eosinophil apoptosis. Furthermore, at higher concentrations of TSA which do cause induction of constitutive eosinophil apoptosis, there was no amplification of dexamethasone mediated modulation of apoptosis. It would be interesting to measure histone acetylation directly to investigate if dexamethasone induced hyperacetylation is involved in the ability of glucocorticoids to induce eosinophil apoptosis. From these experiments it is difficult to draw conclusions and it would be necessary to perform a titration of dexamethasone against TSA to establish if they are acting through a similar mechanism.

SUMMARY

- TSA does not reverse dexamethasone mediated delay of neutrophil apoptosis
- Constitutive eosinophil apoptosis appears to be accelerated by TSA $\geq$ 0.1 μM while constitutive neutrophil apoptosis is unaffected.
- TSA does not modulate dexamethasone mediated induction of eosinophil apoptosis

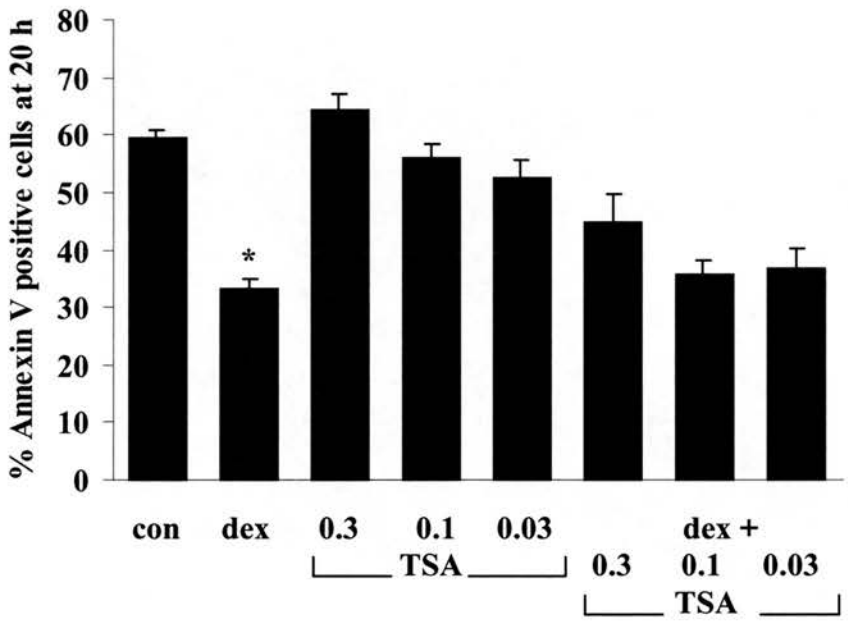


Figure 4.8.1 Effect of trichostatin A on dexamethasone mediated delay of neutrophil apoptosis. Human neutrophils (5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with the indicated concentrations of trichostatin A (TSA) (μM) or without dexamethasone (1 μM). After 20 h, cells were harvested and assessed for apoptosis by Annexin V binding. All values represent mean ± S.E. of n = 4-6 experiments, each performed in duplicate where significant difference from control is represented by *P<0.001.

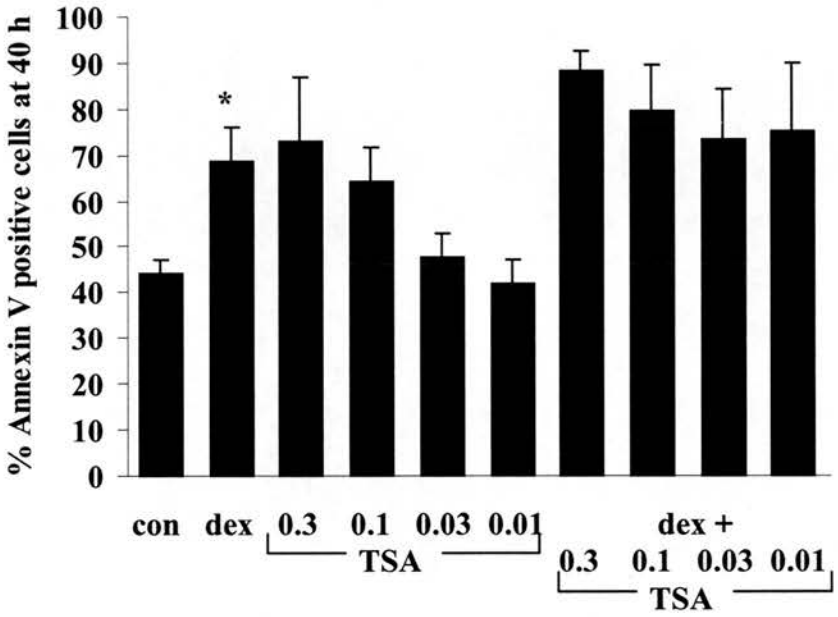


Figure 4.8.2 Effect of trichostatin A on dexamethasone mediated induction of eosinophil apoptosis. Human eosinophils (2.5 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C, and treated with the indicated concentrations of trichostatin A (TSA) (μM) or without dexamethasone (1 μM). After 40 h, cells were harvested and assessed for apoptosis by Annexin V binding. All values represent mean ± S.E. of n = 3-4 experiments, each performed in duplicate where significant difference from control is represented by *P<0.01.

4 DISCUSSION

The studies herein have sought to elucidate the mechanisms by which glucocorticoids regulate granulocyte apoptosis. Neutrophils and eosinophils are closely related myeloid cells, which arise from a common precursor in the bone marrow. Granulocyte apoptosis can be modulated by a variety of inflammatory mediators and cytokines, which commonly elicit similar effects in both eosinophils and neutrophils. However, it has long been observed that glucocorticoids cause a rapid and dramatic reduction in eosinophil numbers *in vivo* yet neutrophil numbers in contrast, are often elevated following glucocorticoid treatment (Schleimer, 1990). We propose that the direct influence of glucocorticoids to differentially modulate granulocyte apoptosis, may be a major factor in the production of this effect.

We have confirmed previous reports that glucocorticoids exert diametrically opposed effects upon the rate of apoptosis in neutrophilic and eosinophilic granulocytes *in vitro*; promoting eosinophil apoptosis while inhibiting neutrophil cell death (Kato *et al.*, 1995; Cox, 1995; Liles *et al.*, 1995; Meagher *et al.*, 1996). It has been widely assumed that neutrophils are unresponsive to the effects of glucocorticoids (Schleimer *et al.*, 1989). Indeed many groups have ignored any possible influence of glucocorticoids on neutrophil behaviour due to reports that functions such as secretion, degranulation and chemotaxis, fail to be suppressed by glucocorticoid treatment (Schleimer *et al.*, 1989). Our results demonstrate that neutrophil longevity is remarkably sensitive to the survival influence of glucocorticoids, with neutrophil apoptosis capable of being delayed by low concentrations of glucocorticoids. Importantly, it appears neutrophils are more sensitive to apoptotic influence of glucocorticoids than eosinophils, with induction of cell death in eosinophils requiring higher concentrations of glucocorticoids compared to those needed to delay neutrophil death. These findings suggest that glucocorticoid regulation of neutrophil behaviour should not be overlooked. The relative inability of glucocorticoids to resolve neutrophilic inflammation has been proposed to relate to the 'unresponsiveness' of neutrophils to glucocorticoid treatment. However, we would suggest that the influence of glucocorticoids in enhancing neutrophil survival plays

an important part in prolonging neutrophilic inflammation. Moreover, differences in the concentrations of glucocorticoid required to modulate apoptosis in neutrophils compared to eosinophils further emphasise the distinct regulatory mechanisms by which these cells respond to apoptotic modulating stimuli.

The glucocorticoid signal transduction cascade and the molecular mechanisms by which glucocorticoids regulate transcriptional activity have been well characterised. However, divergent responses of various cell types to glucocorticoid treatment, not only in terms of their ability to differentially modulate apoptosis, but through their differential actions on secretion and adhesion, make it highly unlikely that a universal mechanism of glucocorticoid action exists. Little is known of the underlying signalling mechanisms that allow glucocorticoids to exert divergent actions on various cell types. Indeed many of the molecules characterised in glucocorticoid signal transduction studies have yet to be implicated in various glucocorticoid effector functions and pertinent to this study, in glucocorticoid regulation of granulocyte cell death.

Glucocorticoids exert their effects by diffusing passively through the cell membrane where they bind to cytoplasmic glucocorticoid receptors (GRs). Glucocorticoid receptors are associated with specific molecular chaperones and other proteins, which prevent them from translocating to the nucleus in the absence of ligand (Pratt *et al.*, 1989; Morishima *et al.*, 2000b). Of the number of molecular chaperones and proteins involved in preventing nuclear localisation of unoccupied GR, hsp90 appears to play a major role. By making use of the hsp90 binding benzoquinoid ansamycin, geldanamycin (GA), we have been able to demonstrate that functional hsp90 is required for both glucocorticoid-mediated delay of neutrophil apoptosis and glucocorticoid induction of eosinophil death. Interestingly, geldanamycin appears to induce constitutive eosinophil apoptosis. Although this was not statistically significant, the data perhaps imply a role for hsp90 in regulating basal eosinophil apoptosis.

Insight into the processes involved in heat shock protein assembly of stable cytoplasmic GR heterocomplexes receptive to ligand binding, has recently been gained through elegant studies by Rajapandi *et al* and Morishima *et al.* It appears hsp70 and hsp90 are required sequentially to activate hormone binding to glucocorticoid receptor with co-chaperones Hop hsp40 and p23 enhancing heterocomplex formation and assembly (Rajapandi *et al.*, 2000; Morishima *et al.*, 2000b). Hsps may also perform a similar role in the nucleus, assembling recycled receptors into hormone binding competent heterotrimeric complexes (Liu and DeFranco, 1999). It would be interesting to investigate if similar molecules, in addition to hsp90, are involved in formation of conformationally mature glucocorticoid receptors in granulocytes.

The requirement of hsp90 activity in both glucocorticoid induction of eosinophil apoptosis and delay of neutrophil death is presumably to allow formation of competent steroid binding glucocorticoid receptors, allowing glucocorticoid signal transduction to follow. However, it has recently come to light that in addition to binding GR, Hsp90 forms complexes with various kinases and transcription factors, assisting their transport within the cell and/or stabilising their conformation required for functional maturity (Pratt, 1998; Mayer and Bukau, 1999). Thus chaperones such as hsp90 may be key regulators of signal transduction cascades through selective transport or segregation of signalling components. Indeed a recent study has reported the direct association of hsp90 with components of the apoptotic signalling pathway. Hsp90 has been shown to bind Apaf-1, preventing the participation of Apaf-1 in forming an active complex with caspase-9, thus inhibiting downstream caspase activation, delaying apoptosis (Pandey *et al.*, 2000). It would be interesting to investigate if glucocorticoid delay of neutrophil apoptosis involves targeted binding of hsp90 to Apaf-1, preventing apoptosome formation and thus inhibiting apoptosis. However, if such a mechanism were also to be implicated in glucocorticoid induction of eosinophil apoptosis, dexamethasone mediated abrogation of hsp90 binding to Apaf-1 would be required to induce cell death.

To elucidate further the mechanisms controlling glucocorticoid modulation of granulocyte apoptosis, we sought to determine GR expression in granulocytes and the glucocorticoid signal transduction cascade activated following ligand binding. Although it has been presumed that glucocorticoid modulation of granulocyte apoptosis requires competent ligand binding receptors, this has not been shown directly. Binding studies using ^{3}H-labelled agonists have indicated that neutrophils and eosinophils exhibit similar numbers of glucocorticoid receptors, with similar affinity for glucocorticoid binding (Peterson *et al.*, 1981). This suggests that differences in receptor number and affinity cannot be responsible for the differential effect of glucocorticoids on granulocyte apoptosis. We wished to investigate if divergence in responsiveness to glucocorticoids among granulocyte types was instead due to differential expression of glucocorticoid receptor isoforms. From binding studies, granulocytes appear to express a single class of glucocorticoid receptor however as GRβ is not thought capable of binding glucocorticoid, the influence of this isoform would not have been taken into account, in these studies (Peterson *et al.*, 1981; Bamberger *et al.*, 1995). GRβ is thought to influence glucocorticoid signalling and transcription through formation of heterodimers with steroid binding GRα (Oakley *et al.*, 1999), which may reduce the transcriptional activity of GRα by denying access to GREs (Oakley *et al.*, 1996). By Western blotting, we have shown GR is expressed in the cytoplasm of both neutrophils and eosinophils. Successful immunoblotting required extensive optimisation of standard protein extraction protocols suggesting GR may be particularly sensitive to proteolytic degradation by granulocyte proteases such as elastase (Distelhorst *et al.*, 1987). Attempts to establish the isoforms of GR expressed in each cell type were hampered due to non-specific binding of available GRβ specific antibodies. Curiously, we did not observe any translocation of GR to the nucleus following treatment of granulocytes with dexamethasone.

