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MAST CELL CHYMASE AS A MEDIATOR OF INFLAMMATION IN THE HUMAN AIRWAYS

BY

RODOLPHE HERVÉ

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ABSTRACT
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The chymotryptic serine protease chymase is an abundant cellular constituent of a major subset of human mast cells. Several substrates have been identified, though its mediator actions are not well defined. Relatively little is known of the distribution of chymase-containing cells in the airways, or on the actions of chymase on the airway epithelium. The aims of this study have been to examine the potential involvement of chymase in inflammatory conditions of the respiratory tract, and in particular to investigate the actions of chymase on cells of the airway epithelium.

Sequentially cut thin sections of bronchial tissues from patients with cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD), or from control tissues, were stained immunohistochemically for mast cell chymase and tryptase, as well as for neutrophil and eosinophil markers. The numbers of chymase- and tryptase-containing cells, as well as those of neutrophils were increased in tissues from CF patients compared with the two other groups of patients, whereas eosinophil counts were little different between groups. Chymase-containing cells were identified throughout the tissues, with large numbers present in the vicinity of glands in all three groups. The proportion of mast cells that contained chymase was increased in airways from CF patients, and particularly in the epithelial layer.

Recombinant human prochymase was produced using a baculovirus expression system, isolated to high purity using heparin-agarose column chromatography and gel filtration, and activated with dipeptidyl peptidase I (DPPI). In addition chymase was purified by similar methods from high salt extracts of human skin tissue. Addition of either form of chymase to 16HBE 14o- epithelial cells stimulated within 3h marked increases in expression of mRNA for IL-6, IL-8, and GM-CSF, as detected by reverse transcription-polymerase chain reaction (RT-PCR). This was followed by increased secretion of IL-6 (maximal at 6h), IL-8 and to some extent GM-CSF (both maximal at 24h). This was not reflected in increased cell numbers, and the rate of proliferation of epithelial cells was actually reduced at the higher concentrations. Chymase appeared to degrade IL-8 to a small extent, though not Il-6 or GM-CSF. The concentrations of chymase employed in this study (10 to 40mU/ml) did not induce cytotoxicity. The actions of chymase were in all cases inhibited by heat inactivation or by addition of the selective chymase inhibitor Y-40018, or chymostatin. Preincubating the cells with a p38α kinase inhibitor, SB203580, reduced markedly the release of IL-6 and IL-8 from 16HBE 14o- cells, suggesting that p38α may be involved in the regulation of cytokine release in these cells.

Chymase at 10 to 40mU/ml induced a progressive dose-related increase in transepithelial electrical resistance (TER) across monolayers of 16HBE 14o- cells grown on inserts. Higher concentrations (up to 160mU/ml) resulted in a dramatic shrinking of the cells, the appearance of gaps in the monolayers, and eventually in detachment of the cells from the side of the inserts. These changes did not appear to alter expression of the tight junction protein, occludin in immunocytochemistry. An inhibitor of protein kinase C (staurosporine) did not significantly alter the response to chymase, while an inhibitor of PI3 kinase (wortmannin) accentuated the increase in TER induced by chymase at 12h, 18h, and 24h. PD 98059, an inhibitor of MEK, was also capable of inducing an increase in TER. The actions of chymase on epithelial monolayers were inhibited by heat inactivation or by addition of a protease inhibitor. Preincubation of the monolayers with pertussis toxin did not prevent the increase in TER induced by chymase, and addition of chymase to epithelial cells did not stimulate calcium flux detected spectrofluorometrically. This would suggest that the effects observed were not mediated by a G protein-coupled receptor.

Chymase could play a key role in inflammation in the airway epithelium, particularly in CF, and could represent an attractive target for therapeutic intervention.

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À mes filleuls, Camille et Noam.

ABBREVIATIONS

AAPFpNA	*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide
Ab	antibody
AEC	3-amino-9-ethyl carazole
Ala	alanine
APRT	adenine phosphoribosyltransferase
APS	ammonium persulphate
Arg	arginine
BAL	bronchoalveolar lavage
BAPNA	*N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride
Bp	base pair
BSA	bovine serum albumin
CCR-3	CC chemokine receptor 3
C3a, C5a	complement component 3a, 5a
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CF	cystic fibrosis
CFTR	CF transmembrane conductance regulator
CGRP	calcium gene-related peptide
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DFP	diisopropyl fluorophosphate
DMEM	Dulbecco-modified Eagle's medium
DMSO	dimethylsulphoxide
DPPI	dipeptidyl peptidase I
EGF	epidermal growth factor
ERK	extracellular signal-related kinase
FCS	foetal calf serum
FEV1	forced expiratory volume in one second
FITC	fluorescein isothiocyanate
Gly	glycine
G-CSF	granulocyte colony stimulating factor

GM-CSF	granulocyte-macrophage colony stimulating factor
H_1, H_2, H_3, H_4	histamine receptor type 1, 2, 3, 4
HBSS	Hank's balanced salt solution
HRP	horseradish peroxidase
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ITS	insulin-selenium-transferrin
kDa	kilodalton
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LT	leukotriene
Lys	lysine
MAP	mitogen-activated protein
MC_T	mast cell containing tryptase, but not chymase
MC_{TC}	mast cell containing tryptase and chymase
MEK	extracellular signal-related kinase-activating kinase
MEM	minimum essential medium
MES	(2-[N-morpholino]) ethanesulfonic acid
MIP-1 & -2	macrophage inflammation protein-1 & -2
MMP	matrix metalloproteinase
MOPS	(3-[N-morpholino]) propanesulfonic acid
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa B
OD	optical density
PAF	platelet activating factor
PAR	protease-activated receptor
PBS	phosphate buffered saline
PEG	polyethylene glycol
PKC	protein kinase C
PTX	pertussis toxin

TEMED	N,N,N',N' tetramethylethylenediamine
PG	prostaglandin
RANTES	regulated on activation, normal T-cell expressed and secreted
SBTI	soybean trypsin inhibitor
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	standard error
Ser	serine
TER	transepithelial electrical resistance
TGF	transforming growth factor
TNF	tumour necrosis factor
Tris-HCl	tris[hydroxymethyl] aminomethane hydrochloryde
Val	valine
VCAM-1	vascular cell adhesion molecule-1
VIP	vasoactive intestinal peptide
ZIGPFM	Z-Ile-Glu-Pro-Phe-CO_2Me

Chapter 1

General Introduction

1 General Introduction

Mast cells can play a key role in the regulation of allergic and inflammatory processes. Besides their contribution to hypersensitivity reactions through the cross-linking of IgE antibodies on specific surface receptors (FcεRI), mast cells can be activated also by non IgE-dependent processes. On activation, mast cells can release a number of important mediators of inflammation, amongst which neutral proteases are found in high quantities. Chymase-containing mast cells have been reported to be prominent in connective tissue, and it has been suggested that there may be increased numbers of this mast cell subtype in certain diseases such as cystic fibrosis. The aim of the present study has been to examine the potential role of mast cell chymase as a mediator of inflammation, and in particular to determine its role in inflammatory conditions of the lung such as cystic fibrosis, chronic obstructive pulmonary disease and bronchial asthma.

1.1 Inflammation

Inflammation involves a cascade of cellular and molecular interactions leading to physiological alterations in the affected tissues. It may be triggered by injury, infection, or other noxious stimuli such as air pollutants and allergens. Different cells may be involved, depending on the nature of the stimulus. The activated cells secrete a number of chemical mediators, including cytokines, which in turn attract and/or activate other inflammatory cells. The immediate consequences of inflammation are dilation and increased permeability of local blood vessels, leading to increased local blood flow and leakage of fluid.

Although inflammation is an essential defence mechanism against pathogens, the fine balance regulating these processes may be altered in a number of chronic inflammatory diseases. Inflammatory mediators such as cytokines and proteases released by mast cells may contribute to prolong the inflammation, increase tissue damage and alter tissue repair processes. This may lead to tissue remodelling and loss of structure and function, as may be seen in chronically inflamed airways. Mast cells, which are widely distributed throughout most tissues, are likely to have a key role in these inflammatory processes.

1.2 The Mast Cell

The term mast cell is derived from the German "mastzellen", meaning well fed cell, for Ehrlich, who first identified mast cells (Erhlich, 1878), thought the large granules contained in their cytoplasm were phagocytic vacuoles. It was shown later that these granules in fact contain pre-formed mediators that may be released by mast cells upon activation. Mast cells are found throughout the body, and are particularly abundant in mucosal tissues and at external interfaces with the environment such as the respiratory and gastrointestinal tracts and skin. Mast cells can release a range of potent mediators of inflammation (Fig. 1.1).

Mast cells possess on their surface FcεRI receptors that bind IgE molecules with high affinity, and are the key effector cells in the allergic response, in which mast cell degranulation is triggered by the cross-linking of two receptors. Neuropeptides such as substance P (SP), which are contained in some sensory neurones, are also able to stimulate mast cell degranulation in skin (Foreman et al., 1982 & 1983; Fewtrell et al., 1982). Lung mast cells have been reported to be unaffected by such neuropeptides. Other mechanisms can lead to mast cell activation. The complement components C3a and C5a can induce mast cell degranulation

in skin tissue (Hartman & Glovsky, 1981; Swerlick et al., 1986), although this has not been shown with lung mast cells (Schulman et al., 1988). Other stimuli include certain eosinophil granule proteins (O'Donnell et al., 1983), histamine-releasing factors (Guranowski et al., 1981), and some muscle relaxants and analgesics (Moss and Rosow, 1983; Stellato et al., 1991).

Mast cells and their mediators are emerging as important initiators and regulators of inflammatory processes, and appear to take part in a number of pathologies involving chronic tissue damage and remodelling.

1.2.1 Origin and Differentiation

Mast cells are derived from CD34-positive progenitor cells in the bone marrow (Bressler et al., 1990; Kirshenbaum et al., 1991). Stem cell factor (SCF) appears to be the main growth factor involved in the differentiation of these stem cells (Irani et al., 1992; Valent et al., 1992; Kirshenbaum et al., 1992), which express the surface receptor c-*kit* (also known as CD117) (Geissler et al., 1988; Chabot et al., 1988). Some cytokines including IL-3, IL-4 and IL-10, have also been shown to stimulate growth and differentiation of mast cells (Rennick et al., 1985; Martin et al., 1990; Thompson-Snipes et al., 1991; Kirshenbaum et al., 1992; Eklund et al., 1997). It is not clear if all human mast cells have one or more different precursors before they migrate into the tissue. If all mast cells are issued from a single lineage, final differentiation may occur before reaching the site of activity, or could rely on local stimuli (Irani et al., 1987; Caughey et al., 1988). The different hypotheses are still subject to debate. It is generally accepted that mature mast cells eventually fall into two main subtypes based on their content of neutral proteases.

1.2.2 Mast Cell Heterogeneity

Mast cells subtypes may be defined on the basis of their protease content (Irani, 1986). Mast cells of the MC_{TC} subtype contain both tryptase and chymase, whereas in MC_T cells tryptase is found but not chymase (Schwartz et al., 1987). The two subtypes have also been described in different parts of the body. It appears that over 90 % of dispersed mast cells from human lung are of the MC_T subtype (Schwartz et al., 1987). In contrast, mast cells found in the human skin are predominantly of the MC_{TC} subtype (Irani et al., 1986). According to these observations, MC_{TC} cells appear to be preferentially located in connective tissues, whereas the MC_T subtype would be predominant in mucosal tissue (table 1.1). It was reported recently that MC_{TC} cells express the surface receptor CCR3, making them susceptible to CCR3-binding chemokines such as eotaxin and RANTES (Romagnani et al., 1999). It is not clear if these chemokines are involved in mast cell differentiation, but they may contribute to the migration of mast cells to sites of inflammation. Recent studies have suggested that all mast cells in culture could express chymase, provided they are stimulated by the right cytokines, particularly IL-4 (Toru et al., 1998; Ahn et al., 2000).

Further differences between the two mast cell subtypes have been reported. Carboxypeptidase, another mast cell protease isolated and characterised from skin mast cell preparations (Goldstein et al., 1987 & 1989), has been detected by immunohistochemistry only in the MC_{TC} subtype (Irani et al., 1991). Similarly, cathepsin G has been reported only in MC_{TC} mast cells (Schechter et al., 1990). Differences in the content of certain other mediators have also been reported in nasal mucosal and bronchial mucosal biopsies, where IL-4 is present in both mast cell subtypes (preferentially in the MC_{TC} subset), whereas Il-5, IL-6, and TNF-α are found only in MC_T cells (Bradding et al., 1993 & 1995).

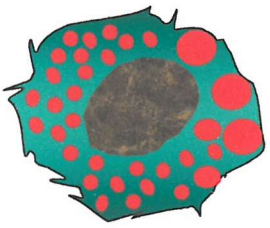
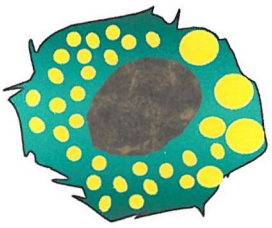
	MC_T	MC_{TC}
		
Protease content	Tryptase	Tryptase Chymase Carboxypeptidase Cathepsin G
Predominant distribution in normal tissue	Mucosal tissue	Connective tissue

Table 1.1 The MC_T and MC_{TC} mast cell subtypes

Interestingly, functional heterogeneity of mast cells appears to be unrelated to their protease content. The response of mast cells to different stimuli of degranulation appears to be related mainly to the tissue of origin. IgE-cross-linking can induce various degrees of histamine release in the different mast cell subtypes (Lowman et al., 1988; Kaminer et al., 1995). In contrast, neuropeptides such as substance P have been reported to stimulate skin mast cells only (Foreman et al., 1982 & 1983; Fewtrell et al., 1982).

The role of the different mast cell subsets in inflammation is not clear. Immunohistological studies demonstrated the accumulation of mast cells in the synovial tissues of patients with rheumatoid arthritis (RA). The

MC_T subtype appears to predominate in these tissues (Tetlow and Woolley, 1995a and 1995b), and elevated levels of tryptase have been reported in the synovial fluid of patients with RA, indicative of activation (Buckley et al., 1997). An immunohistochemical study carried out on a large number of patients with atherosclerotic lesions of the carotid arteries demonstrated that mast cells are found in higher numbers during the onset of inflammation (Jeziorska et al., 1997). This study also indicated that the ratio of MC_{TC} cells that represents approximately 80 to 90% of mast cells detected in normal tissues was transiently reduced to 60 to70 % in early atherosclerotic lesions (types I-III), and increased again to nearly 100% in later stages (types IV-VI) presenting with neovascularization, calcification, and thrombi. Experimental models also suggest the possibility of transient alterations in mast cell phenotype during an inflammatory response. In a murine model, transient alterations in the expression of tryptase and chymase have been described during the course of parasitic infection (Friend et al., 1998).

Different mast cell subsets have the potential to fulfil different roles, depending on the presence or absence of chymase and other mediators. Furthermore, these cells may be subject to a number of further internal and external regulatory processes. Close interactions with their environment may alter protease and cytokine content, and these alterations may be critical in disease.

1.2.3 Mast Cell Mediators

Mast cell granules contain a range of prestored and newly formed mediators released upon IgE dependent or non-immunological activation (Fig. 1.1). The major products of mast cell activation are reviewed here.

1.2.3.1 Proteases

The proteases of human mast cells so far described include chymase, a major protease of MC_{TC} cells, which will be described in detail in a separate section. Tryptase appears to be present in all mast cells in higher quantities than any other mast cell product. Other proteases, carboxypeptidase and cathepsin G, have been reported to be stored exclusively in MC_{TC} cells (Irani et al., 1991; Schechter et al., 1990). Proteases are emerging as major mediators of altered cell function in inflammatory diseases. However, the role of chymase in inflammation of the airways has received little attention.

