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The role of the epidermal growth factor receptor and its ligands in the regulation of the bronchial epithelial phenotype in smoking related lung disease

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A thesis submitted for the award of the degree of
Doctor of Medicine

Division of Infection, Inflammation and Repair
School of Medicine

December 2006

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCE

DIVISION OF INFECTION, INFLAMMATION AND REPAIR

RESPIRATORY CELL AND MOLECULAR BIOLOGY

Doctor of Medicine

The role of the epidermal growth factor receptor and its ligands in the regulation of the bronchial epithelial phenotype in smoking related lung disease

by Timothy John Howell

Cigarette smoking is the major cause of chronic obstructive pulmonary disease (COPD), a condition characterised by airflow limitation that causes significant morbidity and mortality worldwide. A common symptom in this condition is that of chronic mucus hypersecretion (CMH), a regular cough productive of sputum. In those with COPD, CMH is associated with a more rapid decline in lung function, an increased risk of infection, and an increase risk of death. A number of pathological changes are seen in the airways of subjects with COPD and CMH including goblet cell hyperplasia and metaplasia, hypertrophy and hyperplasia of submucosal glands, and inflammation in the lumen of the airway, the bronchial wall, and the submucosal glands. These changes are provoked by cigarette smoke and recent evidence suggests that the epidermal growth factor receptor (EGFR), which is found in bronchial epithelial cells (BECs), plays a key role in mediating these changes. When activated by smoke, or other disease-relevant stimuli, the EGFR promotes release of pro-inflammatory cytokines from BECs and up-regulates mucin gene expression. This is an important mechanism in the process of differentiation of epithelial cells to a mucus secreting goblet cell phenotype. Activation of the EGFR requires cleavage of its ligands by a group of membrane-bound metalloproteinases.

In this work it is shown that any of the ligands for the EGFR found in BECs can produce an increase in expression of the pro-inflammatory cytokine interleukin 8 (IL-8) and the secreted mucin MUC5AC. The time course of this expression is significantly different between the two genes. In addition an auto-induction of EGFR ligand expression is seen which may be important in modulating the effects of chronic exposure to noxious stimuli like smoke. The effects of cigarette smoke on release of EGFR ligands with subsequent EGFR activation is confirmed with smoke promoting IL-8 release and increasing expression of both IL-8 and MUC5AC. Doxycycline, a commonly used antibiotic, is shown to inhibit the release of EGFR ligands in BECs exposed to smoke. This inhibition reduces the release of IL-8 from the cells and attenuates the mucin gene response. These observations suggest that agents which reduce EGFR ligand shedding from BECs may provide a novel therapeutic target for treatment of CMH in smoking related lung disease and other conditions characterised by this phenotype.

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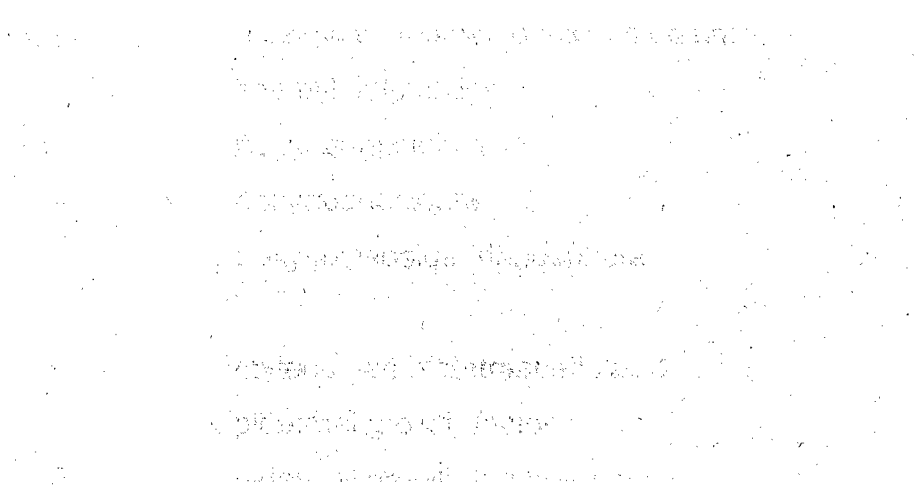
Acknowledgements

I would like to thank all members of the Brooke laboratory who helped me during my time there. Particular thanks though must go to Audrey Richter who taught me the majority of techniques that I have used in producing this work and guided me through the early stages of my thesis. Also Rob Powell, an expert on RT-qPCR, made a vital contribution to this work, teaching me how to use the PCR equipment and more importantly designed all the assays which I used in these experiments. Most importantly my supervisor Donna Davies tolerated yet another medic in her group, guided the direction this work took, and has continued to support me during the inevitably difficult time of writing up.

None of this work though would have been possible without the support of my parents and my wife Helen. They have supported me through everything I have done, tolerated my mood swings whilst I was undertaking this work, and helped me with all the decisions I have had to make it the last year. I would like to thank them for the love they have always given me.



List of abbreviations

A	Adenine
ADAM	A Disintegrin and metalloproteinase
α_1-AT	Alpha-1 arıtitrypsin
AR	Amphiregulin
AP-1	Activating protein 1
BEC	Bronchial epithelial cells
BHR	Bronchial hyperresponsiveness
BSA	Bovine serum albumin
BTC	Betacellulin
C	Cytosine
cDNA	Copy deoxyribonucleic acid
CMH	Chronic mucus hypersecretion
COPD	Chronic obstructive pulmonary disease
CSE	Cigarette smoke extract
Ct	Threshold cycle
DALY	Disability adjusted life years
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EPI	Epigen
EPR	Epiregulin

ERK	Extracellular signal related kinase
FBS	Fetal bovine serum
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
G	Guanine
GOLD	Global initiative for chronic obstructive lung disease
GTP	Guanine triphosphate
HB-EGF	Heparin-binding EGF-like growth factor
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IL	Interleukin
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LTA	Lipotechoic acid
LTB_4	Leukotriene B_4
LTOT	Long term oxygen therapy
LTRA	Leukotriene receptor antagonist
MAPK	Mitogen activated protein kinase
MAPKK/MEK	Mitogen activated protein kinase kinase
MMLV	Murine moloney leukaemia virus
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NCI-H292	National cancer institute H292 cells
Neu	Neuregulin
NF-κB	Nuclear factor kappa B

OR	Odds ratio
PAFR	Platelet activating factor receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PLC-γ	Phospholipase C gamma
PMA	Phorbol 12-myristate 13-acetate
PTB	Phosphotyrosine binding
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RR	Relative risk
rRNA	Ribosomal RNA
RSV	Respiratory syncytial virus
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative PCR
RTK	Receptor tyrosine kinase
SFM	Serum free medium
SH1/2	src homology 1/2
SOD	Superoxide dismutase
T	Thymine
TACE	TNFα converting enzyme
TAPI-1	TNFα proteinase inhibitor 1
TBE	Tris-borate EDTA
TGFα	Transforming growth factor alpha
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumour necrosis factor alpha
VCAM	Vascular cell adhesion molecule

WHO World Health Organisation

1 Chapter 1: Introduction

1.1 Overview

The respiratory condition chronic obstructive pulmonary disease (COPD) is a disease state characterised by airflow limitation that is not fully reversible despite treatment. It is a common condition worldwide and is a major cause of morbidity and mortality. It is the fifth leading cause of death worldwide (1), and in the World Health Organisation's (WHO) Global Burden of Disease study it was the twelfth commonest cause of premature morbidity and mortality (as measured by disability adjusted life years (DALYs)). This position is predicted to rise to make it the fifth commonest cause by 2020 as the prevalence of COPD increases (2).

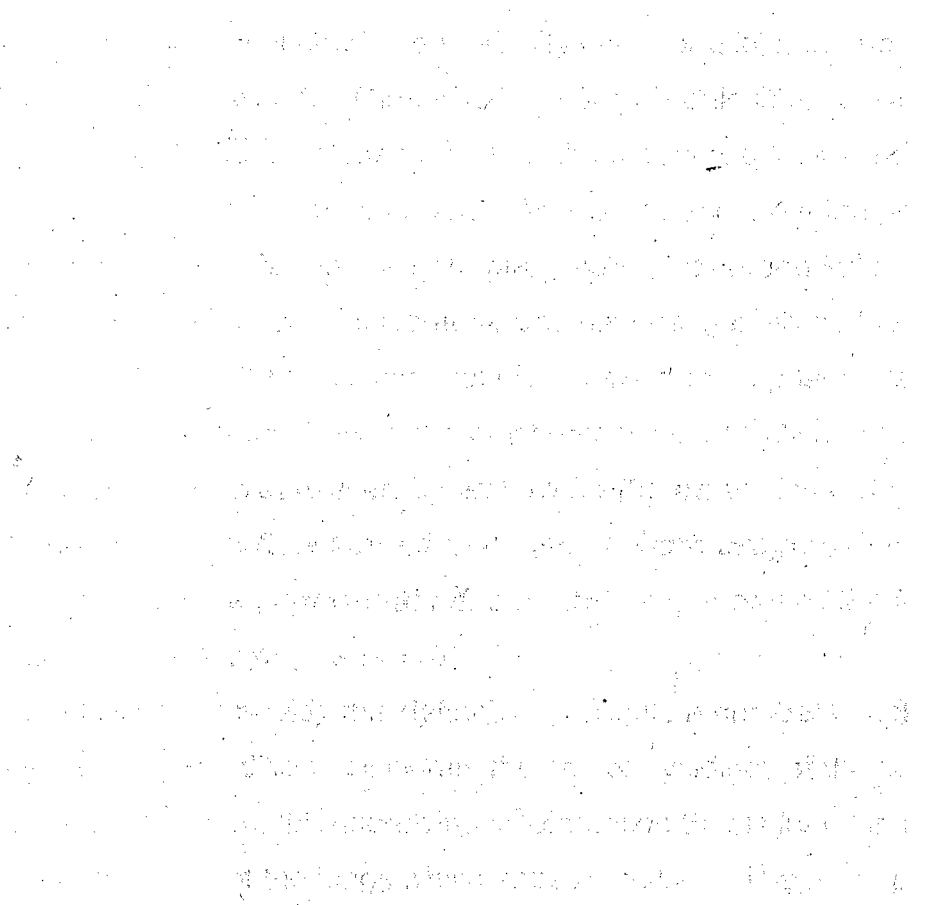
The airflow obstruction found in COPD tends to be progressive and produces a number of symptoms in the sufferer the commonest of which is breathlessness. Symptoms worsen as the disease progresses and the chronic course of the disease can be interrupted by episodes of acute deterioration, often due to infection, which result in the need for an increase in treatment and sometimes hospitalisation.

The major risk factor for development of the condition is smoking although some cases relate to occupational dust exposure or indoor air pollution. In the UK around 25% of all adults continue to smoke although higher rates are seen in younger adults and in certain demographic groups (3).

The majority of treatments for COPD are aimed at providing symptomatic relief, and only long term oxygen therapy which is given to patients with chronic hypoxia has been shown to improve prognosis (4;5). There is much interest in new drugs that may alter the progression of the disease. Recently interest has focused on treatments that reduce the rate of exacerbations as these cause significant morbidity and mortality, and have enormous economic consequences (6). Chronic mucus hypersecretion (CMH) is a term which describes the symptom of a regular productive cough. It is often used synonymously with the term chronic bronchitis and is a common symptom in those with COPD. It seems to play a role in disease progression and exacerbation frequency (7) and currently there are no effective treatments

which reduce its impact. The underlying mechanisms causing it have generally been poorly understood, but in recent years some of the pathways involved in the production of mucus components have been identified and may provide targets for treatment of this condition (8).

In this chapter I will provide an overview of COPD, with a particular focus on the entity of CMH and its effects on morbidity and mortality, before going on to describe what is known about the cellular mechanisms involved in the control of mucin gene expression, the main protein component of secreted mucus.



1.2 Chronic obstructive pulmonary disease

1.2.1 Definition

Over the last fifty years the condition characterised by breathlessness and chronic airflow obstruction has been given a number of different diagnostic labels, often with associated definitions attached. Whilst this has helped characterise the condition in more detail many of these labels have also caused confusion regarding the causes and features of the disease. The various definitions were often produced by national respiratory societies and as such varied between countries. These definitions and some of the debate relating to their usage will be discussed in more depth later in this chapter. More recently an international group has been set up by the WHO and the US National Heart, Lung, and Blood Institute (NHLBI) to promote awareness of COPD with the aim of decrease morbidity and mortality from the condition. This group was called the Global Initiative for Chronic Obstructive Lung Disease (GOLD) and included members from many countries including both the developed and developing world. An expert panel from the group, including health professionals from many fields, has produced a consensus document based on current literature and national guidelines focusing on all aspects of COPD (9). This document is reviewed and updated yearly by the GOLD scientific committee. In the most recent report COPD is defined as '***a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases***' (10).

Although rather unwieldy this definition highlights a number of different aspects of the condition. It includes the pathophysiology of the condition (airflow obstruction), the underlying inflammation that is found in the airways and parenchyma of the lungs which appears to be an important part of the pathogenesis of the condition, and also details the natural history of the condition which is usually of a progressive nature. It is noteworthy that it does not include symptoms due to the condition or other terms such as chronic bronchitis and emphysema both of which have been used in previous

definitions and have caused confusion as to the spectrum of the disease being described.

From the definition it can be seen that the diagnosis of COPD rests on demonstrating airflow obstruction. This is achieved using a spirometer to measure exhaled lung volumes. Measures are made by getting the subject to perform a forced exhalation from total lung capacity. Two values are recorded: the forced expiratory volume in the first second (FEV_1) and the forced vital capacity (FVC) which is the total volume of air exhaled from maximum inspiration to maximum exhalation. From these values the "expiratory ratio" of FEV_1/FVC can be calculated. Airflow obstruction is said to be present when the expiratory ratio is < 0.7 (11) and the FEV_1, which has well defined reference values for different populations, is used to classify the severity of the disease.

The GOLD definition of COPD has been extremely useful for making the diagnosis of COPD both in individual subjects and as part of large epidemiological studies, but there are potential problems with it that have been identified. Perhaps the commonest source of debate relates to using the term COPD when describing other lung conditions which can cause airflow obstruction. Many of these conditions are usually well characterised by other features of the condition and hence labelled as such. For example patients with cystic fibrosis (CF) or non-CF bronchiectasis often have airflow obstruction on spirometry but these conditions are not usually included under the spectrum of COPD, and neither is the condition characterised by airflow obstruction found in some patients with connective tissue diseases due to an obliterative bronchiolitis although they present with the same symptoms as patients with COPD (12;13). There has been much debate around the overlap of COPD with some cases of asthma though. Here airflow obstruction is usually reversible but in a small proportion of patients it becomes fixed and progressive. Many of the clinical features found are the same as for COPD due to smoking. In addition asthma is often characterised by the presence of bronchial hyperresponsiveness (BHR) as measured by bronchial challenge (14). Many subjects with COPD can also be shown to have BHR and there are some groups that feel that BHR contributes to the development of COPD in smokers (15).

One other area where the definition stumbles is in those subjects who have developed smoking related emphysema but do not have airflow obstruction (16). Technically this excludes them from the diagnosis of COPD despite the fact that the aetiological agent is the same, they present in a similar manner clinically, and the majority of patients with COPD have demonstrable emphysema when the lungs are either sampled or imaged. These groups are uncommon though especially when compared to the huge numbers of patients with COPD and as such the current GOLD definition is generally accepted, and increasingly being used in studies of COPD.

As part of their work on the definition of COPD the GOLD group also describe a classification of severity of disease based on the degree of abnormality of FEV_1 (10). Although not validated this approach has also been used in other previous guidelines for the diagnosis and management of COPD (14;17;18). It is important to note that although the severity of disease as defined by severity of airflow obstruction relates to overall prognosis, as has been recognised for many years (19), it does not always correlate with the clinical severity as experienced by the patient. Nonetheless it is useful to subdivide COPD in this manner for descriptive purposes, classification in epidemiological studies, and grouping in clinical treatment trials. The GOLD classification also includes an "at risk" group to help raise awareness of a group where interventions such as smoking cessation may prevent development of disease. The classification of severity is shown in table 1.1.

Based on the above definition and spirometric classification COPD can be easily diagnosed in the majority of cases in conjunction with an appropriate clinical history and examination. Unfortunately by the time COPD causes symptom that are of a severity that cause the sufferer to seek medical advice the disease is already well advanced. At this stage there is little that can be done to reverse the changes that are already present in the lung, although there are treatments that can improve the patient's symptoms and interventions which can alter the progression of the disease such as smoking cessation.

As highlighted in the GOLD report and many other studies a large proportion of COPD in the population remains undiagnosed (9;20). Much of this is at the earlier stages of the disease where smoking cessation may significantly alter

the long term outcome. Some of the features of COPD will be discussed below.

Table 1.1 – GOLD classification of severity of COPD

Stage	Classification	Spirometry and characteristics
0	At risk	Normal spirometry Chronic symptoms (cough, sputum production)
I	Mild COPD	$FEV_1/FVC < 0.7$ $FEV_1 \geq 80\%$ predicted With or without chronic symptoms (cough, sputum production)
II	Moderate COPD	$FEV_1/FVC < 0.7$ $50\% \leq FEV_1 < 80\%$ predicted With or without chronic symptoms (cough, sputum production)
III	Severe COPD	$FEV_1/FVC < 0.7$ $30\% \leq FEV_1 < 50\%$ predicted With or without chronic symptoms (cough, sputum production)
IV	Very severe COPD	$FEV_1/FVC < 0.7$ $FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted plus chronic respiratory failure*
* Respiratory failure defined as an arterial partial pressure of oxygen (PaO_2) less than 8 kPa with or without an arterial partial pressure of carbon dioxide ($PaCO_2$) greater than 6.7 kPa whilst breathing air at sea level		

1.2.2 Epidemiology

Whilst the importance of COPD as a cause of morbidity and mortality across the world is well recognised it has proved difficult to accurately assess the prevalence of COPD in different populations. There are a number of reasons for this.

The definition of COPD has changed a number of times over the years and in addition has varied between countries making direct comparisons between populations difficult and comparison of prevalence at different times problematic. In relation to this point terms such as "chronic bronchitis" and "emphysema" have been included in previous definitions despite the fact that the first, which has an epidemiological definition, can occur in the absence of airflow obstruction, and the second is a pathological definition (21) and hence almost never assessed in population surveys. The diagnosis of both is also affected by cultural factors (22). Diagnosis of COPD using the most recent definition requires the demonstration of airflow obstruction and hence staff trained to perform the tests, and a system to exclude other conditions that cause airflow obstruction. This makes studies using spirometry more expensive to perform. Allowing for these methodological problems many surveys have been performed which give an idea of the prevalence of COPD in different populations.

In the American National Health and Nutrition Examination Survey (NHANES) the prevalence of self-reported or physician diagnosed COPD was around 6% of the total population with higher rates in men than women and higher rates in Caucasians compared to the black population. This represents around 10 million people in the USA. Airflow obstruction measured by spirometry though suggests around 24 million of the American population have a degree of COPD highlighting the under diagnosis of this common condition (23). In a European community study around 12% of the population had respiratory symptoms (cough, sputum, or dyspnoea) with 3.6% having airflow obstruction (24). In the UK based General Practice Research Database the prevalence of physician diagnosed COPD was found to be around 1.5% overall although there was a marked increase in the prevalence of COPD in women over the years studied (25). This result reflects the prevalence in the whole adult

population and it is important to recognise that prevalence of COPD increases with age: in this study the rates of COPD were 0.15% for adults aged 20 – 44 and around 8% for those over 65. Again as for the US survey this rate is undoubtedly an underestimate of the true prevalence amongst adults as many patients in the GP database with COPD will remain undiagnosed in the absence of symptoms in the early stages of the disease.

It can be seen therefore that COPD is a common condition and a significant cause of global mortality. The WHO currently list COPD as the fifth leading cause of death worldwide and it is important to realise that unlike many other chronic diseases mortality rates are continuing to rise. COPD also causes significant morbidity and the WHO global burden of disease project currently rates COPD as the twelfth commonest cause of disability-adjusted life years lost with a predicted rise to the fifth commonest by 2020 (2). The economic significance of this morbidity is massive: The annual cost of COPD to the NHS is estimated to be £982 million when direct and indirect costs are taken into account (18).

1.2.3 Aetiology, pathology, and pathogenesis of COPD

Although the clinical diagnosis of COPD relies on the demonstration of airflow limitation a number of processes can be found in the lungs' of these subjects which contribute to this final phenotype. Broadly speaking three processes are seen: mucous gland hyperplasia which is mainly seen in the large airways (those over 4mm in diameter), inflammation with remodelling and fibrosis in the small airways, and emphysema in the alveolar region of the lung (26). These are found to varying degrees in all patients. It is not clear whether they represent a single process that progresses with time or whether they are associated due to a common aetiological factor such as smoking (27). Their relationship to airflow obstruction is also complex although the small airways disease is probably responsible for the majority of airflow limitation seen in individuals. The aetiological factors responsible for and the pathological mechanisms seen in COPD will be discussed below.

1.2.3.1 Aetiological factors for development of COPD

The single most important aetiological factor in the development of COPD is smoking and this probably accounts for 85-90% of COPD seen in the developed world. Only around 10-20% of smokers develop significant airflow obstruction though (27). This has led to the concept of the "susceptible smoker" i.e. those smokers who go on to develop COPD, but in fact there is a continuum in susceptibility to the effects of cigarette smoke in terms of decline in FEV_1, as was noted in the original work of Fletcher and Peto (28). In their work which examined the rate of decline of FEV_1 in a cohort of middle-aged male workers in London the average yearly decline in FEV_1 was 30ml/year in non-smokers and 60ml/year in smokers (29). Even with this increased rate of FEV_1 decline the majority of smokers would not develop clinically significant airflow obstruction over the course of their life, but amongst the smokers were individuals with much higher rates of decline, often well above 100ml/year, who develop airflow obstruction at an early age. The factors which make an individual susceptible to cigarette smoke are still the subject of much debate but it is likely to be polygenic in origin.

Air pollution has been strongly linked to morbidity and mortality in those already suffering from cardio-respiratory disease. Whether long-term exposure to outdoor air pollution increases the likelihood of developing airflow obstruction is less clear (30). Higher levels of pollution are linked to mucus hypersecretion though. In developing countries exposure to high levels of indoor air pollution, often from open solid-fuel fires and stoves, may be important in the development of COPD. This may be particularly so in women (31). Many of the long term studies addressing these questions have been hampered by the fall in levels of air pollution that has occurred over the last half century as well as other confounding factors.

There is a strong association between COPD and the presence of CMH. This is undoubtedly due to the role cigarette smoking plays in the aetiology of both conditions. There was a belief that COPD developed in stages with the first stage being the development of mucus hypersecretion. This excessive mucus caused an impairment of host defences such that infection of the airways occurred. Infection was then responsible for causing inflammation in the

airways and this resulted in damage to the lungs leading to emphysema and bronchiolar obstruction. This theory was known as the "British hypothesis" for the aetiology of COPD (27). The links between CMH and COPD are still the subject of some debate. It has become increasingly clear that CMH and infection cause significant morbidity in those with COPD though (32), but the relationship of CMH with an accelerated decline in FEV_1 resulting in premature COPD is more contentious. These points will be discussed in more detail later in this chapter.

Deficiency of the antiproteinase alpha-1 antitrypsin (α_1-AT) is a major risk factor for the development of emphysema. It is the only genetic predisposition for COPD that has been definitively identified. α_1-AT is predominantly produced in the liver although bronchial epithelial cells and macrophages in the airway also produce smaller amounts (33;34). It is the major circulating antiproteinase and is important for the inactivation of elastase and other proteolytic enzymes that are released from activated neutrophils. Left unchecked elastase can cause extensive tissue destruction. Individuals with α_1-AT deficiency who smoke develop COPD at an early age. It is felt that the unopposed action of neutrophil elastase is responsible for this, and this has led to the idea that a proteinase/antiproteinase imbalance is responsible for the development of COPD.

α_1-AT is coded for by a single gene on chromosome 14. Numerous alleles are recognised. The commonest allele is PiM (the alleles are defined by the electrophoretic movement of their protein products) and hence the commonest genotype is PiMM. This is found in approximately 85% of the UK population and individuals have normal serum α_1-AT levels. PiS and PiZ are the next two commonest alleles and in heterozygotes produce the genotypes PiMS and PiMZ. These genotypes result in a reduction of serum levels of α_1-AT to around 75% and 57% of normal respectively, but individuals rarely develop COPD even if they smoke. The PiZZ genotype has circulating levels of α_1-AT of 10-20% normal and these individuals are highly likely to develop COPD at a young age if they smoke. The serum levels of α_1-AT are reduced because the Z allele produces a change in the structure of α_1-AT such that the active site of the protein binds other α_1-AT molecules to form long polymer chains. These chains can not be secreted by the cell. α_1-AT deficiency is

found in around 1-2% of patients with COPD but this rate increases if a young population with COPD is examined (35-37).

There are a number of occupations where there is exposure to high levels of dust in the working environment. It is thought that this increases the risk of developing COPD although this is still the subject of some debate. There is evidence that coal miners are at risk of an accelerated decline in FEV_1 even if they are non-smokers, and a similar effect may be seen in cement workers and gold miners (38). Chronic exposure to fumes from cadmium, which is used in a number of industries, has also been associated with an increased risk of COPD (39). Studies of workers in these occupations have often given contradictory results though. This is likely to be due to difficultly in controlling for confounding factor such as smoking, socioeconomic factors, environmental pollution, and nutritional status in the populations studied. It is generally accepted that there is an increased risk of COPD in those working in the professions mentioned above though. In addition high levels of occupational dust exposure are associated with the development of CMH (40).

Airway hyperresponsiveness and allergy may be related to the development of COPD. This was originally suggested by Dutch investigators in the 1960s and has been termed the "Dutch hypothesis". They proposed that those smokers susceptible to developing COPD had an underlying atopic constitution with BHR, as is seen in patients with asthma, even before they started smoking (41). It was the presence of this underlying tendency which led to the accelerated decline in FEV_1 and hence development of airflow obstruction if the individual smoked (15). This hypothesis continues to be a subject for debate. It is recognised that smokers have higher levels of serum IgE (a characteristic finding in atopy) than non-smokers, and certainly some have a detectable sputum eosinophilia, but there are differences in the nature of the BHR seen in subjects with COPD compared to those with asthma. Also rates of atopy do not differ between smokers and non-smokers. Opponents of the hypothesis also point out that COPD and asthma are both common conditions and significant numbers of individuals will have evidence of both processes because of this (42;43).

Other factors suggested to be important in the development of COPD include nutritional status, low birth weight, early childhood respiratory infections, and lower socioeconomic status. It is often difficult to tease out the exact factors which may be important in these areas due to multiple confounders (27).

1.2.3.2 Pathological changes in of COPD

Although the aetiological agents responsible for COPD are mostly well established and the final pathological phenotype seen in COPD clearly described the pathological mechanisms leading to these changes is not fully understood. The structural changes seen in COPD will be described below, and this will be followed by a brief discussion of some of the processes thought to be important in the development of these changes.

Chronic bronchitis or CMH describes the symptoms of a chronic productive cough and is found in many smokers with or without COPD. Pathologically a number of changes are seen in the large airways of smokers with CMH including changes in the submucosal glands and the epithelium. The submucosal glands are the predominant source of airway mucus and in CMH there is an increase in their size. This is due to both an increase in individual cell size (hypertrophy) and an increase in cell numbers (hyperplasia) (44). At the surface epithelium goblet cell hyperplasia occurs and there is a reduction in the number of ciliated cells with focal squamous metaplasia (45). These changes result in an increase in airway mucus volume and an impairment of mucociliary clearance and will be discussed in more detail in the section on mucus hypersecretion below (Chapter 1.2.5)

The small airways (those bronchi and bronchioles below 2-3mm size) are the major site of airflow limitation in individuals with COPD (46). By the time airflow obstruction is clinically detectable a number of irreversible changes to the structure of the small airways has occurred. There is loss of epithelial cells and goblet cell hyperplasia in the epithelium, and smooth muscle hypertrophy, peribronchiolar fibrosis, oedema and inflammation in the airway wall (45). These changes may occur well before the clinical presentation of COPD though (47) and a number of studies have shown that in "healthy" smokers i.e. those with no demonstrable airways disease, some of these structural changes and significant inflammation are already present (48). This

inflammation may be responsible for the development of some of the structural changes. As well as the structural changes excessive mucus production in the small airways may worsen airflow limitation by physically occluding the lumen. It has also been hypothesised that the increase in mucus in the airway lining fluid with a corresponding reduction in surfactant levels results in an alteration of the surface tension of the fluid compared to normal. This makes the airways more susceptible to collapse during expiration again worsening airflow obstruction (49).

Emphysema is defined pathologically as a lesion where there is "an abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls and without obvious fibrosis (21)". Again as for the small airways disease described above this is a final disease state and the steps which result in gross emphysema remain unclear. The development of emphysema takes many years though and early changes can be seen in the lungs of "healthy" smokers. These changes include microscopic emphysema where small defects in the alveolar wall are seen, loss of elastic fibres in alveolar walls, and bronchiolar distortion due to loss of the attachments between the bronchioles and the surrounding lung parenchyma which normally help maintain the patency of the small airways (50;51).

Traditionally emphysema has been sub-classified according to its distribution in the acinus. The acinus is defined as the area of lung supplied by one terminal bronchiole, the last airway before alveoli appear in the bronchiole wall. Although a number of different types of emphysema have been described two predominate in COPD: Centriacinar (or centrilobular) emphysema is characterised by destruction of the central portion of the acinus around the terminal bronchiole whereas panacinar (panlobular) emphysema affects the whole of the acinar unit (44;52). The first of these forms is classically seen in smoking related emphysema and typically affects the upper lobes of the lung whereas the second is often seen in individuals with α_1-AT deficiency and tends to be more severe at the lung bases. Both types can be found in the lungs of smokers though and there is some debate as to whether they represent different pathologies or differing degrees of the same process (53).

Vascular changes are also seen in the lungs of individuals with COPD. In emphysematous areas, along with the destruction of the alveolar wall, there is destruction of the capillary bed although arterioles persist in these abnormal areas. There are also alterations in the walls of small arteries and arterioles in the lung with an increase in smooth muscle in the media and appearance of muscle in the external elastic lamina. In severe disease where there is significant hypoxaemia pulmonary arterial hypertension can occur and ultimately this can lead to right ventricular dysfunction (45).

1.2.3.3 Pathogenesis of COPD

The structural changes in the epithelium, airways, parenchyma, and vasculature described above represent the result of a chronic process which takes several decades to develop. The events leading to these changes are not clear but there are a number of areas which are likely to be important and will now be discussed.

Inflammation is seen in both the large and small airways and in the alveoli of individuals with COPD. It is well established that this develops as a reaction to the inhalation of noxious stimuli such as cigarette smoke or particulate matter (54). This inflammation involves a number of cells and these are likely to play a major role in the development of the pathological changes of COPD (55). In the submucosal glands there is an accumulation of mast cells, macrophages, and neutrophils (56). The airway epithelium contains increased numbers of neutrophils and lymphocytes with the latter being T-cells of the $CD8^+$ type (57). In the airway wall beneath the epithelium increases in lymphocyte and macrophage numbers are seen (58). There does appear to be a correlation between the severity of airflow obstruction and the degree of inflammation seen in the airways (53;59) but many of these inflammatory changes are also found in smokers without COPD. In those with COPD smoking cessation does not seem to lead to a resolution of the inflammatory changes in the airway wall (60).

In the alveoli there is also an ongoing inflammatory process with an increase in the numbers of $CD8^+$ T-cells in alveolar walls (61). Again a correlation has been found between the degree of inflammation and the severity emphysema (62).

The inflammatory cells and other cells in the airway release many proteins which are thought to play a role in mediating tissue damage and further promoting an inflammatory phenotype (63). These include pro-inflammatory mediators such as TNFα, IL-8, and leukotriene B4 all of which are found in increased concentrations in the sputum of subjects with COPD (64;65), and proteinases such as neutrophil elastase and matrix metalloproteinases (MMP) from macrophages. The inflammatory mediators recruit more inflammatory cells into the airway and activate them resulting in release of further pro-inflammatory mediators. The proteinases, particularly neutrophil elastase but also MMPs and cathepsins, are thought to be critical in the development of emphysema (66;67) as described in the hypothesis that there is a proteinase/antiproteinase imbalance in the lung that results in COPD development. This postulates that there is an increased proteinase burden in the lungs of smokers and a relative deficiency of antiproteinases such as α_1-AT, α_1-antichymotrypsin, and secreted leukocyte proteinase inhibitor (SLPI). This imbalance results in excessive tissue destruction in response to pro-inflammatory stimuli such as smoking and infection (68). Animal studies have certainly demonstrated the development of emphysema in rats treated with neutrophil elastase, and MMP-12 deficient mice do not develop emphysema (69), but this simple imbalance model is not the whole picture and much work continues in this area.

More recently the role of oxidant stress in the lung has been investigated as a causative factor for the development of COPD (70). Oxidants are potentially harmful compounds and are found in higher levels in the lungs of subjects with COPD. This may be the result of their introduction into the lungs either from exogenous sources such as cigarette smoke, a complex mixture containing high concentrations of oxidants and other free radicals (71), or from endogenous sources such as neutrophils and other inflammatory cells where they are normally produced as part of the cell's antibacterial defences (72;73). In excess these oxidants can directly damage components of the airway and alveoli, and can inactivate antiproteinases hence worsening proteinase derived damage (70). Similar to the proteinase/antiproteinase imbalance model there may be an imbalance between oxidant burden and antioxidant defences (which include mucins, glutathione, and negatively

charged proteins) such that tissue damage results. Some of the processes involved in the development of COPD are shown in figure 1.1.

A number of other factors are likely to be involved in the pathogenesis of COPD possibly interacting with the proteinase/antiproteinase and oxidant/antioxidant models described above. There appears to be a genetic susceptibility to accelerated decline in FEV_1 in individuals with COPD with higher rates of COPD in first degree relatives of affected individuals (36;74). Whilst the link of COPD with α_1-AT deficiency as discussed above is a clear example of genetic susceptibility to the effects of smoking, there is also evidence from population studies that polymorphisms in other genes may increase the risk of development of COPD. These polymorphisms that have been linked to an accelerated decline in FEV_1 decline often seem to occur in genes encoding proteins that are thought to be important in the pathogenesis of COPD. Examples of this include polymorphisms in genes encoding the matrix metalloproteinases MMP1 and MMP12 (75) and those for the antioxidant enzyme glutathione S-transferase (76). Polymorphisms in the gene encoding for TNFα have also been associated with COPD in a Japanese population (77). Other studies have not shown these associations though. Differences between studies may relate to the ethnicity of the population studied or the disease phenotype studied e.g. pure emphysema vs. COPD. This growing field has been recently reviewed by Molfino (78).

The fact that airway inflammation persists even after stopping smoking (60) has led some to suggest that underlying infection may be driving much of this inflammation. A number of studies have shown evidence of latent infection (persistence of viral DNA and proteins without replication of complete virus) with adenovirus in the lungs of patients with COPD (79;80). This latent infection may result in ongoing production of viral proteins in cells of the bronchial epithelium and lung parenchyma. These proteins may predispose to the development of COPD by altering the inflammatory response to cigarette smoke and up-regulating the production of the neutrophil chemoattractant IL-8 (81). Other groups have found that individuals with COPD who had evidence of persistent infection with Respiratory Syncytial Virus (RSV) had an accelerated decline in FEV_1, and increased airway inflammation as measured

by inflammatory cytokines in sputum, compared to those with lesser degrees of infection (82).

In summary it can be seen that a number of pathological processes are present in individuals with COPD. The mechanisms that produce these changes are complex, occur over many years, and may become self-perpetuating once present. Many questions remain unanswered though and whether these changes are reversible is not known.

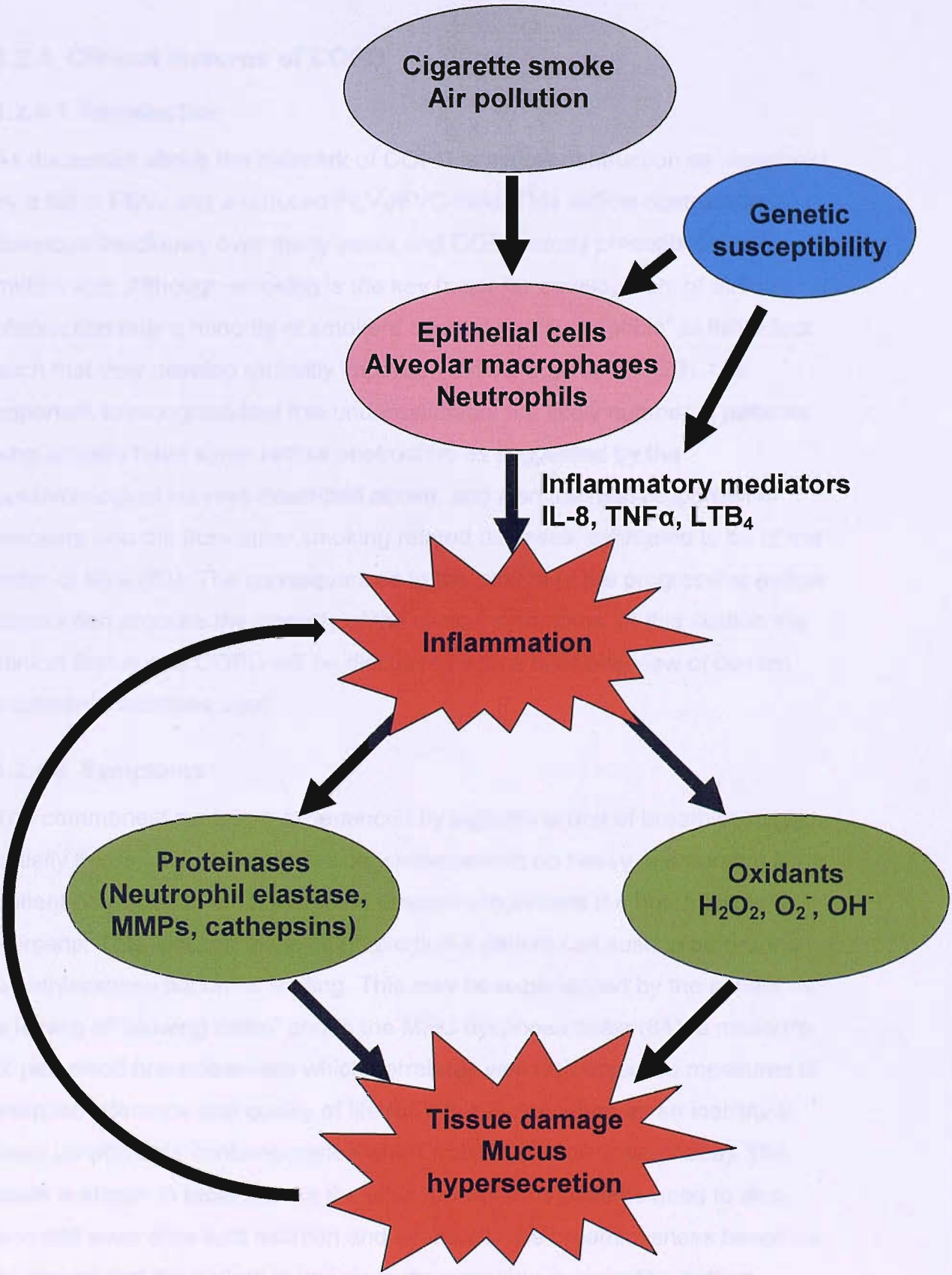


Figure 1.1 – Processes involved in the development of COPD

1.2.4 Clinical features of COPD

1.2.4.1 Introduction

As discussed above the hallmark of COPD is airflow obstruction as measured by a fall in FEV_1 and a reduced FEV_1/FVC ratio. This airflow obstruction develops insidiously over many years and COPD rarely presents before middle age. Although smoking is the key factor for development of airflow obstruction only a minority of smokers seem to be "susceptible" to this effect such that they develop clinically important airflow obstruction (29). It is important to recognise that this underestimates the likely number of patients who actually have some airflow obstruction as suggested by the epidemiological surveys described above, and also the high proportion of smokers who die from other smoking related diseases, estimated to be of the order of 50% (83). The consequences to the patient of the progressive airflow obstruction produce the majority of the clinical symptoms. In this section the clinical features of COPD will be discussed with a brief overview of current treatment modalities used.

1.2.4.2 Symptoms

The commonest symptom experienced by patients is one of breathlessness. Initially this is mild such that it is only experienced on heavy exertion but if the patient continues to smoke and the disease progresses the breathlessness worsens. This reduces the level of exertion a patient can sustain before the breathlessness becomes limiting. This may be experienced by the patient as a feeling of "slowing down" and in the MRC dyspnoea scale (84), a measure of perceived breathlessness which correlates well with objective measures of exercise tolerance and quality of life (85), this is described as an inability to keep up with their contemporaries when walking (Grade 3 dyspnoea). The scale is shown in table 1.2. As the situation worsens patients need to stop and rest even after light exertion and eventually the breathlessness becomes so severe that the patient is unable to do even simple tasks like getting dressed without stopping. At its most extreme the sufferer may even feel breathless at rest. It is breathlessness which usually results in the subject seeking medical help and is usually the most disabling symptom.

Table 1.2 – The MRC Breathlessness Scale

Grade 1	I only get breathless with strenuous exercise
Grade 2	I get short of breath when hurrying on the level or walking up a slight hill
Grade 3	I walk slower than people of the same age on the level because of breathlessness or have to stop for breath when walking at my own pace on the level
Grade 4	I stop for breath after walking 100 yards or after a few minutes on the level
Grade 5	I am too breathless to leave the house or breathless when dressing or undressing

The sensation of dyspnoea is unpleasant and in some individuals causes significant anxiety. This anxiety can alter their breathing pattern worsening their dyspnoea. A vicious circle can develop which limits the sufferer's exercise capacity further. In some cases this anxiety becomes so severe that the individual feels they are unable to leave their house due to the symptoms they experience (86;87).

Besides breathlessness individuals with COPD may suffer from a number of other symptoms. A persistent cough is common and although this may be dry more often it is productive and fulfils the definition of CMH. Patients report that their cough is often worse in the mornings and during the winter months and the sputum volume may increase after upper respiratory tract infections. Cough may precede the development of breathlessness by many years and is often dismissed by the individual particularly if they smoke when it is attributed to a "smokers cough" (1). As highlighted in the GOLD classification of severity (Table 1.1) these people should be considered "at risk" of developing airflow obstruction although there is no good evidence that there

is a progression from cough to airflow obstruction (88), and many patients with severe airway obstruction have never experienced cough or sputum production previously.

As the lung disease progresses complications can occur. Hypoxaemia is common in advanced disease and this can lead to abnormalities of salt and water handling in the kidney and right heart dysfunction. This causes peripheral oedema which the patient usually notices as swollen ankles (89).

It is increasingly recognised that as well as localised inflammation in the lungs there is a significant systemic inflammatory response in individuals with severe COPD (90). This may be responsible for some of the global effects of COPD such as weight loss and fatigue which can be dramatic and disabling in some individuals. This systemic inflammation along with deconditioning from inactivity also results in loss of muscle bulk and hence strength (91;92). This can adversely affect the subject's exercise tolerance.

Whilst there is often a degree of variability in an individual's symptoms, many patients reporting good and bad days, this variation is small compared to that seen in asthma, and overall there tends to be little change in the symptoms over the short term. In the long term if the individual continues to smoke their symptoms invariably progress and complications occur. If they can stop smoking and if interventions such as pulmonary rehabilitation programmes are available there may be some improvement in their symptoms or at least stability (93;94).

All individuals with significant COPD have an increased risk of acute exacerbations of their symptoms usually due to infection or following changes in the environment e.g. increased air pollution (95). These result in an acute deterioration of symptoms over 1 or 2 days which may require a medical intervention. It may take a long time, particularly in those with the most severe disease, for the individual's health status to return to its pre-exacerbation level (96).

1.2.4.3 Clinical findings

Clinical examination in the early stages of COPD is often normal and even in the most advanced disease may surprisingly remain so. Where abnormalities are seen they are usually in the chest with signs of lung hyperinflation, use of

the accessory muscles of respiration even during quiet breathing, and pursed lip breathing with a prolonged expiratory phase. Auscultation of the chest is often normal but there may be a reduction in the intensity of breath sounds due to the parenchymal destruction and wheezes and crackles may be heard (1). In advanced disease the patient may be cyanosed and there may be evidence of pulmonary hypertension and peripheral oedema. There may be evidence of recent weight loss and loss of muscle bulk.

1.2.4.4 Investigations

A likely diagnosis of COPD can often be made from the history and examination alone but a definite diagnosis requires documenting airflow limitation using spirometry (10;97). More detailed pulmonary function tests can be performed to look at the total lung capacity and the residual volume of the lung both of which can help in the diagnosis of disease extent, and the ability of the lung to transfer gases into the blood can be measured using an inhaled mixture of carbon monoxide, helium, and oxygen (98;99). Again this gives more detail regarding the severity of lung function impairment and may be useful in differentiating smoking related COPD from other conditions characterised by airflow limitation (100).

Radiological investigations are not usually required to make the diagnosis of COPD but are useful to exclude other causes of breathlessness, and to look for evidence of other smoking related conditions such as lung cancer. Oxygen levels can be assessed non-invasively using pulse oximetry or by formal arterial blood gas analysis which also allows assessment of CO_2 levels. A number of other tests such as exercise testing or echocardiography can be performed as clinically indicated.

1.2.4.5 Treatment of COPD

The aims of treatment for COPD are to prevent disease progression, relieve symptoms, and reduce complications of the disease (10;97). As the early stages of the disease rarely produce troublesome symptoms individuals do not seek help from medical services. It as at this stage though that evidence of mild disease can be found and they may have symptoms which they have dismissed as insignificant. Although these symptoms rarely need treatment it

is at this stage that smoking cessation may have the largest impact in preventing disease progression. Smoking cessation can be aided by using nicotine replacement products and the help of support groups (101).

By the time symptoms develop a significant amount of lung damage has been sustained and this does not appear to be reversible. Even so stopping smoking at this stage is beneficial to the long term outcomes of the disease: it reduces the rate of decline of the FEV_1, lessens the chance of exacerbations due to respiratory infections, and reduces the risk of other smoking related diseases particularly cardiovascular events (102).

Breathlessness is managed either pharmacologically or non-pharmacologically. Non-pharmacological treatments include pulmonary rehabilitation programmes, physiotherapy input, breathing control techniques, and nutritional advice (94). These will not be discussed further here.

Pharmacological interventions aimed at relieving breathlessness can improve an individual's exercise tolerance and quality of life. The drugs used depend on the severity of the individual's symptoms and the stage of their disease and are often used in combinations supported by non-pharmacological interventions (102).

The mainstay of treatment for breathlessness is bronchodilator therapy. A number of different drugs are available. These include β_2-agonists such as salbutamol, and anticholinergics like ipratropium bromide. Both these classes of drugs are available in short and long acting forms. The short acting bronchodilators improve symptoms and can improve spirometry (103-105). They can be used as needed or given regularly with similar efficacy (106).

Long acting bronchodilators include the β_2-agonists salmeterol and formoterol and the newer long acting anticholinergic tiotropium. As for the short acting bronchodilators these drugs are able to improve symptoms and exercise tolerance in patients with COPD (107). There is a suggestion in some studies that tiotropium may also reduce exacerbation frequency (108;109).

Bronchodilators from the two classes can be combined and this may give better symptom relief. The majority of formulations of the drugs are for inhaled therapy and this is the preferred route of delivery although oral salbutamol can be used.

The methylxanthine theophylline is another agent that can be used for treating dyspnoea. Its bronchodilator effect is less than that of the other agents discussed above but it may have other actions which help patients with COPD such as an anti-inflammatory effect and enhancing diaphragmatic contractility. Given orally it has a narrow therapeutic window and potentially serious cardiac side effects (102).

The place of corticosteroids in the treatment of COPD has been a source of debate for some time. It was hoped that they may slow the progression of airflow limitation due to their anti-inflammatory effect. A number of large trials were performed to test this hypothesis and essentially showed that inhaled corticosteroids do not alter the rate of decline of FEV_1 in patients with COPD (110-113). There was evidence that inhaled steroids improved health status and symptoms, and reduced the frequency of exacerbations in those with more severe airflow obstruction and frequent exacerbations though (112). These effects have been investigated further in more recent studies using inhalers which contain both an inhaled corticosteroid and a long-acting β_2 agonist. There may be benefit of using the 2 drugs in combination (114-117). In view of the impact of exacerbations on quality of life inhaled corticosteroids are recommended in patients with $FEV_1 < 50\%$ predicted and frequent exacerbations (18).

Oxygen therapy is recommended for individuals with COPD who have any of chronic hypoxaemia, nocturnal hypoxaemia or desaturation on exercise. Oxygen used as long term oxygen therapy (LTOT) for a minimum of 15 hours a day to those with a $PaO_2 < 7.3kPa$ is the only intervention apart from smoking cessation that has been shown to improve the prognosis in COPD (4;5).

A number of other pharmacological treatments have been used for COPD in the past including respiratory stimulants, long term oral corticosteroids, and long term prophylactic antibiotics. These treatments do not seem to have any benefit though and are currently not recommended for use (102). Mucolytics will be discussed in more detail later in this chapter.

1.2.5 Chronic mucus hypersecretion and COPD

1.2.5.1 Introduction

A productive cough is a common symptom in many respiratory conditions. When this occurs as part of a self-limiting illness and the sputum production resolves either spontaneously or following treatment it is usually described as acute bronchitis. This nearly always follows an insult to the airways such as a viral infection or inhalation of a noxious substance, and can occur in previously fit individuals or those with underlying lung disease (118). Chronic bronchitis describes a persistent productive cough which tends to be present for many months and often many years. This symptom is seen in many patients with COPD. This close association led to these two terms, as well as the terms emphysema and asthma, being used interchangeably for many years to describe a clinical picture characterised by cough, sputum, and breathlessness (22;119). It has only been in the last half century that the different conditions have been more clearly defined. The confusion that resulted in the use of these terms for the same and different conditions led to a delay in the understanding of the causes of the conditions and the underlying pathological changes associated with them. The background to this situation and the development of the modern definitions will now be outlined.

1.2.5.2 History of chronic bronchitis as a disease entity

The term chronic bronchitis was introduced to medical literature by Charles Badham, a British physician, in the early 1800s. He used the term to describe a chronic cough with sputum and recognised that chronic bronchitis was often a disabling condition (120). Laënnec, the inventor of the stethoscope, described the clinical features of emphysema in his 'Treatise on diseases of the chest' in 1821 outlining the pathological findings in the lungs of patients who had severe breathlessness, cough, and sputum production (121). The association of these same symptoms with abnormalities of pulmonary function became possible in the mid 19th century after the invention of the spirometer by John Hutchinson in 1846, although this only measured vital capacity and its uptake was limited initially (122).

Over the course of the first half of the twentieth century death rates from "chronic bronchitis" were increasing, particularly among lower social classes. This was an era when air pollution was rising, cigarette smoking was increasing, and working conditions in many factories were poor. Despite its high prevalence there was little research into chronic bronchitis and emphysema at this time, but changes were occurring in the definitions of the different conditions and what become known as emphysema included the symptoms of chronic bronchitis as part of the diagnostic criteria.

In 1951 as the number of people suffering from and dying from chronic bronchitis continued to rise the Association of Physicians of Great Britain and Ireland held a major symposium on chronic bronchitis to encourage investigation of this condition. In 1953 Oswald published an account of the clinical features of 1000 cases of chronic bronchitis (123). His paper focused on a condition characterised by cough, sputum, and breathlessness which could not be placed in another diagnostic category such as heart failure or TB, but he noted that 'some difficulty was experienced in determining the common usage of the term "chronic bronchitis" as opposed to "asthma"'. Thus in this study he excluded patients whose breathlessness was episodic with chest tightness which was felt to be more in keeping with a diagnosis of asthma rather than chronic bronchitis alone.

Oswald showed that chronic bronchitis usually presented in middle age or later except in the smaller proportion who had symptoms from childhood (and probably had bronchiectasis by modern day definitions), and became worse in the presence of upper respiratory tract infections (90% of patients describing colds "going down to the chest" compared to 27% of controls) or during the winter months particularly with damp weather. It was associated with breathlessness in 70% of patients of whom 52% had constant breathlessness. It produced a degree of disability in nearly all patients ranging from mild (occasional absences from work but normal in between attacks) to severe (unfit for work or able to do light work only), and many patients felt that their working conditions worsened their symptoms. It is interesting to note that in his cohort of patients with chronic bronchitis 95% of men and 63% of women were smokers. Many of these felt smoking aggravated their bronchitis and over half had cut down or stopped because of their symptoms with

Oswald noting that 'a surprisingly large number had abandoned or reduced the habit with benefit.' In his discussion he interprets the breathlessness experienced by over half the patients as being due to emphysema. He notes that 'emphysema is an almost invariable complication of chronic bronchitis provided the bronchitis persists long enough.' This view was the commonly accepted theory at the time.

1.2.5.3 Investigation of chronic bronchitis 1950 onwards

Further impetus for research into the condition came in December 1952 when a severe dense smog affected London for over a week. Official reports on the smog suggested that in this week there were over 4000 extra deaths in people suffering from chronic respiratory or cardiac disease. More recent studies have calculated that the smog may have caused an excess of over 12000 deaths in the following 2-3 months (124). Levels of air pollution in this 1 week period showed an average total particulate matter concentration of 1400 $\mu g/m^3$ and sulphur dioxide levels of 0.57 ppm. Modern regulations on air quality state a safe upper limit of 83 $\mu g/m^3$ and 0.047 ppm for particulate matter and sulphur dioxide concentrations respectively (124).

The next year the British Medical Research Council set up a committee to specifically promote research into chronic bronchitis. One of the main problems they faced was the lack of uniformity in the labels given to patients who had combinations of symptoms including cough, sputum, and breathlessness, with or without measurable airflow limitation: The same patient could be labelled as suffering from chronic bronchitis, emphysema, advanced or severe chronic bronchitis, chronic bronchitis and emphysema, or even asthma depending on the clinician's preference. There also seemed to be national preferences with British physicians usually describing this sort of patient as having advanced chronic bronchitis without referring to emphysema whereas in America physicians tended to use the diagnostic label emphysema without the term chronic bronchitis (22).

These difficulties in terminology led to the influential CIBA guest symposium on chronic bronchitis and emphysema in 1958 (21) and a similar report from the Committee on Diagnostic Standards of the American Thoracic Society (ATS) in 1962 (125) which produced definitions that form the basis for those

that are in usage today. The CIBA symposium defined emphysema on an pathological basis as 'a condition of the lung characterised by increase beyond normal in the size of air spaces distal to the terminal bronchiole either from dilatation of from destruction of their walls.' This was then subdivided on the distribution of the emphysema and whether there was dilatation or destruction of the alveolar walls. Chronic bronchitis was a clinical definition describing 'the condition of subjects with chronic or recurrent excessive mucus secretion in the bronchial tree' and was one of the conditions included in the group 'Chronic non-specific lung disease' which required exclusion of a number of other conditions which could present in a similar manner e.g. infective lung diseases such as TB or heart failure. They stated that 'chronic' should be defined as 'occurring on most days for at least 3 months in the year during at least two years' (21).