A recent report has suggested that neutrophils contain high constitutive expression of GRβ, which it is postulated, allows neutrophils to escape glucocorticoid induced cell death (Strickland *et al.*, 2001). Immunofluorescence studies by Strickland *et al*

propose neutrophils contain higher levels of GRα and GRβ in comparison to PBMCs. Furthermore, IL-8 is demonstrated to synergise with dexamethasone to decrease neutrophil apoptosis *in vitro,* a response associated with an increase in the ratio of GRβ to GRα. The argument is further strengthened by transfection of mouse neutrophils with GRβ, resulting in a small reduction in the rate of apoptosis in response to dexamethasone. In contrast to previous reports, it is suggested that GRβ is located mainly in the cytoplasm of cells. Curiously, there is no demonstration of ligand dependent accumulation of GR in the nucleus upon dexamethasone treatment, particularly regarding GRα isoform.

The hypothesis that in neutrophils an alternative mode of action of GR exists, which allows neutrophils to reduce their rate of spontaneous apoptosis in response to glucocorticoid, is an attractive one. Strickland *et al.,* argue that glucocorticoid induction of apoptosis proceeds through GRα homodimer transcription via GRE mediated transactivation. In neutrophils, an increase in the ratio of GRβ to GRα in neutrophils, favours the formation of responsive heterodimers, preventing normal GRα homodimer GRE mediated transcription, thereby delaying apoptosis. In contrast PBMCs, shown to have a higher ratio of GRα to GRβ, favouring GRα homodimer formation, are sensitive to apoptosis induction by dexamethasone. It would be extremely useful to examine the ratio of GR isoform expression in neutrophils compared to eosinophils, to test if acceleration of apoptosis by glucocorticoids in this cell type is due to a higher ratio of GRα to GRβ. It is further proposed by Strickland *et al* that IL-8 enhances neutrophil survival by increasing GRβ expression, however it is not revealed whether GR isoform expression in PBMC is also affected by IL-8 treatment. This control would be useful to rule out the possibility that IL-8 non-specifically upregulates GRβ expression in many cell types and would help establish if increased GRβ in neutrophils by IL-8, is specifically responsible for mediating enhanced neutrophil survival in the presence of glucocorticoids.

Our own studies of GR expression in granulocytes and those by Strickland *et al* do not directly demonstrate the necessity for GR activation as an essential requirement for glucocorticoid modulation of apoptosis. Although there may be limitations in the techniques we have used, we could not detect translocation of the glucocorticoid receptor from the cytoplasm to the nucleus following glucocorticoid treatment. Further, Strickland *et al.,* do not reveal in their immunofluorescence studies if GR activation occurs in response to dexamethasone in neutrophils. To address this issue, we elected for an alternative approach, making use of the glucocorticoid receptor antagonist RU 486. While we found that 10 μM RU 486 partially abrogated dexamethasone mediated modulation of both neutrophil and eosinophil apoptosis, lower concentrations of this compound had no effect, even when in significant excess. This may suggest that the antagonist binds to granulocytes with low affinity compared to the agonist or that glucocorticoid modulation of granulocyte apoptosis may not occur through the classical glucocorticoid signal transduction pathway. However, as RU 486 is unable to bind and therefore antagonise GRβ, glucocorticoid modulation of granulocyte apoptosis may still proceed through GRβ. It will be interesting if future studies ascertain if indeed differential GR isoform expression relates to diverging responses to the apoptotic influences of glucocorticoids in these cells.

Glucocorticoid signal transduction, regardless of being mediated via classical GRα homodimers or formation of antagonistic GRα/GRβ heterodimers, is thought to ultimately lead to regulation of transcription of target genes in responsive cells. We found that dexamethasone induced suppression of neutrophil cell death was abrogated upon co-treatment with cycloheximide, confirming gene transcription to be essential for glucocorticoid inhibition of neutrophil apoptosis. One could hypothesise that glucocorticoids therefore delay neutrophil apoptosis through enhanced production of a survival protein(s) or by switching off production of a putative death factor(s) in these cells. If the latter were true, one would expect that treatment of neutrophils with protein synthesis inhibitors would delay constitutive neutrophil apoptosis. However this has been shown not to be the case and in contrast,

protein synthesis inhibitors rapidly accelerate apoptosis in granulocytes (Whyte *et al.*, 1997; Cox and Austin, 1997). This suggests granulocyte cell death may normally held in check by the constant production of inhibitor protein(s). It is possible that glucocorticoids could influence granulocyte apoptosis through differential regulation of possible inhibitor protein(s) however dissection of the mechanisms involved using inhibitors of transcription and translation are inherently problematic due to fact that synthesis of as yet unidentified proteins, appear intrinsically important in regulating constitutive apoptosis. Thus it is difficult to separate the influence of these compounds on glucocorticoid regulation from their additional effects on constitutive cell death. This is particularly pertinent to examination of transcriptional mechanisms involved in glucocorticoid induction of eosinophil apoptosis, as inhibitors of protein synthesis even when titrated to low levels, rapidly accelerate apoptosis in these cells (data not shown).

To examine more specifically the molecular basis of glucocorticoid modulation of granulocyte apoptosis, we investigated whether glucocorticoids regulate transcription via direct GRE mediated transactivation or indirect transrepression in these cells. Further we sought to determine if either of these mechanisms was involved in glucocorticoid regulation of apoptosis. Commonly, GRE mediated transcriptional transactivation is measured by the ability of glucocorticoids to enhance the activity of a glucocorticoid responsive luciferase reporter construct, transfected into cells of interest e.g. the GR responsive mouse mammary tumour virus (MMTV) reporter gene. However experimental transfection protocols of this nature cannot be performed in granulocytes due to the limited lifespan of these cells. For this reason, measurement of glucocorticoid transactivation was investigated by examining the effects of glucocorticoids on endogenous proteins whose expression and synthesis can be modulated through GRE mediated transactivation. One such protein appears to be the antiprotease, secretory leukocyte proteinase inhibitor (SLPI), a potent inhibitor of elastase, shown to be present in a variety of cells including human neutrophils (Abbinante-Nissen *et al.*, 1995; Sallenave *et al.*, 1997).

We found that treatment of both neutrophils and eosinophils with dexamethasone does not result in any detectable upregulation of SLPI. Moreover, in neutrophils, SLPI expression may in fact be downregulated in response to glucocorticoids in neutrophils, although this was not always reproducible. These preliminary results question whether glucocorticoids exert their influence on granulocyte apoptosis through GRE-mediated transactivation of steroid responsive genes. It must be noted however that measurement of SLPI production by Western blotting may not be optimal for detection of quantitative changes in expression of this protein. Development of an ELISA based detection procedure may help more accurately determine and quantitate changes in expression of SLPI. In addition it may have been more pertinent to examine glucocorticoid transcriptional transactivation by looking at glucocorticoid induced changes in levels of gene expression rather than changes in protein expression. Analysis of glucocorticoid induced alterations in a variety of genes using techniques such as real-time PCR or microarray technology may have been more useful in determining whether glucocorticoids modulate granulocyte apoptosis via transcriptional transactivation or repression.

The discovery that many genes down-regulated by glucocorticoids do not contain appropriate GRE sequences or any other GR binding sites, suggested that glucocorticoids may mediate effects on transcription, independent of direct GR DNA binding. Indeed, it has been proposed that many of the anti-inflammatory effects of glucocorticoids involve indirect modulation of gene expression by transcriptional transrepression instead of transactivation of steroid responsive genes. One of the main transcription factors thought to be targeted by GR is NFκB (Scheinman *et al.*, 1995b; Scheinman *et al.*, 1995a). It has been proposed that the anti-inflammatory potential of glucocorticoids resides in their ability to interfere with the activity of this important transcription factor (Caldenhoven *et al.*, 1995). Our studies have shown that glucocorticoids suppressed LPS induced IL-8 secretion in neutrophils in a concentration dependent manner. In contrast, not all concentrations of dexamethasone, shown to modulate eosinophil apoptosis, could down-regulate LPS stimulated IL-8 production in eosinophils. Together these results suggest

glucocorticoid suppression of NFκB activation may be involved in glucocorticoid inhibition of neutrophil apoptosis but may not be essential to glucocorticoid modulation of eosinophil cell death. However, these results should be interpreted with caution due to donor variability in these experiments. Although we have demonstrated glucocorticoids down-regulate the synthesis of an NFκB regulated protein in granulocytes, it does not necessarily follow that glucocorticoids exert their apoptotic influence via this mechanism. Indeed, if glucocorticoids do regulate granulocyte apoptosis via transcriptional transrepression, many other transcription factors in addition to NFκB could potentially be involved.

To directly assess the necessity of glucocorticoid transactivation or transrepression of gene transcription for glucocorticoid regulation of granulocyte apoptosis, we made use of a novel set of synthetic glucocorticoids, which have been published to discriminate between transactivation and transrepression (Vayssiere *et al.*, 1997; Heck *et al.*, 1997). Compared to dexamethasone, transactivating compounds ZK77945 and ZK55740 and transrepressing compounds RU24858 and RU24782 were less effective at modulating granulocyte apoptosis. In neutrophils, transrepressing compounds delayed apoptosis at concentrations similar to dexamethasone whereas higher concentrations of the transactivating compounds, were required to delay neutrophil cell death. In eosinophils, transactivating ZK77945 and transrepressing RU24858 could induce eosinophil death, but again neither compound was effective at accelerating eosinophil apoptosis as dexamethasone. Thus, dissociated glucocorticoids, in which either transrepressing or transactivating ability is deficient, are able to modulate granulocyte apoptosis, though not to as an extent as the classical glucocorticoid, dexamethasone. It has previously been shown that both transactivating and transrepressing capabilities are fully intact in responses mediated by dexamethasone. It would be interesting to test whether combinations of the two types of dissociated steroids could restore the ability of these glucocorticoids to modulate granulocyte apoptosis to levels comparable with dexamethasone.

Interpretation of the results from these experiments must be viewed with a degree of caution. Firstly, it is likely that dissociated glucocorticoids such as the transrepressing compounds RU24858 and RU24782, retain some ability to transactivate. The converse may be true of the transactivating compounds ZK7745 and ZK55740. This may confuse and obscure the responses elicited by transrepression or transactivation alone and must be taken into consideration when viewing these results. Secondly, although these compounds have been demonstrated to discriminate between transactivation and transrepression, we have yet to demonstrate separation of these activities in our system. The necessity of such experiments is illustrated in recent findings by Vanden Berghe *et al* who report RU24782 displays similar transactivating ability as dexamethasone, measured by GRE dependent reporter gene expression, in mouse fibroblast cells (Vanden Berghe *et al.*, 1999). This is in contrast to the original studies in which RU24782 exerted strong AP-1 inhibition, but only weakly activated the GRE based reporter gene (Heck *et al.*, 1997). This illustrates that there may be divergent potencies of dissociated steroids in stimulating GRE dependent transactivation in different cell types. Using these reagents to draw conclusions regarding the mechanism of glucocorticoid transcriptional regulation, requires experiments to test their ability to stimulate GRE mediated transcription in granulocytes and further investigate their capacity to interfere with gene activation driven by a variety of transcription factors such as NFκB and AP-1. Experiments of this kind would elucidate if mechanisms of glucocorticoid transcriptional regulation, examined mainly through overexpression studies in other cell types, were applicable to the mechanisms of glucocorticoid regulation in primary cells such as granulocytes.

In summary, limitations of the prototypic dissociated glucocorticoids as tools to dissect glucocorticoid regulation of transcription must be taken into consideration when elucidating the mechanism of glucocorticoid regulation of granulocyte apoptosis. The development of these compounds as novel glucocorticoids with improved therapeutic benefit is perhaps questionable. Recent findings by Belvisi *et al* have suggested transrepressing glucocorticoids exhibit comparable anti-inflammatory activity to classical glucocorticoids such as budesonide, however they

were also found to have equally potent systemic effects (Belvisi *et al.*, 2001). This suggests that *in vitro* separation of transrepression from transactivation may not be reflected in whole animal physiological studies or that side effects of glucocorticoids, previously attributed to transactivation only, may also be a consequence of transrepression.