1.2.3.1a Tryptase

Tryptase is a tetrameric serine protease, with a molecular weight of about 110 to 130 kDa. It was first purified and characterised from dispersed human lung mast cells (Schwartz et al., 1981). Tryptase, which is present in all mast cells of the lung, skin, and small intestine (Schwartz et al., 1987), has proved valuable as a specific marker for human mast cells (Walls et al., 1990). It is composed of four subunits which all have one catalytic site, though tryptase appears to have little enzymatic activity if not in the tetrameric form. Tryptase may be stabilised in the tetrameric form by binding with heparin or other proteoglycans (Schwartz and Bradford, 1986).

There are a number of alleles encoding for tryptase, but it appears that only some of these are actively transcribed, whereas others are pseudogenes (Caughey et al., 2000; Pallaoro et al., 1999; Wong et al., 1999). Genes corresponding to known mast cell tryptases are all located on the short arm of chromosome 16 (Daniels et al., 2001; Pallaoro et al., 1999). Bacterial artificial chromosomes have been used to restriction-map

tryptase genes, and locate them by fluorescence in situ hybridisation (FISH) (Caughey; 2001). These studies revealed the presence of three loci transcribed into active tryptase, one accepting the α and ßI alleles, the second accepting the ßII and ßIII alleles, and the third encoding for γ tryptase. The allelic relationship between α and βI genes could account for α-tryptase deficiency in some individuals (Soto et al., 2002), though the pathophysiological significance is not known. The α-tryptase appears to be released constitutively in its pro-form, while the β-tryptases are stored in secretory granules and released in their active form upon mast cell degranulation. The γ-tryptase is a membrane-bound enzyme stored in secretory granules, which becomes exposed on the surface of degranulating mast cells (Wong et al., 2002). Genomic sequencing has identified other tryptase-like genes designated δ- and ε-tryptase. The gene for δ-tryptase has been found in a variety of human tissues, and the translated product has been detected in the colon, lung, and inflamed synovium (Wang et al., 2002). The last isoform reported to date, ε-tryptase, is a product of foetal lung epithelial cells.

Differences in substrate specificity between α- and β-tryptases have been reported, which appear to be due in part to a single amino-acid difference located at the active site (Huang et al., 1999). Analysis of purified lung and skin mast cell lysates by 2D gel electrophoresis and subsequent Western blotting revealed multiple forms of tryptase with major differences in size, charge, and variable glycosylation, and the purified secreted tryptase isoforms showed differences in K_m and k_{cat} values for a range of chromogenic substrates (Peng et al., 2003). The classification of tryptases might be subject to further alterations in the future.

Human skin tryptase has been shown to degrade fibronectin, leading to dermal-epidermal separation in vitro (Kaminska et al., 1999). Tryptase also induces the accumulation of neutrophils and eosinophils *in vitro* by up-regulating expression of adhesion molecules such as ICAM-1 and stimulating the release of interleukin-8 from H292 cells (Cairns &

Walls, 1996). Injected into the skin of guinea pigs or into the peritoneum of mice, tryptase induces the infiltration and accumulation of neutrophils and eosinophils at the site of injection (Walls et al., 1995; He et al., 1997). An *in vitro* model using human umbilical vein endothelial cells showed that tryptase can up-regulate mRNA for IL-8 as well as for IL-1β, and stimulate the release of IL-8 in these cells (Compton et al., 1998). It has been reported that tryptase can stimulate the synthesis of type I collagen in the MRC-5 fibroblast cell line (Cairns & Walls, 1997).

The mechanism whereby tryptase can act on specific cellular targets is not clear. However the identification of four protease-activated receptors (PARs) may provide a clue. These G protein-coupled transmembrane receptors are activated by cleavage of a distinct Arg/Ser bond located on the extracellular N-terminal extension of the receptor (Hollenberg, 1996; Déry et al., 1998; Bahou & Schmidt, 1996). Tryptase has been found to activate PAR-2, thrombin targets PAR-1, PAR-3, and PAR-4, and trypsin can activate PAR-2 and PAR-4.

The recent finding that mast cell tryptase may alter epithelial permeability and induce cytokine release in the bronchial epithelium (Perng et al., in press) calls to attention the potential for other mast cell neutral proteases to take part in similar processes.

1.2.3.1b Carboxypeptidase

With a molecular weight of about 34.7 kDa (Goldstein et al., 1987 & 1989), carboxypeptidase has been reported only in MC_{TC} cells (Irani et al., 1991). It is released bound to the same proteoglycans as chymase. It seems that chymase and carboxypeptidase are stored in the same complex within the secretory granules they share with tryptase (Goldstein et al., 1992). Mast cell carboxypeptidase shares some enzymatic activity with pancreatic carboxypeptidase. Its substrates include Leu-enkephalin,

neurotensin, and kinetensin (Goldstein et al., 1991), but in contrast with the pancreatic enzyme, it does not cleave des-Arg bradykinin or substance P.

1.2.3.1c Cathepsin G

The presence of a cathepsin G-like protease in MC_{TC} cells has been demonstrated by Schechter et al (Schechter et al., 1990). Cathepsin G is also present in neutrophils and monocytes. Mast cell cathepsin G has a molecular weight of about 30kDa, as demonstrated by Western blotting. Its chymotryptic activity may be inhibited by aprotinin and by an anti-cathepsin G antibody, and accounts for about 30% of the total chymotryptic activity found in MC_{TC} cells of the human skin (Nilsson & Schwartz, 1995).

1.2.3.2 Histamine

Histidine is taken up passively in mast cells and basophils and converted to histamine by histamine decarboxylase (Schayer, 1963; Bauza & Lagunoff, 1981). Histamine is then stored in secretory granules, ionically bound to proteoglycans, and released upon degranulation. It was one of the first mast cell mediators to be identified (Riley & West, 1956) and thus it has been extensively studied. Histamine is a potent vasodilator and the mast cell has been considered for some time to be simply the key cell in allergic responses and anaphylaxis. Once released, histamine is dissociated from proteoglycans and metabolised within minutes (Schayer et al., 1959), hence its effect is limited to the vicinity of degranulating mast cells. At least four histamine receptors have been reported so far. The H_1-receptor mediates bronchoconstriction, increased permeability and microvascular leakage (Foreman et al., 1983), and activation of sensory

reflexes. The H_2-receptor is involved in vasodilation (Robertson & Greaves, 1978) and mucus secretion. Histamine can also modulate nonadrenergic noncholinergic neural bronchoconstriction through the H_3-receptor (Ichinose & Barnes, 1989). Histamine acts on endothelial cells, stimulating the expression of P-selectin, platelet activating factor (PAF; Lorant et al., 1991), and the secretion of LTB_4 and PGI_2. Histamine also induces the release of IL-6 (Delneste et al., 1994) and IL-8 (Jeannin et al., 1996) from endothelial cells, and therefore may also be involved in the late phase response observed in allergic inflammation.

1.2.3.3 Proteoglycans

Proteoglycans are single-chain proteins with glycosaminoglycan side chains (Stevens et al., 1988). Heparin proteoglycans and chondroitin sulphate E proteoglycans are found in mast cells, associated with proteases, histamine, and acid hydrolases (Stevens et al., 1988; Thompson et al., 1988). Chondroitin sulphate E proteoglycans have also been reported in cultured human macrophages (Koiset et al., 1986). Heparin proteoglycans are essential in stabilising tryptase in its active tetrameric form (Schwartz & Bradford, 1986), but due to their high negative charge, they also remain bound to the other neutral proteases after degranulation.

In addition of stabilising endogenous enzymes of mast cells, proteoglycans have a range of other biological functions. Heparin is a well-known anticoagulant (Damus et al., 1973), and has been reported to have anti-inflammatory properties, regulating histamine activity and acting as an antagonist of bradykinin and prostaglandin E (Carr, 1979).

1.2.3.4 Lipid mediators

Mast cell lipid mediators are derived from arachidonic acid. This is a 20-carbon polyunsaturated fatty acid, which is metabolised through the cyclooxygenase pathway to prostaglandins (PGs) and thromboxanes, or through the lipoxygenase pathway to leukotrienes (LTs) and lipoxins. PGD_2 is the main prostaglandin to be generated after mast cell activation by IgE cross-linking to surface receptors or artificial stimulation *in vitro* by calcium ionophore. PGD_2 can induce smooth muscle contraction (Hardy et al., 1984), vasodilation and increased capillary permeability (Wasserman et al., 1977), and can also alter neutrophil function (Goetzl et al., 1980). Human lung mast cells also produce LTC_4 and LTB_4 (MacGlashan et al., 1982; Peters et al., 1984). LTC_4, the main leukotriene produced by mast cells, is a potent bronchoconstrictor (Dahlen et al., 1980) and can increase microvascular permeability (Dahlen et al., 1981). LTB_4 stimulates leukocyte adhesion and migration (Ford-Hutchinson et al., 1980; Dahlen et al., 1981) and act as a neutrophil chemoattractant in human skin (Soter et al., 1983).

1.2.3.5 Cytokines

A range of cytokines is produced by mast cells. Rodent mast cells have been shown to contain IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IFN-γ, TGF-β, granulocyte-macrophage colony stimulating factor (GMCSF), macrophage inflammatory protein 1-α and 1-β (MIP1-α and 1-β), and T-cell activation antigen-3 (TLA-3) (Burd et al., 1989; Plaut et al., 1989; Gordon et al., 1990; Seder et al., 1991).

Human mast cells have been shown to express IL-4, IL-5, IL-6 (Bradding et al., 1993; Okayama et al., 1995), IL-8 (Moller et al., 1993), IL-13 (Burd et al., 1995; Jaffe et al., 1996), CD40 ligand (Gauchat et al.,

1993), and TNF-α (Walsh et al., 1991; Ohkawara et al., 1992; Bradding et al., 1994). This latter can induce the upregulation of ICAM-1 and VCAM-1 on human endothelial cells (Meng et al., 1995), hence promoting the migration of further inflammatory cells to the site of inflammation (Galli & Costa, 1995). More cytokines are likely to be reported in mast cells in the future, and the resulting direct effects and amplification signals resulting from their release indicate once more that mast cells are at the centre of inflammatory processes.

1.2.3.6 Platelet Activating Factor (PAF)

PAF is a family of low molecular weight phospholipids (Demopoulos et al., 1979), of which the 1-acyl form has been identified in lung mast cell preparations (Triggiani et al., 1990). However, the classification of PAF as a mast cell mediator is subject to controversy, since it shows rapid reuptake from the cells.

1.3 Chymase

1.3.1 Biochemical Properties

Chymase is the principal enzyme accounting for the chymotryptic activity of MC_{TC} cells (Wintroub et al., 1986), though it has been less extensively studied than tryptase. Human chymase is a monomeric serine endopeptidase of about 30 kDa and was first purified and characterised from human skin, (Schechter et al., 1983 & 1986). Chymase activation involves the cleavage of prochymase by dipeptidyl peptidase I (DPPI; McEuen et al., 1998a). The action of DPPI is inhibited by heparin and histamine, suggesting that chymase activation occurs at early stages of

vesicle maturation, when concentrations of heparin and histamine are low and pH close to neutrality (McEuen et al., 1998a). Chymase is stored fully active, though activity is limited at the low pH (5.5) in the secretory granules (McEuen et al., 1995). It appears to be bound to the same proteoglycans as carboxypeptidase (Goldstein et al., 1992), and is released upon IgE-dependent activation. An average content of 4.5pg of chymase per mast cell were measured by radioimmunoassay in human adult foreskin (Schwartz et al., 1987).

Mammalian mast cell chymases have been divided into two phylogenetic groups, α- and β-chymase, based on structural alignments (Caughey, 1995). Only α-chymase has been reported in dog and primates, including humans, whereas both α- and β-chymases are present in rodents (reviewed by Caughey, 2001). The encoding gene for chymase has been located on chromosome 14 (Caughey et al., 1991; Urata et al., 1990). Some polymorphisms have been reported and have been associated with various conditions such as hypertrophic cardiomyopathy (Pfeufer et al., 1996), atopic eczema, and atopic asthma (Mao et al., 1998; Tanaka et al., 1999). A murine model suggested that IL-10 might regulate chymase expression at a post-transcriptionnal level by stabilisation of the mRNA transcript (Xia et al., 1996). Two distinct isoforms of chymase with different affinity for heparin have been isolated from human mast cell preparations (McEuen et al., 1998b). These are likely to reflect polymorphic variations. Heparin is not essential for chymase activity, though it may contribute to prolong the half-life of chymase after mast cell degranulation (Sayama et al., 1987). The actions of many products of mast cell activation on various cell types have been studied, but little is known of the ability of chymase to alter the behaviour of surrounding cells.

1.3.2 Actions of Chymase

Chymase has a chymotrypsin-like activity profile. Its activity may be measurable by the cleavage of the synthetic peptides such as *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPFpNA). In contrast with tryptic enzymes, no specific cellular receptors have been reported yet for chymase or other chymotryptic enzymes. A number of physiological molecules have been identified as substrates of chymase on the basis of *in vitro* and *in vivo* studies.

Human chymase has been shown to convert angiotensin I to angiotensin II approximately fourfold more efficiently than angiotensin converting enzyme (Reilly et al., 1982; Wintroub et al., 1984; Urata et al., 1990). Human skin chymase can cleave the epidermal-dermal junction of human skin *in vitro* (Briggaman et al., 1984), suggesting that chymase may be involved in tissue remodelling processes. The potential action of human chymase as a mediator of tissue remodelling in the human lung is not known.

Chymase can convert the precursors of IL-1β and SCF to their active forms (Mizutani et al., 1991; Longley et al., 1997), and has been shown to degrade IL-4 (Tunon de Lara et al., 1994). Chymase may also mediate inflammatory processes by acting on other cells. Chymase induces the release of latent TGF-β1 from the extracellular matrix of cultured human umbilical vein endothelial cells and amniotic epithelial cells (Taipale et al., 1995). The activity of recombinant human chymase as a potent stimulus for human monocyte and neutrophil recruitment has been demonstrated in a microchemotaxis chamber (Tani et al., 2000). The response of human fibroblasts to thrombin can be inhibited by pretreating the cells with human chymase, suggesting that at least PAR-1 may be susceptible to hydrolysis by chymase (Schechter et al., 1998). The effect of human chymase as a mediator of cytokine release in other tissues where it is present, such as the lung, remains unknown.

Various sources of animal chymase have been employed for *in vitro* studies. The closest known relatives of human chymase, besides that from primates, are dog chymase and mouse chymase 5 (Caughey et al., 1991; McNeil et al., 1991), and for this reason these have been used in a number of experimental models. Dog mast cell chymase (but not tryptase) stimulates the secretion of mucus in cultured bovine serous cells (Sommerhoff et al., 1989; Nadel, 1991). This action of chymase does not seem to involve any transduction mechanism via an intracellular second messenger signal, (Sommerhoff et al., 1996), suggesting that chymase does not bind to a specific surface receptor on these cells. Dog chymase has also been reported to degrade the neuropeptides substance P and VIP (Caughey et al., 1988), and to activate the precursors of the matrix metalloproteinases (MMP) collagenase (MMP1), and stromelysin (MMP3) (Lees et al., 1994).

