

Induction of epidermal growth factor receptor family and mucin expression by eosinophil granule proteins in epithelial cells.

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**Induction of Epidermal Growth Factor Receptor
Family and Mucin Expression by
Eosinophil Granule Proteins in Epithelial Cells**

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A thesis submitted to the National University of Ireland (RCSI) for the
degree of Doctor of Medicine

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June 2010

Declaration

I declare that this thesis, which I submit to the RCSI for examination in consideration of the award of a higher degree MD, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme, this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed: _____

Student Number: 8512728

Date: 15th June 2010

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ABSTRACT

Objectives

To determine the effect of the eosinophil cationic granule proteins Major Basic Protein (MBP) and Eosinophil Peroxidase (EPO) on expression of the epidermal growth factor receptors (EGFR) HER1 and HER2 and consequently mucin gene expression in bronchial epithelial cells.

Eosinophil accumulation and degranulation at local inflammatory sites is a feature of asthma, allergic rhinitis and nasal polyposis. Eosinophils and their granule proteins have been implicated in cell and tissue remodelling and have been shown to communicate with other cell types at inflammatory sites, including epithelial cells and nerve cells. The HER family of receptors, including HER1 and HER2 are activated by EGF ligands. They are upregulated in rhinosinusitis and nasal polyposis and in several human cancers. Activation of HER receptors leads to the production of mucin. We hypothesize that eosinophil cationic granule proteins including MBP and EPO will change the levels of expression and activation of the HER family and consequently of mucin genes.

Materials and Methods

Immortalised Human Bronchial Epithelial Cell line (16HBE14o) was used to investigate the above hypothesis. Cells were exposed in a concentration of 500,000 cells per well, in culture on a 6-well plate to eosinophil granule proteins including MBP/EPO. MBP concentration was 1µg/ml equivalent to 0.37µl per well while EPO concentration was 4µg/ml equivalent to 0.94µl per well. The cells were then harvested at various time points, as previous work suggests that exposure of 1, 4, 18, 24 hours are most useful. Cells were examined for measurable changes in gene and protein expression.

From cultured 16HBE14o cell line, extraction of:

1. RNA by lysing the culture cells using Tri reagent (Sigma) in accordance with manufacturer's instructions. This was followed by exposure to chloroform, isopropanol, ethanol and centrifuging to isolate the RNA as a supernatant for use in preparation of cDNA, which was used for PCR of:
 - (a) Epidermal growth factor receptors HER1 and HER2
 - (b) MUC1, MUC4
2. Cultured 16HBE14o cells were exposed to different inhibitors (Integrin β 1, GM6001 and DPI) in an attempt to understand the signalling pathway of EPO in inducing changes in the gene and protein expression of HER1 and HER2 as well as MUC4 and MUC1.

Results

The eosinophil granule protein EPO induces the expressional upregulation of HER2 in 6HBE14o cells. EPO also induces the expression of MUC4, an endogenous tethered ligand of HER2, in 6HBE14o cells. By contrast, eosinophil MBP reduces MUC4 and does not change HER2 expression.

Conclusion

This is the first study to address EGFR and mucin gene expression under the effect of EPO in epithelial cells. This study suggests a previously unsuspected mechanism whereby EPO could contribute to remodelling, which is a feature of nasal polyposis and asthma. The findings of this study that eosinophil cationic granule protein (EPO) induces transcriptional upregulation in the expression of epidermal growth factor receptor HER2 and MUC4 offers a therapeutic possibility to the problem of mucus hypersecretion in conditions such as nasal polyposis. Prior to this work, findings of EGFR and mucin gene expression in response to EPO were obscure.

Currently, further work is ongoing in the lab to demonstrate increased protein levels along with the mRNA described in this thesis. Understanding the mechanism of mucin expression under the influence of inflammatory mediators may shed more light on the control points of mucin and could provide a new target for therapeutic intervention. Long term, it may be possible to test specific inhibitors of EPO in clinical studies.

ABBREVIATIONS

CCAAT:	Enhancer-Binding Proteins (or C/EBPs)
CCR3:	Chemokine (C-C motif) Receptor 3
CD 11b:	Integrin Alpha M
CD 18:	Integrin Beta 2
CD95 (fas):	FAS Receptor
CD95L (FASL):	Transmembrane of Tumour Necrosis Family
cDNA:	Complementary DNA
COPD:	Chronic Obstructive Pulmonary Disease
ECP:	Eosinophil Cationic Protein
EDN:	Eosinophil Derived Neurotoxin
EGF:	Epidermal Growth Factor
EGFR:	Epidermal Growth Factor Receptor
EoSVs:	Eosinophil Sombrero Vesicles
EPO:	Eosinophil Peroxidase
ESS:	Endoscopic Sinus Surgery
GATA:	Transcription Family
GM-CSF:	Granulocyte-Macrophage Colony-Stimulating Factor
GPCR:	G-Protein Coupled Receptor
HER1, ErbB1:	Human Epidermal Growth Factor Receptor
HER2, ErbB2:	Human Epidermal Growth Factor Receptor 2
IFN- γ :	Interferon Gamma
IL1:	Interleukin 1
IL2:	Interleukin 2
IL3:	Interleukin 3
IL4:	Interleukin 4
IL5:	Interleukin 5

IL 10:	Interleukin 10
IL12:	Interleukin 12
LTC4:	Leukotriene C4
MMPs:	Matrix Metalloproteinases
MUC1:	Mucin 1
MUC4:	Mucin 4
MUC5AC:	Mucin 5-AC
NADP Oxidase:	Nicotinamide Adenine Dinucleotide Phosphate
NP:	Nasal Polyps
PCR:	Polymerase Chain Reaction
PU.1:	Proviral Integration Oncogene spi1
RANTES:	Chemokine (C-C motif) Ligand 5
RNA:	Ribonucleic Acid
ROS:	Reactive Oxygen Species
RSV:	Rous Sarcoma Virus
RT:	Room Temperature
TNF α :	Tumour Necrosis Factor Alpha
VCAM-1:	Vascular Cell Adhesion Molecule-1
VEGF:	Vascular Endothelial Growth Factor
VLA4:	Integrin Receptor, Very Late Antigen-4

CHAPTER 1

INTRODUCTION

1.1 THE EOSINOPHIL

Eosinophils are granulocytic leukocytes notably associated with allergic conditions, anthelmintic host defence and immunoregulatory responses. Eosinophils contain cationic proteins stored within their cytoplasmic granules, including eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN), which are capable of inducing tissue damage and dysfunction (Blanchard and Rothenberg 2009).

Eosinophils comprise less than 5% of the circulating white cell population in healthy individuals and are easily distinguished by their bi-lobed appearance and the characteristic granules that occupy up to one fifth of the cytoplasmic content (Kariyawasam and Robinson 2007). Eosinophils are about 12-17µm in size and can easily be identified because of their strong affinity to the acidic dye eosin in blood and tissues even when they are paraformaldehyde fixed and paraffin embedded (Simon et al. 2004). Eosinophils are released from the bone marrow into the peripheral blood, where they circulate only a few hours before they enter the tissues, where they can survive for at least two weeks (Simon et al. 1997, Simon and Simon 2007, Blanchard and Rothenberg 2009).

1.2 **EOSINOPHIL PRODUCTION**

Eosinophils are generated from pluripotential hematopoietic stem cells in the bone marrow via a process that is highly regulated by transcription factors and cytokines (Tenen et al. 1997). Eosinophil lineage development is dictated by the interaction of three classes of transcription factors including GATA-1 (a zinc family finger member), PU.1 (an ets family member), and C/EBP members (CCAAT/enhancer-binding protein family) (McNaghy and Graf 2002). Although these transcription factors are expressed in a variety of hematopoietic lineages, their mechanism of action in eosinophils is distinctive as GATA-1 and PU.1 antagonize each other in most cell types but have synergistic activity in regulating eosinophil lineage specification and eosinophil granule protein transcription (Du et al. 2002).

1.3 **THE PHYSIOLOGICAL FUNCTIONS OF EOSINOPHILS**

A) **The “Respiratory Burst” and Extracellular Traps**

Eosinophils rapidly release mitochondrial DNA in response to exposure to bacteria, C5a or CCR3 ligands. These DNA traps also contain the granule protein ECP and MBP, and display antimicrobial activity (Yousefi et al. 2008). This suggests a unique mechanism whereby eosinophils participate in innate immunity against bacteria.

B) Cytokine Production and Release

Eosinophils have the propensity to synthesize numerous cytokines and growth factors that have implicated eosinophils in numerous homeostatic processes and inflammatory conditions.

C) Mast Cell Regulation

Eosinophils regulate mast cell functions through the release of granule protein and cytokines. MBP has been demonstrated to induce the release of histamine, PGD-2, GM-CSF, TNF α , and IL-8 from human cord mast cells in vitro (Piliponsky et al. 2002).

D) Antigen Presentation and T-cell Proliferation

In vitro experiments have demonstrated that eosinophils are able to process antigens and express costimulatory molecules and MHC class II molecules, and therefore may function as antigen-presenting cells in stimulating T-cell responses (Shi 2004).

1.4 EOSINOPHIL TRAFFICKING AND TISSUE MIGRATION

At baseline healthy conditions, eosinophils normally account for a small subset of circulating blood cells. Most eosinophils traffic into the gastrointestinal tract where they normally reside within the lamina propria of all segments except the esophagus (Mishra et al. 1999). Eosinophil trafficking from the circulating blood into peripheral tissues is a multi-step process involving rolling, tethering and firm adhesion to the vascular endothelium followed by transendothelial migration into the tissue (Ebnet et al. 1996).

After transmigration through the blood vessel, eosinophils enter the extracellular matrix where they bind to matrix proteins such as fibronectin.

1.5 **EOSINOPHIL SURVIVAL**

Eosinophils are believed to possess a limited ability to survive in tissues in the absence of survival promoting cytokines. There is increasing evidence that eosinophil numbers are regulated, not only by their production in the bone marrow, but also by the amount of programmed cell death or apoptosis (Fulkerson and Rothenberg 2008). Mediators known to inhibit eosinophil apoptosis include survival factors, e.g. IL-5, IL-3, and granulocyte-macrophage colony stimulating factor (GM-CSF) produced by neighbouring cells or by the eosinophils themselves.

Eosinophil death can be triggered not only by the absence of survival factors but also by interaction of the surface death receptors CD95 (Fas) and its ligand, CD95L (FasL), which is expressed by activated T-cells (Hebestreit et al. 1996). Therefore, T-cells, known as the main producers of eosinophil survival factors, may also limit eosinophil expansion within inflammatory sites (Simon 2000).

1.6 **EOSINOPHIL DEGRANULATION**

Upon activation, eosinophils are recruited from the circulation into inflammatory foci and release their granule-derived products (Rothenberg 2006; Gleich 2000). Specific eosinophil proteins can be released rapidly and selectively when these cells are activated. Granule-stored products are released from eosinophils through different modes, i.e. classical exocytosis, cytolysis and piecemeal degranulation. Classical exocytosis entails release of the entire contents of the granules following granule fusion with the plasma membrane, including compound exocytosis, which also involves intracellular granule-granule fusion before extracellular release. Cytolysis involves the extracellular deposition of intact granules upon lysis of the cell and is an important mechanism for allergen-induced granule content release in the upper airways (Moqbel 2006; Erjefalt 1999). Piecemeal degranulation (PMD) is a process of secretion of the contents of intracellular granules mediated by transport vesicles. PMD is a general secretion process implicated in the release of products from activated eosinophils in a range of human diseases, for example in gastric carcinoma (Caruso et al. 2005). During PMD, eosinophil granules undergo progressive emptying of their contents, as secretory vesicles are mobilized to transfer secretory load from granules to the cell surface (Crivellato et al. 2003). PMD enables differential release of eosinophil products, a physiologically relevant event, which is a distinct function of eosinophils. It has now become apparent that the eosinophil secretory pathway is mediated by morphologically distinct, large

pleiomorphic vesiculotubular carriers, termed eosinophil sombrero vesicles (EoSVs), which are responsible for moving proteins between granules and the plasma membrane (Spencer et al. 2006; Melo 2005). These vesicles have been shown to facilitate piecemeal secretion of cytokines (Melo et al. 2008) and most recently of MBP (Melo et al. 2009). Thus, in contrast to previous models which would have envisaged all the eosinophil cationic granule proteins being released simultaneously and exerting purely cytotoxic effects, it is likely that individual eosinophil proteins or different sub-sets are released depending on the circumstances. Indeed, the extent of piecemeal degranulation varies widely among inflammatory conditions, being extensive in nasal allergy conditions such as polyposis and less marked in asthma and inflammatory bowel disease including allergic inflammation (Erjefalt et al. 2001).

1.7 **THE EOSINOPHIL CATIONIC GRANULE PROTEINS**

A) *Eosinophil Cationic Protein (ECP)*

ECP is a small, basic protein found in the matrix of the eosinophil secondary granules. It exhibits cytotoxic, helminthotoxic and ribonuclease activity. Eosinophils store abundant amounts of ECP and may release it upon repetitive stimulation with the same agonist, implying that mature eosinophils do not require significant *de novo* ECP synthesis for secretion (Simon et al. 2000).

B) Major Basic Protein (MBP)

MBP is expressed as two different homologues (MBP-1 and MBP-2) derived from two separate genes. MBP-1 is a small protein that consists of 117 amino acids, with a molecular weight of 13.8 kDa, and a high isoelectric point (Hamann et al. 1991). All of the MBP-1 is stored in crystalloid granules and is synthesized during early eosinophil development prior to maturation. Mature eosinophils lose the ability to synthesize MBP1 (Popken-Harris et al. 1998; Voehringer et al. 2007).

MBP2 is exclusively expressed by eosinophils and may be a more specific marker for elevated eosinophils in patients with eosinophilia than MBP1 (Plager et al. 2006). MBP is highly cytotoxic (Gleich et al. 1979) and plays an essential role in host defence against helminth infection. Because of its cationic nature, it affects the charge of surface membranes resulting in disturbed permeability, disruption and injury of cell membranes with perturbation of the cell surface lipid bilayer (Kroegel et al. 1987; Wasmoen et al. 1988).

MBP has also been shown to be cytotoxic to airways and may be responsible for tissue damage associated with eosinophil infiltration in bronchial mucosa in asthma (Frigas et al. 1980; Furuta et al. 2005; Hisamatsu et al. 1990). At low concentrations, MBP stimulates mediator production by other inflammatory cells.

C) *Eosinophil-derived Neurotoxin (EDN)*

EDN is an eosinophil granule-derived secretory protein with ribonuclease and antiviral activity. EDN has been demonstrated to induce recruitment, migration, maturation and cytokine release by dendritic cells (Yang et al. 2003).

D) *Eosinophil Peroxidase (EPO)*

EPO is localized in the matrix of secondary eosinophil cytoplasmic granules and is composed of two subunits, a heavy chain of 50–57 kDa and a light chain of 11–15 kDa. EPO constitutes 25% of the total protein mass of specific granules. EPO has peroxidase activity and has been shown to catalyze the oxidation of halides, pseudohalides and nitric oxide to form highly reactive oxygen species (hypohalous acids), reactive nitrogen metabolites (nitric dioxide), and peroxynitrate-like oxidants. This action promotes oxidative stress and target cell apoptosis (Agosti et al. 1987; MacPherson et al. 2001; Wu et al. 1999).

1.8 **THE ROLE OF EOSINOPHILS IN NASAL POLYPOSIS**

Nasal polyposis (NP) is a chronic inflammatory airway disease of the nasal and paranasal sinus mucosa. The pathogenesis of NP is multifactorial and NP frequently coexists with asthma and aspirin intolerance (Kramer and Rasp 1999). It had long been assumed that allergy predisposed to nasal polyps as the symptoms of rhinorrhea and mucosal swelling are present in both diseases, as well as abundant eosinophils; however, epidemiological data fails

to show consistent support for this relationship (Fokkens et al. 2007).