Finally, we have examined whether glucocorticoid regulation of granulocyte apoptosis involves changes in gene transcription, through chromatin remodelling. It is thought that actively transcribed genes are associated with acetylation of specific lysine residues on histone proteins, resulting in unwinding of DNA, allowing increased accessibility of transcription factors to nearby promoter sequences. Thus histone acetylation is thought to be correlate with increased transcription (Beato *et al.*, 1996; Wolffe, 1997) in contrast to histone deacetylation, which has been correlated with transcriptional repression and gene silencing (Wolffe, 1997; Ura *et al.*, 1997). GR has been shown to form part of the basal transcriptional machinery through interaction with large co-activator molecules such as CBP (Sheppard *et al.*, 1998). Hormone activated GR may bind to CBP and/or associated molecules, enhancing local histone acetyltransferase activity and increasing gene transcription. Alternatively, GR may reduce gene transcription via deacetylation of histones, causing a tightening of the chromatin structure, reducing the access of transcription factors such as NFκB to their DNA binding sites, thereby transrepressing proinflammatory gene expression. It is possible that firstly, competition between GR and other transcription factors for binding to CBP, could reduce the availability of the CBP associated HAT activity required for transcriptional activation by these transcription factors (Kamei *et al.*, 1996). Alternatively, GR may recruit proteins with histone deacetyltransferase activity to repress histone acetylation, again resulting in transcriptional repression (Ito *et al.*, 2000). Indeed recruitment of HDAC2 by GR has been reported to be essential for maximal transrepression of NFκB stimulated HAT activity by glucocorticoids and may be vitally important for glucocorticoid exertion of their anti-inflammatory effects (Ito *et al.*, 2000; Ito *et al.*, 2001).

Our studies suggest changes in acetylation of histone proteins may be important for regulation of constitutive granulocyte apoptosis. TSA, a potent histone deacetylation inhibitor, at high concentrations could induce eosinophil apoptosis yet could not reverse or enhance glucocorticoid acceleration of apoptosis in these cells. It is possible that histone hyperacetylation and therefore activation of transcription, may be important for induction of eosinophil apoptosis. Indeed, it has been shown in A549 cells, that dexamethasone alone can induce histone acetylation in a concentration dependent manner (Ito *et al.*, 2001). Curiously, in these same studies, low concentrations of dexamethasone repress IL-1β p65-associated histone acetylation which, it is proposed, is due to recruitment of HDAC2 to the p65-HAT complex. Indeed, in the presence of IL-1β, dexamethasone is shown to induce HDAC expression (Ito *et al.*, 2001). It was argued that glucocorticoid transrepression of proinflammatory transcription factors, involves recruitment of proteins with histone deacetyltransferase activity. In eosinophils, we found that low concentrations of dexamethasone could effectively transrepress NFκB regulated IL-8 expression, yet high concentrations, which modulate eosinophil apoptosis, had no effect. It is possible that in eosinophils, dexamethasone may repress NFκB at low concentrations through HDAC expression but more importantly, at high concentrations which are required to modulate eosinophil apoptosis, dexamethasone may itself induces histone acetylation and therefore can no longer inhibit NFκB stimulated histone acetylation. Measurement of histone acetylation directly would help ascertain the extent of the involvement, if any, of chromatin remodelling in glucocorticoid control of eosinophil death.

In neutrophils, dexamethasone delays neutrophil apoptosis over a wide concentration range, which are also able to repress NFκB stimulated IL-8 secretion. However, as TSA does not reverse dexamethasone-mediated delay of neutrophil apoptosis, histone deacetylation and therefore transrepression may not be essential for glucocorticoid modulation of neutrophil cell death. It is important to note however, that high concentrations of dexamethasone (1μM) were used in these experiments

and a full titration of dexamethasone against TSA would be required to rule out the possibility of glucocorticoid associated HDAC activity in the mechanism by which glucocorticoids delay neutrophil apoptosis.

In summary, it is unclear at present if glucocorticoid regulation of granulocyte apoptosis requires glucocorticoid stimulated changes in histone acetylation/deacetylation. Experiments measuring histone acetylation and deacetylation in granulocytes in response to glucocorticoid treatment, will be necessary to provide direct evidence of the existence of such mechanism of glucocorticoid action in these cells. Furthermore, additional investigations would be required to elucidate if changes in histone acetylation correlate with transactivation and histone deacetylation with repression, before implicating either mechanism in glucocorticoid regulation of granulocyte apoptosis. There is some evidence to suggest that hyperacetylation of histones may suppress or be ineffectual in stimulating gene transcription at some promoters (Van Lint *et al.*, 1996; Mizuguchi *et al.*, 2001). Moreover, recent findings by Sheldon *et al,* investigating glucocorticoid regulation of the mouse mammary tumor virus (MMTV) promoter, demonstrate high levels of acetylation when the promoter is inactive, which decreases during hormone activation through histone deacetylation (Sheldon *et al.*, 2001). Therefore it is likely that regulation of gene transcription through changes in histone acetylation may vary between different genes.

Finally, there may be novel mechansims of transcriptional regulation by glucocorticoids, which may be important for the ability of these compounds to modulate granulocyte apoptosis. For example Wallace *et al.*, have recently suggested that transcriptional activation by glucocorticoids may be regulated by proteasome mediated degradation of GR, which may provide a mechanism to terminate glucocorticoid responses (Wallace and Cidlowski., 2001). Whether the ubquitin-proteasome pathway or other novel regulatory mechanisms are involved in glucocorticoid regulation of granulocyte apoptosis is yet to be assessed.

In conclusion, there may be several mechanisms by which glucocorticoids regulate transcriptional activation and future studies should help unravel which of these processes are key in eliciting the potent anti-inflammatory effects of these compounds. At present, little is known of the mechanisms of transcriptional regulation involved in glucocorticoid-mediated control of apoptosis and how glucocorticoids differentially regulate transcriptional activation to exert diverging responses on apoptosis in different cell types. A greater understanding of the signalling mechanisms, by which glucocorticoids differentially regulate granulocyte apoptosis, could potentially lead to the development of novel glucocorticoids with a greater selectivity of action. This could only be achieved through further characterisation of glucocorticoid transcriptional regulation in granulocytes and investigations into how glucocorticoids control of components of the apoptotic signal transduction cascade, to differentially modulate the rate of cell death in these cells.

5 CONCLUSIONS

Apoptosis, concomitant with efficient recognition and clearance by phagocytes, has been proposed as a major mechanism involved in the removal of excess or effete granulocytes from an inflammatory focus. Although granulocytes appear to be 'pre-programmed' or committed to death via apoptosis, it is clear that the life span and functional longevity of these cells can be modulated significantly by a number of inflammatory mediators and cytokines. As a consequence, there is the potential to regulate granulocyte longevity by altering the balance between pro- and anti-apoptotic stimuli at the inflammatory site. The therapeutic induction of apoptosis as an anti-inflammatory strategy may be successful if developed in parallel with upregulation of mechanisms that drive efficient clearance of apoptotic cells. Although closely related in ontogeny, there are major differences in the apoptotic control mechanisms of neutrophils and eosinophils, which may additionally provide the opportunity to induce apoptosis selectively in these inflammatory cells.

The work presented in this thesis has sought to further define the signalling mechanisms by which glucocorticoids and cyclic AMP regulate granulocyte apoptosis. We have observed that elevation of cyclic AMP profoundly delays constitutive neutrophil apoptosis and are the first to demonstrate that cyclic AMP delays apoptosis through initiation of a novel signal transduction mechanism, which contrary to expectations is independent of PKA activation. Further, we have shown that cyclic AMP exerts control of signalling components which may be key to initiation and execution of apoptosis in these cells. We are the first to report that dissipation of mitochondrial transmembrane potential occurs during constitutive neutrophil apoptosis. Furthermore, protection from apoptosis, afforded by cyclic AMP, appears to be upstream of the changes in mitochondrial transmembrane potential. Mitochondria play an integral role in apoptotic signal transduction and activation of apoptosis in response to internal insults or extracellular cues is thought to converge on the mitochondria, leading to commitment to death. It has yet to be demonstrated whether mitochondria perform a similar regulatory role to control granulocyte apoptosis and furthermore whether early dissipation of mitochondrial

transmembrane potential is indicative of granulocyte irreversible commitment to cell death. We have also demonstrated that cyclic AMP delays neutrophil apoptosis upstream of activation of executioner caspases. The mechanisms by which this second messenger exerts influence over the apoptotic signal transduction machinery, to delay granulocyte apoptosis, have still to be fully defined.

To our surprise, cyclic AMP acts independently of PKA activation to delay constitutive neutrophil apoptosis. Furthermore, PI-3 kinase and MAP kinase activation do not appear to be required for the survival effects of cyclic AMP. Our work suggests that cyclic AMP delays neutrophil cell death via initiation of a rapid, reversible and transcriptionally independent signalling pathway. We have demonstrated that proteasome activity in the neutrophil is vitally involved in the powerful ability of cyclic AMP to delay constitutive neutrophil apoptosis and together our data suggests cyclic AMP may induce post-translational modifications of a previously uncharacterised protein(s), to promote survival. Alteration of the balance between pro- and anti-apoptotic proteins may be a key mechanism by which cyclic AMP modulates neutrophil death. We have also shown that cyclic AMP inhibits acceleration of neutrophil apoptosis induced by a variety of death receptor stimuli. Intriguingly however, powerful death stimuli may eventually overwhelm the capacity of cyclic AMP to inhibit neutrophil apoptosis. Future identification of novel targets of cyclic AMP regulation and elucidation of the point at which cyclic AMP can no longer rescue cells from irreversible commitment to death, may lead to a better understanding of the signalling mechanism by which this second messenger modulates granulocyte cell death.

The work in this thesis has also given novel insight into the signal transduction pathways by which glucocorticoids modulate granulocyte apoptosis. Glucocorticoids exert differential effects on granulocyte apoptosis; causing induction of apoptosis in eosinophils while delaying neutrophil cell death. In contrast to previous reports, we have demonstrated that neutrophils are not unresponsive to the effects of glucocorticoids. Indeed, we have shown that neutrophil longevity is remarkably sensitive to the inhibitory influence of glucocorticoids which may have important

implications for the use of glucocorticoids in treatment of chronic inflammatory conditions associated with neutrophilic infiltration. Interestingly, it appears that eosinophils are less responsive to the apoptotic influence of glucocorticoids and this further emphasises the distinct signalling mechanisms by which glucocorticoids modulate apoptotic regulation in these closely related cells. We are the first to report the requirement for hsp90 in both glucocorticoid induction of eosinophil apoptosis and delay of neutrophil death. The requirement of hsp90 as a molecular chaperone for GR, to allow the formation of competent steroid binding receptors in granulocytes, awaits future confirmation. We have attempted to further characterise the glucocorticoid signal transduction pathway controlling apoptosis in granulocytes by examining GR isoform expression and activation following glucocorticoid treatment. Although we have shown GR to be present in both neutrophils and eosinophils, problems in immunoblotting with isoform specific antibodies meant we were not able to characterise if differential expression of GR isoforms in granulocytes, relates to the divergent effect of glucocorticoids in modulating apoptosis in these cells. It has been suggested that glucocorticoid delay of neutrophil apoptosis may be as a result of preferential expression of GRβ, allowing neutrophils to escape from glucocorticoid induction of apoptosis. However, it has not yet been demonstrated whether eosinophils, sensitive to induction of apoptosis by glucocorticoids, exhibit lower expression of GRβ compared to neutrophils or indeed whether acceleration of apoptosis by glucocorticoids in this cell type, requires GRα homodimer transactivation. Future studies, using specific antibodies or quantitative PCR, involving a direct comparison of GR isoform expression in neutrophils compared to eosinophils, would help ascertain whether the outcome of glucocorticoid treatment on granulocyte apoptosis specifically correlates to differential expression of GR isoforms in these cells.

Studies were undertaken to dissect the complex transcriptional regulatory mechanisms that may be involved in glucocorticoid modulation of granulocyte apoptosis. We have demonstrated that glucocorticoids transrepress NFκB regulated IL-8 production in granulocytes. Furthermore, glucocorticoids appear not to be

capable of transcriptional transactivation, however due to the limitations in our ability to measure GRE-mediated transactivation in granulocytes, these results should be viewed with caution. Considering the implication of NFκB as an important survival factor in granulocytes, it is possible that in eosinophils, glucocorticoids block the synthesis of a NFκB regulated survival protein by transrepression to induce apoptosis. Obviously the same mechanism could not apply to neutrophils as dexamethasone, although transrepressing NFκB regulated IL-8 production in neutrophils, enhances cell survival. Through the use of dissociated glucocorticoids, we have shown that transactivating and transrepressing glucocorticoids can both induce eosinophil apoptosis and delay neutrophil cell death. Importantly however, neither set of compounds appear as efficient as dexamethasone in modulating granulocyte apoptosis. This suggests both transactivation and repression by glucocorticoids to some extent, may be implicated in glucocorticoid regulation of granulocyte apoptosis. Further research investigating the complex mechanisms of transcriptional regulation by glucocorticoids, including the involvement of chromatin remodelling, is required to fully elucidate the aspects of transcriptional regulation important for glucocorticoid modulation of apoptosis. The development of more selective reagents leading to a greater understanding of the glucocorticoid signal transduction cascade in granulocytes, may help ascertain if differential responsiveness to glucocorticoids in neutrophils and eosinophils, results from divergence in transcriptional regulation by glucocorticoids, in each cell type.