The relevance of airflow obstruction was noted with the term 'Generalised obstructive lung disease' being introduced. This included subgroups of irreversible and intermittent obstruction i.e. what would now be considered COPD and asthma respectively. The symposium concluded that patients should be classified on the components they had e.g. irreversible obstructive lung disease (with or without emphysema) or chronic bronchitis with obstructive lung disease (with or without emphysema). The ATS definition for chronic bronchitis was essentially the same as the CIBA symposium but the definition of emphysema required destruction of the alveolar walls to be present rather than dilatation or destruction (125). The term 'Generalised obstructive lung disease' never came into common usage with the terms chronic obstructive pulmonary, lung, or airways disease (COPD, COLD, COAD) being used more often. These definitions allowed greater uniformity in the diagnostic labels given to diseases when clinical, pathological, physiological, and radiological measurements were being reported.

In 1966 Burrows and Fletcher reported on the clinical spectrum of disease in a group of patients from chest clinics in Chicago and London all of whom had a significantly reduced FEV_1 (126). They described patients as being 'Type A' with pure emphysema and 'Type B' with predominantly bronchitic symptoms and cor pulmonale. Their report highlighted the clinical similarities between patients on both sides of the Atlantic who had previously been given different

diagnostic labels, and the spectrum of clinical phenotypes associated with airflow limitation.

Despite these improved and more exact definitions the older diagnostic labels tended to persist in common usage and continue to do so.

1.2.5.4 The natural history of CMH and airflow obstruction

It was in the 1950s and 1960s that clear links between smoking and disease were being established as a result of numerous epidemiological studies (127). The link was particularly strong for respiratory disease: Lung cancer, chronic bronchitis, and obstructive lung disease were all strongly associated with smoking habits. In addition the natural history of established obstructive lung disease was being described by workers including Burrows who showed the link between severity of airflow obstruction and mortality from the condition (19). The early events that led to this final stage were not clear though. There were a number of possible hypotheses with perhaps the main theories being known as the British and Dutch hypotheses as described above (Chapter 1.2.3).

The British hypothesis and the role of chronic bronchitis in the development of airflow obstruction were investigated in the seminal work of Fletcher and Peto in the 1960s and 1970s (28). This provided a clearer understanding of the effects of cigarette smoke on FEV_1 decline and the role of mucus hypersecretion in this regard. Much of the subsequent debate over mucus hypersecretion and airflow obstruction stems from this work as will now be described.

At the time of Fletcher's study it was recognised that individuals with CMH had a higher frequency of chest illnesses and lower lung function than those without. It was also clear that those most disabled by COPD had had symptoms for many years before getting to this advanced stage (123;128). Many concluded from this that the development of airflow limitation resulted from the long term effects of CMH and recurrent chest illnesses. Much of the evidence to support this notion came from retrospective studies and reports based on patient completed questionnaires. These were potentially subject to bias. Moreover it had been noted that many patients with end-stage emphysema had never had the symptoms of CMH thus casting some doubt

on the validity of the British hypothesis (28). To answer some of the questions regarding the natural history of CMH and airflow obstruction a prospective study was performed.

Fletcher's study planned to measure lung function over a five year period collecting information on the presence of chest symptoms, chest illnesses, sputum volumes and purulence, and a number of other factors at regular intervals during this time. The study recruited a cohort of around 1100 men from 2 places of work: the London Transport workshops and the Post Office Savings Bank. Smokers, non-smokers, and those with chronic bronchitis symptoms were included. Although originally planned to last 5 years the study actually ran for 8 years.

The results were striking. They showed that compared to the mean rate of decline in FEV_1 of 30-40 ml/year in non-smokers the group of smokers had a mean rate of decline of 60-80 ml/year. They identified that some smokers were 'susceptible' to the effects of smoking and that this was a continuum of effect with some smokers having rates of decline of FEV_1 of well over 100ml/year. It was also found that if those 'susceptible' smokers stopped smoking their rate of FEV_1 decline returned to that of the non-smokers although their lung function did not improve (29).

As important as these findings was the fact that neither the presence of CMH or episodes of chest illness had an affect on the rate of FEV_1 decline even though those with CMH suffered from more chest illnesses. From these findings they concluded that smoking was causing two unrelated disease processes: firstly an obstructive pulmonary disease and secondly a hypersecretory disorder presenting as chronic bronchitis/CMH. Subsequent studies by workers in other countries including a cohort study of 575 French workers supported these findings (129;130).

Peto and colleagues also reported on 20 year mortality relating to airflow obstruction and respiratory symptoms pooling data from 5 British surveys which had been investigating these factors (131). They showed that, as expected, there was a strong association between degree of airflow obstruction on spirometry and mortality from COPD, but also that airflow obstruction was associated with mortality from other causes including vascular disease and lung cancer. Although there was an association

between CMH and mortality it disappeared when FEV_1 was taken into account, and conversely the link between FEV_1 and mortality remained even when adjusted for presence of CMH.

These studies seemed to agree that although causing troublesome symptoms and a tendency to infection CMH was not involved in the development of airflow obstruction and did not increase mortality. This implied that the British hypothesis was incorrect and as a result there was a loss of interest in CMH as a disease entity. It is of note though that all these studies were of selected cohorts rather than a randomly selected population, did not include those with significant airways disease, and did not include any women at all. These factors may have had a significant effect on the results seen.

1.2.5.5 Recent studies on CMH and health

The studies described above relegated the symptoms of CMH to a bit part in the natural history of COPD progression for many years. Recently a number of groups have reported studies which suggest that CMH may not be such an innocent bystander after all, and this has led to renewed interest in treatment options for patients with this condition. Data suggesting this initially came from a report of the long term health outcomes of the cohort of French workers mentioned above (132). This study reported mortality data 22 years after the initial survey was completed. In the original study (129) the findings agreed with the conclusions of Fletcher and colleagues regarding CMH and rates of FEV_1 decline showing no effect of the former on the latter. The later report demonstrated a link between level of FEV_1 and mortality, as has been documented by other groups, but also found an increase risk of death in those individuals who had CMH (132). This association persisted even after adjustment for age and FEV_1 level with the relative risk of death being of the order of 1.5. Although this risk is relatively low it applied to a large proportion of the population studied as around 40% of the group reported CMH as a symptom. Due to the way deaths are recorded in France it was not possible to see what the cause of death was in those that had died.

Since this report other large studies have also found an increase risk of death in those individuals with CMH. A study of mortality in a population sample of over 8500 people from 6 American cities found that in the presence of cough

or sputum there was a significant increase in the odds ratio for death from COPD in men and women. This effect seemed greatest in women when the sexes were compared (133). The odds ratio for mortality from COPD in those with CMH was of the order of 3.75 in men and 11 in women. This is clearly a significant increase but is relatively small when compared to the odds ratios for mortality in those with poor lung function which were 48 and 26 for men and women respectively with the division of good and poor lung function being made by comparing those in the lowest quartile of FEV_1 values with the other 3 quartiles combined.

Data from a Danish study, the Copenhagen City Heart Study, has added weight to the argument that CMH is a serious symptom in those with COPD. This large study recruited a randomly selected population of over 14000 subjects. Lange et al found that the presence of CMH was associated with an increase risk of death from all causes, albeit weakly so with the relative risk of death being 1.1 and 1.3 for men and women respectively (134). The presence of CMH had a greater impact on the risk of death from COPD though. This risk increased depending on the level of lung function with a relative risk of death of 4.2 for those with $FEV_1 < 40\%$ predicted compared to 1.2 for $FEV_1 > 80\%$ predicted. The same group have also reported an increase risk of lung cancer in those with CMH (RR 1.5) (135).

The cause for this increase in mortality is still not completely clear. As described above it is well recognised that those individuals with CMH have an increase risk of respiratory infection and a large proportion of deaths from COPD follow infective exacerbations of the disease (136). These deaths are usually in those with more severe COPD, and again this is associated with increased rates of CMH. This certainly provides a clinically plausible explanation for the findings, and this explanation would be supported by other data from the Copenhagen population study which showed that the increase in COPD related mortality in those with CMH was due to an increased risk of death associated with pulmonary infection (RR of death 3.5). In those COPD deaths where infection played no part there was no increase in the risk of death in the group with CMH (137). The group were only able to get data on the cause of death for 50% of the deaths though and used presence of mucus

as one of the diagnostic criteria of infection which may potentially bias the results.

The reports described above have focused on mortality as an outcome measure. COPD is a chronic illness causing significant morbidity which worsens as the level of FEV_1 declines. Other studies have addressed the morbidity associated with CMH and FEV_1 decline as will now be described. Concerning FEV_1 decline in those with CMH, data from the Copenhagen City Heart Study group were used to compare rates of FEV_1 decline in those with and without CMH (138). There was a statistically significant increase in the rate of FEV_1 decline in those with CMH with an excess decline of 23ml/year in men and 13ml/year in women compared to those without CMH after adjusting for smoking and other important variables. This excess decline did not include an adjustment for initial level of FEV_1 and when this was included the association was significantly weakened, especially in women, with excess declines in FEV_1 (+/- 95% CI) of 18ml/year (3.3 – 32.7) and 7.9ml/year (-4.1 – 19.8) for men and women respectively. Whilst statistically significant it could be argued that this excess decline is not clinically relevant. This same consideration applies to another study which found an excess decline in FEV_1 of 4.5ml/year in the presence of CMH. This was only apparent in men (139). More recently with the publication of the GOLD classification of severity of COPD Vestbo and colleagues have examined the likelihood of progression to COPD in a group of smokers with or without a productive cough (the features of GOLD stage 0 in smokers with normal lung function). They found that the odds ratios for development of COPD in those with or without CMH were 1.1 (0.9 – 1.4) and 1.2 (0.9 – 1.6) respectively after adjusting for age, gender, and smoking (88). This finding supports the original conclusions of Fletcher and colleagues that CMH is an innocent disorder in early smoking related disease, but is at odds with the same group's findings of an association of CMH and accelerated FEV_1 decline as discussed above (138). This earlier study included individuals with COPD though and there may be differences in the effect of CMH depending on the severity of the disease.

With regards to morbidity Vestbo and colleagues also showed that CMH was associated with an increase risk of hospitalisation from COPD in men and women (Relative risk of hospitalisation 5) (138). Even after adjustment for

FEV_1 level which weakened the association there was still an increased risk of hospitalisation (RR 2.5). These findings supported those of an earlier study by the same author that followed up a smaller cohort of men over a 12 year period and found that there was an increased risk of hospitalisation from either COPD (OR 4.16-5.75) or respiratory disease (OR 2.56-3.29) in individuals with airflow limitation and CMH compared to those without CMH (32;140).

The reasons behind the different findings of these later studies compared to the earlier ones are not clear. They may reflect differences in the populations studied with the later studies tending to use random population samples rather than specific age limited cohorts, often of men only, who were working in environments containing high burdens of occupational dust. Also over this time period smoking rates and individual levels of smoking (as measured by pack years) have tended to fall which may have affected the results found. Air pollution has also fallen and it is of note that in some of the earlier studies the prevalence of CMH was often of the order of 20-40% compared to prevalence in the population studies of around 12% (28;129;130;138).

Based on all the above data it is reasonable to conclude that the effect of CMH on rate of FEV_1 decline is still unclear. On the other hand the presence of CMH does seem to confer significant morbidity with increased rates of hospitalisation from either COPD or respiratory illness. CMH also seems to have an effect on mortality particularly from respiratory disease which may be due to an increased risk of death from infection.

Hospitalisation is clearly an important health and economic outcome in patients with COPD and may be a better outcome measure than mortality in those with lesser degrees of airflow limitation. Whether these hospitalisations and other respiratory illnesses of lesser severity are important in the decline of FEV_1 is still unclear though.

There are putative mechanisms which might explain some of the above findings. It is well recognised that those with CMH and/or COPD are more likely to have chronic colonisation of the normal sterile tracheo-bronchial tree with bacteria (141). Bacteria have a number of important effects in the airway including stimulation of mucus production, impairment of mucociliary clearance, and epithelial cell damage (142). Where there is bacterial

colonisation of the airways there is a greater degree of inflammation, as measured by inflammatory cytokines and cells in BAL, than in those non-colonised subjects (143), and inflammation increases during acute exacerbations (144). This chronic ongoing inflammation may therefore predispose to lung damage as has been suggested in other lung diseases which are characterised by CMH and bacterial colonisation such as cystic fibrosis (145).

Currently there are no longitudinal studies that have investigated the link between chronic airway infection and FEV_1 decline. Some recent evidence though suggests this could be a pathogenic mechanism. Seemungal and colleagues investigated a group of patients with moderate to severe COPD during and after exacerbations (146). They found that following an exacerbation there was a significant proportion of patients whose lung function did not return to pre exacerbation levels. Many of these patients experienced further exacerbations during their period of recovery hence accelerating the decline in FEV_1 (146). The same group though were not able to show a correlation between inflammatory cytokine levels and lung function during exacerbations (147). In a small group of stable COPD patients Stănescu and colleagues showed an association of airway neutrophilia with an accelerated decline in FEV_1 (148).

Any relationship is likely to be complex and other factors such as smoking and severity of disease are likely to interact. For instance in the Lung Health Study an accelerated decline in FEV_1 due to lower respiratory illnesses was only seen in smokers (149).

1.2.5.6 Pathological changes in chronic bronchitis

In the 1950s, at the time the clinical features of chronic bronchitis were being clarified, work was being performed to describe the pathological changes associated with chronic bronchitis. Reid showed that in patients with chronic bronchitis there was an increase in the thickness of the submucosal glands and in the size of the acini which composed them (150). These glands are found in all bronchi which have cartilage in their wall i.e. the larger airways. She went on to show that the ratio of the thickness of the gland layer compared to the thickness of the airway wall was increased in chronic

bronchitis (151). This ratio became known as the Reid index. In patients with chronic bronchitis almost two thirds of the thickness of the wall was composed of the mucus gland layer compared to around a quarter in normal subjects. Reid and other groups noted that goblet cell numbers in the large airways were also increased (152), but it was felt that the submucosal glands were the more important source of airway mucus as their volume was over 40 times that of the goblet cells in these airways (151). In the bronchioli where goblet cells are normally sparse significant increases in goblet cell numbers were often seen in chronic bronchitics. This increase in cell numbers tended to occur in a patchy distribution which may have accounted for the different findings in a number of early studies reporting goblet cell numbers (44). In some cases the increase in goblet cell numbers was such that they formed the majority of the epithelial surface. This finding coupled with the finding that many of the small airways in patients with COPD were blocked with mucus suggested that increased secretion from goblet cell hyperplasia and metaplasia was responsible for some of the airflow limitation found in individuals with chronic bronchitis (153).

Reid suggested that although mucus gland hypertrophy was probably responsible for most of the mucus found in the larger airways the goblet cell changes and mucus seen in the smaller airways could be responsible for much of the disability by blocking these airways and hence reducing airflow. This would explain why some patients could have marked sputum production but little breathlessness and vice versa. This suggestion certainly fitted with the finding that the majority of resistance to airflow in the lungs of patients with COPD occurs in the small airways rather than the large airways as is the normal finding (46).

Other workers found that CMH could occur even in the absence of mucus gland hypertrophy (154) and different pathological processes could be seen in patients who had identical clinical features such as airflow limitation: pathologically there could be emphysema and no chronic bronchitis in some patients and chronic bronchitis but no emphysema in others. This suggested other factors were involved in the production and release of excessive mucus. Although called bronchitis the role of inflammation in CMH remains somewhat unclear. In the studies described above where hypertrophy of the mucus

glands was seen without an inflammatory cell infiltrate the hypertrophy was attributed to the irritant effect of noxious substances such as smoke on the airways. When inflammatory cells were seen their presence was felt to reflect the presence of infection. As discussed above it was believed that this infection resulted in worsening of mucus hypersecretion and irreversible damage to the lung thus causing emphysema.

More recent studies have expanded the understanding of the role of inflammation in CMH. As discussed above mucus gland hypertrophy was not found in all patients with CMH and many patients had inflammatory cell infiltrates in the airway wall (154). It was shown that the severity of inflammation correlated better with the presence and severity of CMH than gland hypertrophy *per se* (56;155). Newer immunohistochemical techniques have allowed the inflammatory cell population to be characterised further. When smokers with CMH and airflow obstruction were compared to "healthy" smokers (those with no CMH or airflow obstruction) it was found that there was a significant infiltration of the bronchial glands with neutrophils and macrophages in the former (58). In addition although there was no difference in absolute T-lymphocyte numbers between the groups there was a reduction in the $CD4^+/CD8^+$ ratio in the symptomatic smokers (58). The same group subsequently showed that there were increased numbers of $CD8^+$ cells in the peripheral airways of symptomatic smokers compared to those without symptoms (156).

Other groups have also found increased numbers of inflammatory cells associated with the submucosal glands in smokers with CMH with increased numbers of these cells expressing IL-4. The cells appeared to be plasma cells (157). It is likely that the inflammatory cells in the bronchial glands play a role in CMH as both neutrophil products and IL-4 are potent stimuli for mucus secretion (158;159).

1.2.5.7 Role and composition of mucus

The lining fluid of the airways can be divided into a watery periciliary layer (the sol layer) on which floats a layer of viscous mucus (the gel layer). These layers have a variety of functions including trapping and removing particles, maintenance of hydration of the epithelium, humidification of air, and anti-

pathogen activity (160). To achieve these different roles mucus contains a number of molecules including mucin glycoproteins, proteins, lipids, and defence molecules such as lysozyme and defensins. In addition cells such as neutrophils and macrophages are present in this lining fluid.

Whilst there has been increasing research into the factors which control the composition of the airway lining fluid in health and disease much of these processes remain unclear. One of the important properties of the mucus layer though is its viscoelasticity. This allows it to be moved efficiently up the tracheo-bronchial tree by the action of the underlying cilia, and to withstand the forces produced during coughing which clears sputum from the larger airways. A number of interactions between molecules within mucus occur to give it this viscoelasticity. These are mostly dependent on the presence of mucin glycoproteins in the mucus (161).

The mucins are high molecular weight glycoproteins. They consist of a peptide backbone produced from translation of the mucin gene product with multiple sugar (oligosaccharide) side chains attached. These side chains make up about 80% of the mucin's weight and are of numerous types (162). Hydrogen bonds form between the sugar units on neighbouring mucins and due to their size entanglements also occur. Disulphide bonds are also formed between the glycoprotein subunits to join them into extended chains. These interactions result in the visco-elastic properties of mucus. The sugar side chains also have a role in defence by provide multiple binding sites for bacterial attachment. This causes the bacteria to be trapped in the mucus layer allowing its removal via the mucociliary escalator rather than colonisation of the epithelium (162).

Many mucin genes have been identified and their products are found on most epithelial surfaces including the lung, intestine, and cervix. Some of the mucin gene products remain membrane bound whereas others are secreted. In the airway the mucin genes MUC2, MUC5AC, and MUC5B have been found (163). The latter two appear to be the dominant mucins in mucus and localisation studies have shown that MUC5AC is predominantly synthesised in the epithelial goblet cells and MUC5B in the submucosal glands (164;165).

Many different stimuli have been shown to increase airway mucus production. These stimuli, such as neutrophil elastase, particulate matter, and bacterial

products, all cause an increase in mucin gene expression in airway epithelial cells. A number of intracellular pathways may be responsible for this gene response, but recently it has been shown that activation of the transmembrane epidermal growth factor receptor (EGFR) is a key event in the up-regulation of mucin gene expression (166). This will be discussed in more detail below (Chapter 1.3).

1.2.5.8 CMH as a major unmet clinical need

Whilst there is still a debate regarding the role of CMH in progression of COPD there is good evidence that individuals with CMH are more likely to experience respiratory infections. These infections can have serious consequences, precipitating respiratory failure in those with advanced COPD, but even in individuals with less severe disease they have a significant impact on health status (96) and significant economic effects on health services (18). Currently there are no effective treatments which reduce the symptoms of CMH or significantly reduce its potential for complications. It is only in recent years with a better understanding of the mechanisms of mucus production that potential therapeutic targets are being identified (167). There are several potential difficulties in designing trials using muco-active drugs. These include how best to assess the presence of CMH in an individual, what outcome measures to use, and how to assess response to the therapeutic agent. For example FEV_1 decline, infection rate, quality of life scores, measures of sputum purulence, volume or inflammatory cytokine content could all be considered as suitable outcome measures. There are advantages and disadvantages to each of these: FEV_1 decline is an important marker of disease progression but would require a large cohort and long term follow-up to assess whereas sputum measures can be assessed easily and quickly but may not correlate with measures of airflow limitation or infection rates. In addition clinical CMH usually reflects a large increase in mucus production above normal but lesser degrees of mucus hypersecretion where the sputum is not expectorated may be as important in terms of causation of morbidity. As yet there is no easy and reliable way to measure this. It is also worth noting though that mucus has many important actions in the airways and so any

treatment would need to be aimed at restoring the level of production to that of a healthy airway rather than abolishing production altogether (163). Currently the only agents that have been studied in detail in smoking related CMH are the mucolytics. These include drugs such as carbocisteine, iodinated glycerol, and N-acetylcysteine. They are felt to act by disrupting disulphide bridges between mucin molecules reducing the viscosity of sputum, but it is important to recognise that N-acetylcysteine is also an antioxidant and hence may have other important effects. Many trials have been carried out using these agents, but they are often small and underpowered. A systematic review of mucolytics trials was published in 2001 (168;169). This concluded that mucolytics had no significant effect on lung function but did appear to reduce the risk of exacerbation: weighted mean difference -0.07 per month, 95% confidence intervals -0.08 to -0.05. In the studies there was an annual exacerbation rate of 2.7 events per year and so this reduction amounts to a 29% decrease in exacerbation rate per year. As well as being small many of the trials included in the meta-analysis did not specify COPD as an inclusion criterion or measure lung function and were short lasting making definite conclusions difficult. More recently a large trial of over 500 individuals with COPD was carried out comparing N-acetylcysteine to placebo over a 3 year period (170). The primary outcome measures were rate of decline of FEV_1 and exacerbation frequency. No difference was found in these outcomes between the active and placebo arms of the trial. In this trial N-acetylcysteine was being used for its antioxidant properties and it is not known what percentage of the patients enrolled had CMH.

The effect of other respiratory drugs on mucus production is not clear. Corticosteroids reduce mucus hypersecretion in asthmatics. This is likely to be an indirect effect as they reduce the level of cytokines which stimulate mucus production, such as IL-13 and IL-4, in the airway rather than by an effect on the mucin gene pathway (8). β_2-agonists can improve mucociliary clearance by an effect on ciliary beat frequency but are not known to affect mucus production (171).

Potential targets for muco-active drugs include inhibitors of the many cytokines and proteases which have been shown to increase mucus production such as IL-4 or neutrophil elastase. Agents that affect EGFR

activation either directly or by inhibition release of its activating ligands may also have potential roles. This will be discussed in more detail below. As yet there is no clinical evidence supporting the role of any of these agents in the treatment of CMH.

1.3 Epidermal growth factor receptor and mucin gene expression

1.3.1 The EGFR and its ligands

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase and is the prototype member of the ErbB family of receptors. This family consists of ErbB1 (EGFR/HER1), ErbB2 (Neu/HER2), ErbB3 (HER3), and ErbB4 (HER4). The receptors are expressed in a variety of tissue of epithelial, mesenchymal, and neuronal origin. EGFR is a 170kDa protein that can be considered to comprise of 3 main regions: an extracellular domain where ligand binding occurs, a transmembrane domain that makes a single pass through the plasma membrane, and an intracellular domain. The intracellular domain includes a region with protein tyrosine kinase activity and a cytoplasmic tail which has specific tyrosine residues in it. These tyrosine residues are auto-phosphorylated by the protein tyrosine kinase region of the receptor and when phosphorylated interact with secondary messengers within the cell (172).

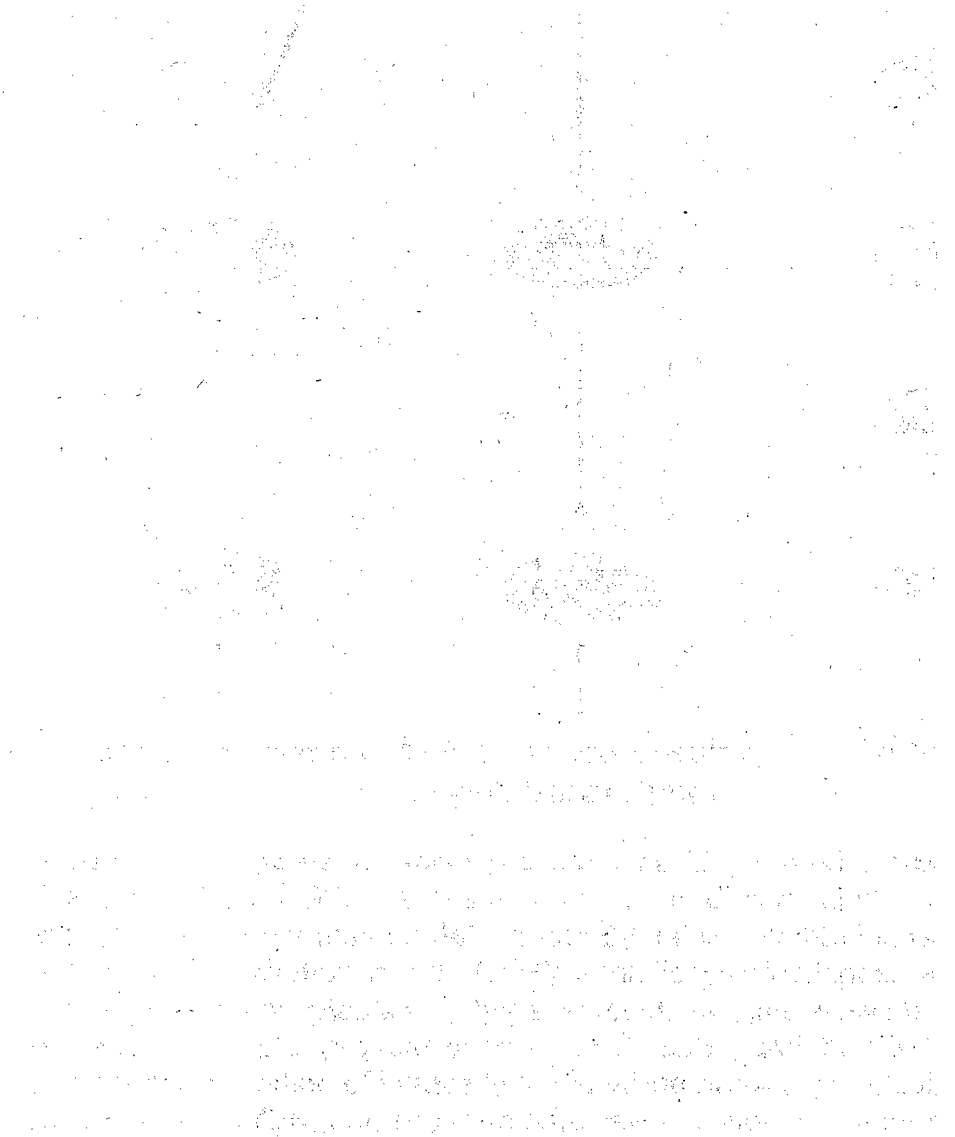
Currently 11 ligands have been identified that can bind to the ErbB family: EGF, which was first identified as a growth factor by Cohen in 1962 before he and co-workers identified the EGFR (173), transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HBEGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR), epigen (EPI), and neuregulins 1 – 4 (NRG1 – 4) (174). EGF and the other ligands acting via the ErbB receptors have a number of roles, both in foetal development and in the adult. They are involved in proliferation, differentiation, and apoptosis of cells (175). The ligands are all characterised by the presence of a three-looped structure which is formed by disulphide bonds between 6 cysteine residues in the protein (174). This structure appears to be vital for receptor binding, and may give the proteins resistance against proteolytic attack. All the ligands are formed as membrane-bound precursors and although some have biological activity in this bound state they usually require cleavage by metalloproteinases for activity (176).

In its inactive state the EGFR is found as a monomer. Binding of ligand causes a conformational change in the receptor such that receptor dimers form. These dimers may be homodimers e.g. ErbB1/ErbB1 or heterodimers e.g. ErbB1/ErbB2. In fact it appears that ErbB2 which has no identified ligand is the preferred dimerisation partner for all the ErbB receptors (177). The pattern of dimerisation that occurs post ligand binding may affect the subsequent intracellular signalling pathway. In addition the ligands for the ErbB family have specific binding patterns for the receptors: EGF, TGFα, and AR only bind to ErbB1 (EGFR) whereas BTC, HB-EGF, and EPR can bind to ErbB1 and ErbB4. NRG1 and 2 bind to ErbB3 and ErbB4 but NRG3 and 4 only bind to ErbB4 (177). Again which ligand binds to the receptor may determine the dimerisation partner and the pattern of phosphorylation that occurs and hence the downstream signalling pathway which is activated (174).

Dimerisation of the receptor causes activation of its intracellular protein tyrosine kinase domain. This causes phosphorylation of specific tyrosine residues in the C-terminal end of the receptor. The phosphorylation of these residues allows the receptor to interact with second messenger proteins in the cell such as the enzyme phospholipase C gamma (PLCγ). These can then begin the transmission of the signal to the cytoplasm or the nucleus. The receptor/messenger interaction is dependent on the presence of structures such as *src* homology-2 (SH2) or phosphotyrosine binding (PTB) domains in the messenger which allow it to interact with and be phosphorylated by the active receptor (178). These second messengers then interact with a number of different intracellular pathways. Pathways that have been shown to be activated following EGFR activation include the MAP kinase pathway and the phosphatidylinositol 3-kinase (PI3K)/protein kinase C pathway which results in activation of the nuclear transcription factor NF-κB (174). The interaction between these different pathways is not fully understood but it appears that activation of multiple pathways may be required for some biological effects whereas activation of just one pathway may produce multiple responses (Figure 1.2).

Post activation of the EGFR the receptor/ligand complex is rapidly internalised from the cell surface into endosomes. From here both the

receptor and the ligand may be degraded or, if the ligand dissociates from the receptor, the receptor may be recycled to the cell surface and the ligand degraded as seems to occur following TGFα binding to the EGFR (179). This and other inhibitory factors post EGFR activation allows the signal due to EGFR ligand binding to be attenuated.



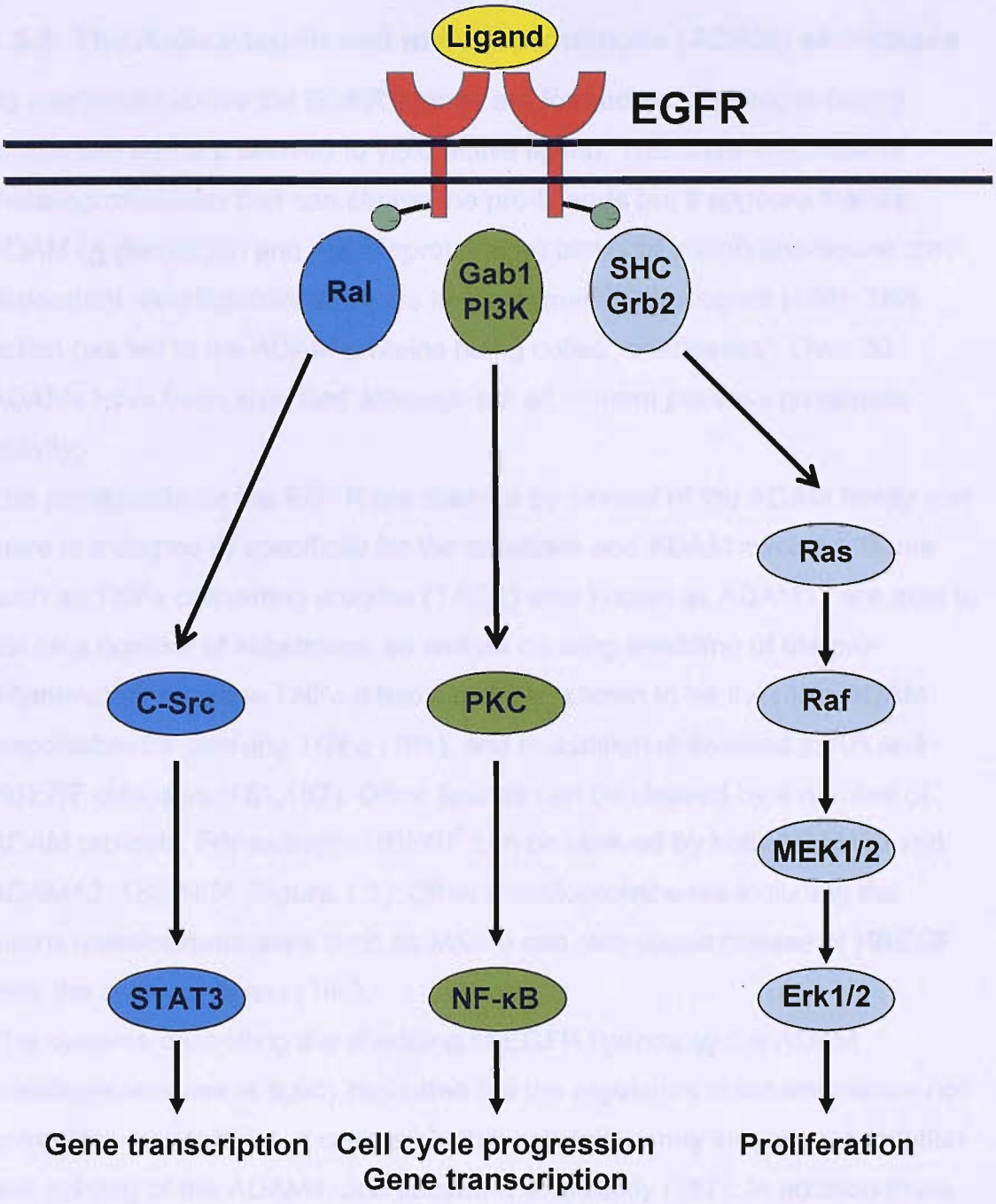


Figure 1.2 – Schematic representation of EGFR signalling pathways
Ligand binding to EGFR causes homo or heterodimerisation. The dimerisation partner may be determined by the ligand that binds to the EGFR. The resulting activation of the EGFR's intrinsic tyrosine kinase activity causes phosphorylation of cytoplasmic tyrosine residues. This allows binding and activation of adaptor proteins such as SHC, Gab1, and the GTPase Ral with subsequent activation of intracellular signalling pathways including those involving PKC, MEK, and Src. The pathway activated will determine the cell response and includes cell proliferation, anti-apoptotic effects, and gene transcription which involves the transcription factor NF-κB and members of the STAT family.

1.3.2 The A-disintegrin and metalloproteinase (ADAM) sheddases

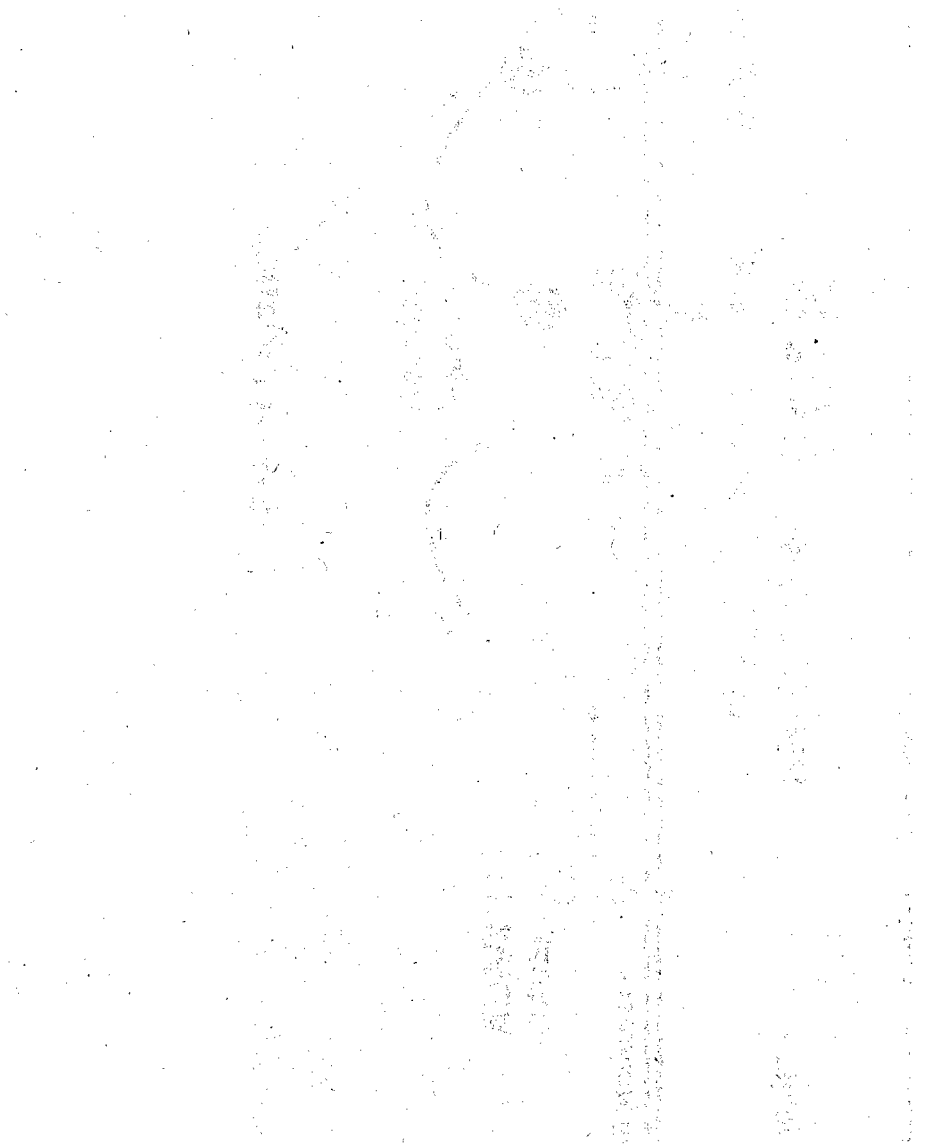
As mentioned above the EGFR ligands are formed as membrane-bound precursors and are cleaved to yield active ligand. There are a number of metalloproteinases that can cleave the pro-ligands but it appears that the ADAM (A disintegrin and metalloproteinase) family of membrane-bound zinc-dependent metalloproteinases are key enzymes in this regard (180). This action has led to the ADAM proteins being called "sheddases". Over 30 ADAMs have been identified although not all of them possess proteinase activity.

The pro-ligands for the EGFR are cleaved by several of the ADAM family and there is a degree of specificity for the substrate and ADAM involved. Some such as TNFα converting enzyme (TACE) also known as ADAM17 are able to act on a number of substrates: as well as causing shedding of the pro-inflammatory cytokine TNFα it has also been shown to be the main ADAM responsible for cleaving TGFα (181), and in addition is involved in AR and HBEGF cleavage (181;182). Other ligands can be cleaved by a number of ADAM proteins. For example HBEGF can be cleaved by both ADAM10 and ADAM12 (183-185) (Figure 1.3). Other metalloproteinases including the matrix metalloproteinases such as MMP3 can also cause release of HBEGF from the cell membrane (186).

The systems controlling the shedding of EGFR ligands by the ADAM metalloproteinases is tightly regulated but the regulatory mechanisms are not completely understood. It is possible that regulation may involve transcription and splicing of the ADAMs, and substrate availability (187). In addition there is evidence that there is spatial segregation of ligand and receptor in epithelial cells which would also affect receptor activation (188). The cytoplasmic domain of the ADAM protein contains a number of potential sites for interaction with signalling proteins and adaptors and this may influence their extracellular cleavage activity (180).

Despite the uncertainties regarding regulation of sheddase activity it is clear that the activity of the ADAMs is crucially important in development (189). Mice with a germ-line mutation in ADAM17 exhibit perinatal lethality and have defects in maturation and organisation of the respiratory and digestive

systems (190). In addition the EGFR appears to play a vital role in responses to a range of disease relevant stimuli with effects on pro-inflammatory responses and mucin gene expression as will be discussed below. The need for ligand release in this process identifies members of the ADAM family of metalloproteinases as a potential therapeutic target in respiratory disease.



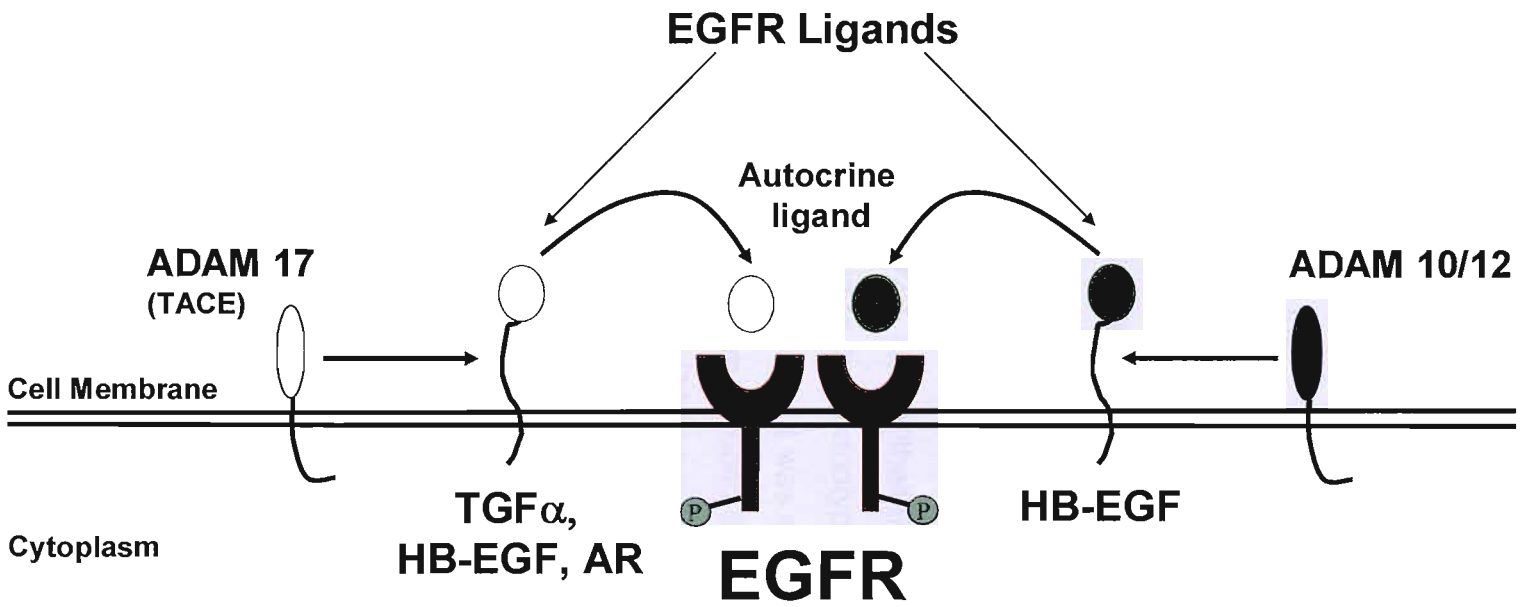


Figure 1.3 – Activation of EGFR following cleavage of its ligands by ADAM sheddases

The ligands for the EGFR include TGFα, AR, HB-EGF, and EGF. They are found as membrane-bound precursors. Cleavage releases the active ligand which can then bind to the EGFR with resulting receptor dimerisation and activation of tyrosine kinase. Cleavage is performed by ADAM metalloproteinases which are also membrane-bound. ADAM 17 or TNFα converting enzyme (TACE) has been shown to cleave AR, TGFα, and HB-EGF. HB-EGF has also been shown to be the substrate for other ADAMs such as ADAM 10 and 12. Matrix metalloproteinases including MMP3 may also cause release of active ligand from the membrane-bound precursors. The mechanisms controlling the activation of the ADAM sheddases has not been fully established.

Figure 1.3 – Activation of EGFR following cleavage of its ligands by ADAM sheddases

1.3.3 EGFR and mucin expression

As discussed above (Chapter 1.2.5) many mucin genes have been identified. In the airway wall MUC2, MUC5AC, and MUC5B are found, but only the latter two gene products appear to be found in mucus where they are the main mucin constituents (162). The pathways involved in regulation of mucin genes in airway epithelial cells have only recently begun to be elucidated and it appears that the EGFR plays a pivotal role.

Early suggestions for a potential role for EGFR in regulation of the airway epithelial phenotype came from observations relating to the effects of EGF on the airway epithelium. EGF promoted the maturation of the lungs of lamb foetuses (191) and appeared to be responsible for the excessive mucus production seen in epithelial cells grown at an air-liquid interface (a way of culturing cells such that an approximation of the pseudo-stratified epithelium of the normal airway is produced (192)). The epithelial phenotype seen in these cultures was often very different to the phenotype seen in the animal that provided the cells used for these cultures. It was shown that reducing the concentration of EGF in the culture medium reduced the degree of mucus secretion and MUC5AC expression (193). Other groups had shown that EGF stimulated the production of mucus glycoproteins from other epithelial surfaces such as gastric mucosa where mucus provides a defence against the acidic gastric contents (194).

The role of the EGFR in controlling the expression of mucin genes in the bronchial epithelium was investigated further by Takeyama and colleagues. They showed that whereas EGFR was only sparsely expressed in normal airways the addition of TNFα caused an increase in EGFR expression (166). The addition of EGF or TGFα to a bronchial epithelial cell line *in vitro* caused an increase in MUC5AC mRNA and protein, an effect that was enhanced by pre-treatment with TNFα. Selective inhibitors of the EGFR including tyrphostin (AG1478) prevented this increase whereas inhibitors of other receptor protein kinases did not. It was also shown *in vivo* that exposure to TNFα prior to EGFR ligand stimulation caused an increase in goblet cell numbers in the bronchial epithelium of rats, but not an increase in total epithelial cell

numbers. This suggested that the increase was due to differentiation of cells rather than division.

Perrais and colleagues also showed that TGFα and EGF up-regulated MUC5AC gene and protein expression in H292 cells (195). They also found that the EGFR ligands caused an increase in MUC2 gene and protein expression. Although they demonstrated the presence of MUC5B mRNA in these cells there did not appear to be any translational product present, and its gene expression did not increase with EGFR ligand treatment. Unlike Takeyama (166) they did not find a synergistic effect on mucin expression with stimulation in the presence of TNFα, but again demonstrated that the increase in mucin gene expression could be blocked by inhibition of EGFR using tyrphostin.

To investigate possible downstream signalling pathways they also stimulated cells in the presence of inhibitors of the secondary messengers Ras and Raf and ERK. These inhibitors attenuated the mucin gene response but did not block it completely suggesting that more than one pathway is involved. In this report the authors also investigated which nuclear transcription factors were involved in the gene response, and showed that there was an EGF response element 200 base pairs (bps) upstream from the MUC5AC gene. The response of this element was mediated by the transcription factor Sp1 whereas the transcription factor Sp3 had a negative effect on MUC2 and MUC5AC expression (195).

EGFR mRNA is found in bronchial epithelial cells (196) but EGFR protein expression is sparse in healthy individuals and pathogen free laboratory animals. Where found it is normally localised to goblet cells and the basal cells of the epithelium (197-199). In individuals with asthma there is a change in the expression of EGFR compared to normal. Increased EGFR protein is found in areas of damage in the asthmatic airway and in increased levels compared to healthy individuals (198;200). Although few studies have been performed examining EGFR expression in individuals with CMH it is interesting to note that a number of disease relevant stimuli increase EGFR expression such as smoking (201;202) and the inflammatory cytokine TNFα (166). Increased TNFα is found in the sputum of patients with COPD (64) and it is released from a number of inflammatory cells which are found in

increased numbers in the airways of patients with CMH such as macrophages (203) and neutrophils (204) as well as being released by epithelial cells (205). Following the identification of the EGFR as a key regulator of mucin gene expression many diverse stimuli that are relevant to airways disease have been shown to activate the EGFR and increase mucin gene expression. The mechanisms that they act through may vary and it is possible that some of the stimuli activate the EGFR directly whereas others cause ligand shedding, probably by activation of ADAM proteinases, which then leads to ligand-dependent EGFR activation as will now be discussed.

1.3.4 Cigarette smoke and mucin gene expression

Cigarette smoke is the main cause of COPD and is associated with CMH. Takeyama and colleagues demonstrated that cigarette smoke solution caused an increase in EGFR mRNA expression and tyrosine phosphorylation of the EGFR (202). This resulted in an increase in MUC5AC mRNA and protein product in H292 cells. A similar response was seen when TGFα was added to the cells. A selective EGFR tyrosine kinase inhibitor prevented the increase in both EGFR phosphorylation and MUC5AC increase. A potential role for oxidative stress in this response was identified: DMSO and superoxide dismutase (SOD) which scavenge oxygen free radicals attenuated MUC5AC synthesis in response to cigarette smoke solution but did not abolish it completely. This suggested that other factors in cigarette smoke may be involved in the response. It is important to recognise that cigarette smoke is an extremely complex mixture containing many active substances some of which, such as acrolein an aldehyde, have been shown to increase MUC5AC expression (71;206;207). *In vivo* it was shown that the EGFR tyrosine kinase inhibitor BIBX1522 prevented the increase of goblet cells numbers and MUC5AC expression in rat tracheas that was seen when the rats were exposed to cigarette smoke (202).

The mechanism of activation of EGFR by cigarette smoke is not completely understood. EGFR activation occurs following ligand binding to the receptor but it has also been shown that some substances cause EGFR tyrosine phosphorylation in the absence of exogenous ligand, so called ligand-independent or transactivation of the receptor. Oxidative stress (208), UV light

(209), and G-protein-coupled receptor activation (178) have all been demonstrated to cause EGFR activation in this way.

More recently it has been recognised that what was thought to be ligand-independent EGFR activation does actually involve ligand release and binding. Preformed membrane-bound EGFR ligands can be released in response to a variety of stimuli with binding to and autocrine activation of the EGFR. For example it was demonstrated that cigarette smoke extract (CSE) caused release of EGFR ligands and up-regulation of their gene expression from bronchial epithelial cells (210). The resulting activation of EGFR caused an increase in IL-8 release from the cells. The presence of an anti-EGFR antibody which prevented binding of the ligands to the receptor reduced the CSE mediated IL-8 release by nearly half. This suggests that autocrine release of EGFR ligands is playing a major role in pro-inflammatory responses to CSE in bronchial epithelial cells and challenges the assumption that exogenous ligand is required for EGFR activation in cell cultures. Results from this work also suggested that TGFα may be the predominant biologically active ligand involved in EGFR activation. The G-protein-coupled receptor transactivation of EGFR (178) was also initially thought to be independent of EGFR ligand but was subsequently shown to involve metalloproteinase mediated shedding of the EGFR ligand HB-EGF (211). In this study activation of G-protein-coupled receptor by its ligands resulted in activation of a metalloproteinase which cleaved HB-EGF. This was able to bind to and activate EGFR. The identity of the metalloproteinase could not be elucidated in this work but it shows another route by which extracellular signals may feed into the EGFR pathway.

TGFα, like the other EGFR ligands, is formed as a membrane-bound precursor and is cleaved by TACE (ADAM17) to release the active protein. Shao reported that exposure of H292 cells to PMA, a phorbol ester that is known to cause ectodomain shedding of TGFα (212), increased MUC5AC expression and production (213). This effect was reduced by inhibitors of EGFR tyrosine phosphorylation, anti-EGFR antibodies, and anti-TGFα antibodies suggesting that EGFR activation following binding of active TGFα is essential for MUC5AC expression. They went on to show that inhibition of TACE by the metalloproteinase inhibitors TNFα proteinase inhibitor 1 (TAPI-

1) or tissue inhibitor of metalloproteinase 3 (TIMP-3) also blocked EGFR phosphorylation and MUC5AC expression as did the inhibition of protein kinase C which has been implicated in TACE activation. The inhibitory effects of metalloproteinase inhibition could be reversed by the addition of exogenous TGFα. The metalloproteinase inhibitors in these experiments had broad effects and so to investigate the role of TACE further its expression was knocked down to around 10% of normal using siRNA against it. This also prevented TGFα release, EGFR phosphorylation, and MUC5AC expression in response to PMA.

Having identified TGFα shedding as an important step in mucin gene expression the same group went on to show that cigarette smoke induced EGFR phosphorylation and MUC5AC expression were also dependent on shedding of TGFα (214). By knocking out the expression of the EGFR ligands individually they showed that TGFα was the ligand responsible for the mucin gene response to cigarette smoke. Preventing TGFα release with metalloproteinase inhibitors or TGFα binding with anti-EGFR or anti-TGFα antibodies prevented the up-regulation of MUC5AC expression in response to cigarette smoke solution exposure. As for their results with PMA this highlighted the role of the sheddases in producing active EGFR ligands to mediate the cellular responses to cigarette smoke. By specifically knocking out TACE with siRNA there was no TGFα release, EGFR tyrosine phosphorylation, or MUC5AC expression in response to cigarette smoke. Other groups have also shown that cigarette smoke causes activation of TACE and shedding of EGFR ligands from bronchial epithelial cells which results in EGFR phosphorylation and cellular responses (215;216).

Lemjabbar and colleagues were investigating cell proliferation in response to cigarette smoke and demonstrated that this required an EGFR ligand-receptor interaction and that ligand release was dependent on the activity of TACE. In contrast to Takeyama they identified the EGFR ligand responsible for this proliferation as AR which can also be cleaved by TACE (181). Again TACE activation appeared to be dependent on reactive oxygen species which they showed to be generated intracellularly by a low activity NADPH oxidase rather than diversion of oxygen species from mitochondria electron transport (215).

Gensch and colleagues also confirmed the up-regulation of MUC5AC expression in response to cigarette smoke and went on to investigate the downstream signalling pathways involved in this response and the mechanisms of gene transcription (216). In contrast to Perrais who had found that EGF and TGFα induced mucin gene transcription involved an EGF response element ~200 bps upstream of the MUC5AC gene which was positively regulated by the transcription factor Sp1 (195) the authors demonstrated that cigarette smoke induced MUC5AC mRNA transcription was mediated by an AP-1 binding site ~ 3000 bps upstream of the gene. It appeared that a heterodimer of Jun and Fos family members (JunD and Fra2 respectively) was binding to the AP-1 site. The Jun family of intracellular messenger proteins are activated by JNK dependent phosphorylation whereas the Fos family are activated by ERK dependent phosphorylation. Both JNK and ERK phosphorylation occurs secondary to cigarette smoke exposure. In turn JNK activation can occur via Src family members which are activated by reactive oxygen species produced by exposure of cells to cigarette smoke. The smoke induced activity of JNK was not inhibited by EGFR inhibition though suggesting EGFR is not involved in JunD activation. Fra2 which appeared to be the other protein involved in the AP-1 binding complex is activated by ERK and this is activated by EGFR signalling through Ras/Raf and MEK (MAPKK).

These results show that cigarette smoke causes an increase in mucin gene expression and protein production. This involves a number of steps beginning with activation of membrane-bound sheddases which cleave ligands for the EGFR. The EGFR ligands bind to their receptor causing receptor dimerisation and activation with auto-phosphorylation of tyrosine residues on the cytoplasmic portion of the EGFR. This activation allows signalling through secondary messengers and intracellular signalling pathways such as the JNK and ERK pathways which activate proteins of the Jun and Fos family. These proteins form a heterodimer which binds to an AP-1 binding site to promote transcription of the MUC5AC gene as shown in figure 1.4. The increase in MUC5AC transcription results in MUC5AC protein production and may lead to a phenotype change of the cell into a mucus secreting form.