Tissue eosinophilia is a consistent feature in NP, and activation and enhanced survival of eosinophils may affect polyp formation and growth (Simon et al. 1997). The chronic eosinophilic inflammatory changes in NP are similar to those observed in the bronchial mucosa in asthma and are a feature of polyp tissue whether or not an allergy is present (Ediger et al. 2005). The degree of tissue eosinophilia within the sinonasal mucosa in chronic rhinosinusitis has been found to predict extensive disease, as measured by computed tomographic (CT) scan stage (Ediger et al. 2005, Bhattacharyya et al. 2001). Other data suggests that tissue eosinophilia may be predictive of success or failure of endoscopic sinus surgery (ESS) (Elwany et al. 2002). Activated eosinophils are known to secrete eosinophilic cationic protein (ECP), which plays a role in the inflammatory alteration of the mucosa. ECP is a well-described and standardized marker of tissue eosinophilia. ECP is strongly implicated in the pathophysiology of upper airway inflammation observed in several conditions including asthma and allergy (Niimi et al. 1998). ECP levels in nasal secretions are significantly higher than serum levels and are better related to disease activity and clinical symptoms, and should therefore be preferred over ECP serum measurements (Rasp et al. 1994). Elevated levels of ECP in nasal secretions of patients with NP have been documented in several studies (Di Lorenzo et al. 2001, Kramer et al. 2004). Histological studies of NP have revealed that there are significantly more activated eosinophils in polyp tissue compared with nasal mucosa. Activated

eosinophils release inflammatory substances such as the major basic protein and eosinophil peroxidase, which damage nasal and sinus mucosa, leading to oedema and inflammation. In addition to these inflammatory substances, the activated eosinophils found in polyp tissue were shown to produce a number of inflammatory cytokines including GM-CSF, IL-3 and IL-5. These cytokines are also potent inflammatory mediators and are thought to contribute to the significant mucosal inflammation seen in NP (Bachert et al. 2003). Eosinophils are pivotal in the ongoing inflammation and tissue damage of NP. Nasal polyposis is frequently associated with asthma and this relationship may imply a shared pathophysiology. The clinical relationship of NP to asthma or aspirin intolerance has been well described (Alobid et al. 2005).

Patients with asthma or aspirin intolerance have more extensive disease involvement of the sinuses and a higher recurrence rate from surgical treatments for NP than those without asthma or aspirin intolerance (Alobid et al. 2005, Amar et al. 2000). Activated eosinophils within the nasal mucosa and nasal polyp in the patients with NP were more prominent in patients with asthma than in patients without asthma (Ediger et al. 2005, Haruna et al. 2004). Bilateral NP is particularly common in patients with aspirin intolerant asthma, in whom cyclooxygenase inhibitors can trigger activation of eosinophils and mast cells (Kramer and Rasp 1999). More extensive eosinophilic infiltration and elevated ECP levels have been reported in the polyp tissues of aspirin intolerant asthma patients compared with aspirin

tolerant asthma patients (Suh et al. 2004). However, little is known concerning the relationship between ECP levels in nasal secretions, the disease severity of NP and recurrence after ESS. It has recently been shown that ECP levels in nasal secretions of patients with NP correlated with the presence of asthma or aspirin intolerance and disease severity determined by CT score. ECP levels in nasal secretions post-operatively declined with mucosal healing and rose in patients with recurrence of NP (Sun et al. 2009).

1.9 **THE ROLE OF EOSINOPHILS IN ASTHMA**

There is extensive literary evidence implicating blood and pulmonary tissue eosinophilia in the development of asthma. Levels of eosinophils and their products in blood, sputum, bronchoalveolar lavage fluid and bronchial biopsies have been found to correlate with disease severity (Wardlaw et al. 2000). During asthmatic inflammation, eosinophils interact with lymphocytes, mast cells, dendritic cells, macrophages and neutrophils, as well as with resident tissue cells, such as epithelial cells, endothelial cells, smooth muscle, fibroblasts and nerve cells (Jacobsen 2007).

The cytotoxic effects of the eosinophil cationic granule proteins MBP, EPO, ECP and EDN result in extensive epithelial desquamation and destruction leading to airway dysfunction (Jacobsen 2007). Histological samples from the airways of severe asthmatics, as well as post mortem samples following fatal asthma attacks have immunohistochemically localized MBP to the sites of epithelial damage. High levels of MBP have also been detected in induced

sputum samples from asthmatics (Gleich 2000). Eosinophils from asthmatic patients contain significantly higher amounts of EDN compared to healthy controls (Sedgwick 2004). ECP levels in blood, sputum and bronchoalveolar lavage fluid of patients with asthma have been reported to correlate with the degree of airway obstruction and disease activity (Venge 1999). EPO also plays a key role in mucosal damage in the airways, and excessive levels of reactive oxygen species have been produced in vitro from eosinophils obtained from the airways of asthmatic patients (Kariyawasam 2006). MBP and EPO have been shown to augment the cholinergic phenotype of airway nerves by binding to the muscarinic M₂ receptors and causing loss of negative feedback to acetylcholine release at nerve terminal resulting in vagal overstimulation and bronchoconstriction (Jacoby et al. 1993). MBP is also known to induce histamine release from mast cells and basophils, further potentiating bronchoconstriction (O'Donnell et al. 1983).

Airway remodelling in asthma is characterized by structural changes in the lungs including epithelial hypertrophy, subepithelial deposition of extracellular matrix proteins, mucus gland hypertrophy, airway smooth muscle hypertrophy and vascular changes. Many of the factors implicated in remodelling are expressed by eosinophils. These include fibroblast growth factor (FGF)-2, IL-4, IL-11, IL-13, IL-17, nerve growth factor (NGF), and vascular endothelial growth factor (VEGF).

Eosinophil granule proteins play a role in tissue remodelling. For example, MBP interacting with IL-1 and TGF- β stimulates lung fibroblasts. MBP has also been shown to inhibit neurite outgrowth in airway nerve cells in dose dependent fashion in a manner similar to in vitro studies with whole eosinophils.

Eosinophils are known to secrete metalloproteinases, especially matrix metalloproteinase (MMP-9), which has been found to be increased in severe persistent asthma and following allergen challenge.

1.10 **ANTI-EOSINOPHILIC THERAPIES IN ASTHMA**

Multifaceted approaches targeting eosinophil mediated processes in asthma and their clinical efficacy are testimonial to the integral role played by the eosinophil in the pathogenesis of this condition. These therapeutic targets are geared towards either reducing eosinophil production and migration into inflamed tissues, increasing eosinophil apoptosis, preventing eosinophil activation or blocking of eosinophil mediators.

A. Corticosteroids

Corticosteroids are widely used in the treatment of bronchial asthma and COPD. They are also the most common agents used to reduce eosinophilia (Rothenberg 1998). They increase the rate of eosinophil apoptosis by inhibiting cytokine dependent survival (Schleimer and Bochner 1994).

B. Anti-IL-5 Monoclonal Antibodies

Humanized anti-IL-5 decreases eosinophil load in humans, dramatically lowering eosinophil levels in the blood and decreases eosinophil activation and to a lesser extent eosinophil levels in the inflamed lung (Flood-Page et al. 2003; Leckie et al. 2000; Stein et al. 2008).

1.11 THE ROLE OF EOSINOPHILS IN OTHER DISEASES

A) Chronic Obstructive Pulmonary Disease

The presence and role of eosinophils in COPD is controversial (Tetley 2005). Airway eosinophilia has been reported to occur during acute exacerbations of COPD as well as in patients with stable disease (Balzano et al. 1999). Also, ECP, EPO levels are raised in the sputum and bronchoalveolar lavage fluid in patients with COPD during exacerbations (Keatings and Barnes 1997).

B) Infection

Eosinophils have been shown to play a pivotal role in the defence against helminthic infection. They have been demonstrated to mediate antibody and complement host responses to infection as well as to aggregate and degranulate in areas of infection in the vicinity of damaged parasites.

- Fungal Infection

Eosinophils release their cytotoxic granule proteins into the extracellular milieu and onto the surface of fungal organisms and kill fungi in a contact-dependent manner

- Bacterial Infection

Eosinophils release extracellular traps consisting of mitochondrial DNA and cytotoxic cationic proteins ECP and MBP in response to exposure to bacteria, C5a or CCR3 ligands.

1.12 **LAB WORK TO DATE**

Eosinophils localise to airway nerves

Eosinophils localize to specific tissue structures under the coordinated influence of specific chemoattractants and through interactions with adhesion molecules at sites of inflammation. Direct cell-to-cell contact between eosinophils and airway nerves has been demonstrated microscopically in patients with asthma. Adhesion leads to degranulation of the eosinophils and release of mediators, including MBP, EPO, EDN, ECP and leukotriene C₄, and to the generation of reactive oxygen species (ROS) within the nerve cells through an NADPH oxidase-dependent mechanism. In animal models, eosinophil MBP is associated with the development of vagally mediated hyperreactivity of cholinergic nerves; this is due to

inhibition by MBP of protective M₂ muscarinic autoreceptor activity (Costello et al. 2000).

Eosinophils induce neurite retraction in parasympathetic nerves and in the IMR32 nerve cell line. Eosinophil MBP has been shown to inhibit neurite outgrowth in a dose-dependent manner. The mechanism of this effect is unknown, however part of the action of MBP may be related to its cationic charge, since poly-L-lysine had a similar effect.

M₂ Receptor Dysfunction

The dominant innervation of airway smooth muscle is by the parasympathetic nerves of the vagus. Stimulation of these nerves causes release of acetylcholine onto M₃ muscarinic receptors to mediate bronchoconstriction, production of mucus and dilation of the bronchiolar vasculature. This mechanism is subject to feedback control by M₂ muscarinic receptors present on the parasympathetic cholinergic terminals.

Airway hyper-reactivity has been shown to result from M₂ receptor dysfunction which is prevented by inhibiting eosinophil localization to the airways. The eosinophil protein major basic protein (MBP) is an allosteric antagonist at M₂ muscarinic receptors *in vitro* (Jacoby et al. 1993). Neutralizing MBP prevents both M₂ receptor dysfunction and antigen-induced hyperreactivity *in vivo*.

Eosinophils Cause Cell Signalling

Co-culture of eosinophils with IMR32 nerve cells leads to activation of neuronal transcription factors by adhesion-dependent mechanisms. Adhesion of eosinophils to IMR-32 cells induces activation of the transcription factors NF- κ B and AP-1 both within 5 minutes of co-incubation with a return to baseline by 2 hours (Morgan et al. 2004). This is then followed by a second surge in activity between 3 to 24 hours.

The intracellular intermediates involved in NF- κ B and AP-1 activation involve the MAP kinases ERK1/2 and p38, both of which are rapidly and transiently activated in IMR32 cells in response to eosinophil adhesion (Walsh et al. 2004).

Eosinophils Protect Nerve Cells from Apoptosis

Eosinophils protect cholinergic IMR-32 nerve cells against apoptosis induced by serum deprivation or by the cytokines IL-1 β , TNF- α , and IFN- γ (Morgan et al. 2004). Co-incubation of IMR32 cells with whole eosinophils as well as eosinophil membranes prevents neuronal apoptosis. These *in vitro* findings are consistent with the *in vivo* models demonstrating increased cholinergic nerve mediated effects in conditions such as asthma and allergic rhinitis despite the presence of inflammation which might be expected to lead to an increase in nerve cell death in these tissues.

Eosinophil Cationic Proteins Cause Intracellular Signalling

The cationic eosinophil granule protein Major Basic Protein 1 (MBP1) has been demonstrated to protect IMR-32 cholinergic nerves from an apoptosis induced by serum deprivation (Morgan et al. 2004). This finding was not duplicated with Eosinophil Derived Neurotoxin (EDN). The explanation for this lies in differences in intracellular signalling induced by the two proteins. While both MBP1 and EDN activate NF κ B, they do so over different time courses. MBP1 induced a prolonged activation of NF- κ B, extending from 10 min to 12 hours and declining by 24 hours.

CHAPTER 2

2.1 **EPIDERMAL GROWTH FACTOR FAMILY**

EGFR is the prototypical member of the ErbB family, which comprises four receptors: EGFR/ErbB1/human epidermal growth factor receptor HER1; ErbB2/Neu/HER2; ErbB3/HER3 and ErbB4/HER4 (Holbro and Hynes 2004).

These are transmembrane tyrosine kinases receptors. Upon activation by their cognate ligands, ErbB receptors form dimers (either homodimers or heterodimers), leading to activation of the intrinsic kinase domain, which results in phosphorylation of specific tyrosine residues in the intracellular domain. EGFR (HER1), HER2 and HER3 are expressed in human airway epithelium. (Polosa et al. 1999, O'Donnell et al. 2004). EGFR expression was found to be weak or absent in the airway epithelium of healthy subjects (Amishima et al. 1998, Polosa et al. 2002), but it is increased in the airway epithelium of asthmatics (Polosa et al. 2002, Massion et al. 1995), smokers with normal lung function, patients with COPD (O'Donnell et al. 2004, de Boer et al. 2006), and Cystic Fibrosis (CF) patients (Voynow et al. 2005). This data suggests that EGFR activation may play more obvious roles in inflammatory states than in healthy individuals. In humans, the airway epithelium expresses EGFR ligands, including EGF, TGF- α , HB-EGF, amphiregulin, heregulin and betacellulin (de Boer et al. 2006, Polosa et al. 1999). Expression of several EGFR ligands has been investigated in diseases such as asthma, COPD and CF (Amishima et al. 1998, Fan et al. 2004). These studies showed that EGFR ligands are expressed in normal and diseased

epithelia but their expression pattern and distribution are variable. It is suggested that activation of EGFR by different ligands results in changes in the activation of different intracellular pathways, which causes different responses. These proteins are important in orchestrating the epithelial repair process via induction of epithelial migration, proliferation, differentiation and extracellular matrix synthesis. However, excessive expression of EGF-like factors or their receptors may lead to squamous metaplasia or epithelial hyperplasia (de Boer et al 2006).

2.1.1 EGFR Signalling and Mucin Production

It has long been known that some chronic airway diseases, e.g. asthma, COPD and cystic fibrosis, are associated with mucous hypersecretion. Among the major components of human mucus are large-sized glycoproteins, gel-forming mucins (Rose and Voynow 2006). Understanding of mucous hypersecretion began with the cloning of these mucin genes (Li et al. 1998, Meezaman et al. 1994). Multiple studies reported that various stimuli cause mucin production via EGFR activation in human airway epithelial cell lines, normal human bronchial epithelial cells and in animals *in vivo* (Burgel and Nadel 2004). Studies of bronchial biopsies obtained from healthy volunteers and subjects with mild-to-moderate asthma have found a positive correlation between EGFR immunoreactivity and MUC5AC mucin staining in the epithelium of healthy and asthmatic subjects, suggesting a causal relationship (Takeyama et al. 2001). Also, studies

of the expression of several members of the EGFR family of receptors in relation to mucin expression, comparing non-smokers with current smokers with or without COPD, concluded that long-term cigarette smoking is associated with enhanced expression of EGFR and mucin MUC5AC in the epithelium of proximal airways (O'Donnell et al. 2004). Another study found a positive correlation between mucin and EGFR staining in the small airway epithelium of subjects with advanced CF (Burgel et al. 2007). These studies are compatible with the hypothesis that EGFR activation is responsible for increased mucin production in airway diseases.

2.1.2 **Ligands involved in EGFR activation**

EGFR ligands including epidermal growth factor (EGF), transforming growth factor (TGF)- α , heparin-binding EGF, amphiregulin, betacellulin and epiregulin, bind to EGFR causing direct activation of the receptors (Citri and Yarden 2006). Many inflammatory cells produce these ligands, including eosinophils (Wong et al. 1990, Burgel et al 2001), neutrophils (Calafat et al. 1997), mast cells and macrophages (Calafat et al. 1997; Rappolee et al. 1988). Activated eosinophils (Burgel et al. 2001) have been shown to induce mucin synthesis in airway epithelial cells via the release of EGFR ligands.