There is accumulating evidence of the importance of apoptosis as a major means of eliminating extravasated granulocytes from inflamed sites. It has been proposed that persistent inflammatory responses may arise from failure or inefficiency in the phagocytic clearance mechanisms to remove apoptotic cells, which as a consequence undergo secondary necrosis, releasing toxic cell contents thus amplifying inflammation. The majority of evidence supporting the hypothesis that granulocyte apoptosis provides a mechansim for resolution of inflammation, has mainly come from *in vitro* experimentation. In a recent report, Erjefalt *et al.* have questioned the *in vivo* relevance of apoptosis as a tissue-injury limiting mechanism during

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inflammation, particularly with regard to the role of eosinophil apoptosis in regulation and resolution of lung inflammation (Erjefalt and Persson, 2000; Uller *et al.*, 2001). Ejefalt *et al* suggest eosinophil apoptosis may occur as a secondary event following eosinophil extrusion into the airway lumen from the lung tissue. Furthermore, they argue that lack of evidence of apoptosis of airway tissue-residing eosinophils, suggests that an alternative pathway of luminal extrusion may be involved in clearance of eosinophils during lung inflammation. The difficulty in obtaining biopsy and tissue samples, with particular regard to human studies, has meant that it has been difficult to directly demonstrate apoptosis and subsequent clearance of tissue dwelling granulocytes during the inflammatory response. Furthermore, *in vitro* data suggests engulfment of apoptotic cells usually occurs within minutes of contact, thus it may be difficult to visualise the extent of this process occuring in tissue samples, due to the rapid kinetics of phagocytosis. Many of the *in vivo* studies of apoptotic cell clearance during inflammation have numbers of apoptotic cells obtained from BAL fluid or sputum samples. In a model of acute lung injury, Cox *et al.* report the appearance of apoptotic neutrophils and macrophage engulfment in BAL fluid to be temporally correlated with the resolution of pulmonary inflammation (Cox *et al.*, 1995). Wedi *et al* report that eosinophils derived from patients with inhalent allergy and atopic dermatitis have a reduced rate of apoptosis compared to nonatopic subjects (Wedi *et al.*, 1997). As mentioned previously, Woolley *et al* have demonstrated that glucocorticoid treatment of asthmatic patients is associated with an increase in the number of apoptotic eosinophils in the airways and eosinophil products inside macrophages (Woolley *et al.*, 1996). In addition, it has been demonstrated that administration of an anti-Fas antibody to the lungs following allergen induced eosinophilia, caused a marked reduction in the number of eosinophils in the airways (Tsuyuki *et al.*, 1995). Furthermore human airways inflammation has been shown to be associated with ingestion of apoptotic neutrophils by macrophages through measurement of apoptotic cells and macrophages in BAL fluid from neonates (Grigg *et al.*, 1991). There are also a small number of studies which present *in vivo* evidence that apoptosis in tissue granulocytes may provide an important mechanism for limitation of the inflammatory response. For example, Ying *et al.* report association of granulocyte

apoptosis and clearance by macrophages with resolution of allergen induced cutaneous late phase response (Ying *et al.*, 1997). In addition, Kawabori *et al* have demonstrated increased eosinophil apoptosis and phagocytosis in corticosteroid treated intestine (Kawabori *et al.*, 1991) and Davidsson *et al* report apoptosis of tissue dwelling eosinophils and their engulfment by macrophages, in sinonasal polyps (Davidsson *et al.*, 2000). Furthermore, Vignola *et al.* have demonstrated an inverse correlation between the number of apoptotic eosinophils and clinical severity of asthma in mucosal biopsy specimins (Vignola *et al.*, 1999). In a murine model, adminstration of anti-Fas antibody induced apoptosis of infiltrated eosinophils and abolished the augmentation of airway hyperresponsiveness in response to ovalbumin sensitisation (Ohta *et al.*, 2001). Furthermore, Kodama *et al* report increased apoptosis in the lung, following ovalbumin challenge in a murine model of allergic airway inflammation (Kodama *et al.*, 1998) It is not unlikely that apoptosis and removal by macrophages and other phagocytes is an injury limiting disposal mechanism for the extravasated neutrophil. With the development of sophisticated imaging techniques, it may in the future be possible to track the migration of granulocytes into the tissues and therefore ultimately determine the fate of these cells and their removal, during the resolution of inflammation.

The *in vitro* data presented in this thesis suggests that the rate of granulocyte apoptosis may be powerfully modulated by glucocorticoids and cyclic AMP. These findings may have potentially important implications for the effectiveness of existing anti-inflammatory treatments or the development of new intervention strategies which favour the resolution of inflammation. Given that selective phosphodiesterase (PDE) inhibitors and β2 adrenoceptor agonists may exert their anti-inflammatory effects through elevation of cyclic AMP, there is the potential that in some instances inflammation may not resolve due to enhanced granulocyte longevity and inhibited macrophage clearance mechanisms in such treatments. Furthermore, given the ability cyclic AMP to interfere with the signal transduction pathways of inflammatory cytokines such as GM-CSF, cyclic AMP may have opposing effects depending on the influence of other mediators at an inflammatory focus. Further exploration of possible cross-talk between the cyclic AMP signal transduction cascade and other

signalling pathways, may give a greater comprehension of the potential outcomes of elevation of this second messenger *in vivo*. In addition, a better understanding of the mechanisms which underlie differential steroid responsivenss in granulocytes, could potentially be exploited for the development of novel approaches for treatment of allergic inflammation. Currently, there is little known of the mechansims by which glucocorticoids integrate with the apoptotic signal transduction machinery to differentially modulate granulocyte apoptosis. Further definition of the signalling mechanims involved in glucocorticoid responsivenss may be useful not only for the development of novel glucocorticoids which have the ability induce apoptosis in selective cell populations, but may aid production of glucocorticoids with fewer systemic side effects, that currently limits their use.

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Cyclic AMP Regulation of Neutrophil Apoptosis Occurs via a Novel Protein Kinase A-independent Signaling Pathway*

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Morag C. Martin‡, Ian Dransfield, Christopher Haslett, and Adriano G. Rossi

From the Medical Research Council Centre for Inflammation Research, Respiratory Medicine Unit, University of Edinburgh Medical School, Edinburgh EH8 9AG, United Kingdom

The second messenger molecule cyclic AMP dramatically modulates the apoptotic program in a wide variety of cells, accelerating apoptosis in some and delaying the rate of apoptosis in others. Human neutrophil apoptosis, a process that regulates the fate and numbers of these potentially histotoxic cells in inflammatory sites, is profoundly delayed by the cell-permeable analog of cyclic AMP, dibutyryl-cAMP. We have investigated the mechanisms underlying cyclic AMP-mediated delay of neutrophil apoptosis, and we show that cyclic AMP inhibits loss of mitochondrial potential occurring during constitutive neutrophil apoptosis. Furthermore, we demonstrate that cyclic AMP also suppresses caspase activation in these inflammatory cells. Despite increasing protein kinase A activity, this kinase is unlikely to mediate the effect of cyclic AMP on apoptosis because blockade of protein kinase A activation did not influence the survival effects of cyclic AMP. Further investigation of the signaling mechanism demonstrated that the delay of apoptosis is independent of phosphoinositide 3-kinase and MAPK activation. Our results suggest cyclic AMP delays neutrophil apoptosis via a novel, reversible, and transcriptionally independent mechanism. We show that proteasome activity in the neutrophil is vitally involved in this process, and we suggest that a balance of pro-apoptotic and anti-apoptotic proteins plays a key role in the powerful ability of cyclic AMP to delay neutrophil death.

The neutrophil is a terminally differentiated phagocytic cell that plays a key role in first line defense against invading bacteria. Neutrophils are rapidly recruited to inflamed sites in response to infection and, following phagocytosis of the invading organism, release a variety of toxic granule contents into the phagolysosome containing the engulfed microorganisms (1, 2). The neutrophil normally has a short life span, and senescent neutrophils must be prevented from releasing their cytotoxic cell contents into the surrounding milieu because such liberation will lead to local tissue damage. To avoid this undesirable and inappropriate response, the neutrophil undergoes a regulated process of programmed cell death or apoptosis (3–5), allowing shutdown of secretory capacity (6) and phagocytic removal of the intact effete cell by a mechanism that does not incite an inflammatory response (7–9).

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‡ To whom correspondence should be addressed. Tel.: 44-131-651-1323/1572; Fax: 44-131-651-4384; E-mail: moragmartin@hotmail.com.

The execution of the apoptotic program generally involves the activation of a family of cysteine proteases, collectively referred to as the caspases, that are ultimately responsible for the structural dismantling of the cell (10, 11). In addition, the mitochondria play a central role through their ability to integrate anti-apoptotic or pro-apoptotic signals from Bcl-2 family members with coordinated activation of downstream caspases and nucleases (12, 13). In many cell types it has been documented that apoptosis is accompanied by an early dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$) with increased permeability of the outer mitochondrial membrane allowing release of apoptosis-inducing factors such as cytochrome *c* (12–14). Neutrophils are thought to contain very few mitochondria, and it has not yet been fully established whether they have the capacity to play a functional role in regulation of neutrophil apoptosis (15, 16).

Neutrophils undergo constitutive apoptosis during *in vitro* culture and exhibit the classic changes associated with apoptosis including cytoplasmic condensation, internucleosomal cleavage of DNA by endogenous endonucleases, and exposure of phosphatidylserine on the outer leaflet of the plasmalemma (3). Although the apoptotic program in neutrophils is an intrinsic cell process, the rate of apoptosis can be altered dramatically by a number of agents (17). In particular, we and others (18–20) have shown that elevated levels of the second messenger cyclic AMP can prolong neutrophil longevity by delaying apoptosis.

The cyclic AMP-dependent signaling transduction pathway is a multienzyme cascade that regulates a diverse array of biological processes. Specific ligation of appropriate G-protein-coupled receptors followed by adenylate cyclase activation leads to the production of cyclic AMP. Cyclic AMP then binds to cytoplasmic protein kinase A, a tetrameric structure composed of two regulatory (R) and two catalytic (C) subunits, resulting in dissociation of the C subunits and subsequent phosphorylation of target proteins (21). Although most components of this signaling cascade are well characterized, the molecular mechanisms underlying cyclic AMP-mediated modulation of apoptosis remain to be elucidated. The signaling mechanism(s) used by cyclic AMP to control these events is (are) likely to be complex and cell type-specific. For example, in contrast to the profound delay in the engagement of the apoptotic process in neutrophils (18–20), cyclic AMP elevation induces apoptosis in thymocytes (22) and leukemic cell lines (23, 24). The signaling mechanism determining this ability to differentially influence apoptosis in diverse cell types remains to be elucidated.

In the present study we show that elevated cyclic AMP inhibits activation of caspase-3 and loss in mitochondrial potential ($\Delta\Psi m$) when neutrophils are aged *in vitro*, *i.e.* effects that appear to be associative rather that causative. Although