Chymase from rat peritoneal mast cells has been reported to digest the constituents of the extracellular matrix, fibronectin and vitronectin, in cultures of the rat thyroid cell line FRTL-5 (Banovak & De Forteza, 1992). Rat chymase 2 has been shown to affect the distribution of tight junction proteins ZO-1 and occludin, thus altering the permeability of canine kidney epithelial cell monolayers (Scudamore et al., 1998). However, rodents possess a number of different chymotryptic enzymes with potentially different substrates, and which are susceptible to induce different responses *in vivo*, so these data must be taken with caution, particularly with rat chymase, which shares little homology with the human chymase (Caughey et al., 1995).

The effect of purified human chymase was studied to some extent in animal models. The activity of human skin chymase as a potent stimulus for neutrophil and eosinophil accumulation was demonstrated in the skin of guinea pigs and in the peritoneum of mice (He and Walls, 1998), indicating that chymase could itself contribute to the amplification

of the inflammatory response *in vivo*. Specific inhibitors of chymase have been shown to suppress IgE-dependent degranulation of suspended skin mast cells, suggesting that chymase could be involved in initial amplification of mast cell degranulation (He et al., 1999b).

1.3.3 Inhibitors of Chymase Activity

The activity of human chymase can be inhibited by intracellular and circulating inhibitors of serine proteases, α_1-antichymotrypsin, α_1-protease inhibitor, and α_2-macroglobulin (Sayama et al., 1987; Schechter et al., 1989). Rapid inactivation of chymase activity has been reported in the skin after allergen challenge. This inactivation is associated with the release of histamine, and could be due to the presence of chymase inhibitors in the mast cell itself (Saarinen et al., 2001). A study of chymase isolated from hamster cheek pouch tissues revealed the presence of a 10kDa molecular weight protein in a complex with chymase, capable of inhibiting the enzyme (Takao et al., 2001).

A number of protease inhibitors of non-mammalian origin such as chymostatin, soybean trypsin inhibitor (SBTI), or pharmaceutical compounds may also be used to inhibit chymase (Aoyama et al., 2000a & 2000b). The specific chymase inhibitor Z-Ile-Glu-Pro-Phe-CO_2Me (ZIGPFM; Bastos et al., 1995) is about 3 times more potent than SBTI and 10 times more potent than chymostatin as an inhibitor of chymase activity as assessed by the IC_{50} values (He et al., 1999b). Other inhibitors include L-tosylamide-2-phenylethyl chloromethyl ketone, diisopropyl fluorophosphate, and peptide boronic acid inhibitors. Suramin, an anti-cancer drug, has been found to have an inhibitory action on the activity of purified human chymase *in vitro* (Takao et al., 1999). More recently, nonpeptidic heterocycles have been developed that show better bioavailability in animal models, and increased selectivity for chymase

over other proteases, especially chymotrypsin, (Akahoshi et al., 2001a&b). Specific and non-toxic inhibitors of chymase may become important pharmacological compounds in the future.

Chymase-containing mast cells are abundant cellular constituents of the bronchial submucosa, and mast cells may even be found within the airway epithelium or in the lumen. Activation of mast cells could have the potential to alter epithelial function.

1.4 The Airway Epithelium

1.4.1 General Description

The bronchial epithelium acts as a barrier between the external environment and the underlying tissues. It contributes also a great deal to airway function by allowing the diffusion and active transport of ions and molecules, ensuring the clearance of airways thanks to mucus secretion and ciliary function (particularly in the large airways). A number of pharmacologically potent mediators are also secreted that can regulate muscle tone or stimulate the accumulation of inflammatory cells (Martin et al., 1997; Polito and Proud, 1998). The structure and function of the bronchial epithelium may be altered by exogenous or endogenous stimuli, such as air pollutants, allergens, pathogens, and certain inflammatory mediators.

The role of mast cell proteases has been little studied in inflammatory conditions of the lung, though mast cells have been described in great numbers in those tissues. The findings that tryptase may alter epithelial permeability and induce cytokine release from cultured bronchial epithelial cells (Perng et al., in press) strongly indicate that activation of mast cells has the potential to alter epithelial function.

1.4.2 Structure of the Airway Epithelium

At least 22 distinct cell types have been identified in the airways of mammals, of which 13 are found in the epithelium (Breeze & Wheeldon, 1977). Columnar ciliated cells are the most numerous cells found along the bronchial epithelium. Mucus secreting goblet cells are found amongst ciliated cells along the respiratory tract, though in smaller numbers, and these tend to decrease in numbers in the peripheral airways (Mercer et al., 1994). In large airways, the epithelium appears pseudostratified with the ciliated cells adjacent to basal cells, which are attached to the basement membrane. The epithelium is reduced to a single layer of ciliated and secretory goblet cells in terminal bronchioles. Other secretory cells include serous cells and clara cells, the latter being found in terminal bronchioles (Widdicombe and Pack, 1982).

Epithelial integrity is assured by multiple intercellular connecting structures (Fig 1.2). The barrier properties are mainly due to the presence of tight junctions joining the cells near their apical side (Godfrey et al., 1992 & 1997). Cells are further held together by occludens junctions containing the adhesion molecule E-cadherin, located below tight junctions, and they are attached to basal cells by desmosomes (Montefort et al., 1993). Hemidesmosomes containing integrins attach basal cells to the basement membrane (Stepp et al., 1990). Integrins are linked to the cytoskeleton via actin filaments. Diesel exhaust particles (DEPs) have been reported to alter the link between actin and the extracellular matrix, suggesting that they could facilitate the detachment of airway epithelial cells (Doornaert et al., 2003).

1.4.3 Permeability of the Airway Epithelium

The airway epithelium acts as a physical barrier between the airway lumen and underlying tissues. Alterations in permeability allow selective exchange of fluid, ions, hydrophilic molecules and cells between the two environments. There are two pathways involved in these exchanges. The cellular route is used by some ions and molecules, which diffuse passively or are taken actively across the cell membrane, are transported through the cell and released on the other side. This route is usually characterised by a certain specificity of the channels or vesicles involved. The paracellular route involves the diffusion of fluid, ions, molecules, or the migration of inflammatory cells between epithelial cells. As mentioned earlier, tight junctions located on the apico-lateral border of epithelial cells have a major role in regulating the paracellular exchanges. The ultrastructure and morphology of human airway tight junctions have been investigated by freeze electron microscopy, and measurements of strand number, junctional depth and indexes of junctional complexity revealed considerable morphological variation between individual tight junctions (Elia et al., 1988; Carson et al., 1990; Godfrey et al., 1992).

1.4.3.1 Molecular structure of tight junctions

Tight junctions are made of several proteins, which are organised, in a functional structure termed the apical junction complex. Four transmembrane proteins are involved in the assembly of tight junctions, occludin, claudin-1 and claudin-2 (Furuse et al., 1998), and junctional adhesion molecule (JAM; Martìn-Padura et al., 1998). These proteins are responsible for the extracellular structure of tight junctions, forming seals and channels between epithelial cells.

Occludin is a 65kDa protein with four transmembrane domains, a large C-terminal cytosolic domain and two extracellular loops. It is found in tight junctions of epithelial and endothelial cells (Furuse et al., 1993). Occludin expression correlates with the number of tight junction fibrils observed in monolayers of MDCK cells, and upregulation of occludin expression results in increased TER (Fujimoto, 1995), and an increase in tight junction strands (McCarthy et al., 1996). Binding of a peptide to occludin extracellular loop 2, but not loop 1, results in reduced TER and increased permeability to molecules up to 40 kDa (Wong and Gumbiner, 1997). This indicates that occludin is essential for creating tight junctions and has an important part in maintaining epithelial permeability. However, a more recent study has reported that embryonic stem cells deficient in occludin by gene depletion through homologous recombination, were able to establish tight junctions. These tight junctions contain fibrils that are able to form a barrier to low molecular weight tracers (Saitou et al., 1998), suggesting the possible existence of another protein with functions similar to occludin.

Two intracellular membrane-associated guanylate kinase (MAGUK) proteins, ZO-1 and ZO-2 (zonula occludens 1 and 2), connect occludin to the cytoplasmic plaque (Stevenson et al., 1986; Gumbiner et al., 1991; Anderson et al., 1995; Beatch et al., 1996). It is believed that ZO-1 could act as an organisational scaffold, anchoring occludin molecules into the membrane, thus allowing lateral occludin-occludin interactions to create a stable structure and promote further polymerisation of occludin into linear fibrils (Mitic and Anderson, 1998). Other proteins such as cingulin, symplekin, 7H6 and ZA1TJ have been associated with tight junctions, and could play a role in their assembly and regulation. A specific antibody directed against E-cadherin can induce a fall in transepithelial electrical resistance (TER) of monolayers of the 16HBEo- cell line, especially when this antibody binds to E-cadherin located at the apical side, in the vicinity of tight junctions, suggesting the requirement of

E-cadherin for the formation and maintenance of these junctions (West et al., 2002).

A number of molecules involved in intracellular signalling pathways may also play a role in the building up of tight junctions. These include G proteins, phospholipase C, protein kinase C (PKC; Mulin et al., 1986 and 1988; Karczewski and Groot, 2000), calmodulin (Balda et al., 1991), and the GTPase Rab13 (Marzesco et al., 2002).

1.4.3.2 Alterations in epithelium permeability

Under normal conditions, permeability results in a difference in electrical potential between the apical and basolateral sides of the epithelium. This is due to the selective transport of ions through the cells and through tight junctions (Claude, 1978). The epithelium thus acts as a barrier limiting the migration of ions, and transepithelial electrical resistance (TER) can be measured *in vitro* with specially designed electrodes to assess the permeability, and thus the integrity, of an epithelium. Some structural changes can also be observed using fluorescence-labelled antibodies and confocal laser microscopy.

Many factors have been reported to alter the permeability of the airway epithelium. Chemical mediators or physical damage may result in a temporary loss of permeability and leakage between the lumen and the subepithelial compartments, a situation that may be seen in lung inflammatory conditions. Inflammatory cell mediators have been reported to modulate epithelial permeability In the lung, pertussis toxin (PTX) can induce cytoskeletal reorganization, seemingly via p38 MAP kinase activation, resulting in a decrease in TER (Garcia et al., 2002). The cytokines IL-1β, TNF-α, and IFN-γ can alter the function of tight junctions, a response that can be blocked by inhibitors of protein kinase C (Coyne et al., 2002).

In the 16HBEo- cell line, hydrogen peroxide has been found to decrease TER (Chapman et al., 2002), and other oxidants or cationic proteins (poly-L-arginine) also increase permeability, as well as airway responsiveness (Hulsmann et al., 1996). Rhinovirus infection has been shown to increase the permeability of cultured human tracheal epithelium (Ohrui et al., 1998), and common allergens such as Der p1, which is found in house dust mite faecal pellets (HDMFP), have the same effect (Herbert et al., 1990 & 1995). Der p1 is a cysteine peptidase capable of cleaving tight junctions (Wan et al., 1999), a property shared by other serine peptidases found in HDMFP (Wan et al., 2001). It is probable that the alterations in epithelium integrity and transepithelial resistance observed in infections are partly caused by the resulting inflammatory response.

Interestingly, trypsin is capable of altering the epithelial barrier function (Lewis et al., 1995). The action of proteolytic allergens and trypsin calls attention to the potential of endogenous proteases such as neutral proteases released by activated mast cells to induce similar effects *in vivo*. Indeed mast cell mediators may be involved in alterations of epithelial structure and function. Histamine has been reported to increase airway permeability *in vivo* (Braude et al., 1984; Chan et al., 1987), although it had no effect on epithelial monolayers *in vitro* (Devalia et al., 1994). It was suggested that the increase in permeability observed with patients could be due to the increase in submucosal hydrostatic pressure, which causes the opening of tight junctions. Recently, human mast cell tryptase was shown to alter the permeability of epithelial monolayers (Perng et al., in press). The effect of mast cell chymase on epithelial structure and function in the lung remains unknown.

1.4.4 Inflammatory Mediators Produced by Epithelial Cells

The airway epithelium can mediate inflammatory events in response to environmental insults such as injury and infection (Holgate et al., 1999). Epithelial cells can generate a range of cytokines including Il-1, IL-3, Il-5, IL-6, IL-8, IL-10, IL-11, RANTES, GM-CSF, G-CSF, MCP-1, TNFα, TGF_{β}, and also various other mediators such as PGE_2, $PGF_{2\alpha}$, endothelin, nitric oxide, and oxygen radicals (Cromwell et al., 1992; Knobil and Jacoby, 1998; Park and Wallace, 1998). Some cytokines (e.g. GM-CSF, IL-8, and TNFα) are capable of specifically attracting and activating inflammatory cells such as macrophages, neutrophils and eosinophils. It is believed that further release of cytokines contributes to maintaining the epithelium in a pro-inflammatory state, and is responsible for recurrent chronic inflammation, tissue damage and remodelling of the airways. Early increases in the expression of IL-1β, IL-6 and TNFα have been reported following exposure to airborne particles (Finkelstein et al., 1997). King et al. (1998) reported that house dust mite cysteine and serine proteolytic allergens, Der p1 and Der p9, respectively, induce concentration-dependent increases in the release of IL-6, IL-8 and GM-CSF as well as an increase in the expression of IL-6 mRNA in the BEAS-2B cell line. This again calls to attention the potential for mast cell proteases to induce similar responses from epithelial cells.

Epithelial cells can express adhesion molecules that allow the migration of inflammatory cells from the capillaries. Intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin (Ig) superfamily, is expressed constitutively by the airway epithelium. Its expression can be induced by cytokines such as IFN-γ and TNF-α (Bloemen et al., 1993; Look et al., 1992; Tosi et al., 1992), by infection with parainfluenza virus (Tosi et al., 1992), and to a small extent by human mast cell tryptase (Cairns and Walls, 1996). Upregulation of ICAM-1 is associated with

increased influx and binding of both neutrophils (Tosi et al., 1992) and eosinophils (Wegner et al., 1990).

The epithelium may also regulate airways smooth muscle tonus. PGE_2 can inhibit the release of acetylcholine from airway parasympathetic nerves, which is responsible for airway smooth muscle contraction (Walters et al., 1984). In addition, epithelial cells may also produce endothelin (Black et al., 1989; MacCumber et al., 1989) and $PGF_{2\alpha}$ (Churchill et al., 1989; Widdicombe et al., 1989), thus directly mediating bronchoconstriction. Epithelial cell derived cytokines which were investigated in the present study are reviewed below.

1.4.4.1 Interleukin 6 (IL-6)

Human IL-6 is a 26kDa pleiotropic cytokine, with a predicted structure as a folded four alpha-helix bundle with up-up-down-down topology. Receptor binding properties indicate that at least two regions, the first in helix D, and the second incorporating part of the putative connecting loop AB through to the beginning of helix C are critical for efficient binding to the IL-6 receptor (Hammacher et al., 1994). A number of cell types produce IL-6, including endothelial cells, fibroblasts, keratinocytes, mesangial cells, T- and B- cells, monocytes, and several tumour cells. IL-6 is implicated in the growth and differentiation of various cells involved in the immune system, haematopoiesis, and inflammation (Ishibashi et al., 1989; Kishimoto, 1989; Kelley. 1990). IL-6 induces final maturation of B-cells into antibody-producing cells, stimulates T-cells growth and cytotoxic T-cell differentiation, and is a potent growth factor for myeloma and plasmacytoma cells. IL-6 is known as one of the endogenous pyrogens (with IL-1 and TNFα), and can induce acute phase reactions in response to tissue injury (Hack et al., 1992) or inflammation (Heinrich et al., 1990). IL-6 is a crucial cytokine for mast

cell maturation. In vitro, the differentiation of human cord blood CD34+ cells into mast cells, induced by stem cell factor (SCF), leads in presence of IL-6 to increases in frequency of chymase positive cells, and intracellular histamine levels when compared with cells treated with SCF alone (Conti et al., 2002). The multiple actions of IL-6 are integrated within a complex cytokine network in which IL-6 has synergistic or antagonistic activities with other cytokines, which include IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF and IFN-γ, suggesting that IL-6 may be involved in a number of inflammatory processes.