2.2 Mucus Function

The mucus covering of the luminal surfaces of epithelial organs forms a gel that serves as a selective physical barrier between the extracellular surroundings on one side and the plasma membrane and cell interior on the other side. The most important functions of the mucus gel are protection, lubrication and transport. In the respiratory tract, mucus performs an important protective function by entrapping inhaled foreign debris and bacteria and clearing them from the airways by ciliary action. In the gastrointestinal tract, the mucus barrier prevents the epithelial cells from autodigestion. Cervical mucus protects the uterine cavity and controls the survival and penetrability of the spermatozoa. Lacrimal mucus stabilizes the preocular liquid film that prevents the corneal surface from drying. Normal mucus secretion is probably an essential element for survival; however, abnormal mucus secretion can be an irritating or life-threatening condition, as in simple common cold and cystic fibrosis (CF), respectively. Normal airway epithelium contains a small number of surface goblet cells and a moderate number of submucosal glands. However, in inflammatory airway diseases such as asthma and COPD, the surface goblet cells expand through mucous cell hyperplasia and metaplasia, while the submucosal gland frequency and size increase (Bergeron and Boulet 2006). The increase in surface goblet cells and submucosal glands in these diseases may be what causes the excessive mucus secretion seen in these disorders (Kuyper et al. 2003, Lange et al. 1990).

The thickness of the mucus layer depends on the luminal conditions and functional requirements of the secreting surface. In the respiratory tract, the optimal mucociliary clearance requires a thin mucus layer that can be transported along with the entrapped inhaled foreign particles. The gel-like properties of mucus secretions depend primarily on their content of the high molecular weight glycoproteins, named mucins. Mucins are secreted in vesicles derived from the Golgi apparatus and released by exocytosis. Mucus gel is formed on the surface of mucosa when the secreted mucins absorb water. On hydration of mucins, their volume increases by several hundred times in a few seconds. Mucus (goblet) cells in the superficial epithelium and submucosal glands (SMG) represent the cellular stores of mucins. In the lungs of healthy individuals, goblet cells represent 5% to 15% of the columnar cell population and their proportion decreases in smaller airways. In bronchioles less than 2mm diameter, goblet cells are rare. Because of extensive innervation of SMG, it appears that these glands supply the greater volume of mucin in intact tissues.

The paranasal sinuses, as a part of the respiratory tract, are lined with pseudostratified columnar ciliated epithelium with fairly evenly distributed goblet cells. In the adult, the density of goblet cells ranges from 5,700 to 11,000 cells/mm². Maxillary sinus mucosa is thinner than in the nose and contains goblet cells at a concentration of approximately 10,000 cells/mm² (Ali and Pearson 2007).

2.2.1 Mucins

Mucins are heavily glycosylated proteins that establish a selective molecular barrier at the epithelial surface and engage in signal transduction pathways that regulate morphogenesis (Hollingsworth and Swanson 2004, Hudson et al. 2001). In addition, mucins influence many cellular processes including growth, differentiation, transformation, adhesion, invasion and immune surveillance (Hollingsworth and Swanson 2004, Moniaux et al. 2004).

Historically, mucins were thought to have the sole function of protecting and lubricating epithelial surfaces. As the diversity of mucin structures became evident, a variety of functions were assigned accordingly. At present, a total of 21 genes have received the appellation MUC: *MUC1-2*, *MUC3A*, *MUC3B*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC6-13*, *MUC15-20* (Hollingsworth and Swanson 2004, Moniaux et al. 2001).

Mucins can be further grouped into two subfamilies: secreted and membrane-bound. Typically, secreted mucins are expressed exclusively by specialised epithelial cells, are secreted in the mucus and display a restricted expression pattern within the human body. As the composition of gel-forming mucins differs among tissues according to the types of mucins expressed, the rheologic properties of the mucus will be different and specific.

Because the nasal and paranasal sinus mucosa is a modified respiratory epithelium, it is reasonable to expect that a similar pattern of mucin gene expression may exist in the nose and paranasal sinuses. Using in situ hybridization, Northern blotting and immunohistochemistry, Aust (Aust et al. 1997) and Kim (Kim et al. 2000) reported MUC2 expression, in addition to MUC1, MUC4, MUC5AC, MUC5B, MUC7 and MUC8, in the normal nasal mucosa. Immunohistochemistry has shown abundant MUC5B staining in submucosal mucus glands and epithelial goblet cells in normal human nasal mucosa, whereas MUC5AC was identified in surface epithelium goblet cells only.

2.2.2 **MUC1**

MUC1 can mediate signal transduction responses through Ras, β -catenin, and p53. Signalling through these pathways may affect the apoptotic state of the cell, which may explain the greater expression of MUC1 in neoplastic cells (Singh and Hollingsworth 2006). MUC1 is regulated by inflammatory mediators in noncancerous airway cells.

2.2.3 **MUC4**

MUC4, on the other hand, is one of the largest human membrane-anchored mucins identified to date.

A: MUC4 Structural Features

MUC4 is a high-molecular-weight glycoprotein with multi-domain organization (Moniaux et al 1999). The deduced full-length amino acid sequence of the MUC4 apoprotein shows the presence of a leader peptide, a serine and threonine rich nontandem repeat region, central large tandem repeat domain containing 16-amino acid repetitive units, regions rich in potential N-glycosylation sites, two cysteine-rich domains, a putative GDPH proteolytic cleavage site, three epidermal growth factor-like domains, a hydrophobic transmembrane domain and a short cytoplasmic tail. MUC4 mucin shares many structural similarities with the sialomucin complex (SMC/ratMUC4), which has previously been shown to facilitate tumor progression by multiple mechanisms (Moniaux et al. 2004, Moniaux et al. 1999, Komatsu et al 2000). MUC4 possesses two subunits: an extracellular mucin-like subunit, MUC4 α , and a growth factor-like transmembrane subunit, MUC4 β , containing three epidermal growth factor-like domains (Moniaux et al. 2004). The SMC/MUC4 is known to act as an intramembrane ligand for the receptor tyrosine kinase ErbB2/HER2/neu, inducing its limited phosphorylation via one of the epidermal growth factor domains (Price-Schiavi et al. 2005, Carraway et al. 2003, Carraway et al. 2000). The

EGF-like domains present in MUC4 contain conserved cysteine residues. These domains are present in a variety of extracellular proteins (Hsuan et al. 1989, McInnes and Sykes 1997). These cysteine-rich domains serve in homodimerization or oligomerization of the MUC4 mucin with itself as well as with other members of the mucin family. MUC4 has a short cytoplasmic tail of 22 amino acids that contains one tyrosine and three serine residues as potential phosphorylation sites and hence may participate in signal transduction events.

B: MUC4 Expression in Different Tissues

MUC4 is present on the surface epithelium of the eye, vagina, ectocervix, trachea and salivary gland, providing lubrication and protection to the ducts and lumen (Gipson 2001, Juusola and Ballantyne 2005). The expression of both the transmembrane and secretory forms of MUC4 has been described in a variety of epithelial cells (Buisine et al. 1999, Liu et al. 2002). The transmembrane forms of the MUC4 mucin are hypothesized to provide localized protection to the cell surface while the secretory forms are thought to be implicated in lubrication and provide protection to the luminal surfaces by trapping foreign particles and pathogens. In the lungs, MUC4 is the first mucin to be expressed, even before organogenesis takes place (Buisine et al. 1999). It is expressed

6.5 weeks post gestation followed by MUC1 and MUC2 after 9.5 weeks of gestation. Between weeks 8 and 12, MUC4 mRNA is present only in the trachea; however, its expression increases progressively in small bronchi and bronchioles after week 12 (Lopez-Ferrer et al. 2001).

C: MUC4 Expression in Inflammatory Diseases and Malignancy

An abnormal expression of MUC4 has been reported in various inflammatory diseases and cancers. Changes in MUC4 expression level have been reported in inflammatory diseases of the airways such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) in humans (Lamblin et al. 2001, Leikauf et al. 2002). MUC4 expression has also been reported in major and minor salivary gland mucoepidermoid carcinoma (Handra-Luca et al. 2005). High-grade salivary gland tumours have a trend for reduced MUC4 expression in comparison to the low-grade and intermediate-grade tumours. Also, MUC4-expressing salivary gland mucoepidermoid tumours are associated with improved patient survival and a longer disease free interval as compared with patients whose tumors were diagnosed negative for MUC4 expression (Alos et al. 2005, Weed et al. 2004). Recent studies have shown that MUC4 interacts with HER2 and modulates its expression and it is important to understand the

biological significance of MUC4-HER2 interaction (Chaturvedi et al. 2008). Recent studies concluded that the MUC4 gene is an important mucin gene expressed in nasal polyposis (Bai et al. 2007, Ali et al. 2007).

2.3 **Cell Adhesion and Integrins**

2.3.1 **Cell Adhesion**

Cell adhesion is crucial for the assembly of individual cells into the three-dimensional tissues of animals. A variety of cell adhesion mechanisms are responsible for assembling cells together. Cell adhesions are multiprotein complexes made up of three general classes of protein; cell adhesion molecules/adhesion receptors, extracellular matrix (ECM) proteins and cytoplasmic plaque/peripheral membrane proteins (Gumbiner 1996).

2.3.2 **Integrins**

Integrins are one of the major families of cell adhesion receptors. They are heterodimers of noncovalently associated α and β subunits, each of which is a singlepass type I transmembrane protein (Humphries et al. 2006, Hynes 2002). Integrin activation is a widespread phenomenon that is important in many cell types, in which it regulates matrix remodelling, angiogenesis, tissue formation and cell migration (Calderwood 2004). Integrin activation is

controlled by intracellular signals which, through their action on integrin cytoplasmic domains, induce conformational changes in integrin extracellular domains resulting in increased affinity for the ligand.

These heterodimeric receptors bridge the cytoplasmic actin cytoskeleton with proteins present in the extracellular matrix and/or on adjacent cells. The clustering of integrins on a cell surface leads to the formation of focal contacts. Interactions between integrins and the extracellular matrix lead to activation of signal transduction pathways and regulation of gene expression. Talin is a final common step in integrin activation pathways (Calderwood 2004). Integrin receptors are involved in the regulation of a variety of important biological functions, including embryonic development, wound repair, hemostasis and prevention of programmed cell death.

2.3.3 Inhibitors of Integrin Activation

Several proteins can inhibit integrin activation by competing with Talin for binding to the β -integrin tail.

2.3.4 Integrin β 1 (CD 29)

Integrin β 1, also known as CD29, is a 130kDa transmembrane glycoprotein associated with a very late antigen receptor that forms noncovalent complexes with various integrin alpha subunits to form the functional receptors that bind to specific extracellular matrix

proteins. The family of beta1 integrins includes receptors for vascular cell adhesion molecule-1 (VCAM-1), extracellular matrix (ECM) components such as collagen (COL), fibronectin (FN), laminin (LM) and vitronectin (VN).

2.3.5 Antibody against Integrin β 1 (CD29)

Monoclonal antibody reacts with integrin β 1 causing changes in the affinity of $\alpha\beta$ integrin heterodimers and their capability of mediating a variety of cellular responses including adhesion, trafficking, proliferation and differentiation.

2.4 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) have been implicated in the transactivation of the epidermal growth factor receptor (EGFR) induced by G-protein coupled receptor (GPCR) agonists (Santiskulvong and Rozengurt 2003). The broad-spectrum matrix metalloprotease inhibitor galardin (GM6001, Ilomastat) is a hydroxamic acid originally synthesized as an inhibitor of human skin collagenase (Grobelny et al. 1992) and has been shown to block MMP-1, -2, -3, and -9 (Agren et al. 2001, Hao et al. 1999). It has been shown that MMP products are necessary for Src-mediated phosphorylation of EGFR at Tyr-845. Sustained MMP activity is required for GPCR-induced DNA synthesis and MMPs also generate the sustained release and build-up of EGFR ligands (Santiskulvong and Rozengurt 2003).

2.4.1 Galardin (GM6001)

Also known as GM6001, N-[(2R)-2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan Methylamide. A potent, cell-permeable, broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases (MMPs); prevents the release of TNF- α *in vivo* and *in vitro* and abrogates endotoxin-induced lethality in mice.

2.5 Reactive Oxygen Species (ROS)

Aerobic organisms, which derive their energy from the reduction of oxygen, are susceptible to the damaging actions of the small amounts of $\bullet\text{O}_2^-$, $\bullet\text{OH}$ and H_2O_2 that inevitably form during the metabolism of oxygen, particularly in the reduction of oxygen by the electron transfer system of mitochondria. These species, together with unstable intermediates in the peroxidation of lipids, are referred to as Reactive Oxygen Species (ROS) (Mugesh et al. 2001). H_2O_2 is converted in a spontaneous reaction catalyzed by Fe^{2+} (Fenton reaction) to the highly reactive hydroxyl radical ($\bullet\text{OH}$). The hydroxyl radical reacts instantaneously with any biological molecule (RH) from which it can abstract a hydrogen atom, $\text{OH}\bullet + \text{R-H} \rightarrow \text{H}_2\text{O} + \text{R}\bullet$. The resulting free radical ($\text{R}\bullet$) is more stable and hence longer-lived than the hydroxyl radical.

ROS modify the structure and function of proteins and individual nucleotide bases. Single-strand breaks and cross-linking are the typical effects of

reactive oxygen species on nucleic acids. Alzheimer's and Parkinson's diseases are some of the disorders linked to damage from ROS as a result of an imbalance between radical-generating and radical-scavenging systems - a condition called *oxidative stress*.

2.5.1 NADPH Oxidase (nicotinamide adenine dinucleotide phosphate-oxidase)

NADPH Oxidase is a membrane-bound enzyme complex which catalyzes the production of superoxide. The importance of the NADPH oxidase is illustrated by the inherited condition Chronic Granulomatous Disease (CGD) in which a component of NADPH oxidase is absent or defective. The production of ROS in the body is controlled by several antioxidative mechanisms. The first mechanism is at the level of production itself thus inhibition of NADPH oxidase represents an attractive therapeutic target for the treatment of many diseases (Curtiss 2009).

2.5.2 Diphenyleneiodonium (DPI)

Diphenyleneiodonium (DPI) is widely used as an uncompetitive inhibitor of flavoenzymes. Firstly identified as a hypoglycemic agent able to block gluconeogenesis in rat liver (Holland et al. 1973), DPI has been subsequently shown to inhibit the activity of NADPH oxidase (Cross and Jones 1986, Doussiere and Vignais 1992).

2.6 **Hypothesis**

Our data suggests that eosinophil granule proteins upregulate and engage the EGF receptor family in IMR32 neuroblastoma cells. In this study, we will further investigate the effects of eosinophil granule proteins on EGF receptor family expression and signalling in epithelial cells.

We hypothesise that eosinophil cationic granule proteins such as major basic protein (MBP) and eosinophil peroxidase (EPO) in the proximity of bronchial epithelial cells lead to increased expression of epidermal growth factor receptor family members, HER1 and HER2, which may lead to changes in mucin gene expression.