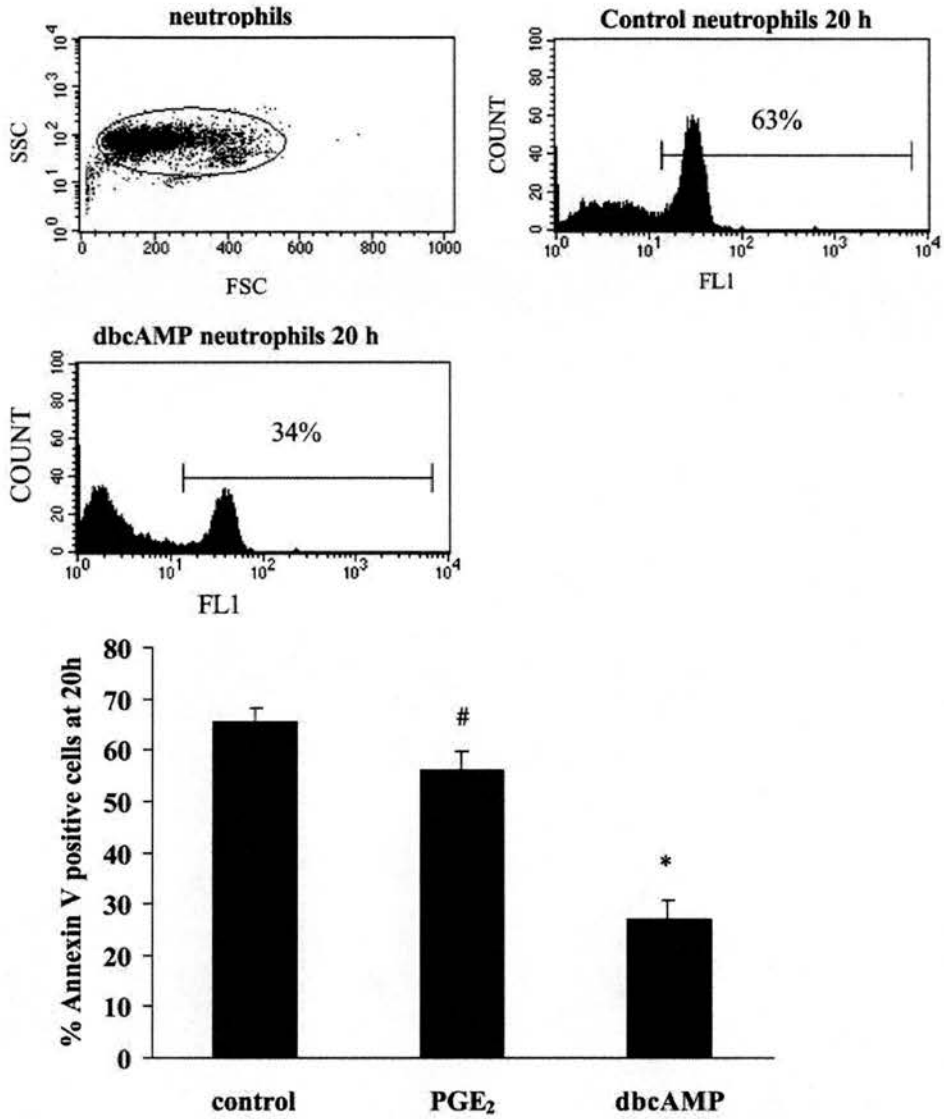

FIG. 1. **Effect of cyclic AMP elevation on human neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum and treated with Bt_2cAMP (*dbcAMP*) (0.2 mM) or PGE_2 (10 μM). After 20 h, the cells were incubated with FITC-labeled recombinant human annexin V to determine phosphatidylserine expression. The cells were then assessed by flow cytometry on a FACSCalibur and analyzed on associated CellQuest software. Data from a minimum of 5000 cells were analyzed for each condition. All values represent mean ± S.E. of $n = 5$–8 experiments, each performed in duplicate where significant difference from control is represented by *, $p < 0.001$, and #, $p < 0.05$. Similar results were found by morphological assessment of apoptosis (data not shown).

we could demonstrate that cyclic AMP rapidly elevates endogenous PKA[1] activity in cultured neutrophils, blockade of PKA activation did not influence the observed delay in neutrophil apoptosis induced by cyclic AMP elevation. We also show that cyclic AMP elevation delays neutrophil apoptosis via a transcriptionally independent and reversible pathway, which does not require PI 3-kinase and MAPK activity. Together these data point to a novel mode of action for the major retardation of neutrophil apoptosis induced by cyclic AMP elevation.

[1] The abbreviations used are: PKA, protein kinase A; Bt_2cAMP, dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; GM-CSF, granulocyte macrophage-colony-stimulating factor; JC-1 [5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazocarbocyaniniodide; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphoinositide 3-kinase; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; FITC, fluorescein isothiocyanate.

EXPERIMENTAL PROCEDURES

Granulocyte Isolation and Culture

Neutrophils were purified from the peripheral blood of normal donors by dextran sedimentation (Sigma) followed by centrifugation on discontinuous Percoll™ (Amersham Pharmacia Biotech) gradients as described previously (25, 26). Only neutrophil preparations with a purity of >98% were used. Cells were cultured in flat-bottomed Falcon flexible wells (Becton Dickinson, Oxford, UK) at 37 °C in a 5% CO_2 atmosphere at a concentration of 5×10^6/ml in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 100 units/ml penicillin/streptomycin (Life Technologies, Inc.) and 10% (v/v) autologous serum. As an index of necrosis, cell membrane integrity was assessed by the ability of cells to exclude the vital dye trypan blue (Sigma). Under all experimental conditions, greater than 99% of the cells consistently excluded trypan blue.

Assessment of Granulocyte Apoptosis

Morphology—Cells were cyto-centrifuged, fixed in methanol, stained with Diff-Quik™ Gamidor Ltd. (Abingdon, Oxon, UK), and counted

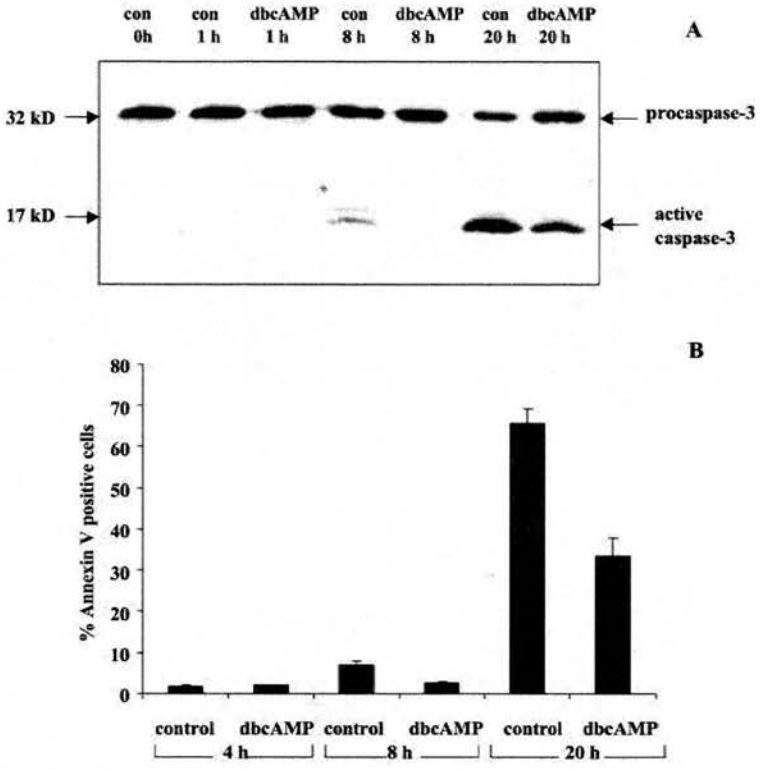

FIG. 2. **Time course for the effect of Bt_2cAMP (dbcAMP) on caspase-3 expression during human neutrophil apoptosis.** *A*, Western blot of cytoplasmic extracts from neutrophils treated with control buffer or Bt_2cAMP (0.2 mM) for the time points indicated. Cell lysates were prepared and immunoblotted as described under "Experimental Procedures." Lysates were prepared from equivalent numbers of cells and subjected to SDS-polyacrylamide gel electrophoresis/immunoblot analysis using a rabbit polyclonal antibody specific for caspase-3. The caspase-3 antibody recognizes both the 32-kDa pro-caspase-3 and the 17-kDa subunit of active caspase-3. The 17-kDa caspase-3 cleavage product is faintly visible in control (*con*) lysates at 8 h becoming more apparent by 20 h. There appears to be less active caspase-3 in Bt_2cAMP (*dbcAMP*)-treated cell lysates compared with control cell lysates. The gel is representative of three experiments. *B*, human neutrophils were treated with or without Bt_2cAMP (0.2 mM) for the time points indicated under equivalent culture conditions as the cells used for caspase-3 expression assessment above. Cells were assessed for apoptosis by measurement of phosphatidylserine expression using annexin V FITC. Data from a minimum of 5000 cels were analyzed for each condition. All values represent mean ± S.E. of $n = 3$ experiments, each performed in duplicate.

using oil immersion microscopy to determine the proportion of cells with distinctive apoptotic morphology (3, 26). At least 500 cells were counted per slide with the observer blinded to the experimental conditions. The results were expressed as the mean percent apoptosis ± S.E.

Annexin V Binding—A separate and independent assessment of apoptosis was performed by flow cytometry using annexin V binding (annexin V-FLUOS, Roche Molecular Biochemicals) to measure phosphatidylserine exposure on the surface of apoptotic cells. A working solution of annexin V-FLUOS was made from stock annexin V-FLUOS (0.1 μg/μl) diluted 1:3000 in Hanks' balanced salt solution (Sigma) supplemented with 2.5 mM $CaCl_2$. Neutrophils (20 μl of 5×10^6/ml) were added to 200 μl of the working solution of annexin V-FLUOS before being assessed by flow cytometry on a FACSCalibur (Becton Dickinson, Oxford, UK) and analyzed on associated CellQuest (Becton Dickinson) software. All experiments were performed at least three times unless otherwise indicated.

Measurement of PKA Activity

PKA activity was measured using Promega's SignaTECT™ cAMP-dependent Protein Kinase (PKA) Assay System, which utilizes biotinylated Kemptide (LRRASLG), a peptide substrate derived from the *in vivo* substrate pyruvate kinase. Neutrophils (5×10^6 cells) were preincubated with control buffer or 10 μM H89 (Calbiochem) for 1 h in PBS with Ca^{2+}/Mg^{2+} (or for 19 h in DMEM Iscove's with 10% autologous serum) at 37 °C before being stimulated with 0.2 mM Bt_2cAMP or 1 μM PGE_2 (both from Sigma) for 30 min at 37 °C. Following one wash in ice-cold PBS, neutrophils were resuspended in 0.5 ml of cold extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 (Sigma)). The lysates were centrifuged (5 min at 4 °C; 14,000 × *g*) and the supernatants retained. The PKA reaction mixture consisting of 5 μl of 5× PKA Assay Buffer, 5 μl of cyclic AMP (0.025 mM), 5 μl of PKA-biotinylated peptide substrate (0.5 mM), 5 μl of [γ-^{33}P]ATP mixture (5 μl of 0.5 mM ATP and 0.05 μl of [γ-^{33}P]ATP (3,000 Ci/mmol) 10 μCi/μl) was mixed gently and preincubated at 30 °C for 5 min (Promega, Southampton, UK). A control reaction without substrate was performed to determine background counts. The PKA activity reaction was initiated by adding 5 μl of the lysates to the reactants and incubated at 30 °C for 5 min. The reaction was terminated by adding 12.5 μl of Termination Buffer to each sample (Promega, Southampton, UK). Aliquots (10 μl) from each terminated reaction sample were spotted onto prenumbered SAM^{2TM} membrane squares (Promega, Southampton, UK). The SAM^{2TM} membrane squares containing the spotted samples were then washed 1 time for 30 s with 200 ml of 2 M NaCl (Sigma) followed by 3 washes for 2 min with 200 ml of 2 M NaCl and then 4 washes for 2 min with 200 ml of 2 M NaCl in 1% H_3PO_4. Finally the Membrane squares were quickly washed in deionized water before being allowed to dry. PKA activity was measured by scintillation counting.

Measurement of Mitochondrial Dissipation

Changes in mitochondrial potential were measured in neutrophils following stimulation using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazocarbocyaniniodide (Molecular Probes), a cationic dye that exhibits potential dependent accumulation in mitochondria indicated by a fluorescence emission shift from green (525 nm) to red (590 nm) (27). Mitochondrial depolarization is therefore indicated by a decrease in the red/green fluorescence intensity ratio. JC-1 (10 μg/ml) was diluted in PBS from stock JC1 (5 mg/ml in Me_2SO) and added to neutrophils (1×10^6/ml) for 10 min at 37 °C. Neutrophil mitochondria labeled with JC-1 were examined by confocal fluorescent microscopy together with TO-PRO-3 (1 μM) (Molecular Probes) (28) to assess neutrophils with necrotic morphology. Alternatively, neutrophils labeled with JC-1 were assessed by flow cytometric analysis using FACSCalibur (Becton Dickinson, Oxford, UK) and analyzed on associated CellQuest (Becton Dickinson) software. Non-apoptotic neutrophils were removed using immunomagnetic separation with sheep anti-mouse IgG-Dynabeads (Dynabeads M-450, Dynal, Mersyside, UK) coated with the murine anti-neutrophil antibody 3G8 (anti-CD16; a gift from Dr. J. Unkeless, Mount Sinai Medical School, New York). Cells were mixed with washed antibody-coated magnetic beads on a rotary mixer at 4 °C for 20 min, and the beads removed magnetically by two 3-min stationary magnetic contacts (Dynal Magnetic Particle Concentrator, MPC-1) to yield an apoptotic neutrophil preparation. After purification, the apoptotic neutrophils were labeled with JC-1 as described previously.