1.4.4.2 Interleukin 8 (IL-8)

IL-8 is a member of the CXC chemokine family, which have two cysteine (C) residues separated by a non-conserved residue (X) at the N-terminal region. Also known as neutrophil-activating peptide, it is a chemoattractant protein for neutrophils, and there is evidence that it can also function as a chemotactic factor for cytokine-primed eosinophils (Shute, 1994; Erger and Casale, 1995). The IL-8 gene is known to be activated by AP-1, NFκB like factors and C/EBP like factors, but the relative importance of these transcriptional factors varies from cell to cell. In the 16HBE cell line, upregulation of IL-8 by neutrophil elastase appears to be mediated by interleukin-1 receptor-associated kinase (IRAK), and involves the signal transducing molecules MyD88 and TRAF-6, and NFκB (Walsh et al., 2001). IL-8 is secreted by epithelial cells, endothelial cells, fibroblasts, monocytes, neutrophils, and mitogen-stimulated lymphocytes (Alam, 1997). It is considered to be the most potent chemotactic factor for neutrophils in the lung, and IL-8 levels have been found to increase in patients with cystic fibrosis (Koller et al., 1997) and severe asthma (Shute et al., 1997). Antibodies against IL-8 can block neutrophilic inflammation in various models (Kunkel et al., 1991; Alam, 1997). IL-8 is also found in

most patients with sepsis (Hack et al., 1992), and its levels correlate with some inflammatory, biochemical and clinical parameters. Neutrophilia, which has been reported in CF and COPD patients, is likely to be related to the expression of IL-8 in inflamed airways

1.4.4.3 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

The human granulocyte-macrophage colony-stimulating factor (GM-CSF) core structure consists of a four alpha-helix bundle, and a number of residues located on the B and C helices are postulated to interact with the alpha chain of the GM-CSF two sub-unit receptor. GM-CSF is produced by macrophages, endothelial cells, and activated T cells as well as epithelial cells. GM-CSF can enhance the expression of adhesion molecules on epithelial cells and endothelial cells, prime granulocytes to release substantial amounts of mediators on stimulation, and prolong survival of inflammatory cells (Lopez et al., 1986; Soloperto et al., 1991; Wislez et al., 2001), thereby promoting the infiltration with inflammatory cells and their local activation. Human tracheal epithelial cells constitutively release GM-CSF, which is inhibited by glucocorticoids (Churchill et al., 1992). Recombinant GM-CSF is used clinically to accelerate haematopoietic recovery following chemotherapy or radiotherapy and bone marrow transplantation (Costello, 1993).

1.5 Mast Cells in Inflammatory Conditions of the Lung

Mast cells are found in high numbers in normal lung tissues, and they have the potential to secrete a range of potent mediators of inflammation. Upon mast cell degranulation, the products released by different mast cell subtypes are likely to have a range of effects on the

neighbouring cells, due in part to their differences in neutral protease and cytokine content, but also in their occurrence throughout the lung tissues. Although early studies reported that MC_T cells predominate in lung tissues, the presence of the MC_{TC} subtype has also been reported (Bradding et al., 1995; Matin et al., 1992; Irani et al., 1986b), suggesting that these cells may contribute to inflammation of the airways.

Some characteristics of inflammation in the lung include hyperresponsiveness, mucus hypersecretion, tissue damage and remodelling. Chymase can induce mucus hypersecretion from bovine airway submucosal glands (Sommerhoff et al., 1989), and chymase-containing cell have been reported mostly in the vicinity of submucosal glands in normal lung tissues (Matin et al., 1992). The pro-inflammatory actions of mast cell tryptase in the airways and its potential to induce tissue damage and remodelling have been described in section 1.2.3.1a. Besides its potential to induce mucus secretion, little is known about the effect of chymase in the lung.

Most research on lung inflammation has been directed towards asthma. Interestingly, chymase-containing mast cells have been found in higher numbers in the submucosa of asthmatic subjects (Bradding et al., 1995), but the involvement of mast cells in other pathologies has been poorly studied. It is well established that there is a genetic background to asthma, which may predispose patients to developing airways with an inflammatory phenotype. Cystic fibrosis (CF), which is caused by a genetic mutation, is another disease associated with inflammation of the lung. Another common lung pathology is chronic obstructive pulmonary disease (COPD), which is induced by air pollutants such as cigarette smoke, and characterized by chronic inflammation. In the studies reported in chapter 3, we have sought to characterise in detail the distribution of cells in these conditions, an area of study that has received relatively little attention to date.

1.5.1 Pathogenesis of Cystic Fibrosis

Cystic fibrosis was first described in 1938. Early in the study of CF, excessive mucus secretions were recognised as a major pathophysiological feature of the disease, also called "mucoviscidosis".

The genetic mutation responsible for CF has been identified on the long arm of chromosome 7 (7q31; Rommens et al., 1989; Boat et al., 1989). The gene encoding for the chloride channel termed "CF transmembrane conductance regulator" (CFTR), which is located on the apical membrane of epithelial cells, is mutated, preventing the secretion of chloride ions. In addition, sodium absorption is increased by up to threefold (Welsh, 1990). This seems to be associated with the particular texture of the secreted mucus. The ionic imbalance would lead to a dehydration of the epithelium surface, thus thickening the excessive secretions. Mucocillary clearance of the mucus is also altered, leading eventually to the plugging of affected organs. Nearly every mucus-secreting epithelium is pathologically altered in CF (Gerken and Gupta, 1993). In the lung of CF patients, the ongoing accumulation of infected viscous secretions and chronic inflammation lead to lung damage, tissue remodelling and eventually fatal loss of function.

When first described, 80% of CF patients died within a year of birth. Nowadays, with improved disease management, many patients survive to adulthood, though there is still a very high morbidity and mortality. The commonest cause of death is respiratory failure due to bronchopulmonary sepsis. The failure of clearance and subsequent accumulation of mucus facilitates the colonisation of the respiratory epithelium by a range of microorganisms (particularly *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Staphylococcus aureus*). Chronic inflammatory responses lead to temporary elimination of the microorganism, but also to destruction of structural molecules of the

extracellular matrix (ECM) and eventually irreversible abnormal repair, termed tissue remodelling.

So far, most reports on inflammatory cells found in CF lung have concentrated on neutrophils and eosinophils. In normal lung, the epithelium is protected from the action of neutrophil elastase by two antiproteases, α1-antitrypsin (α1AT) and secretory leukocyte protease inhibitor (SLPI). Western blot analysis of epithelial lining fluid from patients with CF demonstrated that these proteases inhibitors were in complexes, or were degraded, suggesting that protease/anti-protease imbalance may be a major cause of extracellular matrix degradation in children as early as one year of age (Birrer et al., 1994). Neutrophils have been implicated in this process through the release of enzymes such as neutrophil elastase (NE), collagenase, gelatinase, heparanase and plasminogen activator (reviewed in Walls, 2000a). Various metalloproteases are produced also by macrophages, which are present in greater number later in chronic inflammatory processes). IL-8 expression from epithelial cells appears to be selectively upregulated in the airways of CF patients (Tabary et al., 1998), which could induce leukocyte migration towards the epithelial layers and further inflammation and tissue remodelling. Neutrophil counts, as well as neutrophil elastase and IL-8 levels in BAL fluid of 4 week-old CF children are found to be increased as compared with control infants (Khan et al., 1995), indicating that inflammation is already present at early stages of life (for review see Puchelle et al., 2000). Macrophages, which are a potential early source of interleukin-8, could play a role in this early influx of neutrophils in the lungs of CF children.

Eosinophils may contribute to the pathophysiology of CF, as a number of their secreted products, including eosinophil cationic protein (ECP) are found in significantly higher amounts in the serum of CF patients (Koller et al., 1997; Irani, 1995; Venge et al., 1999). Eosinophils also secrete platelet-activating factor (PAF), which is implicated in the

pathophysiology of airway inflammation by acting as a mucus secretagogue (Dwaine Rieves et al., 1992; Smith et al., 1998).

Although the potential role of neutrophil elastase and eosinophil cationic protein in CF strongly suggests that other endogenous enzymes such as mast cell proteases may play a key role in airway inflammation in CF, this has received little attention.

1.5.2 Pathogenesis of Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is defined as a disorder characterized by reduced maximum expiratory flow and slow forced emptying of the lungs, features which do not change markedly over several months (Siafakas et al., 1995). The inflammation and proteolysis observed in COPD appears to be the consequence of normal inflammatory responses to chronic exposure to cigarette smoke. Early stages of COPD are often asymptomatic, but may present as asthma, chronic bronchitis, emphysema or combinations (Petty, 2002).

In contrast with cystic fibrosis, there is no defined specific genotype associated with COPD, but animal models indicate that genetic factors are critical in the predisposition to emphysema from cigarette smoke exposure (Cavarra et al., 2001). In COPD, chronic inflammation leads to fixed narrowing of small airways and alveolar wall destruction (emphysema). This is characterized by increased numbers of alveolar macrophages, neutrophils, cytotoxic T lymphocytes, and the release of multiple inflammatory mediators (lipids, chemokines, cytokines, growth factors). There is increased elastolysis and probable involvement of matrix metalloproteinases (Hogg and Senior, 2002), and also a high level of oxidative stress, which may amplify this inflammation (for review, see Barnes, 2003). As early as in 1963, Laurell and Ericksson observed a high prevalence of pulmonary emphysema in families with low concentrations

of serum alpha 1 antitrypsin (Laurell and Ericksson, 1963). The local synthesis of alpha 1 protease inhibitor (α_1-PI) may provide a partial mechanism for neutrophil elastase regulation during inflammation. Airway epithelial cells can increase their production of α_1-PI when stimulated by specific inflammatory mediators, including IL-1 and oncostatin M (Cichy et al., 1997). Thus alpha 1 protease inhibitor deficiency has been linked to pulmonary emphysema and COPD for many years (Turino, 2002), although a recent study failed to detect a preponderance of this phenotype in patients with chronic lung disease when compared with the normal population (Wencker et al., 2002). Neutrophilia is an important feature of COPD, since elevated numbers of neutrophils are present in patients with severe COPD (Di Stefano et al., 1998), where it appears to correlate with periods of COPD exacerbation (Aaron et al., 2001). Increased numbers of eosinophils and macrophages have also been reported in the airways of COPD patients (Zhu et al., 2001; Hospers et al., 1999; Gibson et al., 1998).

Elevated histamine and tryptase levels have been reported in BAL fluid from smokers (Kalendarian et al., 1988), suggesting that mast cells are likely to have a role in the pathophysiology of COPD. In addition, increased numbers of tryptase-positive mast cells have been reported in the airway epithelium of COPD patients (Grashoff et al., 1997). Atopy has been identified as a risk factor for COPD (Weiss, 2000), and it has been reported that production of specific IgE enhances bronchial hyperresponsiveness in patients with COPD (Mitsunobu et al., 2001). A recent study reported the presence of increased levels of tryptase in the induced sputum of some COPD patients, suggesting that mast cell activation may also take part in the inflammatory processes in COPD lung (Louis et al., 2002).

Mast cells are likely to play a key role in the regulation of the inflammation, and they are found abundantly in the lung tissue, where they are potentially amongst the first cells to be involved in inflammatory

processes. Compounds released from mast cells, of which proteases are the most abundant, can induce the influx of further inflammatory cells as described in section 1.2.3, and are likely to have a role in the pathophysiology of inflammatory diseases of the airways. However, a relatively small number of studies have concentrated on the potential for mast cell proteases to take part in inflammatory processes in the human lung, and so far most studies concentrated on tryptase. The role of chymase, an essential constituent of MC_{TC} cells, remains unclear.

1.6 Aims

The purpose of these studies was to investigate the hypothesis that mast cell chymase has a key role in inflammatory conditions of the human airways. The aims were:

1 To investigate the expression of chymase in the respiratory tract in cystic fibrosis and chronic obstructive pulmonary disease,

2 To assess the potential of mast cell chymase as a stimulus for inflammation and repair in the bronchial epithelium,

3 To examine the actions of mast cell chymase on the structural integrity and permeability of the bronchial epithelium.

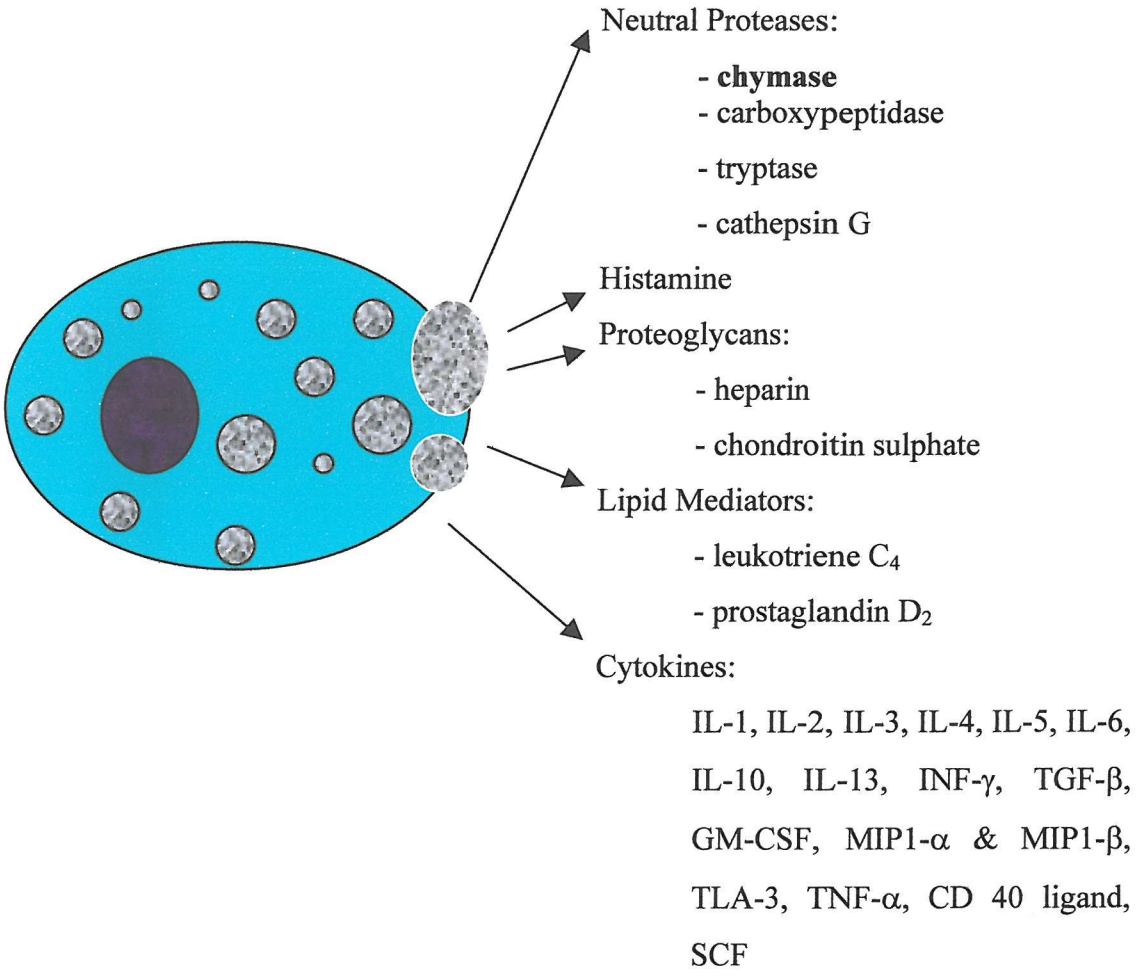


Figure 1.1 Mediators released by mast cells.