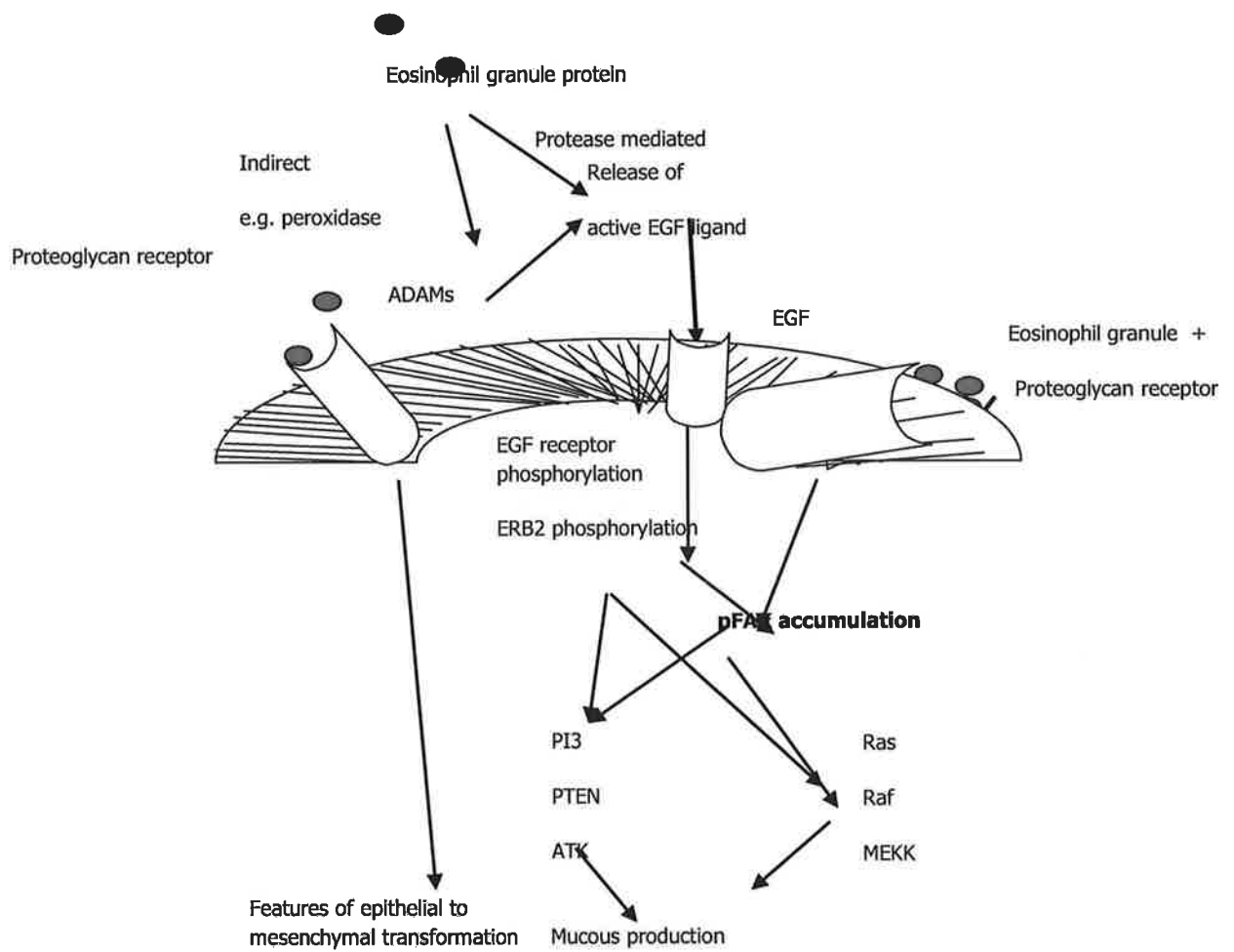
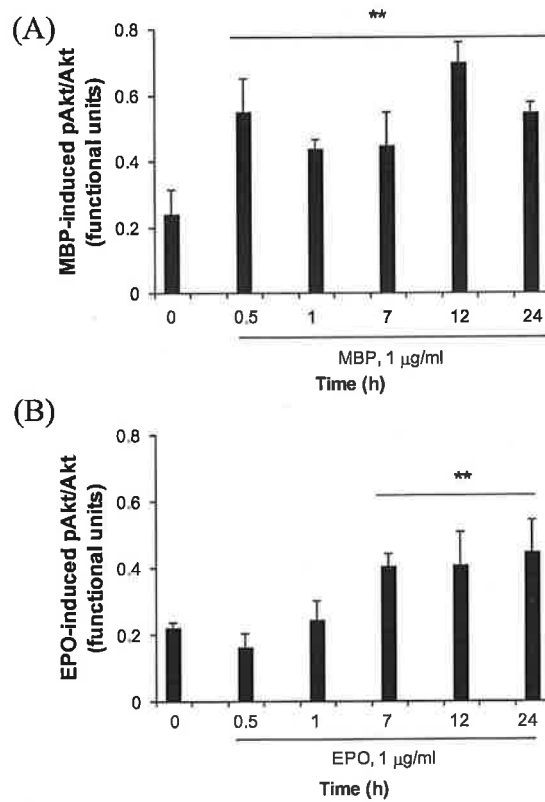


Fig. 1 Pathway of mucus production via ERB2 phosphorylation

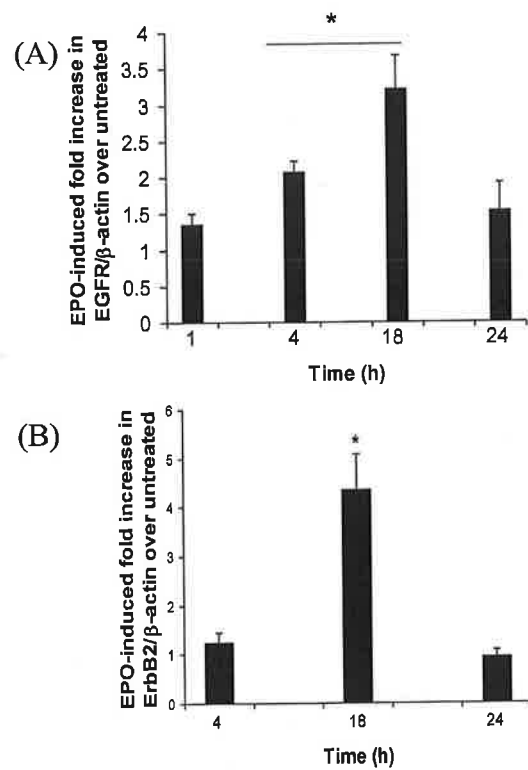
Our preliminary data in IMR32 neuroblastoma cells shows that both EPO and MBP differentially induce Akt signalling (Fig.2).



Both n=3, mean \pm sem. **, $p<0.01$, significantly greater than untreated

Fig. 2 MBP (1 μ g/ml; panel A) and EPO (1 μ g/ml; panel B) induce up-regulation of p-Akt normalised to total Akt protein at the indicated time points by Western blotting

MBP and EPO up-regulate transcriptional expression of various growth factors in epithelial cells (Pegorier et al. 2006). Our preliminary data suggests that EPO (1 μ g/ml) induces transcriptional up-regulation of both HER1 (EGFR) (Figure 3A), consistent with published data (Pegorier et al. 2006), and of HER2 (ErbB2) (Figure 3B) in IMR32 cells.



Both $n=3$; mean \pm sem, fold change induced by EPO (1 μ g/ml) over untreated; * $p<0.05$

Fig. 3 EPO (1 μ g/ml) induces up-regulation of HER1 (panel A) and HER2 (panel B) normalised to β -actin at the indicated time points by real-time PCR

We have also shown that EPO (Figure 4A) and MBP (Figure 4B) induce differential changes in subcellular localisation of FAK and of the cyclin dependant kinase inhibitor p27^{kip} (Fig 4). EGFR family signalling has been shown to be mediated by FAK, which can physically bind to EGFR, and which integrates EGFR and integrin signalling (Sieg et al. 2000). Also, subcellular localisation of the p27^{kip} is influenced by EGF receptor family signalling in various cancers (Le et al. 2005).

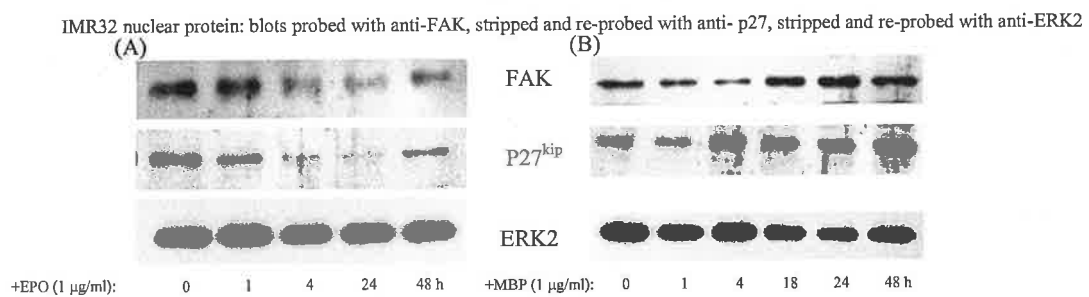


Fig. 4 EPO (1µg/ml; panel A) induces down-regulation of both FAK and p27^{kip} (normalised to ERK2) in nuclear protein fractions of IMR32 cells whereas MBP (1µg/ml; panel B) induces up-regulation of both FAK and p27^{kip} (normalised to ERK2) in nuclear protein fractions of IMR32 cells, by Western blotting

Specific Aims

We aim to test our hypothesis by carrying out the following specific aims:

1. Stimulation of human bronchial epithelial cell line (16HBE14o) with varying concentration of the eosinophil cationic granule proteins MBP and/or EPO.
2. Measurement of HER1 and HER2 expression by real time polymerase chain reaction (PCR).
3. Measurement of Mucin 5AC (MUC5AC), Mucin 4 (MUC4) and Mucin 1 (MUC1) in 16HBE14o cell line by real time PCR.

Chapter 3

3.1 **Materials and Methods**

3.1.1 **Cell Line**

The immortalised cell line 16HBE14o was a kind gift of the University of Vermont, USA. (16: Clone number 16; HBE: human bronchial epithelial cell line [normal bronchus, first bifurcation]; 14: 14th sample received by the University of Vermont; o: refers to cell transfection with the pSVori-plasmid an endogenous WT CFTR genotype). This cell line retains differentiated epithelial morphology and functions and cell cultures show the presence of tight junctions and cilia (Cozens et al. 1994).

3.1.2 **Cell Thawing**

Solutions:

Proliferation Medium

MEM + Glutamax (500ml)

Fetal Calf serum (50ml)

Penicillin G-streptomycin (5ml)

Procedure:

16HBE14o cells were thawed quickly by placing the cryovial in a 37°C incubator. The contents of the cryovial were then transferred into 5ml proliferation medium (pre-warmed to 37°C) in a sterile 15ml conical tube. Cells were collected by centrifugation at 1100rpm for 5 minutes at room temperature (RT). Supernatant was discarded and cells were resuspended in 1ml proliferation medium (pre-warmed to 37°C). Cells were then transferred to culture flask (T75) containing 10ml of proliferation medium (pre-warmed to 37°C) and incubated at 37°C in 5% CO₂.

3.1.3 Cell Passaging:

The culture media was discarded and the flask was then washed with D-PBS buffer solution (pre-warmed to 37°C). The D-PBS was discarded and 5ml of Trypsin (5%) was added to the flask. The flask was incubated at 37°C and 5% CO₂ for 5 minutes or until cells lifted off the bottom of the flask with gentle tapping. 5ml of proliferation medium was added to stop the trypsinization process. Cells were re-suspended in the proliferation medium by repeated pipetting up and down. Medium with suspended cells was transferred into sterile 15ml conical tube for cell collection by centrifugation at 1100rpm for 5 minutes at room temperature (RT). Supernatant was discarded and the cell pellet was resuspended in 1ml of proliferation media (pre-

warmed to 37°C). 10µl of cells were transferred into a sterile 1.5ml microcentrifuge tube and diluted 1:10 in proliferation medium for counting. Based on the counts obtained, cells were transferred to flasks and plates at appropriate numbers (see below: 3.2: Cell Treatments).

3.1.4 **Cell Freezing:**

Cells were trypsinised, collected by centrifugation and counted as above (see 3.1.3: Cell Passaging). Based on cell count, cells were resuspended in Freezing Mix (90% FCS; 10% DMSO) at approximately 5×10^6 per ml and 1ml was placed in each cryotube. Cryotubes were labelled and placed at -80°C overnight, insulated in polystyrene, before being transferred to liquid nitrogen for storage.

3.2 **CELL TREATMENTS**

3.2.1 **Plating Conditions:**

16HBE14o cells were seeded into 6-well plates at a density of 5×10^5 cells/well in proliferation medium. After 24 hours, medium was changed to serum free medium and cells were exposed to the appropriate eosinophil granule protein treatment at different time points including 0, 1, 4, 18, 24 hours and 48 hours. In some experiments, cells were pre-treated and co-exposed to a variety of

inhibitors including GM6001, DPI and anti- β 1 integrin (CD29) antibody.

3.2.2 Eosinophil Granule Proteins:

The human eosinophil cationic proteins eosinophil peroxidase (EPO) and major basic protein (MBP1) were kind gifts from Prof. G.J. Gleich, University of Utah, and were isolated from eosinophils as previously described (Gleich et al. 1979, Gleich et al. 1986).

3.3 **MOLECULAR BIOLOGY**

3.3.1 RNA ISOLATION

TRI REAGENT (SIGMA) was used in the isolation of RNA. The procedure was carried out according to the manufacturer's instructions as follows:

Following treatment, cells were washed in ice-cold 1x PBS and the PBS was discarded. Cells were lysed in the well in TRI reagent (1ml per well of 6-well culture dish), pipetted up and down repeatedly to obtain a homogeneous lysate and transferred to sterile microcentrifuge tubes. Tubes were left to stand at room temperature for 5 min to ensure complete cell lysis. Chloroform (200 μ l per 1ml TRI reagent) was then added to each tube followed by vigorous shaking for 15 seconds. After standing at room temperature for 10 min, the tubes were centrifuged at 12000g for 15 min at 4°C to separate the

mixture into three phases: an aqueous phase containing the RNA, the interphase containing DNA and an organic phase containing proteins. The aqueous phase was then transferred to a fresh tube and isopropanol (0.5ml per 1ml Tri reagent) was added and mixed by inversion several times. The sample was allowed to stand for 10 minutes at room temperature and centrifuged at 12000g for 10 minutes at 4°C. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet was washed by adding 1ml of 75% ethanol. The sample was vortexed and then centrifuged at 12,000 x g for 5 minutes at 4°C. The ethanol was removed and discarded and the wash was repeated. Following removal of as much ethanol by pipette as possible, the RNA pellet was air-dried for 5 minutes at room temperature. The pellet was dissolved by pipetting up and down in 50µl of nuclease-free water. The RNA samples were stored at -80C until further use.

3.3.2 Quantification and Determination of Purity of RNA by Nanodrop

The NanoDrop 8000 spectrophotometer (Thermo Scientific) checks for concentration (ng/ul) and quality of RNA based on the the absorbant at 260nm, and the selected analysis constant.

3.3.3 cDNA Synthesis

cDNA was synthesised by reverse transcription from total RNA using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions as follows:

Step 1: Elimination of Genomic DNA

1µg RNA was incubated in a 0.5ml sterile microcentrifuge tube in the presence of Genomic DNA Wipeout Buffer (Qiagen) at 42⁰C for 2 min on a thermal cycler to effectively remove contaminating genomic DNA.

Step 2: Reverse Transcription

Following genomic DNA elimination, the RNA sample was subjected to reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix (Qiagen) at 42⁰C for 15 min followed by 95⁰C for 2 min to inactivate the enzyme. In contrast to other methods, additional steps for RNA denaturation, primer annealing and RNase H digestion are not necessary.

Quantiscript Reverse Transcriptase has a high affinity for RNA and is optimized for efficient and sensitive cDNA synthesis from 10pg to 1µg of RNA. This high RNA affinity, in combination with Quantiscript RT Buffer, enables high cDNA yields.

RT Primer Mix ensures cDNA synthesis from all regions of RNA transcripts, even from 5' regions. This allows high yields of cDNA template for real-time PCR analysis regardless of where the target region is located on the transcript.