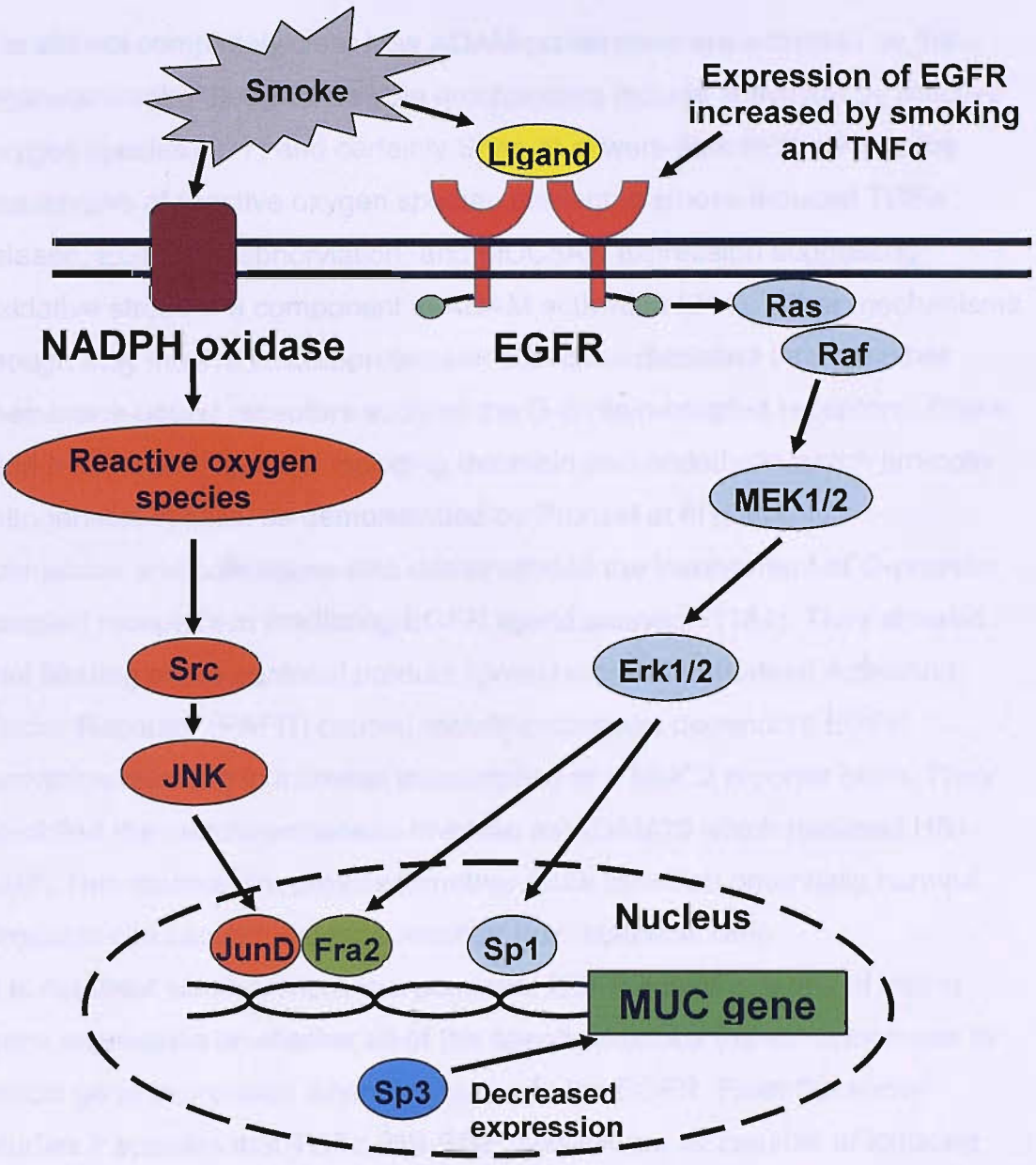


Figure 1.4 – Intracellular pathways responsible for mucin gene expression in response to EGFR activation

EGFR expression in the airway is increased by both cigarette smoke and pro-inflammatory cytokines such as TNFα. Cigarette smoke causes EGFR activation by promoting release of active EGFR ligands from their membrane-bound precursors and in addition is responsible for generation of reactive oxygen species via NADPH oxidase. These may also promote EGFR ligand release by TACE activation. Perrais et al showed that activation of EGFR by its ligands caused signalling via MEK/ERK pathway initiated by Ras/Raf activation. EGF and TGFα stimulated mucin gene expression involved binding of the transcription factor Sp1 to a promoter site upstream of the mucin genes. Gensch and colleagues found that smoke caused activation of both the MEK/ERK and JNK pathways resulting in production of a Jun/Fos heterodimer (JunD/Fra2) which bound to an AP-1 site to increase MUC5AC expression. The production of reactive oxygen species by cigarette smoke appeared to be the initiating stimulus for activation of the JNK pathway.

It is still not completely clear how ADAM proteinases are activated by the cigarette smoke though. Possible mechanisms include activation by reactive oxygen species (217) and certainly Shao et al were able to show that the scavengers of reactive oxygen species prevented smoke induced TGFα release, EGFR phosphorylation, and MUC5AC expression suggesting oxidative stress is a component in ADAM activation (214). Other mechanisms though may involve metalloproteinase activation mediated through other membrane-bound receptors such as the G-protein-coupled receptors. These bind a number of ligands, including thrombin and endothelin which promote mitogenesis in cells, as demonstrated by Prenzel et al (211).
Lemjabbar and colleagues also demonstrated the involvement of G-protein-coupled receptors in mediating EGFR ligand cleavage (184). They showed that binding of the bacterial product lipotechoic acid to Platelet Activating Factor Receptor (PAFR) caused metalloproteinase dependent EGFR activation resulting in increase transcription of a MUC2 reporter gene. They identified the metalloproteinase involved as ADAM10 which released HB-EGF. This mechanism provides another route by which potentially harmful products can cause a defence reaction from epithelial cells.
It is not clear whether there is a dominant EGFR ligand in terms of mucin gene expression or whether all of the ligands produce the same increase in mucin gene expression when they activate the EGFR. From the above studies it appears that TGFα, HB-EGF, and AR are all capable of inducing mucin gene expression but different ligands may be shed depending on the activating stimulus.

1.3.5 Neutrophils and mucin gene expression

Neutrophils appear to play a role in the pathogenesis of CMH and COPD (58) releasing a number of products in response to activation that may be responsible for this. These include neutrophil elastase and other proteinases which cause tissue destruction as well as reactive oxygen species which, although normally involved in bacterial killing, can also be harmful to healthy tissue. Neutrophil elastase causes secretion of mucus from goblet cells (218;219) and since the identification of the role of EGFR in mucin gene expression a number of studies have shown that neutrophil products are

capable of inducing mucin genes in bronchial epithelial cells as will now be discussed.

Voynow and co-workers (220) showed that neutrophil elastase increased MUC5AC mRNA levels in bronchial epithelial cells. This increase required the proteolytic activity of elastase and appeared to be due to an increase stability of MUC5AC mRNA rather than new transcription. As a result of this increase there was a rise in the amount of MUC5AC glycoprotein in the cells.

Takeyama and colleagues, following on from their work relating to cigarette smoke, demonstrated that H_2O_2 increased MUC5AC mRNA and protein in H292 cells (221). Activated neutrophils caused a similar increase whereas inactive ones did not and this increase appeared to be due to a component found in the supernatant from the activated neutrophils. Further work showed that EGFR activation was involved in the neutrophil provoked response although the degree of EGFR tyrosine phosphorylation in response to H_2O_2 or activated neutrophils was less than that induced by addition of the EGFR ligand TGFα to the cells. The anti-oxidant N-acetylcysteine inhibited EGFR tyrosine phosphorylation and MUC5AC synthesis by either H_2O_2 or supernatant from activated neutrophils suggesting that reactive oxygen species produced by the activated neutrophils were responsible for this effect. This finding was supported by the absence of EGFR ligands in the neutrophil supernatant and the absence of effect of N-acetylcysteine on TGFα stimulated EGFR phosphorylation and MUC5AC synthesis.

Interestingly although a specific tyrosine kinase inhibitor prevented EGFR activation by the neutrophil supernatant an anti-EGFR antibody which blocked ligand binding did not. This suggests a ligand-independent mechanism was involved in the EGFR activation seen. The downstream signalling pathway activated in this response involved the MAP kinase kinase (MEK) and specific MEK inhibitors stopped the increase in MUC5AC expression but did not prevent EGFR phosphorylation by H_2O_2 or neutrophil supernatant.

Neutrophils also release elastase and as described above Voynow and colleagues demonstrated that elastase caused an increase in MUC5AC mRNA (220). The mechanism of elastase induced MUC5AC increase is unclear though. Fischer and colleagues showed that neutrophil elastase increased MUC5AC expression both in a cell line and primary human

bronchial epithelial cells and that this response could be inhibited by the free radical scavenger DMTU or desferrioxamine (which prevents iron induced free radical formation) (222). The authors demonstrated that elastase treatment generated intracellular reactive oxygen species and again this was prevented by the antioxidants.

Kohri et al also demonstrated that human neutrophil elastase caused an increase in MUC5AC production, but found that this action was dependent on its proteolytic activity and was not inhibited by the presence of free radical scavengers (158). In their model selective inhibition of EGFR or MEK activation prevented the MUC5AC response showing the importance of EGFR activation and downstream signalling in mucin gene expression. They found that the EGFR activation was dependent on elastase induced TGFα shedding and binding to the EGFR i.e. ligand-dependent activation. Neutrophil elastase has previously been demonstrated to induce cleavage of pro-TGFα from cell membranes (223) and this action was also found by Kohri. The reasons for the different findings by these two groups are unclear. The groups were using different cell lines and Fischer also used primary human bronchial epithelial cells. It is possible that the actions of neutrophil elastase were different in these different cells. In addition the antioxidants were present for different times in the experiments.

More recent work by Shao and co-workers has supported the role of reactive oxidant species in the mucin gene response caused by neutrophil elastase (224). Again this study focused on the central role of the EGFR and its ligand TGFα in mediating mucin gene expression in response to neutrophil elastase. As found in their work with cigarette smoke (214) activation of TACE (ADAM17) was required for TGFα shedding and subsequent ligand-dependent EGFR activation. Unlike Kohri (158) though, Shao and colleagues found that the antioxidants DMTU and nPG did inhibit elastase induced TGFα shedding and subsequent EGFR activation and MUC5AC production. This suggests that a mechanism involving reactive oxygen species was producing the neutrophil elastase induced mucin gene response. The role of reactive oxygen species was clarified further by showing that TACE activity was not increased by elastase directly whereas H_2O_2 did increase its activity. This supports the hypothesis described above (Chapter 1.3.4) (217) that reactive

oxygen species activate ADAM metalloproteinases which may have been the mechanism of action in this model.

Protein kinase C (PKC) has previously been implicated in the generation of intracellular oxygen radicals (225) and Shao and colleagues went on to show that neutrophil elastase activated PKC (224). This caused the generation of oxygen radicals as measured by increased H_2O_2 production which could be prevented by inhibition of PKC. The inhibition of PKC also resulted in a reduction in TGFα shedding suggesting that it was required for TACE activation. The presence of PKC or TACE inhibitors whilst blocking the MUC5AC effect of neutrophil elastase did not prevent the increase in MUC5AC production when exogenous TGFα was added to the cells.

Other neutrophil products have also been shown to increase mucin gene expression via EGFR activation and MEK signalling during the process of epithelial repair (226), and neutrophil elastase can also increase the induction of other mucin genes such as the membrane bound MUC4 (227) and MUC1 (228). They may also be important in mediating the goblet cell metaplasia and MUC5AC increase seen when animals are exposed to the cytokines involved in allergic responses such as IL-13 (229).

Taken together these data suggest that neutrophil products can increase mucin gene expression and this action is dependent on EGFR activation following ADAM mediated processing of TGFα. Activation of the ADAM metalloproteinase may be dependent on generation of reactive oxygen species by protein kinase C. The extracellular and intracellular pathways involved in this response are similar to those described for cigarette smoke induced mucin gene expression. Figure 1.5 shows some of the routes by which neutrophils, smoke, and bacteria may promote EGFR ligand shedding with subsequent EGFR activation and up-regulation of mucin gene expression.

Figure 1.5 – Pathways used by disease relevant stimuli which result in activation of EGFR

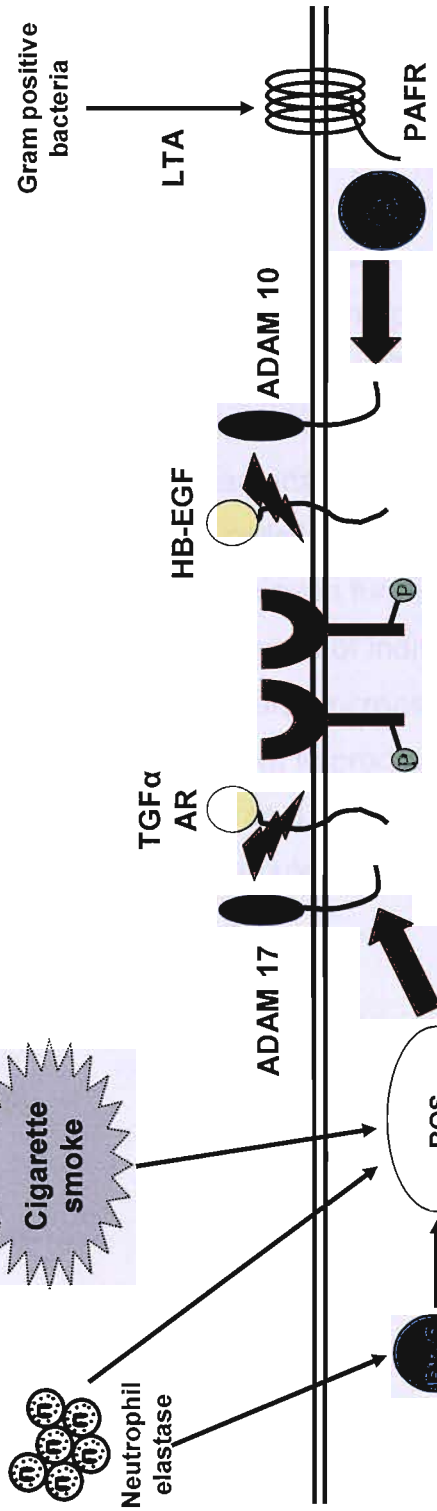


Figure 1.5 – Schematic of possible pathways used by disease relevant stimuli which result in activation of EGFR

1.3.6 EGFR activation and mucin production by other stimuli

Whilst cigarette smoke and neutrophils are key factors in CMH and produce an increase in mucin production via EGFR activation other stimuli have also been shown to act in a similar fashion. Many of these stimuli are relevant to other respiratory diseases where mucus hypersecretion is seen such as asthma, cystic fibrosis, and bronchiectasis. Examples of these stimuli include the bacterial products lipoteichoic acid and lipopolysaccharide which are released from Gram positive and Gram negative bacteria (184;213). Bacteria such as Pseudomonas aeruginosa and Haemophilus influenzae are often found in the sputum of patients with CMH and COPD and also in the airways of individuals with cystic fibrosis. As well as a direct effect on mucin gene expression (230) these and other bacterial products cause recruitment of neutrophils and other inflammatory cells into the airways which as described above may increase mucin production even further. Eosinophils which are found in increased numbers in the airways of individuals with asthma and during some exacerbations of COPD also increase MUC5AC expression via TGFα release and EGFR activation, but to produce this effect the eosinophils must be activated by cytokines such as IL-3 and IL-5 (231). The leukotrienes, another group of asthma related molecules, can also increase MUC5AC expression via the EGFR (232).

1.4 Doxycycline as a metalloproteinase inhibitor

Doxycycline is a member of the tetracycline group of bacteriostatic antibiotics. It is widely used in clinical practice as it has a broad spectrum of activity against organisms which can cause infections of the respiratory tract, the genito-urinary system, and the gut as well as being an effective treatment for systemic infections such as Lyme disease, caused by the spirochaete Borrelia burgdorferi, and the rickettsial infections Q fever and tick-borne typhus (233). With particular regard to respiratory disease it is useful in the treatment of community acquired pneumonia and exacerbations of COPD due to its activity against Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae, and Legionella pneumophilia. The first two of these are commonly isolated from the sputum during exacerbations of COPD (142).

The antibacterial effect of doxycycline and the other tetracycline antibiotics is due to the inhibition of bacterial protein synthesis. This is achieved by its binding to the bacteria's 30S ribosomal subunit thereby preventing aminoacyl tRNA binding to the acceptor site on the mRNA-ribosomal complex. This action requires the intracellular entry of doxycycline which occurs in two ways: it is lipid soluble thus allowing it to pass through the bacterial cell wall lipid bilayer by passive diffusion and there is also an energy-dependent transport system which moves the drug across the inner cytoplasmic membrane. High intracellular concentrations of doxycycline will also inhibit protein synthesis in mammalian cells but these cells lack the active transport system found in bacteria and hence do not achieve the intracellular concentrations needed for inhibition (233). The normal adult therapeutic dose consists of 200mg on day 1 and 100mg once daily hereafter. This gives a peak serum concentration of around 3μg/ml (~5.8μM) 2 hours post dose which is well above the minimum inhibitory concentration of doxycycline of 0.8μg/ml. Some tissues including the lung and the kidney may attain higher concentrations of doxycycline than that found in the serum.

In addition to its antibiotic effect doxycycline has been shown to inhibit members of the matrix metalloproteinase family of enzymes such as MMP-2, MMP-8, and MMP-9 (234-236). These enzymes, which play an important part

in the normal process of tissue remodelling, appear to play a major part in the pathogenesis of many diseases including arthritis, periodontal disease, atherosclerosis, and respiratory diseases such as COPD, bronchiectasis and pulmonary fibrosis (236-245). Different members of the MMP family are released from a diverse range of cells including epithelial cells, fibroblasts, neutrophils, and macrophages and it is their release from bronchial epithelial cells and inflammatory cells in the airway which is felt to play a part in the pathogenesis of COPD as discussed above (Chapter 1.2.3).

The mechanism by which doxycycline inhibits the metalloproteinases is not completely clear. It may be due to chelation of the divalent cations calcium and zinc which are required both for initial activation of the MMP which occurs by cleavage of their proenzymes and for MMP action (246;247) or by causing a conformational change in the metalloproteinase such that it is more susceptible to enzymatic degradation (234).

The MMP inhibitory action of doxycycline has led to its use in periodontal disease (248) and in trials to prevent progression of abdominal aortic aneurysms (249). No trials of long term doxycycline use to prevent progression of respiratory disease have been performed in humans. There have been trials of long term prophylactic antibiotics including tetracyclines to prevent exacerbations of COPD though. Many of these date from the 1950s and 1960s (250;251) when there were difficulties with disease definitions as described earlier and generally antibiotic use in this manner is not recommended (18). There is evidence though that doxycycline attenuates the collagenolytic activity of the MMPs MMP-1, MMP-8, and MMP-13 which are found in increased levels in the BAL fluid of patients with bronchiectasis another respiratory disease where progression is felt to be related to uncontrolled inflammation and tissue destruction (236;252).

The action of doxycycline on the membrane-bound zinc-dependent ADAM sheddases has not previously been studied. It is the metalloproteinase activity of ADAM proteins which is required for release of active ligands for the EGFR (180). In view of the inhibitory action of doxycycline on other metalloproteinases it is likely that doxycycline may inhibit members of the ADAM sheddase family. If this were so it would decrease EGFR ligand release in response to stimuli such as CSE, and this may result in a reduction

in EGFR activation. This could attenuate the pro-inflammatory response generated by CSE much of which is dependent on EGFR activation, and possibly alter mucin gene expression. If this was the case this could identify a potential target for drugs which could lead to beneficial clinical effects in patients with COPD and mucus hypersecretion and may alter the structural changes that are seen in patients with CMH.

1.5 Conclusions

Cigarette smoking is a major cause of morbidity and mortality in the developed world. It is the leading cause of respiratory diseases including COPD (1). CMH is often seen in individuals with COPD, but can also occur in smokers with no airflow obstruction and in other respiratory diseases. In the setting of COPD it appears that CMH may accelerate further the increased decline in lung function seen in smokers, and increase the chance of developing chest infections (137;138). These are associated with significant morbidity and mortality particularly in those with more advanced COPD.

A number of pathological changes are seen in the airways of individuals with CMH including bronchial mucosal gland hypertrophy and goblet cell hyperplasia (44;166). These changes appear to occur as a result of EGFR activation (166).

The EGFR and its ligands play a key role in mucin gene expression and in promoting airway inflammation which worsens mucus hypersecretion (166;210). An increasingly broad range of stimuli have been shown to activate the EGFR and increase mucin gene expression and mucin production (166;201;220;224). This activation may be via a number of different mechanisms but it appears that one of the key processes involved is activation of the ADAM metalloproteinases which cause the release of active EGFR ligands from their membrane-bound precursors (214).

The ADAMs that are activated may vary depending on the stimulus and hence different ligands be released (184). This may provide a mechanism for different signalling pathways to be activated in response to different stimuli or provide a means of convergence of diverse stimuli into a final pathway resulting in mucin gene expression. It is not clear whether one of the EGFR ligands is most important in mediating mucin gene expression or whether all the ligands produce this response. Different groups have found contradictory results in this regard (210;214;215).

Currently there are no effective treatments to attenuate the symptoms of CMH. The pathway involving the EGFR and its ligands may provide a useful therapeutic target in this respect. As it appears that several of the ligands produce mucin gene responses when they bind to the EGFR an inhibitor of

the ADAM metalloproteinases which would prevent cleavage of the pro-ligands may be clinically useful. As different ligands are cleaved by different ADAMs (181;183-185) an inhibitor which is not completely specific for one ADAM may potentially be the most useful agent. Decreasing pro-inflammatory and mucin responses in cells exposed to cigarette smoke may offer a potential treatment to modulate the natural history of COPD and CMH and also have relevance for other conditions associated with excessive mucus production.

1.6 Hypotheses

Differing patterns of gene expression will be seen in bronchial epithelial cells exposed to different members of the family of epidermal growth factor receptor ligands

Doxycycline, acting as an inhibitor of EGFR ligand shedding, will attenuate the pro-inflammatory and mucin gene responses seen in bronchial epithelial cells exposed to cigarette smoke extract

1.7 Aims

In these studies the main aims are as follows:

To investigate the effects of different EGFR ligands on expression of genes involved in inflammation and mucin production and on autocrine expression of EGFR ligands in bronchial epithelial cells

To investigate the effects of a CMH/COPD disease relevant stimuli (cigarette smoke) on mucin gene expression and on release and expression of the neutrophil chemoattractant IL-8 in the same cells

To investigate whether the effects of cigarette smoke on the cells involve EGFR ligand release and gene expression

To investigate whether inhibition of metalloproteinases reduces EGFR ligand shedding and whether this alters the cellular response to cigarette smoke

2 Chapter 2: Materials and methods

2.1 Materials

2.1.1 Cell culture reagents and disposables

The following reagents were used for tissue culture and were purchased from Invitrogen, Paisley, UK.

RPMI 1640 medium without L-glutamine

Hank's balanced salt solution (HBSS) without calcium

L-glutamine 200mM (100x) solution

Penicillin – streptomycin 100x solution

Foetal bovine serum (Gamma-irradiated)

Typsin-EDTA 10x solution

The following reagents were used for tissue culture and were purchased from Biowhittaker, Wokingham,UK.

Ultraculture tissue culture medium

Dulbeccos modified Eagles medium (DMEM) liquid

Culture flasks, Petri dishes, and 6, 12, and 24 well culture trays were from Nunc (Fisher Scientific), Loughborough, UK.

2.1.2 Reagents and antibodies for treatment of cell cultures

The following compounds and proteins were used for stimulation/inhibition of the cell cultures.

Doxycycline hydrochloride (Calbiochem, Nottingham, UK)

Recombinant human amphiregulin (long form), recombinant human heparin-binding EGF like growth factor (HB-EGF), and recombinant human TGFα were purchased from R&D systems (Abingdon, Oxfordshire, UK)

Polyclonal sheep anti-EGFR antibody. The antibody used had been previously produced in-house by raising antibodies against affinity-purified EGF receptors derived from A431 squamous carcinoma cell membranes and partially purified by $(NH_4)_2SO_4$ precipitation and diethylaminoethyl ion exchange chromatography.

2.1.3 Cigarette smoke extract

The following materials were used for the production of cigarette smoke extract.

Kentucky 1R4F research cigarette (University of Kentucky, Lexington, KY, USA)

DMEM (Biowhittaker, Wokingham,UK)

Ultraculture (Biowhittaker, Wokingham,UK)

0.22µm Millex-GS filter (Millipore, Watford, UK)

2.1.4 ELISAs and LDH assay

The following buffers and reagents were used for the amphiregulin and IL-8 ELISAs.

Coupling Buffer – Na_2CO_3 (1.59g/l), $NaHCO_3$ (2.93g/l), and NaN_3 (0.2g/l) were dissolved in distilled water. The pH was corrected to 9.6 and the buffer stored at room temperature.

Blocking buffer (10x) – NaCl (80g/l), $Na_2HPO_4.2H_2O$ (14.2g/l), KH_2PO_4 (2g/l), KCl (2g/l), and BSA fraction V (50g/l) were dissolved in distilled water. The pH was corrected to 7.4 and the buffer stored in 5ml aliquots at -20°C. The buffer was diluted with distilled water to a working concentration of 1x for use.

PBS (10x) – Na_2HPO_4 (12.8g/l), NaCl (85g/l), and $Na2HPO_4.2H_2O$ (1.56g/l) were dissolved in distilled water. The pH was corrected to 7.4. The buffer was stored at room temperature and diluted with distilled water to a working concentration of 1x for use.

Wash buffer – 1x PBS with 0.05% Tween-20 (0.5ml/l). The pH was corrected to 7.4.

Assay buffer – 1x blocking buffer with 0.1% Tween-20 (1ml/l)

Chromagen diluent – sodium acetate.$3H_2O$ (13.6g/l) was dissolved in distilled water. The pH was corrected to 6.0 with 0.1M citric acid. The diluent was autoclaved to prevent bacterial/fungal growth during storage and stored at 4°C.

TMB stock solution – tetra-methyl benzidine (30mg) was dissolved in 5ml dimethyl sulphoxide (DMSO). This solution was stored at room temperature protected from light. In this environment it was stable for 1 month.

Chromagen solution – 200µl TMB stock solution and 1.2µl 30% H_2O_2 were added to 12ml chromagen diluent. This was prepared immediately prior to use.

The following reagents and solutions were used for the LDH assay and purchased from Sigma (Dorset, UK).

βNADH, reduced form (10mg/vial).

Sodium pyruvate solution (0.75mM pH 7.5).

Colour reagent – 2,4 dinitrophenylhydrazine in 1M HCl.

Substrate solution – 10ml sodium pyruvate solution was added to 1 vial of βNADH. This is an unstable solution and hence made up immediately before use.

Stop solution – 1.6g NaOH were diluted in 100ml distilled water to make a 0.4M solution. This was made up immediately before use.

2.1.5 Reagents and disposables for RNA extraction and reverse transcription

The following products were used for RNA extraction and reverse transcription.

RNase/DNase free water

Trizol reagent (Invitrogen, Paisley, UK)

Molecular grade chloroform (Sigma, Dorset, UK)

Molecular grade isopropanol (Sigma, Dorset, UK)

Molecular grade ethanol (Sigma, Dorset, UK)

High purity analytical grade agarose (Bio-rad, Hertfordshire, UK)

Ethidium bromide (Sigma, Dorset, UK)

Tris-borate EDTA (TBE) buffer (Invitrogen, Paisley, UK)

DNase kit (Ambion, Cambridgeshire, UK)

MMLV reverse transcription kit (Promega, Hampshire, UK)

Random hexamers and dNTP (MWG Biotech UK, Milton Keynes, UK)

RNase zap, RNase/DNase free microfuge tubes (Sigma, Dorset, UK)

RNase/DNase free barrier pipette tips (ART, Promega, Hampshire, UK)

2.1.6 Reverse transcription qPCR

The following additional products were used for RT-qPCR.

RT-qPCR master mix (Eurogentech, Seraing, Belgium)

Primers and probes for RT-qPCR assays were designed by Dr Rob Powell in the Brooke Laboratory, Southampton General Hospital, Southampton and manufactured by Oswel DNA service (Eurogentech, Seraing, Belgium) except 18S ribosomal RNA primers and probes which were purchased direct from Eurogentech.

iCycler iQ™ RT-qPCR machine (Bio-rad, Hertfordshire, UK)

96 well optical plates and plate sealer (Bio-rad, Hertfordshire, UK)

2.2 Methods

2.2.1 Cell culture of bronchial epithelial cell lines

The mucoepidermoid bronchial epithelial cell line NCI-H292 (American type culture collection (ATCC)) was used routinely for cell culture work. This is an adenocarcinoma cell line which constitutively expresses epidermal growth factor receptor (EGFR). The cells were maintained in $80cm^3$ Nunc tissue culture flasks using RPMI-1640 cell medium which had been supplemented with 10% heat-inactivated foetal bovine serum (FBS), 50iu/ml penicillin, 50μg/ml streptomycin and 2mM L-glutamine. Cells were grown in an humidified Heraeus incubator at 37°C, 5% CO_2. The cultures were fed every 48 hours until confluent. All culture techniques were routinely performed in a Microflow class II biological safety cabinet using sterile techniques. All media and reagents were warmed to room temperature prior to use.

2.2.1.1 Trypsinisation of the confluent cell monolayer

When the H292 cells were confluent they were passaged using 1x trypsin-EDTA to detach the cells from the flask surface. This was prepared by diluting 10x trypsin stock 1:10 in HBSS.

Trypsin is inactivated by calcium and magnesium salts and therefore after removal of the culture medium the cell monolayer was washed twice using 8mls HBSS. 1.5mls of 1x trypsin was then added to the flask which was incubated for up to 5 minutes to allow detachment of the cells. Flasks were tapped sharply to detach any cells remaining adherent to the surface. Once all the cells were detached on visual inspection 10mls of RPMI/FBS was added to the flask to inactivate the trypsin. The cell suspension formed was diluted 1:10 in a new $80cm^3$ flask for routine passage or used either to create a cell line stock for storage or counted and diluted for plating into experimental trays.

2.2.1.2 Cryogenic storage and regeneration of cell stocks

Post trypsinisation the cell suspension could be frozen in liquid nitrogen to create a stock of the cell line for use in future experiments. The cell suspension was spun in a Sigma 3-10 desktop centrifuge at 150g for 5

minutes. The supernatant was discarded and the cell pellet re-suspended in culture medium containing 10% (v/v) dimethylsulphoxide (DMSO), which acts as a cryoprotectant, to give a final density of $1x10^6$ cells/ml. 1ml aliquots of the cell suspension were placed in cryotubes and frozen at -80°C for 24 hours before being placed into a liquid nitrogen freezer at -180°C. This method allows a gradual freezing process to occur making it less likely that ice crystals will form within the cells.

To regenerate the frozen stock small volumes of culture medium were added to the cryotube to thaw the cells as quickly as possible. The cells were then added to an $80cm^3$ flask containing 30mls of culture medium and placed in an incubator at 37°C for 6 hours to allow the cells to adhere to the flask. At this time the medium was removed and replaced with fresh culture medium so as to remove traces of DMSO.

2.2.1.3 Cell counting

Where cells were to be used for experiments the cell density was measured using a Neubauer haemocytometer. The cell suspension was examined visually and, if necessary, any large cell clumps were carefully disrupted by aspirating the suspension through a 25 gauge needle to give a suspension of single cells. 50μl of this suspension was taken and added to 50μl of trypan blue dye. The dye is only able to enter those cells that do not have an intact cell membrane, an indicator of cell death, turning these cells blue. This method allows the counting of viable (non-blue) cells only.

A pipette was used to introduce a small volume of the suspension to either side of the haemocytometer. This has a depth of 0.1mm and a counting area of $1mm^2$ (volume $0.1mm^3$). The number of viable cells per ml is then calculated by multiplying the mean cell count for the 2 squares by 2 (dilution factor with trypan blue) and then by 10^4.

2.2.1.4 Plating of cells for treatments

For stimulation experiments the H292 cells were prepared and trypsinised to form cell suspensions as described above. The experiments were performed in 6, 12 or 24 well culture trays. For 24 well trays the cells were plated at a density of $2x10^5$/ml by diluting the cell suspension as necessary. For 12 and 6 well trays the cells were plated at densities of $2.75x10^5$/ml and $3.75x10^5$/ml

respectively. At these densities the cell monolayer would become 90% confluent at 24-48 hours. At this stage cells were rendered quiescent by the replacement of culture medium with serum free medium (SFM-Ultraculture) for 24 hours. After 24 hours of incubation cells were ready for the various stimulation experiments as described below.

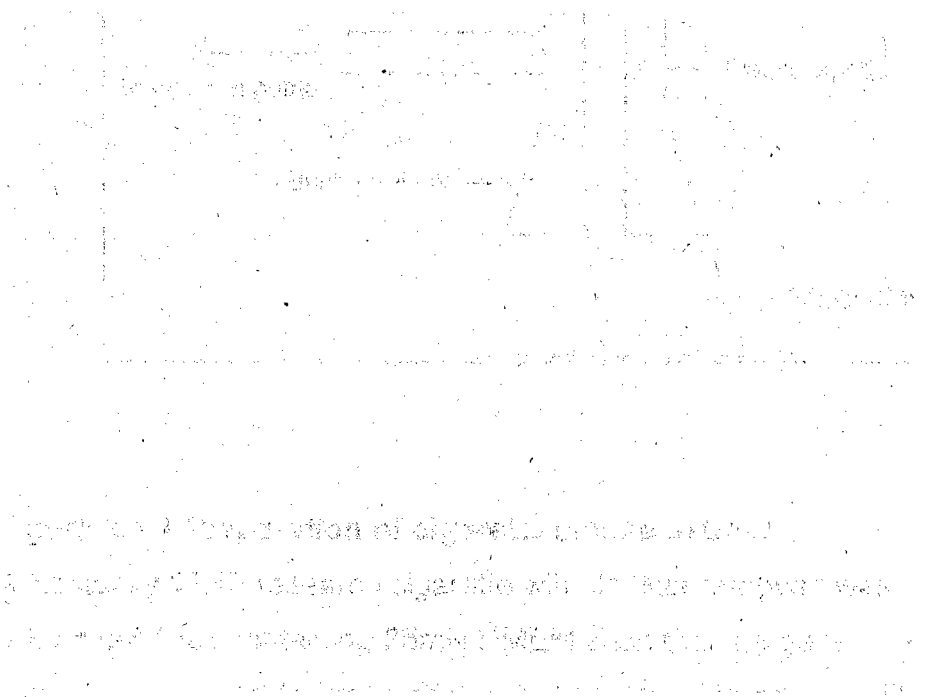
Following treatment and subsequent incubation the cell supernatant was removed and stored at -80°C for later analysis by ELISA. The remaining cell monolayer if needed was then treated with TriZol for RNA extraction as described below.

For stimulation experiments with EGFR ligands 12 well trays were prepared as above. Following removal of SFM EGFR ligand diluted in SFM as required was added to the cells which were then incubated for 0 (baseline control), 2, 6, 12, or 24 hours before the supernatant was removed and the cells treated for RNA extraction. Concentrations of ligand used are as shown in table 2.1.

To investigate the effects of doxycycline on stimulation of cells with CSE 6 well trays were prepared. After 24 hours incubation the SFM was removed and replaced by either SFM (control) or doxycycline solution to give a final concentration of either 10μM or 25μM doxycycline. Once this had been done fresh CSE smoke extract was prepared and added to half the wells on the tray to give a final concentration of 5% CSE. The time from addition of doxycycline/SFM to stimulation with CSE was 15 minutes. Where TGFα release was to be measured an anti-EGFR antibody was added to the wells at a concentration of 0.5mg/ml to prevent binding and internalisation of the TGFα/EGFR complex. This was added 15 minutes prior to stimulation with CSE. Where exogenous TGFα was added to the cultures for stimulation this was added at the same time as CSE i.e. 15 minutes after doxycycline/SFM had been added. The concentrations of substances used are shown in table 2.1.

Table 2.1 - Compounds used in cell culture experiments

Protein/compound	Supplier	Concentrations used
Amphiregulin (AR)	R & D Systems	0.5, 5, 10, 25, 50 ng/ml
Heparin-binding EGF like growth factor (HBEGF)	R & D Systems	0.5, 5, 10, 25, 50 ng/ml
Transforming growth factor alpha (TGFα)	R & D Systems	0.025, 0.25, 0.5, 1, 2.5 ng/ml
Anti EGFR antibody	Prepared in house	0.5mg/ml
Doxycycline hydrochloride	Calbiochem	10µM, 25µM



2.2.2 Preparation of cigarette smoke extract (CSE)

CSE was prepared by connecting one Kentucky 1R4F research cigarette with its filter removed to a Buchner flask via a short piece of tubing. Using a vacuum pump the cigarette was smoked through 25ml DMEM over 1-2 minutes (Figure 2.1) to produce 100% CSE. The CSE was filtered through a 0.22µM Millex-GS filter and diluted using Ultraculture serum free medium. The CSE was prepared for each experiment as necessary and used immediately. It could not be stored.

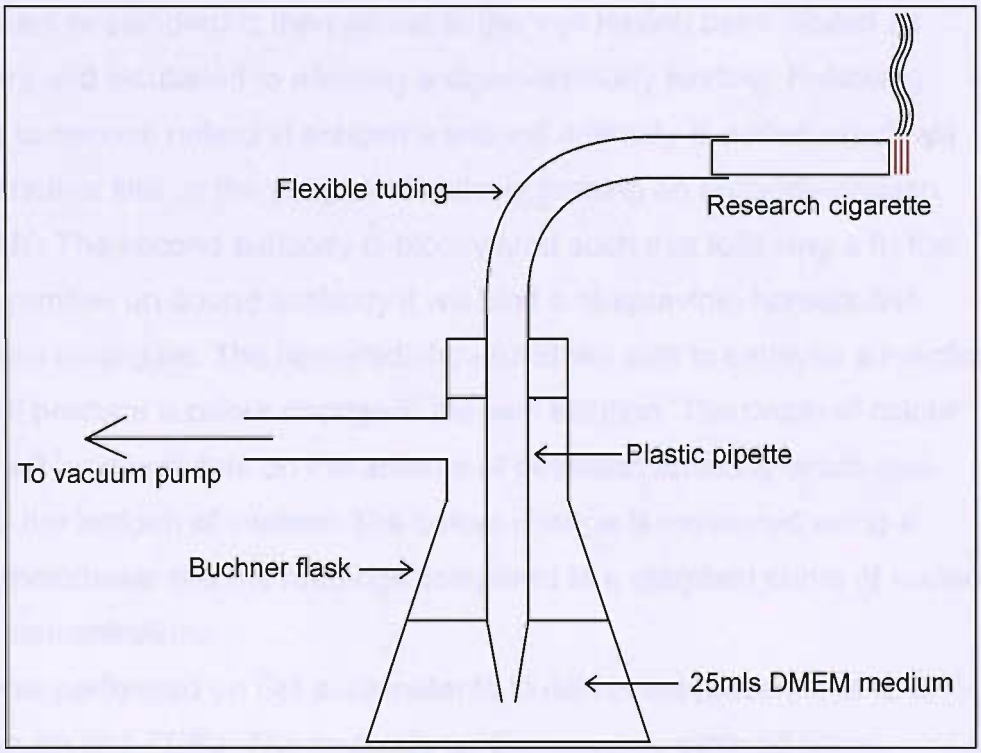


Figure 2.1 – Preparation of cigarette smoke extract

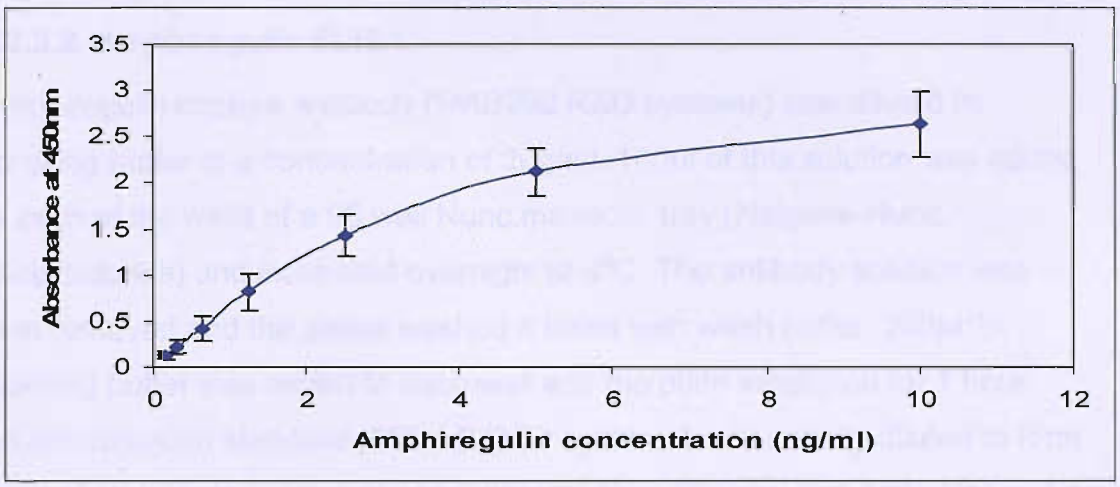
A Kentucky 1R4F research cigarette with its filter removed was connected to a Buchner flask containing 25mls DMEM such that the gases from the cigarette were bubbled through the DMEM. The cigarette was lit and the vacuum pump started. This procedure caused the cigarette to be 'smoked' over 1-2 minutes. The DMEM was then filtered and diluted for use

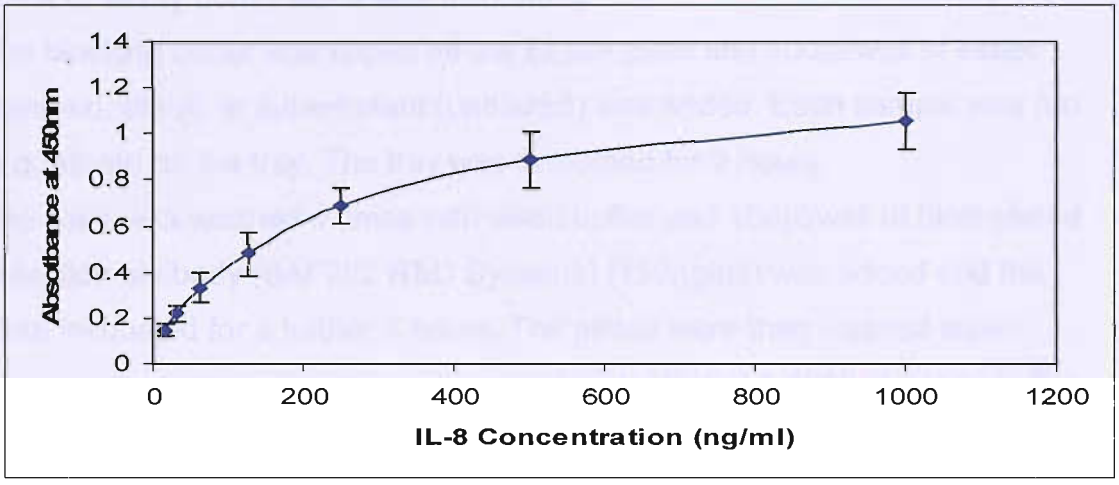
2.2.3 Enzyme-linked immunosorbent assay (ELISA)

2.2.3.1 ELISA principles

The ELISA method of antigen detection relies on the specificity of antibodies to bind to antigens present in the cell supernatant. The sandwich ELISA technique uses 2 antibodies which bind to non-overlapping epitopes on the antigen of interest. The first antibody, known as the capture antibody, is bound to the floor of the reaction well coming either pre-bound as part of the manufacturer's kit or by overnight incubation onto absorbent trays. The well is then washed and blocked with BSA to prevent non-specific binding. The supernatant or standard is then added to the well having been diluted as necessary and incubated to allowing antigen-antibody binding. Following washing to remove unbound antigen a second antibody is added which will bind to another site on the antigen effectively forming an antibody-antigen 'sandwich'. The second antibody is biotinylated such that following a further wash to remove un-bound antibody it will bind a streptavidin-horseradish peroxidase conjugate. The horseradish peroxidase acts to catalyse a reaction which will produce a colour change in the well solution. The depth of colour change will be dependent on the amount of detection antibody which has bound to the antigen of interest. The colour change is measured using a spectrophotometer and the readings compared to a standard curve of known antigen concentrations.

ELISA was performed on cell supernatants to detect the presence of IL-8, amphiregulin and TGFα. The methods for these are as outlined below. Typical standard curves for AR and IL-8 are shown in figure 2.2.





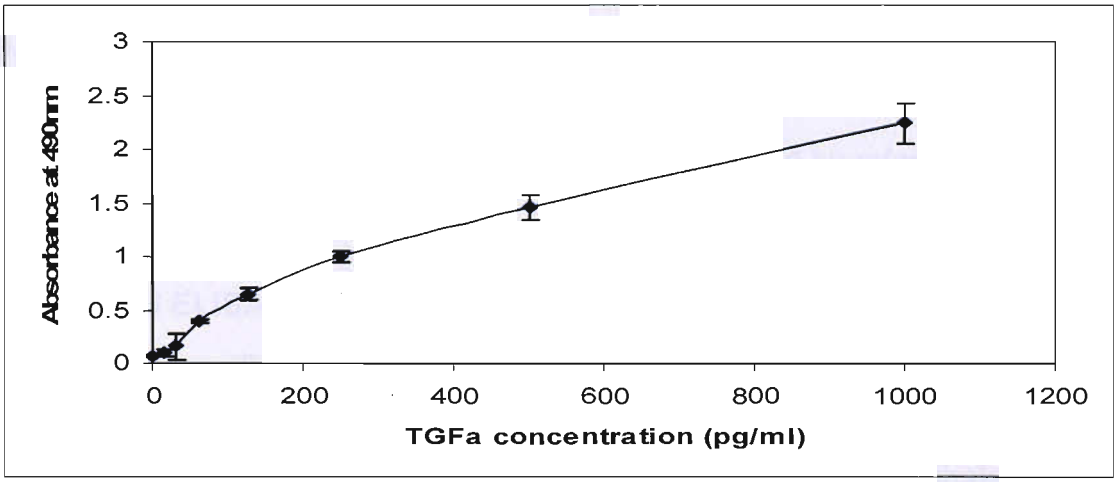


Figure 2.2 – Representative standard curves for amphiregulin, IL-8, and TGFα ELISAs

Data are the mean +/- SD of n=10 standard curves except for TGFα where n=4

2.2.3.2 Amphiregulin ELISA

Amphiregulin capture antibody (MAB262 R&D systems) was diluted in coupling buffer to a concentration of 2µg/ml. 100µl of this solution was added to each of the wells of a 96 well Nunc maxisorb tray (Nalgene-Nunc, Herefordshire) and incubated overnight at 4°C. The antibody solution was then removed and the plates washed 4 times with wash buffer. 200µl 1x blocking buffer was added to each well and the plate incubated for 1 hour.
An amphiregulin standard (262-AR R&D systems) was serially diluted to form a set of standards ranging in concentration from 10ng/ml to 0.156ng/ml and a blank of assay buffer alone was also made.
The blocking buffer was tipped off the ELISA plate and 100µl/well of either standard, blank, or supernatant (undiluted) was added. Each sample was run in duplicate on the tray. The tray was incubated for 2 hours.
The plate was washed 4 times with wash buffer and 100µl/well of biotinylated detection antibody (BAF262 R&D Systems) (150ng/ml) was added and the plate incubated for a further 2 hours. The plates were then washed again.
Streptavidin-horseradish peroxidase conjugate diluted 1:1000 in assay buffer was added to the plate (100µl/well) and incubated for 30 minutes. The plate was washed and 100µl/well of chromagen solution was added and incubated for 10-30 minutes in the dark at room temperature. The reaction was then stopped by the addition of 50µl/well of 2M sulphuric acid.
The plate could then be read on a microplate spectrophotometer at 450nm wavelength. Sample concentrations were then calculated by reference to the standard curve.

2.2.3.3 IL-8 ELISA

An IL-8 cytoset (Biosource International, Camarillo, CA) was used for this ELISA. The capture antibody was diluted in coupling buffer to a concentration of 1µg/ml. 100µl/well were added to a Nunc maxisorb tray and incubated overnight at 4°C.
The following morning the capture antibody was tipped off and the trays washed 4 times with wash buffer. 200µl 1x blocking buffer was added to each tray and incubated at room temperature for 2 hours.

An IL-8 standard curve was generated by making serial dilutions of the IL-8 standard antibody in the kit with a concentration range from 1ng/ml to 0.02ng/ml.

The trays were washed to remove the blocking buffer and 100µl/well of standard or cell supernatant added to the tray with duplicate samples being run. The cell supernatant was diluted with assay buffer as necessary to give concentrations of 1:2 – 1:20. Following addition of the sample 50µl/well of biotinylated detection antibody at a concentration of 0.1µg/ml was added to each well. The tray was left to incubate for 2 hours to allow antigen-antibody binding.

After 2 hours the supernatant/standard and detection antibody was tipped from the tray and the tray washed with wash buffer as above. 100µl of streptavidin-horseradish peroxidase conjugate diluted 1:2500 in assay buffer was added to each well. The tray was incubated for 30 minutes.

The tray was washed again and 100µl of chromagen solution added to each well. This was left on for 5-10 minutes before the reaction was stopped using 50µl of 2M sulphuric acid.

The plates were read on the spectrophotometer at a wavelength of 450nm with the sample concentrations of IL-8 being calculated by reference to the standard curve.

2.2.3.4 TGFα ELISA

The TGFα ELISA came as a prepared kit from Oncogene research products. The assay plates are pre-coated with polyclonal TGFα antibodies and stored with the other reagents at 4°C prior to use. The reagents are warmed to room temperature before commencing the assay.

Rinse and wash buffer was prepared as per the manufacturers instructions. TGFα standard was reconstituted to give a stock solution of 1000pg/ml and serial dilutions performed to give a range of concentrations down to 62.5pg/ml with a sample of assay buffer alone for 0pg/ml.

The reporter antibody was diluted 1:20 with assay buffer and 50µl added to each well. 50µl of standard/sample was then added on top of the reporter antibody with each standard/sample being run in duplicate. The plate was sealed and incubated at room temperature for 3 hours.

At the end of incubation the tray was washed 3 times with wash buffer followed by 1 wash with rinse buffer which should not remain on the plate for more than 5 minutes. The rinse buffer was tipped off and 100µl of 1x peroxidase conjugate, which was prepared from the manufacturers supplied 100x solution by dilution with assay buffer, was added to each well. The tray was then incubated for 30 minutes.

At the end of this time the peroxidase conjugate was removed and the trays washed 3 times with wash buffer and once with rinse buffer. This was tipped off and 100µl of substrate solution added to each well. The substrate solution had been prepared by diluting 1 O-Phenylenediamine (OPD) tablet in 4ml substrate diluent to give enough solution for 100µl/well. It is light sensitive and thus stored in the dark and used within 30 minutes of preparation. The tray was then incubated in the dark for 30 minutes and the reaction then stopped by the addition of 100µl of stop solution (2.5M sulphuric acid) to each well. The plate was read on a microplate spectrophotometer using a wavelength of 490nm. Sample concentrations were calculated with reference to the standard curve.

2.2.4 Lactate dehydrogenase assay

Lactate dehydrogenase is an intracellular enzyme which is released into the cell supernatant when the cell undergoes necrosis. The LDH activity in the supernatant can be assayed to give an indication of the extent of cell necrosis caused by any of compound of interest. The lactate dehydrogenase assay was run in a 24 well tray using fresh supernatant taken directly from the cell cultures. The reagents used are described in the materials section above. 100µl of substrate solution was added to each well of a 24 well plate. 10µl of sample was added to each well with each sample being run in duplicate. The tray was then incubated for 30 minutes at 37°C.

100µl of colour reagent was added to each well and the tray incubated for a further 20 minutes at room temperature. The reaction was then stopped by the addition of 1ml/well of 0.4M NaOH.

At the same time a standard curve was prepared by mixing sodium pyruvate solution and distilled water in the volumes shown below. 100µl of colour reagent was added to each well and the tray incubated for 20 minutes at

room temperature. The reaction was stopped by the addition of 1ml 0.4M NaOH to each well.

300µl from each well of the samples or standards was taken and added to a 96 well tray to be read on the microplate spectrophotometer at a wavelength of 450nm. The samples' LDH activity in Berger-Broide units/ml was calculated by reference to the standard curve.

Volumes for the standard curve preparation are as shown in the table 2.2 below.

Table 2.2 – Volumes of reagents used for preparation of the LDH activity standard curve

	Well1	Well 2	Well 3	Well 4	Well 5	Well 6
Pyruvate solution	100µl	80µl	60µl	40µl	20µl	10µl
Distilled water	10µl	30µl	50µl	70µl	90µl	100µl
LDH Activity (B-B units/ml)	0	280	640	1040	1530	2000

2.2.5 RNA Preparation from bronchial epithelial cells

2.2.5.1 RNA extraction precautions

RNA extraction was performed on the cell monolayers post treatment. RNA is easily degradable and as such special precautions need to be taken to prevent contamination of samples particularly with RNase. RNA/DNA work was carried out in a dedicated area which was cleaned with RNase-zap prior to use. Sterile DNase and RNase free tubes and sterile barrier pipette tips were used. All reagents used were certified DNase/RNase free and MilliQ quality distilled water (Millipore) was used.

2.2.5.2 Preparation of samples into Trizol

After removal of the cell supernatant Trizol was added to the well. Trizol reagent is a monophasic solution of phenol and guanidine isothiocyanate and can be used for a single step extraction of RNA. 750µl/well was added for 6 well trays and 500µl/well for 12 well trays. To ensure that all cells were lysed and homogenised the Trizol was pipetted up and down onto the cell monolayer several times. The cells were left for 5 minutes at room temperature to allow cell components to dissolve and for the dissociation of soluble proteins from the RNA. The samples were then collected and stored in RNase/DNase free microfuge tubes. The samples could be stored in this state at -80°C for later RNA extraction.

For subsequent steps the samples were thawed if necessary and 200µl chloroform per 1ml Trizol added to each sample. The tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 10 minutes. The tubes were then spun at 12000g for 15 minutes at 4°C.

The centrifugation separates the sample into a top aqueous fraction containing the RNA, an interphase containing DNA, and a phenol fraction containing protein. The RNA containing aqueous layer was carefully pipetted off into a sterile microfuge tube aiming to take as much of the layer as possible without carry over from the interphase. 500µl of isopropanol was then added to each sample and the samples mixed by vigorous shaking.