Western Blotting

Human neutrophils (5×10^6/ml) were cultured with or without Bt_2cAMP (0.2 mM) at 37 °C for various time points as detailed under "Results." Cytoplasmic extracts were then prepared from equivalent numbers of cells (10×10^6 cells). To minimize problems with proteolysis, lysates were prepared using methods normally used for electrophoretic mobility shift assay preparations (26, 29) with the addition of 1 mM phenylmethylsulfonyl fluoride. Samples were loaded onto a 12.5%

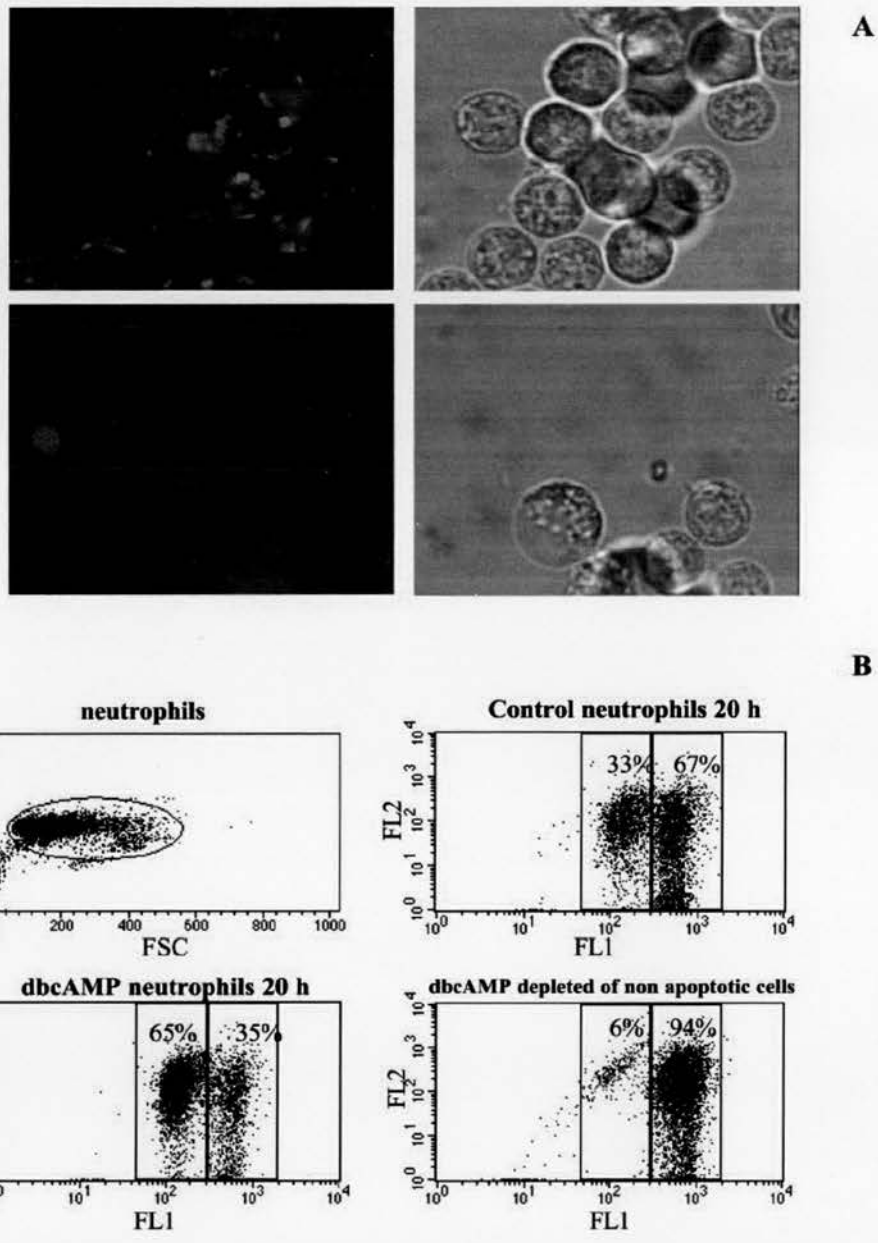

FIG. 3. *A*, determination if neutrophils contain mitochondria. Human neutrophils (1×10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum. Neutrophils were labeled with JC-1, a mitochondrial specific dye, and examined by confocal fluorescent microscopy as described under "Experimental Procedures." *Bottom panels* show TO-PRO-3 staining for neutrophils with necrotic morphology (*blue*). *B*, effect of Bt_2cAMP (dbcAMP) on dissipation of mitochondrial transmembrane potential during human neutrophil apoptosis. Human neutrophils (5×10^6/ml) were cultured for 20 h at 37 °C in Iscove's DMEM containing 10% autologous serum with or without Bt_2cAMP (dbcAMP) (0.2 mM). Cells were then labeled with the mitochondrial specific dye JC-1 as described under "Experimental Procedures" before flow cytometric analysis of mitochondrial membrane potential using a FACSCalibur and associated CellQuest software. Non-apoptotic neutrophils (*bottom right panel*) were removed by anti-CD16 immunodepletion before the remaining cells were labeled with JC-1. Shown is one representative experiment.

Tris-HCl polyacrylamide mini-gel under reducing conditions and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech) at 60 V for 1 h before overnight incubation at 4 °C with an antibody specific to caspase-3 (catalog number 65906E, PharMingen). After washing, blots were incubated with donkey anti-rabbit horseradish peroxidase conjugate (Amersham Pharmacia Biotech) diluted 1:2000 and developed using a commercial chemiluminescence detection system (ECL, Amersham Pharmacia Biotech).

Further Materials

Further specific materials were obtained as follows: (R_p)-8-Br-cAMPS, PD98059, SB203580, and cycloheximide (Calbiochem); lactacystin and epoxomicin (Affiniti, Mamhead, UK); and LY294002 (New England Biolabs, Hertfordshire, UK).

Statistical Analysis

Statistical analysis was performed using the Student's t test or by analysis of variance with comparisons between groups made using the Newman-Keuls procedure. Differences were considered significant when $p < 0.05$.

RESULTS

Elevation of Cyclic AMP Delays Neutrophil Apoptosis—To examine the effects of cyclic AMP on apoptosis induction, neutrophils were exposed to Bt_2cAMP, a membrane-permeant cyclic AMP analog and the receptor-directed stimulus PGE_2, for 20 h. Both Bt_2cAMP and PGE_2 delay neutrophil apoptosis, determined by standard morphological criteria (data not

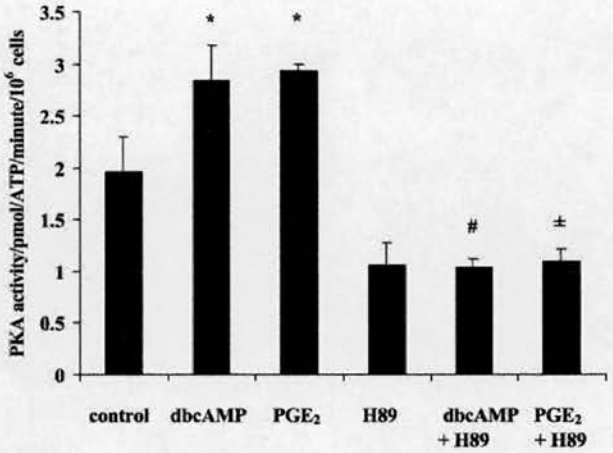

FIG. 4. **Measurement of PKA activation by elevators of cyclic AMP in human neutrophils.** Human neutrophils (5×10^6/ml) were preincubated with 10 μM H89 for 1 h before being stimulated with Bt_2cAMP (*dbcAMP*) (0.2 mM) or PGE_2 (1 μM) for 30 min at 37 °C. PKA activity was measured as described under "Experimental Procedures." All values represent mean ± S.E. of $n = 3$ experiments where significant difference from control values is represented by *, $p < 0.05$. Significant difference from Bt_2cAMP alone is represented by #, $p < 0.001$, and significant difference from PGE_2 alone is represented by ±, $p < 0.001$.

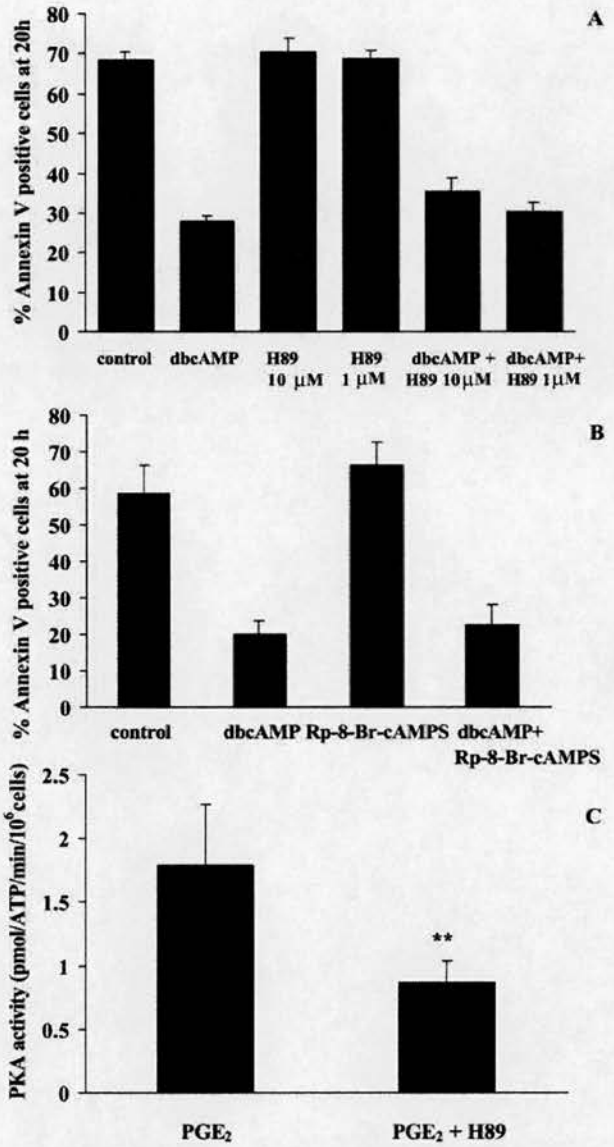

FIG. 5. **The effect of pharmacological blockade of PKA activity on Bt_2cAMP-mediated delay of neutrophil apoptosis.** *A* and *B*, human neutrophils (5×10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C were preincubated for 30 min with H89 (10 μM) (*A*) or (R_p)-8-Br-cAMPS (100 μM) (*B*) before stimulation with Bt_2cAMP (*dbcAMP*) (0.2 mM). After a further 20 h in culture, the cells were incubated with FITC-labeled recombinant human annexin V to determine phosphatidylserine expression. The cells were then analyzed by flow cytometry using a FACSCalibur and associated CellQuest software. Data from a minimum of 5000 cells were analyzed for each condition. All values represent mean ± S.E. of $n = 3$ experiments, each performed in duplicate. Similar results were found when cells were assessed for apoptosis by morphological examination (data not shown). *C*, human neutrophils (5×10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C were preincubated with 10 μM H89 for 19 h before being stimulated with PGE_2 (1 μM) for 1 h at 37 °C. PKA activity was measured as described under "Experimental Procedures." All values represent mean ± S.E. of $n = 5$ experiments where significant difference from PGE_2 alone is represented by **, $p < 0.01$.

shown) and annexin V binding (Fig. 1). It is interesting to note that maximal concentrations of Bt_2cAMP (0.2 mM) were more effective at delaying neutrophil apoptosis compared with maximal concentrations of PGE_2 (10 μM) (Fig. 1).

Bt_2cAMP Inhibits Caspase Activation but Is Unlikely to Act Directly as an Inhibitor of Caspases—It is widely believed that caspases act as the main executioners of apoptosis, with their activation resulting in chromatin condensation and DNA fragmentation. Whether cyclic AMP delays constitutive neutrophil apoptosis by directly suppressing caspase activation in neutrophils has not been examined. Therefore, we investigated the effect of cyclic AMP on activation of caspase-3 during constitutive apoptosis by immunoblotting. Neutrophils were found to express active caspase-3 (17 kDa) which closely correlates with the exposure of phosphatidylserine during constitutive neutrophil apoptosis (Fig. 2, *A* and *B*). By 20 h there is significant caspase-3 activity (17 kDa) which can be inhibited by Bt_2cAMP. The appearance of some caspase-3 activity in the presence of Bt_2cAMP at 20 h probably reflects the presence of some apoptotic cells in the population (Fig. 2*B*).

Apoptosis in Neutrophils Is Accompanied by Dissipation of Mitochondrial Transmembrane Potential That Can Be Inhibited by Bt_2cAMP—In many cell models, apoptosis is accompanied by an early dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$). Previous data (16) have indicated that neutrophils do not respire, and it was thought unlikely that they contained mitochondria. By using confocal microscopy and flow cytometry, we have been able to demonstrate that neutrophils do contain mitochondria (*orange*), which during overnight culture exhibit loss of mitochondrial potential as indicated by an increase in green fluorescence (Fig. 3*A*). Our studies also reveal that Bt_2cAMP inhibits changes in mitochondrial potential occurring during constitutive neutrophil apoptosis (Fig. 3*B*). As the number of cells showing loss of mitochondrial potential appeared to correlate with the number of apoptotic cells measured by annexin V positivity in previous experiments (Fig. 1), we examined directly if loss in mitochondrial potential occurred in those neutrophils undergoing apoptosis. It is well established that neutrophils lose cell surface expression of CD16 during the process of apoptosis (30). Immunodepletion of non-apoptotic neutrophils using anti-CD16 magnetic beads demonstrated apoptotic neutrophils were indeed positive for loss of mitochondrial potential (Fig. 3*B*) indicating that dissipation of mitochondrial membrane potential occurs in neutrophils undergoing programmed cell death.