3.3.4 Design of PCR Primers

All primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

All Primers were designed using primer3 software (<http://www.basic.nwu.edu/biotools/Primer3>). PCR primers were designed against published gene sequences downloaded from Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed/>). In designing primers, certain criteria were considered. In particular, the overall balance of purine versus pyrimidines was important; where possible, at least half the bases were kept as G or C as this makes binding of oligonucleotides to template cDNA more stable. However, runs of three or more G's or C's were avoided as this can promote mispriming at GC rich regions. Also, care was taken to ensure that the two oligonucleotides designed to be used together did not contain significant complementary regions. In particular, if the 3' ends are complementary, primer dimers may be the main PCR products obtained. In general, PCRs were found to be more successful when oligonucleotide pairs of 20bp each in length were used. Also, it was

ensured that PCR products were no greater than 150bp in size for the purpose of analysis on the LightCycler.

3.3.5 Quantitative PCR using the Lightcycler[™]

A. QuantiTect SYBR Green PCR kit

Amplification of cDNA was carried out by quantitative PCR on the Light Cycler[™] 1.0 (Roche) using HotStart Taq DNA polymerase containing the double-stranded DNA binding dye SYBR Green 1 from the QuantiTect SYBR Green PCR kit (Qiagen), a ready-to-use Hot Start reaction mix for PCR, according to the manufacturer's instructions. The addition of the HotStart *Taq* DNA polymerase to the reaction mix minimizes non-specific amplification products that contaminate the desired product.

The HotStart *Taq* DNA polymerase is a modified form of QIAGEN *Taq* DNA polymerase. It is inactive at room temperature and has no enzymatic activity at ambient temperature. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step leading to high PCR specificity and accurate quantification. The enzyme is activated at the start of a reaction by a 15-minute 95°C incubation step. The hot

start enables reactions to be set up rapidly at room temperature (www.qiagen.com/HB/).

B. Setting up real-time PCR

Amplification of cDNA by Lightcycler

A master mix was prepared with the following components; 4mM MgCl₂, 0.5mM forward and reverse primers (specific to gene of interest), 1X LightCycler Fast Start DNA Master to a final volume of 18ml with PCR grade H₂O. 18ml of master mix was pipetted into a pre-cooled capillary tube in cooling block. 2ml of cDNA was added and the capillary was sealed with a stopper and centrifuged at 1000 rpm for 5 seconds in a benchtop centrifuge. The capillary was then placed in the rotor of the LightCycler instrument. The sample was cycled as described below.

C. Real-time PCR Cycling Parameters

The samples were continuously monitored during the PCR and fluorescence was acquired every 0.1°C. The samples were denatured at 95°C for 15 min followed by 35-40 cycles of denaturation, annealing and extension at 95°C for 15 seconds, 55°C for 25 seconds, and 72°C for 11 seconds (HER1, HER2, MUC4, MUC1, MUC5AC and MUC5B), or 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds (β-actin).

Characteristic melting curves were obtained at the end of amplification by cooling the samples to 65°C for 15 seconds followed by further cooling to 40°C for 30 seconds. Serial 10-fold dilutions were prepared from individual PCR products, which were then used as standards to plot against the unknown samples. Quantification of data was analyzed using the LightCycler™ analysis software, and values were normalized to the level of β -actin expression for each sample on the same template cDNA.

D. Melting Curve Analysis

A melting curve is produced subsequently to PCR (Appendix 12, 13 and 14). Hereby, the fluorescence of the samples is monitored while the temperature is increased. Due to the melting behavior of DNA, fluorescence will decrease with the increase of temperature. In case of the SYBR Green 1 format, this is due to the separation of double strands and consequently the release of SYBR Green 1 molecules, resulting in a drop in fluorescence. The purpose of melting curve analysis is to determine the characteristic melting temperature of a target cDNA. This temperature supplies information useful for product identification and identification of unwanted by-products such as primer dimers. Display and analysis of this data is performed using the melting curve

analysis tool of the LightCycler software (www.qiagen.com/HC/primerAssay).

Table 1**Primer Pairs Used**

<u>PRIMER</u>	<u>SEQUENCE</u>	<u>T_m (°C)</u>
β-actin F	5'- GGACTTCGAGCAAGAGATGG -3'	59.4
β-actin R	5'- AGGAAGGAAGGCTGGAAGAG-3'	59.4
HER2 F	5'- CAGCAGAGGATGGAACACAG -3'	59.4
HER2 R	5'- ACTCCTGGATATTGGCACTG -3'	57.3
HER1 F	5'- GCTCAACTGGTGTGTGCAGA -3'	59.4
HER1 R	5'- GATCTTGACATGCTGCGGTG -3'	59.4
MUC 4 F	5'- CTGTGTCTCTGCCTCCTTCC -3'	61.4
MUC 4 R	5'- TTGTTGAGCCTGTTGAGGTG -3'	57.3
MUC 1 F	5'- AGACGTCAGCGTGAGTGATG -3'	59.4
MUC 1 R	5'- GACAGCCAAGGCAATGAGAT -3'	57.3
MUC 5 AC F	5'- GTCCTCATGAAGGTGGATGG-3'	59.4
MUC 5- AC R	5'- CTGCTGCTCTGCTGAATGAG -3'	59.4
MUC 5 -B F	5'- CAAATACGCCAACCAGAC CT-3'	57.3
MUC 5-B R	5'- CCATCCAACCTTCTGCAGGTT -3'	57.3

3.4 Conditions for Inhibitors

Mouse Anti-Human CD29 Antibody:

16HBE14o cells were cultured as mentioned above and treated under the following conditions:

The cells were seeded into two 6-well plates at a density of 5×10^5 cells per well in proliferating medium for 24 hours. Medium was then changed to serum free medium on all wells two hours prior to the 18h EPO time point. CD29 inhibitor at a concentration of $1 \mu\text{g/ml}$ was added only to the second plate to achieve a 2 hour pre-treatment.

EPO at a concentration of $4 \mu\text{g/ml}$ was added at the two time points (4h and 18h) to both 6-well plates. This was mixed well by gently swirling the plates. RNA isolation, cDNA synthesis and amplification of cDNA by LightCycler were performed, as mentioned above.

Galardin (GM6001):

16HBE14o cells were cultured as mentioned above and treated under the following conditions:

The cells were seeded into two 6-well plates at a density of 5×10^5 cells per well in proliferating medium for 24 hours. Medium was then changed to serum free medium on all wells two hours prior to the 18h EPO time point. GM6001 inhibitor at a concentration of $10 \mu\text{M}$ was added only to the second plate to achieve a 2 hour pre-treatment.

EPO at a concentration of 4µg/ml was added at the two time points (4h and 18h) to both 6-well plates. This was mixed well by gently swirling the plates. RNA isolation, cDNA synthesis and amplification of cDNA by LightCycler were performed, as mentioned above.

Diphenyleneiodonium DPI

16HBE14o cells were cultured as mentioned above and treated under the following conditions:

The cells were seeded into two 6-well plates at a density of 5×10^5 cells per well in proliferating medium for 24 hours. Medium was then changed to serum free medium on all wells two hours prior to the 18h EPO time point. DPI inhibitor at a concentration of 1µM was added only to the second plate to achieve a 2 hour pre-treatment.

EPO at a concentration of 4µg/ml was added at the two time points (4h and 18h) to both 6-well plates. This was mixed well by gently swirling the plates. RNA isolation, cDNA synthesis and amplification of cDNA by LightCycler were performed, as mentioned above.

3.5 **STATISTICAL ANALYSIS**

All values are shown as mean \pm SEM from the number of experiments indicated unless otherwise indicated. Data was compared using ANOVA; a p value of <0.05 was considered significant. All statistical tests were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, USA).

3.6 **SOURCE OF MATERIALS USED**

DMEM plus Glutamax, M-199 medium, phosphate-buffered saline (PBS) and penicillin/streptomycin solution were purchased from GIBCO/BRL Life Technologies (Paisley, UK). Trypan Blue, guanidine hydrochloride, PIPES, Tri reagent, chloroform, isopropanol, interleukin (IL)- 8, lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and all common buffer constituents, except where otherwise stated, were obtained from Sigma (Poole, UK). Anti-rabbit and anti-goat IgG horseradish peroxidase (HRP) conjugates and 100 bp DNA marker were obtained from Promega (Madison, WI, USA). QuantiTect Reverse Transcription kit from (Qiagen) and HRP LumiGlo detection solution was purchased from Cell Signaling Technology (Beverly, MA, USA). Ficoll-Paque PLUS was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK).

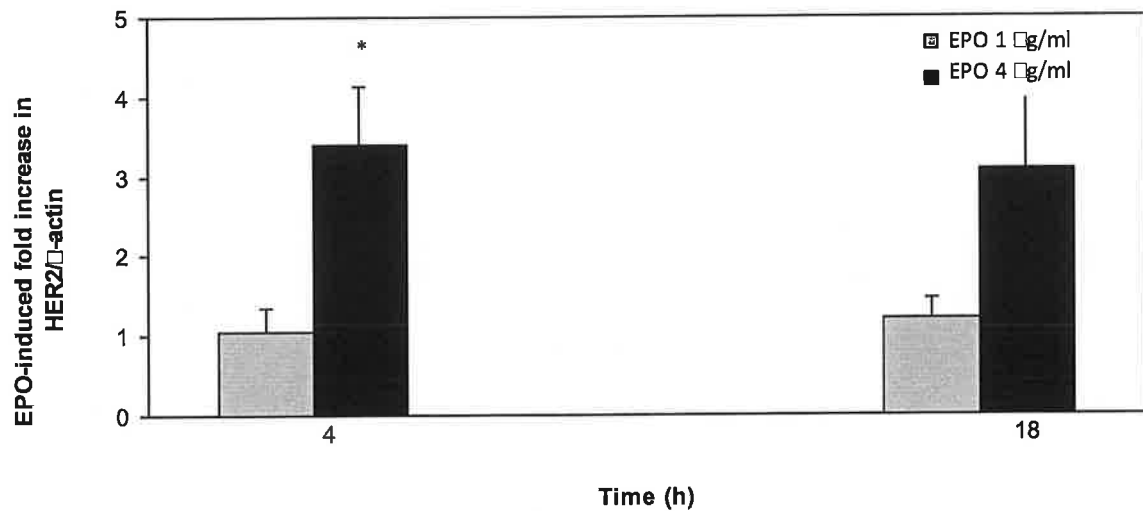
CHAPTER 4

4.1 **RESULTS**

The first project was to examine if HER2 expression was affected by EPO. The experimental design and procedures for cell culture and treatment with eosinophil granule proteins are described in the previous Methods chapter. EPO at a concentration of 4 μ g/ml was added in duplicate wells to 16 HBE14o cells for varying time points over 1-24 hours. Having extracted the mRNA and reverse transcribed the mRNA, the samples were subjected to PCR as described in the Methods chapter. I then normalized the results to the housekeeping gene β -actin on three independent experiments, each of which was a duplicate. The results are shown in Figure 5 (See individual data in Appendix 1). Having established that a specific concentration of EPO has an effect on the expression of HER2, I went on to investigate in further detail a time course of the effect of EPO on HER2 expression using a concentration of 4 μ g/ml.

Fig. 5

EPO effect on HER2 at two different concentrations

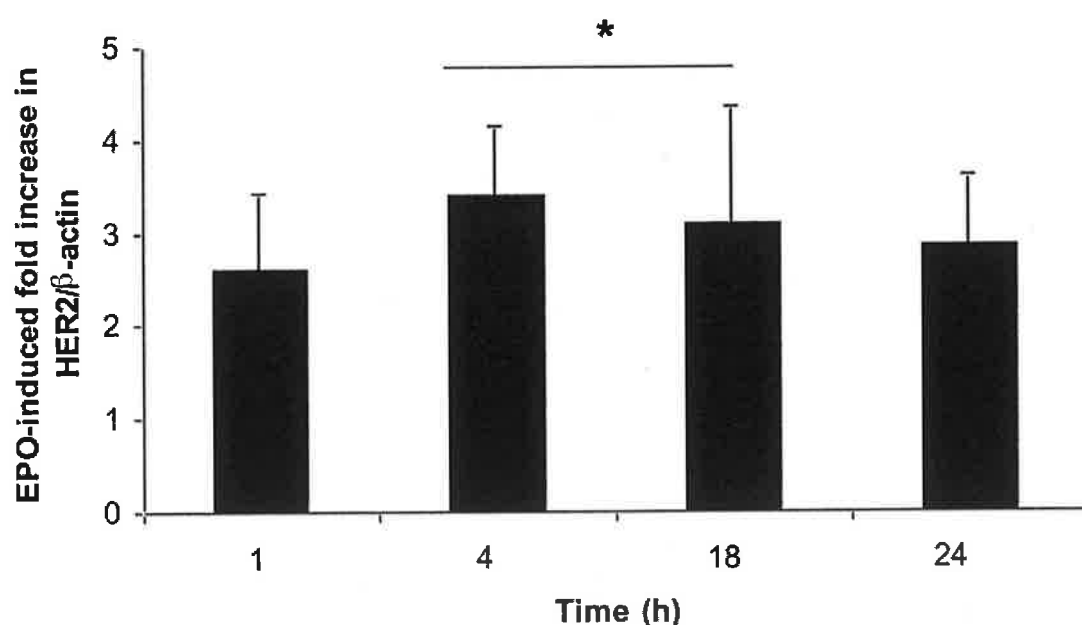


EPO at 4 µg/ml shows marked expression of HER2 in 16HBE14o cells compared to the effect of EPO at 1 µg/ml concentration. The increased expression of HER2 occurred at both 4 and 18 hours with a statistical significance demonstrated at 4 hours. Results are expressed as fold change induced by EPO compared to untreated cells (n=3).

In the following experiments, I incubated 16HBE14o cells with EPO (4 μ g/ml) for varying time points 1-24 hours. Having extracted the mRNA and subjected it to reverse transcription, I then assayed the samples by real time PCR. I then examined the samples and normalized the results to the housekeeping gene β -actin. The experiments show that there is an early rise in HER2, as early as 1 hour, and that this is statistically significant at 4 hours and at 18 hours and is still raised but not statistically significant at 24 hours (Fig. 6) (See individual data in Appendix 2).

Fig. 6

Eosinophil Peroxidase increases the expression of HER2 in 16HBE14o cells

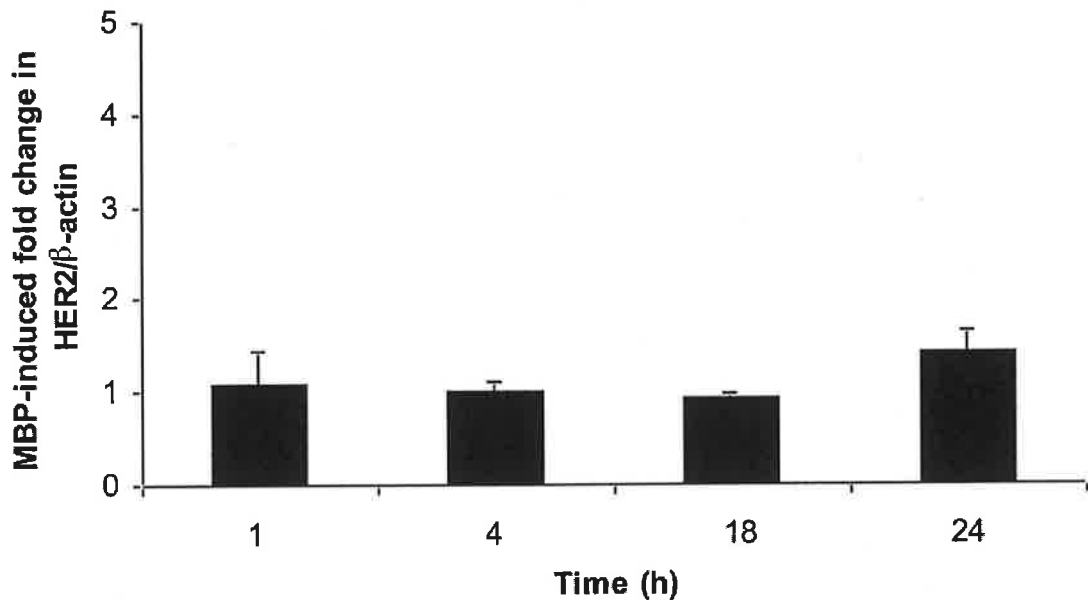


16HBE14o cells were treated with EPO at 4 μ g/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for HER2 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by EPO compared to untreated cells. (mean \pm sem, n=3, *p<0.05, when compared to untreated control).