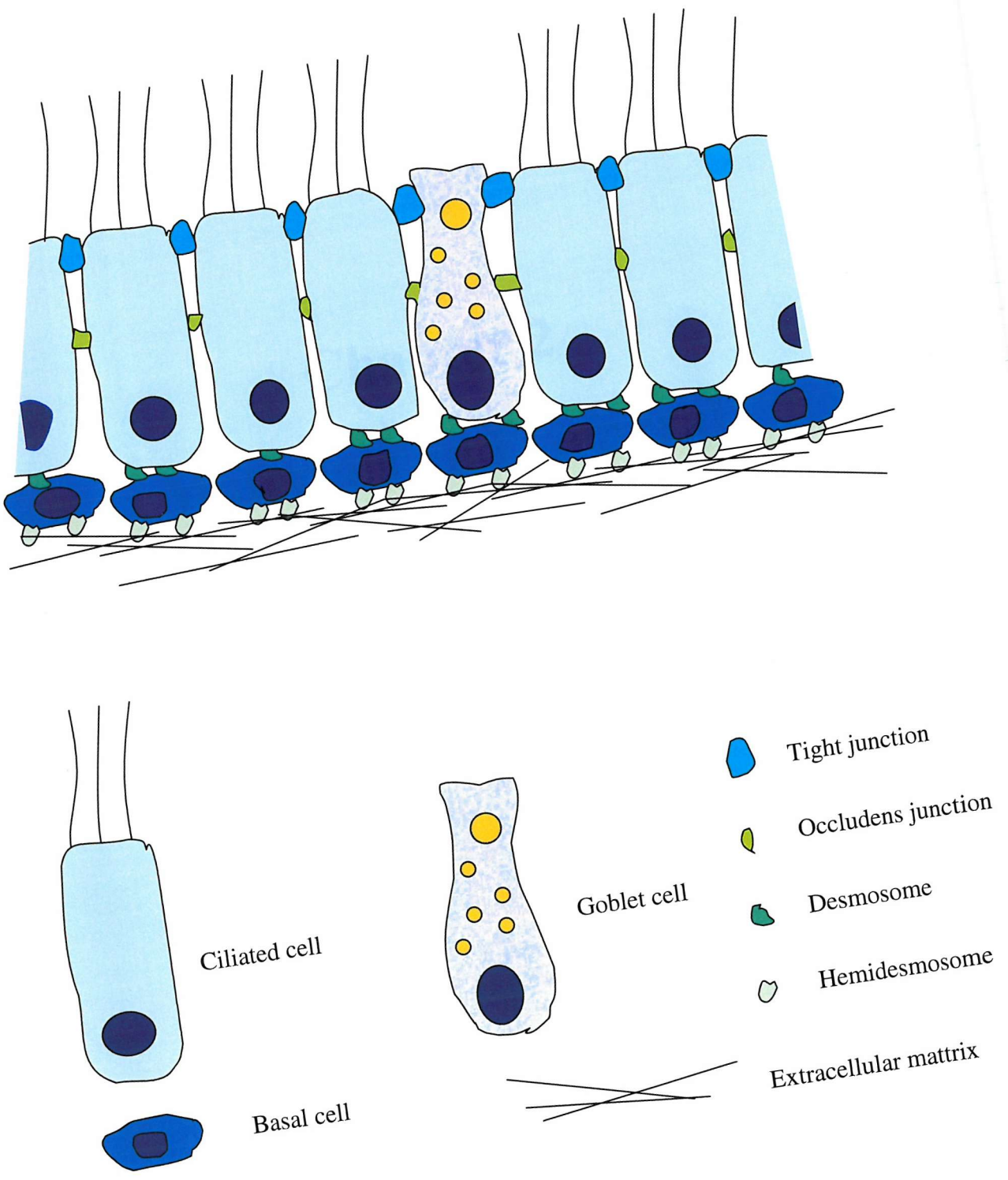


Figure 1.2 Structure of the airway epithelium

Chapter 2

Materials and General Methods

2 Materials and General Methods

The methods described in this chapter were employed in generating the data presented in chapters 3, 4, and 5. Particular techniques that were specific to an individual chapter are described in the methods section of the relevant chapter.

2.1 Materials and Reagents

2.1.1 Cells

2.1.1.1 Cell lines

The 16HBE 14o- cell line, kindly provided by Dr. D.C. Gruenert (Cardiovascular Research Institute, University of California, San Francisco, CA) was employed in a model of the normal human airway epithelium. This cell line is derived from the surface epithelium of mainstream, second-generation bronchi (Cozens et al., 1994). 16HBE 14o- can form tight junctions, generating polar monolayers that express a number of features characteristic of native epithelium such as ion transport, secretion, production of metabolic enzymes and adhesion molecules. They are also able to generate cilia under defined growth conditions (Gruenert et al., 1995).

The High Five insect cell line (Invitrogen; San Diego, CA) was used to express recombinant human chymase.

2.1.1.2 Primary cultures

Normal human bronchial epithelial cells (NHBE) were purchased from Clonetics (San Diego, CA). When possible, primary human bronchoepithelial cells, isolated from surgical resections, were used in some experiments. Findings were compared to those with the 16HBE 14o- cell line.

2.1.2 Chymase

A baculovirus expression system (strain PVL 1393, Invitrogen) was used to produce recombinant human chymase. Methods for the expression, purification and characterisation of recombinant chymase are described below in the corresponding section.

Human skin excised from amputated limbs was used as a source of native chymase. Pieces of tissue were stored at -80° until extraction. Procedures for extraction, purification and characterisation of native chymase are detailed in section 2.2.3 below.

2.1.3 Other Materials

Heparin agarose, soybean trypsin inhibitor (SBTI), Sephacryl S-200 and S-300, bovine serum albumin (BSA), insulin-selenium-transferrin cell culture supplement (ITS), chymostatin, collagen type IV, sodium chloride, N,N,N',N' tetramethylethylenediamine (TEMED), Tween-20 (polysorbitol monolaurate), (2-[N-Morpholino]) ethanesulfonic acid (MES), (3-[N-Morpholino]) propanesulfonic acid (MOPS), and polyethylene glycol (PEG) 4000 were obtained from Sigma Chemicals Co. (Poole, Dorset, UK); Coomassie Blue protein assay from Pierce (Chester, UK); molecular

weight markers (prestained SDS-PAGE standards), silver staining reagent and electrophoresis grade agarose from BioRad Laboratories (Hemel Hempstead, Herts, UK); ultrafilters YM10 and YM30 from Amicon (Stonehouse, UK); acetic acid, ethanol, and methanol from Fisher Scientific International Company.

Minimum essential medium (MEM), Dulbecco-modified Eagle medium (DMEM), RPMI 1640, LHC-9, Hank's balanced salt solution (HBSS), heat-inactivated foetal calf serum (FCS), phosphate-buffered saline (PBS), trypsin-EDTA, growth supplement Ultrosur G, penicillin, streptomycin and glutamine from Gibco BRL Life Technologies (Paisley, UK); EX-CELL 400 culture medium for insect cells from JRH Biosciences (Lenexa, KS); bronchial epithelial basal medium (BEBM) for primary cells and supplements including bovine pituitary extract, hydrocortisone, human recombinant epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, 3,3',5-triiodo-L-thyronine, gentamicin, and amphotericin B, from Clonetics (San Diego, CA); Falcon cell culture plates and inserts were obtained from Becton Dickinson Life Technologies (Oxford, UK); EVOM micro volt-ohm-meter from World Precision Instruments (Owslebury, UK); mouse monoclonal anti-occludin antibody from Zymed Laboratories (San Francisco, CA); biotinylated sheep anti-mouse IgG antibody, horse radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody, avidin-biotin-horseradish peroxidase, and goat anti-mouse IgG antibody conjugated to FITC from DAKO (Copenhagen, Denmark); chemiluminescent substrate SuperSignal West Femto from Pierce (Rockford, IL. USA). Anti-human tryptase (AA1, AA5) and anti-human chymase (CC1) mouse monoclonal antibodies were prepared as previously reported (Walls et al., 1990; Buckley et al., 1999).

2.2 General methods

2.2.1 Cell Culture

2.2.1.1 16HBE 14o- cell line

16HBE cells were grown in MEM supplemented with 10% FCS, 100 U/ml penicillin, 100μg/ml streptomycin, and 2 mM glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Cells grown in 75 cm^2 culture flasks became confluent every 4-5 days and were transferred to a new culture flask for subculturing or seeded on culture inserts (24 well plates) for experiments.

To dissociate cells, spent culture media was discarded and cells washed twice with 5ml Hank's balanced salt solution without calcium and magnesium, followed by 10 to 15 minutes incubation with 2ml of a 0.05% trypsin-EDTA solution. When cells were observed under an inverted light microscope to have rounded up, they were dislodged by gently tapping the flask. The enzymatic activity of trypsin was stopped by adding 10 ml of culture medium and the suspension was centrifuged at 150 g for 10 min. Supernatant was then discarded and cells resuspended in 12 ml culture medium. Depending on cell density, 2 to 3 ml of suspension was transferred into a new 75 cm^2 culture flask containing 9 or 10 ml culture medium (for a final volume of 12 ml) and replaced at 37°C in a humidified atmosphere containing 5% CO_2.

For experiments, cells were seeded on culture inserts in 24 well plates at a density of 10^5 cells/ml and cultured until confluency under the same conditions as above.

2.2.1.2 Primary cultures of bronchial epithelial cells

Primary cells from human bronchus were obtained frozen from the manufacturer. The whole batch of cells was gently thawed and after decontaminating the vial with alcohol, immediately transferred into a 25cm^2 flask containing 6ml bronchial epithelial growth medium (BEGM: BEBM medium supplemented according to the manufacturer's protocol). A sample of suspended cells was taken and viable cells were counted using Trypan blue exclusion. Cells were then seeded on two 25cm^2 flasks at a density of 1.10^5 viable cells/cm^2. Medium was replaced every 72h, and cells were further subcultured using trypsin-EDTA as described above.

2.2.1.3 Insect cell line

An insect cell line (High Five) culture was initiated from frozen stock. Initial growth was carried out in culture flasks until cells reached log phase and viability was over 95%. The culture was then expanded to supernatant culture with heparin added in the medium (20 U/ml) to prevent clumping. Cells were then progressively weaned from heparin. Once a satisfactory volume was obtained (500ml of suspension culture containing 2x10^6 cells/ml), the culture was transfected with a baculovirus preparation containing the gene encoding for human prochymase. Prochymase can be activated by dipeptidyl peptidase I (DPPI) to form active chymase (McEuen et al., 1998a). Prochymase production could therefore be assessed daily by measuring the chymotryptic activity in a 10µl sample incubated with 10µg/ml DPPI. When levels of activable chymase were sufficient for purification, the whole suspension was harvested and centrifuged. The supernatant, containing the recombinant prochymase was stored at –20°C.

2.2.2 Extraction and Purification of Mast Cell Chymase and Tryptase from Human Skin

Skin was removed from storage at -80°C and transferred to 4°C the day prior to extraction. Pieces were ground and suspended in NaCl-free buffer (10 mM MOPS, pH 6.1) to disrupt red blood cells. After subsequent centrifugation, the supernatant was kept in ice to be assayed for chymotryptic and tryptic activity, and the pellet was resuspended in low salt buffer (0.15M NaCl, 10mM MOPS, pH 6.1). Two similar cycles of extractions were carried out with the low salt buffer, followed by 3 cycles using high salt buffer (2.5M NaCl in the first one, to compensate for the low salt content of the pellet at that stage, then 2M NaCl; MOPS was always used at the same concentration of 10 mM, pH 6.1, throughout the whole procedure).

Extracts presenting chymotryptic activity were sequentially filtered (last filtration through 0.4μm pore-size filters) and dialysed against 10 mM MOPS (pH 6.1) to 0.3M NaCl, prior to loading on a heparin-agarose affinity column. Later we decided to load the chymotryptic enzyme (about 80% left at that stage) on a SBTI (soybean trypsin inhibitor) column. Columns were washed with low salt buffer (NaCl 0.4M, MOPS 10mM) and each wash was checked for activity to confirm stable binding.

The tryptic enzyme and chymase were eluted from the heparin-agarose columns using a NACl gradient (0.4M to 2M). Chymase was eluted from the SBTI column using HCl 0.1M, and collected 5ml fractions were immediately neutralised in 0.5ml of a Tris-HCl buffer (pH 8.0) as described earlier (Schechter et al., 1986). Tryptase and chymase-containing fractions were pooled separately and concentrated to less than 1ml through Centricon ultrafilters YM30 (cut-off at 30,000 kDa) and YM10 (cut-off at 10,000 kDa) respectively. Each sample was then loaded on a Sephacryl S-200 column and eluted with a low salt buffer (NaCl 0.4M, MOPS 10mM). Chymase and tryptase-containing fractions were

then pooled in two separate lots and reconcentrated as described above before being stored at -80°C.

2.2.3 Purification of Recombinant Human Chymase

After transfection on day 0, cell viability and prochymase production were monitored daily. A sample was activated with DPPI to determine the content of activable prochymase in the batch (Fig. 4.3). When the decrease in cell viability started to reduce prochymase production, PEG 4000 was added to 0.01% to prevent adhesion of the prochymase to plastic containers, and the batch was harvested and stored at -20°C until purification. For each batch of supernatant containing recombinant prochymase, NaCl was added to reach a concentration of 0.4M and then the whole batch was sequentially filtered (last filtration through 0.4μm pore-size filters) prior to being loaded on a newly made heparin-agarose affinity column. The column was then washed with low salt buffer (NaCl 0.4M, MOPS 10mM) and wash checked for chymotryptic activity to confirm stable binding. Recombinant chymase was then eluted with a gradient of NaCl (from 0.4M up to 4M). Chymase-containing fractions were pooled and concentrated on Centricon YM10 ultrafilters and loaded on a S-200 column as described above. The recombinant enzyme was then eluted with low salt buffer and chymase-containing fractions pooled and reconcentrated prior to storage at -80°C as described above.

2.2.4 Determination of Protein Content

To determine protein content, UV spectroscopy and Coomassie blue assay (Bradford technique) were used. For UV spectroscopy, a UVIKON 930 spectrophotometer (Kontron Instruments, Watford, UK) was

used to read absorbance of tested samples at 280 nm. For the end point Coomassie blue protein assay, 90 µl of Coomassie blue reagent was added to 10 µl of sample in a microtitre plate (Greiner) and absorbance read at 595 nm (Thermomax microplate reader, Molecular Devices). For each Coomassie blue assay, a calibration curve was made using BSA as a standard.

2.2.5 Determination of Enzymatic Activity

Enzymatic activity was expressed in milliunits per millilitre (mU/ml), one unit being equal to 1µmol of substrate hydrolysed per minute at 37°C.