Although the samples can then be used immediately overnight incubation at -20°C improves RNA yield and so this method was routinely used.
The following morning the samples were warmed to room temperature and incubated at this temperature for 15 minutes. The tubes were centrifuged at 12000g for 30 minutes at -4°C. This produces a pellet of RNA at the tip of the tube which can be seen visually when there is a significant quantity of RNA. The supernatant was removed and discarded with careful pipetting to prevent disruption of this pellet and 1ml ethanol added to each tube for washing.
The tubes with ethanol were spun in the centrifuge for 5 minutes at 12000g - 4°C and the ethanol was removed by pipetting and pulse spinning of the tube to remove all traces of ethanol. The remaining pellet was air-dried for around 5-10 minutes. This produces a translucent pellet which can then be re-suspended for the next stage of extraction. It is important that no traces of ethanol remain as this can inhibit some of the down stream reactions.

2.2.5.3 DNase treatment of RNA sample

During the RNA extraction it is possible that traces of genomic DNA will still be present in the sample. It is necessary to remove this contaminating DNA so that the only DNA present after reverse transcription will be copy DNA (cDNA) produced from the RNA in the sample. This process requires treatment of the RNA pellet with a DNase.
The DNase treatment was performed according to the manufacturer's instructions. In brief each RNA sample was re-suspended using 17µl RNase free water with 1 unit DNase (1µl volume) and 2µl 10x DNase buffer. The sample was gently agitated to re-suspend the pellet and then incubated for 1 hour at 37°C.
The DNase enzyme was inactivated by the addition of a neutralising reagent. 5µl of the neutralisation slurry were added to each sample. The samples were mixed by gentle tapping to disperse the slurry and then incubated at room temperature for 2 minutes followed by a further mix and 2 minutes incubation. The samples were pulse spun to send the slurry to the bottom of the tube and in this state could be stored at -80°C.

2.2.6 Quantification of RNA

The RNA extracted from the cell monolayers needs to be quantified prior to reverse transcription and also to be examined to look for evidence of significant degradation or contamination with proteins or DNA. This can be done in one step using a 1% agarose gel. Again as with RNA extraction it is essential that the equipment used is cleaned and washed with RNase zap prior to use to prevent degradation of the RNA during analysis.

The gel apparatus and combs were cleaned as described and masking tape used to form the sides of the wells. The agarose gel was formed by the addition of 0.75g agarose to 100ml 1x tris-borate-EDTA (TBE) buffer which was heated in a microwave oven on medium power to dissolve the agarose. This solution was then cooled to around 55°C and 10µl ethidium bromide added. This stains the RNA to allow visualisation of the bands. The agarose solution is poured into the tray, the combs inserted, and left to set. Once set the masking tape was removed from the ends of the tray and the tray placed in a horizontal gel tank which was filled with 1x TBE buffer to cover the gel by approximately 3mm. The combs were then carefully removed from the gel to prevent air bubbles getting into the wells.

The wells were filled with a mixture of 2µl RNA sample and 8µl 2x loading buffer (30% glycerol, 0.5% bromophenol blue, 70% 1x TBE buffer). In addition an RNA standard curve was produced using 0.2µg/µl stock RNA. This RNA had previously been extracted from bronchial epithelial cells, assessed for absence of degradation and quantified using a spectrophotometer. The curve was produced by the addition of 0, 1, 2, 3 and 4µl of standard to 2x loading buffer to give a final volume of 10µl per well giving standards of 0ng, 200ng, 400ng, 600ng, and 800ng RNA.

When all samples and standards were loaded into the gel it was run at a constant current of 80mAmps for 30 minutes or until the loading dye had moved down the gel by around 4cm. This allows sufficient progress of the RNA for quantification and quality assessment. The tray was then removed from the tank and the gel transferred carefully to a UV light visualiser. The samples were examined for the presence of 2 discrete bands representing the 18S and 28S ribosomal subunits. mRNA appears as a smear

predominantly between these 2 bands. Significant blurring of the bands and smearing down the gel compared to the standards indicates significant degradation of RNA. Significant smearing up the gel indicates genomic DNA contamination. A typical gel is shown below (Figure 2.3).
If the RNA was of sufficient quality the quantity in each sample was assessed using the "GeneSnap" protocol and the "Gene Genius" bioimaging system. Essentially this involved assigning each sample/standard to a track on the imaging software and then assessing the pixel intensity of each of the 18S and 28S bands. A standard curve was produced by plotting the standard pixel intensities against the RNA quantity for each standard using Microsoft™ Excel. Sample RNA quantities could then be calculated by reference to this standard curve.

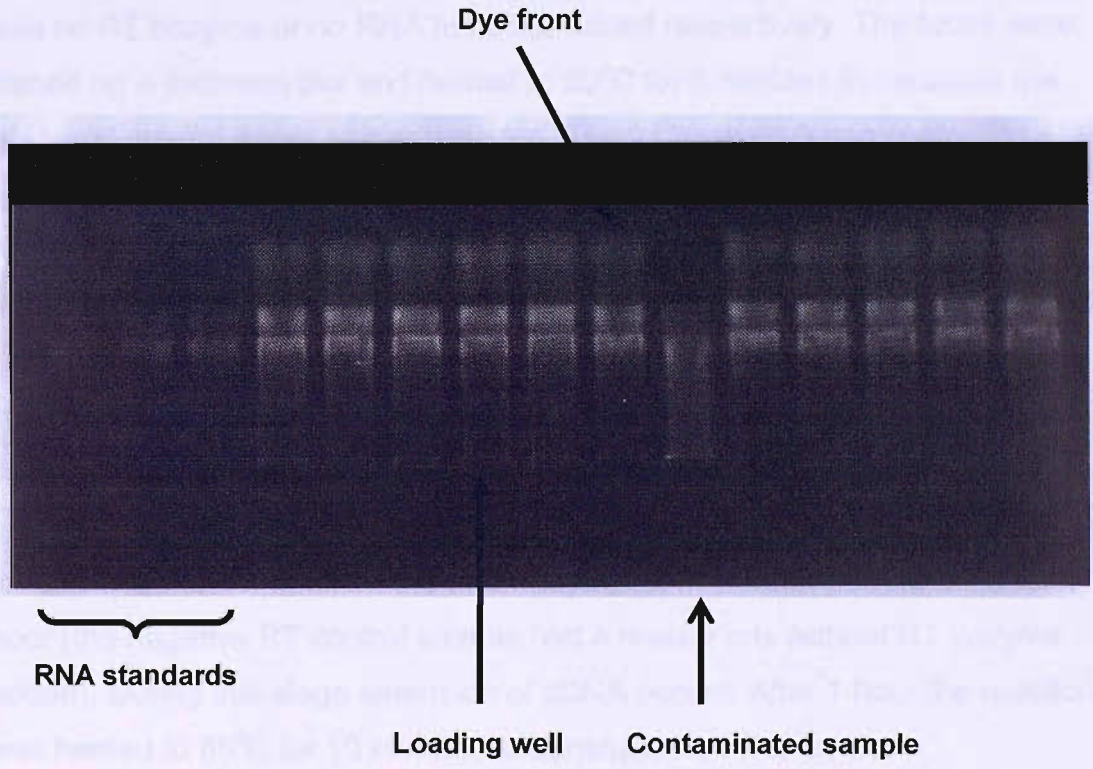


Figure 2.3 – Quantification and qualitative analysis of RNA using agarose gel

2.2.7 Reverse transcription of RNA to cDNA

To provide a DNA template for reverse-transcription quantitative PCR (RT-qPCR) it is necessary to form cDNA from the extracted RNA by the process of reverse transcription (RT). This process has 2 stages: Annealing when the primer is bound to the template, and extension to form new single stranded DNA. The method used was as per the manufacturer's instruction and is outlined in brief below.

The reaction was carried out in sterile RNase/DNase free thin walled 200µl microtubes. For each reaction 1µg RNA sample (based on calculations from the agarose gel analysis) was added to the tube with 1µl random hexamers (3µg/ml), 1µl dNTP mix (10mM) and water to make a final volume of 10µl. A negative RT control and negative template control were included where there was no RT enzyme or no RNA template added respectively. The tubes were placed on a thermocycler and heated to 80°C for 5 minutes to denature the RNA and ensure it was single stranded. The tubes were "snap cooled" in a dry ice/ethanol bath.

An RT master mix was made to be added to each tube. Moloney murine leukaemia virus (MMLV) reverse transcriptase was used for the reaction. This enzyme is an RNA dependent DNA polymerase that allows first strand synthesis of cDNA from RNA. A master mix containing 0.5µl MMLV RT (100 units), 4µl MMLV transcription buffer, and 5.5µl water per reaction was prepared. 10µl of this master mix was added to each RT tube and the RT tubes placed either back on the thermocycler or in a water bath at 37°C for 1 hour (the negative RT control sample had a master mix without RT enzyme added). During this stage extension of cDNA occurs. After 1 hour the reaction was heated to 85°C for 10 minutes to denature the RT enzyme.

The aim of the procedure was to produce approximately 1µg of cDNA. This product is relatively stable and can be stored at -20°C until needed.

2.2.8 Reverse-transcription quantitative Polymerase Chain Reaction (RT-qPCR)

2.2.8.1 RT-qPCR principles

The polymerase chain reaction can be used to detect the presence of a target gene/specific DNA fragments in a sample of DNA (which may be genomic double-stranded DNA or DNA produced by reverse transcription from RNA) by a stepwise amplification of the region of interest. To allow the PCR to occur a nucleotide sequence for the area of interest must be known. This allows primers that are specific for the target region to be designed. The primers are nucleotide sequences of 60-150 bases that bind to the 2 complementary DNA strands being known as the forward and reverse primers. When designing primers for cDNA if possible they should span 2 exons of the gene of interest. This method prevents binding of the primers to any genomic DNA which may have contaminated the sample. Genomic DNA contains introns in the gene sequence which are regions of the gene that are normally removed during processing of the mRNA produced during gene transcription.

As well as the primers a heat stable DNA polymerase enzyme is required and a supply of nucleotides. The reaction occurs in a mixture of the above with buffers and essential salts. The PCR reaction proceeds by a series of steps where initially the sample is heated to denature any double stranded DNA to single strands. The sample is then cooled to allow annealing of the primers and then incubated for extension to occur. The sample is then reheated to denature the new DNA products and the cycle begins again. Usually the cycle will be repeated around 40 times. As each cycle produces 2 copies of the region of interest this process has the potential to produce up to 2^{40} copies of the target. In reality the reaction usually becomes less efficient nearer the end of the cycle due to depletion of reagents and enzyme fatigue.

The final copy number of the target DNA will be proportional to the starting copy number. This quantity can be calculated (end point quantification) using gel analysis with staining to show the product bands and densitometry to quantify the bands. The final amount could then be calculated either by

comparing to a known standard or assessing relative expression by comparing different samples. When using real-time analysis of the PCR reaction as can be done using the Bio-Rad iCycler iQ™ the accumulation of the DNA products from the area of interest can be monitored each cycle. This is achieved by using fluorescence based technology. There are 2 common methods for this. The first is to add a dye which binds to double stranded DNA and fluoresces once bound. As the DNA product accumulates there will be a progressive increase in fluorescence. An example of this is the intercalating dye SYBR green™. The other option is to use a probe based system where a probe as well as the primers are added to the reaction. The probe is designed to bind to another region in the area of interest which is located between the binding sites for the forward and reverse primers. This probe is an oligonucleotide of around 20-25 bases and is labelled with a fluorescent reporter dye on its 5' end and a quencher dye on its 3' end. On the probe these two dyes are in close proximity and hence no fluorescence is seen. The probe binds to the sense strand during the annealing stage of PCR. When polymerisation occurs the polymerase enzyme moves along the strand in a 3' – 5' direction adding bases to the 3' end of the primer. The DNA polymerase used in PCR has 5' exonuclease activity. This means any nucleotides bound to the template strand in front of the 3' end of the new DNA product will be cleaved as the polymerisation progresses. This cleavage separates the fluorescent dye from the quencher and hence fluorescence occurs as shown in figure 2.4.

There are advantages and disadvantages to both methods. These include that intercalating dyes tend to be cheaper but the fluorescence is non-specific so will detect amplification of mis-primed sequences. Conversely the probe based systems are more expensive and harder to design but add specificity to the system and the 3' end of the probe can be designed so as to block further extension of the DNA product hence further lessening the chance of non-specific amplification.

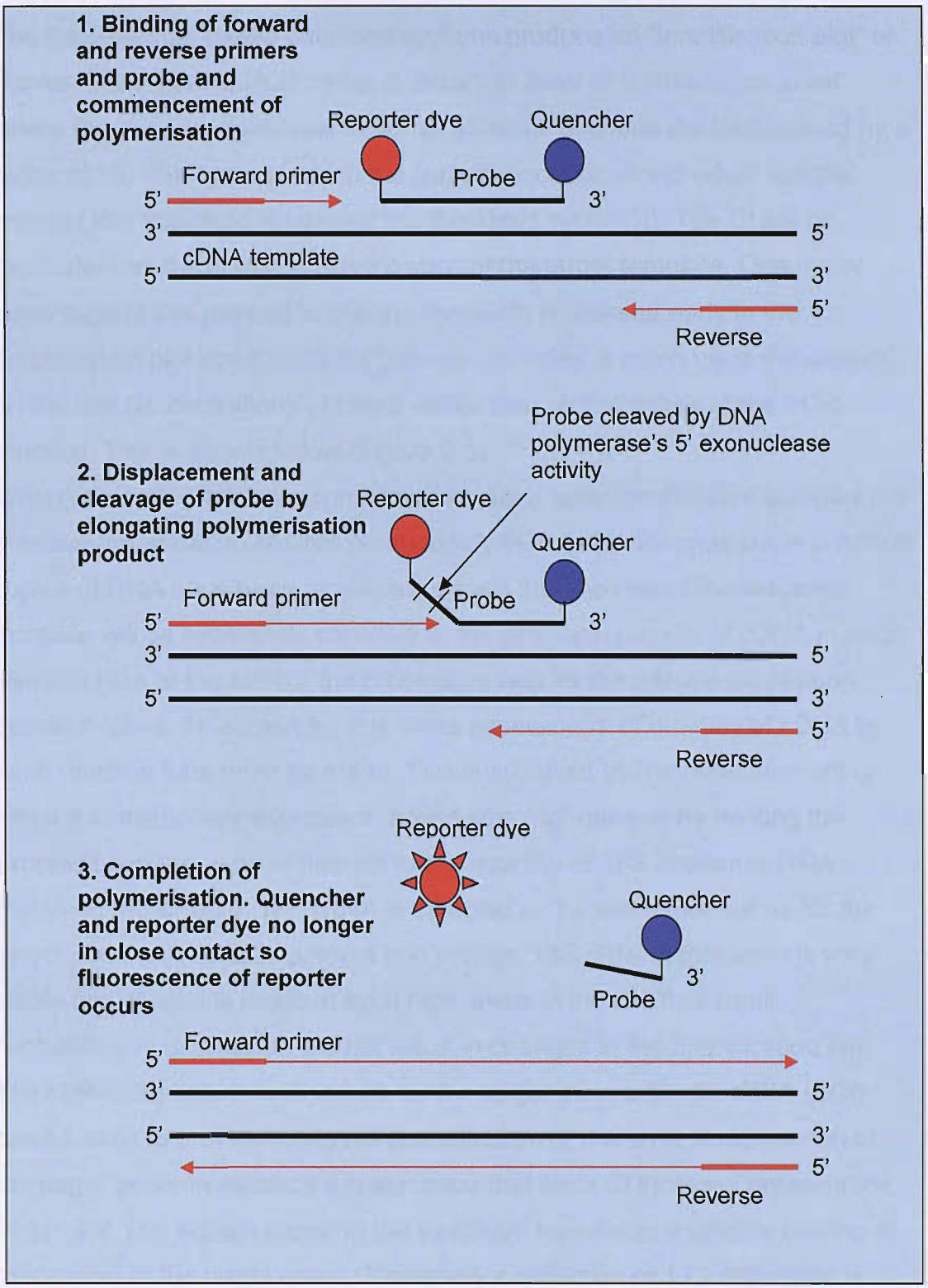


Figure 2.4 – Principles of RT-qPCR
Following the initial denaturing step of the PCR cycle the sample is cooled to allow annealing of the forward and reverse primers and the probe. The sample is then incubated and polymerisation occurs. During this process the probe is cleaved from the cDNA template separating the quencher and reporter dye allowing the latter to fluoresce. Once polymerisation is complete the level of fluorescence is measured and the sample reheated to denature the double stranded PCR products and allow the cycle to begin again

The fluorescence based detection systems produce an "amplification plot" of fluorescence against PCR cycle. A threshold level of fluorescence is set where the average fluorescence for all samples exceeds the background by a factor of 10. The cycle at which the fluorescence for an individual sample crosses this threshold is labelled the threshold cycle (Ct). The Ct will be dependent on the starting concentration of the target template. One major advantage of this method is that the threshold is crossed early in the amplification plot when variation between samples is much more dependent on starting concentrations of target rather than technicalities of the PCR reaction. This is shown below (Figure 2.5).

When comparing relative expression of a gene between different samples the massive amplification of DNA produced by PCR (after 20 cycles over 2 million copies of DNA have been produced) means that the rate of fluorescence increase will be exquisitely sensitive to the absolute quantity of cDNA in each reaction tube at the start of the process as well as the relative expression between tubes. To correct for this some assessment of quantity of cDNA in each reaction tube must be made. This is achieved by the measurement of either a constitutively expressed "house keeping" gene or by relating the expression of the gene of interest to the quantity of 18S ribosomal RNA (rRNA) in the sample. 18S rRNA is detected in the same manner as for the target gene with specific primers and probes. 18S rRNA expression is very stable though and is found at such high levels in the cell that small fluctuations in expression will not result in changes in the amplification plot. The difference between Ct values for the target gene and 18S rRNA (ΔCt) can be used to provide a relative quantification of the level of expression of the target gene. In essence it is assumed that each Ct increase between the target and 18S signals crossing the threshold equates to a relative halving of expression of the target gene. Conversely a reduction of 1 Ct difference is due to a doubling of expression. As the level of 18S rRNA is so high it is usually easier to designate one of the samples as 1 by subtracting its ΔCt from itself to give a ΔΔCt value of 0. The same value is then subtracted from the other ΔCt for the other samples. The target gene expression in these samples will then be represented in more manageable numbers. A worked example of the ΔCt and ΔΔCt methods of quantification is shown below

(Table 2.4). Note the addition of a minus in front of the ΔΔCt value as a reduction in this value represents an increase in gene expression.

2.2.8.2 Primer and probe design

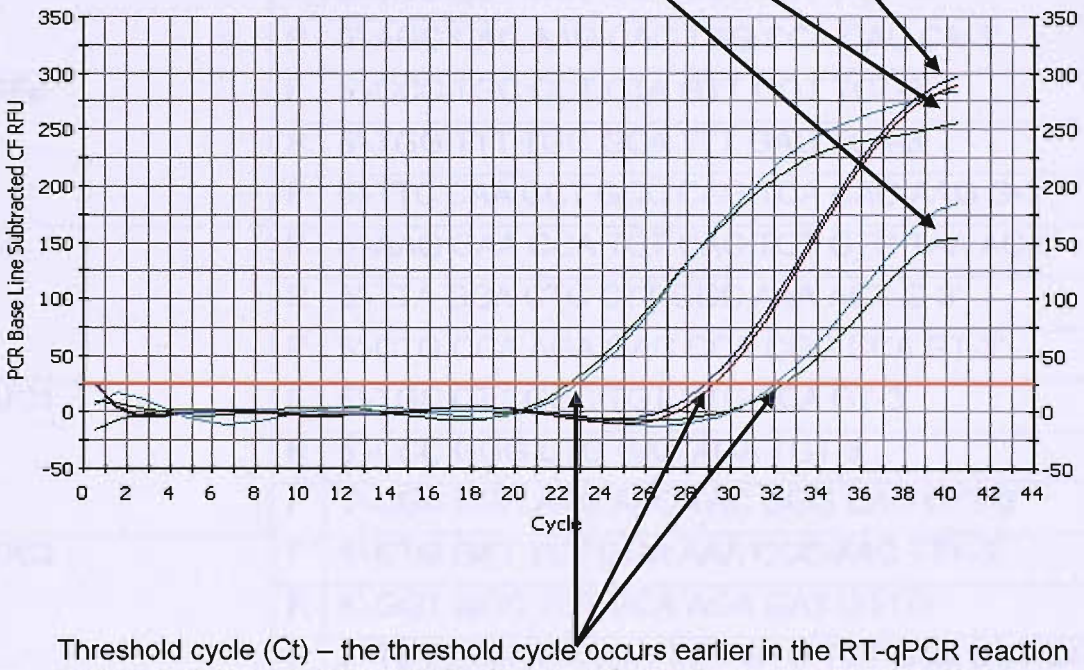
For RT-qPCR quantification to be valid there are a number of factors to take into account. As well as the starting concentration of cDNA in the reaction as outlined above it is important that all assays are optimised for maximal efficiency so that different assays can be compared. This is achieved by careful primer and probe design with stringent testing of the assays prior to use. The technicalities of primer and probe design require software programmes to aid in their design. The primers and probes used in my experiments were designed by Dr Rob Powell using Beacon Designer II™ software. The sequences are detailed in the table 2.3.

A number of factors must be taken into account when designing primers and probes for RT-qPCR as will now be detailed. Generally primers and probes are designed to contain a guanine/cytosine (G/C) content of between 20 – 80%. This ensures that they have a melting temperature (Tm – the temperature at which denaturisation of the cDNA/primer strand occurs) of approximately 58°C. This ensures that denaturing occurs during thermal cycling and prevents non-specific interactions. Primers containing a high proportion of adenine/thymine (A/T) sequences need to be longer to reach the recommended Tm but sequences that are too long tend to be less efficient in the PCR reaction due to primer-dimer formation and intra-primer binding. The last 5 bases of the primer 3' end should not contain more than 2 G or C residues. This lessens the chances of non-specific interactions.

The probe needs to have a melting temperature around 10°C higher than the primers to ensure that if is fully bound during the extension phase of the PCR reaction. Also it must not have a G residue at the 5' end as this causes quenching of the probe even after cleavage and distancing from the quencher.

End of PCR fluorescence – at the end of the PCR reaction significant variations in fluorescence (representing amount of PCR product) are seen for duplicate samples. This is due to the reaction being affected by "fatigue" of the DNA polymerase and exhaustion of the substrate rather than on initial concentration of cDNA product

In these wells the PCR fluorescence curves the Ct and the end of reaction fluorescence remain well paired



Threshold cycle (Ct) – the threshold cycle occurs earlier in the RT-qPCR reaction and as such there is less variation between the replicate samples. The Ct is dependent on the quantity of the gene of interest at the start of the PCR reaction and the reaction is less affected by other factors such as quantity of substrate and enzyme efficiency

Figure 2.5 – Typical RT-qPCR traces

This plot shows fluorescence against cycle number for 3 different samples each run in duplicate. The threshold is shown by the orange bar and the threshold cycle cycle (Ct) for each sample indicated by the arrows. As described the fluorescence for each sample crosses the threshold level early in the PCR reaction and the cycle number will be determined by starting concentration of cDNA product and less affected by inefficiencies in the PCR reaction.

Table 2.3 – Primer and probe sequences for the RT-qPCR assays

Target Gene	Sequence (F=Forward R=Reverse P=Probe)	
18S Ribosomal RNA	F	Obtained as a pre-developed assay from Eurogentec
	R	Obtained as a pre-developed assay from Eurogentec
	P	Obtained as a pre-developed assay from Eurogentec
Amphiregulin	F	5'-GTG GTG CTG TCG CTC TTG ATA C-3'
	R	5'-GCT TCC CAG AGT AGG TGT CAT TG-3'
	P	5'-TCC AAT CCA GCA GCA TAA TGG CCT GA-3'
HB-EGF	F	5'-GAT CTG GAC CTT TTG AGA GTC ACT T-3'
	R	5'-TCC CGT GCT CCT CCT TGT T-3'
	P	5'-AGC CAC AAG CAC TGG CCA CAC CA-3'
TGF*α*	F	5'-CCC TGC CCT CTA GTT GGT TCT-3'
	R	5'-TGG TTT TGG GCA TTT GAG TCA-3'
	P	5'-TTC CAA CCT GCC CAG TCA CAG AAG G-3'
IL-8	F	5'-AAG GAA CCA TCT CAC TGT GTG TAA AC-3'
	R	5'-TTA GCA CTC CTT GGC AAA ACT G-3'
	P	5'-CTG CCA AGA GAG CCA CGG CCA GT-3'
MUC1	F	5'-TGC CTT GGC TGT CTG TCA GT-3'
	R	5'-CCC GGG CTG GAA AGA TGT-3'
	P	5'-CGC CGA AAG AAC TAC GGG CAG CTT-3'
MUC2	F	5'-CTG GAT TCT GGA AAA CCC AAC TTT-3'
	R	5'-GGT GGC TCT GCA AGA GAT GTT-3'
	P	5'-TCT CCA ATC AAT TCT GTG TCT CCA CCT GGT-3'
MUC4	F	5'-CAG TCA ACA ACC GAA TTG CTG T-3'
	R	5'-ATC GAA GAC GCC ATT CCT GT-3'
	P	5'-CGC CTC AGC CAG TCA TGG TGC C-3'
MUC5AC	F	5'-ATG ACA AAC GAG ATC ATC TTC AAC A-3'
	R	5'-CGG GAT GGT CGC GTA CAT-3'
	P	5'-TCT CGC GCA TCG GCG TCA AT-3'
MUC5B	F	5'-GCA GCT ACG TTC TGT CCA AGA-3'
	R	5'-GTC ACC GCT TTC AGG CAG TT-3'
	P	5'-CGT TGT CCG TCA GCC CGC ACC TC-3'

All probes were labelled with a 5' FAM dye and 3' TAMRA quencher except the 18S ribosomal RNA probe which had a 5' VIC dye so could be used in multiplex reactions

2.2.8.3 Validating primer design

Having designed primers for the target gene it is essential that they are validated before they can be used for RT-qPCR. This process is to ensure that the assay has maximal efficiency over the range if template concentrations likely to be found in the experimental samples. If the efficiency varies from the maximum it is essential to investigate the reason why. It may be because of the presence of primer-dimer formation, due to the primer forming hairpin structures that interfere with primer annealing of because the amplicon (the strand of DNA formed by the PCR reaction) forms a secondary structure which inhibits primer annealing.

The efficiency of the assay and the presence of primer-dimer formation can be assessed in one procedure using serial dilutions of cDNA template for a PCR reaction followed by a melt curve which will show up the presence of primer-dimers.

The PCR reactions for my experiments were carried out on a Bio-rad iCycler iQ™ machine and the data analysed using the iCycler iQ™ optical system software provided by Bio-rad. The RT-qPCR reactions were carried out in Bio-rad iCycler optical 96 well reaction plates using a reaction volume of 12.5µl per well.

For primer validation the reaction mix contained 5µl of cDNA template. The template was initially diluted 1 in 10 using dH_2O. This concentration was defined as 1 for the standard curve. A dilution series halving the cDNA concentration each time was then formed to give a range of dilutions down to 1:128. The rest of the reaction mix contained 6.5µl of 2x qPCR master mix (This master mix was made using the following volumes: 10ml 10x RT buffer, 10ml 25mM $MgCl_2$, 5ml 2.5mM dNTP, 625µl Hot goldstar Taq polymerase enzyme, and 26.5ml water), and 1µl forward and reverse primers which had been diluted to 15pmol concentration. No probe is used during primer validation and SYBER green dye was used to detect the presence of amplification products by its property of fluorescing when bound to double stranded DNA (This was added to the master mix with a volume of 1µl per 100µl). For the validation of assays each condition was run in triplicate.

The RT-qPCR reaction protocol consisted of an initial 10 minutes at 90°C to ensure that any contaminating DNase was denatured and then 40 cycles consisting of 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 15 seconds. Fluorescence is measured for each well at the end of each cycle. At the end of the 40 cycles a melt curve is performed on the reactions. This requires the reactions to be heated to 95°C for 60 seconds then cooled to 55°C for 60 seconds followed by an incremental increase in temperature from 55 to 95°C at a rate of 0.1°C per second. The rate of change of fluorescence is recorded by the iCycler.

The software generated amplification curves will show the threshold cycle (Ct) for each well during the PCR reaction. As each cDNA sample for primer validation was diluted 1:2 i.e. an effective halving of the template each serial dilution should have a Ct 1 cycle greater than the previous. A graph of log(dilution) against Ct is plotted. At maximum efficiency this should have a gradient of -3.3. Examples of some of the standard curves for assays used in my work are shown below (Figure 2.6). The gradient of the standard curve will be altered if the primers form dimers or secondary structures which impair annealing. It is possible that at very low concentrations of template the reaction efficiency will be impaired so an assessment needs to be made as to whether the Ct of that curve falls within the expected range of expression found in the biological samples to be used and over what range of Cts the assay is useful e.g. Ct of 20-36.

The melt curve is a plot of temperature (x-axis) against rate of change of fluorescence (y-axis) and is used to look for the presence of primer dimers. When the melting point of the PCR products is reached there will be a rapid decrease in the level of fluorescence as bound SYBR green which fluoresces is released from the double stranded DNA back into solution where its fluorescence is not as intense. Where there are a number of PCR products of different lengths a number of peaks in rate of change of fluorescence will be detected with those at lower temperatures representing the presence of the shorter primer-dimers. It is important to ensure that there is no significant primer-dimerisation across the range of template concentrations likely to be found in the actual assays. Examples of melt curves for some of the assays used in my work are shown in figure 2.7 below.

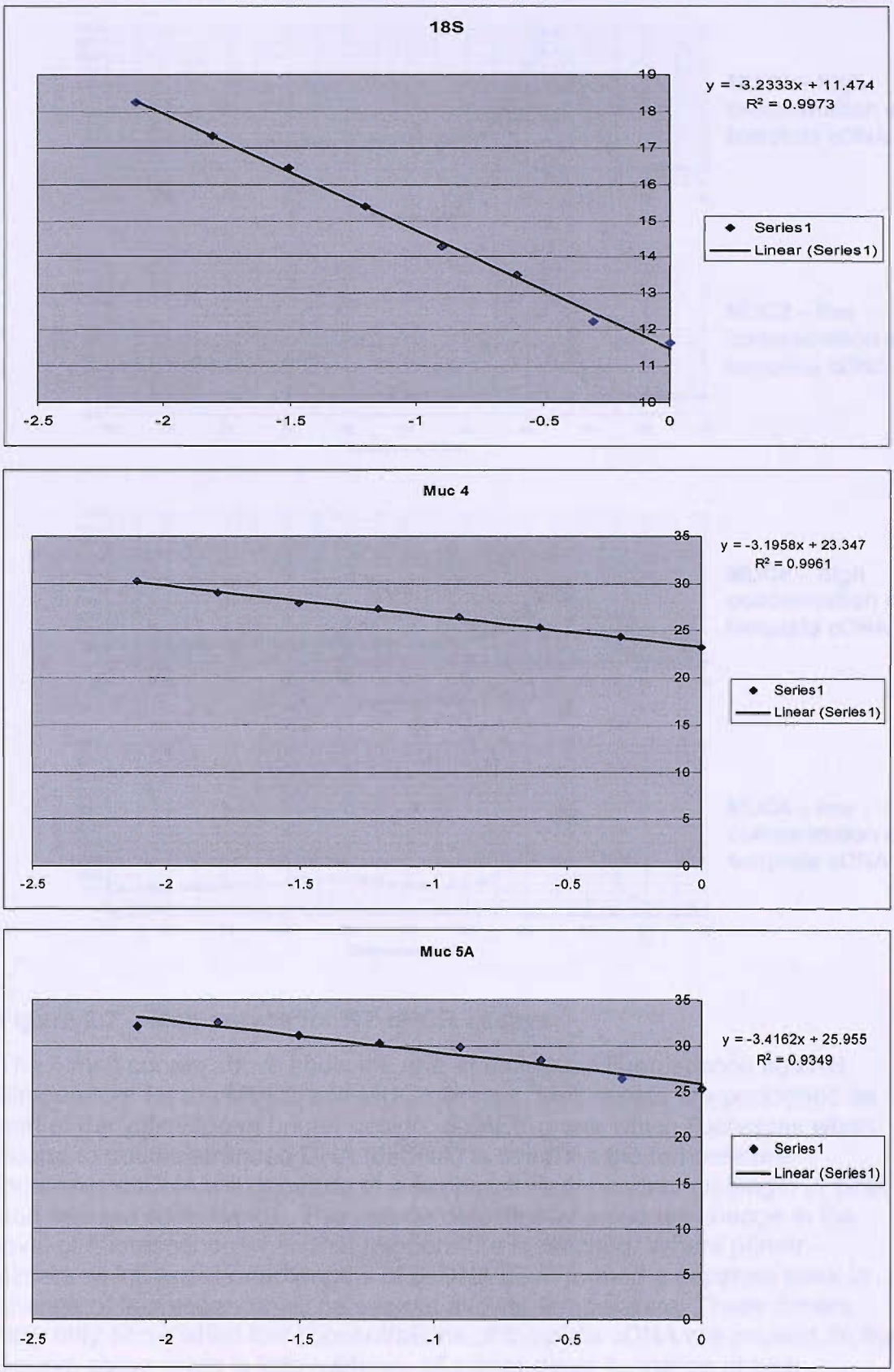
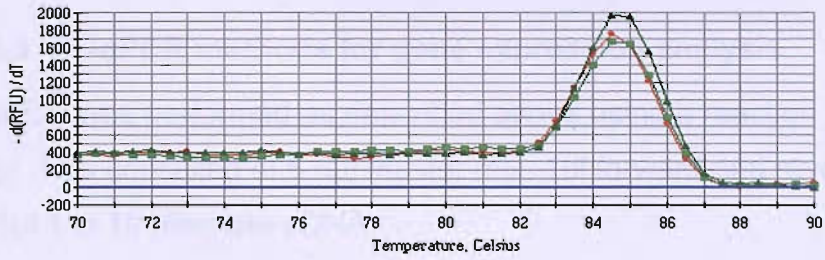
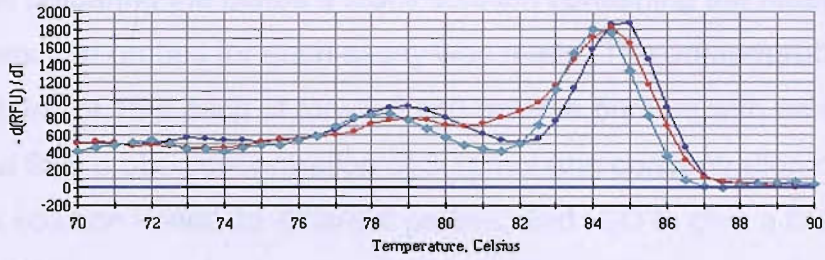


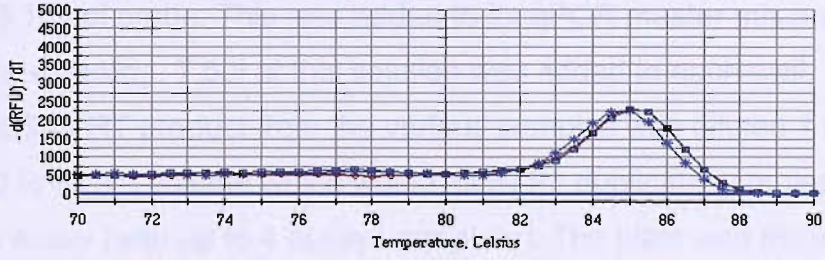
Figure 2.6 – Standard curves for the primers for 18S, MUC4, and MUC5 assays used in RT-qPCR



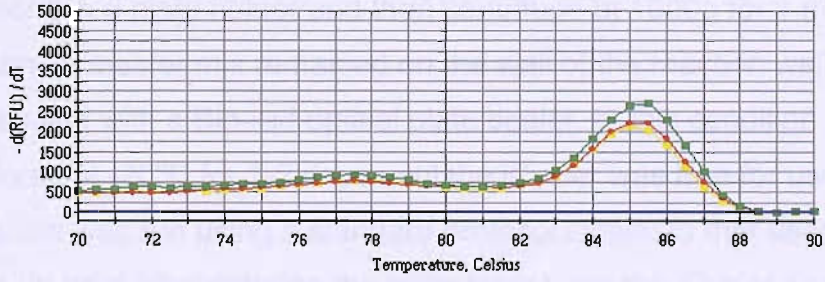
MUC2 – high concentration of template cDNA



MUC2 – low concentration of template cDNA



MUC4 – high concentration of template cDNA



MUC4 – low concentration of template cDNA

Figure 2.7 – Melt curves for RT-qPCR assays

The 4 melt curves above show the rate of change of fluorescence against temperature for the MUC2 and MUC4 assays. Melt curves are performed as part of the validation of primer design. SYBER green which fluoresces when bound to double stranded DNA (dsDNA) is used. As the temperature increases dsDNA will denature at a temperature dependent on length of DNA and its base constituents. This can de detected by a sudden change in the level of fluorescence when that temperature is reached. Where primer – dimers which are shorter lengths of dsDNA have formed a separate peak in change of fluorescence will be seen at a lower temperature. These dimers may only occur when low concentrations of template cDNA are present. In the assays above there is little evidence of primer-dimer formation at high concentrations of template but the MUC2 assay shows that dimers are forming when there is a low concentration of template as evidenced by the small extra peak at 79°C compared to the peak at 84°C which represents denaturing of the PCR product

2.2.8.4 RT-qPCR methods for gene expression analysis

RT-qPCR was performed as described above using a reaction volume of 12.5µl. This consisted of 6.5µl master mix, 1µl forward and reverse primers, and 5µl 1 in 10 template cDNA.

When preparing the plates a stock solution containing the master mix and primers and probes for each assay was made. The primer/probe mix consisted of 15µl each of forward and reverse primers with probe added to give a final probe concentration of 3.1pmol (the concentration of the probe stock solution varied for different probes) and H_2O to give a final volume of 100µl. This gave a final concentration of 15pmol forward and reverse primers and 3.1pmol probe. This was added to 2x qPCR master mix and mixed by gentle vortexing. 7.5µl of this solution was added to each well.

The cDNA RT product from the various samples was diluted 1 in 10 with dH_2O to give a volume which would allow for duplicate samples to be run for each assay (with up to 4 assays per plate). The plate was mixed by gentle vortexing in a plate holder and then centrifuge at 1000g for 1 minute to ensure no sample/master mix remained on the wall of the reaction well. The plate was sealed with a Bio-rad optical plate sealer. In this condition the plate could be stored at -20°C for 1-2 days until the iCycler was free for use.

The plate was run using a standard protocol similar to that used for the melt curve. In brief after entering the plate layout into the iCycler and specifying which fluorescent dyes were being used the PCR programme was run. This involved an initial incubation at 90°C for 10 minutes followed by 40 cycles made up of 15 seconds at 95°C, 60 seconds at 60°C, and 15 seconds at 72°C. The fluorescence at the end of each cycle was read automatically. At the end of the programme the iCycler software calculated a baseline threshold based on a fluorescence of 10x the baseline value averaged for all wells and calculated the Ct for each samples. The amplification plots for each sample could then be examined ensure good correlation between duplicates and adequacy of the amplification curve.

2.2.8.5 Quantification of relative gene expression using the ΔΔCt method

As described in chapter 2.2.8.1 the ΔΔCt method was used to analyse the RT-qPCR results. For all experiments a control well, prepared in duplicate, was included on each plate. The target gene expression in the cDNA produced from one of these wells was assigned a value of 1 using the ΔΔCt method. This means that the target gene expression for the other samples was expressed as a fold increase/decrease relative to this. An example is given below (Table 2.4).

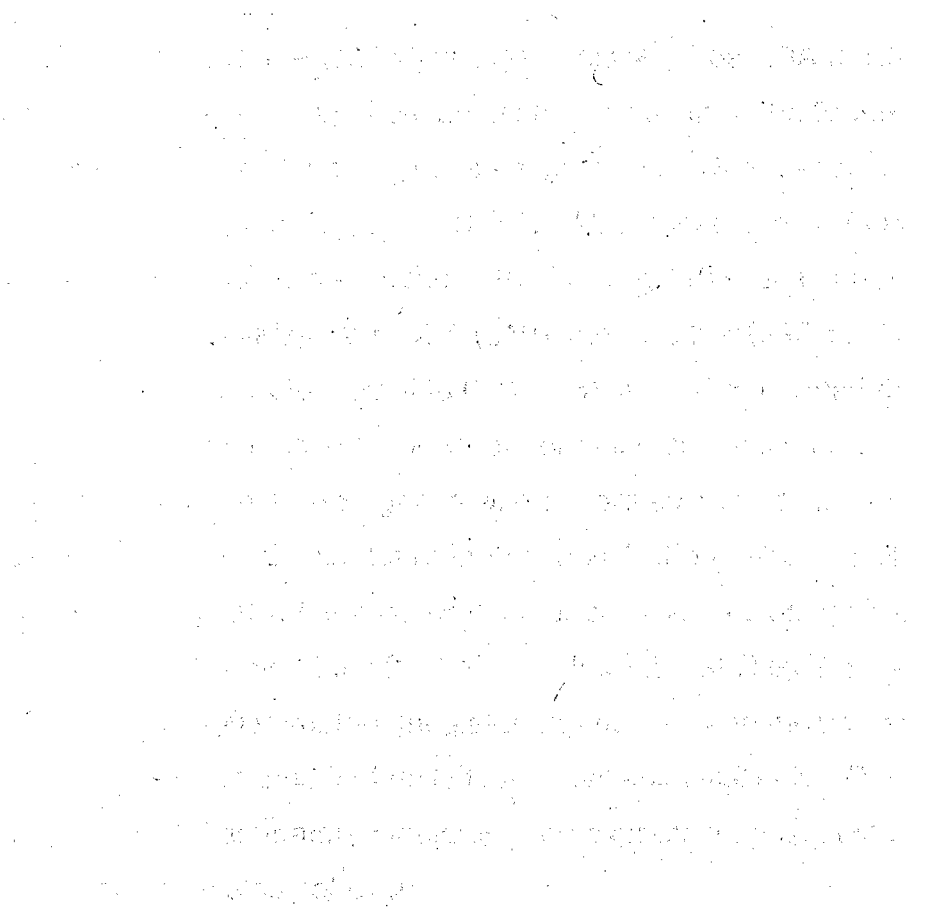
In this example the expression of IL-8 in cell cultures treated with cigarette smoke extract (CSE) in the presence of absence of doxycycline (DOX) is being investigated. 4 conditions were run in cell culture and following RNA extraction and reverse transcription qPCR was performed with each sample being run in duplicate. Column A and B show the Ct for the IL-8 assay and 18S rRNA assay respectively. The duplicates are well matched with only one duplicate having a Ct difference of more than 0.5 from its pair. The 18S Ct gives a normalising signal as its expression is very high and small fluctuations will not affect its Ct value greatly. The average 18S Ct for each experimental condition is then subtracted from the IL-8 Ct as shown in column D. This is the ΔCt between assays. This data is hard to use and therefore one of the conditions is used as a control. In this example it is the untreated control cells as indicated in column I. The average ΔCt for these duplicates is then subtracted from each of the individual ΔCt the results being shown in column F. This value is the ΔΔCt value. As a difference in Ct value of 1 represents a halving/doubling of template concentration the ΔΔCt from column F is added to the equation $2^{-(x)}$ noting the initial minus as a reduction in ΔΔCt represents an increase in template. The values for the duplicates are averaged given relative expression of the gene of interest compared to the control samples (Column G). In this experiment there was a 6.5 fold increase in IL-8 expression in cells exposed to cigarette smoke extract which was reduced to baseline in a dose-dependent manner by the addition of Doxycycline.

Table 2.4 – Quantification of gene expression using the ΔΔCt method

Column A	Column B	Column C	Column D	Column E	Column F	Column G	Column I
Ct value (IL-8 assay)	Ct value (18S assay)	Average 18S Ct for duplicates	(IL-8 Ct)-(18S Ct) (ΔCt)	ΔCt – designated control (ΔΔCt)	$2^{-(x)}$ (Where x = ΔΔCt value)	Average	Experimental Condition
30.7	18	18.05	12.65	0.25	0.840896	1.015052	Control
30.2	18.1		12.15	-0.25	1.189207		
28.7	18.8	18.8	9.9	-2.5	5.656854	6.560559	CSE
28.3	18.8		9.5	-2.9	7.464264		
28.9	19	18.55	10.35	-2.05	4.14106	4.14106	CSE+10 DOX
28.9	18.1		10.35	-2.05	4.14106		
30.4	18.6	18.55	11.85	-0.55	1.464086	1.182668	CSE+25 DOX
31.1	18.5		12.55	0.15	0.90125		

2.2.9 Statistical methods

The data from all experiments was subjected to statistical analysis. The cytokine release data was analysed using the paired t-test in Microsoft Excel™ as was the gene expression data for the cigarette smoke stimulation/doxycycline experiments. The time course and dose-response of gene expression in the presence of EGFR ligands was analysed with ANOVA using the SPSS™ statistical package. For all experiments a p value of 0.05 or lower was considered statistically significant.



3 Chapter 3: The kinetics of the EGFR gene response

3.1 Introduction

As described in chapter 1 it is apparent that many stimuli relevant to COPD and CMH such as cigarette smoke and neutrophil and bacterial products are able to induce expression of mucin genes in bronchial epithelial cells. This response is dependent on activation of the EGFR by its ligands. These are shed from their membrane-bound precursors by ADAM proteinase mediated cleavage. Different groups have suggested that different EGFR ligands may be responsible for the mucin gene response and that different stimuli may cause different patterns of ligand release (215). Recently it has also been demonstrated that what was initially thought to be ligand-independent activation of EGFR by stimuli such as cigarette smoke does in fact involve EGFR ligand release and binding. Again this requires ADAM activation to cleave ligands which can effect autocrine activation of the EGFR (211).
The EGFR ligands which have been most studied with respect to mucin gene expression are TGFα, AR, and HB-EGF. All of these can be found in bronchial epithelial cells from the normal airway (196) and can be up-regulated by stimulation with CSE (210) and by other EGFR ligands, a process known as auto-induction (210;253). It has been noted that the time course of EGFR ligand auto-induction following stimulation with CSE may vary between the different ligands with a more sustained increase in expression of HB-EGF compared to AR and TGFα (210). A similar time-dependency has also been noted in other cell lines exposed to different stimuli (254). However the effect of the different EGFR ligands on ligand gene auto-induction, inflammatory mediator expression, and mucin gene response has not been described in detail in bronchial epithelial cells. The time course of mucin and inflammatory mediator gene expression post stimulation with EGFR ligands is also not clear.
To investigate some of these issues further I examined the responses of H292 cells stimulated with AR, HBEGF or TGFα over a 24 hour period, including a dose-response element in the experiments. The doses of ligand chosen for the stimulation experiments were based on their expected

biological activity as stated in the manufacturer's data sheet for each ligand (255). It is recognised that TGFα has greater binding affinity for the EGFR than AR (256) and the dose range used for TGFα was in the order of 10 fold lower than the dose range for AR and HB-EGF but was comparable to doses used by other groups investigating mucin gene expression (166;195).

3.2 Aims

1) To investigate the kinetics of the gene responses for H292 cells stimulated with different EGFR ligands.
2) To investigate whether EGFR ligands have a dose-dependent effect on the EGFR responsive genes.
3) To investigate whether the profile of gene expression differs for the different EGFR ligands: AR, HB-EGF and TGFα.
4) To find suitable time points to measure EGFR ligand, mucin gene and IL-8 expression for subsequent experiments using CSE as a stimulant

3.3 EGFR ligand gene response in H292 cells stimulated with EGFR ligands

Richter and colleagues showed that H292 cells exposed to CSE release AR and TGFα. Although it is likely that HB-EGF is also released this could not be detected due to lack of a suitable assay (210). This release was accompanied by an auto-induction of genes for the EGFR ligands. It is not clear whether this response was due to the release and subsequent binding to the EGFR of one of the ligands alone or whether it required the presence of all the ligands. In this series of experiments I exposed H292 cells to one of AR, HB-EGF, or TGFα alone across a range of concentrations and measured gene expression for the three ligands. The results are shown in figures 3.1 – 3.3. As can be seen, exposure to any of the ligands up-regulated EGFR ligand gene expression both for the stimulating ligand and the other two ligands. The effects of exposure to each ligand and for each gene response will be described in more detail below. The maximum induction of gene expression for each gene and the time it occurred for stimulation with each ligand is shown in table 3.1.

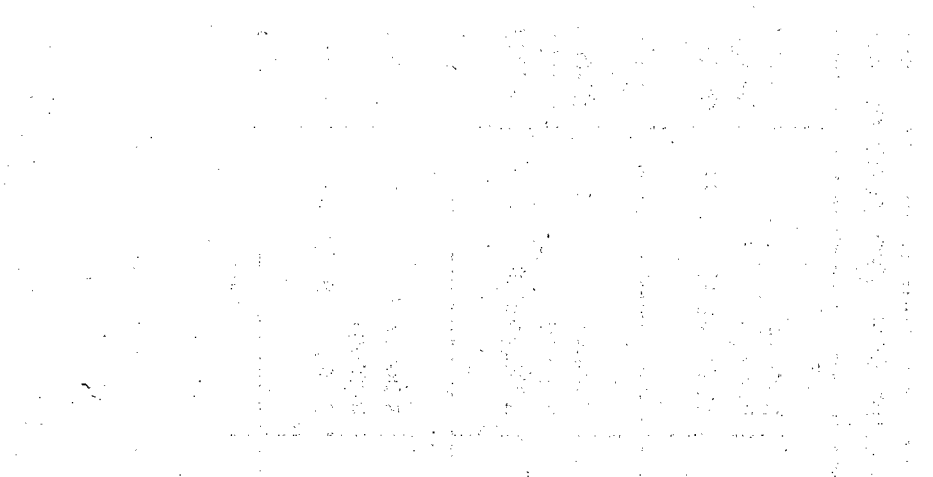


Table 3.1 – Induction of genes in H292 cells in response to stimulation with AR, HBEGF, or TGFα

STIMULATING LIGAND	GENE				
	AR	HBEGF	TGFα	IL-8	MUC5AC
Amphiregulin (50ng/ml)	68.87 (42.9 – 94.83) p<0.001 2 hours	19.45 (13.09 – 25.81) p<0.001 2 hours	6.28 (4.27 – 8.29) p<0.001 6 hours	36.00 (27.77 – 44.23) p<0.001 6 hours	10.5 (7.11 – 13.89) p<0.001 24 hours
HBEGF (50ng/ml)	76.53 (45.75 – 107.3) p<0.001 2 hours	26.51 (20.57 – 32.44) p<0.001 2 hours	18.25 (6.96 – 29.55) p=0.003 6 hours	36.04 (12.78 – 59.31) p=0.004 6 hours	13.65 (9.28 – 18.01) p<0.001 24 hours
TGFα (2.5ng/ml)	73.59 (56.37 – 90.81) p<0.001 2 hours	33.72 (27.77 – 39.67) p<0.001 2 hours	4.32 (2.97 – 5.68) p<0.001 2 hours	28.35 (21.69 – 35.01) p<0.001 2 hours	11.39 (7.6 – 15.19) p<0.001 12 hours

Results show the mean fold induction with lower & upper 95% C.I. above untreated cells and the time at which this response was measured. Statistical significance by ANOVA with respect to untreated cells at the same time point. The results are the mean from 3 separate experiments in which each condition was run in duplicate

Table 3.1 – Induction of genes in H292 bronchial epithelial cells in response to stimulation with AR, HB-EGF, or TGFα

3.3.1 AR gene responses in H292 cells stimulated with AR, HB-EGF, or TGFα

The maximum induction of AR expression was seen at 2 hours for stimulation with any ligand, and had returned to basal levels of expression by 24 hours. The vast majority of this fall back towards basal expression had occurred by 6 hours when AR or TGFα were used as the stimulus, but there was the suggestion (Figure 3.1b) that the increase in expression may persist for longer following exposure to HB-EGF. Using univariate ANOVA for statistical analysis, ligand dose had a statistically significant effect on the level of the gene response ($p<0.001$) but as seen in figures 3.1a-c all but the lowest dose of ligand brought about a similar maximal increase in expression. The degree of induction was large with around a 70 fold increase in AR expression relative to control and was similar for each of the ligands despite the differences in dose. TGFα was the most potent ligand with a similar gene response being produced for a stimulating dose around 10 times lower than that of amphiregulin and HB-EGF which had similar potencies.
Figure 3.1d shows the effects of the maximum dose of each ligand on AR expression and highlights the similarity in response whichever ligand was used for stimulation with the large induction at 2 hours.

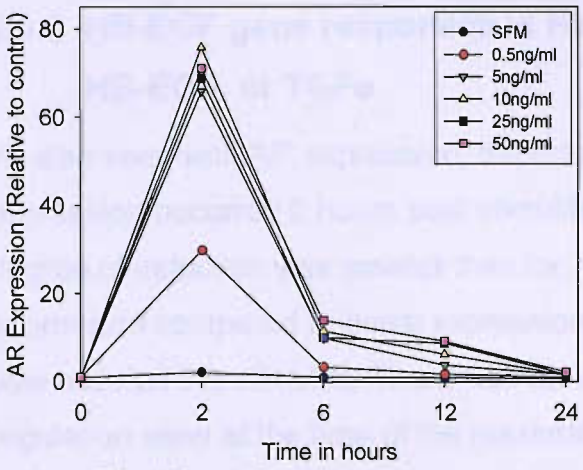


Fig 3.1a – AR

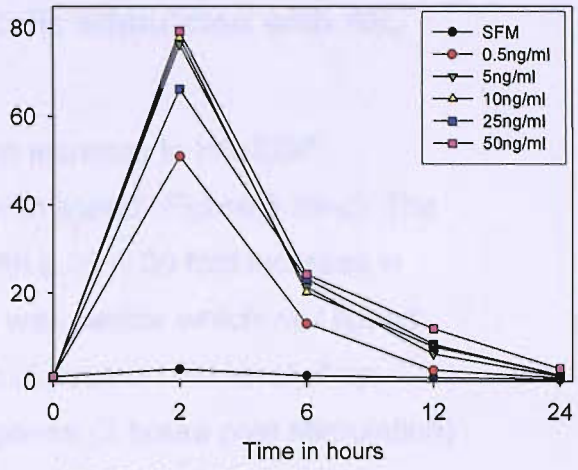


Fig 3.1b – HB-EGF

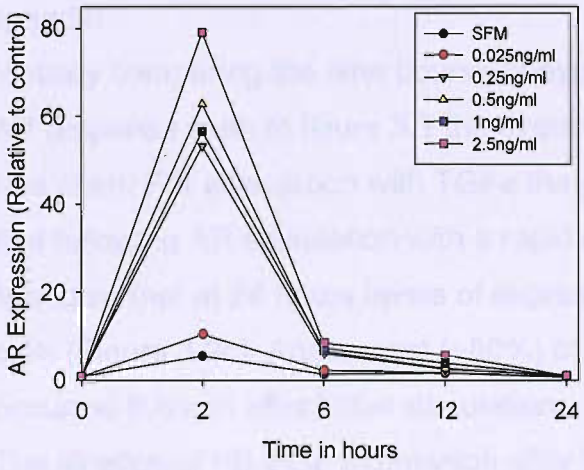


Fig 3.1c - TGFα

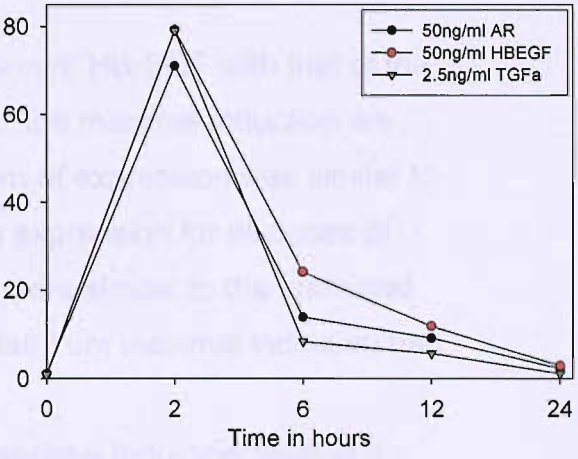


Fig 3.1d

Figure 3.1 – The effects of AR (a), HB-EGF (b), or TGFα (c) on AR expression in H292 cells

H292 cells were cultured for 2, 6, 12, or 24 hours in the presence of either one of the EGFR ligands (AR, HB-EGF, TGFα) or serum free medium (SFM) at the doses shown in the figures. mRNA expression for amphiregulin was determined using RT-qPCR with data being normalised to 18S rRNA. Units are the fold increase in expression compared to baseline expression at t=0. **Figure 3.1d** shows the effect of the maximum stimulating dose of AR (50ngml), HB-EGF (50ng/ml), or TGFα (2.5ng/ml) on amphiregulin expression.