I then proceeded to assess the effect of the eosinophil Major Basic Protein on the mRNA levels of HER2. The experiments were performed as described in the Materials and Methods section. Samples were subjected to real time PCR and the levels normalized to the housekeeping gene β -actin. In contrast to the effect of EPO, MBP had no effect at all on the expression of HER2 at any of the time points assessed (Fig.7). Note that the experiments were performed four times and that there was little statistical deviation between the experiments (See individual data in Appendix 3).

Fig. 7

Eosinophil Major Basic Protein induces no change in the expression of HER2

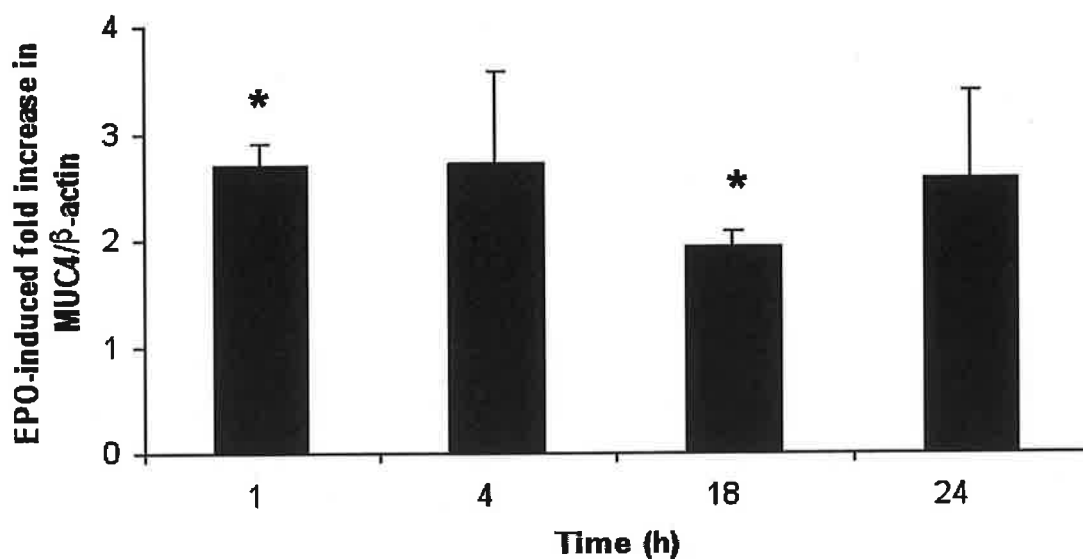


*16HBE14o cells were treated with MBP at 1 μ g/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for HER2 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by MBP compared to untreated cells. (mean \pm sem, n=4, *p<0.05, when compared to untreated control).*

Since MUC4 is a major gene involved in the mucus production associated with asthma and nasal polyps and is also a tethered ligand to HER2, as discussed in the introduction to this thesis, I next studied the effect of EPO on MUC4 gene expression. The experiments were performed as described previously; EPO (4µg/ml) was added to 16HBE14o cells at the varying time points between 1 and 24 hours. The samples were extracted and mRNA was reverse transcribed to cDNA and then subjected to real time PCR. The results of the experiment are shown in Fig.8. The results indicate, as was the case with HER2, that EPO induces the expression of MUC4 as early as 1 hour and that this is sustained through 24 hours (see individual data in Appendix 4).

Fig. 8

EPO increases MUC4 expression in 16HBE14o cells



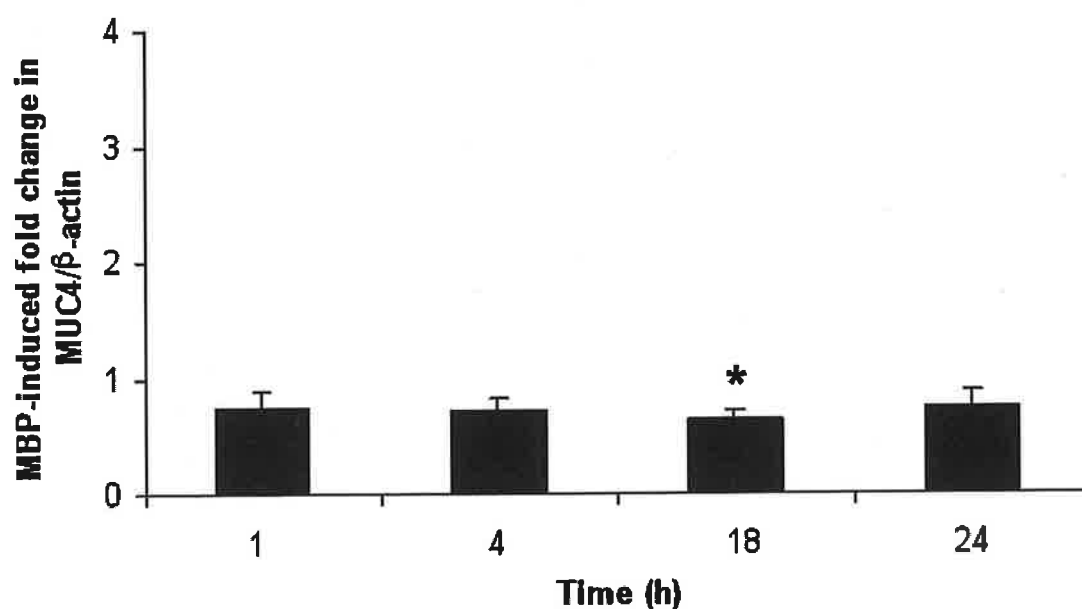
*16HBE14o cells were treated with EPO at 4 μ g/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for MUC4 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by EPO compared to untreated cells. (mean \pm sem, n=3, *p<0.05, when compared to untreated control).*

Having shown that EPO induced MUC4 expression, I then assessed if MBP had an effect on MUC4. As in the previous experiment (Fig.7), MBP at a concentration of 1 μ g/ml had no effect on MUC4, as had been the case with HER2 (See individual data in Appendix 5).

It should be noted that this concentration of MBP has a significant effect on cell signalling, e.g. signalling via ERK and p38 (MT Walsh 2004).

Fig. 9

MBP reduces MUC4 expression in 16HBE14o cells

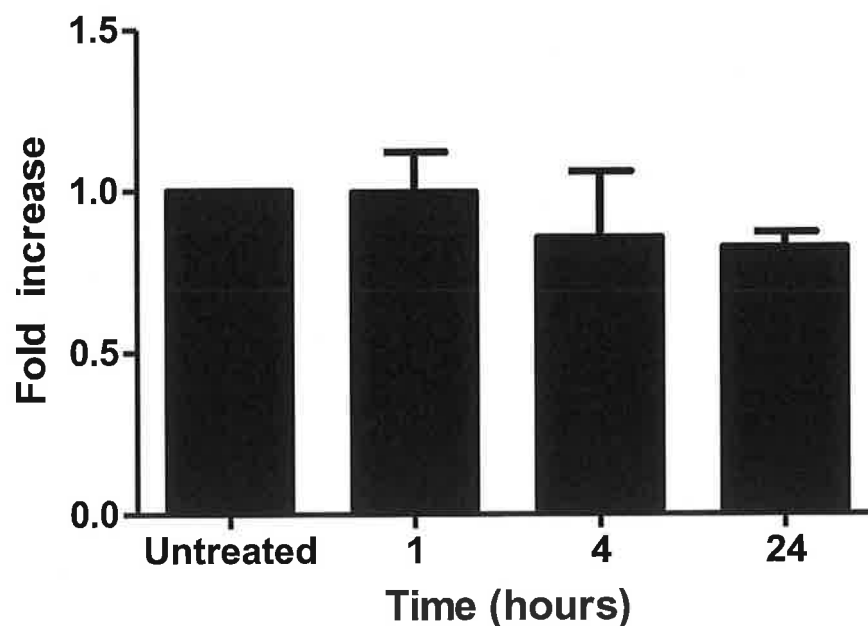


16HBE14o cells were treated with MBP at 1 μ g/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for MUC4 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by MBP compared to untreated cells (mean \pm sem, n=4, *p<0.05, when compared to untreated control).

Having shown that EPO induces the expression of HER2, I then proceeded to investigate the effect of EPO at a concentration of 4 μ g/ml on HER1 for varying time points. Having extracted the mRNA and subjected it to reverse transcription, I then assayed the samples by real time PCR and normalized them to the housekeeping gene β -actin. The experiments show that EPO has no effect at all on the expression of HER1 at any of the time points assessed (Fig.10) (See individual data in Appendix 6).

Fig. 10

EPO induces no change in HER1 expression in 16HBE14o cells

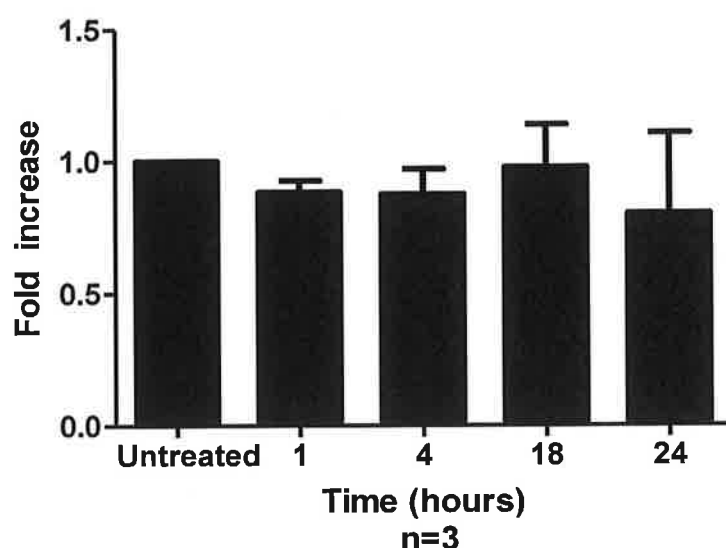


16HBE14o cells were treated with EPO at 4 μ g/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for HER1 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by EPO compared to untreated cells. (n=3)

Having shown that EPO induces no expression of HER1, I then proceeded to investigate the effect of EPO at a concentration of 4 μ g/ml on MUC1 for varying time points. Having extracted the mRNA and subjected it to reverse transcription, I then assayed the samples by real time PCR and normalized them to the housekeeping gene β -actin. The experiments show that EPO has no effect at all on the expression of MUC1 at any of the time points assessed (Fig.11) (See individual data in Appendix 7).

Fig.11

EPO induces no change in MUC1 expression in 16HBE14o cells

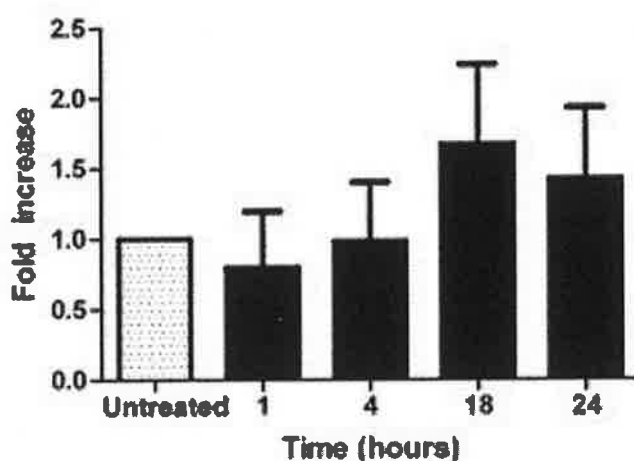


16HBE14o cells were treated with EPO at 4 μ g/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for MUC1 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by EPO compared to untreated cells. (n=3).

As EPO has induced no expression of HER1, I then proceeded to investigate the effect of MBP at 1mg/ml on MUC1 for varying time points. Having extracted the mRNA and subjected it to reverse transcription, I then assayed the samples by real time PCR and normalized them to the housekeeping gene β -actin. The experiments show that MBP had a slight increase on the expression of MUC1 at 18h (Fig.12) (See individual data in Appendix 8).

Fig. 12

MBP increases MUC1 expression in 16HBE14o cells



*16HBE14o cells were treated with MBP at 1µg/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for MUC1 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by MBP compared to untreated cells. (mean±sem, n=3, *p<0.05, when compared to untreated control).*

MUC5AC and MUC5B Expression in 16HBE14o Cell Line

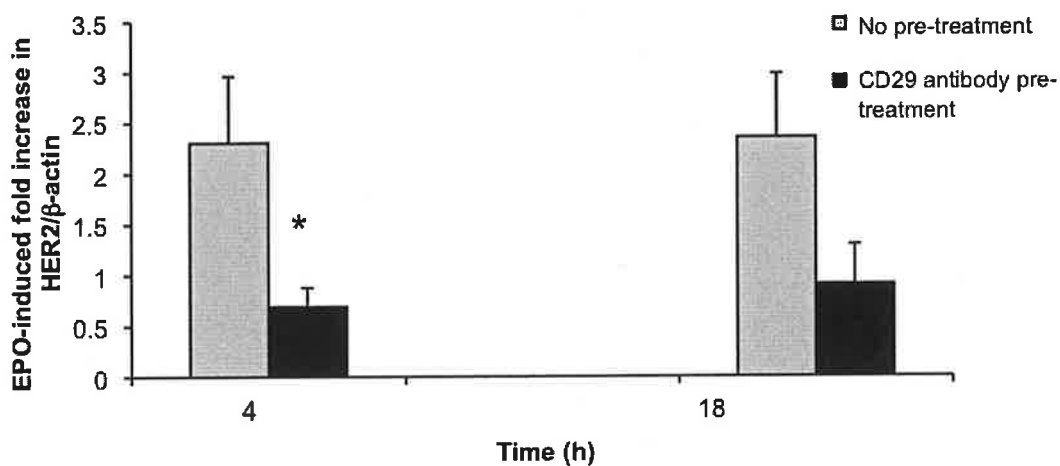
As the expression of MUC5AC and MUC5B has been shown to be induced by activated eosinophils in airway epithelial cells via EGFR, I proceeded to demonstrate their expression in the 16HBE14o cell line by extracting mRNA and reverse transcription to cDNA. This was followed by real time PCR. The experiments show no PCR product, indicating the absence of MUC5AC and MUC5B from the cell line.

To examine the underlying mechanism of action of EPO, I investigated if integrin binding was required to activate the increased expression of HER2. As discussed in the introduction, integrins are often clustered as part of the growth factor's activation of HER2.

Antibody against Integrin $\beta 1$ (CD29) at $1\mu\text{g/ml}$ for two time points, 4 and 18 hours, was added to the 16HBE14o cells two hours prior to treating cells with EPO. Having extracted the mRNA and subjected it to reverse transcription, I then assayed the samples by real time PCR and normalized them to the housekeeping gene β -actin. The experiments show that anti-CD29 has inhibited the effect of EPO expression on HER2 at both time points with statistical significance at 4 hours (Fig.13) (See individual data in Appendix 9).