2.2.5.1 Assay for chymase

Chymotryptic activity was determined by hydrolysis of the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-NH-Np) on a microtitre plate. 10µl samples were applied per well and 90µl of chymase assay buffer (1.5M NaCl, 0.79% dimethylsulphoxide, 0.3M Tris base, pH=8) containing 0.7mM Suc-Ala-Ala-Pro-Phe-NH-Np was added immediately prior to reading on an ELISA plate reader (programmed to read every 10 seconds at 450 nm for 10 minutes).

2.2.5.2 Assay for tryptase

Tryptic activity was determined by hydrolysis of the substrate *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) on a microtitre plate. 10µl samples were applied per well and 90µl of tryptase assay buffer (100mM Tris base, 1M glycerol, pH=8) containing 1mM BAPNA was added

immediately prior to reading on an ELISA plate reader (programmed to read every 10 seconds at 450 nm for 10 minutes).

2.2.6 Assessment of Enzyme Purity and Identity

2.2.6.1 Gel electrophoresis

The purity of each preparation was assessed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Stacking gel and running gel were prepared in duplicate with 4% and 10% acrylamide respectively. Enzyme-containing samples were dissolved to about 1μg protein in 50μl buffer containing 0.1% SDS, 8% glycerol, 2% mercaptoethanol, and 0.01% bromophenol blue. Each mix was boiled for 5 min, and 10μl layered on the stacking gel. 5μl of prestained marker were also applied on each gel. Electrophoresis was then performed at 160 V for about 1 hr.

2.2.6.2 Silver staining

One gel was silver stained according to the instructions provided by the manufacturer (kit from Bio-Rad Laboratories, Hemel Hempstead, UK). The gel was incubated in a first fixative (40% methanol, 10% acetic acid; v/v) for 30 min or overnight. It was then transferred to a second fixative (10% ethanol, 5% acetic acid; v/v) for 15 min. This was followed by incubation in the provided oxidiser solution for 5 min. The gel was then washed several times in deionised water for 5 min, and incubated with the provided silver reagent for 20 min. After a last wash of 1 min in deionised water, the gel was incubated with a 3.2% developing solution (w/v) until

bands were clearly visible. The gel was then transferred into a 5% acetic acid solution to stop the development.

2.2.6.3 Western blotting

The second gel was incubated for 30 min in a transfer buffer consisting of 39mM glycine, 38mM Tris, 20% methanol, and 0.0375% SDS. A nitrocellulose membrane was then applied on the gel and these were sandwiched in a hinged cassette between slices of blotting paper pre-humidified in transfer buffer. The cassette was then placed in a Trans-Blot apparatus, and electrophoresis carried out overnight using a 30V current. The nitrocellulose membrane was then washed in PBS-T (1ml of tween-20 added to 500ml PBS) for 5 min and blocked with BLOTTO + T (5% skimmed milk powder, 0.05% sodium azide, 49ml PBS, 49ml PBS-T) for 1h or overnight. After a 5 min wash in PBS-T, the membrane was incubated with monoclonal antibody AA1 or AA5 (1/200 dilution in BLOTTO + T) for tryptase, or CC1 (1/500 dilution in BLOTTO + T) for chymase, for 1h or overnight. The membrane was then washed four times with PBST and incubated with horseradish peroxidase-conjugated anti mouse antibody for 1h. After four washes in PBS-T and two washes with PBS (5 min for each wash), the membrane was incubated with a chemoluminescent substrate for 5 min. After incubation, excess of chemoluminescent substrate was removed from the membrane, which was then sealed in cling film and placed in an autoradiographic cassette. In a dark room, a film (Hyperfilm ECL) was placed in the cassette for a first exposure of 30 sec. Depending on the result, further exposures were carried out for various time if required until a neat band was visible on the film.

Chapter 3

Distribution of Chymase Containing Mast Cells in the Airways of Patients with CF and COPD

3 Distribution of Chymase Containing Mast Cells in the Airways of Patients with CF and COPD

3.1 Introduction

The mast cell has been implicated as playing a key role in allergic inflammation, and can induce mucus hypersecretion and participate in tissue remodelling (reviewed in Walls et al., 2000a). The primary focus of most studies has been in the context of allergic conditions of the airways. The potential involvement of this cell type in the inflammatory processes in CF and COPD has received little attention. Alterations in the ratio of MC_T/MC_{TC} cells phenotype have been reported in a preliminary study of BAL fluid cells from CF patients (Irani et al., 1986b). This feature has been little investigated since, though more recent reports also suggest alterations in mast cell subsets in the airways of asthmatic subjects (Walls et al., 2000a).

It has been reported that chymase-containing mast cells are concentrated in the proximity of glands and smooth muscle tissue in the normal human bronchus (Matin et al. 1992). This is of interest because canine chymase has been reported to act on bovine airways submucosal glands as a potent mucus secretagogue (Sommerhoff et al., 1989). However the distribution of mast cell chymase in inflammatory conditions of the lung has not been studied.

The aim of this study has been to investigate relative numbers and the locations of mast cells and their subtypes, as well as neutrophils and eosinophils in bronchial tissues obtained at surgical resection from CF and COPD patients, and to compare them with macroscopically normal airways tissue from carcinoma resections. We report significant differences in the numbers and subtype of mast cells found in these different conditions.

3.2 Materials and Methods

Materials and methods that were common to other chapters of results are described in chapter 2. Materials and methods specific to this study are described below.

3.2.1 Tissues

This study was performed with the approval of the local Ethics Committee, and written consent was obtained from patients or their relatives. Bronchial tissues were obtained from 19 CF patients (14 males aged 17 to 44, and 5 females aged 21 to30), and 18 COPD patients (11 males aged 44 to 60, and 7 females aged 32 to 58) undergoing lung transplantation at the Alfred and Monash Memorial Hospital, Prahran, Australia. Macroscopically normal bronchial tissues were obtained also from 10 subjects without inflammatory airway disease undergoing lung resection for bronchial carcinoma, whose other details were not revealed under the terms of the Ethics Committee approval. All tissues were resections from second or third generation bronchi. Tissues were fixed in formalin for 30 mins, then progressively dehydrated through a gradient of alcohol, and embedded in paraffin. Blocks in paraffin were stored at 4°C until use.

3.2.2 Immunohistochemistry on Paraffin-Embedded Lung Sections

Sequential transverse sections of 2μm thickness were prepared to allow co-localisation of structures and positive cells. Paraffin was removed by two washes in xylene for 5 min each, then sections were rehydrated through a decreasing gradient of alcohol and finally washed in Tris-

buffered saline (TBS). Hydrogen peroxide was applied for 10 mins to inhibit endogenous peroxidase, and antigens were retrieved by incubating with pronase for 10 mins at room temperature (RT). Endogenous biotin was also inhibited by an avidin/biotin blocking stage (20 mins each, separated by three washes at room temperature). Sections were immunostained for tryptase, chymase, neutrophil elastase and eosinophil cationic protein (ECP) using the monoclonal antibodies AA1, CC1, NOE, and EG2, respectively. All primary antibodies were left on sections at appropriate dilutions overnight at 4°C. Biotinylated secondary antibody (rabbit anti-mouse antibody from DAKO) was applied for 2 hours at room temperature, and sections were stained using the streptavidin-biotin complex method, with AEC or DAB as chromogens. Sections were counterstained in Mayer's haematoxylin solution, drained, Crystal mount (Biomedia) applied and dried at 80°c for 10 min. Slides were then cooled and mounted in DPX.

3.2.3 Cell Counting and Localisation

Nucleated stained cells were counted on an image analyser (Colourvision, Macintosh), and the number of cells per square millimetre determined. Three tissue compartments were defined: epithelium (between the airway lumen and the basement membrane), subepithelial layer (the submucosal layer, excluding the submucosal glands), and glandular tissue (submucosal glands, including an area approximately 20μm around glands corresponding to the maximum diameter of a mast cell). Co-localisation was carried out using a camera lucida system to identify cells stained by both anti-chymase and anti-tryptase antibodies.

3.2.4 Statistical Analysis

This study was performed double blind. For each tissue compartment, for each category of patient (control, CF and COPD), the mean number of cells per square millimetre was determined. Statistical analysis was performed using the SPSS software for Windows. The counts for each cell types and the ratio of MC_{TC} cells in each compartment of control, CF and COPD tissues are shown as median and range. For each cell type, where Kruskal-Wallis analysis indicated significant differences between groups, the Mann-Whitney *U* test was used to compare counts in each compartment. In addition, the relative distribution of mast cells and the ratio of MC_{TC} cells in the different compartments within each group were analysed using the Wilcoxon signed ranks test. Values of $P \leq 0.05$ were considered significant.

3.3 Results

3.3.1 Tissue Structure

The structure of the tissues studied was well preserved, though the epithelial layer was partially or totally absent in about a quarter of them. This may have occured during tissue collection or processing. The thickness of the epithelium varied between 50 and 150μm, and appeared unrelated to the disease group. The submucosal glands closest to the epithelium were approximately 100μm away from the basement membrane of the epithelium. A number of tissue specimen from CF and COPD patients exhibited hyperplasia of gland cells, and thick mucus was visible inside the acini. The epithelium was absent or reduced to the basal cell layer in more than half of CF or COPD tissues, and fibrous filaments were sometimes observed directly below the basement membrane of COPD tissues, though this did not appear to be related to increased numbers of any of the cell types examined. The 2μm sequential sections facilitated colocalisation between cells stained for chymase and those stained for tryptase. Tryptase-containing mast cells and neutrophils represented the predominant inflammatory cells, and these two cell types were present in high numbers in all tissues examined. In contrast eosinophils were found in a relatively small numbers in most CF and COPD patients, but only one or two cells were occasionally detected in control tissues. Cell numbers and distribution are detailed below.

3.3.2 Alterations in Numbers and Tissue Distribution of Mast Cells

In the three groups of patients studied, most inflammatory cells were located between the epithelium and the submucosal glands, which is why this area was defined as one of the compartments of interest. Mast

cells were quite evenly distributed in each compartment. Virtually all mast cells contained immunoreactive tryptase. Interestingly, in the tissue from one control patient a few cells in the vicinity of submucosal glands were detected with the anti-chymase antibody (Fig. 3.3; B) which were not detected with the anti-tryptase antibody on the adjacent section (Fig. 3.1; B). However, this was rare, it was assumed that the great majority of mast cells identified in the present studies belonged to either the MC_T or the MC_{TC} subtype.

The total number of mast cells, as assessed by staining for tryptase (Fig. 3.1), was increased in the epithelial layer of CF tissues when compared with controls (Fig. 3.2; P= 0.001). Mast cell counts appeared also increased in the submucosa and in the vicinity of submucosal glands of CF tissues, though statistical significance was not achieved. The general distribution of chymase-containing cells was similar to that of tryptase-containing cells (Fig. 3.3). Numbers of chymase-containing mast cells were significantly increased in the epithelium of CF patients (P= 0.014), indicating that mast cell hyperplasia affected the MC_{TC} subtype too. In contrast, no significant increase in numbers of chymase- or tryptase-containing cells were observed in any of the three compartments of tissues from COPD patients.

When the counts of tryptase-positive cells in the different compartments were compared within each group, it appeared that the general distribution of mast cells remained unchanged in tissues from CF and COPD patients when compared with that from controls (Fig. 3.5), with cells located mostly in the submucosa, and about three times fewer cells were found in the vicinity of submucosal glands. Mast cell numbers were increased throughout the whole area of the tissue examined from CF patients, without evidence of the pattern of cell distribution being affected. However, the increase in cell numbers was more pronounced in the epithelium.

The ratio of chymase-containing cells to total mast cells, expressed as a percentage for the different compartments, showed clear differences related to the area of tissue, and to the disease group (Fig. 3.6). We found that the percentage of MC_{TC} cells was increased in the epithelium of CF patients when compared with controls (P= 0.029), indicating that mast cell hyperplasia observed in the CF epithelium affected predominantly the cells of the MC_{TC} subtype. There was also a trend for an increase in the percentage of MC_{TC} cells present in the submucosa of tissues from CF patients when compared with that of control tissues, though this was not statistically significant. The percentage of MC_{TC} cells in the vicinity of submucosal glands showed great variability in the three groups of patients studied, but no difference between groups. It appeared that most cells of the MC_{TC} subtype were located in the vicinity of submucosal glands in all tissues examined. Tissues from some COPD patients appeared to exhibit an increase in the percentage of MC_{TC} cells in the submucosa when compared with the submucosa of control tissues, but this trend did not reach statistical significance for the group as a whole. The proportion of MC_{TC} cells identified in the epithelium of COPD tissues was negligible.

3.3.3 Neutrophil Counts

Neutrophils were present in all three compartments of each group of patients, where they appeared often in clusters of cells. These clusters were located close to the epithelium, inside or in close proximity to blood vessels, and were also observed in the airway lumen of some CF and COPD patients (Fig. 3.7). Intra-group variability was more pronounced than with mast cell counts, especially in CF tissues where some patients had four times more cells in the epithelium than others. The general tissue distribution was quite similar to that of tryptase-containing mast cells, with about twice as many neutrophils in the submucosa and in the epithelium,

and about half the number in the vicinity of submucosal glands (Fig. 3.8). Alterations in neutrophil counts appeared similar to those of mast cells. Numbers of neutrophils were increased in the epithelium and the submucosa of CF tissues when compared with the same compartments of control tissues. Interestingly, the submucosa of COPD tissues also presented a significant increase in neutrophil numbers compared with controls.

3.3.4 Eosinophil Counts

Eosinophils were relatively scarce in the various tissues examined (Fig. 3.9). The general distribution was similar to that of mast cells and neutrophils, with most cells found in the submucosa. A few isolated cells were occasionally detected in the epithelium or in the vicinity of submucosal glands. Alterations in eosinophil counts did not show any similarity with those observed with the other cell types examined. Although total numbers in each compartment were much lower than those of other cell types, eosinophils were found in greater numbers in the submucosa of CF and COPD tissues when compared with controls (Fig. 3.10). Interestingly, eosinophilia in the submucosa was a striking feature in some of the COPD patients, though it did not distinguish them from the CF group.