All experiments were performed in triplicate with duplicate wells of each condition used in each experiment.

3.3.2 HB-EGF gene responses in H292 cells stimulated with AR, HB-EGF, or TGF*α*

As also seen with AR expression, the maximum increase in HB-EGF expression occurred 2 hours post stimulation with ligand (Figure 3.2a-c). The degree of induction was smaller than for AR with a 20 – 30 fold increase in expression compared to basal expression, but was similar whichever ligand was used as the stimulus. There was an effect of dose on the level of up-regulation seen at the time of the maximal response (2 hours post stimulation) which was statistically significant ($p<0.001$ on univariate ANOVA for all ligands).

Visually comparing the time course of expression of HB-EGF with that of the AR response seen in figure 3.1 the events after the maximal induction are less clear. For stimulation with TGF*α* the pattern of expression was similar to that following AR stimulation with a rapid fall in expression for all doses of ligand so that at 24 hours levels of expression were similar to the untreated cells (Figure 3.2c). Again most (~80%) of this fall from maximal induction had occurred 6 hours after initial stimulation.

The kinetics of HB-EGF expression after the maximal induction seen at 2 hours was less clear when the stimulating ligand was AR or HB-EGF. For doses of 10ng/ml or lower the expression of HB-EGF fell in a more gradual manner compared to stimulation with TGF*α*. At 6 hours the average fall from maximal induction was around 50% with a 75% fall occurring at 12 hours. The pattern for the 2 higher concentrations of ligand (25ng/ml and 50ng/ml of AR or HB-EGF) appeared to be different: after an initial fall in expression from the maximal value seen at 2 hours the increase in expression of the gene appeared to plateau with similar increases in expression above those of the untreated cells at both 6 and 12 hours (Figure 3.2a & b). For stimulation with AR the expression of HB-EGF then fell towards basal expression again but remained significantly raised at 24 hours with levels 3.2 and 7.6 fold that of the expression at t=0 for the 25ng/ml and 50ng/ml concentration of AR respectively ($p<0.002$ vs. control) (Figure 3.2a). For stimulation with HB-EGF at these higher concentrations the increase in HB-EGF expression remained and did not fall further after 6 hours. In fact there even appeared to be a slight

increase in expression again at 24 hours for the cultures exposed to 50ng/ml HB-EGF (Figure 3.2b).

The effects of stimulation with the three different ligands can be compared directly in figure 3.2d which shows the expression of HB-EGF over 24 hours following stimulation with the maximum dose of each of the three ligands. This shows the differences in the kinetics of expression after the maximum induction at 2 hours for the different ligands. It is also of note that there appears to be a difference in potency for the three ligands in terms of the maximum increase in HB-EGF expression which was not seen with expression of AR (Figure 3.1d). The fold increase in expression of HB-EGF at 2 hours for the different ligands was 36, 28, and 20 for TGFα, HBEGF, and AR respectively. As for the AR gene response this again shows that TGFα is the most potent ligand.

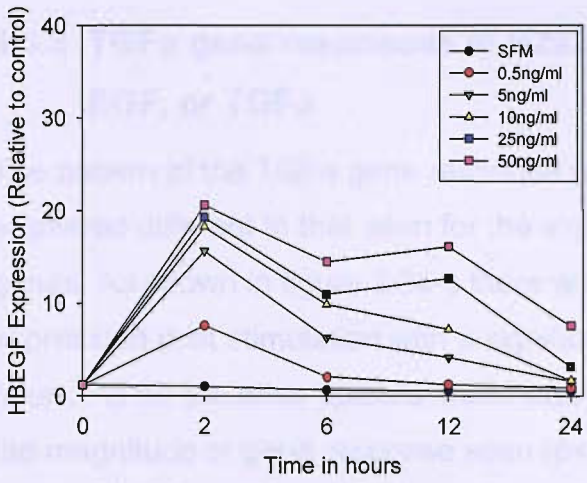


Fig 3.2a – AR

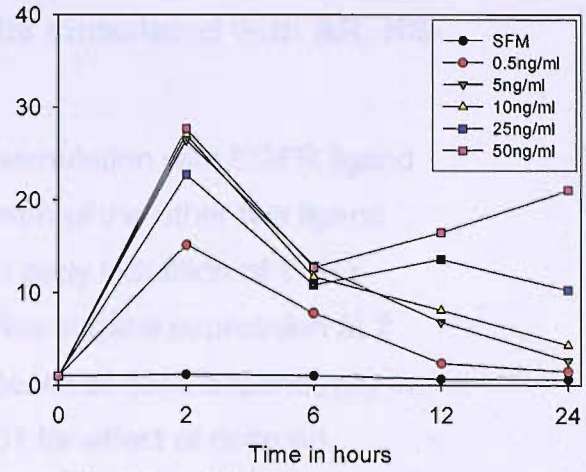


Fig 3.2b – HBEGF

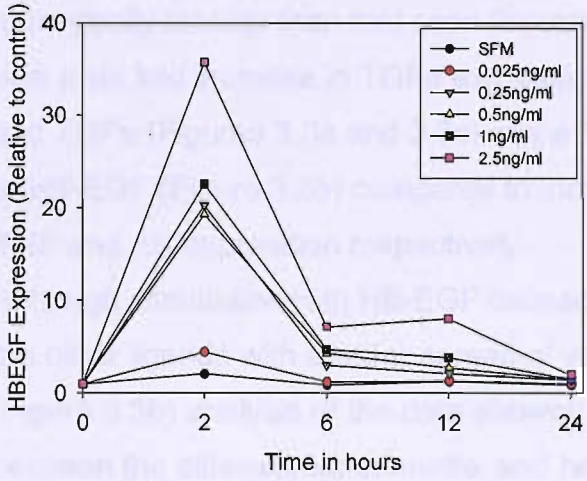


Fig 3.2c – TGFα

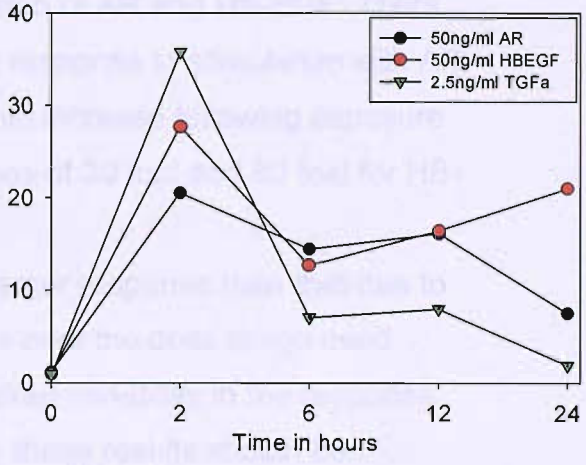


Fig 3.2d

Figure 3.2 – The effects of AR (a), HB-EGF (b), or TGFα (c) on HB-EGF expression in H292 cells

H292 cells were cultured for 2, 6, 12, or 24 hours in the presence of either one of the EGFR ligands (AR, HB-EGF, TGFα) or serum free medium (SFM) at the doses shown in the figures. mRNA expression for HB-EGF was determined using RT-qPCR with data being normalised to 18S rRNA. Units are the fold increase in expression compared to baseline expression at t=0. **Figure 3.2d** shows the effect of the maximum stimulating dose of AR (50ngml), HB-EGF (50ng/ml), or TGFα (2.5ng/ml) on HB-EGF expression. All experiments were performed in triplicate with duplicate wells of each condition used in each experiment.

3.3.3 TGFα gene responses in H292 cells stimulated with AR, HB-EGF, or TGFα

The pattern of the TGFα gene response post stimulation with EGFR ligand appeared different to that seen for the expression of the other two ligand genes. As shown in figure 3.3a-c there was an early induction of TGFα expression post stimulation with a significant rise in gene expression at 2 hours. As for the other ligands, there was evidence of dose-dependency in the magnitude of gene response seen ($p<0.001$ for effect of dose on response). The degree of up-regulation of TGFα gene expression was numerically smaller than that seen for expression of AR and HB-EGF: There was a six fold increase in TGFα expression in response to stimulation with AR and TGFα (Figures 3.3a and 3.3c) and a 14 fold increase following exposure to HB-EGF (Figure 3.3b) compared to increases of 30 fold and 80 fold for HB-EGF and AR expression respectively.

Although stimulation with HB-EGF caused a larger response than that due to the other ligands with a wider spread of values over the dose range used (Figure 3.3b) analysis of the data showed marked variability in the response between the different experiments, and hence these results should be interpreted with caution as there may have been other factors influencing them.

Unlike the pattern of expression for the other two EGFR ligands the kinetics of the TGFα response after 2 hours was less clear. For stimulation with AR and TGFα gene expression seemed to remain increased over the next 10 hours. After this time there was a fall back towards baseline expression in those cells exposed to TGFα whereas expression remained increased in those cells exposed to AR (Figures 3.3a and 3.3c). In the cultures stimulated with HB-EGF after 2 hours there was a fall from the maximum fold increase in expression seen at 2 hours, although as discussed above the level of induction at 2 hours may have been artificially high. As for stimulation with AR expression remained increased above baseline up to 24 hours post stimulation (Figure 3.3b).

The pattern of TGFα response for stimulation with the 3 different ligands can be seen in figure 3.3d which shows TGFα expression relative to control for

stimulation with the maximum doses of each of the ligands. As for the other genes TGFα was the most potent ligand but for TGFα response HB-EGF may have been more potent than AR whereas for the other 2 gene responses assayed these two ligands appeared equipotent.

3.3.4 The effects of EGFR ligand stimulation on EGFR ligand expression in H292 bronchial epithelial cells

The data discussed above shows that in bronchial epithelial cells stimulation with any of the EGFR ligands causes an up-regulation of gene expression for that ligand (auto-induction) as well as the other two EGFR ligands studied. In the following figures the effect of each ligand on its own and the other two ligands' expression is demonstrated (Figures 3.4a-c). It can be seen that stimulation with any one of the three ligands causes a large rise in amphiregulin expression at 2 hours which falls rapidly. This 70-80 fold induction in AR expression tends to distract from the smaller increase in expression seen for HB-EGF and TGFα, but when the cells are stimulated with AR or HB-EGF this rise in expression of HB-EGF and TGFα appears to persist over the 24 hour period. This suggests that there may be a mechanism which perpetuates the effect of the ligand on the expression of these genes.

When TGFα is used as the stimulus the initial response seen is similar to that following exposure to AR or HB-EGF with an increase in expression for all three genes 2 hours post stimulation. TGFα is more potent though being used at concentrations a tenth that of the other 2 ligands but producing an equivalent gene response. In addition the kinetics of the gene responses seen appear subtly different to those following stimulation with either AR or HB-EGF. In these experiments gene expression for the three ligands falls back to baseline after 12 hours in all cases whereas post stimulation with AR or HB-EGF gene expression for TGFα and HB-EGF remain increased at 24 hours (Figure 3.4c). The significance of these differences is unclear.

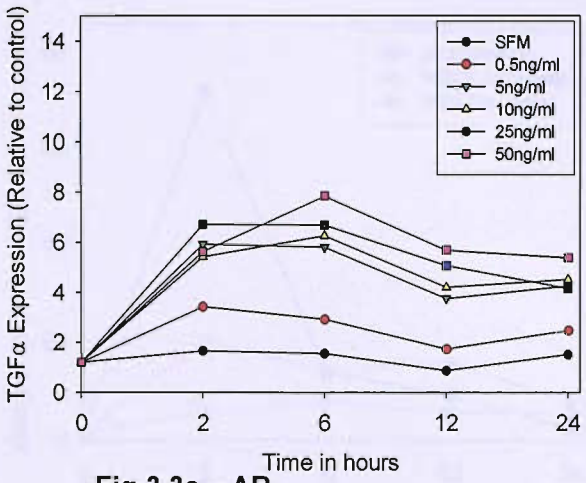


Fig 3.3a – AR

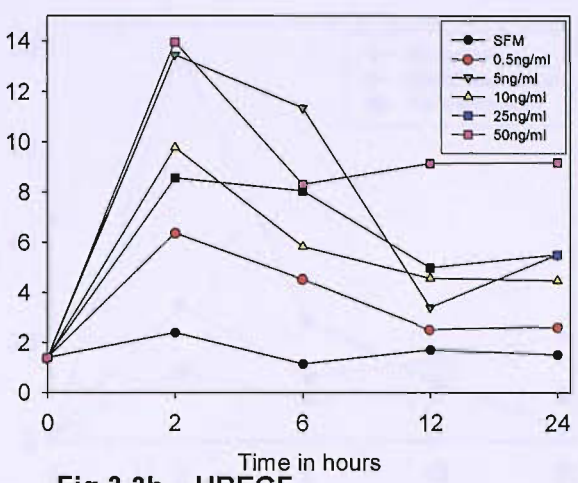


Fig 3.3b – HBEGF

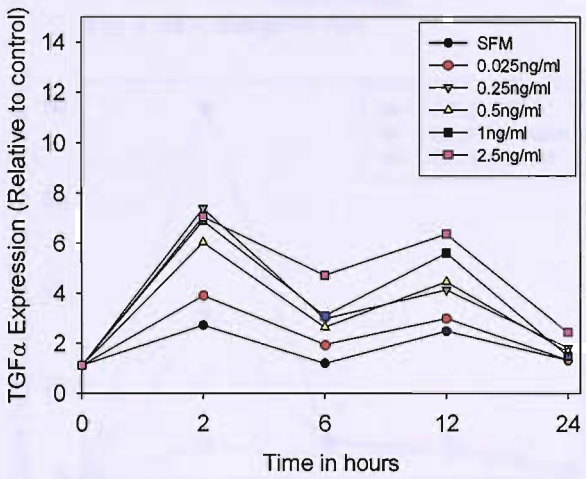


Fig 3.3c – TGFα

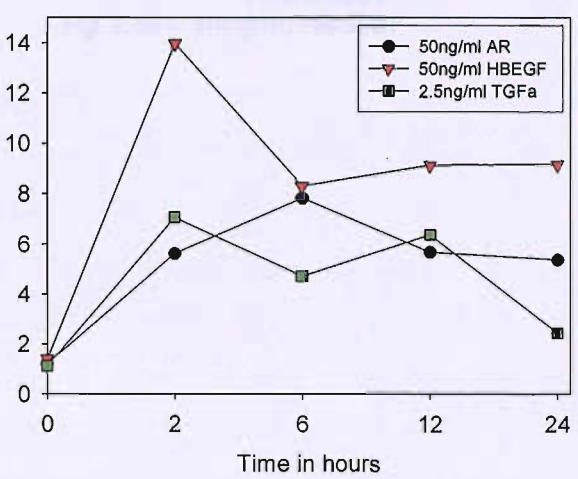


Fig 3.3d

Figure 3.3 – The effects of AR (a) , HB-EGF (b), or TGFα (c) on TGFα expression in H292 cells

H292 cells were cultured for 2, 6, 12, or 24 hours in the presence of either one of the EGFR ligands (AR, HB-EGF, TGFα) or serum free medium (SFM) at the doses shown in the figures. mRNA expression for TGFα was determined using RT-qPCR with data being normalised to 18S rRNA. Units are the fold increase in expression compared to baseline expression at t=0. **Figure 3.3d** shows the effect of the maximum stimulating dose of AR (50ngml), HB-EGF (50ng/ml), or TGFα (2.5ng/ml) on TGFα expression. All experiments were performed in triplicate with duplicate wells of each condition used in each experiment.

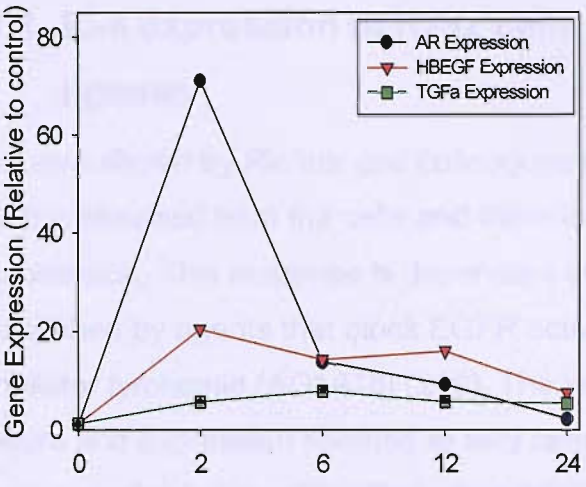


Fig 3.4a – 50ng/ml AR

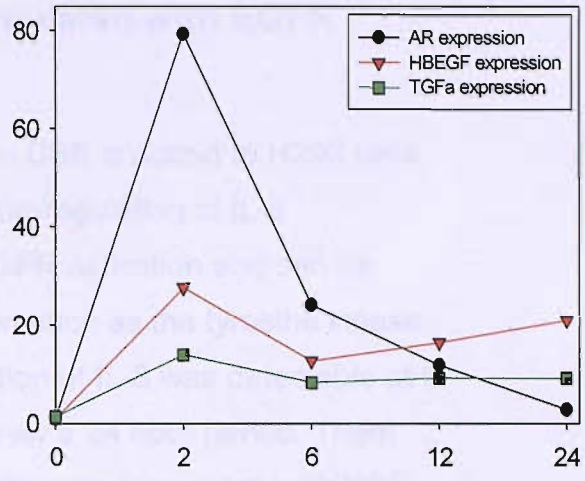


Fig 3.4b – 50ng/ml HB-EGF

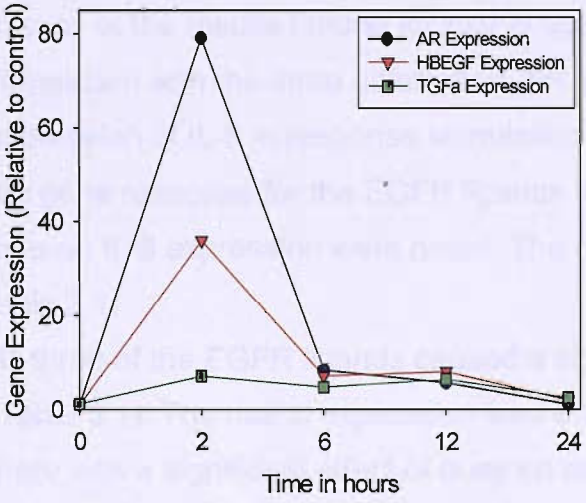


Fig 3.4c – 2.5ng/ml TGFα

Figure 3.4 – The effects of AR, HB-EGF, or TGFα on AR, HB-EGF, and TGFα gene expression in H292 cells

H292 cells were cultured for 2, 6, 12, or 24 hours in the presence of one of the EGFR ligands.
Figure 3.4a – AR 50ng/ml
Figure 3.4b – HB-EGF 50ng/ml
Figure 3.4c – TGFα 2.5ng/ml
mRNA expression for the individual ligands was determined using RT-qPCR with data being normalised to 18S rRNA. Units are the fold increase in expression compared to baseline gene expression at t=0.
The three figures show the pattern of gene expression with time for the three ligands in response to stimulation with one of those ligands alone.
All experiments were performed in triplicate with duplicate wells of each condition used in each experiment. Data shown are the mean response.

3.4 IL-8 expression in H292 cells stimulated with EGFR ligands

As was shown by Richter and colleagues when CSE is added to H292 cells IL-8 is released from the cells and there is an up-regulation of IL-8 expression. This response is dependent on EGFR activation and can be abolished by agents that block EGFR activation such as the tyrosine kinase inhibitor tyrphostin (AG1478) (210). The induction of IL-8 was detectable at 6 hours and expression seemed to stay raised over a 24 hour period. There was a similar but smaller effect when H292 cells were stimulated with EGF alone.

In view of the results I found for EGFR ligand gene expression following stimulation with the three different EGFR ligands I investigated the pattern of expression of IL-8 in response stimulation with any of the three ligands. As for the gene response for the EGFR ligands the effects of stimulating dose and time on IL-8 expression were noted. The results are shown in figure 3.5 and table 3.1.

All three of the EGFR ligands caused a significant increase in IL-8 expression (Table 3.1). The rise in expression was evident by 2 hours for all ligands and there was a significant effect of dose on the level of induction ($p<0.001$ for effect of dose on response by univariate ANOVA). After 2 hours the pattern of induction varied depending on which ligand was used as the stimulus: For AR and HB-EGF the increase in expression seemed to be maximal 6 hours post stimulation before falling back towards basal levels of expression by 24 hours (Figure 3.5a and 3.5b). The only exceptions to this pattern were for exposure to the 2 highest doses of HB-EGF where expression continued to rise to 12 hours post stimulation before falling (Figure 3.5b). At 24 hours there was still a significant increase in IL-8 expression compared to that of the untreated cells for the cells stimulated with HBEGF (Figure 3.5b) but the expression of IL-8 in the cells stimulated with AR was no longer raised except for in the cells that were exposed to the highest concentration of AR (Figure 3.5a).

In those cells stimulated with TGFα there was an up-regulation of IL-8 expression which was maximal at 2 hours post stimulation. After this time

there was a steady fall back towards baseline expression from this maximum increase. At 24 hours there was no increase in IL-8 expression compared to baseline whichever stimulating dose of TGFα was used (Figure 3.5c).
The effects of the maximum stimulating dose of each ligand on IL-8 expression can be seen in figure 3.5d. As for the ligand gene responses described above TGFα was the most potent ligand with AR and HB-EGF being equipotent.

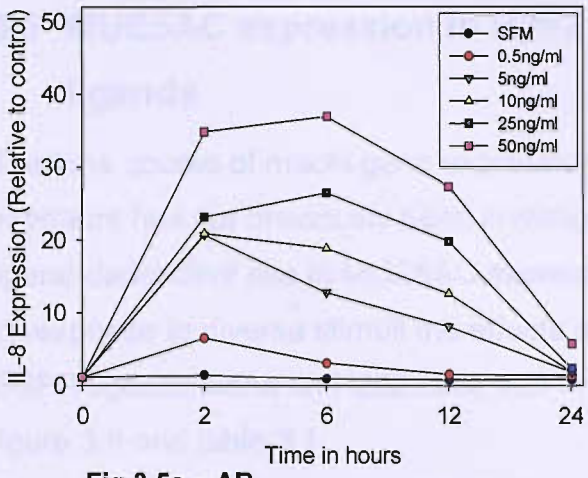


Fig 3.5a – AR

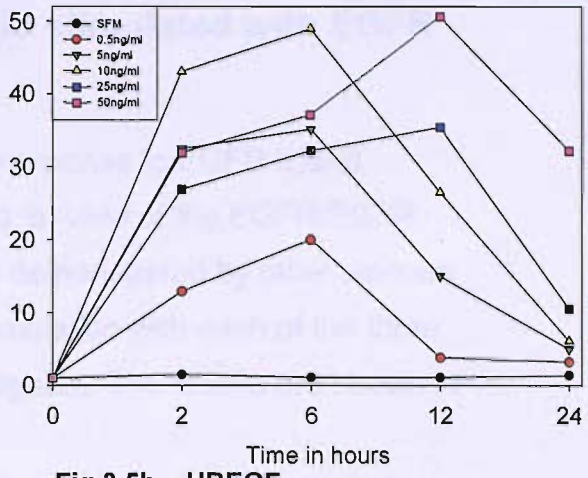


Fig 3.5b – HBEGF

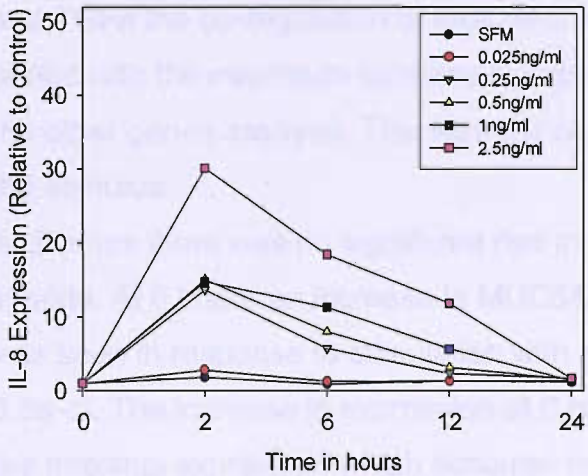


Fig 3.5c – TGFα

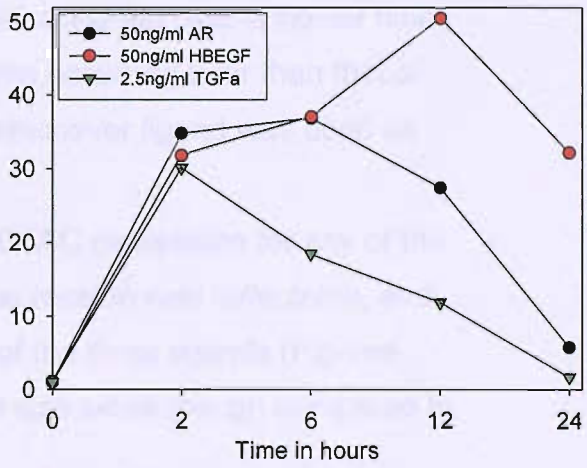


Fig 3.5d

Figure 3.5 – The effects of AR (a) , HB-EGF (b), or TGFα (c) on IL-8 expression in H292 cells

H292 cells were cultured for 2, 6, 12, or 24 hours in the presence of one of the EGFR ligands (AR, HB-EGF, TGFα) or serum free medium (SFM) at the doses shown in the figures. mRNA expression for IL-8 was determined using RT-qPCR with data being normalised to 18S rRNA.

Units are the fold increase in expression compared to baseline expression at t=0.

Figure 3.5d shows the effect of the maximum stimulating dose of AR (50ngml), HB-EGF (50ng/ml), or TGFα (2.5ng/ml) on IL-8 expression. All experiments were performed in triplicate with duplicate wells of each condition used in each experiment.

3.5 MUC5AC expression in H292 cells stimulated with EGFR ligands

The time course of mucin gene expression in response to EGFR ligand exposure has not previously been investigated. In view of the EGFR/EGFR ligand dependent rise in MUC5AC expression demonstrated by other workers in response to diverse stimuli the effects of stimulation with each of the three EGFR ligands alone on H292 cells was investigated. The results are shown in figure 3.6 and table 3.1.

Compared to the time course of gene expression seen for IL-8, AR, HB-EGF and TGFα the up-regulation of MUC5AC mRNA occurred over a slower time period with the maximum increase in expression occurring later than that of the other genes assayed. This was the case whichever ligand was used as the stimulus.

At 2 hours there was no significant rise in MUC5AC expression for any of the ligands. At 6 hours an increase in MUC5AC expression was detectable, and was seen in response to stimulation with any of the three ligands (Figures 3.6a-c). The increase in expression at 6 hours was small though compared to the maximal expression which occurred later.

The expression of MUC5AC continued to rise up to 12 hours post stimulation for all of the ligands, but as for the results seen for IL-8 expression there did appear to be differences in the timing of the maximal gene response depending which ligand had been used as stimulus. For stimulation with TGFα the maximal induction of MUC5AC was seen at 12 hours (Figure 3.6c) after which expression began to return to basal levels. For AR and HB-EGF the expression of MUC5AC continued to rise after this time as was maximal at 24 hours (Figure 3.6a and figure 3.6b). The rise in MUC5AC expression was dose dependent for all ligands ($p<0.001$ for effect of dose on response by univariate ANOVA). The lowest concentrations of AR and TGFα did not provoke a significant MUC5AC gene response (Figures 3.6a and 3.6c). Again TGFα was the most potent ligand with AR and HB-EGF being equipotent.

It is not known whether the expression of MUC5AC would continue to rise after 24 hours in response to stimulation with AR and HB-EGF. Further

experiments with extended time points would need to be performed to answer this question. It is interesting to note though that the IL-8 and MUC5AC gene responses in the H292 cells followed a broadly similar pattern albeit over different time scales with the maximal expression being earlier when the cells were stimulated with TGFα compared to stimulation with AR or HB-EGF. It is possible therefore that MUC5AC expression would follow this pattern with a fall back towards baseline expression after 24 hours in the cells stimulated with AR and HB-EGF. The fact that TGFα is the most potent ligand and produces a MUC5AC and IL-8 gene response over a shorter time period than the response following stimulation with AR or HB-EGF (Figure 3.6d and 3.5d) does suggest that TGFα is the dominant ligand mediating these gene responses in the H292 cells.

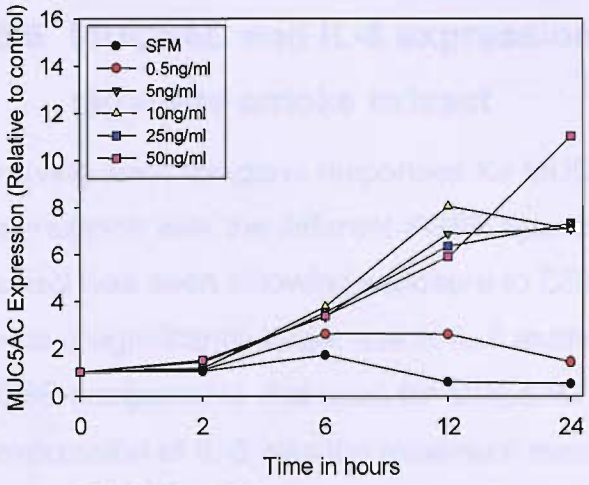


Fig 3.6a – AR

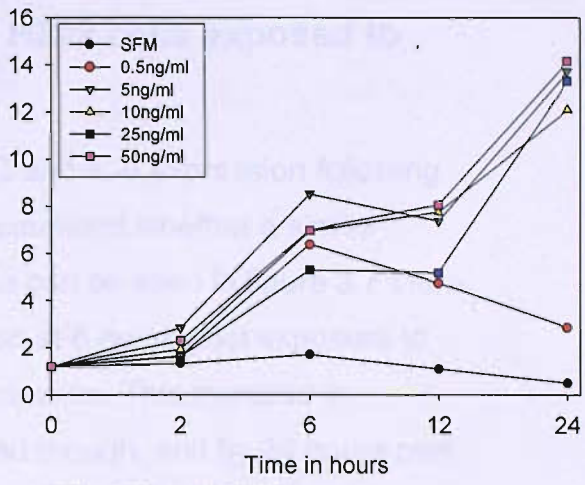


Fig 3.6b – HB-EGF

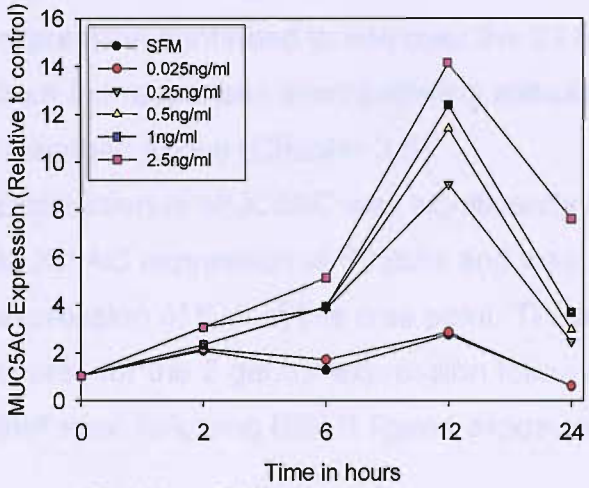


Fig 3.6c – TGFα

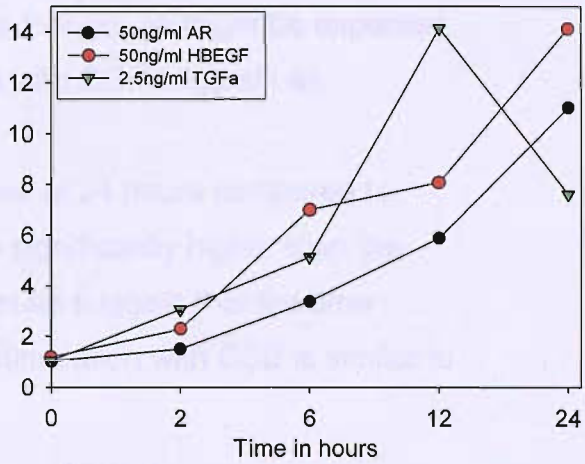


Fig 3.6d

Figure 3.6 – The effects of AR (a) , HB-EGF (b), or TGFα (c) on MUC5AC expression in H292 cells

H292 cells were cultured for 2, 6, 12, or 24 hours in the presence of one of the EGFR ligands (AR, HB-EGF, TGFα) or serum free medium (SFM) at the doses shown in the figures. mRNA expression for MUC5AC was determined using RT-qPCR with data being normalised to 18S rRNA.

Units are the fold increase in expression compared to baseline expression at t=0.

Figure 3.6d shows the effect of the maximum stimulating dose of AR (50ngml), HB-EGF (50ng/ml), or TGFα (2.5ng/ml) on MUC5AC expression. All experiments were performed in triplicate with duplicate wells of each condition used in each experiment.

3.6 MUC5AC and IL-8 expression in H292 cells exposed to cigarette smoke extract

Having seen the gene responses for MUC5AC and IL-8 expression following stimulation with the different EGFR ligands I examined whether a similar effect was seen following exposure to CSE. As can be seen in figure 3.7 there was a significantly larger rise in IL-8 expression at 6 hours post exposure to CSE compared to that seen for MUC5AC expression. This increase in expression of IL-8 was the maximum measured though, and by 24 hours post exposure to CSE gene expression was returning to baseline. MUC5AC expression continued to rise over the 24 hours though, as might be expected from the responses seen following stimulation with EGFR ligands as described above (Chapter 3.5).

Expression of MUC5AC was significantly higher at 24 hours compared to MUC5AC expression at 6 hours and was also significantly higher than the expression of IL-8 at this time point. These results suggest that the time course for the 2 genes' expression following stimulation with CSE is similar to that seen following EGFR ligand exposure.

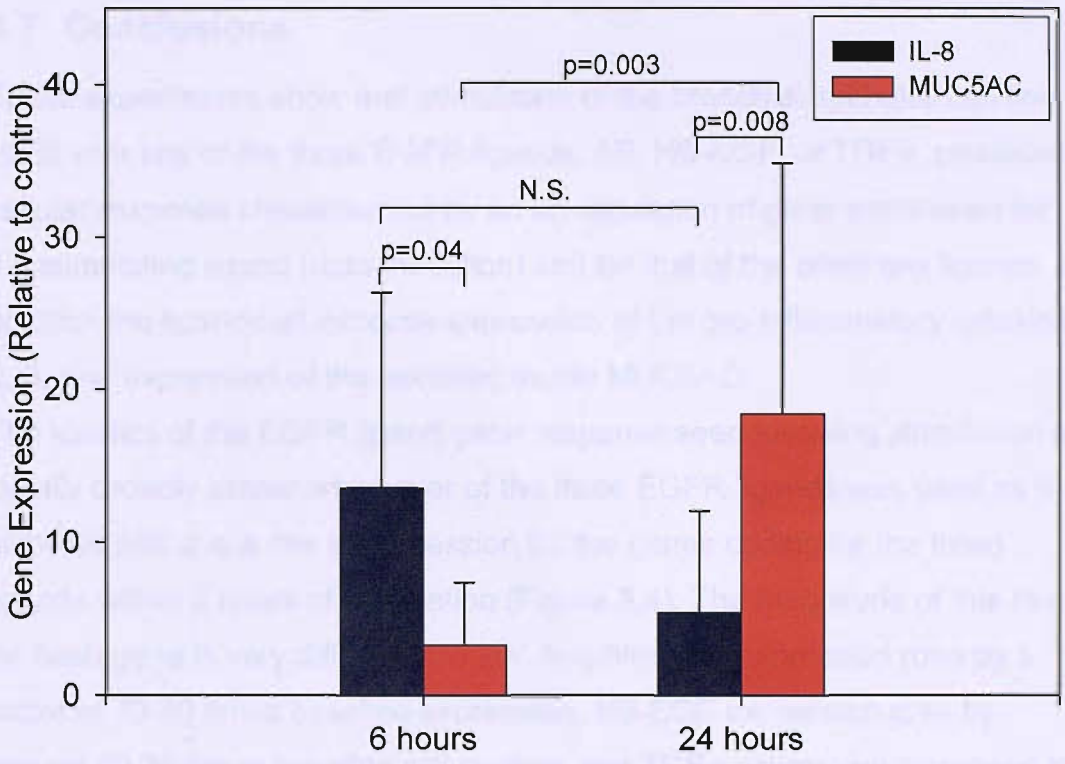


Figure 3.7 – The effect of CSE on MUC5AC and IL-8 expression in H292 bronchial epithelial cells

H292 cells were cultured until confluent at which point cell medium was removed and replaced with serum free medium for 24 hours to render the cells quiescent. This was then removed and replaced by either serum free medium (control) or 5% cigarette smoke extract (CSE). The supernatant was removed and cells processed for RNA extraction at either 6 hours or 24 hours post exposure to CSE. Data above show the effects of CSE on IL-8 expression (black bars) or MUC5AC expression (red bars) at 6 and 24 hours. Gene expression is expressed relative to control (non-CSE exposed) cultures at the same time point and represents the mean + SD fold induction from 5 separate experiments.

3.7 Conclusions

These experiments show that stimulation of the bronchial epithelial cell line H292 with any of the three EGFR ligands, AR, HB-EGF, or TGFα, produces a cellular response characterised by an up-regulation of gene expression for the stimulating ligand (auto-induction) and for that of the other two ligands. In addition the ligands all increase expression of the pro-inflammatory cytokine IL-8, and expression of the secreted mucin MUC5AC.

The kinetics of the EGFR ligand gene response seen following stimulation is initially broadly similar whichever of the three EGFR ligands was used as the stimulus with a is a rise in expression for the genes coding for the three ligands within 2 hours of stimulation (Figure 3.4). The magnitude of this rise for each gene is very different though. Amphiregulin expression rose by a factor of 70-80 times baseline expression, HB-EGF expression rose by around 20-30 times baseline expression, and TGFα expression increased to values 6-8 fold that of baseline expression, although in response to stimulation with HB-EGF this rise was somewhat larger (14 fold baseline expression).

The pattern of expression after 2 hours differed for each of the three EGFR ligand genes studied. Amphiregulin expression fell rapidly back towards baseline expression after 2 hours and was not significantly different from baseline expression 24 hours post stimulation (Figure 3.1d). The fall in AR expression may have been slightly slower when HB-EGF was the stimulating ligand compared to stimulation with AR or TGFα (Figures 3.1a-c). The HB-EGF gene response post stimulation with TGFα looked similar to that seen for AR gene expression with a sharp rise in expression at 2 hours, a large fall from this raised level 6 hours post stimulation, and then a further steady fall back to baseline expression over the next 18 hours (Figure 3.2c). HB-EGF expression in response to stimulation with AR or HB-EGF differed in that, although there was some regression towards baseline expression 6 hours post stimulation, gene expression remained raised over the 24 hours period (Figure 3.2a and 3.2c).

TGFα expression post stimulation of the H292 cells also exhibited a longer period of up-regulation of expression post stimulation, albeit at a lower level of

increase above baseline expression compared to that seen for the other 2 ligands. The expression remained above expression at t=0 for the full 24 hours when AR or HB-EGF was the stimulating ligand (Figure 3.3a and 3.3b), but stimulation with TGFα increased TGFα expression from baseline for only 12 hours with expression returning to baseline by 24 hours (Figure 3.3c). These results support the previously reported findings that activation of EGFR causes an up-regulation of gene expression for its own ligands (210;253;254), but also demonstrates that this response is not dependent on which of its ligand has bound to and activated the EGFR. The results also show that the size and time course of the ligand gene response varies between the different EGFR ligand genes. The significance of this will be discussed further below.

The kinetics of the IL-8 and MUC5AC gene response following stimulation of these cells with EGFR ligands was very different: There was an early up-regulation of IL-8 expression with marked increases in expression seen 2 hours post stimulation. This increase in expression was maintained for 12 hours post stimulation with AR and HB-EGF before expression fell back towards baseline expression at 24 hours (Figure 3.5a and 3.5b). A similar degree of up-regulation was seen when TGFα was used as the stimulus but this rise was not maintained beyond 2 hours (Figure 3.5c). Compared to these findings the pattern of the MUC5AC gene response was very different. No significant increase in expression of MUC5AC was seen 2 hours post stimulation and only a small increase above baseline expression had occurred at 6 hours. After this expression rose significantly and peaked at 12 hours in response to TGFα stimulation and 24 hours in response to stimulation with AR or HB-EGF (Figure 3.6a-c). A similar pattern of response was seen when cigarette smoke was used as the stimulus as seen in figure 3.7. This supports the hypothesis that EGFR ligand release and subsequent EGFR activation is responsible for the gene response seen post CSE exposure.

For all genes assayed there was evidence that the magnitude of up-regulation was dose-dependent and this effect was statistically significant ($p<0.001$ measured by ANOVA for each of the three ligands). Visually this can be seen in many of the plots with the lowest concentrations of ligand either producing a smaller maximal induction of gene expression or not producing a response

at all. Where an increase was seen at lower concentrations of stimulus there was often not a prolonged plateau in increase of expression as was seen with the higher concentration of stimulus e.g. figure 3.5a and 3.6b showing IL-8 expression in response to AR and MUC5AC expression in response to HBEGF respectively. Possible explanations for this are that the clearance mechanisms for removal of the stimulus, such as internalisation of the ligand/receptor complex with degradation of the ligand and recycling of the receptor, become saturated or above a certain stimulating concentration other factors come into play which perpetuates the gene response. These possibilities need further investigation as they may explain why some of the changes in the airways seen in cigarette smokers don't regress with smoking cessation.

It appears that TGFα is the most potent ligand with similar degrees of up-regulation of ligand gene expression occurring in response to stimulation of cells with doses of ligand 10 times lower than that of AR and HB-EGF (These 2 ligands have similar molecular masses and hence were used in approximately equimolar concentrations. The mass of TGFα is around half that of the other 2 ligands. It was used at concentrations twenty times smaller than those used for the other ligands giving molar concentrations around 10 times lower than those of the other 2 ligands). AR and HB-EGF have lower but approximately similar potencies. This may explain the marked difference in the magnitude of the gene response seen for the different ligands as described above with a need for larger quantities of AR and HB-EGF to be produced by the cells to compensate for their lower potency. This finding would be expected based on the known higher binding affinity of TGFα for binding to the EGFR compared to AR and HBEGF, and also matched the quoted biological activity in the ligand supplier's datasheet (255;257-259). In addition the gene response seen when TGFα was used as the stimulus tended to occur over a shorter time period compared to that seen following stimulation with AR or HB-EGF: The rise and fall in expression of the genes assayed was mostly complete by 24 hours when TGFα was the stimulus. This again suggests that TGFα is the most important of the EGFR ligands for producing the described responses.

It is important to note that this work was aiming to be primarily hypothesis generating. A large number of outcomes were measured at a small number of time points to get an impression of the patterns of gene expression post stimulation of H292 cells with EGFR ligands. In addition many variables were introduced into the model with three different ligands used for stimulation, each being used in a wide range of doses. This makes formal statistical analysis of the results seen problematic due to the effects of multiple comparisons. As such only limited statistical analysis could be performed on the results. Where this could be tested though there was evidence of statistically significant effect of dose on the magnitude of up-regulation seen for each gene assayed with each ligand. Also the increase in expression for all genes was statistically significant when compared to baseline expression for the highest dose of stimulating ligand used as shown in table 3.1.

To investigate the kinetics of the gene responses further these experiments would need to be repeated using more time points and fewer concentrations of ligand. In addition the experiments would need to be focused towards the first few hours after stimulation to assess EGFR ligand gene responses and later to look at MUC5AC expression particularly.

As discussed above it does appear that TGFα may be the most important ligand in terms of producing a cellular response. This needs to be investigated further and future work could include the stimulation of bronchial epithelial cells in the presence of antibodies against the various ligands to prevent binding to the EGFR or by the use of siRNA to prevent production of protein from the EGFR ligand mRNA hence testing the hypothesis that AR and HBEGF need TGFα to produce their response. Work also needs to be done to look at the effect of ligands in combination.

The experiments described above only examined gene expression in response to EGFR ligand stimulation and demonstrate differences in magnitude of the gene response for the EGFR ligands. The magnitude of rise though does not necessarily relate to quantity of protein produced by translation of the gene as many other factors are involved in this process, and in addition the protein product may not be transported to the cell membrane or be cleaved from its membrane bound inactive form. Thus the magnitude of gene response is not necessarily a guide to the importance of the protein

product. This work would need to be repeated with measurement of ligand release into the cell supernatant or cellular protein production using techniques such as Western blotting to get an impression of the events post gene transcription.

In summary the above results show that stimulation with EGFR ligands or CSE provoke a gene response in H292 cells with up-regulation of EGFR ligand expression and up-regulation of Il-8 and MUC5AC. These results are broadly similar whichever stimulus is used. They also suggest that H292 cell cultures can be used for future experiments to assess the effects of CSE on EGFR ligand release and cellular pro-inflammatory and mucin gene responses. In terms of measuring outcomes it would appear that EGFR ligand and IL-8 expression can be measured 6 hours post stimulation with CSE. At this time although the peak of expression of AR and HBEGF has passed they are both still substantially raised compared to baseline and TGFα and IL-8 are both raised in their plateau phases at this point. MUC5AC expression would need to be measured later though and in view of the above results 24 hours post exposure to CSE would seem to be an appropriate time point.

4 Chapter 4: The effect of doxycycline on EGFR ligand shedding and expression in bronchial epithelial cells exposed to cigarette smoke extract

4.1 Introduction

As previously discussed cigarette smoking causes COPD. Many patients with COPD complain of chronic mucus hypersecretion and pathologically a number of changes occur in the bronchial epithelium of patients with CMH including goblet cell metaplasia and hyperplasia and an increase in the size and number of sub-mucosal mucus secreting glands (54). CMH does not appear to be an harmless symptom and has been associated with an increase rate of decline in lung function (138) and with an increase risk of infection and death in patients with COPD (137). Currently there are no effective treatments to reduce CMH. Although the exact mechanisms responsible for the airway changes remains unclear the epidermal growth factor receptor (EGFR) seems to be a key regulator of mucin gene expression and may be important in the determination of the epithelial phenotype with EGFR activation causing an increase in mucus secreting cells in the airways of animals (166) and in cell cultures (193).

The ligands for the EGFR TGFα, AR, and HB-EGF are found as membrane-bound precursor molecules which require cleavage to release the active ligand (176). Cleavage is mediated by another group of membrane-bound proteins: the ADAM family of metalloproteinases, also known as sheddases. This group includes tumour necrosis factor-α converting enzyme (TACE) or ADAM17 which is known to cleave TGFα and AR (180;181). Cigarette smoke extract (CSE) causes activation of the EGFR and mucin gene expression by a process that involves ADAM activation and EGFR ligand shedding (210;214). As was demonstrated in chapter 3 any of the three EGFR ligands, AR, HB-EGF, or TGFα, can produce a pro-inflammatory and mucin gene response in bronchial epithelial cells as well as an auto-induction of EGFR ligand genes. I postulated that agents that inhibit shedding of EGFR ligands would alter the EGFR-dependent cellular responses IL-8 release and mucin gene expression

that occur when bronchial epithelial cells are exposed to noxious agents such as CSE. As discussed in chapter 1.4 the tetracycline antibiotic doxycycline, which is widely used in respiratory medicine, is known to inhibit a number of matrix metalloproteinases including MMP2, MMP8, and MMP9 (234;252) by a mechanism which may involve chelation of ions required for normal MMP function (246). In view of this I considered it likely that doxycycline may also inhibit the zinc-dependent ADAM sheddases thereby decreasing EGFR ligand release in response to CSE. This could result in a useful reduction in EGFR activation thus altering the pro-inflammatory response caused by CSE and possibly affecting mucin gene responses. If this were the case it would identify a potential target for drugs which may lead to clinically beneficial effects in patients with COPD and mucus hypersecretion. I therefore conducted a series of experiments to examine the effects of CSE and doxycycline on H292 bronchial epithelial cells, initially to ensure no significant toxic effects were seen and to identify suitable doses of both substances for future experiments, and secondly to investigate the effects of doxycycline on EGFR ligand shedding and gene expression.

4.2 Aim

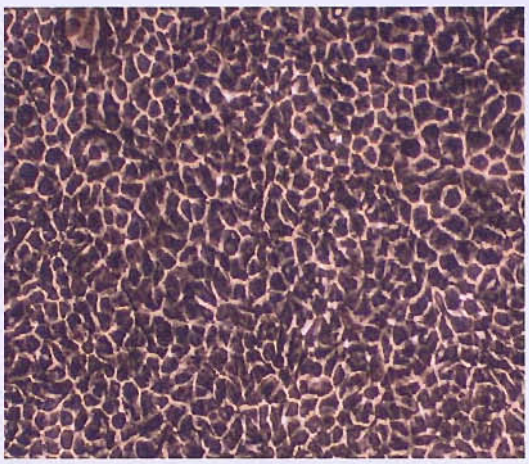
1) To investigate the toxic effects of CSE and doxycycline on bronchial epithelial cells
2) To investigate the effects of doxycycline on EGFR ligand shedding and gene expression in bronchial epithelial cells exposed to cigarette smoke.

4.3 The effect of cigarette smoke on NCI-H292 bronchial epithelial cells

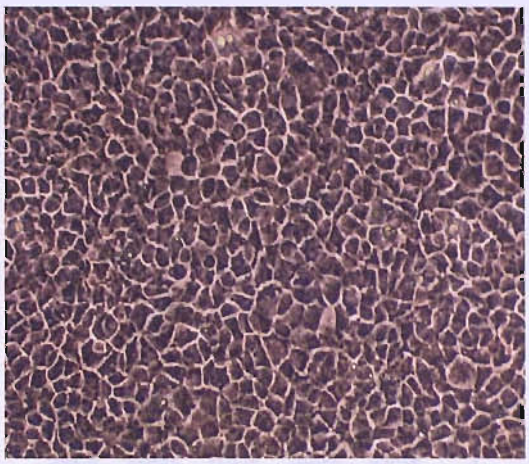
Cigarette smoke extract is a complex mixture containing numerous toxic compounds which can have adverse effects on cells. These include oxygen free radicals, complex hydrocarbons, and acrolein some of which have been shown to cause activation of the EGFR (71;206;207). Therefore it was important initially to establish a dose of CSE which would activate the EGFR but not cause significant toxic effects on the cells. A dose-response experiment was performed using doses of CSE from 2% to 15% which were added to the H292 cell monolayer for 24 hours. Toxicity was assessed by visual inspection of the cells at the end of the incubation and by measuring LDH in the supernatant which is released from cells when they die and undergo necrosis.

As can be seen in figure 4.1 there was a dose-dependent increase in toxicity with higher concentrations of CSE causing significant morphological change in the cells and increasing degrees of detachment from the culture well surface. At the highest dose of CSE used the majority of cells had detached or appeared abnormal on visual inspection.

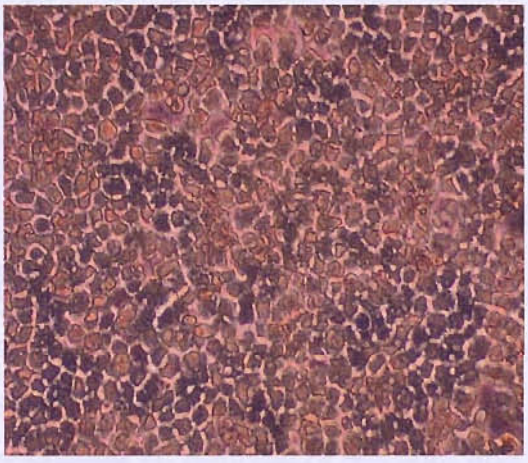
The level of LDH activity in the supernatant is shown in figure 4.2. This shows that there is a small background level of LDH release into the supernatant which falls slightly at lower doses of CSE probably due to activation of the EGFR increasing cell survival. Above 6% CSE there was progressive toxicity to the cells with significant necrosis. This fits with previous experiments by our group (210) and with the visual inspection of the cells.



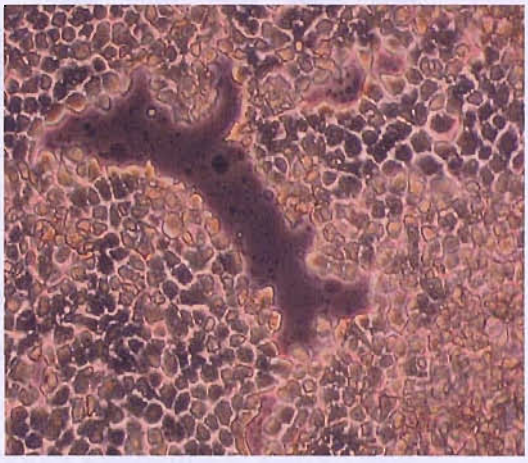
a – serum free medium



b – 5% CSE



c – 8% CSE



d – 12.5% CSE

Figure 4.1 – Toxicity of CSE to H292 cells

H292 cells were grown to confluence in 24 well trays and then rendered quiescent by removal of medium and 24 hours exposure to serum free medium (SFM – Ultraculture). The SFM was then removed and replaced either by fresh SFM (Control) or CSE in a range of concentrations from 2 to 12.5% with each condition being run in duplicate. Cells were visually inspected for toxicity at 24 hours and supernatant removed for LDH analysis. The pictures above are representative for some of the different concentrations of CSE.