Fig. 13

Inhibitory effect of Anti CD29 on EPO expression of HER2



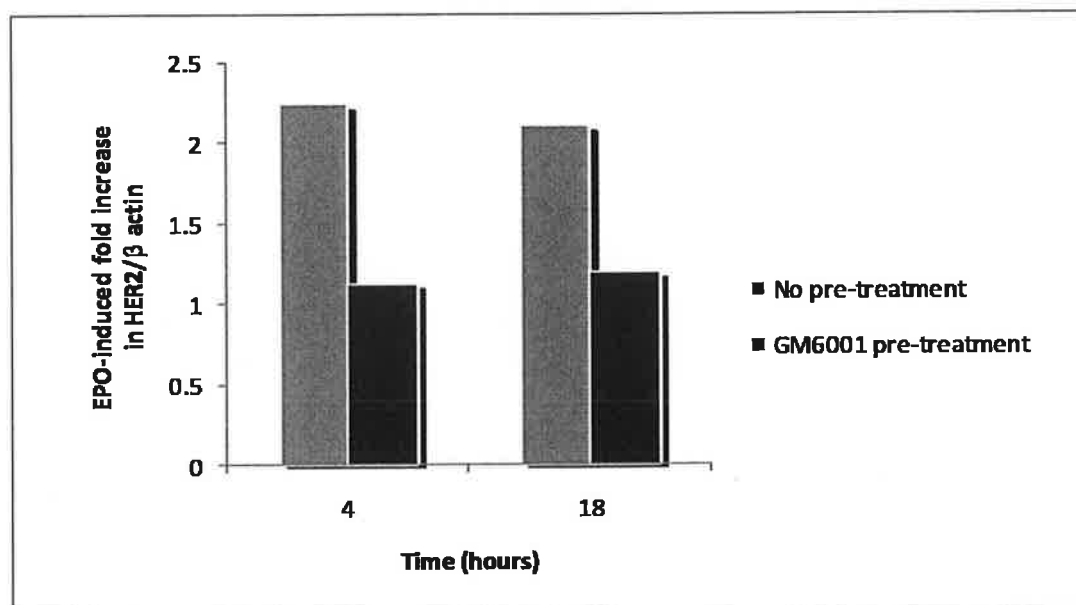
16HBE14o cells were treated with EPO at $4\mu\text{g/ml}$ and AntiCD29 at 1mg/ml for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for HER2 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by EPO compared to untreated cells. (n=3)

Matrix metalloproteinases (MMPs) have been implicated in the transactivation of the epidermal growth factor receptor (EGFR) (Santiskulvong and Rozengurt 2003), and MMPs generate the sustained release and build-up of EGFR ligands (Santiskulvong and Rozengurt 2003).

Galadrin (GM6001), a potent, cell-permeable, broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases (MMPs); at a concentration of 10 μ M for two time points, 4 and 18 hours, was added to the 16HBE14o cells two hours prior to treating cells with EPO. Having extracted the mRNA and subjected it to reverse transcription, I then assayed the samples by real time PCR and normalized them to the housekeeping gene β -actin. The experiments show that GM6001 inhibited the effect of EPO expression on HER2 at both time points (Fig.14) (See individual data in Appendix 10).

Fig. 14

Inhibitory effect of GM6001 on EPO expression of HER2



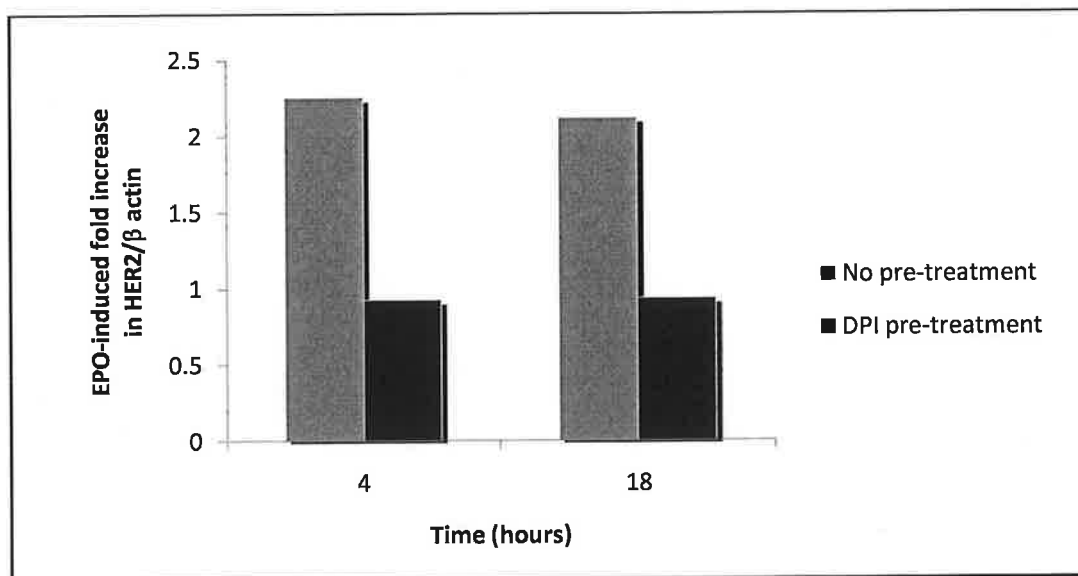
16HBE14o cells were treated with EPO at 4μg/ml and GM6001 at 10μM for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for HER2 and the results normalized to the housekeeping gene β-actin. Results are expressed as fold change induced by EPO compared to untreated cells. (n=2).

The production of ROS in the body is controlled by several antioxidative mechanisms. The first mechanism is at the level of production itself, thus inhibition of NADPH oxidase by DPI inhibits ROS effect on modifying the structure and function of proteins, and also inhibits ROS effect on modifying individual nucleotide bases.

Diphenyleneiodonium (DPI) at a concentration of 1 μ M for two time points, 4 and 18 hours, was added to the 16HBE14o cells two hours prior to treating cells with EPO. Having extracted the mRNA and subjected it to reverse transcription, I then assayed the samples by real time PCR and normalized them to the housekeeping gene β -actin. The experiments show that DPI has inhibited the effect of EPO expression on HER2 at both time points (Fig.15) (See individual data in Appendix 11).

Fig. 15

Inhibitory effect of DPI on EPO expression of HER2



16HBE14o cells were treated with EPO at 4 μ g/ml and DPI at 1 μ M for the indicated times and RNA and cDNA were extracted. Real-time PCR was carried out using primers specific for HER2 and the results normalized to the housekeeping gene β -actin. Results are expressed as fold change induced by EPO compared to untreated cells. (n=2).

4.2 **DISCUSSION**

4.2.1 Overview

In this project, I have shown that EPO but not MBP induces the expression of HER2 and MUC4 in 16HBE14o cells. In contrast, MBP has a modest effect on MUC1. The mechanism of action of EPO seems to be multi-factorial involving β -Integrin clustering, generation of reactive oxygen species and glycosaminoglycan binding. This work suggests a link between the observed presence of eosinophils in rhinitis and the development of nasal polyps.

The major findings of this study relate to EPO and show for the first time that an eosinophil granule protein causes upregulation of expression of a member of the EGFR family, namely HER2 (Fig.6), and of a tethered mucin, MUC4 (Fig.8). These results on upregulation of both HER2 and MUC4 are complementary, as MUC4 is an endogenous tethered ligand of HER2 which facilitates HER2 activation and signalling. Upregulation of both HER2 and MUC4 in epithelial cells has potential clinical relevance in a range of conditions in which eosinophils have been implicated, including nasal polyposis, asthma and cancer.

4.2.2 Eosinophils and their Granule Proteins in Remodelling

Eosinophils were long considered end-stage cells involved in host protection against parasites. However, recent evidence suggests that eosinophils are multifunctional leukocytes involved in initiation and propagation of inflammatory responses (Hogan et al. 2008). Accumulation and degranulation of eosinophils, with release of granule contents such as EPO and MBP at local inflammatory sites, is a hallmark of allergic and inflammatory diseases including allergic rhinitis, asthma and inflammatory bowel disease. Eosinophils are implicated in airway epithelial remodelling including increased expression of mucins (Burgel et al. 2001). In our study, we sought to specifically examine the role of eosinophil derived cationic proteins on epithelial cell remodelling, in particular the expression of growth factor receptors and mucins.

Eosinophils exert many of their inflammatory effects in allergic disorders by degranulation and release of intracellular mediators including cytokines, leukotrienes and reactive oxygen species. They also release a set of cationic granule proteins which include major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN). Both EPO and MBP have high arginine content but differ substantially in size and structure and this may well explain the different results seen in this thesis between MBP and EPO, where EPO shows increase in the

expression of HER2 (Fig.6) and MUC4 (Fig.8) in epithelial cells, while MBP induces no change in the expression of HER2 (Fig.7) and shows a reduction in MUC4 expression in epithelial cells (Fig.9). Eosinophils most commonly release their granular contents in a piecemeal fashion making it likely that MBP and EPO may be released separately at the inflammatory site. Therefore, eosinophils may exert differential effects on HER2, MUC4 and MUC1 expression depending on the combination of eosinophil protein release under individual circumstances.

In lower concentrations, eosinophil granule proteins exert effects on airway cells which contribute to diseases such as rhinitis and asthma, for example sensitisation of airway sensory nerves by inhibition of neuronal M₂ auto-receptors (as we and others have shown). These effects can be mimicked by poly-L-arginine or prevented by heparin which inhibits the cationic nature of the proteins. More recently however, other properties of eosinophil granule proteins have been recognized. For example, MBP and EPO upregulate transcriptional expression of various growth factors in airway epithelial cells. These effects could only be partially mimicked by poly-L-arginine, implying that properties of MBP and EPO beyond their cationic charge are responsible for their observed effects on remodelling (Pegorier et al. 2006).

The results of this study, showing up-regulation of HER2 and MUC4 in human bronchial epithelial cells by EPO, are consistent with published studies and further suggest a role for eosinophil granule proteins in the cellular and tissue remodelling associated with epithelium in airway disease. Thus, eosinophil granule proteins have diverse effects on cell growth, differentiation and survival factor expression, factors which are central to conditions ranging from allergy and inflammatory bowel disease to cancer. Our observations on EPO mediated upregulation of the EGFR family member HER2 suggest a mechanism whereby eosinophils and their granule proteins can impact on airway remodelling.

4.2.3 Eosinophils and their Granule Proteins in Cancer

Eosinophils are important in the pregnant and the estrous uterus and promote ductal morphogenesis in normal mammary development. Eosinophilia has also been associated with malignant disorders, including breast and lung cancer. However, it remains unclear whether eosinophils in cancer tend to promote or inhibit progression; much depends on the tumour type and the local inflammatory milieu.

There is evidence of deposition of EPO in breast cancer tissue as opposed to neighbouring healthy breast tissue. We have shown that EPO upregulates the expression of HER2 and MUC4. HER2

upregulation is associated with approximately 30% of breast cancer cases and MUC4 over-expression is also associated with cancer. Our results suggest a possible contributory role for EPO in dysregulation of HER2 and MUC4 in tumours.

The wide range of conditions in which eosinophils play a role are all characterised by increased cell turnover. Eosinophils are now considered to be important regulators of repair and/or remodelling. An additional role for the eosinophil granule protein EPO in promoting tissue turnover is suggested by the observations in this thesis, showing that MUC4 and HER2 genes are expressed. This involvement of eosinophils and their protein in epithelial cell remodelling would have particular relevance in nasal polyposis.

4.2.4 Eosinophils and their Granule Proteins in Nasal Polyposis and Asthma

Histological studies of nasal polyposis have revealed that there are significantly more activated eosinophils in polyp tissue than in nasal mucosa. Activated eosinophils release inflammatory substances such as major basic proteins and eosinophil peroxidase, which damage nasal and sinus mucosa, leading to oedema and inflammation (Sun et al. 2009). In addition to these inflammatory substances, the activated eosinophils found in polyp tissue were shown to produce a number of inflammatory cytokines including GM-CSF, IL-3 and IL-5. These

cytokines are also potent inflammatory mediators and are thought to contribute to the significant mucosal inflammation seen in nasal polyposis. Eosinophils are pivotal in the ongoing inflammation and tissue damage of nasal polyposis, hence it is important to understand the link between nasal polyps and eosinophils.

Nasal polyposis is frequently associated with asthma and this relationship may imply a shared pathophysiology. In patients with nasal polyposis, activated eosinophils within the nasal mucosa and nasal polyps are more prominent in those patients with asthma than in those patients without asthma (Ediger et al. 2005). The clinical relationship of nasal polyposis to asthma or aspirin intolerance has been well described (Alobid et al. 2005). Patients with asthma or aspirin intolerance have more extensive involvement of the sinuses and a higher recurrence rate from surgical treatments for nasal polyposis than those without asthma or aspirin intolerance (Alobid et al. 2005). Bilateral nasal polyposis is particularly common in patients with aspirin intolerance and asthma, in whom cyclooxygenase inhibitors can trigger activation of eosinophils. Thus, the results in this study, which show up-regulation of HER2 and MUC4 in response to EPO, a mediator released by activated eosinophils, are likely to be relevant in asthma patients with nasal polyposis and aspirin intolerance.

Nasal polyposis is a chronic inflammatory airway disease of the nasal and paranasal sinus mucosa. Epithelial proliferation and differentiation have been implicated in the pathogenesis of nasal polyposis (Coste et al. 1996), but the underlying mechanisms are unknown, and as a result medical and surgical therapy is not completely effective (Lund 1995). Clinical features of nasal polyposis include nasal obstruction and rhinorrhea; the epithelium of nasal polyps shows morphologic changes, including goblet cell hyperplasia and mucus hypersecretion which contributes to these symptoms. Polyps are reported to contain inflammatory cells (Hamilos et al. 1996) and these cells could play important roles in the disease.

Mucus secretion has a protective role in the normal human respiratory tract. However, in patients with chronic inflammatory airway diseases, excessive mucus secretions accumulate and can contribute to the pathogenesis of nasal polyps, allergic rhinitis, acute asthma, cystic fibrosis, and chronic obstructive pulmonary disease (Aikawa et al. 1992, Berger et al. 1999, Piquette et al. 2000, Burgel et al. 2000, Brihaye et al. 1997). To date, no notably effective treatments for mucus hypersecretion have been found in these diseases.

Since several studies have postulated that EGFR activation is responsible for increased mucin production in airway diseases (Takeyama et al. 2001, O'Donnell et al. 2004, Burgel et al. 2007) and as tissue eosinophilia is a constant feature in nasal polyps, we investigated the hypothesis that eosinophil cationic granule proteins such as major basic protein (MBP) and eosinophil peroxidase (EPO) in the proximity of bronchial epithelial cells lead to increased expression of epidermal growth factor receptor family members, HER1 and HER2, which may lead to changes in mucin gene expression.

The finding of this work that eosinophil cationic granule protein (EPO) induces transcriptional upregulation in the expression of epidermal growth factor receptor HER2 and MUC4 offers a therapeutic possibility to the problem of mucus hypersecretion in conditions such as nasal polyposis. Prior to this work, findings of EGFR and mucin gene expression in response to EPO were obscure.

This is the first study to address EGFR and mucin gene expression under the effect of EPO in epithelial cells. This study suggests a previously unsuspected mechanism whereby EPO could contribute to remodelling, which is a feature of nasal polyposis and asthma.

4.2.5 Concentration Dependence of EPO Effect on HER2 and MUC4

Our data shows that the effect of EPO on HER2 (Fig.5) and MUC4 is concentration dependant. We used a concentration of 1 μ g/ml, which corresponds to 15.22nm, and 4 μ g/ml, which corresponds to 60nm, and found that the higher concentration was necessary to exert the observed effects on HER2 and MUC4 (Fig.5). EPO is cytotoxic to respiratory epithelial cells and to parasites in the absence of peroxidase substrates. This is related to induction of membrane permeability of cells and is dose-dependent with a Michaelis-Menten constant of 113nM (Kleine et al. 1999). EPO has previously been measured in the sputum of patients with asthma and chronic obstructive pulmonary disease (Keatings and Barnes 1997). The EPO concentration ranged from undetectable to ~10nM, with a mean of ~4nM in asthma. It is likely that the tissue concentration of EPO is considerably higher than that of the sputum. It is worth noting that it is difficult to definitively state the concentration of EPO in localized areas of inflammation or overall in tissue, as its cationicity tends to cause it to adhere firmly to cell membranes.