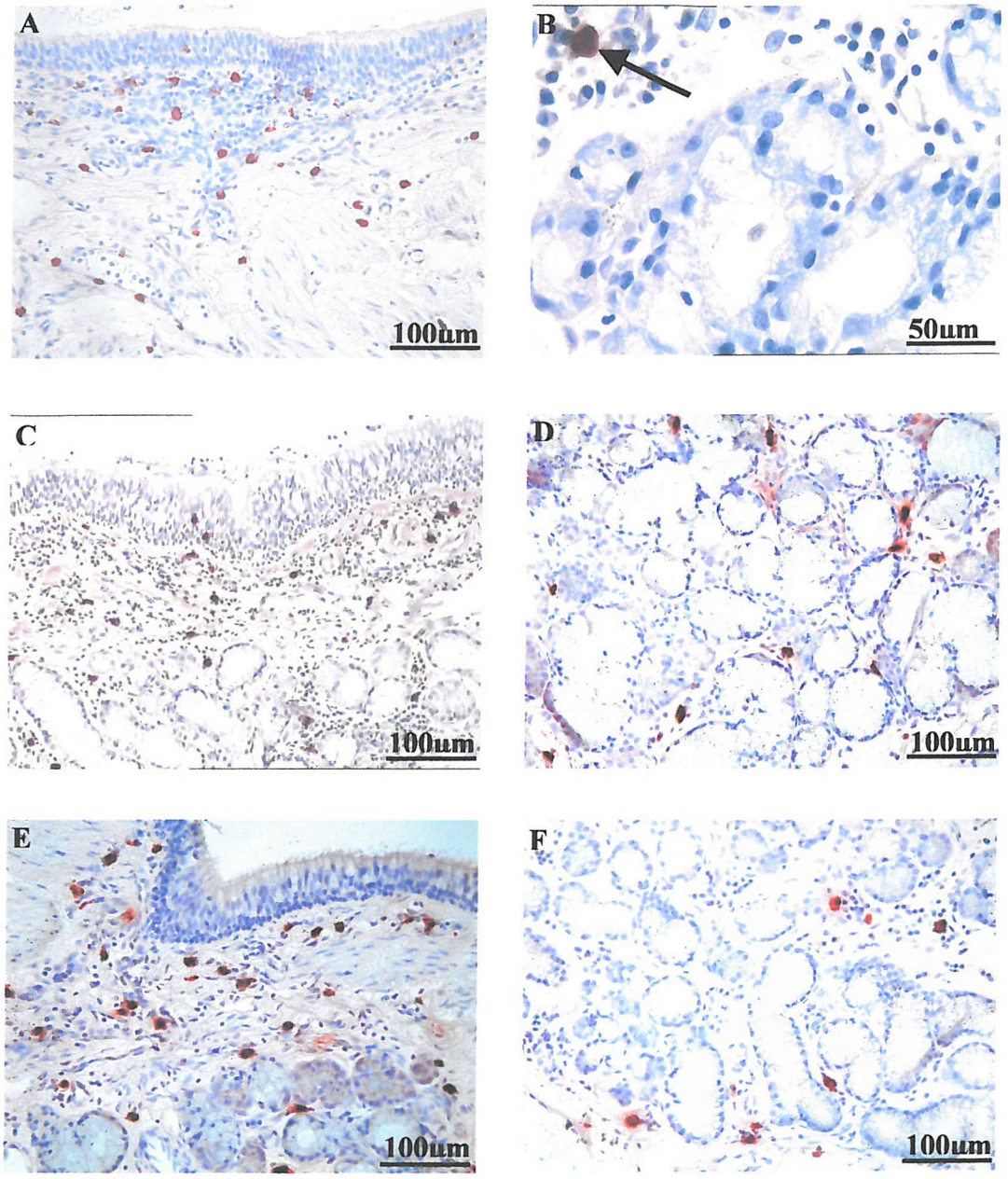
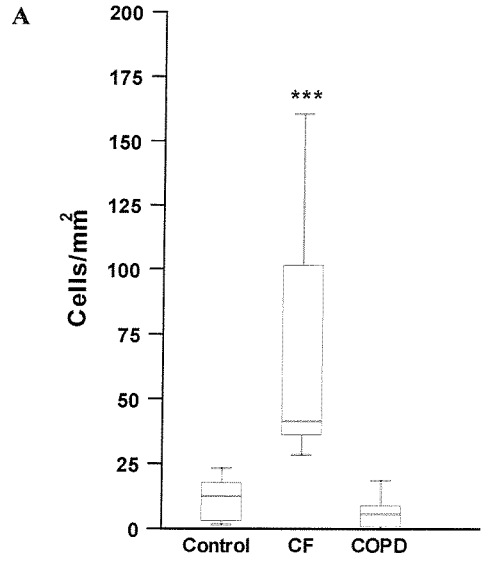


Figure 3.1 Cells stained for mast cell tryptase in tissues from (A & B) control, (C & D) CF, and (E & F) COPD patients. Tryptase-positive cells were identified by immunohistochemistry with monoclonal antibody AA1 and with AEC, and Mayer's haematoxylin was employed for counterstaining.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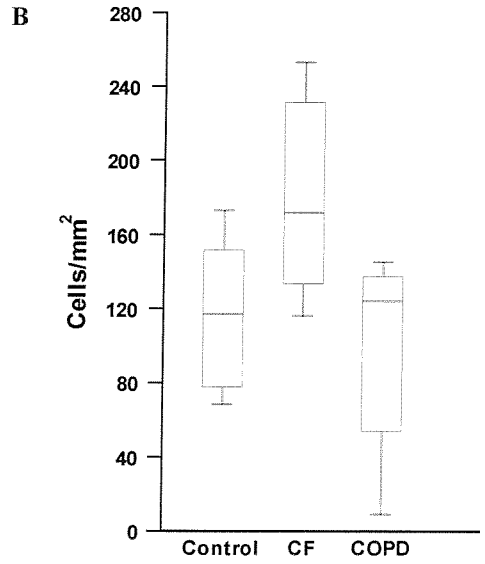
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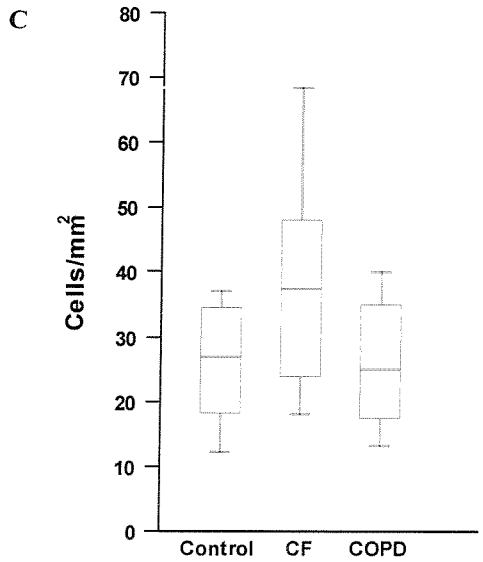
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Figure 3.2 Numbers of cells positive for tryptase in (A) the epithelial layer of control (n=7), CF (n=8), and COPD (n=7) tissues, (B) the submucosal layer of control (n=8), CF (n=10), and COPD (n=7) tissues, and (C) the vicinity of subepithelial glands of control (n=10), CF (n=14), and COPD (n=13) tissues. Data are displayed as a boxplot showing the median value, interquartile range, and the tenth and ninetieth percentiles (*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$).

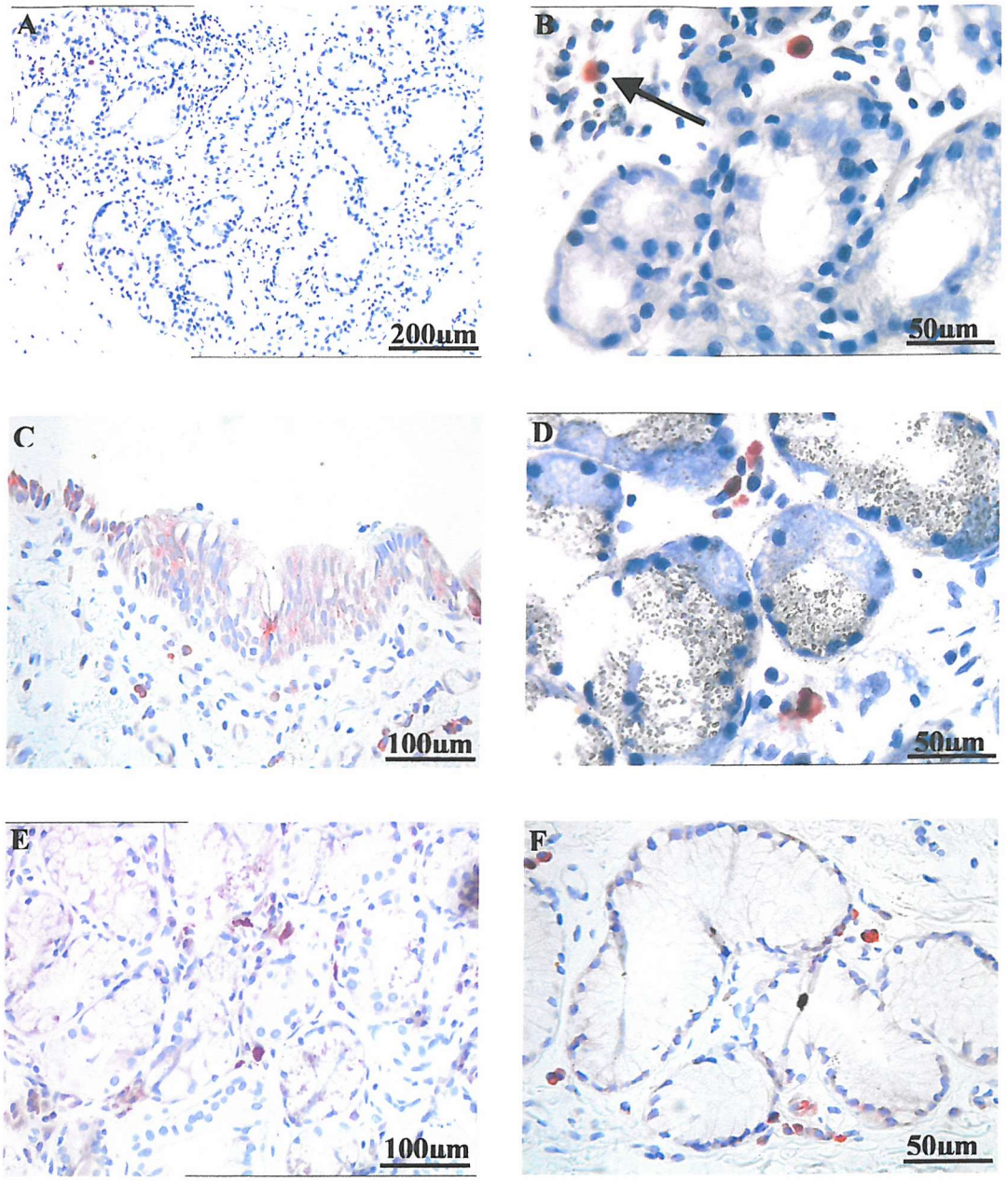
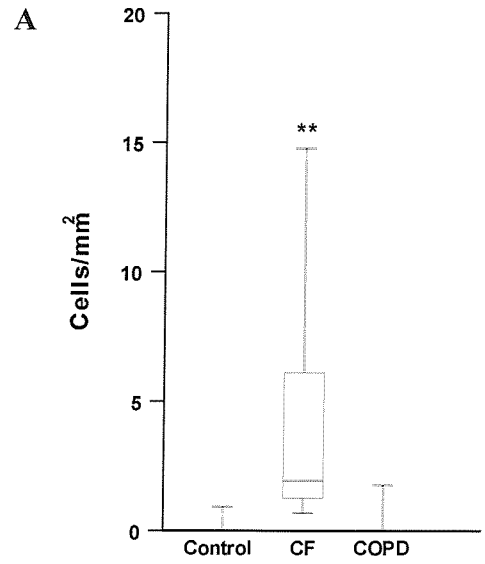


Figure 3.3 Cells stained for mast cell chymase in tissues from (A & B) control, (C & D) CF, and (E & F) COPD patients. Chymase-positive cells were identified by immunohistochemistry with monoclonal antibody CC1 and with AEC, and Mayer's haematoxylin was employed for counterstaining.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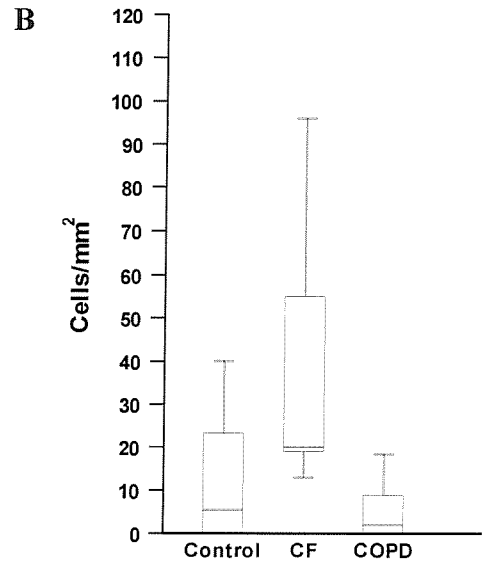
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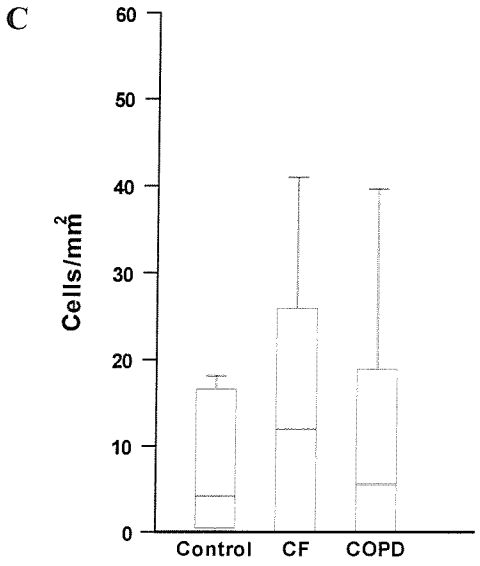
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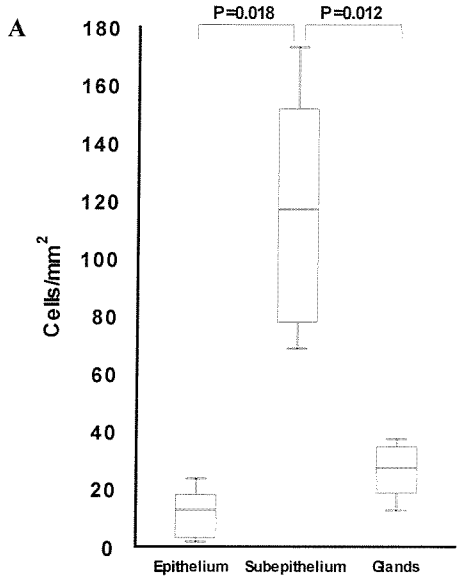
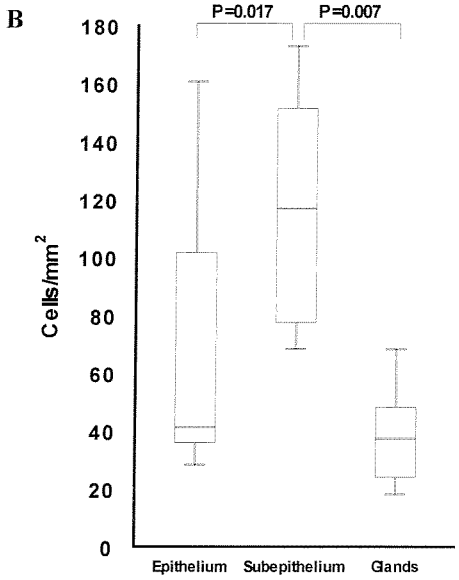
CF
COPD

Figure 3.4 Numbers of cells positive for chymase in (A) the epithelial layer of control (n=7), CF (n=8), and COPD (n=7) tissues, (B) the submucosal layer of control (n=8), CF (n=10), and COPD (n=7) tissues, and (C) the vicinity of subepithelial glands of control (n=10), CF (n=14), and COPD (n=13) tissues. Data are displayed as a boxplot showing the median value, interquartile range, and the tenth and ninetieth percentiles (*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$).