It has been shown previously (31) that inhibitors of the

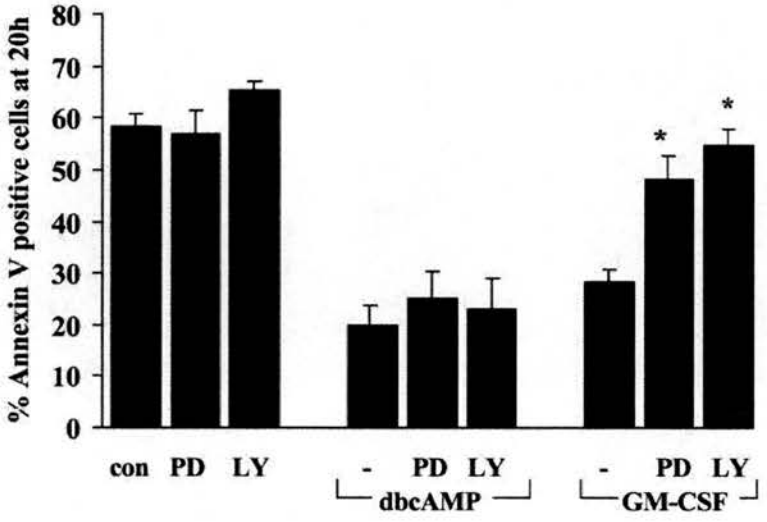

FIG. 6. **Effect of PI 3-kinase and MAPK inhibition on Bt_2cAMP (dbcAMP)-mediated delay of neutrophil apoptosis.** Human neutrophils (5×10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C were treated with LY294002 (*LY*, 10 μM) or PD98059 (*PD*, 10 μM) for 30 min prior to stimulation by Bt_2cAMP (0.2 mM). After a further 20 h in culture, the cells were incubated with FITC-labeled recombinant human annexin V to determine phosphatidylserine expression. The cells were then analyzed by flow cytometry using a FACSCalibur and associated CellQuest software. Data from a minimum of 5000 cells were analyzed for each condition. All values represent mean ± S.E. of $n = 3$ experiments, each performed in duplicate where significant difference from GM-CSF alone is represented by *, $p < 0.01$. *con*, control.

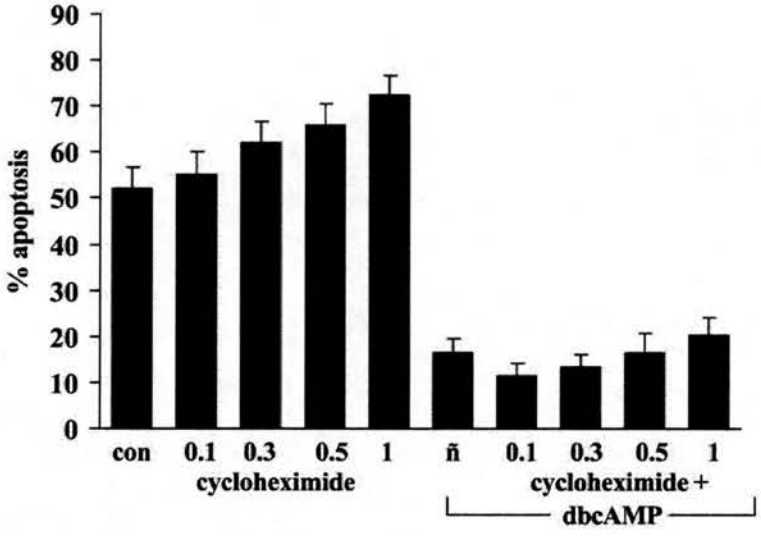

FIG. 7. **Effect of protein synthesis inhibition by cycloheximide on Bt_2cAMP (*dbcAMP*)-mediated delay of neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured in Iscove's DMEM containing 10% autologous serum at 37 °C and treated with the indicated concentrations of cycloheximide (μg/ml) with or without Bt_2cAMP (0.2 mM). After 20 h, cells were harvested and assessed morphologically for apoptosis. All values represent mean ± S.E. of n = 10 experiments, each performed in triplicate. Similar results were found when cells were assessed for apoptosis by annexin V binding (data not shown). *con*, control.

mitochondrial respiratory chain do not affect constitutive neutrophil apoptosis, raising the question of the source of their $\Delta\Psi m$. It may be the case that the neutrophil maintains a transmembrane gradient by a functional F_1,F_0-ATPase; however, this needs to be investigated in more detail.

Cyclic AMP Elevation Stimulates PKA Activation in Neutrophils, an Effect That Is Blocked by Pharmacological Inhibitors—To elucidate further the mechanism by which cyclic AMP regulates neutrophil apoptosis, we examined its downstream signaling pathway. The effects of cyclic AMP are thought to be mediated through binding of cyclic AMP to the intracellular kinase, PKA. This leads to the dissociation of PKA into regulatory and catalytic subunits, which can consequently lead to phosphorylation events of proteins such as the cyclic AMP-response element-binding protein (21). To explore whether cyclic AMP suppresses apoptosis and apoptotic signaling via activating the PKA pathway in neutrophils, we examined the effects of Bt_2cAMP on endogenous PKA activation (Fig. 4). We found rapid activation of PKA when cells were treated with both Bt_2cAMP and PGE_2. Furthermore, the activation of PKA upon stimulation of neutrophils with cyclic AMP elevators could be blocked by the pharmacological PKA inhibitor H89 (32) (Fig. 4).

Activation of the PKA Pathway Does Not Account for Cyclic AMP-mediated Retardation of Apoptosis—To investigate whether PKA activation by cyclic AMP was necessary for cyclic AMP-mediated delay of apoptosis, neutrophils were incubated with the PKA inhibitor H89 before being stimulated with Bt_2cAMP and assessed for apoptosis. Surprisingly, whereas pre-treatment with H89 prevented activation of PKA, it did not prevent the inhibition of apoptosis by Bt_2cAMP (Fig. 5*A*). Additionally, the highly specific inhibitor of PKA, (R_p)-8-Br-cAMPS (100 μM), also failed to block Bt_2cAMP inhibition of neutrophil apoptosis (Fig. 5*B*). This suggests that cyclic AMP elevation stimulates PKA activity but PKA does not play a major role in the anti-apoptotic effect of cyclic AMP elevation in neutrophils.

The ability of H89 to block PKA activity, as shown by direct measurement of kinase activity, suggests a lack of involvement of PKA in the anti-apoptotic effect of cyclic AMP in neutrophils. Most importantly, we investigated whether H89 could block PKA activity for the full overnight culture period and under identical culture conditions that we use for our apoptosis assay. Neutrophils were therefore cultured in serum-supplemented Iscove's DMEM for 19 h in the presence or absence of H89 before stimulation with PGE_2 for 1 h. PKA activity was then measured as described under "Experimental Procedures." We found that H89 could still block PGE_2-stimulated PKA activity

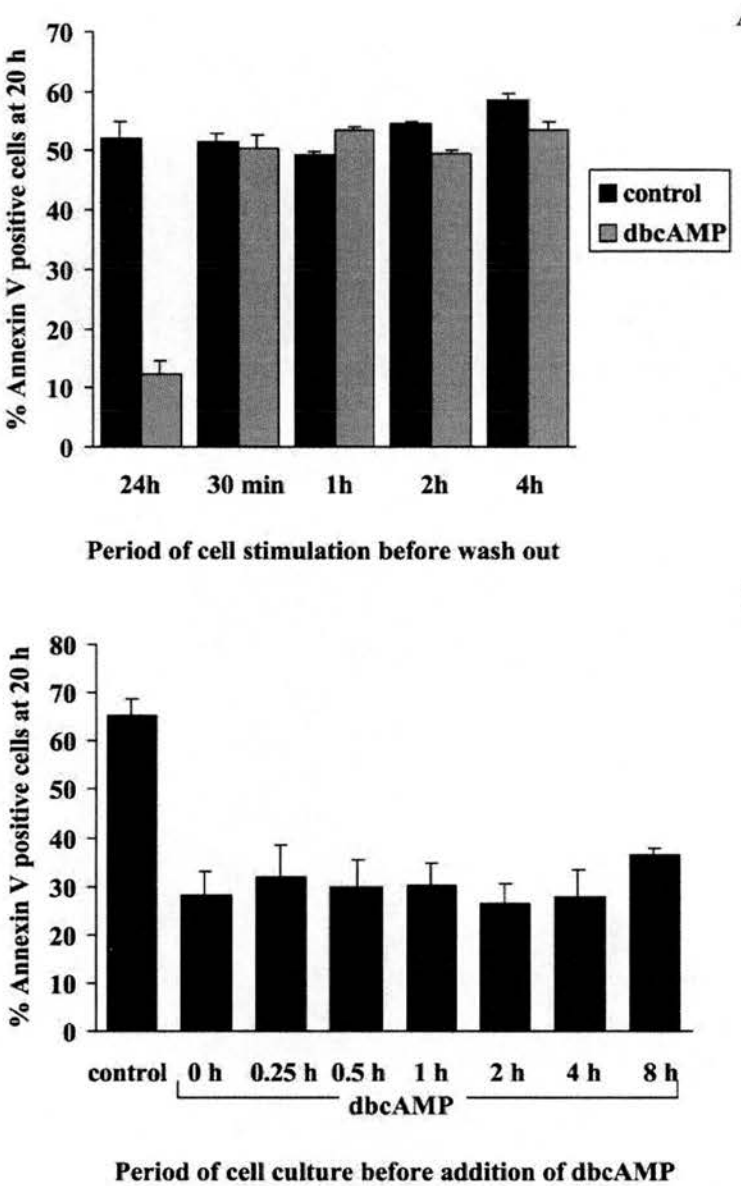

FIG. 8. **Loss of Bt_2cAMP (dbcAMP)-mediated delay of neutrophil apoptosis by washing and rescue of cultured neutrophils from apoptosis by delayed addition of Bt_2cAMP.** *A*, human neutrophils (5×10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C were treated with or without Bt_2cAMP (*dbcAMP*) (0.2 mM) for the time points indicated before the cells were washed 2 times in PBS to remove Bt_2cAMP and returned to culture. Cells were cultured in Iscove's DMEM containing 10% autologous serum until 20 h when the cells were resuspended and incubated with FITC-labeled recombinant human annexin V to determine phosphatidylserine expression. The cells were then assessed by flow cytometry on a FACSCalibur and analyzed on associated CellQuest software. Data from a minimum of 5000 cells were analyzed for each condition. All values represent mean ± S.E. of $n = 3$ experiments, each performed in duplicate. *B*, human neutrophils (5×10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum at 37 °C for the time points indicated before addition of Bt_2cAMP (0.2 mM). At 20 h, cells were resuspended and incubated with FITC-labeled recombinant human annexin V to determine phosphatidylserine expression. The cells were then assessed by flow cytometry on a FACSCalibur and analyzed on associated CellQuest software. Data from a minimum of 5000 cells were analyzed for each condition. All values represent mean ± S.E. of $n = 3$ experiments, each performed in duplicate.

at 20 h (Fig. 5*C*). This is very important because it demonstrates that the inability of H89 to reverse cyclic AMP-mediated delay of neutrophil apoptosis is not due to degradation of H89 during the overnight culture period. Furthermore, it also demonstrates that H89 is not inactivated by autologous serum that is used in our apoptosis assay.

Activation of Akt/PI 3-Kinase or Mitogen-activated Kinase Pathways Does Not Account for the Bt_2cAMP-mediated Delay of Neutrophil Apoptosis—The phosphoinositide 3-kinase/Akt pathway plays an essential role in cell survival in various cell types (33) and may be involved in the cyclic AMP-signaling cascade. For example, it has been reported that cyclic AMP requires PI 3-kinase activation for DNA synthesis induced by insulin-like growth factor I in FRTL-5 cells (34) and is involved in the ability of cyclic AMP to attenuate chemoattractant-induced respiratory burst in neutrophils (35). Therefore, we examined whether PI 3-kinase is involved in the signaling pathway mediating the protective effect of cyclic AMP on neutrophil survival. Cells were preincubated with the specific PI 3-kinase inhibitor LY294002 (36) prior to exposing them to Bt_2cAMP or GM-CSF (Fig. 6). We found that the PI 3-kinase inhibitor suppressed GM-CSF-mediated delay of neutrophil apoptosis, which has been reported previously (37), yet had no effect on suppression of apoptosis by Bt_2cAMP.