Figure 4.1a – SFM (Control)
Figure 4.1b – 5% CSE
Figure 4.1c – 8% CSE
Figure 4.1d – 12.5% CSE

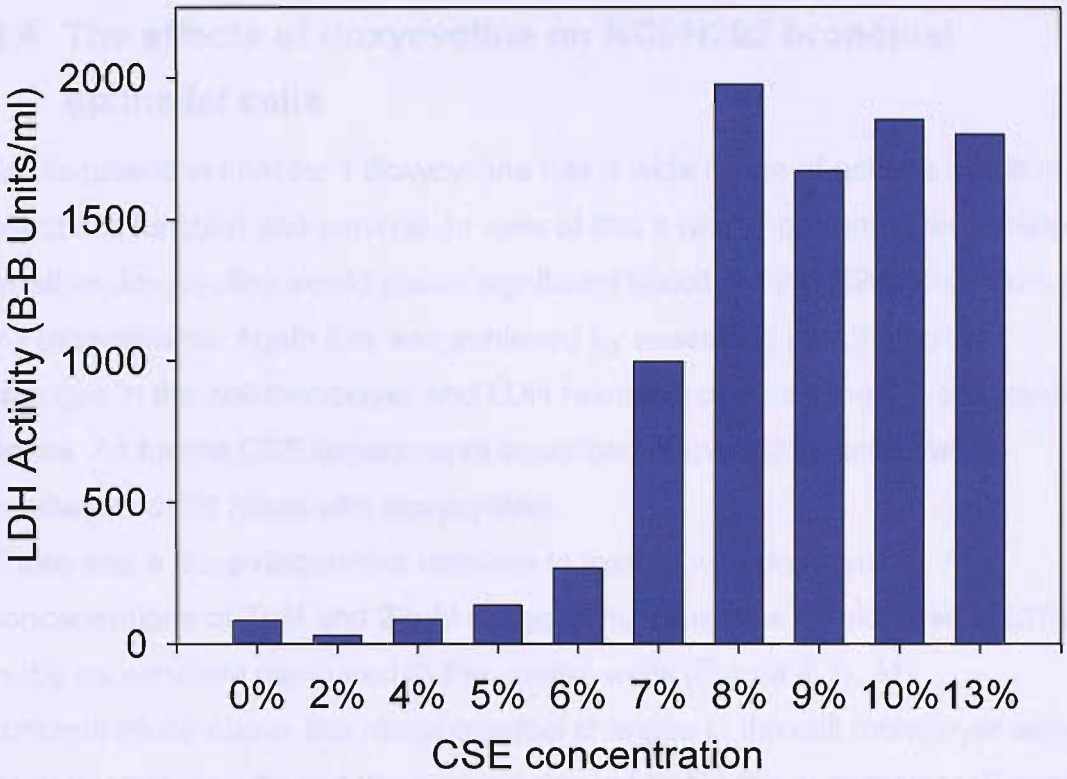


Figure 4.2 – Toxicity of CSE to H292 cells as measured by LDH activity in supernatant 24 hours post exposure to CSE

H292 cells were grown to confluence in 24 well trays and then rendered quiescent by removal of medium and 24 hours exposure to serum free medium (SFM). The SFM was then removed and replaced by either fresh SFM (Control) or CSE in concentrations ranging from 2% to 12.5% with each condition being run in duplicate on the tray. Cells were visually inspected for toxicity at 24 hours and supernatant removed for LDH analysis.

n=1

4.4 The effects of doxycycline on NCI-H292 bronchial epithelial cells

As discussed in chapter 1 doxycycline has a wide range of actions which may affect cell function and survival. In view of this it was important to determine whether doxycycline would cause significant toxicity to the H292 cells during my experiments. Again this was achieved by assessing morphological changes in the cell monolayer and LDH release across a range of doxycycline doses. As for the CSE experiments described above H292 cells were incubated for 24 hours with doxycycline.

There was a dose-dependent increase in toxicity with doxycycline. At concentrations of 7μM and 22μM doxycycline there was no increase in LDH in the supernatant compared to the control wells (Figure 4.3). At concentrations above this morphological changes in the cell monolayer were seen microscopically and there was a rise in LDH in the supernatant (Figure 4.4).

As well as measuring cellular toxicity I was concerned that doxycycline may produce a pro-inflammatory response from the H292 cells which could interfere with the expected results from exposure to CSE. To assess this I measured IL-8 release by ELISA using the supernatant of cells exposed to increasing doses of doxycycline for 24 hours. As shown in figure 4.5 there was no increase in IL-8 release from the H292 cells at doxycycline concentrations of 67μM or below. In fact there appears to be a decrease in IL-8 release which may be due to an inhibition of background shedding of the EGFR ligands with a subsequent reduction in EGFR activation.

On the basis of these results I elected to use two concentrations of doxycycline for further experiments namely 10μM and 25μM. These are a little higher than the levels found in the serum of patients taking doxycycline routinely, although as discussed in chapter 1 higher levels may be found in lung tissue, but they correspond to the levels found in a trial looking at the effect of doxycycline on abdominal aortic aneurysm progression where a higher dose of doxycycline was used for long term treatment giving mean serum levels of 4.6μg/ml (~9μM) (249).

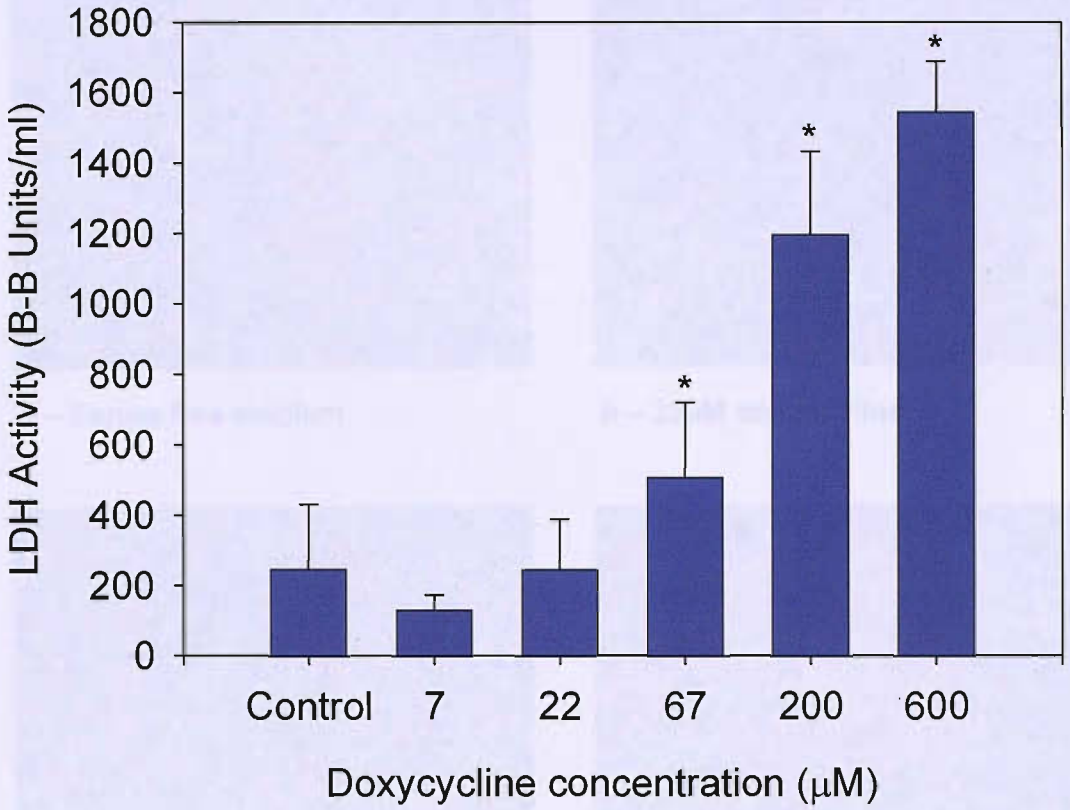
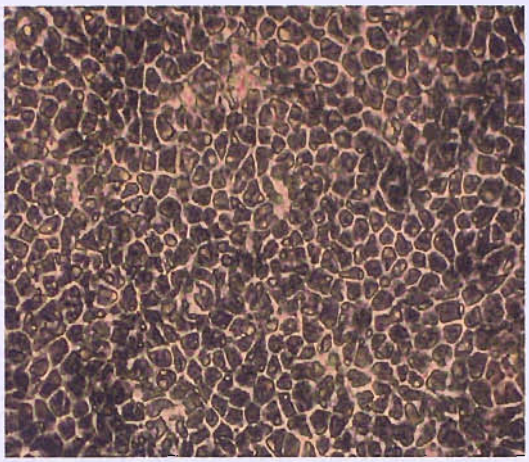


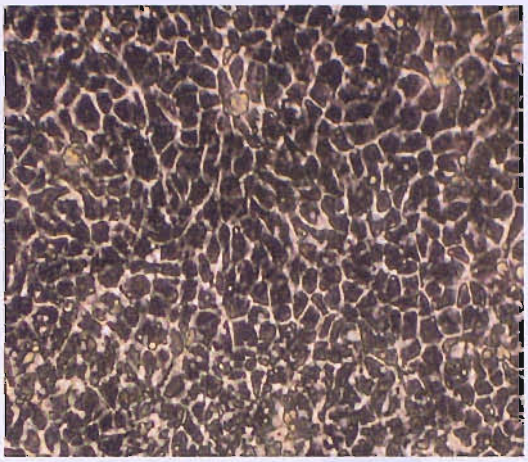
Figure 4.3 – Toxicity of doxycycline to H292 cells as measured by LDH activity in supernatant 24 hours post exposure to doxycycline

H292 cells were grown to confluence in 24 well trays and then rendered quiescent by removal of medium and 24 hours exposure to serum free medium (SFM). The SFM was then removed and replaced by either fresh SFM (Control) or doxycycline in concentrations ranging from 7.4μM to 600μM with each condition being run in duplicate on the tray. Cells were visually inspected for toxicity at 24 hours and supernatant removed for LDH analysis. The results shown are the mean and standard deviation of the results from 3 experiments with duplicate wells run each time.

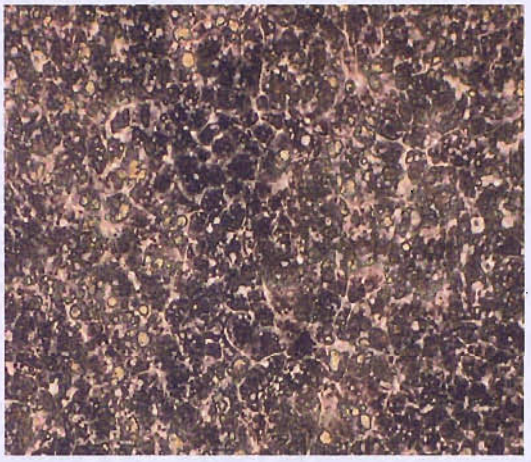
* $p<0.005$ vs. control



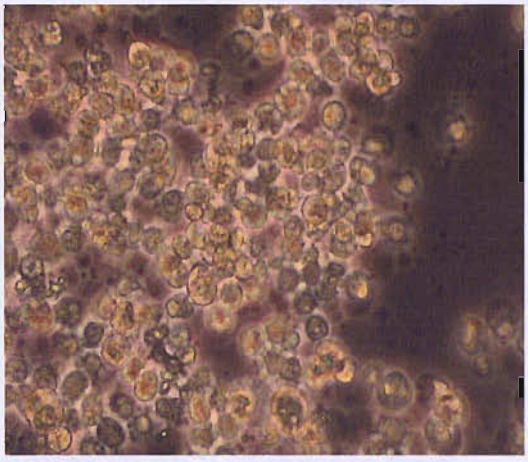
a – Serum free medium



b – 22µM doxycycline



c – 67µM doxycycline



d – 200µM doxycycline

Figure 4.4 – Toxicity of doxycycline to H292 cells

H292 cells were grown to confluence in 24 well trays and then rendered quiescent by removal of medium and 24 hours exposure to serum free medium (SFM). The SFM was then removed and replaced by either fresh SFM (Control) or doxycycline concentrations ranging from 7.4µM to 600µM with each condition being run in duplicate on the tray. Cells were visually inspected for toxicity at 24 hours and supernatant removed for LDH analysis. The pictures above are representative of the results seen from n=3 experiments

Figure 4.4a – Control (SFM)

Figure 4.4b – 22µM doxycycline

Figure 4.4c – 67µM doxycycline

Figure 4.4d – 200µM doxycycline

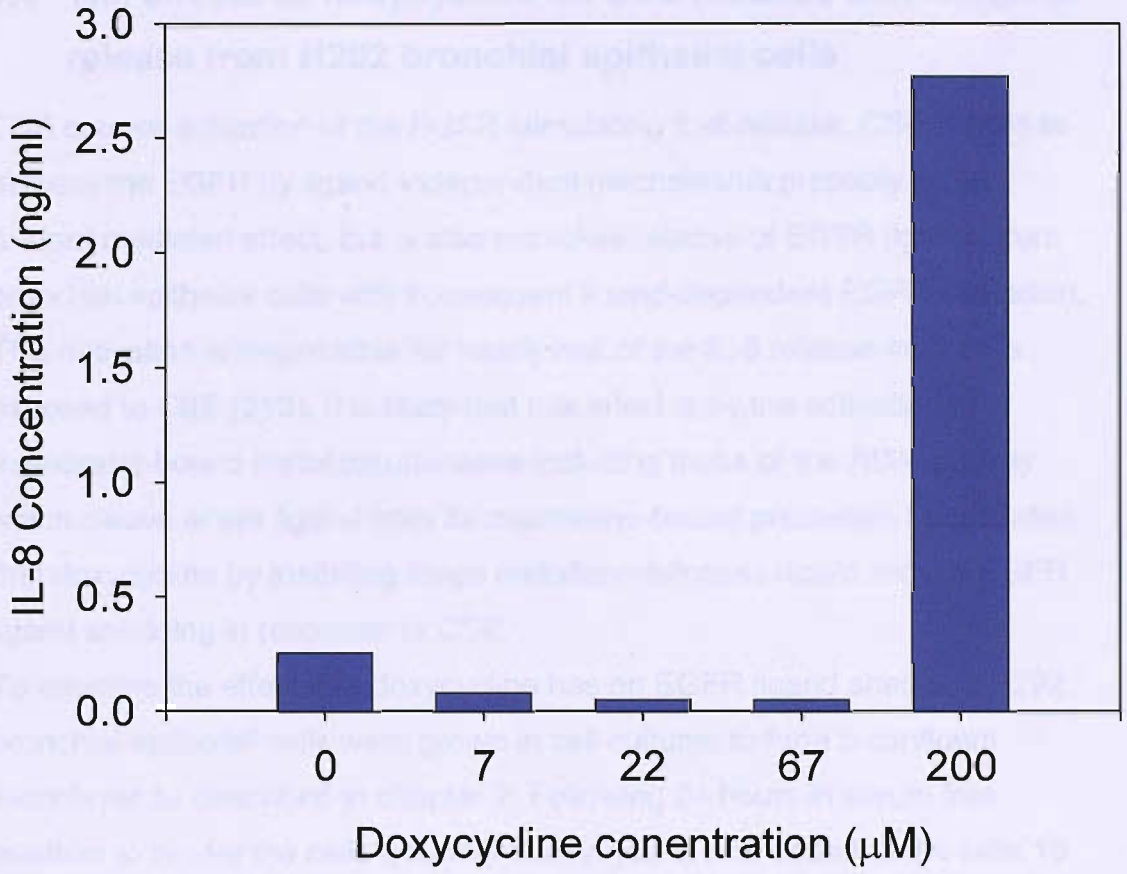


Figure 4.5 – Effect of doxycycline concentration on IL-8 release from H292 cells

H292 cells were grown to confluence in 24 well trays and then rendered quiescent by removal of medium and 24 hours exposure to SFM. This was then removed and replaced by either fresh SFM (Control) or doxycycline in concentrations ranging from 7.4μM to 600μM with each condition being run in duplicate on the tray. Cells were visually inspected for toxicity at 24 hours and supernatant removed for assay of IL-8 by ELISA

n=1

4.5 The effects of doxycycline on CSE induced EGFR ligand release from H292 bronchial epithelial cells

CSE causes activation of the EGFR stimulating IL-8 release. CSE is able to activate the EGFR by ligand-independent mechanisms probably by an oxidant mediated effect, but is also provokes release of EGFR ligands from bronchial epithelial cells with subsequent ligand-dependent EGFR activation. This activation is responsible for nearly half of the IL-8 release from cells exposed to CSE (210). It is likely that this effect is by the activation of membrane-bound metalloproteinases including those of the ADAM family which cleave active ligand from its membrane-bound precursor. I postulated that doxycycline by inhibiting these metalloproteinases would reduce EGFR ligand shedding in response to CSE.

To examine the effect that doxycycline has on EGFR ligand shedding H292 bronchial epithelial cells were grown in cell cultures to form a confluent monolayer as described in chapter 2. Following 24 hours in serum free medium to render the cells quiescent doxycycline was added to the cells 15 minutes prior to their exposure to CSE. CSE was added to give a final concentration of 5% CSE and doxycycline was added to give final concentrations of 10μM and 25μM. I measured the release of amphiregulin, HB-EGF, and TGFα in the supernatant by ELISA. For measurement of TGFα a neutralising anti-EGFR antibody must be added to the cell medium. This competes with the ligand for binding to the EGFR. Previous work has shown that when measured in the presence of the EGFR antibody TGFα levels rise significantly when cells are exposed to CSE. In the absence of the antibody little rise is seen despite evidence of EGFR activation suggesting that the TGFα is binding to the receptor and being internalised. Such a pronounced effect is not seen with AR suggesting that TGFα preferentially binds to the EGFR reflecting its higher affinity for the receptor (210). Ligand release was measured at 2, 6, and 24 hours post addition of doxycycline/CSE.

Initial experiments showed that there was a rise in EGFR ligand release in those cells treated with CSE which was noticeable at 2 and 6 hours post CSE exposure but the absolute levels of ligand in the supernatant were at the

lower limit of the sensitivities of the assays used to measure them (data not shown). As such I elected to measure ligand release into the supernatant after 24 hours exposure to CSE for further experiments. I was unable to detect HB-EGF in the supernatant of any of the cell cultures including those treated with CSE. The ELISA I used had sensitivity down to 0.2ng/ml. This may be because HB-EGF is not found in sufficient levels in these cells or because the sheddases responsible for its release are not activated by CSE.

The effect of doxycycline on TGFα release in H292 cells stimulated with CSE is shown in figure 4.6. There was a three-fold increase in TGFα in the supernatant compared to the untreated cells ($p<0.001$ vs. control). Doxycycline reduced this increase in a dose-dependent manner such that in the presence of 25μM doxycycline there was only a 1.5 fold increase in TGFα release when cells were exposed to CSE ($p=0.004$ vs. CSE alone). In those cells exposed to the two concentrations of doxycycline without exposure to CSE there was a reduction in the background shedding of TGFα such that in the presence of 25μM doxycycline TGFα release was reduced by half ($p<0.001$ vs. control).

Similar results were seen for AR (Figure 4.7) with CSE producing a 3.5 fold increase in AR release compared to untreated control cells ($p<0.05$ vs. control). Doxycycline attenuated this ligand shedding response in a dose-dependent manner. In the presence of 10μM doxycycline AR release in response to CSE rose to just under twice (1.88) the control value (p=ns vs. control) an halving of the ligand shedding seen due to CSE, but 25μM doxycycline prevented the rise altogether (AR release relative to control = 0.96, $p<0.05$ vs. CSE).

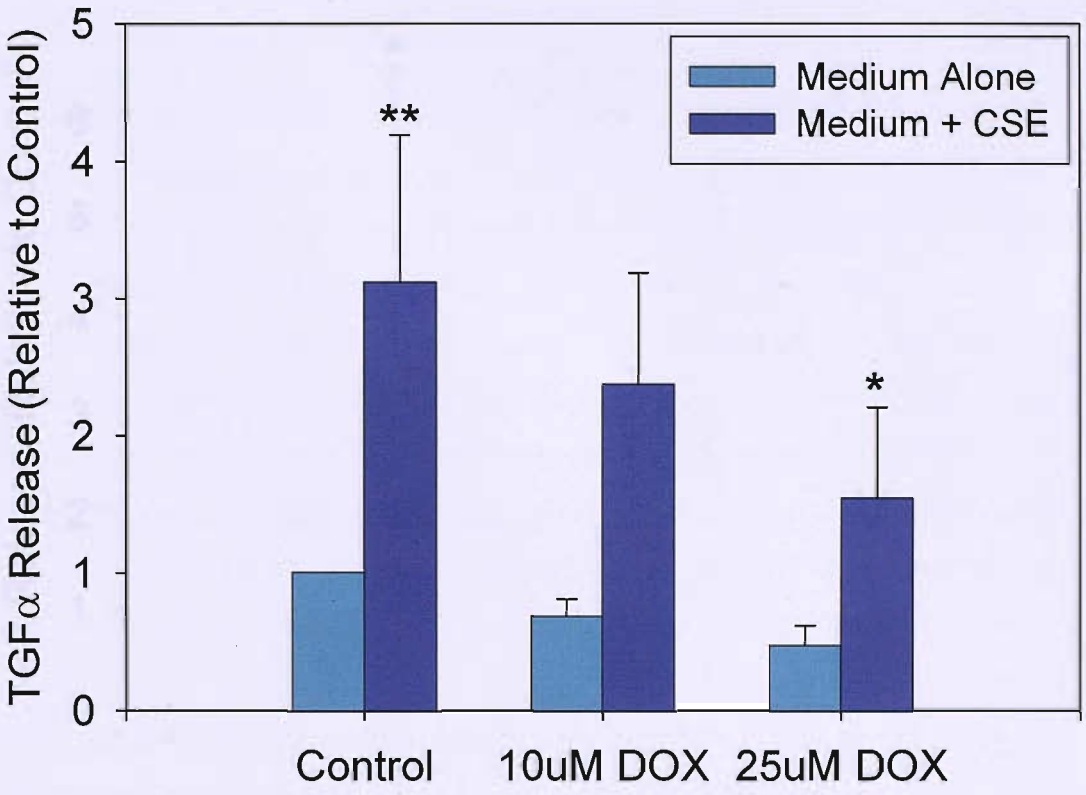


Figure 4.6 – Doxycycline inhibits release of TGFα from H292 cells stimulated with CSE

Confluent cultures of H292 cells were rendered quiescent with 24 hours incubation in SFM. Medium was removed and then replaced either with SFM (cyan bars) or 5% CSE (blue bars) with doxycycline being added in the concentrations shown 15 minutes prior to stimulation. All wells had an anti-EGFR antibody added (0.5mg/ml) at the time the medium was changed to prevent binding and internalisation of the EGFR/TGFα complex. Cells were then incubated for a further 24 hours at which time the supernatant was removed and TGFα in the supernatant measured by ELISA.

Data are expressed as mean and SD of TGFα release from n=4 experiments

* p=0.004 vs. CSE

** p=0.001 vs. control

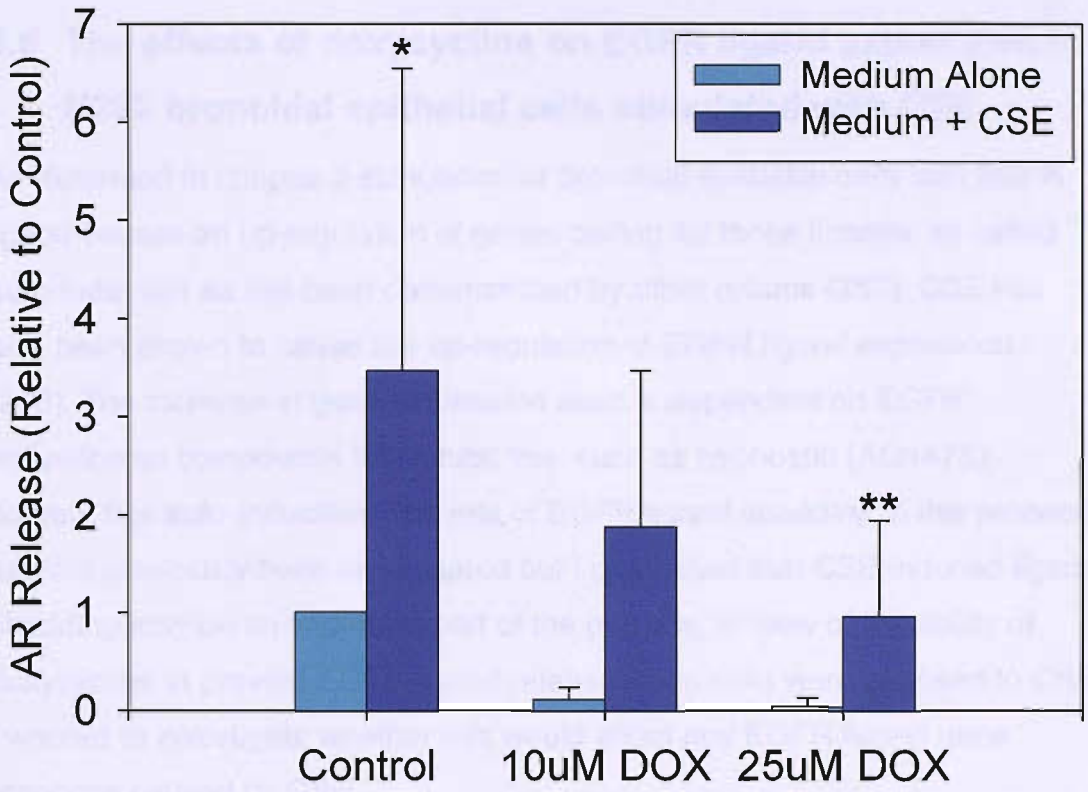


Figure 4.7 – Doxycycline inhibits release of amphiregulin from H292 cells stimulated with CSE

Confluent cultures of H292 cells were rendered quiescent with 24 hours incubation in SFM. Medium was removed and then replaced either with SFM (cyan bars) or 5% CSE (blue bars) with doxycycline being added in the concentrations shown 15 minutes prior to stimulation. Cells were incubated for a further 24 hours and the supernatant then removed. Amphiregulin (AR) in the supernatant was measured by ELISA.

Data are expressed as mean and SD of AR release from n=4 experiments

* $p<0.05$ vs. control

** $p<0.05$ vs. CSE

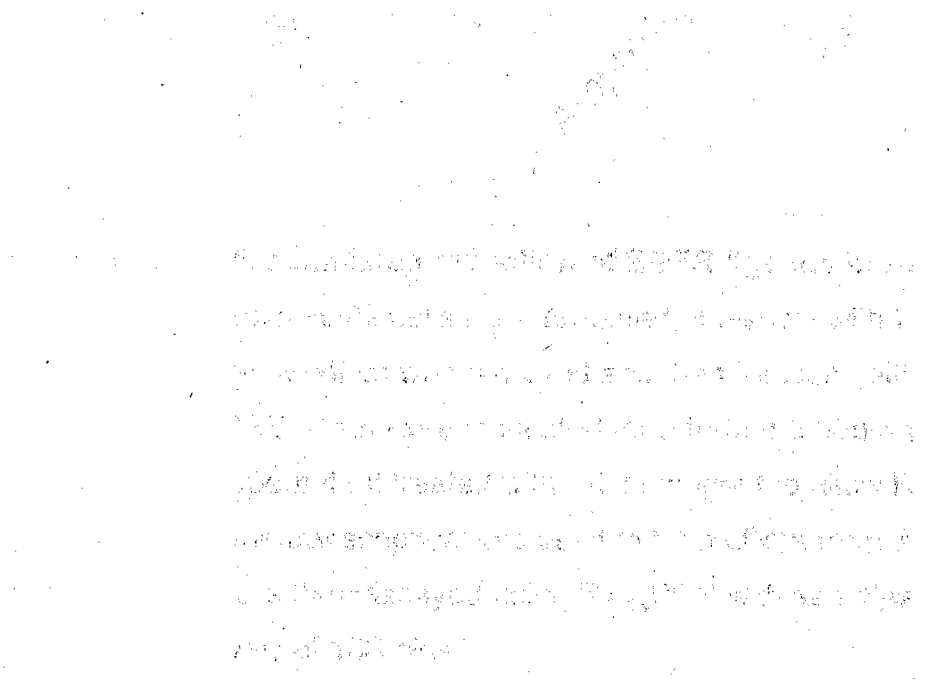
4.6 The effects of doxycycline on EGFR ligand expression in H292 bronchial epithelial cells stimulated with CSE

As illustrated in chapter 3 stimulation of bronchial epithelial cells with EGFR ligand causes an up-regulation of genes coding for those ligands, so called auto-induction as has been demonstrated by other groups (253). CSE has also been shown to cause this up-regulation of EGFR ligand expression (210). The increase in gene expression seen is dependent on EGFR activation as compounds that inhibit this, such as tyrphostin (AG1478), prevent this auto-induction. The role of EGFR ligand shedding in this process has not previously been investigated but I postulated that CSE-induced ligand shedding may be an important part of the process. In view of the ability of doxycycline to prevent EGFR ligand release when cells were exposed to CSE I wanted to investigate whether this would affect any EGFR ligand gene response caused by CSE.

A series of experiments was performed as for the investigation of EGFR ligand release described above with either 10μM or 25μM doxycycline being added to the cell medium prior to their exposure to CSE. The cell medium was removed at 6 hours and the cell monolayer treated with Trizol for RNA extraction. This time point was chosen based on the work discussed in chapter 3 which showed that at this time an increase in gene expression for all the EGFR ligands was detectable when cells had been stimulated by any one of the ligands. Although the peak up-regulation of AR and TGFα occurred earlier than 6 hours this peak followed direct stimulation with ligand at time 0. In these experiments EGFR ligand levels in the supernatant build up over a longer time post CSE exposure thereby shifting the time course of expression backwards. Even if this were not the case one would expect to find raised levels of AR, HB-EGF, and TGFα expression at this time as shown in figure 3.4.

The results are shown in figures 4.8 and 4.9. As expected the addition of CSE to the medium caused a significant rise in the expression of AR, HB-EGF, and TGFα with a 10 fold, 7 fold and 4 fold increase in expression relative to control respectively ($p<0.001$ vs. control for each) (Figure 4.8). The addition of

doxycycline to the medium prior to CSE exposure caused a dose-dependent reduction in the degree of gene up-regulation (Figure 4.9). In the presence of 25μM doxycycline the induction of AR, HB-EGF and TGFα gene expression in response to CSE exposure was 3.3, 4.4, and 2.5 fold that of the control cells respectively (Figure 4.9). This reduction was statistically significant when compared to the induction brought about by CSE for AR and HB-EGF ($p=0.005$ and $p=0.03$ respectively). The reduction of TGFα expression seen when 25μM doxycycline was added to the medium did not reach statistical significance ($p=0.06$ vs. CSE). Even in the presence of 25μM doxycycline there was still an increase in EGFR ligand gene expression in response to CSE compared to the untreated cells. It is likely that this reflects ligand-independent activation of the EGFR by compounds in the CSE such as oxidants.



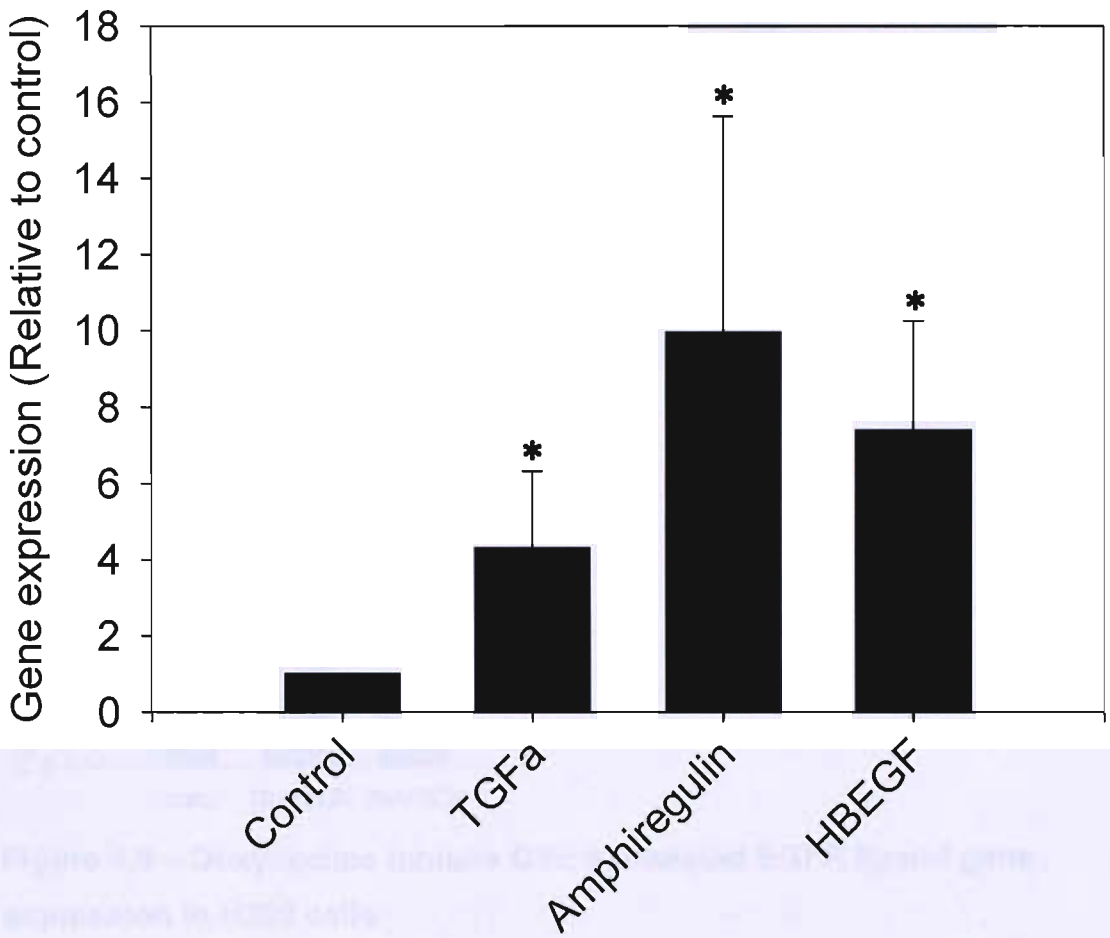


Figure 4.8 – CSE stimulates induction of EGFR ligands in H292 cells

Confluent cultures of H292 cells were rendered quiescent with 24 hours incubation in SFM. Medium was removed and then replaced either with SFM (control) or 5% CSE. Cells were incubated for a further 6 hours and following removal of the supernatant treated with Trizol reagent to allow RNA extraction. Reverse transcription was used to form cDNA from the samples and the genes were then assayed using RT-qPCR with samples being normalised to levels of 18S rRNA.

Data are expressed as mean and SD of gene expression relative to expression in the control cultures. n=5

* $p<0.001$ vs. control

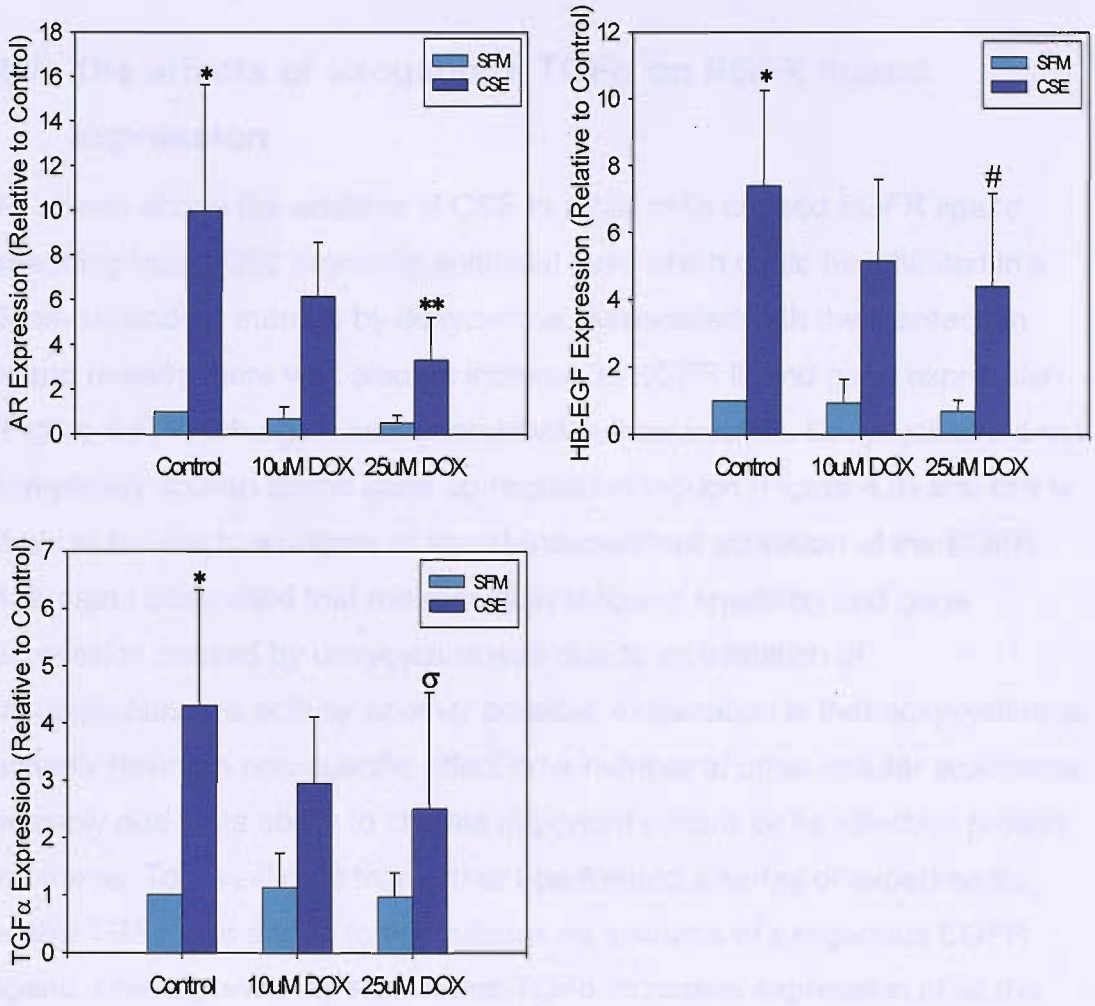


Figure 4.9 – Doxycycline inhibits CSE stimulated EGFR ligand gene expression in H292 cells

Confluent cultures of H292 cells were rendered quiescent with 24 hours incubation in SFM. Medium was removed and then replaced either with SFM (cyan bars) or 5% CSE (blue bars) with doxycycline being added in the concentrations shown 15 minutes prior to stimulation. Cells were incubated for a further 6 hours and following removal of the supernatant treated with Trizol reagent to allow RNA extraction. This was analysed for AR, HB-EGF, and TGFα expression by RT-qPCR with samples being normalised to levels of 18S rRNA. Data are expressed as mean and SD of gene expression relative to expression in the control cultures. n=5

* $p<0.001$ vs. control

** $p=0.005$ vs. CSE

$p=0.03$ vs. CSE

σ $p=0.06$ vs. CSE (ns)

4.7 The effects of exogenous TGFα on EGFR ligand expression

As shown above the addition of CSE to H292 cells caused EGFR ligand shedding from H292 bronchial epithelial cells which could be inhibited in a dose-dependent manner by doxycycline. Associated with the increase in ligand release there was also an increase in EGFR ligand gene expression (Figure 4.8) which again was attenuated by doxycycline. Doxycycline did not completely abolish ligand gene up-regulation though (Figure 4.9) and this is likely to be due to a degree of ligand-independent activation of the EGFR. Although I postulated that the reduction in ligand shedding and gene expression caused by doxycycline was due to an inhibition of metalloproteinase activity another possible explanation is that doxycycline is actually having a non-specific effect on a number of other cellular processes, possibly due to its ability to chelate important cations or its effect on protein synthesis. To investigate this further I performed a series of experiments where TGFα was added to the cultures as a source of exogenous EGFR ligand. I have previously shown that TGFα increases expression of all the EGFR ligands (Figure 3.4c) and so this would be expected to replicate the effects of CSE even in the presence of the highest concentration of doxycycline if this was primarily acting as a metalloproteinase inhibitor.
As shown in figure 4.10 the addition of TGFα to the medium caused an increase in EGFR ligand gene expression compared to control wells that was numerically similar to that caused by CSE with a fold increase in expression above control of 5.8, 5.3 and 4.0 for AR, HB-EGF and TGFα expression respectively ($p=0.01$, 0.004, 0.02 vs. control respectively). Even in the presence of 25μM doxycycline which attenuated the gene response to CSE TGFα caused an 8 fold increase in AR and HB-EGF expression and a 4 fold increase in TGFα expression (all statistically significant) in those cells exposed to CSE. This supports the hypothesis that the effects of CSE on EGFR ligand expression are due to shedding of EGFR ligands which then bind to and activate the EGFR. The predominant action of doxycycline in this model appears to be the inhibition of ligand shedding.

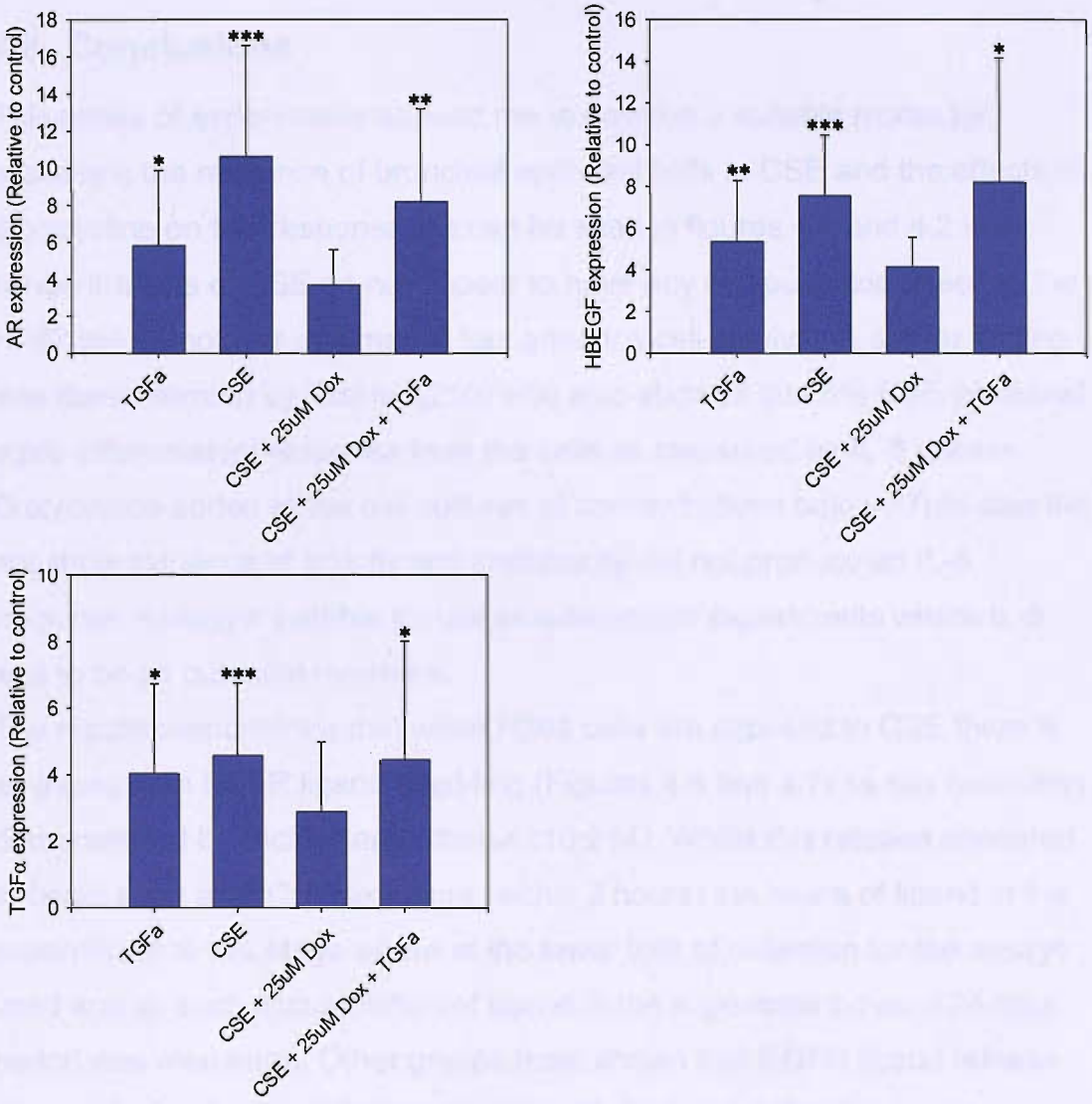


Figure 4.10 – TGFα replicates the effects of CSE on EGFR ligand expression in H292 cells even in the presence of 25μM doxycycline

Confluent cultures of H292 cells were rendered quiescent with 24 hours incubation in SFM. Medium was removed and replaced with SFM (control), TGFα (0.5ng/ml) or 5% CSE in the presence or absence of 25μM doxycycline which was added 15 minutes prior to stimulation. Cells were then incubated for a further 6 hours and following removal of the supernatant treated with Trizol reagent to allow RNA extraction. RNA was analysed for AR, HB-EGF, and TGFα expression by RT-qPCR with samples being normalised to levels of 18S rRNA.
Data are expressed as mean and SD of gene expression relative to expression in the control cultures. n=4
* $p<0.03$ vs. control
** $p=0.005$ vs. control
*** $p=0.003$ vs. control

4.8 Conclusions

This series of experiments allowed me to develop a suitable model for assessing the response of bronchial epithelial cells to CSE and the effects of doxycycline on this response. As can be seen in figures 4.1 and 4.2 low concentrations of CSE do not appear to have any obvious toxic affect on the H292 cell monolayer and may in fact enhance cell survival. A similar finding was demonstrated by Richter (210) who also showed that 5% CSE produced a pro-inflammatory response from the cells as measured by IL-8 release. Doxycycline added to the cell cultures at concentrations below 67μM also did not show evidence of toxicity and importantly did not produce an IL-8 response making it suitable for use in subsequent experiments where IL-8 was to be an outcome measure.

The results demonstrate that when H292 cells are exposed to CSE there is an increase in EGFR ligand shedding (Figures 4.6 and 4.7) as has been also demonstrated by Richter and others (210;214). Whilst this release appeared to begin soon after CSE exposure (within 2 hours) the levels of ligand in the supernatant at this stage where at the lower limit of detection for the assays used and as such accumulation of ligand in the supernatant over a 24 hour period was measured. Other groups have shown that EGFR ligand release does occur early after EGFR activation with Fan and colleagues, using immunoprecipitation techniques and detection of radioactively labelled TGFα, showing a rise in TGFα cleavage 20 minutes after stimulation of an epithelial cell line with EGF or other growth factors (260). More recently Shao and co-workers have demonstrated significant release of TGFα from H292 cells after 2 hours of exposure to cigarette smoke (214). This early rise makes it likely that it is cleavage of pre-formed ligand that is occurring rather than new protein production. The up-regulation of EGFR ligand gene expression which occurs following EGFR activation either by CSE or direct stimulation with EGFR ligand, as shown in figure 4.9 and figure 3.4, may provide a means of replacing used ligand or to modulate the long term effects of EGFR activation. Doxycycline, possibly acting as a metalloproteinase inhibitor, was able to reduce both the background shedding of EGFR ligands from H292 cells and

the shedding of ligands in response to CSE (Figure 4.6 and 4.7). This finding has not been demonstrated previously.

Since this work was completed other metalloproteinase inhibitors such as TNFα proteinase inhibitor, which selectively inhibits TACE/ADAM17, and the non-specific metalloproteinase inhibitor GM6001 have also been shown to reduce TGFα release from H292 cells exposed to cigarette smoke (214) and to other agents which can promote mucin gene expression such as neutrophil elastase (224). In this model it is not possible to determine whether the effect of doxycycline on EGFR shedding is due to a specific inhibition of TACE/ADAM17, which cleaves both TGFα and AR (181), or a more general inhibition of metalloproteinases. Unfortunately as I was unable to measure HB-EGF release it is not possible to say whether other ADAM metalloproteinases such as ADAM10 which cleaves HBEGF (184) are also inhibited. This could be investigated further by specific assays of ADAM protein activity in the presence of doxycycline as has been done for assessing the metalloproteinase inhibitory effect of other proteins such as the tissue inhibitors of metalloproteinases (TIMPs) (261).

The addition of TGFα to the cell medium replicates the effects of CSE on EGFR ligand gene expression even in the presence of doxycycline. This suggests that the reduction in CSE induced ligand shedding caused by doxycycline, shown in figure 4.6, causes a significant reduction in EGFR activation such that subsequent intracellular signalling to the nucleus is reduced. If this effect is also seen for other genes such as those relating to inflammatory responses and mucus production then this identifies the ADAM sheddases as a potential therapeutic target for treatment of smoking related lung disease.

5 Chapter 5: The effect of doxycycline on the pro-inflammatory and mucin gene responses of bronchial epithelial cells exposed to cigarette smoke extract

5.1 Introduction

As was described in chapter 4, when bronchial epithelial cells are exposed to CSE a number of events involving the EGFR and its ligands occur. These include activation of ADAM sheddases which cleave active EGFR ligand from its membrane-bound precursor, and the subsequent activation of the EGFR. EGFR activation occurs by both ligand-dependent and independent mechanisms and, as I have shown, the ligand-dependent activation can be modulated by doxycycline acting as a sheddase inhibitor. EGFR activation causes an up-regulation in the expression of genes coding for its own ligands. Thus post transcription this could provide a source of new substrate for the sheddases. The significance of this auto-induction is unclear although it may be related to preventing cell death when exposed to noxious stimuli, for cellular differentiation or for paracrine signalling.

Doxycycline reduced EGFR ligand shedding both in unstimulated cells and when cells were exposed to CSE (Figures 4.6 and 4.7). This resulted in a reduction of EGFR activation as shown by a decrease in the extent of EGFR ligand gene up-regulation in response to CSE exposure (Figure 4.9). Replacement of active ligand by the addition of TGFα to the cell medium replicated the effect of CSE even in the presence of the higher dose of doxycycline (Figure 4.10).

Activation of the EGFR is a key part of the cell's defence mechanisms against noxious stimuli. Activation of the EGFR causes IL-8 release from the cell (Figure 3.5 & (262). This cytokine is a potent neutrophil chemoattractant and is found along with increased neutrophil numbers in the sputum of patients with smoking related COPD (64). Neutrophils are part of the innate defence mechanisms of the immune system being involved in phagocytosis and destruction of bacteria. This is achieved by the production of substances such

as reactive oxygen species which are directly toxic to the bacteria and also by release of a number of cytokines which promote an influx of other inflammatory cells into the lung. Neutrophils also have a role in the repair of epithelial damage releasing compounds such as defensins which enhance epithelial repair (226), a mechanism that requires activation of the EGFR. In excess activated neutrophils may damage the lung due to release of compounds such as elastase and collagenase which can degrade lung tissue. These enzymes are believed to play an important role in the pathogenesis of emphysema.

As discussed in previous chapters the EGFR also plays a key role in mucin gene expression (166). Mucin gene products form the protein backbone of secreted mucus which is another key part of the lung's defence mechanisms. Mucus can trap inhaled particles and prevent bacterial adhesion to epithelial cells such that they can be removed by the mucocilary escalator formed by the cilia of the bronchial epithelium. There are a number of different mucin gene products some of which are membrane bound e.g. MUC1 and MUC4 and others which make up the major component of secreted mucus e.g. MUC2, MUC5AC, and MUC5B. MUC5AC appears to be the main secreted mucin in the airways and is found predominantly in the goblet cells of the surface epithelium. MUC5B is localised predominantly to the sub-mucosal glands of the airways and MUC2 is found in both sites (162).

A number of stimuli have been shown to up-regulate mucin gene expression such as bacterial products like lipopolysaccharide, neutrophil products including elastase, and cigarette smoke (184;214;224). Most of these stimuli seem to have their greatest effect on MUC5AC expression but some groups have seen increases in MUC2 in response to stimuli such as IL-4 (159). The increase in mucin gene expression is dependent on activation of the EGFR.

In view of the results presented in chapter 4 I postulated that doxycycline by inhibiting EGFR ligand shedding would affect the pro-inflammatory and mucin gene responses of H292 cells exposed to CSE.

5.2 Aim

1) To investigate the effects of doxycycline on IL-8 release and expression in bronchial epithelial cells exposed to CSE.
2) To investigate the effects of doxycycline on mucin gene responses in bronchial epithelial cells exposed to CSE.
3) To investigate whether any effects of doxycycline are due to inhibition of EGFR ligand shedding.

5.3 The effect of doxycycline on IL-8 release and expression in H292 bronchial epithelial cells exposed to CSE

Bronchial epithelial cells are one of the first lines of defence in the human airway. When exposed to noxious stimuli they respond by producing cytokines which promote an inflammatory response in the lung, and alter the expression of proteins on the cell surface to affect cell responses. IL-8 is one of the key cytokines involved in this response and its release involves EGFR activation. It has been shown that oxidants in CSE are able to cause IL-8 release from bronchial epithelial cells (263) and EGFR ligands are also able to produce a similar response as described in chapter 3. Some of the effect of CSE on IL-8 release is by ligand-independent mechanisms but it has been shown that nearly half the IL-8 response is due to EGFR ligand release and receptor binding which can be blocked by an antibody against the EGFR (210).

To investigate the effects of doxycycline on IL-8 release confluent H292 cells were exposed to 5% CSE in the presence or absence of either 10μM or 25μM doxycycline. Conditioned medium was removed and its IL-8 content measured by ELISA. Preliminary experiments showed that an increase in IL-8 release could be detected within 2 hours of exposure to CSE, but, as for the EGFR ligands discussed in chapter 4, the absolute levels of IL-8 release particularly in the untreated or doxycycline exposed cultures was at the lower limit of detection of the IL-8 assay. In view of this and to allow uniformity with the EGFR ligand data IL-8 release was measured 24 hours post exposure to CSE.

As can be seen in figure 5.1 5% CSE caused a significant increase in IL-8 release from the H292 cells to almost 3 times that of the untreated cells ($p=0.001$ vs. control). This response was blocked in a dose-dependent manner by doxycycline: 10μM doxycycline reduced the CSE stimulated IL-8 response to a 1.5 fold increase above control ($p=0.017$ vs. CSE) and 25μM doxycycline prevented the rise altogether (IL-8 levels 1.1 times that of control. $p=0.002$ vs. CSE treated cultures and $p=0.7$ vs. control). As may be expected the background level of IL-8 release also decreased in the cultures with

doxycycline in. 25μM doxycycline reduced the basal release of IL-8 to a fifth that of the untreated cells ($p<0.001$ vs. control).