4.2.6 Inhibitors Effect on Upregulation of HER2 and MUC4

Effect of anti-CD29

The use of antibody against Integrin $\beta 1$ (CD29) to detect the knockdown effect of Integrin $\beta 1$, showed a remarkable decrease in the upregulation of HER2 in bronchial epithelial cells treated with EPO (Fig.13). The same effect was observed on the expression of MUC4.

Effect of Galardin (GM6001)

GM6001 has inhibited the effect of EPO expression on HER2 at both time points (Fig.14), suggesting a role for Matrix Metalloproteinases (MMPs) in mediating EPO induced effects on epithelial cells resulting in upregulation of MUC4 and the epidermal growth factor receptor HER2. This could imply that EPO effects on HER2 and MUC4 are mediated for example by transactivation of the EGFR due to increased release of EGFR ligand (Santiskulvong and Rozengurt 2003). A definitive explanation of the effect of the inhibitor GM6001 would however require further studies.

Effect of Diphenyleneiodonium (DPI)

DPI has inhibited the effect of EPO expression on HER2 at both time points, suggesting a role for ROS in the downstream signalling pathway (Fig.15). Eosinophil peroxidase could cause the production of ROS in the epithelial cells due to its peroxidase activity.

4.3 **CONCLUSION**

This is the first study to address EGFR and mucin gene expression under the effect of EPO in epithelial cells. This study suggests a previously unsuspected mechanism whereby EPO could contribute to remodelling, which is a feature of nasal polyposis and asthma. The findings of this study that eosinophil cationic granule protein (ECP) induces transcriptional upregulation in the expression of epidermal growth factor receptor HER2 and MUC4 offers a therapeutic possibility to the problem of mucus hypersecretion in conditions such as nasal polyposis. Prior to this work, findings of EGFR and mucin gene expression in response to ECP were obscure.

Currently, further work is ongoing in the lab to demonstrate increased protein levels along with the mRNA described in this thesis. Understanding the mechanism of mucin expression under the influence of inflammatory mediators may shed more light on the control points of mucin and could provide a new target for therapeutic intervention. Longterm, it may be possible to test specific inhibitors of ECP in clinical studies.

Appendix 1

EPO at 1mg/ml		EPO at 4mg/ml	
4h	18h	4h	18h
1.60	1.10	2.99	2.27
0.97	0.85	4.84	5.56
0.60	1.70	2.38	1.43

Appendix 2

1h	4h	18h	24h
1.95	2.99	2.27	1.91
4.27	4.84	5.56	4.32
1.62	2.38	1.43	2.34

Appendix 3

1h	4h	18h	24h
0.760	1.050	0.870	1.000
1.780	0.830	1.050	1.460
0.720	1.160	0.860	1.810
0.749	0.622	0.689	0.931

Appendix 4

1h	4h	18h	24h
4.200	6.100	1.800	5.800
2.560	2.210	4.430	2.130
1.879	1.586	1.393	1.194

Appendix 5

1h	4h	18h	24h
1.080	0.960	0.770	0.930
0.350	0.540	0.450	0.420
0.780	0.700	0.560	0.960
0.725	0.719	0.763	0.698

Appendix 6

1h	4h	24h
0.87	0.65	0.78
1.12	1.06	0.87
0.82	0.83	0.95

Appendix 7

1h	4h	18h	24h
0.80	0.69	0.70	0.19
0.95	0.97	1.26	1.08
0.90	0.78	0.97	1.13

Appendix 8

1h	4h	18h	24h
1.570	0.530	1.370	2.420
0.247	1.821	2.771	1.072
0.572	0.595	0.871	0.782

Appendix 9

No Inhibitor		CD29	
4h	18h	4h	18h
1.64	1.12	0.03	0.03
1.27	2.46	0.67	1.93
1.12	4.06	0.71	0.89

Appendix 10

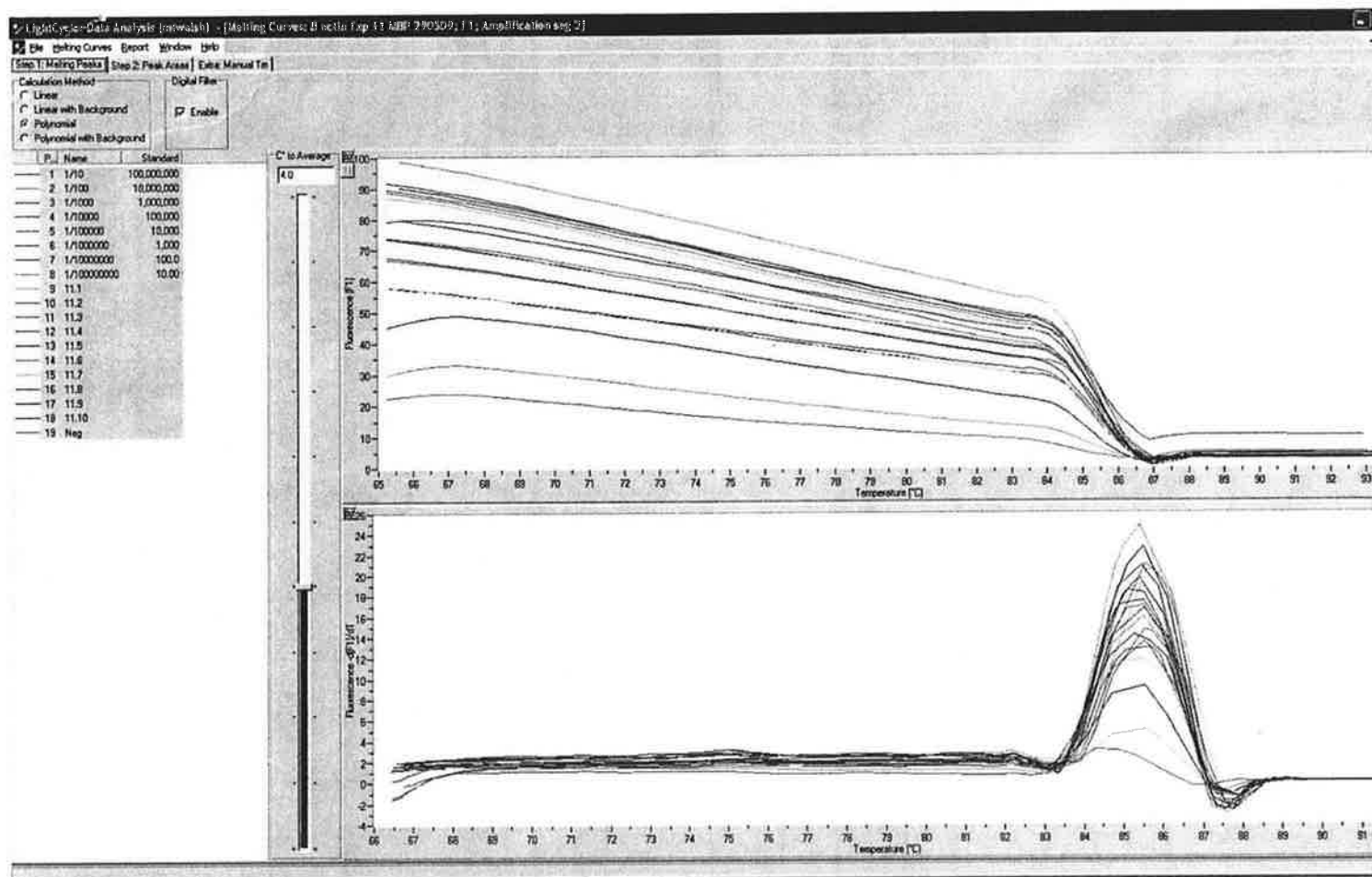
No Inhibitor		GM6001	
4h	18h	4h	18h
2.87	2.46	1.22	1.21
1.64	1.78	1.05	1.20

Appendix 11

No Inhibitor		DPI	
4h	18h	4h	18h
2.87	2.46	1.01	1.15
1.64	1.78	0.84	0.72

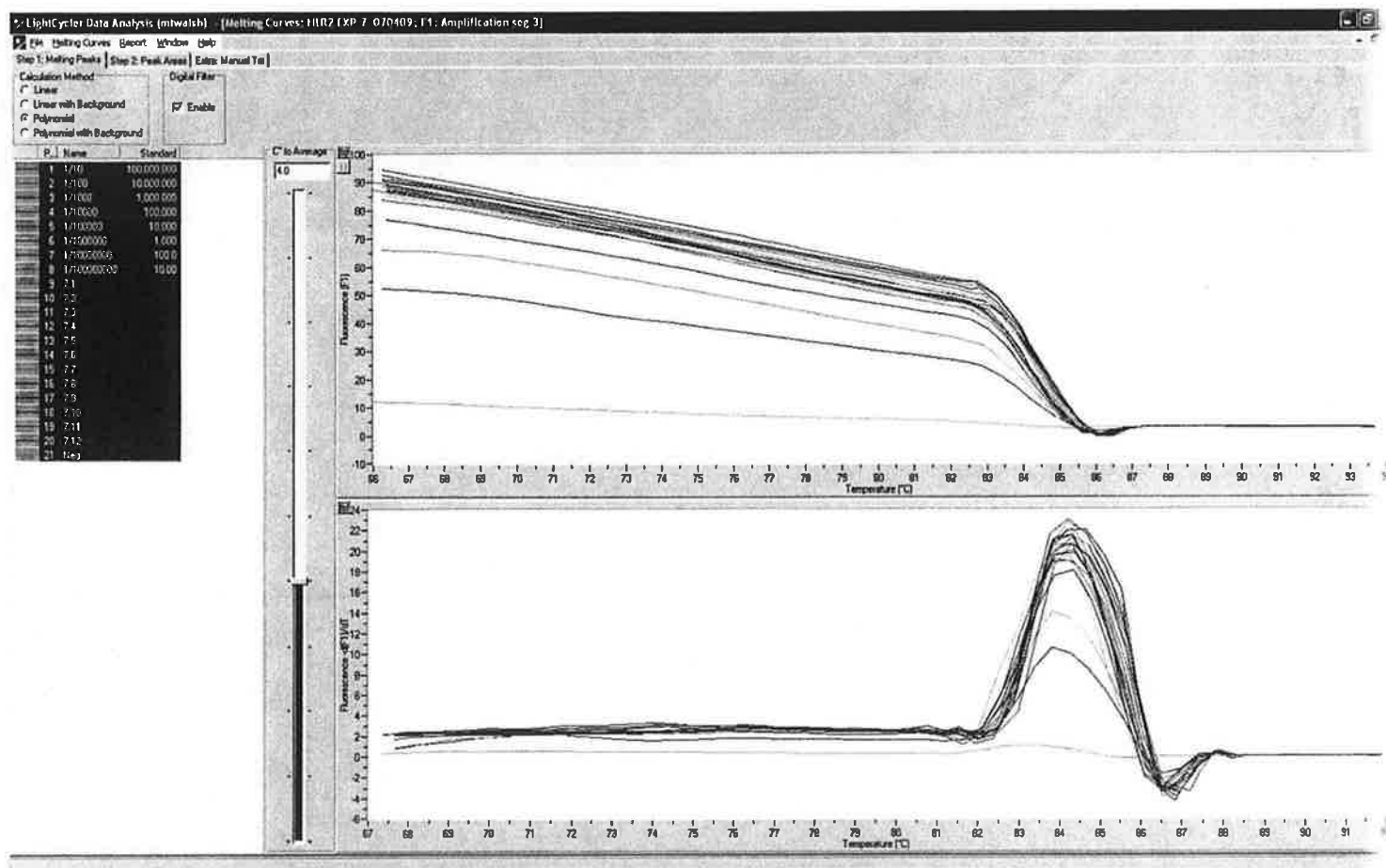
Appendix 12

β -actin Melting Curve



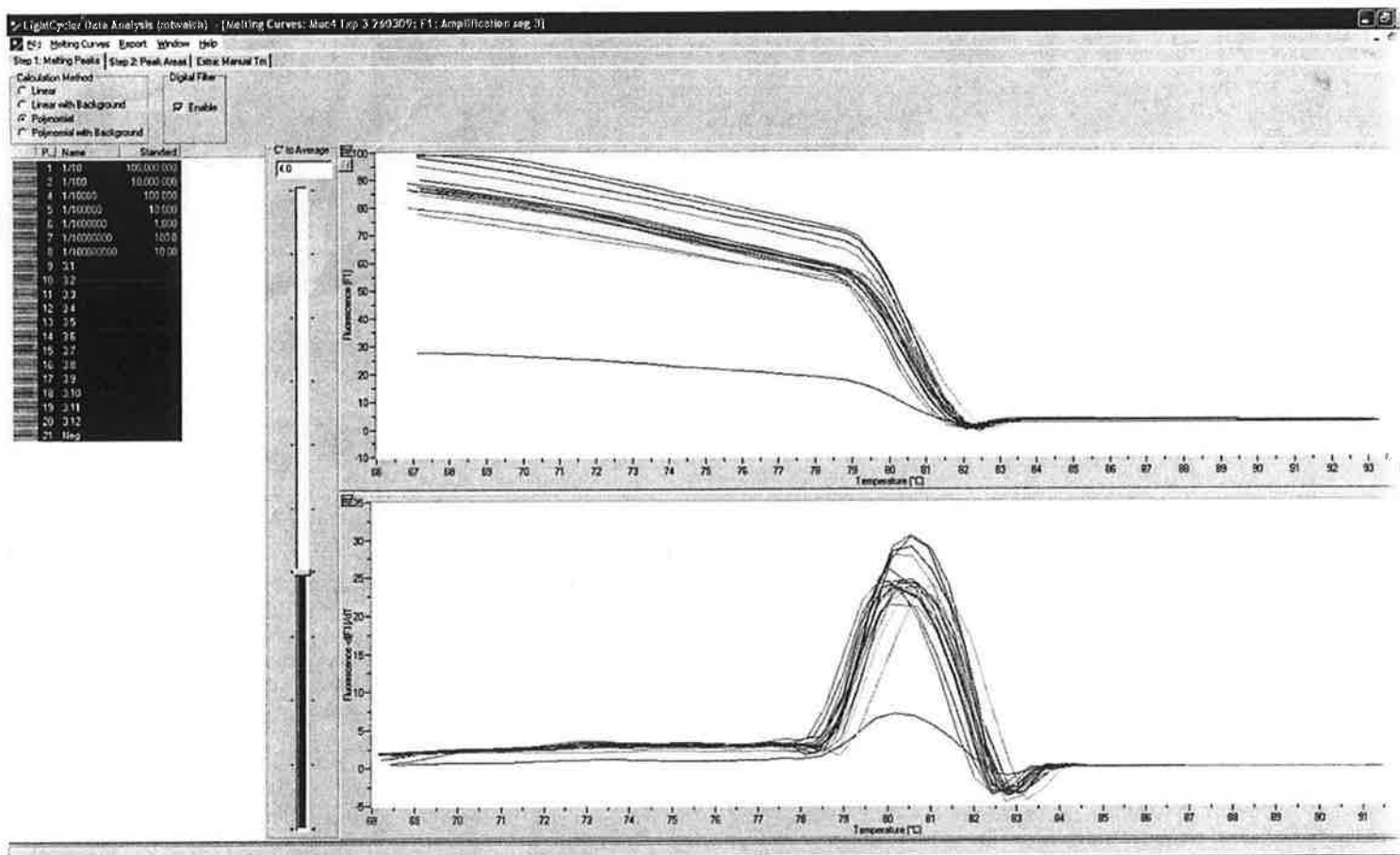
Appendix 13

HER2 Melting Curve under EPO influence



Appendix 14

MUC4 Melting Curve under EPO influence



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