We also investigated whether cyclic AMP could be acting through the MAPK signaling pathway to delay neutrophil apoptosis. Activation of extracellular signal-regulated kinase has been implicated in a number of systems to contribute as a negative regulator of apoptosis (38, 39). Elevation of cyclic AMP levels is also known to either inhibit or activate MAPK in a cell type- and stimulus-specific manner (40, 41). The protective effect of cAMP-elevating agents does not appear to act through the MAPK pathway in our system because the p42/p44 MAPK kinase inhibitor PD98059 had no effect on the anti-apoptotic functions of cyclic AMP in neutrophils, yet reversed the anti-apoptotic functions of GM-CSF treatment in neutrophils (Fig. 6). Similarly the p38 MAPK inhibitor SB203580 (42) did not reverse Bt_2cAMP-mediated delay of neutrophil apoptosis (data not shown).

Retardation of Neutrophil Apoptosis by Bt_2cAMP Does Not Require New Protein Synthesis—Our results suggest Bt_2cAMP suppresses neutrophil apoptosis via a previously uncharacterized signaling mechanism. We therefore determined whether Bt_2cAMP stimulated a novel signaling pathway that would require transcriptional activation to suppress neutrophil apoptosis.

To block protein synthesis cycloheximide was titrated to low concentrations to minimize the induction of neutrophil apoptosis that has been reported by this compound (43). Apoptosis was assessed by morphology and annexin V binding following overnight culture of neutrophils with Bt_2cAMP and cycloheximide (Fig. 7). Cycloheximide failed to reverse the suppression of apoptosis by cyclic AMP. It did however block glucocorticoid-mediated suppression of neutrophil apoptosis at these concentrations (Ref. 44 and data not shown), suggesting that gene transcription is not necessary for the suppression of neutrophil apoptosis by Bt_2cAMP.

We next examined the possibility that Bt_2cAMP might activate a rapid and reversible signaling pathway rather than stimulate new protein synthesis, which would occur over several hours. Cells were cultured in the presence of Bt_2cAMP for the time points indicated before Bt_2cAMP was removed from culture by gently washing in PBS and then returned to normal culture conditions. Bt_2cAMP was required to be continually present in culture to suppress neutrophil apoptosis (Fig. 8*A*). However, Bt_2cAMP rescued neutrophils from apoptosis when added at later time points (Fig. 8*B*). We are investigating whether the effects of cyclic AMP occur when the rate of apoptosis is high (between 8 and 20 h). However, during constitutive apoptosis, cells at different stages of the apoptotic program are present in the population at any one time point. We are currently investigating whether synchronous apoptosis triggered by Fas ligation, tumor necrosis factor-α (26), or temperature shift (15) can be modulated by cyclic AMP. Taken together, our results suggest that Bt_2cAMP exerts a powerful direct signaling mechanism, independent of new protein synthesis, to suppress neutrophil apoptosis, and this suppression is rapidly lost when Bt_2cAMP is removed from culture.

Proteasome Inhibitors Are Able to Reverse Retardation of Neutrophil Apoptosis by Bt_2cAMP—The ubiquitin/proteasome system plays an important role in the degradation of cellular proteins that regulate various cellular processes, including apoptosis. The observations above reveal Bt_2cAMP delays neutrophil apoptosis independently of new protein synthesis, suggesting that Bt_2cAMP is unlikely to stimulate the production of a survival protein. Thus we examined whether alternatively Bt_2cAMP was accelerating the degradation or modification of pro-death proteins within the neutrophil to increase survival. Neutrophils were co-incubated with Bt_2cAMP and the irreversible proteasome inhibitors lactacystin (10 μM) (45) and epoxomicin (10 μM) (46) for 20 h (Fig. 9, *A* and *B*). Both proteasome inhibitors eliminated the delay of neutrophil apoptosis induced by Bt_2cAMP.

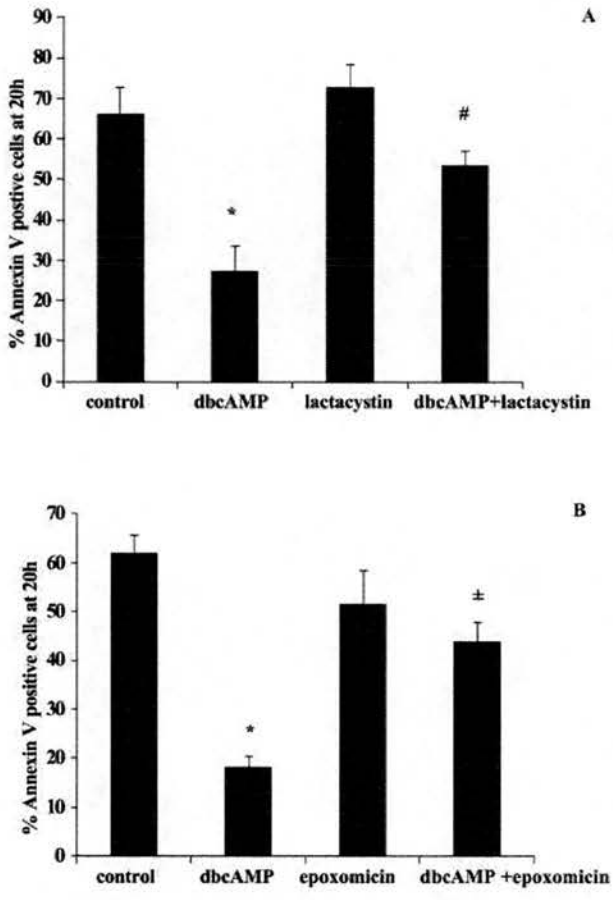

FIG. 9. **Effects of proteasome inhibitors on Bt_2cAMP (db-cAMP)-mediated delay of neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured in Iscove's DMEM containing 10% autologous serum at 37 °C and treated with lactacystin (10 μM, *A*) or epoxomicin (10 μM, *B*) with or without Bt_2cAMP (0.2 mM). After 20 h in culture, the cells were incubated with FITC-labeled recombinant human annexin V to determine phosphatidylserine expression. The cells were then analyzed by flow cytometry using a FACSCalibur and associated CellQuest software. Data from a minimum of 5000 cells were analyzed for each condition. All values represent mean ± S.E. of $n = 3$ experiments, each performed in duplicate where significant difference from control is represented by $*p < 0.001$. Significant difference from Bt_2cAMP alone represented by $\#p < 0.001$ or $\pm p < 0.01$.

DISCUSSION

Human neutrophils undergo apoptosis, a process that is centrally important in the resolution of inflammation. It has been shown previously that cyclic AMP is an important regulator of neutrophil apoptosis (18–20), yet little is known of the signaling mechanism by which by cyclic AMP controls neutrophil cell death. The studies herein have established that cyclic AMP acts upstream of caspase-3 activation to inhibit the apoptotic pathway in neutrophils. For the first time, it was also demonstrated that neutrophils contain a small but significant number of mitochondria, which exhibit a loss of membrane potential during constitutive apoptosis, which can be delayed by cyclic AMP elevation. We are currently investigating whether loss of mitochondrial membrane potential occurs before other indices of apoptosis in neutrophils, such as phosphatidylserine exposure and nuclear condensation. This would help ascertain whether loss of mitochondrial potential during neutrophil apoptosis, shown to trigger apoptosis in other cell types, has a similar function in neutrophils and whether Bt_2cAMP can directly affect loss of mitochondrial potential to delay neutrophil apoptosis.

It has been suggested that PKA plays an important role in cyclic AMP-mediated delay of neutrophil apoptosis (18–20). It is known that cyclic AMP analogs, which selectively activate type I PKA, attenuate neutrophil apoptosis, compared with analogs that preferentially activate type II PKA suggesting that that type I PKA is necessary and sufficient to mediate the cyclic AMP-induced delay in human neutrophil apoptosis (19). We suggest, alternatively, that PKA activation by cyclic AMP is not responsible for the major apoptosis-retarding influences of cyclic AMP in neutrophils. Indeed, we have demonstrated directly that cyclic AMP elevation in neutrophils stimulates an

increase in PKA activity, which is blocked by pharmacological inhibitors. Importantly, however, blockade of PKA was not sufficient to reverse the anti-apoptotic effect of cyclic AMP, implying that this molecule has little or no role in the cyclic AMP signaling pathway responsible for delay of neutrophil apoptosis.

Previous publications (18, 19) have implicated a role for PKA in cyclic AMP regulation of neutrophil apoptosis using concentrations of H89 greater than 10 μM. The specificity of H89 at these concentrations is questionable, and it has been published (47) that H89 may inhibit several other kinases, some with potency similar to or greater than that for PKA. We propose that failure to directly measure PKA activity together with the use of high and possibly nonspecific concentrations of H89 could have led to misinterpretation of previous data. We have demonstrated that 10 μM H89 is sufficient to block PKA activity for extended culture periods and is active in the presence of autologous serum. The failure therefore of both H89 and (R_p)-8-Br-cAMPS, a highly specific inhibitor of PKA, to reverse cyclic AMP-mediated delay of neutrophil apoptosis points to a novel signaling pathway used by cyclic AMP to inhibit neutrophil apoptosis, which is independent of PKA activation.

There have been a few studies reporting PKA-independent effects of cyclic AMP; however, little has been elucidated of the alternative signaling pathways downstream of cyclic AMP. Pharmacological blockade of the MAPK and PI 3-kinase signaling cascades in this study suggest that neither of these signaling pathways are likely to be important in the cyclic AMP-mediated delay of neutrophil apoptosis. There has been interest in the discovery that cyclic AMP can bind specifically to and activate small guanine nucleotide exchange factors which, when bound by cyclic AMP, activate the small Ras-like GTPase, Rap1 (48, 49). The biological function of Rap1 is still unclear, but it has been proposed that activation of this small GTPase may feed into MAPK signaling pathways (50). As an approach to establishing if Rap1 has a role in cyclic AMP-mediated delay of neutrophil apoptosis, we have blocked Rap1 activity using the *Clostridium sordellii* lethal toxin, which has been reported to inhibit specifically the small GTPases Rap1, Ras, and Rac (51). Furthermore, we have tested GGTI-286, a geranylgeranyltransferase inhibitor, which blocks geranylgeranylation required by Rap1 to achieve its mature, biologically active form (52). Thus our preliminary experiments suggest that Rap1 is not involved in cyclic AMP-mediated delay of neutrophil apoptosis (data not shown): however, this area of research is still under investigation. Our studies are in accord with a very recent publication that demonstrates that cyclic AMP-dependent inhibition of interleukin-5 from human T lymphocytes is not mediated by PKA or by the Rap1 signaling pathway (53).

Regulation of neutrophil apoptosis is thought to depend on the balance between pro-apoptotic and anti-apoptotic death factors expressed in the cell (17, 54). Neutrophils contain death regulator proteins, including Bax and Bad, and also express some members of the anti-apoptotic family such as Mcl-1 and Bcl-x_L but not Bcl-2 (17, 54). It has been proposed that neutrophil longevity may be prolonged by the synthesis of anti-apoptotic proteins such as Mcl-1 (55). However, it is unlikely that cyclic AMP effects are mediated by such a mechanism in the retardation of neutrophil apoptosis since we have demonstrated that cyclic AMP-mediated delay of neutrophil apoptosis does not require gene transcription. Furthermore, "wash out" experiments have revealed that retardation of neutrophil apoptosis is rapidly lost when Bt_2cAMP is removed from culture, even after incubation periods that should permit new protein synthesis.

Together, these data suggest a mechanism whereby cyclic AMP does not stimulate production of a survival protein but may alternatively induce post-transitional modifications in the neutrophil to promote survival. One potential mechanism for cyclic AMP-mediated retardation of neutrophil apoptosis may involve cyclic AMP specifically targeting a death protein(s) to the proteasome for degradation. We have demonstrated that blockade of proteasome activity results in a dramatic loss of the pro-survival effect of cyclic AMP. We speculate that cyclic AMP may be involved in the post-translational modification of a death protein, which targets the neutrophil proteasome. If cyclic AMP stimulation is removed or proteasome activity is blocked, then the accumulation of a death protein(s) would be predicted to permit the constitutive death pathway of neutrophils to be reconstituted. Further characterization of proteasome activity in this signaling pathway and possible death protein targets of cyclic AMP are currently under investigation.

In conclusion, cyclic AMP delays neutrophil apoptosis via a novel, reversible, and transcriptionally independent mechanism. Our results contest the dogma that cyclic AMP exerts is physiological functions almost entirely through activation of PKA, and we are currently investigating the involvement of novel downstream signaling pathways in cyclic AMP regulation of neutrophil cell death.

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