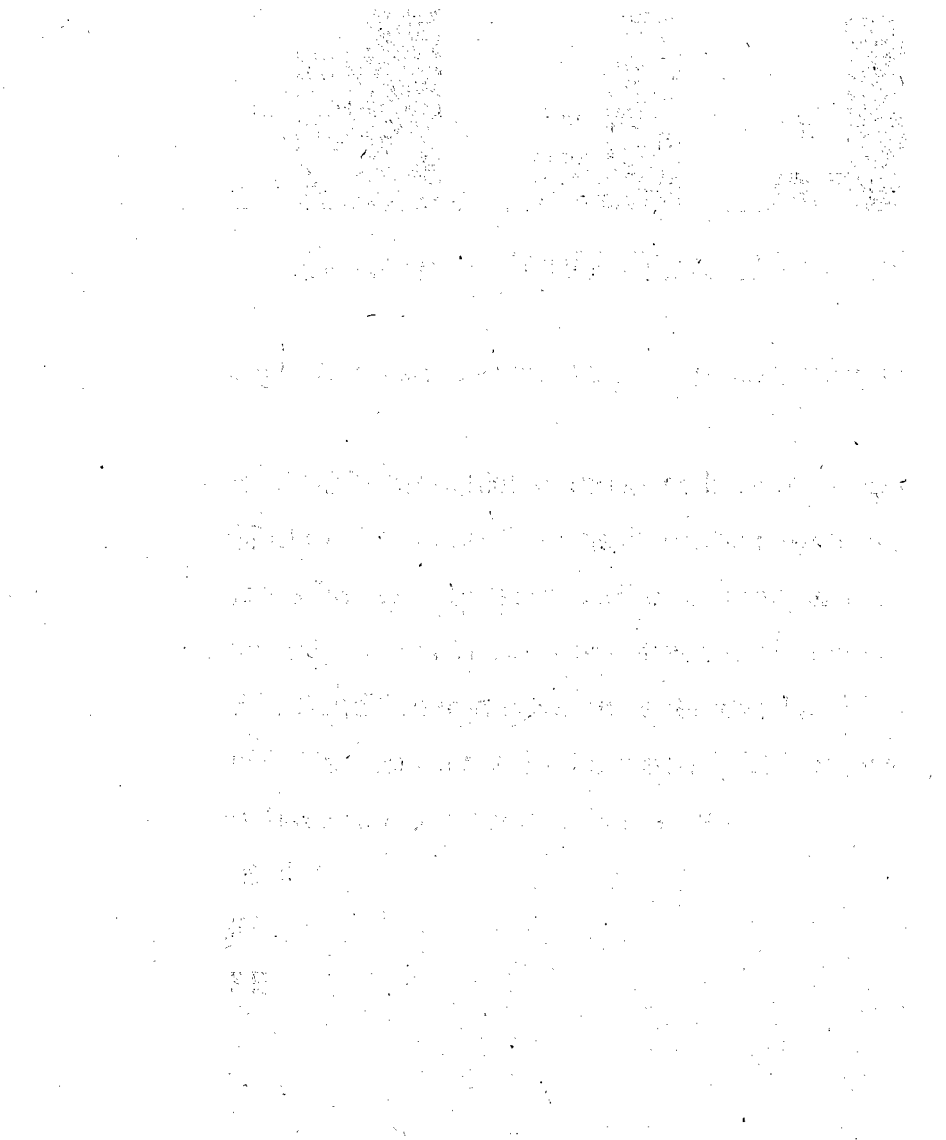
Exposure to CSE also increases IL-8 gene expression. Therefore I went on to examine the effects of doxycycline on this response. For these experiments the cells were exposed to CSE in the presence or absence of doxycycline as described above but the medium was removed at 6 hours and the cells treated for RNA extraction. This time was used based on the data shown in chapter 3 that IL-8 expression rises over 6 hours in response to EGFR ligand stimulation and is higher at 6 than 24 hours post CSE exposure (Figure 3.5 and 3.7). This time point also allows direct comparison with the ligand gene responses presented in chapter 4. The results are shown in figure 5.2.

There was an 8 fold increase in IL-8 gene transcription in those cells exposed to CSE compared to the unexposed cells ($p=0.002$ vs. control). Doxycycline reduced the up-regulation of IL-8 expression seen in response to CSE; 10μM doxycycline reduced the CSE stimulated induction of IL-8 to 3 times that of control ($p=0.01$ vs. CSE treated cells) and 25μM doxycycline reduced it to only 2 times the background expression ($p=0.005$ vs. CSE).

The actions of CSE on both the EGFR and the membrane bound sheddases are complex and involve ligand dependent and independent mechanisms. To ensure that the inhibition in IL-8 release and expression brought about by doxycycline could be reversed TGFα (0.5ng/ml) was added to the medium after the addition of doxycycline and CSE. As seen in figure 5.3 the addition of either CSE or TGFα increased IL-8 expression. The increase in expression resulting from CSE exposure was larger than that due to TGFα exposure alone (8.8 fold versus 4.6 fold rise) but both were statistically significant when compared to control ($p=0.01$ and $p=0.02$ respectively). Stimulation with TGFα in the presence of 25μM doxycycline and CSE still produced a 4 fold increase in IL-8 expression compared to expression in untreated cultures($p=0.05$ vs. control) which was similar in magnitude to the rise caused by TGFα alone. There was no statistical difference between the increases in IL-8 expression brought about by CSE, TGFα alone or TGFα in the presence of CSE and doxycycline.

These results show that the reduction of EGFR ligand shedding in response to CSE caused by doxycycline, shown in chapter 4 (Figure 4.6 and 4.7), also

results in a significant attenuation of the pro-inflammatory response as measured by IL-8 release from H292 bronchial epithelial cells and also a reduction in the degree of CSE induced IL-8 gene expression.



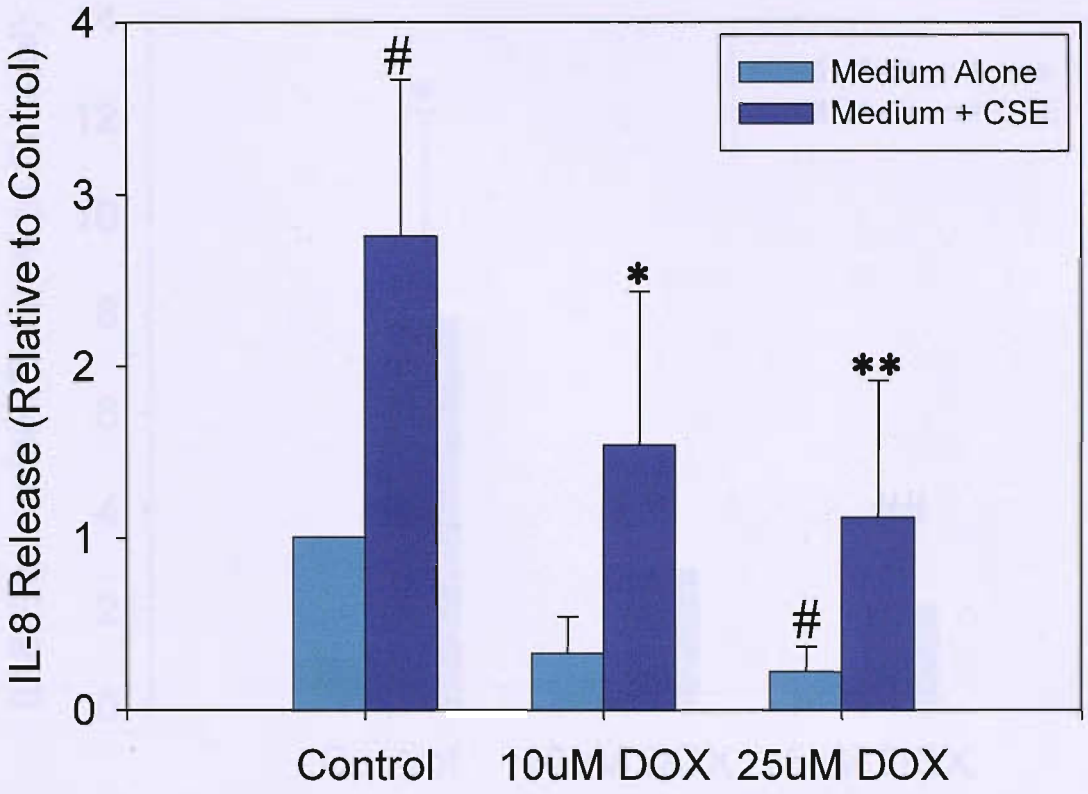


Figure 5.1 – Doxycycline reduces the release of IL-8 from H292 cells exposed to CSE

Confluent cultures of H292 bronchial epithelial cells were rendered quiescent by incubation in SFM for 24 hours. This medium was then removed and replaced either with SFM (cyan bars) or CSE (blue bars) with doxycycline being added to the cell cultures in the concentrations shown 15 minutes prior to exposure to CSE. Conditioned medium was removed at 24 hours and IL-8 measured by ELISA. Results represent the mean (SD) increase in IL-8 release relative to that in the untreated cultures. n=4

$p \leq 0.001$ vs. control

* $p=0.017$ vs. CSE

** $p=0.002$ vs. CSE

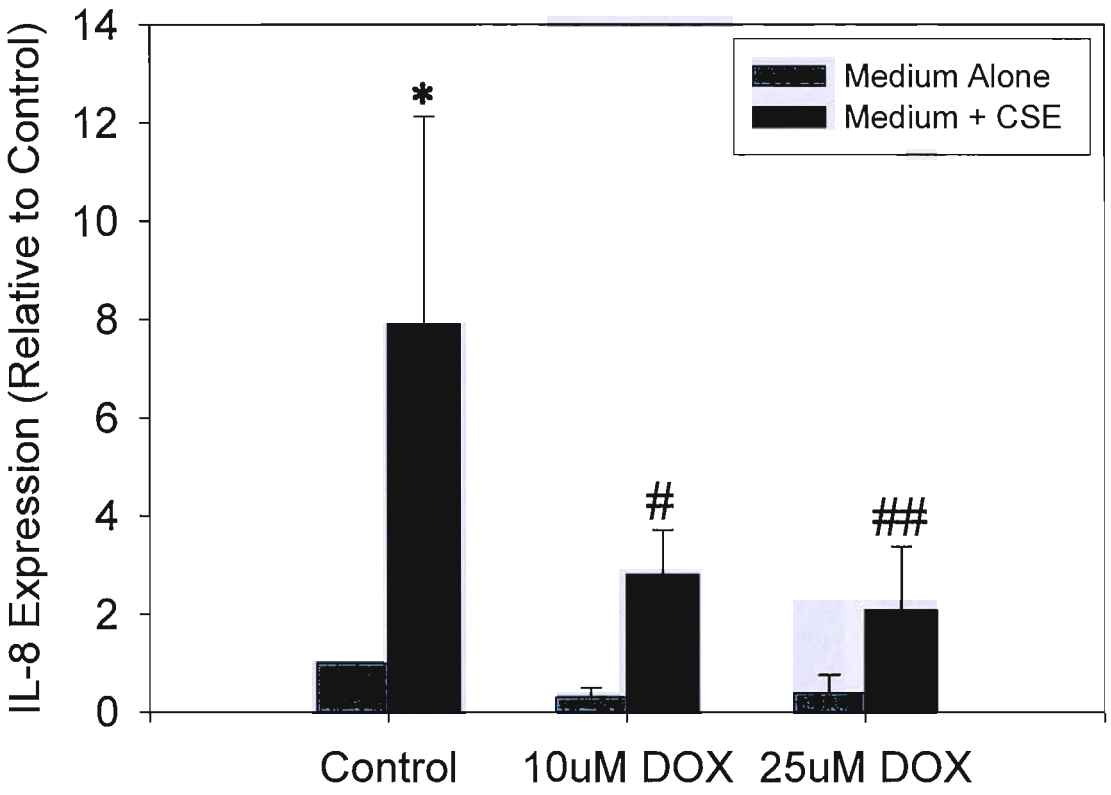


Figure 5.2 – Doxycycline inhibits the up-regulation of IL-8 expression in H292 cells stimulated with CSE

Confluent cultures of H292 bronchial epithelial cells were rendered quiescent by incubation in SFM for 24 hours. This medium was removed and replaced either with SFM (cyan bars) or CSE (blue bars) with doxycycline being added to the cell cultures in the concentrations shown 15 minutes prior to exposure to CSE. Supernatant was removed at 6 hours and the cells treated for mRNA extraction. IL-8 expression was assayed using RT-qPCR with samples being normalised to 18S rRNA. Results represent the mean (SD) increase in IL-8 expression relative to that in the control (untreated) cultures. n=4

* p=0.002 vs. control

p=0.01 vs. CSE

p=0.005 vs. CSE

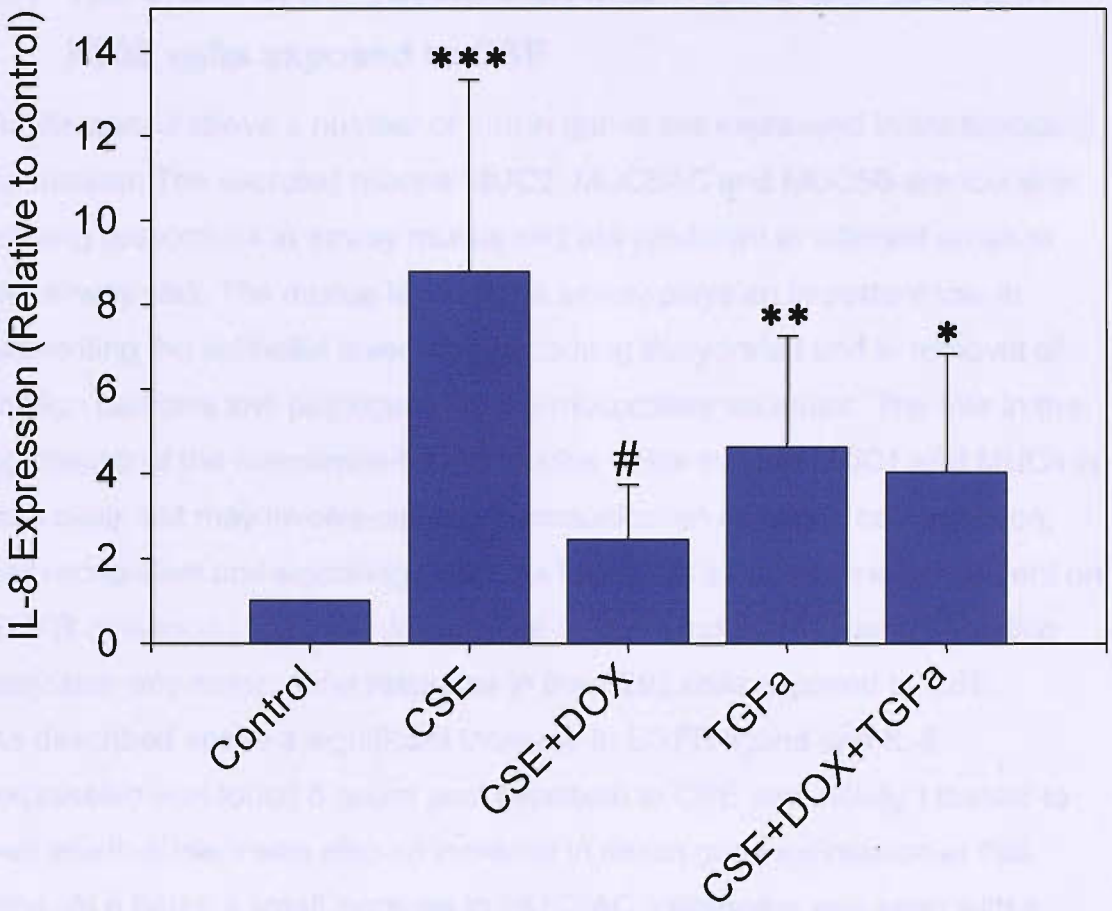


Figure 5.3 – The addition of TGFα partially replicates the effect of CSE on IL-8 expression in H292 cells even in the presence of 25μM doxycycline

Confluent cultures of H292 bronchial epithelial cells were rendered quiescent by incubation in SFM for 24 hours. This medium then replaced with one of SFM, CSE, CSE with doxycycline 25μM, TGFα (0.5ng/ml), or CSE with doxycycline 25μM and TGFα (0.5ng/ml). Doxycycline was added 15 minutes prior to stimulation with CSE +/- TGFα. Medium was removed at 6 hours and the cells treated for RNA extraction. IL-8 expression was assayed using RT-qPCR samples being normalised to 18S rRNA. Results represent the mean (SD) increase in IL-8 expression relative to that in the control (untreated) cultures. n=3

* p=0.05 vs. control
** p=0.02 vs. control
*** p=0.01 vs. control
# p=0.02 vs. CSE

5.4 The effect of doxycycline on mucin gene expression in H292 cells exposed to CSE

As discussed above a number of mucin genes are expressed in the bronchial epithelium. The secreted mucins MUC2, MUC5AC and MUC5B are found in varying proportions in airway mucus and are produced in different areas of the airway wall. The mucus layer of the airway plays an important role in preventing the epithelial layer from becoming dehydrated and in removal of foreign particles and pathogens via the mucociliary escalator. The role in the epithelium of the membrane bound mucins which include MUC1 and MUC4 is less clear, but may involve cell-cell communication including cell adhesion, cell recognition and signalling (162). As MUC5AC expression is dependent on EGFR activation (166;214) doxycycline by reducing EGFR ligand shedding may alter any mucin gene response in the H292 cells exposed to CSE.

As described above a significant increase in EGFR ligand and IL-8 expression was found 6 hours post exposure to CSE and initially I looked to see whether there was also an increase in mucin gene expression at this time. At 6 hours a small increase in MUC5AC expression was seen with a mean (SD) increase in expression of 3 (3.8) times that of the unexposed cells (n=5, p=0.12 vs. control) (Table 5.1). Preliminary experiments though showed that expression of MUC5AC in response to CSE exposure continued to rise over a longer period of time. At 24 hours the up-regulation of MUC5AC was significantly greater than at 6 hours with a mean (SD) increase in expression of 25 (17.7) times that of untreated cells which was highly statistically significant (n=4, p=0.006 vs. control). These results fit with the data shown in chapter 3 (Figure 3.6) which demonstrated that the maximum increase in MUC5AC expression in H292 cells stimulated with EGFR ligand occurs at a later time than that for IL-8 and EGFR ligand.

I also examined the expression of MUC2, MUC5B, MUC1 and MUC4 at 6 hours. Although these genes were all expressed in the H292 cells I could not detect any changes in the level of expression following exposure to CSE. In view of the rising expression of MUC5AC over the course of 24 hours I looked at the expression of the secreted mucins MUC2 and MUC5B at this time

point, but again there appeared to be no induction of gene expression in response to stimulation with CSE. The expression of the different mucin genes post CSE exposure can be seen in table 5.1.

Examining the RT-qPCR traces for the different mucin gene assays there did appear to be differences in the quantities of cDNA for the different mucin genes. I used the ΔCt method (see chapter 2.2.12.5) to compare the relative abundance of the other mucin genes to that of MUC5AC. The results can be seen in table 5.2. These show that mRNA for MUC2 is present at around the same quantity as MUC5AC mRNA in the H292 cells. MUC5B mRNA is less abundant, the level of expression being around a quarter of that for MUC5AC. Background gene expression for the membrane bound mucins MUC1 and MUC4 was much higher level than that for MUC5AC in these cells. mRNA for MUC1 was almost 9 times more prevalent than MUC5AC and that for MUC4 was present in quantities 50 times that of MUC5AC. This relative abundance of mRNA for these two mucin genes may make small changes in their expression in response to CSE undetectable using RT-qPCR.

On the basis of these results I elected to focus on MUC5AC expression as a marker of cell response to CSE and to measure its expression 24 hours after exposure to CSE. As shown in figure 5.4 there was a 25 fold increase in MUC5AC mRNA 24 hours post stimulation with CSE compared to the untreated cells ($p=0.006$ vs. control). Doxycycline reduced this increase in a dose-dependent manner. In the presence of 10μM doxycycline there was still a significant increase in MUC5AC expression in the cells exposed to CSE (8 fold increase above control ($p=0.01$ vs. control)) but this represents a reduction of 68% of the up-regulation induced by CSE ($p=0.03$ vs. CSE exposed cells). In the presence of 25μM doxycycline there was only a non-significant increase in expression of MUC5AC of 2.5 times that of the untreated cells ($p=0.28$ vs. control) which represents a reduction in the MUC5AC response of 90% compared to the 25 fold increase seen when the cells were exposed to CSE. This was highly statistically significant ($p=0.009$ vs. CSE treated cells).

Table 5.1 – The expression of mucin genes in H292 bronchial epithelial cells 6 and 24 hours post exposure to CSE

Mucin Gene	Mean (SD) expression 6 hours post exposure to CSE relative to control	Expression 24 hours post exposure to CSE relative to control
MUC1	0.43 (0.18)	
MUC4	0.94 (0.34)	
MUC2	1.26 (0.85)	1.38 (1.11)
MUC5AC	3.05 (3.86)	25.00 (17.7)
MUC5B	1.32 (0.64)	0.78 (0.49)

Table 5.2 – Expression of different mucin genes in unstimulated H292 bronchial epithelial cells relative to expression of MUC5AC

Mucin gene	Mean (SD) expression relative to MUC5AC
MUC1	8.68 (9.96)
MUC4	49.42 (50.37)
MUC2	1.76 (1.76)
MUC5B	0.26 (0.33)

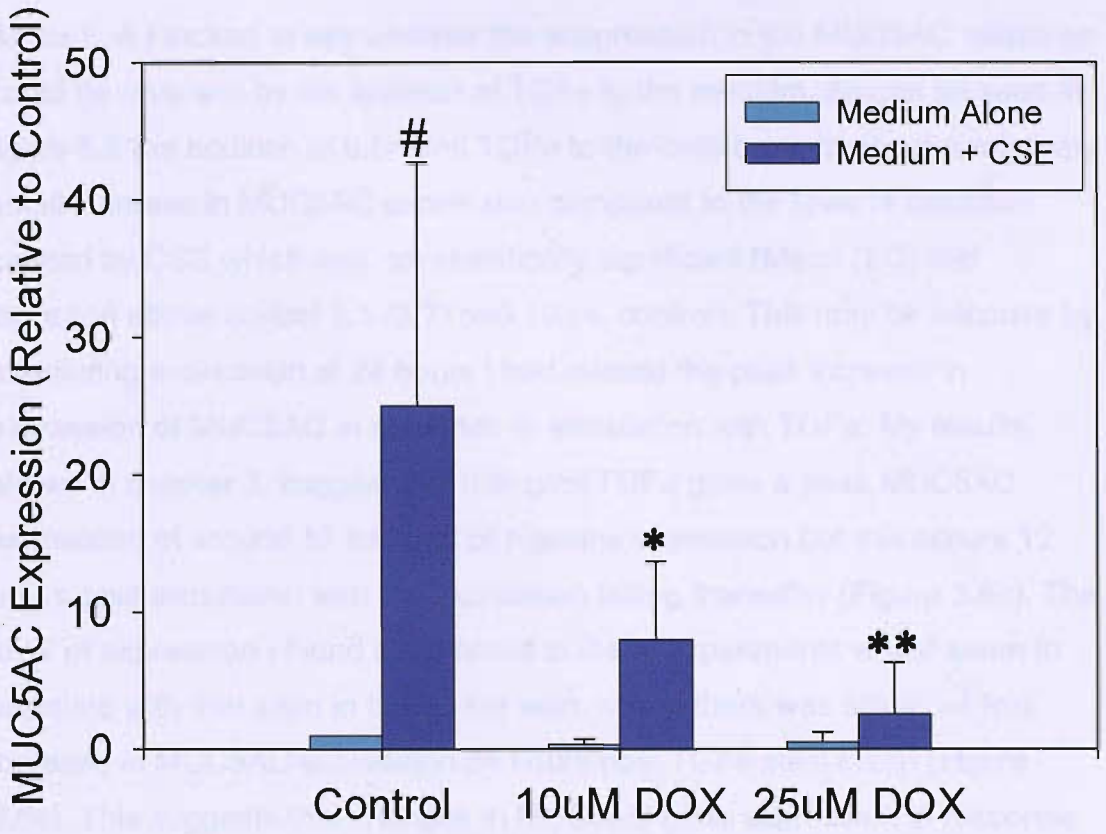


Figure 5.4 – The effect of doxycycline on MUC5AC expression in H292 cells exposed to CSE

Confluent cultures of H292 bronchial epithelial cells were rendered quiescent by incubation in SFM for 24 hours. This medium was removed and replaced either with SFM (cyan bars) or CSE (blue bars) with doxycycline being added to the cell cultures in the concentrations shown 15 minutes prior to exposure to CSE. Conditioned medium was removed at 24 hours and the cells treated for mRNA extraction. MUC5AC expression was assayed using RT-qPCR with 18S rRNA used to normalise data. Results represent the mean (SD) increase in MUC5AC expression relative to that in the control (untreated) cultures. n=4

p=0.006 vs. control

* p=0.03 vs. CSE

** p=0.009 vs. CSE

As for IL-8 I looked to see whether the suppression in the MUC5AC response could be reversed by the addition of TGFα to the medium. As can be seen in figure 5.5 the addition of 0.5ng/ml TGFα to the cells brought about a relatively small increase in MUC5AC expression compared to the level of induction caused by CSE which was not statistically significant (Mean (SD) fold induction above control 3.1 (3.7) p=0.16 vs. control). This may be because by measuring expression at 24 hours I had missed the peak increase in expression of MUC5AC in response to stimulation with TGFα. My results, shown in chapter 3, suggest that 0.5ng/ml TGFα gives a peak MUC5AC expression of around 10 fold that of baseline expression but this occurs 12 hours post stimulation with the expression falling thereafter (Figure 3.6c). The level of expression I found at 24 hours in these experiments would seem to correlate with that seen in the earlier work where there was still a 3-4 fold increase in MUC5AC expression 24 hours post TGFα stimulation (Figure 3.6c). This suggests that changes in MUC5AC gene expression in response to CSE exposure may either be delayed compared to those seen when EGFR ligand alone is used as the stimulus, possibly due to the need for sufficient active ligand to be released from the cell surface before a MUC5AC response is seen, or may involve the other EGFR ligands in the response.

Despite the possible limitations in the data discussed above even in the presence of CSE and 25μM doxycycline the addition of TGFα to the medium still produced an increase in MUC5AC expression that was numerically similar to the increase seen in the cultures where TGFα was added on its own. There was a mean (SD) fold increase in MUC5AC expression of 4.8 (3.6) that of the untreated cells when TGFα was added to the cultures exposed to CSE in the presence of 25μM doxycycline (Figure 5.5) which was statistically significant (p=0.01 vs. control).

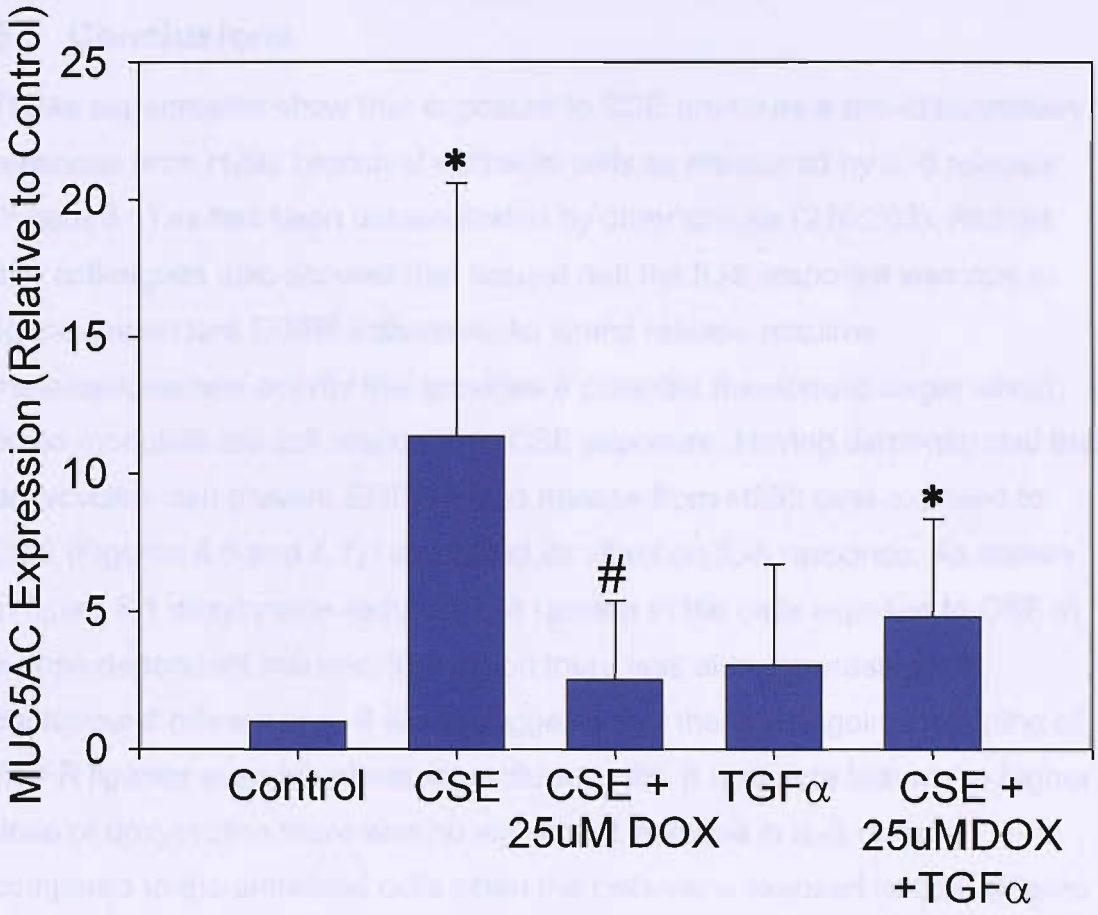


Figure 5.5 – The effects of TGFα on H292 bronchial epithelial cells exposed to CSE in the presence of doxycycline

Confluent cultures of H292 cells were rendered quiescent by incubation in SFM for 24 hours. This medium was then replaced with one of SFM (control), CSE, TGFα (0.5ng/ml), or CSE with doxycycline 25μM and TGFα (0.5ng/ml) with doxycycline being added 15 minutes prior to stimulation with CSE +/- TGFα. Medium was removed at 24 hours and the cells treated for mRNA extraction. MUC5AC expression was assayed using RT-qPCR data with 18S rRNA used to normalise data. Results represent the mean (SD) increase in MUC5AC expression relative to that in the control (untreated) cultures. n=4

* p=0.01 vs. control

p=0.02 vs. CSE

5.5 Conclusions

These experiments show that exposure to CSE provokes a pro-inflammatory response from H292 bronchial epithelial cells as measured by IL-8 release (Figure 5.1) as has been demonstrated by other groups (210;263). Richter and colleagues also showed that around half the IL-8 response was due to ligand-dependent EGFR activation. As ligand release requires metalloproteinase activity this provides a potential therapeutic target which could modulate the cell response to CSE exposure. Having demonstrated that doxycycline can prevent EGFR ligand release from H292 cells exposed to CSE (Figures 4.6 and 4.7) I examined its affect on IL-8 response. As shown in figure 5.1 doxycycline reduced IL-8 release in the cells exposed to CSE in a dose-dependent manner. In addition there was also suppression of background release of IL-8 which suggests that there is ongoing shedding of EGFR ligands even in untreated confluent cells. It is of note that at the higher dose of doxycycline there was no significant increase in IL-8 release compared to the untreated cells when the cells were exposed to CSE (Figure 5.1). This might not be expected if there was a degree of ligand-independent activation of the EGFR by CSE but in fact compared to the cells treated with doxycycline but not exposed to CSE the level of IL-8 release was increased by a factor of 5 which would support the hypothesis that CSE has both ligand dependent and independent actions on the EGFR.

The reduction in CSE-stimulated IL-8 release by doxycycline was matched by a reduction in IL-8 expression (Figure 5.2). There was not such a clear dose-dependency on doxycycline for this effect with similar degrees of suppression of the IL-8 gene response for both 10μM and 25μM doxycycline. This may be because a certain level of EGFR activation is required for the up-regulation in IL-8 expression to occur and persist and the reduction in ligand shedding brought about by both 10μM and 25μM doxycycline reduces the degree of EGFR activation below this level. This would fit with the data shown in figure 3.5a-c which shows that the expression of IL-8 in response to stimulation by any of the EGFR ligands does occur in a dose-dependent manner with there being no IL-8 response at the lowest stimulating dose of TGFα or AR. This would again make EGFR ligand shedding a potentially attractive therapeutic

target as it may not be necessary to completely block EGFR activation to prevent pro-inflammatory responses to noxious stimuli such as CSE.
Similar to the results shown in chapter 4, the effects of doxycycline could be reversed by the addition of exogenous TGFα (Figure 5.3) which although not as potent a stimulus for IL-8 expression as CSE brought about a similar increase in IL-8 expression when it was added to cells in the presence of 25μM doxycycline as it did on its own.
Along with the increase in IL-8 expression CSE also caused an increase in MUC5AC gene expression (Figure 5.4). This has been found by other groups who have also shown that this effect is dependent on EGFR activation (202;214). My results suggest that the majority of this effect is due to EGFR ligand shedding. Doxycycline reduced the level of up-regulation of MUC5AC expression in response to exposure to CSE in a dose-dependent manner with the increase in MUC5AC expression in CSE stimulated cells being of the order of 2.5 fold that of background expression in the presence of 25μM doxycycline compared to 25 fold in its absence (Figure 5.4).
The effects of replacement of EGFR ligand to this system using TGFα are not as clear as those seen for the IL-8 response. As discussed above and shown in figure 5.5 stimulation with TGFα alone only gave a small increase in expression of MUC5AC (3 fold that of untreated cells) which was not statistically significant. This may be because by measuring the expression at 24 hours I had missed the peak of the MUC5AC gene response which does seem to occur earlier when TGFα is used as the stimulus compared to AR or HBEGF (Figure 3.6a-c). The dose of TGFα I used in the above experiments did produce a level of expression of MUC5AC at 24 hours that was of a similar magnitude to that seen in my earlier experiments at this time (Figure 3.6c). Other possibilities though are that the CSE mediated effect on mucin gene expression is via other EGFR ligands such as AR which causes a later rise in MUC5AC expression, or that although the response to CSE is mediated by TGFα the time course is delayed due to the requirement for shedding of TGFα from the cell surface before there is sufficient EGFR activation to cause the downstream signalling that occurs to up-regulate MUC5AC expression.

It is important to note though that even in the presence of 25μM doxycycline an increase in MUC5AC expression of similar magnitude to that seen with TGFα alone occurred. This supports the hypothesis that the effects of doxycycline are not due to a general affect on cell function.

CSE did not appear to induce the expression of the other secreted mucins (MUC2, MUC5B) or the membrane associated mucins (MUC1, MUC4). These may be constitutively expressed and non-inducible although other groups have seen increases in MUC2 expression in response to other stimuli (159;195). The relative abundance of MUC1 and MUC4 expression compared to that of MUC5AC expression in the H292 cells (Table 5.2) may mean that small alterations in expression of these gene products are not easily detectable using RT-qPCR. This does not seem to be the case for MUC2 and MUC5B which are expressed in H292 cells in approximately the same amount as MUC5AC.

The results for the MUC5AC relate to gene expression. I have not measured the protein product either in the cell or in the supernatant. It is possible that the increase in MUC5AC expression may not lead to an increase in secreted mucin product. The events relating to the post-transcriptional fate of the mucin gene products are not well understood. It has been shown though that there is a correlation between MUC5AC secretion and mRNA expression in H292 cells (264) and increase in MUC5AC mRNA is associated with the appearance of goblet cell metaplasia in a mouse model of allergic airway inflammation (265) suggesting that increases in MUC5AC mRNA do cause an increase in production of the protein product. This would need to be confirmed in this model though and the effects of doxycycline on mucin protein production studied in more detail.

6 Chapter 6: Discussion

6.1 Summary of findings

This work has focused primarily on the role of the EGFR and its ligands in the mucin gene and pro-inflammatory response that is seen when bronchial epithelial cells are exposed to cigarette smoke. These areas are of increasing interest due to the role they play in the development of the smoking related lung disease COPD, and of chronic mucus hypersecretion which has an adverse effect on the clinical course of COPD.

It has been recognised for some years that the EGFR is a key receptor for the control of expression and production of mucins, the protein backbone for the heavily glycosylated molecule that is secreted into the airways to give mucus its gel-like properties, but there has been much debate as to which of the various EGFR ligands are involved in this response. As discussed in chapter 1 some authors have suggested that TGFα is the key ligand responsible whereas others have implicated AR and HB-EGF in this response (184;214;215). I have shown that stimulation of bronchial epithelial cells with any of these ligands, all of which are found in these cells, can promote a MUC5AC and IL-8 gene response (Figures 3.5 and 3.6). Following stimulation there were differences in the time course of the gene response for the two genes measured: IL-8 expression increased early (by 2 hours) and following a short plateau fell back towards baseline expression by 24 hours whereas MUC5AC expression did not begin to rise until 6 hours and continued to increase over the subsequent 18 hours. Biologically this may be important as IL-8 is a potent neutrophil chemoattractant and influx of these cells may be part of a rapid first line of defence against harmful stimuli in the airways whereas there is a significant quantity of mucus already present in the airways being stored in goblet cells and submucosal glands. These stores can be released immediately in response to noxious stimuli and can then be replaced by new protein production. Also mucus acts more slowly to clear molecules from the airway via the mucociliary escalator and as such rapid changes in quantities in the airway are not so important.

The results from these experiments also suggest that TGFα is the most important of the ligands. It has the highest potency of the three ligands tested producing a similar magnitude of gene response at concentrations around 10 times less than that of AR and HB-EGF. This would fit with its known higher binding affinity for the EGFR and its equivalent biological activity to the other ligands at these lower concentrations (256). In addition the kinetics of the gene response to stimulation with TGFα occur over a shorter time period than those following stimulation with AR and HB-EGF with an earlier peak in gene expression before the fall back to baseline expression (Figures 3.5 and 3.6). This may be because the other two ligands require a second event for their effect on gene expression, possibly cleavage of TGFα following ADAM sheddase activation.

My results also show that stimulation of bronchial epithelial cells by any of the three EGFR ligands results in an up-regulation of gene expression for all the EGFR ligands used i.e. auto-induction (Figures 3.1 – 3.3). This may provide a mechanism to replace the membrane-bound ligand which is shed when the EGFR is activated or may be important in altering the cell response to persistent EGFR activation by altering the relative expression of the different ligands at the cell surface. It is interesting to note that the magnitude of the gene response varied for the three ligands assayed with AR expression increasing around 80 fold above baseline expression and TGFα only increasing by a factor of 6-8 that of baseline expression. This response was similar whichever ligand was used as the stimulus. Further work needs to be performed to investigate ligand protein production to gain further insights into the importance of this response as will be discussed later (Chapter 6.2.1).

I was able to show that CSE, a disease-relevant stimulus, had a similar effect on IL-8 and MUC5AC expression as that seen for stimulation with EGFR ligand alone. Again there was an early rise in IL-8 expression which then fell over 24 hours and a slower but progressive rise in MUC5AC expression (Figure 3.7). This was expected as it has previously been shown that CSE causes EGFR ligand shedding and activation of the EGFR (210).

As mentioned, EGFR activation follows shedding of EGFR ligands from their membrane-bound precursors. This is achieved via the ADAM family of metalloproteinases which are also membrane bound. This ligand-dependent

activation of the EGFR appears to be responsible for around half of the pro-inflammatory IL-8 release from bronchial epithelial cells exposed to CSE (210). It is also increasingly being recognised that what was thought to be ligand-independent activation of the EGFR actually involves autocrine activation of the EGFR again by shedding of EGFR ligands with oxidative stress activating the ADAM sheddases (266). The ADAM sheddases therefore appear to be a key component in the EGFR response to those external stimuli such as CSE or bacterial products which are potentially harmful to the cell. It has also been shown that the ADAM sheddases can be activated via other transmembrane receptor pathways such as G protein coupled receptors again resulting in EGFR activation (174). This provides a mechanism to extend the repertoire of external stimuli that provoke a pro-inflammatory or mucin gene response in the bronchial epithelial cell. The sheddases involved in EGFR ligand shedding include ADAM17 (TACE), ADAM10, and ADAM12. ADAM17 appears to cleave a broad range of substrates including the three EGFR ligands TGFα, AR, and HB-EGF as well as the pro-inflammatory cytokine TNFα. ADAM10 and ADAM12 cleave HB-EGF (181;184;267). The mechanisms involved in regulating ADAM activity are not clear, but may involve reactive oxygen species and protein kinase C (224;266). Also the ADAM activated may depend on the stimulus: Neutrophil products and cigarette smoke have been shown to cause activation of ADAM17, whereas bacterial products, acting via PAFR and G protein coupled receptors, may activate ADAM10 (214;268). Some of these pathways are outlined in figure 1.3.4 (Chapter 1).

The tetracycline antibiotic doxycycline has previously been shown to inhibit matrix metalloproteinases (234). Due to the similarities between these molecules and the membrane-bound ADAM metalloproteinases I postulated that doxycycline may also prevent EGFR ligand shedding from bronchial epithelial cells exposed to CSE. This was the case with doxycycline causing a dose-dependent reduction in EGFR ligand shedding (Figures 4.6 and 4.7). This was matched by a reduction in the EGFR ligand gene response suggesting that the reduction in ligand shedding was bringing about an important reduction in EGFR activation (Figure 4.9). To ensure that the effect of doxycycline was due to the observed reduction in ligand shedding alone I

replaced EGFR ligand into the system by the addition of TGFα to the cell cultures. This restored the EGFR ligand gene response (Figure 4.10).
Having confirmed the ability of doxycycline to inhibit EGFR ligand shedding in bronchial epithelial cells exposed to CSE I went on to examine the effect this had on IL-8 release and mucin gene expression. As expected, exposure to CSE produced a marked inflammatory response with a 3 fold increase in IL-8 release from the bronchial epithelial cells. Doxycycline reduced this response in a dose-dependent manner (Figure 5.1) such that in the presence of 25μM doxycycline there was almost no increase in IL-8 release in response to CSE. In addition doxycycline reduced the background activation of the EGFR with a reduction in IL-8 from those cells not exposed to CSE. As also seen for the EGFR ligands there was also a reduction in the up-regulation IL-8 expression in response to CSE in the presence of doxycycline (Figure 5.2), and again this effect could be reversed by the addition of TGFα to the cell culture (Figure 5.3).
A number of mucin genes are expressed in the bronchial epithelial cell line that I used for these experiments (NCI-H292 cells). These include the membrane bound mucins MUC1 and MUC4, and the secreted mucins MUC5AC, MUC5B, and MUC2. I used RT-qPCR to investigate the mucin gene response to CSE exposure and the effects of doxycycline on this response. As has also been found by other groups, CSE increased the expression of MUC5AC over a time course that would suggest that this response was dependent on EGFR ligand shedding and subsequent EGFR activation. Doxycycline attenuated this response in a dose-dependent manner (Figure 5.4). Although it was not seen as clearly as the response seen with IL-8 expression, the addition of exogenous ligand did restore the MUC5AC response even in the presence of 25μM doxycycline. The other mucin genes examined did not appear to be inducible in response to CSE as has been seen by other groups.
These results are summarised graphically in figure 6.1. Taken together, they show that the EGFR receptor is a key molecule in the cellular response to CSE. This response is dependent on the shedding of active EGFR ligands from their membrane-bound precursors, a process mediated by ADAM metalloproteinases. Agents which inhibit the action of the metalloproteinases

attenuate the pro-inflammatory and mucin gene response to CSE and may prove a useful therapeutic target in smoking related lung disease.

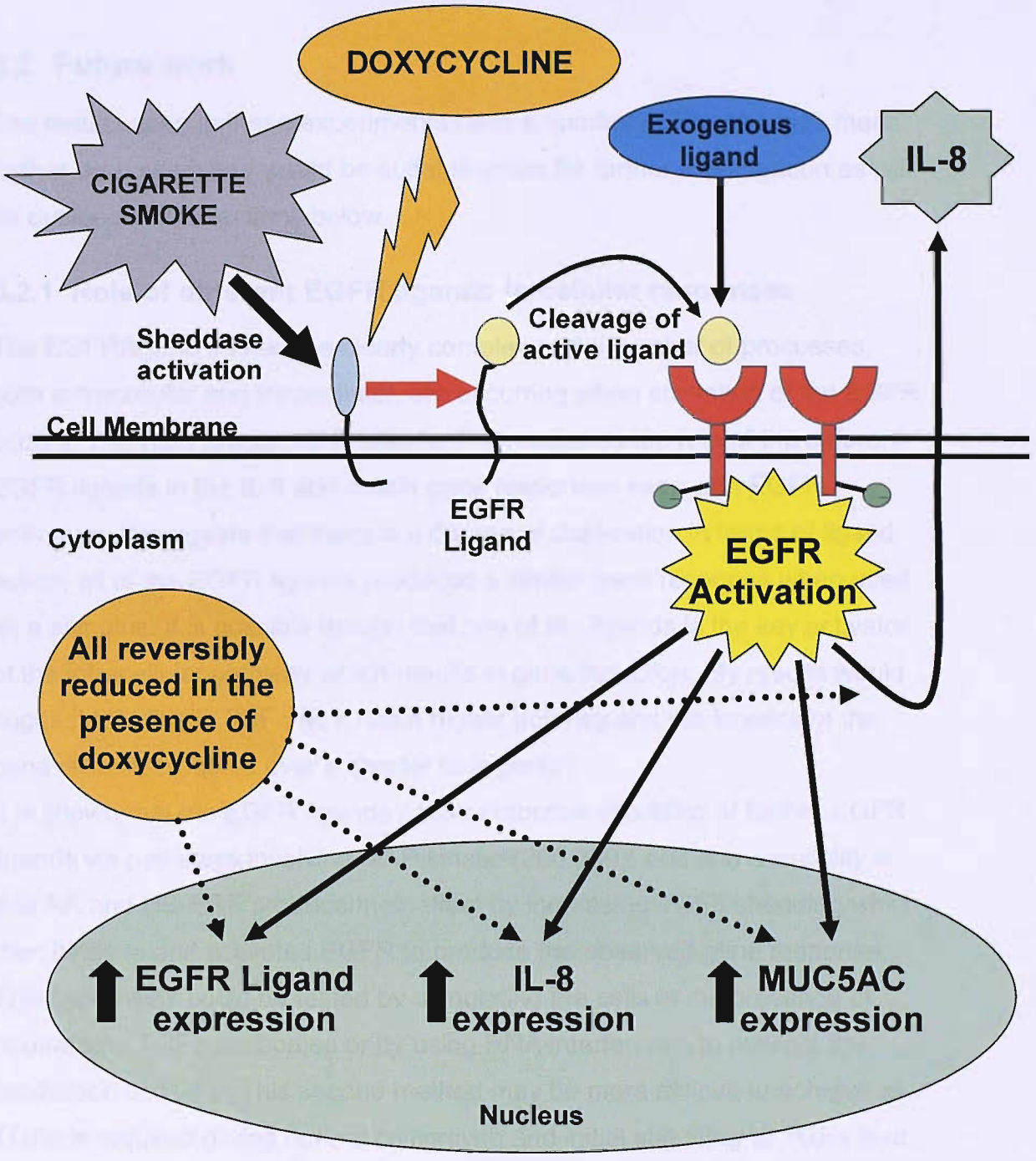


Figure 6.1 – The effects of EGFR ligand, CSE, and doxycycline on EGFR activation

Ligands for the EGFR or cigarette smoke extract, which increases EGFR ligand release by activation of sheddases, cause activation of the EGFR. This results in IL-8 release from the cell and up-regulation of the genes coding for EGFR ligands, IL-8, and MUC5AC. Doxycycline reduces EGFR ligand shedding in response to CSE. This results in an attenuation of the gene responses which can be reversed by the addition of exogenous EGFR ligand to the system.

6.2 Future work

The results seen in these experiments raise a number of issues which merit further discussion and would be suitable areas for further investigation as will be outlined in the sections below.

6.2.1 Role of different EGFR ligands in cellular responses

The EGFR/ligand pathway is clearly complex and a number of processes, both extracellular and intracellular, are occurring when activation of the EGFR occurs. The work presented in chapter 3 investigated the role of the different EGFR ligands in the IL-8 and mucin gene responses seen with EGFR activation. It suggests that there is a degree of duplication in terms of ligand action; all of the EGFR ligands produced a similar gene response when used as a stimulus. It is possible though that one of the ligands is the key activator of the intracellular pathway which results in gene induction. My results would suggest that this is TGFα as it has a higher potency and the kinetics of the gene response occurs over a shorter time period.

It is known that the EGFR ligands cause autocrine shedding of further EGFR ligands via pathways involving MAP kinase (260;269), and one possibility is that AR and HB-EGF produce their effect by increasing TGFα shedding which then binds to and activates EGFR to produce the observed gene response. This hypothesis could be tested by stimulating the cells in the presence of neutralising TGFα antibodies or by using RNA interference to prevent the production of TGFα. This second method may be more difficult to achieve as TGFα is required during normal cell growth and initial shedding of TGFα is of pre-formed ligand rather than new production.

The question as to which ligand is most important for the biological responses mediated via the EGFR is an important question particularly when potential therapeutic targets are being investigated. Recently much research has examined ligand shedding and activation of ADAM metalloproteinases as part of the mucin gene pathway. Different groups using the same stimuli to promote a mucin gene response have found contradictory results in terms of the ligands involved despite demonstrating activation of the same ADAM metalloproteinase. For example cigarette smoke induced mucin production

involves activation of ADAM17/TACE and the EGFR, but the ligand cleaved to produce this response has been identified as amphiregulin by one group (268) and TGFα by another (214). There are a number of possible explanations for this apparently contradictory finding: It has been shown that to measure TGFα release it is necessary to prevent it binding to EGFR and being internalised (210;213) which the former group did not do, and there is also evidence that where autocrine activation of a receptor is occurring there may be no measurable ligand in the extracellular medium despite evidence of receptor activation (270).

It is also recognised with other members of the ErbB family of receptors and ligands that the ligand which binds to the receptor may determine the profile of the gene response that occurs (271;272). This may be achieved by each of the ligands causing a different pattern of phosphorylation on the intracellular portion of the receptor such that different signalling pathways are activated (273). This may provide an explanation for the different findings by the groups described above as well as a mechanism that a 'suitable' biological response occurs depending on the type of stimulus applied to the cells. This is certainly an area which merits further investigation.

6.2.2 Effects of doxycycline in different cell culture systems

My results suggest that doxycycline acts as a metalloproteinase inhibitor, and is able to attenuate the pro-inflammatory and mucin gene response in bronchial epithelial cells exposed to the noxious stimuli cigarette smoke. This is an important finding. It confirms the findings of others that inhibition of ADAM metalloproteinase activity, either by specific inhibitors of metalloproteinases such as GM-6001 or by inhibition of ADAM production using siRNA, reduces the release of IL-8 (274) and MUC5AC (214) in response to inflammatory stimuli such as TNFα or cigarette smoke, but it uses a drug which is known to be safe in humans and has not been investigated in this role previously.

One of the problems of interpretation of these results in terms of further development of metalloproteinase inhibitors as a therapeutic target is that the cells used were a bronchial epithelial adenocarcinoma cell line which expresses EGFR constitutively. In the normal airways although the EGFR is

found (196) it is expressed at low levels. Its expression increases though in response to the inflammatory mediator TNFα (166), and it is found in higher levels in the airways of smokers (both with and without COPD) (201) and in the airways of asthmatics (200;275). It will be important to repeat this work using primary human bronchial epithelial cells (HBECs) obtained from normal subjects as well as from smokers, with or without COPD, and asthmatics to see whether the results can be replicated.

Along similar lines to this point the cell monolayer used in my experiments, whilst being suitable for preliminary experiments, does not necessarily reflect the events that occur in the normal airway where there is a ciliated pseudo-stratified epithelium consisting of a number of different cell types with tight intercellular junctions between them. An approximation of this airway can be obtained by growing human bronchial epithelial cells at an air-liquid interface (ALI) (192). In this form the cells are more resilient to insults. For example in the monolayer cell cultures used in these experiments CSE causes significant toxicity to the cells at concentrations above 7-8% (Figure 4.2), a finding also shown by Richter (210), whereas cells grown at an ALI can withstand much higher concentrations of CSE (>20%) without toxic effects (Personal communication – A Richter). Growth at an ALI takes around 21-28 days and this generates a suitable model to investigate both the effects of acute stimulation of a differentiated epithelium by CSE and the effects doxycycline has on this response, but also provides an opportunity for evaluation of chronic exposure to disease triggering stimuli either during the process of differentiation or once differentiation has occurred. Our group has done similar work differentiating ALI cultures in the presence of low levels of IL-13 to investigate the role of this asthma-related cytokine on epithelial phenotype. In these experiments chronic exposure to IL-13 produced a goblet-cell rich phenotype rather than the normal ciliated epithelial phenotype (Personal communication – S Puddicombe). Future work to investigate COPD relevant stimuli could include the growth of ALI cultures in the presence of the pro-inflammatory cytokines IL-8 or TNFα both of which are increased in the sputum and plasma of patients with COPD (64;276). Both these molecules cause ADAM activation to promote EGFR ligand shedding (274;277) and as such the effects of doxycycline on ALI cultures grown in this milieu should be

investigated. It would also be appropriate to grow ALI cultures in the presence of CSE and again investigate the effects of doxycycline on the phenotype formed.

6.2.3 Clinical studies

Doxycycline is widely used in respiratory disease to treat both acute exacerbations of COPD and community acquired pneumonia due to its spectrum of activity against the bacterial organisms that are often the cause of these infections. It is well tolerated with few important side-effects in older patients who present with these conditions. Like other classes of antibiotics, particularly the macrolides, doxycycline also has non-antibiotic actions and its activity against metalloproteinases has been recognised for a number of years (234-236) and has been investigated further in my work. Despite the known role of matrix metalloproteinases in the development of COPD the effects of doxycycline in this regard have not been investigated, although in horses with COPD doxycycline has been shown to inhibit the MMP collagenase in aspirates of respiratory tract secretions (278). My results suggest that doxycycline may have a role in patients with COPD particularly those with chronic mucus hypersecretion, and this requires further investigation.

There are a number of ways that these hypotheses could be tested in clinical trials. In initial studies I would want to investigate the effects of chronic administration of doxycycline on the levels of pro-inflammatory cytokines in airway secretions, both sputum and BAL, and on the structure of the bronchial epithelium. This could be achieved by taking biopsies from the large airways at bronchoscopy, a well established research technique for examining the bronchial epithelium in airways disease (279). Histological examination could look at the composition of the epithelium in terms of the ratio of mucus secreting goblet cells to ciliated cells and assess changes in this ratio post doxycycline treatment. In addition immunohistochemical techniques could assess the expression of EGFR, its ligands, and mucin genes in the epithelial biopsies. These have been shown to be increased in current smokers whether they have COPD or not (201) and it would be interesting to assess the effects of doxycycline on this increase.

One of the difficulties of using doxycycline for trials is trying to separate out any effect due to ADAM or matrix metalloproteinase inhibition from that due to its antibacterial action. Significant proportions of patients with COPD have chronic colonisation of the airways with bacteria (142) which can be difficult to detect by sputum culture analysis alone. This chronic colonisation contributes to persistent airway inflammation which may accelerate lung damage in COPD. Doxycycline would reduce this colonisation which is often due to organisms that are sensitive to the antibacterial action of doxycycline such as Haemophilus influenzae or Moraxella catarrhalis. This is likely to result in a reduction in airway inflammation and mucus hypersecretion. A possible way round this would be for all subjects to be studied to receive a prolonged course of an alternative antibiotic to eradicate infection prior to investigation. One other common difficulty with subject choice in trials relating to COPD is grouping the subjects according to smoking status and severity of airflow obstruction. It is recognised that even 'healthy' smokers i.e. those without airflow obstruction have increased airway inflammation, but there is a correlation between the severity of airflow obstruction and the degree of inflammation (55). This means that comparator studies require careful subject selection and matching.

Despite these problems preliminary studies such as those described above would be relatively straight forward to perform. Progression from there could involve more prolonged clinical studies using randomised parallel groups to assess the long term effects of doxycycline administration to subjects with COPD. Suitable outcome measures would include lung function parameters, exacerbation rates, and symptom scores as well as laboratory data from analysis of sputum/bronchoscopic samples. Measurement of mucus hypersecretion is difficult. The clinical entity of CMH likely represents the extreme end of mucus hypersecretion but there is no reliable way at present to measure lesser degrees of mucus hypersecretion which may be affected by doxycycline administration.

Doxycycline has been shown to be safe when administered chronically (249) and although concerns are raised regarding bacterial resistance when antibiotics are given for prolonged periods this doesn't seem to be a particular problem in clinical practice where doxycycline has been used to reduce

exacerbation frequency and sputum purulence in patients with chronic suppurative lung diseases such as bronchiectasis (personal experience). One of the attractive features of doxycycline for this sort of trial is that it is likely to affect many of the processes that cause inflammation and mucus hypersecretion in the airway. Using asthma as an example of another inflammatory airway disease inhaled corticosteroid remains the mainstay of treatment. Corticosteroids have numerous effects on different cell types such as eosinophils and lymphocytes which make them effective in the treatment of asthma. More targeted therapies such as agents against single cytokines such as anti IL-4 or IL-5 antibodies have not been successful in clinical trials. Other agents such as the anti-leukotriene receptor antagonists montelukast and zafirlukast which are targeting a specific group of pro-inflammatory molecules also only have significant clinical effects in a small proportion of patients. As COPD is such a heterogeneous condition doxycycline could prove an interesting compound to examine in trials such as those described above.

6.3 Conclusions

The epidermal growth factor receptor and its ligands play an important role in the production of inflammatory cytokines in the bronchial epithelium and in the regulation of the bronchial epithelial phenotype with activation producing an increase in production of MUC5AC the main secreted mucin. I have shown that stimulation of bronchial epithelial cells with any of the three EGFR ligands that are found in these cells produces this pro-inflammatory and mucin gene response, as dose exposure to cigarette smoke. These ligands require cleavage from their membrane-bound precursors to produce active ligand, an action that is mediated by the membrane-bound ADAM metalloproteinases or sheddases. Doxycycline, a member of the tetracycline antibiotic group, is known to inhibit matrix metalloproteinases. In this work I have demonstrated that doxycycline inhibits EGFR ligand release possibly by an action on EGFR ligand sheddases. This action attenuates the pro-inflammatory and mucin gene responses that occur when bronchial epithelial cells are exposed to cigarette smoke. This is a disease-relevant stimulus being the major cause of COPD and CMH in the developed world. This identifies the sheddases as a potential therapeutic target for the prevention or amelioration of smoking related lung diseases, and suggests that further studies should be performed using doxycycline as a therapeutic agent.

7 Chapter 7: References

(1) Pauwels RA, Rabe KF. Burden and clinical features of chronic obstructive pulmonary disease (COPD). Lancet 2004 August 14;364(9434):613-20.

(2) Murray CJ, Lopez AD. Evidence-based health policy--lessons from the Global Burden of Disease Study. Science 1996 November 1;274(5288):740-3.

(3) Department of Health. Statistics on smoking: England, 2003. London, UK: The Stationary Office; 2003 Nov. Report No.: 2003/21.

(4) Long term domiciliary oxygen therapy in chronic hypoxic cor pulmonale complicating chronic bronchitis and emphysema. Report of the Medical Research Council Working Party. Lancet 1981 March 28;1(8222):681-6.

(5) Continuous or nocturnal oxygen therapy in hypoxemic chronic obstructive lung disease: a clinical trial. Nocturnal Oxygen Therapy Trial Group. Ann Intern Med 1980 September;93(3):391-8.

(6) Barnes PJ. New approaches to COPD. European Respiratory Review 2005 September 1;14(94):2-11.

(7) Vestbo J. Epidemiological studies in mucus hypersecretion. Novartis Found Symp 2002;248:3-12; discussion 12-9, 277-82.:3-12.

(8) Barnes PJ. Current and future therapies for airway mucus hypersecretion. Novartis Found Symp 2002;248:237-49.

(9) Pauwels RA, Buist AS, Calverley PMA, Jenkins CR, Hurd SS. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease . NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop Summary. Am J Respir Crit Care Med 2001 April 1;163(5):1256-76.

(10) GOLD Workshop. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease (Updated 2005). 2006.

(11) Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A et al. Standardisation of spirometry. Eur Respir J 2005 August 1;26(2):319-38.

(12) Mysliwiec V, Pina JS. Bronchiectasis: the 'other' obstructive lung disease. Postgrad Med 1999 July;106(1):123-31.

(13) Anaya JM, Diethelm L, Ortiz LA, Gutierrez M, Citera G, Welsh RA et al. Pulmonary involvement in rheumatoid arthritis. Semin Arthritis Rheum 1995 February;24(4):242-54.

(14) Siafakas NM, Vermeire P, Pride NB, Paoletti P, Gibson J, Howard P et al. Optimal assessment and management of chronic obstructive pulmonary disease (COPD). The European Respiratory Society Task Force. Eur Respir J 1995 August 1;8(8):1398-420.

(15) Sluiter HJ, Koeter GH, de Monchy JG, Postma DS, de VK, Orie NG. The Dutch hypothesis (chronic non-specific lung disease) revisited. Eur Respir J 1991 April;4(4):479-89.

(16) Gelb AF, Hogg JC, Muller NL, Schein MJ, Kuei J, Tashkin DP et al. Contribution of emphysema and small airways in COPD. Chest 1996 February 1;109(2):353-9.

(17) Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society. Am J Respir Crit Care Med 1995 November;152(5 Pt 2):S77-121.

(18) National Institute for Clinical Excellence (NICE). Chronic obstructive pulmonary disease: National clinical guideline for management of chronic obstructive pulmonary disease in adults in primary and secondary care. Thorax 2004;59(Suppl 1):S1-S232.

(19) Burrows B, Earle RH. Course and prognosis of chronic obstructive lung disease. A prospective study of 200 patients. N Engl J Med 1969 February;%20;280(8):397-404.

(20) Coultas DB, Mapel D, Gagnon R, Lydick E. The Health Impact of Undiagnosed Airflow Obstruction in a National Sample of United States Adults. Am J Respir Crit Care Med 2001 August 1;164(3):372-7.

(21) Ciba Guest Symposium. Terminology, definitions, and classification of chronic pulmonary emphysema and related conditions. A report of the conclusions of a Ciba guest symposium. Thorax 1959;14:286-99.

(22) Pride NB, Vermeire P, Allegra L. Diagnostic labels applied to model case histories of chronic airflow obstruction. Responses to a questionnaire in 11 North American and Western European countries. Eur Respir J 1989 September 1;2(8):702-9.

(23) Mannino DM, Homa DM, Akinbami LJ, Ford ES, Redd SC. Chronic obstructive pulmonary disease surveillance--United States, 1971-2000. MMWR Surveill Summ 2002 August 2;51(6):1-16.

(24) de Marco R, Accordini S, Cerveri I, Corsico A, Sunyer J, Neukirch F et al. An international survey of chronic obstructive pulmonary disease in young adults according to GOLD stages. Thorax 2004 February 1;59(2):120-5.

(25) Soriano JB, Maier WC, Egger P, Visick G, Thakrar B, Sykes J et al. Recent trends in physician diagnosed COPD in women and men in the UK. Thorax 2000 September 1;55(9):789-94.

(26) Jeffery P, Saetta M. Pathology of chronic obstructive pulmonary disease. In: Gibson J, Geddes D, editors. Respiratory Medicine. Third ed. London: Saunders; 2003. p. 1141-54.

(27) MacNee W. Aetiology and pathogenesis of chronic obstructive pulmonary disease. In: Gibson J, Geddes D, editors. Respiratory Medicine. Third ed. London: Saunders; 2003. p. 1121-40.

(28) Fletcher C, Peto R, Tinker CM, Speizer FE. The natural history of chronic bronchitis and emphysema. 1 ed. Oxford: Oxford University Press; 1976.

(29) Fletcher C, Peto R. The natural history of chronic airflow obstruction. Br Med J 1977 June 25;1(6077):1645-8.

(30) Lebowitz MD. Epidemiological studies of the respiratory effects of air pollution. Eur Respir J 1996 May;9(5):1029-54.

(31) Varkey AB. Chronic obstructive pulmonary disease in women: exploring gender differences. Curr Opin Pulm Med 2004 March;10(2):98-103.

(32) Vestbo J, Knudsen KM, Rasmussen FV. The value of mucus hypersecretion as a predictor of mortality and hospitalization. An 11-year register based follow-up study of a random population sample of 876 men. Respir Med 1989 May;83(3):207-11.

(33) Cichy J, Potempa J, Travis J. Biosynthesis of alpha1-proteinase inhibitor by human lung-derived epithelial cells. J Biol Chem 1997 March 28;272(13):8250-5.

(34) Mornex JF, Chytil-Weir A, Martinet Y, Courtney M, LeCocq JP, Crystal RG. Expression of the alpha-1-antitrypsin gene in mononuclear phagocytes of normal and alpha-1-antitrypsin-deficient individuals. J Clin Invest 1986 June;77(6):1952-61.

(35) DeMeo DL, Silverman EK. α1-Antitrypsin deficiency - 2: Genetic aspects of α1-antitrypsin deficiency: phenotypes and genetic modifiers of emphysema risk. Thorax 2004 March 1;59(3):259-64.

(36) DeMeo DL, Carey VJ, Chapman HA, Reilly JJ, Ginns LC, Speizer FE et al. Familial aggregation of FEF(25-75) and FEF(25-75)/FVC in families with severe, early onset COPD. Thorax 2004 May;59(5):396-400.

(37) Lomas DA, Parfrey H. {alpha}1-Antitrypsin deficiency * 4: Molecular pathophysiology. Thorax 2004 June 1;59(6):529-35.

(38) Oxman AD, Muir DC, Shannon HS, Stock SR, Hnizdo E, Lange HJ. Occupational dust exposure and chronic obstructive pulmonary disease. A systematic overview of the evidence. Am Rev Respir Dis 1993 July;148(1):38-48.

(39) Nemery B. Metal toxicity and the respiratory tract. Eur Respir J 1990 February;3(2):202-19.

(40) Becklake MR. Occupational exposures: evidence for a causal association with chronic obstructive pulmonary disease. Am Rev Respir Dis 1989 September;140(3 Pt 2):S85-S91.

(41) Orie NGM, Sluiter HJ, De Vries K, Tammeling GJ, Witkop J. The host factor in bronchitis. In: Orie NGM, Sluiter HJ, editors. Bronchitis.Assen, The Netherlands: Royal Van Gorcum; 1961. p. 43-59.

(42) Barnes PJ. Against the Dutch Hypothesis: Asthma and Chronic Obstructive Pulmonary Disease Are Distinct Diseases. Am J Respir Crit Care Med 2006 August 1;174(3):240-3.

(43) Kraft M. Asthma and Chronic Obstructive Pulmonary Disease Exhibit Common Origins in Any Country! Am J Respir Crit Care Med 2006 August 1;174(3):238-40.

(44) Reid L. The Pathology of Emphysema. First ed. London: Lloyd-Luke (Medical books) Ltd; 1967.

(45) Jeffery PK. Structural and inflammatory changes in COPD: a comparison with asthma. Thorax 1998 February;53(2):129-36.

(46) Hogg JC, Macklem PT, Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. N Engl J Med 1968 June;278(25):1355-60.

(47) Niewoehner DE, Kleinerman J, Rice DB. Pathologic changes in the peripheral airways of young cigarette smokers. N Engl J Med 1974 October 10;291(15):755-8.

(48) O'Donnell RA, Peebles C, Ward JA, Daraker A, Angco G, Broberg P et al. Relationship between peripheral airway dysfunction, airway obstruction, and neutrophilic inflammation in COPD. Thorax 2004 October;59(10):837-42.

(49) Macklem PT, Proctor DF, Hogg JC. The stability of peripheral airways. Respir Physiol 1970 January;8(2):191-203.

(50) Gillooly M, Lamb D. Microscopic emphysema in relation to age and smoking habit. Thorax 1993 May;48(5):491-5.

(51) Lamb D, McLean A, Gillooly M, Warren PM, Gould GA, MacNee W. Relation between distal airspace size, bronchiolar attachments, and lung function. Thorax 1993 October;48(10):1012-7.

(52) Snider GL. Distinguishing among asthma, chronic bronchitis, and emphysema. Chest 1985 January;87(1 Suppl):35S-9S.

(53) Saetta M, Finkelstein R, Cosio MG. Morphological and cellular basis for airflow limitation in smokers. Eur Respir J 1994 August 1;7(8):1505-15.

(54) Maestrelli P, Saetta M, Mapp CE, Fabbri LM. Remodeling in Response to Infection and Injury . Airway Inflammation and Hypersecretion of Mucus in Smoking Subjects with Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2001 November 15;164(10):76S-80.

(55) O'Donnell R, Breen D, Wilson S, Djukanovic R. Inflammatory cells in the airways in COPD. Thorax 2006 May 1;61(5):448-54.

(56) Mullen JB, Wright JL, Wiggs BR, Pare PD, Hogg JC. Reassessment of inflammation of airways in chronic bronchitis. Br Med J (Clin Res Ed) 1985 November 2;291(6504):1235-9.

(57) Fournier M, Lebargy F, Le Roy LF, Lenormand E, Pariente R. Intraepithelial T-lymphocyte subsets in the airways of normal subjects and of patients with chronic bronchitis. Am Rev Respir Dis 1989 September;140(3):737-42.

(58) Saetta M, Turato G, Facchini FM, Corbino L, Lucchini RE, Casoni G et al. Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. Am J Respir Crit Care Med 1997 November;156(5):1633-9.

(59) Di SA, Turato G, Maestrelli P, Mapp CE, Ruggieri MP, Roggeri A et al. Airflow limitation in chronic bronchitis is associated with T-lymphocyte and macrophage infiltration of the bronchial mucosa. Am J Respir Crit Care Med 1996 February;153(2):629-32.

(60) Willemse BWM, ten Hacken NHT, Rutgers B, Lesman-Leegte IGAT, Postma DS, Timens W. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. Eur Respir J 2005 November 1;26(5):835-45.

(61) Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F et al. CD8+ve Cells in the Lungs of Smokers with Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 1999 August 1;160(2):711-7.

(62) Finkelstein R, Fraser RS, Ghezzo H, Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. Am J Respir Crit Care Med 1995 November;152(5 Pt 1):1666-72.

(63) Hiemstra PS, van Wetering S, Stolk J. Neutrophil serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium. Eur Respir J 1998 November 1;12(5):1200-8.

(64) Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am J Respir Crit Care Med 1996 February 1;153(2):530-4.

(65) Hill A, Bayley D, Stockley RA. The Interrelationship of Sputum Inflammatory Markers in Patients with Chronic Bronchitis. Am J Respir Crit Care Med 1999 September 1;160(3):893-8.

(66) Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, FitzGerald MX et al. Matrix metalloproteinase expression and production by

alveolar macrophages in emphysema. Am J Respir Crit Care Med 1997 July;156(1):240-7.

(67) Shapiro SD. The macrophage in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999 November;160(5 Pt 2):S29-S32.

(68) Tetley TD. New perspectives on basic mechanisms in lung disease. 6. Proteinase imbalance: its role in lung disease. Thorax 1993 May;48(5):560-5.

(69) Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. Science 1997 September 26;277(5334):2002-4.

(70) MacNee W, Rahman I. Oxidants and antioxidants as therapeutic targets in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999 November;160(5 Pt 2):S58-S65.

(71) Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. Environ Health Perspect 1985 December;64:111-26.:111-26.

(72) Renkema TE, Postma DS, Noordhoek JA, Sluiter HJ, Kauffman HF. Influence of in vivo prednisolone on increased in vitro O2- generation by neutrophils in emphysema. Eur Respir J 1993 January;6(1):90-5.

(73) Hampton MB, Kettle AJ, Winterbourn CC. Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing. Blood 1998 November 1;92(9):3007-17.

(74) Silverman EK, Chapman HA, Drazen JM, Weiss ST, Rosner B, Campbell EJ et al. Genetic epidemiology of severe, early-onset chronic obstructive pulmonary disease. Risk to relatives for airflow obstruction and chronic bronchitis. Am J Respir Crit Care Med 1998 June;157(6 Pt 1):1770-8.

(75) Joos L, He JQ, Shepherdson MB, Connett JE, Anthonisen NR, Pare PD et al. The role of matrix metalloproteinase polymorphisms in the rate of decline in lung function. Hum Mol Genet 2002 March 1;11(5):569-76.

(76) He JQ, Ruan J, Connett JE, Anthonisen NR, Pare PD, Sandford AJ. Antioxidant Gene Polymorphisms and Susceptibility to a Rapid Decline in Lung Function in Smokers. Am J Respir Crit Care Med 2002 August 1;166(3):323-8.

(77) Sakao S, Tatsumi K, Igari H, Shino Y, Shirasawa H, Kuriyama T. Association of Tumor Necrosis Factor {alpha} Gene Promoter Polymorphism with the Presence of Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2001 February 1;163(2):420-2.

(78) Molfino NA. Genetics of COPD. Chest 2004 May;125(5):1929-40.

(79) Matsuse T, Hayashi S, Kuwano K, Keunecke H, Jefferies WA, Hogg JC. Latent adenoviral infection in the pathogenesis of chronic airways obstruction. Am Rev Respir Dis 1992 July;146(1):177-84.

(80) Hogg JC. Latent adenoviral infection in the pathogenesis of emphysema: the Parker B. Francis Lectureship. Chest 2000 May;117(5 Suppl 1):282S-5S.

(81) Retamales I, Elliott WM, Meshi B, Coxson HO, Pare PD, Sciurba FC et al. Amplification of inflammation in emphysema and its association with latent adenoviral infection. Am J Respir Crit Care Med 2001 August 1;164(3):469-73.

(82) Wilkinson TMA, Donaldson GC, Johnston SL, Openshaw PJM, Wedzicha JA. Respiratory Syncytial Virus, Airway Inflammation, and FEV1 Decline in Patients with Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2006 April 15;173(8):871-6.

(83) Doll R, Peto R, Boreham J, Sutherland I. Mortality in relation to smoking: 50 years' observations on male British doctors. BMJ 2004 June 26;328(7455):1519.

(84) Fletcher CM. Standardised questionnaire on respiratory symptoms: a statement prepared and approved by the MRC Committee on the Aetiology of Chronic Bronchitis (MRC breathlessness score). BMJ 1960;2:1665.

(85) Bestall JC, Paul EA, Garrod R, Garnham R, Jones PW, Wedzicha JA. Usefulness of the Medical Research Council (MRC) dyspnoea scale as a measure of disability in patients with chronic obstructive pulmonary disease. Thorax 1999 July 1;54(7):581-6.

(86) Bailey PH. The dyspnea-anxiety-dyspnea cycle--COPD patients' stories of breathlessness: "It's scary /when you can't breathe". Qual Health Res 2004 July;14(6):760-78.

(87) Brenes GA. Anxiety and chronic obstructive pulmonary disease: prevalence, impact, and treatment. Psychosom Med 2003 November;65(6):963-70.

(88) Vestbo J, Lange P. Can GOLD Stage 0 provide information of prognostic value in chronic obstructive pulmonary disease? Am J Respir Crit Care Med 2002 August 1;166(3):329-32.

(89) de Leeuw PW, Dees A. Fluid homeostasis in chronic obstructive lung disease. Eur Respir J 2003 November 2;22(46_suppl):33S-40.

(90) Oudijk EJD, Lammers JWJ, Koenderman L. Systemic inflammation in chronic obstructive pulmonary disease. Eur Respir J 2003 November 2;22(46_suppl):5S-13.

(91) Jagoe RT, Engelen MPKJ. Muscle wasting and changes in muscle protein metabolism in chronic obstructive pulmonary disease. Eur Respir J 2003 November 2;22(46_suppl):52S-63.

(92) Couillard A, Prefaut C. From muscle disuse to myopathy in COPD: potential contribution of oxidative stress. Eur Respir J 2005 October 1;26(4):703-19.

(93) Scanlon PD, Connett JE, Waller LA, Alatose MD, Bailey WC, Sonia Buist A et al. Smoking Cessation and Lung Function in Mild-to-Moderate Chronic Obstructive Pulmonary Disease . The Lung Health Study. Am J Respir Crit Care Med 2000 February 1;161(2):381-90.

(94) Troosters T, Casaburi R, Gosselink R, Decramer M. Pulmonary Rehabilitation in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2005 July 1;172(1):19-38.

(95) Sapey E, Stockley RA. COPD exacerbations - 2: Aetiology. Thorax 2006 March 1;61(3):250-8.

(96) Spencer S, Jones PW. Time course of recovery of health status following an infective exacerbation of chronic bronchitis. Thorax 2003 July;58(7):589-93.

(97) BTS Guidelines for the Management of Chronic Obstructive Pulmonary Disease. Thorax 1997 December 1;52(90005):1S-28.

(98) Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F et al. Standardisation of the measurement of lung volumes. Eur Respir J 2005 September 1;26(3):511-22.

(99) MacIntyre N, Crapo RO, Viegi G, Johnson DC, van der Grinten CPM, Brusasco V et al. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. Eur Respir J 2005 October 1;26(4):720-35.

(100) Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R et al. Interpretative strategies for lung function tests. Eur Respir J 2005 November;26(5):948-68.

(101) Molyneux A. Nicotine replacement therapy. BMJ 2004 February 21;328(7437):454-6.

(102) Rennard SI. Treatment of stable chronic obstructive pulmonary disease. Lancet 2004 August 28;364(9436):791-802.

(103) Sestini P, Renzoni E, Robinson S, Poole P, Ram FS. Short-acting beta 2 agonists for stable chronic obstructive pulmonary disease. Cochrane Database Syst Rev 2002;(4):CD001495.

(104) O'Donnell D, Lam M, Webb K. Spirometric Correlates of Improvement in Exercise Performance after Anticholinergic Therapy in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 1999 August 1;160(2):542-9.

(105) Vincken W. Bronchodilator treatment of stable COPD: long-acting anticholinergics. European Respiratory Review 2005 September 1;14(94):23-31.

(106) Cook D, Guyatt G, Wong E, Goldstein R, Bedard M, Austin P et al. Regular Versus As-Needed Short-Acting Inhaled {beta}-Agonist Therapy for Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2001 January 1;163(1):85-90.

(107) Appleton S, Poole P, Smith B, Veale A, Bara A. Long-acting beta2-agonists for chronic obstructive pulmonary disease patients with poorly reversible airflow limitation. Cochrane Database Syst Rev 2002;(3):CD001104.

(108) Casaburi R, Mahler DA, Jones PW, Wanner A, San Pedro G, ZuWallack RL et al. A long-term evaluation of once-daily inhaled tiotropium in chronic obstructive pulmonary disease. Eur Respir J 2002 February 1;19(2):217-24.

(109) Dusser D, Bravo ML, Iacono P, on behalf the MISTRAL study group. The effect of tiotropium on exacerbations and airflow in patients with COPD. Eur Respir J 2006 March 1;27(3):547-55.

(110) Pauwels RA, Lofdahl CG, Laitinen LA, Schouten JP, Postma DS, Pride NB et al. Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. European Respiratory Society Study on Chronic Obstructive Pulmonary Disease. N Engl J Med 1999 June 24;340(25):1948-53.

(111) Vestbo J, Sorensen T, Lange P, Brix A, Torre P, Viskum K. Long-term effect of inhaled budesonide in mild and moderate chronic obstructive

pulmonary disease: a randomised controlled trial. Lancet 1999 May 29;353(9167):1819-23.

(112) Burge PS, Calverley PM, Jones PW, Spencer S, Anderson JA, Maslen TK. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: the ISOLDE trial. BMJ 2000 May 13;320(7245):1297-303.

(113) The Lung Health Study Research Group. Effect of Inhaled Triamcinolone on the Decline in Pulmonary Function in Chronic Obstructive Pulmonary Disease. N Engl J Med 2000 December 28;343(26):1902-9.

(114) Mahler DA, Wire P, Horstman D, Chang CN, Yates J, Fischer T et al. Effectiveness of Fluticasone Propionate and Salmeterol Combination Delivered via the Diskus Device in the Treatment of Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2002 October 15;166(8):1084-91.

(115) Calverley P, Pauwels R, Vestbo J, Jones P, Pride N, Gulsvik A et al. Combined salmeterol and fluticasone in the treatment of chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 2003 February 8;361(9356):449-56.

(116) Calverley PM, Boonsawat W, Cseke Z, Zhong N, Peterson S, Olsson H. Maintenance therapy with budesonide and formoterol in chronicobstructive pulmonary disease. Eur Respir J 2003 December 1;22(6):912-9.

(117) Jones PW, Willits LR, Burge PS, Calverley PMA. Disease severity and the effect of fluticasone propionate on chronic obstructive pulmonary disease exacerbations. Eur Respir J 2003 January 1;21(1):68-73.

(118) Morice AH, McGarvey L, Pavord I, on behalf of the British Thoracic Society Cough Guideline Group. Recommendations for the management of cough in adults. Thorax 2006 September 1;61(suppl_1):i1-24.

(119) Fletcher CM, Pride NB. Definitions of emphysema, chronic bronchitis, asthma, and airflow obstruction: 25 years on from the Ciba symposium. Thorax 1984 February;39(2):81-5.

(120) Badham C. An essay on bronchitis: with a supplement containing remarks on simple pulmonary abscess. Second ed. London: J Callow; 1814.

(121) Laennec R. A treatise on the diseases of the chest (trans. with a preface and notes by John Forbes). Second ed. London: TG Underwood; 1821.

(122) Petty TL. John Hutchinson's mysterious machine revisited. Chest 2002 May;121(5 Suppl):219S-23S.

(123) Oswald NC, Harold JT, Martin WJ. Clinical pattern of chronic bronchitis. Lancet 1953 September 26;265(6787):639-43.

(124) Bell ML, Davis DL. Reassessment of the lethal London fog of 1952: novel indicators of acute and chronic consequences of acute exposure to air pollution. Environ Health Perspect 2001 June;109 Suppl 3:389-94.:389-94.

(125) Committe on diagnostic standards for nontuberculous respiratory diseases ATS. Definitions and classification of chronic bronchitis, asthma, and pulmonary emphysema. Am Rev Respir Dis 1962;85:762-9.

(126) Burrows B, Fletcher CM, Heard BE, Jones NL, Wootliff JS. The emphysematous and bronchial types of chronic airways obstruction. A clinicopathological study of patients in London and Chicago. Lancet 1966 April 16;1(7442):830-5.

(127) Royal College of Physicians of London. Smoking and health : a report of the Royal College of Physicians [Committee on Smoking and Atmospheric Pollution] on smoking in relation to cancer of the lung and other diseases. London: Pitman Medical; 1962.

(128) Burrows B, Niden AH, Barclay WR, Kasik JE. Chronic obstructive lung disease II. Relationship of clinical and physiologic findings to the severity of airways obstruction. Am Rev Respir Dis 1965 May;91:665-78.

(129) Kauffmann F, Drouet D, Lellouch J, Brille D. Twelve years spirometric changes among Paris area workers. Int J Epidemiol 1979 September;8(3):201-12.

(130) Higgins MW, Keller JB, Becker M, Howatt W, Landis JR, Rotman H et al. An index of risk for obstructive airways disease. Am Rev Respir Dis 1982 February;125(2):144-51.

(131) Peto R, Speizer FE, Cochrane AL, Moore F, Fletcher CM, Tinker CM et al. The relevance in adults of air-flow obstruction, but not of mucus hypersecretion, to mortality from chronic lung disease. Results from 20 years of prospective observation. Am Rev Respir Dis 1983 September;128(3):491-500.

(132) Annesi I, Kauffmann F. Is respiratory mucus hypersecretion really an innocent disorder? A 22-year mortality survey of 1,061 working men. Am Rev Respir Dis 1986 October;134(4):688-93.

(133) Speizer FE, Fay ME, Dockery DW, Ferris BG, Jr. Chronic obstructive pulmonary disease mortality in six U.S. cities. Am Rev Respir Dis 1989 September;140(3 Pt 2):S49-S55.

(134) Lange P, Nyboe J, Appleyard M, Jensen G, Schnohr P. Relation of ventilatory impairment and of chronic mucus hypersecretion to mortality from obstructive lung disease and from all causes. Thorax 1990 August;45(8):579-85.

(135) Lange P, Nyboe J, Appleyard M, Jensen G, Schnohr P. Ventilatory function and chronic mucus hypersecretion as predictors of death from lung cancer. Am Rev Respir Dis 1990 March;141(3):613-7.

(136) Roberts CM, Lowe D, Bucknall CE, Ryland I, Kelly Y, Pearson MG. Clinical audit indicators of outcome following admission to hospital with acute

exacerbation of chronic obstructive pulmonary disease. Thorax 2002 February 1;57(2):137-41.

(137) Prescott E, Lange P, Vestbo J. Chronic mucus hypersecretion in COPD and death from pulmonary infection. Eur Respir J 1995 August;8(8):1333-8.

(138) Vestbo J, Prescott E, Lange P. Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. Am J Respir Crit Care Med 1996 May;153(5):1530-5.

(139) Sherman CB, Xu X, Speizer FE, Ferris BG, Jr., Weiss ST, Dockery DW. Longitudinal lung function decline in subjects with respiratory symptoms. Am Rev Respir Dis 1992 October;146(4):855-9.

(140) Vestbo J, Rasmussen FV. Respiratory symptoms and FEV1 as predictors of hospitalization and medication in the following 12 years due to respiratory disease. Eur Respir J 1989 September;2(8):710-5.

(141) Monso E, Rosell A, Bonet G, Manterola J, Cardona PJ, Ruiz J et al. Risk factors for lower airway bacterial colonization in chronic bronchitis. Eur Respir J 1999 February;13(2):338-42.

(142) Sethi S, Murphy TF. Bacterial Infection in Chronic Obstructive Pulmonary Disease in 2000: a State-of-the-Art Review. Clin Microbiol Rev 2001 April 1;14(2):336-63.

(143) Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. Am J Med 2000 September;109(4):288-95.

(144) Sethi S, Muscarella K, Evans N, Klingman KL, Grant BJB, Murphy TF. Airway Inflammation and Etiology of Acute Exacerbations of Chronic Bronchitis. Chest 2000 December 1;118(6):1557-65.

(145) Doring G, Hoiby N. Early intervention and prevention of lung disease in cystic fibrosis: a European consensus. J Cyst Fibros 2004 June;3(2):67-91.

(146) Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ, Wedzicha JA. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2000 May;161(5):1608-13.

(147) Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. Thorax 2000 February;55(2):114-20.

(148) Stanescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM et al. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. Thorax 1996 March;51(3):267-71.

(149) Kanner RE, Anthonisen NR, Connett JE. Lower respiratory illnesses promote FEV(1) decline in current smokers but not ex-smokers with mild chronic obstructive pulmonary disease: results from the lung health study. Am J Respir Crit Care Med 2001 August 1;164(3):358-64.

(150) Reid L. Pathology of chronic bronchitis. Lancet 1954 February 6;266(6806):274-8.

(151) Reid L. Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis. Thorax 1960 June;15:132-41.

(152) Glynn A, Michaels L. Bronchial biopsy in chronic bronchitis and asthma. Thorax 1960;15:142-53.

(153) Matsuba K, Thurlbeck WM. Disease of the small airways in chronic bronchitis. Am Rev Respir Dis 1973 April;107(4):552-8.

(154) Thurlbeck WM, Angus GE. A Distribution curve for chronic bronchitis. Thorax 1964 September;19:436-42.:436-42.

(155) Mullen JB, Wright JL, Wiggs BR, Pare PD, Hogg JC. Structure of central airways in current smokers and ex-smokers with and without mucus

hypersecretion: relationship to lung function. Thorax 1987 November;42(11):843-8.

(156) Saetta M, Di SA, Turato G, Facchini FM, Corbino L, Mapp CE et al. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998 March;157(3 Pt 1):822-6.

(157) Zhu J, Majumdar S, Qiu Y, Ansari T, Oliva A, Kips JC et al. Interleukin-4 and interleukin-5 gene expression and inflammation in the mucus-secreting glands and subepithelial tissue of smokers with chronic bronchitis. Lack of relationship with CD8(+) cells. Am J Respir Crit Care Med 2001 December 15;164(12):2220-8.

(158) Kohri K, Ueki IF, Nadel JA. Neutrophil elastase induces mucin production by ligand-dependent epidermal growth factor receptor activation. Am J Physiol Lung Cell Mol Physiol 2002 September;283(3):L531-L540.

(159) Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. J Immunol 1999 May 15;162(10):6233-7.

(160) Widdicombe JH, Bastacky SJ, Wu DX, Lee CY. Regulation of depth and composition of airway surface liquid. Eur Respir J 1997 December;10(12):2892-7.

(161) Kim KC, McCracken K, Lee BC, Shin CY, Jo MJ, Lee CJ et al. Airway goblet cell mucin: its structure and regulation of secretion. Eur Respir J 1997 November;10(11):2644-9.

(162) Leikauf GD, Borchers MT, Prows DR, Simpson LG. Mucin apoprotein expression in COPD. Chest 2002 May;121(5 Suppl):166S-82S.

(163) Rose MC, Voynow JA. Respiratory Tract Mucin Genes and Mucin Glycoproteins in Health and Disease. Physiol Rev 2006 January 1;86(1):245-78.

(164) Hovenberg HW, Davies JR, Carlstedt I. Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. Biochem J 1996 August 15;318(Pt 1):319-24.

(165) Wickstrom C, Davies JR, Eriksen GV, Veerman EC, Carlstedt I. MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem J 1998 September 15;334(Pt 3):685-93.

(166) Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF et al. Epidermal growth factor system regulates mucin production in airways. Proc Natl Acad Sci U S A 1999 March 16;96(6):3081-6.

(167) Miyata T, Kai H, Isohama Y, Takahama K. Current opinion of muco-active drug research: strategies and problems. Eur Respir J 1998 February;11(2):480-91.

(168) Poole PJ, Black PN. Oral mucolytic drugs for exacerbations of chronic obstructive pulmonary disease: systematic review. BMJ 2001 May 26;322(7297):1271-4.

(169) Poole PJ, Black PN. Mucolytic agents for chronic bronchitis or chronic obstructive pulmonary disease. Cochrane Database Syst Rev 2003;(2):CD001287.

(170) Decramer M, Rutten-van MM, Dekhuijzen PN, Troosters T, van HC, Pellegrino R et al. Effects of N-acetylcysteine on outcomes in chronic obstructive pulmonary disease (Bronchitis Randomized on NAC Cost-Utility Study, BRONCUS): a randomised placebo-controlled trial. Lancet 2005 April 30;365(9470):1552-60.

(171) Bennett WD. Effect of beta-adrenergic agonists on mucociliary clearance. J Allergy Clin Immunol 2002 December;110(6 Suppl):S291-S297.

(172) Riese DJ, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 1998 January;20(1):41-8.

(173) Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J Biol Chem 1962 May;237:1555-62.:1555-62.

(174) Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer 2001 March;8(1):11-31.

(175) Fischer OM, Hart S, Gschwind A, Ullrich A. EGFR signal transactivation in cancer cells. Biochem Soc Trans 2003 December;31(Pt 6):1203-8.

(176) Bazley LA, Gullick WJ. The epidermal growth factor receptor family. Endocr Relat Cancer 2005 July;12 Suppl 1:S17-27.:S17-S27.

(177) Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000 July 3;19(13):3159-67.

(178) Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. Curr Opin Cell Biol 1999 April;11(2):184-9.

(179) Wells A. EGF receptor. Int J Biochem Cell Biol 1999 June;31(6):637-43.

(180) Seals DF, Courtneidge SA. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 2003 January 1;17(1):7-30.

(181) Sunnarborg SW, Hinkle CL, Stevenson M, Russell WE, Raska CS, Peschon JJ et al. Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. J Biol Chem 2002 April 12;277(15):12838-45.

(182) Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J et al. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol 2004 March 1;164(5):769-79.

(183) Yan Y, Shirakabe K, Werb Z. The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. J Cell Biol 2002 July 22;158(2):221-6.

(184) Lemjabbar H, Basbaum C. Platelet-activating factor receptor and ADAM10 mediate responses to Staphylococcus aureus in epithelial cells. Nat Med 2002 January;8(1):41-6.

(185) Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T et al. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. Nat Med 2002 January;8(1):35-40.

(186) Suzuki M, Raab G, Moses MA, Fernandez CA, Klagsbrun M. Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. J Biol Chem 1997 December 12;272(50):31730-7.

(187) Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I, Ortiz RM. Shedding light on ADAM metalloproteinases. Trends Biochem Sci 2005 July;30(7):413-22.

(188) Vermeer PD, Einwalter LA, Moninger TO, Rokhlina T, Kern JA, Zabner J et al. Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. Nature 2003 March;%20;422(6929):322-6.

(189) Kheradmand F, Werb Z. Shedding light on sheddases: role in growth and development. Bioessays 2002 January;24(1):8-12.

(190) Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC et al. An essential role for ectodomain shedding in mammalian development. Science 1998 November 13;282(5392):1281-4.

(191) Sundell HW, Gray ME, Serenius FS, Escobedo MB, Stahlman MT. Effects of epidermal growth factor on lung maturation in fetal lambs. Am J Pathol 1980 September;100(3):707-25.

(192) Gray TE, Guzman K, Davis CW, Abdullah LH, Nettesheim P. Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. Am J Respir Cell Mol Biol 1996 January;14(1):104-12.

(193) Guzman K, Randell SH, Nettesheim P. Epidermal growth factor regulates expression of the mucous phenotype of rat tracheal epithelial cells. Biochem Biophys Res Commun 1995 December 14;217(2):412-8.

(194) Kelly SM, Hunter JO. Epidermal growth factor stimulates synthesis and secretion of mucus glycoproteins in human gastric mucosa. Clin Sci (Lond) 1990 November;79(5):425-7.

(195) Perrais M, Pigny P, Copin MC, Aubert JP, Van S, I. Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. J Biol Chem 2002 August 30;277(35):32258-67.

(196) Polosa R, Prosperini G, Leir SH, Holgate ST, Lackie PM, Davies DE. Expression of c-erbB receptors and ligands in human bronchial mucosa. Am J Respir Cell Mol Biol 1999 May;20(5):914-23.

(197) Takeyama K, Fahy JV, Nadel JA. Relationship of epidermal growth factor receptors to goblet cell production in human bronchi. Am J Respir Crit Care Med 2001 February;163(2):511-6.

(198) Burgel PR, Escudier E, Coste A, Dao-Pick T, Ueki IF, Takeyama K et al. Relation of epidermal growth factor receptor expression to goblet cell hyperplasia in nasal polyps. J Allergy Clin Immunol 2000 October;106(4):705-12.

(199) Aida S, Tamai S, Sekiguchi S, Shimizu N. Distribution of epidermal growth factor and epidermal growth factor receptor in human lung: immunohistochemical and immunoelectron-microscopic studies. Respiration 1994;61(3):161-6.

(200) Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST et al. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. FASEB J 2000 July;14(10):1362-74.

(201) O'Donnell RA, Richter A, Ward J, Angco G, Mehta A, Rousseau K et al. Expression of ErbB receptors and mucins in the airways of long term current smokers. Thorax 2004 December;59(12):1032-40.

(202) Takeyama K, Jung B, Shim JJ, Burgel PR, Dao-Pick T, Ueki IF et al. Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. Am J Physiol Lung Cell Mol Physiol 2001 January;280(1):L165-L172.

(203) Gosset P, Lassalle P, Vanhee D, Wallaert B, Aerts C, Voisin C et al. Production of tumor necrosis factor-alpha and interleukin-6 by human alveolar macrophages exposed in vitro to coal mine dust. Am J Respir Cell Mol Biol 1991 November;5(5):431-6.

(204) Arnold R, Werner F, Humbert B, Werchau H, Konig W. Effect of respiratory syncytial virus-antibody complexes on cytokine (IL-8, IL-6, TNF-alpha) release and respiratory burst in human granulocytes. Immunology 1994 June;82(2):184-91.

(205) Arnold R, Humbert B, Werchau H, Gallati H, Konig W. Interleukin-8, interleukin-6, and soluble tumour necrosis factor receptor type I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. Immunology 1994 May;82(1):126-33.

(206) Borchers MT, Wert SE, Leikauf GD. Acrolein-induced MUC5ac expression in rat airways. Am J Physiol 1998 April;274(4 Pt 1):L573-L581.

(207) Deshmukh HS, Case LM, Wesselkamper SC, Borchers MT, Martin LD, Shertzer HG et al. Metalloproteinases mediate mucin 5AC expression by epidermal growth factor receptor activation. Am J Respir Crit Care Med 2005 February 15;171(4):305-14.

(208) Goldkorn T, Balaban N, Matsukuma K, Chea V, Gould R, Last J et al. EGF-Receptor Phosphorylation and Signaling Are Targeted by H2O2 Redox Stress. Am J Respir Cell Mol Biol 1998 November 1;19(5):786-98.

(209) Rosette C, Karin M. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. Science 1996 November 15;274(5290):1194-7.

(210) Richter A, O'Donnell RA, Powell RM, Sanders MW, Holgate ST, Djukanovic R et al. Autocrine ligands for the epidermal growth factor receptor mediate interleukin-8 release from bronchial epithelial cells in response to cigarette smoke. Am J Respir Cell Mol Biol 2002 July;27(1):85-90.

(211) Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 1999 December 23;402(6764):884-8.

(212) Chen N, Ma WY, She QB, Wu E, Liu G, Bode AM et al. Transactivation of the epidermal growth factor receptor is involved in 12-O-tetradecanoylphorbol-13-acetate-induced signal transduction. J Biol Chem 2001 December 14;276(50):46722-8.

(213) Shao MX, Ueki IF, Nadel JA. Tumor necrosis factor alpha-converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. Proc Natl Acad Sci U S A 2003 September 30;100(20):11618-23.

(214) Shao MX, Nakanaga T, Nadel JA. Cigarette smoke induces MUC5AC mucin overproduction via tumor necrosis factor-alpha-converting enzyme in human airway epithelial (NCI-H292) cells. Am J Physiol Lung Cell Mol Physiol 2004 August;287(2):L420-L427.

(215) Lemjabbar H, Li D, Gallup M, Sidhu S, Drori E, Basbaum C. Tobacco smoke-induced lung cell proliferation mediated by tumor necrosis factor

alpha-converting enzyme and amphiregulin. J Biol Chem 2003 July 11;278(28):26202-7.

(216) Gensch E, Gallup M, Sucher A, Li D, Gebremichael A, Lemjabbar H et al. Tobacco smoke control of mucin production in lung cells requires oxygen radicals AP-1 and JNK. J Biol Chem 2004 September 10;279(37):39085-93.

(217) Zhang Z, Oliver P, Lancaster JR, Jr., Schwarzenberger PO, Joshi MS, Cork J et al. Reactive oxygen species mediate tumor necrosis factor alpha-converting, enzyme-dependent ectodomain shedding induced by phorbol myristate acetate. FASEB J 2001 February;15(2):303-5.

(218) Nadel JA. Role of Neutrophil Elastase in Hypersecretion During COPD Exacerbations, and Proposed Therapies. Chest 2000 May 1;117(90052):386S-389.

(219) Nadel JA. Mechanisms of airway hypersecretion and novel therapy. Chest 2000 May;117(5 Suppl 1):262S-6S.

(220) Voynow JA, Young LR, Wang Y, Horger T, Rose MC, Fischer BM. Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. Am J Physiol 1999 May;276(5 Pt 1):L835-L843.

(221) Takeyama K, Dabbagh K, Jeong SJ, Dao-Pick T, Ueki IF, Nadel JA. Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. J Immunol 2000 February 1;164(3):1546-52.

(222) Fischer BM, Voynow JA. Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. Am J Respir Cell Mol Biol 2002 April;26(4):447-52.

(223) Mueller SG, Paterson AJ, Kudlow JE. Transforming growth factor alpha in arterioles: cell surface processing of its precursor by elastases. Mol Cell Biol 1990 September;10(9):4596-602.

(224) Shao MX, Nadel JA. Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-alpha-converting enzyme. J Immunol 2005 September 15;175(6):4009-16.

(225) Datta R, Yoshinaga K, Kaneki M, Pandey P, Kufe D. Phorbol ester-induced generation of reactive oxygen species is protein kinase cbeta - dependent and required for SAPK activation. J Biol Chem 2000 December 29;275(52):41000-3.

(226) Aarbiou J, Verhoosel RM, Van WS, De Boer WI, Van Krieken JH, Litvinov SV et al. Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro. Am J Respir Cell Mol Biol 2004 February;30(2):193-201.

(227) Fischer BM, Cuellar JG, Diehl ML, deFreytas AM, Zhang J, Carraway KL et al. Neutrophil elastase increases MUC4 expression in normal human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 2003 April 1;284(4):L671-L679.

(228) Kuwahara I, Lillehoj EP, Hisatsune A, Lu W, Isohama Y, Miyata T et al. Neutrophil elastase stimulates MUC1 gene expression through increased Sp1 binding to the MUC1 promoter. Am J Physiol Lung Cell Mol Physiol 2005 August;289(2):L355-L362.

(229) Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K et al. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. Am J Physiol Lung Cell Mol Physiol 2001 January;280(1):L134-L140.

(230) Dohrman A, Miyata S, Gallup M, Li JD, Chapelin C, Coste A et al. Mucin gene (MUC 2 and MUC 5AC) upregulation by Gram-positive and Gram-negative bacteria. Biochim Biophys Acta 1998 April 28;1406(3):251-9.

(231) Burgel PR, Lazarus SC, Tam DC, Ueki IF, Atabai K, Birch M et al. Human eosinophils induce mucin production in airway epithelial cells via

epidermal growth factor receptor activation. J Immunol 2001 November 15;167(10):5948-54.

(232) Vargaftig BB, Singer M. Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR. Am J Physiol Lung Cell Mol Physiol 2003 October;285(4):L808-L818.

(233) Doxycycline hyclate. In: Dollery C, editor. Therapeutic Drugs. 2nd ed. Edinburgh, UK: Churchill Livingstone; 1999. p. D229-D232.

(234) Smith GN, Jr., Mickler EA, Hasty KA, Brandt KD. Specificity of inhibition of matrix metalloproteinase activity by doxycycline: relationship to structure of the enzyme. Arthritis Rheum 1999 June;42(6):1140-6.

(235) Boyle JR, McDermott E, Crowther M, Wills AD, Bell PR, Thompson MM. Doxycycline inhibits elastin degradation and reduces metalloproteinase activity in a model of aneurysmal disease. J Vasc Surg 1998 February;27(2):354-61.

(236) Sepper R, Konttinen YT, Ding Y, Takagi M, Sorsa T. Human neutrophil collagenase (MMP-8), identified in bronchiectasis BAL fluid, correlates with severity of disease. Chest 1995 June;107(6):1641-7.

(237) Keeling WB, Armstrong PA, Stone PA, Bandyk DF, Shames ML. An overview of matrix metalloproteinases in the pathogenesis and treatment of abdominal aortic aneurysms. Vasc Endovascular Surg 2005 November;39(6):457-64.

(238) Perlstein TS, Lee RT. Smoking, metalloproteinases, and vascular disease. Arterioscler Thromb Vasc Biol 2006 February;26(2):250-6.

(239) Elkington PT, Friedland JS. Matrix metalloproteinases in destructive pulmonary pathology. Thorax 2006 March;61(3):259-66.

(240) Sorsa T, Tjaderhane L, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. Oral Dis 2004 November;10(6):311-8.

(241) Murphy G, Knauper V, Atkinson S, Butler G, English W, Hutton M et al. Matrix metalloproteinases in arthritic disease. Arthritis Res 2002;4 Suppl 3:S39-49. Epub;%2002 May 9.:S39-S49.

(242) Schlesinger C, Koss MN. The organizing pneumonias: an update and review. Curr Opin Pulm Med 2005 September;11(5):422-30.

(243) Parks WC. Matrix metalloproteinases in lung repair. Eur Respir J Suppl 2003 September;44:36s-38s.:36s-8s.

(244) Selman M, Pardo A. The epithelial/fibroblastic pathway in the pathogenesis of idiopathic pulmonary fibrosis. Am J Respir Cell Mol Biol 2003 September;29(3 Suppl):S93-S97.

(245) Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. Am J Respir Cell Mol Biol 2003 January;28(1):12-24.

(246) Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA, Rifkin BR. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. Crit Rev Oral Biol Med 1991;2(3):297-321.

(247) Smith GN, Jr., Brandt KD, Hasty KA. Activation of recombinant human neutrophil procollagenase in the presence of doxycycline results in fragmentation of the enzyme and loss of enzyme activity. Arthritis Rheum 1996 February;39(2):235-44.

(248) Reddy MS, Geurs NC, Gunsolley JC. Periodontal host modulation with antiproteinase, anti-inflammatory, and bone-sparing agents. A systematic review. Ann Periodontol 2003 December;8(1):12-37.

(249) Baxter BT, Pearce WH, Waltke EA, Littooy FN, Hallett JW, Jr., Kent KC et al. Prolonged administration of doxycycline in patients with small asymptomatic abdominal aortic aneurysms: report of a prospective (Phase II) multicenter study. J Vasc Surg 2002 July;36(1):1-12.

(250) Moyes E, Kershaw R. Long-continued treatment with tetracycline and prednisolone in chronic bronchitis; a controlled trial. Lancet 1957 December 14;273(7007):1187-91.

(251) Francis RS, May JR, Spicer CC. Chemotherapy of bronchitis. Influence of penicillin and tetracycline administered daily, or intermittently for exacerbations. A report to the Research Committee of the British Tuberculosis Association by its Bronchitis Subcommittee. Br Med J 1961 October 14;2(5258):979-85.

(252) Sepper R, Prikk K, Tervahartiala T, Konttinen YT, Maisi P, Lopes-Otin C et al. Collagenase-2 and -3 are inhibited by doxycycline in the chronically inflamed lung in bronchiectasis. Ann N Y Acad Sci 1999 June 30;878:683-5.:683-5.

(253) Coffey RJ, Jr., Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL et al. Production and auto-induction of transforming growth factor-alpha in human keratinocytes. Nature 1987 August 27;328(6133):817-20.

(254) Barnard JA, Graves-Deal R, Pittelkow MR, DuBois R, Cook P, Ramsey GW et al. Auto- and cross-induction within the mammalian epidermal growth factor- related peptide family. J Biol Chem 1994 September 9;269(36):22817-22.

(255) Marquardt H, Hunkapiller MW, Hood LE, Todaro GJ. Rat transforming growth factor type 1: structure and relation to epidermal growth factor. Science 1984 March 9;223(4640):1079-82.

(256) Adam R, Drummond DR, Solic N, Holt SJ, Sharma RP, Chamberlin SG et al. Modulation of the receptor binding affinity of amphiregulin by modification of its carboxyl terminal tail. Biochim Biophys Acta 1995 April 6;1266(1):83-90.

(257) Jones JT, Akita RW, Sliwkowski MX. Binding specificities and affinities of egf domains for ErbB receptors. FEBS Letters 1999 March 26;447(2-3):227-31.

(258) Neelam B, Richter A, Chamberlin SG, Puddicombe SM, Wood L, Murray MB et al. Structure-function studies of ligand-induced epidermal growth factor receptor dimerization. Biochemistry 1998 April 7;37(14):4884-91.

(259) Lax I, Johnson A, Howk R, Sap J, Bellot F, Winkler M et al. Chicken epidermal growth factor (EGF) receptor: cDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. Mol Cell Biol 1988 May;8(5):1970-8.

(260) Fan H, Derynck R. Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. EMBO J 1999 December 15;18(24):6962-72.

(261) Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ et al. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 1998 September 11;435(1):39-44.

(262) Hamilton LM, Torres-Lozano C, Puddicombe SM, Richter A, Kimber I, Dearman RJ et al. The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma. Clin Exp Allergy 2003 February;33(2):233-40.

(263) Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, Rennard SI. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. Am J Respir Crit Care Med 1997 May 1;155(5):1770-6.

(264) Berger JT, Voynow JA, Peters KW, Rose MC. Respiratory Carcinoma Cell Lines . MUC Genes and Glycoconjugates. Am J Respir Cell Mol Biol 1999 March 1;20(3):500-10.

(265) Zuhdi Alimam M, Piazza FM, Selby DM, Letwin N, Huang L, Rose MC. Muc-5/5ac Mucin Messenger RNA and Protein Expression Is a Marker of Goblet Cell Metaplasia in Murine Airways. Am J Respir Cell Mol Biol 2000 March 1;22(3):253-60.

(266) Shao MX, Nadel JA. Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. Proc Natl Acad Sci U S A 2005 January 18;102(3):767-72.

(267) Lee DC, Sunnarborg SW, Hinkle CL, Myers TJ, Stevenson MY, Russell WE et al. TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase. Arın N Y Acad Sci 2003 May;995:22-38.:22-38.

(268) Basbaum C, Li D, Gensch E, Gallup M, Lemjabbar H. Mechanisms by which gram-positive bacteria and tobacco smoke stimulate mucin induction through the epidermal growth factor receptor (EGFR). Novartis Found Symp 2002;248:171-6; discussion 176-80, 277-82.:171-6.

(269) Baselga J, Mendelsohn J, Kim YM, Pandiella A. Autocrine Regulation of Membrane Transforming Growth Factor-alpha Cleavage. J Biol Chem 1996 February 9;271(6):3279-84.

(270) DeWitt AE, Dong JY, Wiley HS, Lauffenburger DA. Quantitative analysis of the EGF receptor autocrine system reveals cryptic regulation of cell response by ligand capture. J Cell Sci 2001 June;114(Pt 12):2301-13.

(271) Sweeney C, Fambrough D, Huard C, Diamonti AJ, Lander ES, Cantley LC et al. Growth factor-specific signaling pathway stimulation and gene expression mediated by ErbB receptors. J Biol Chem 2001 June 22;276(25):22685-98.

(272) Crovello CS, Lai C, Cantley LC, Carraway KL, III. Differential signaling by the epidermal growth factor-like growth factors neuregulin-1 and neuregulin-2. J Biol Chem 1998 October 9;273(41):26954-61.

(273) Sweeney C, Carraway KL, III. Ligand discrimination by ErbB receptors: differential signaling through differential phosphorylation site usage. Oncogene 2000 November;%20;19(49):5568-73.

(274) Chokki M, Mitsuhashi H, Kamimura T. Metalloprotease-dependent amphiregulin release mediates tumor necrosis factor-alpha-induced IL-8

secretion in the human airway epithelial cell line NCI-H292. Life Sci 2006 May 22;78(26):3051-7.

(275) Polosa R, Puddicombe SM, Krishna MT, Tuck AB, Howarth PH, Holgate ST et al. Expression of c-erbB receptors and ligands in the bronchial epithelium of asthmatic subjects. J Allergy Clin Immunol 2002 January;109(1):75-81.

(276) Vernooy JH, Kucukaycan M, Jacobs JA, Chavannes NH, Buurman WA, Dentener MA et al. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. Am J Respir Crit Care Med 2002 November 1;166(9):1218-24.

(277) Itoh Y, Joh T, Tanida S, Sasaki M, Kataoka H, Itoh K et al. IL-8 promotes cell proliferation and migration through metalloproteinase-cleavage proHB-EGF in human colon carcinoma cells. Cytokine 2005 March 21;29(6):275-82.

(278) Koivunen AL, Maisi P, Konttinen YT, Prikk K, Sandholm M. Collagenolytic activity and its sensitivity to doxycycline inhibition in tracheal aspirates of horses with chronic obstructive pulmonary disease. Acta Vet Scand 1997;38(1):9-16.

(279) Busse WW, Wanner A, Adams K, Reynolds HY, Castro M, Chowdhury B et al. Investigative Bronchoprovocation and Bronchoscopy in Airway Diseases. Am J Respir Crit Care Med 2005 October 1;172(7):807-16.