A
P=0.018
P=0.012
180
160
140
120
100
80
60
40
20
0
Cells/mm2
Epithelium
Subepithelium
Glands


B
P=0.017
P=0.007
180
160
140
120
100
80
60
40
20
0
Cells/mm2
Epithelium
Subepithelium
Glands

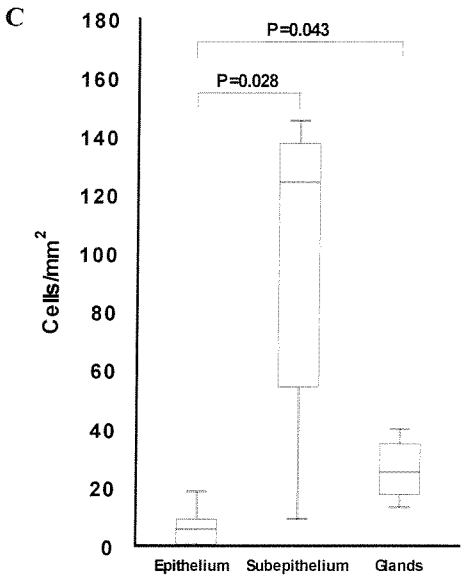
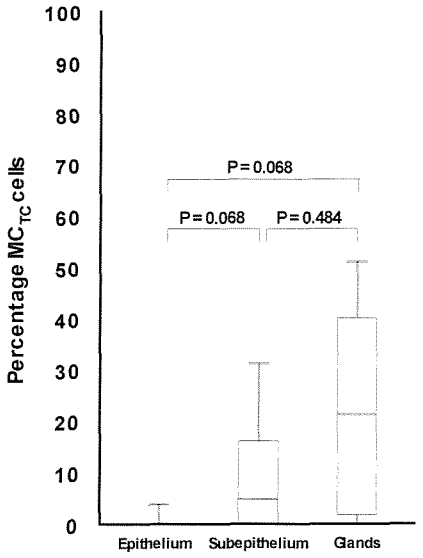
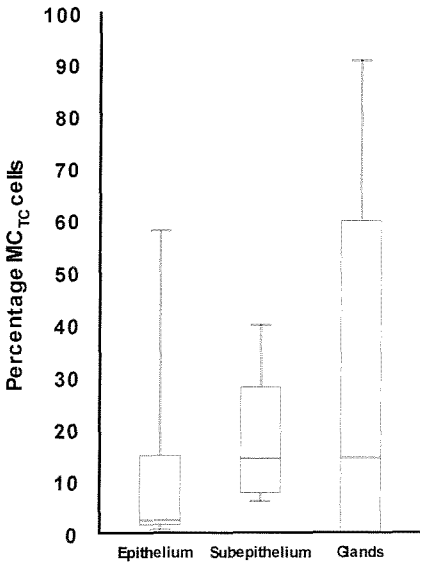

C
P=0.043
P=0.028
180
160
140
120
100
80
60
40
20
0
Cells/mm2
Epithelium
Subepithelium
Glands

Figure 3.5 Distribution of mast cells (tryptase-positive cells) in the different compartments of (A) control, (B) CF, and (C) COPD tissues. Data are displayed as a boxplot showing the median value, interquartile range, and the tenth and ninetieth percentiles.

A


100
90
80
70
60
50
40
30
20
10
0
Percentage MC$_{TC}$ cells
P=0.068
P=0.068
P=0.484
Epithelium
Subepithelium
Glands

B


100
90
80
70
60
50
40
30
20
10
0
Percentage MC$_{TC}$ cells
Epithelium
Subepithelium
Glands

C

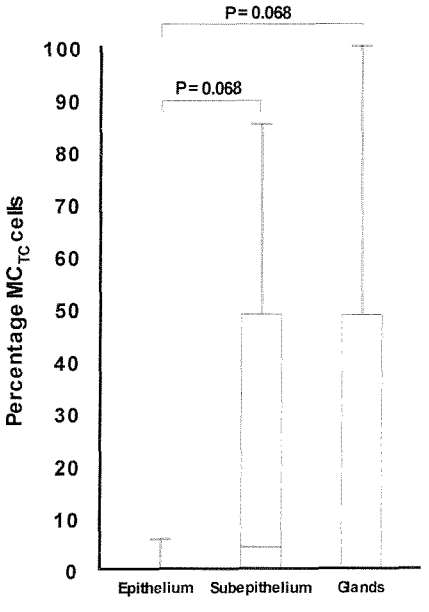

P=0.068
P=0.068
100
90
80
70
60
50
40
30
20
10
0
Percentage MC$_{TC}$ cells
Epithelium
Subepithelium
Glands

Figure 3.6 Ratio of MC_{TC} mast cells in the different compartments of (A) control, (B) CF, and (C) COPD tissues. Data are displayed as a boxplot showing the median value, interquartile range, and the tenth and ninetieth percentiles.

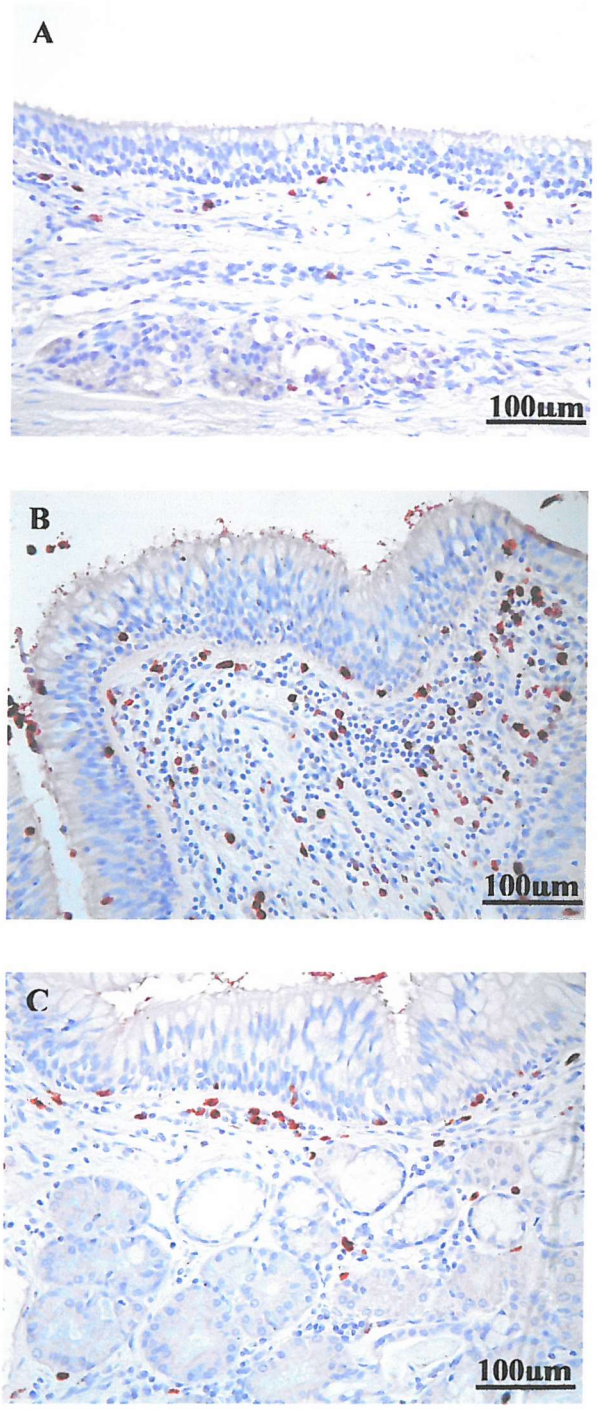
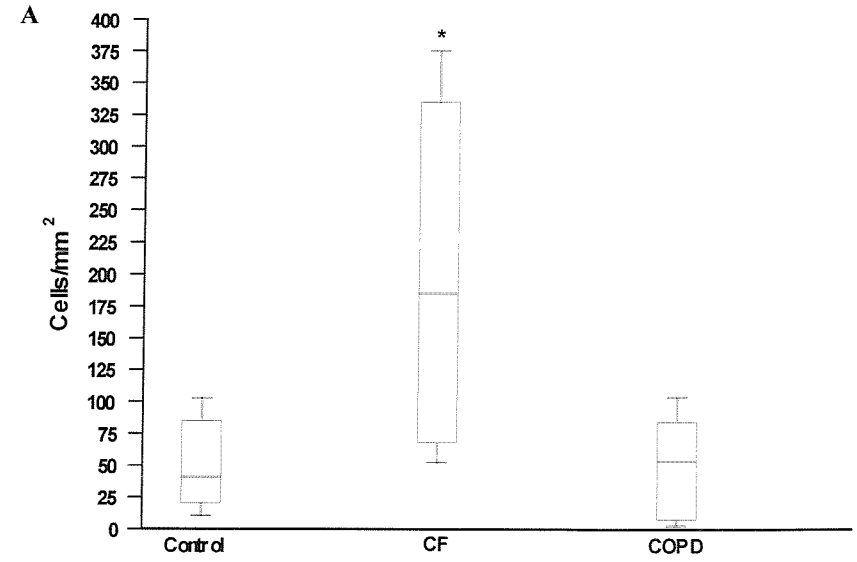
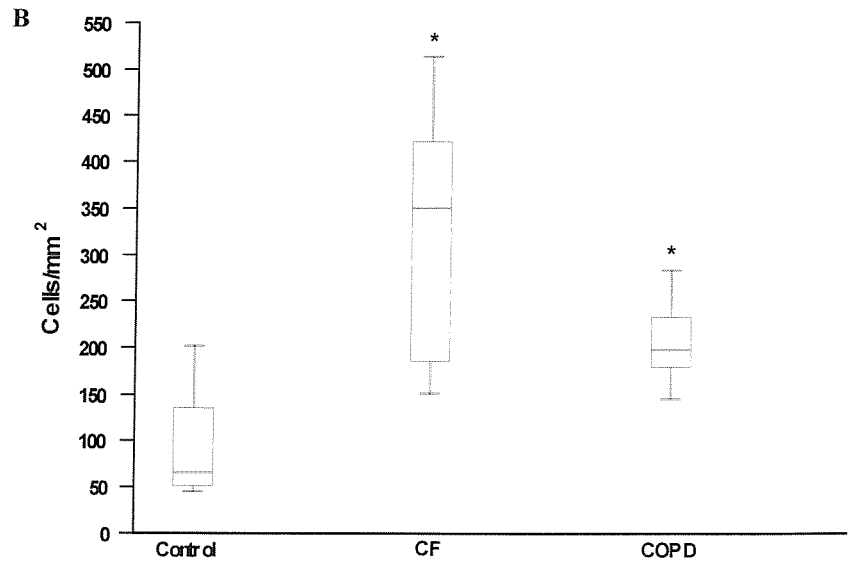


Figure 3.7 Cells stained for neutrophil elastase (NE) in tissues from (A) control, (B) CF, and (C) COPD patients. NE-positive cells were identified by immunohistochemistry with monoclonal antibody NOE and with AEC, and Mayer's haematoxylin was employed for counterstaining.


A
Cells/mm 2
400
375
350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
*
Control
CF
COPD


B
Cells/mm 2
550
500
450
400
350
300
250
200
150
100
50
0
*
*
Control
CF
COPD

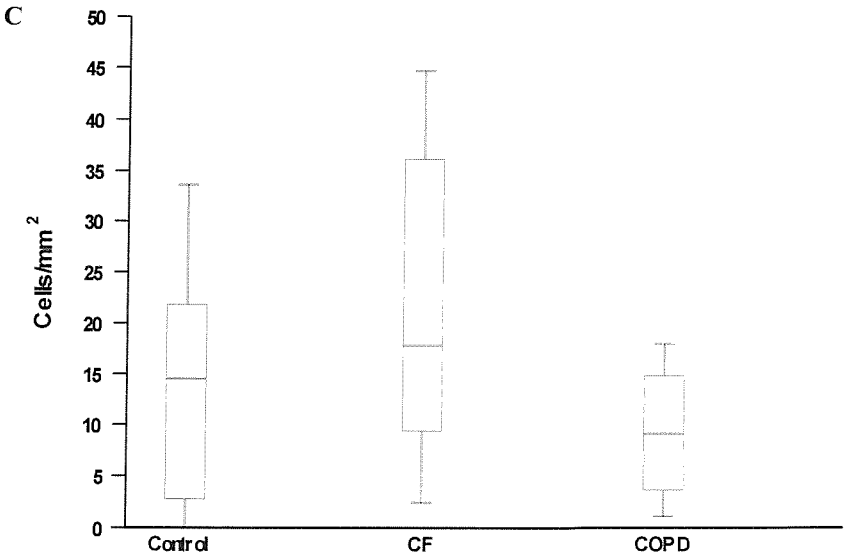

C
Cells/mm 2
50
45
40
35
30
25
20
15
10
5
0
Control
CF
COPD

Figure 3.8 Numbers of neutrophils in (A) the epithelial layer, (B) the submucosal layer, and (C) the vicinity of subepithelial glands of control, CF, and COPD tissues (same numbers as in Fig 3.1). Data are displayed as a boxplot showing the median value, interquartile range, and the tenth and ninetieth percentiles (*: $P \leq 0.05$).

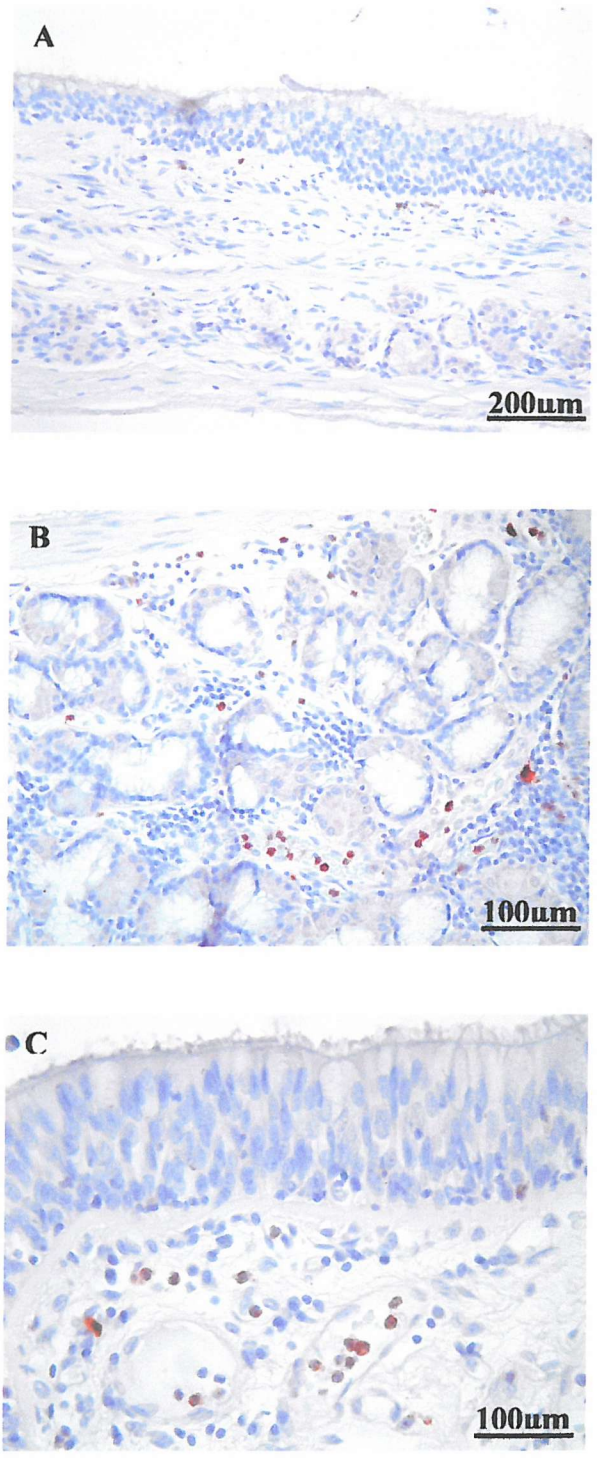


Figure 3.9 Cells stained for eosinophil cationic protein (ECP) in tissues from (A) control, (B) CF, and (C) COPD patients. ECP-positive cells were identified by immunohistochemistry with monoclonal antibody EG2 and with AEC, and Mayer's haematoxylin was employed for counterstaining.

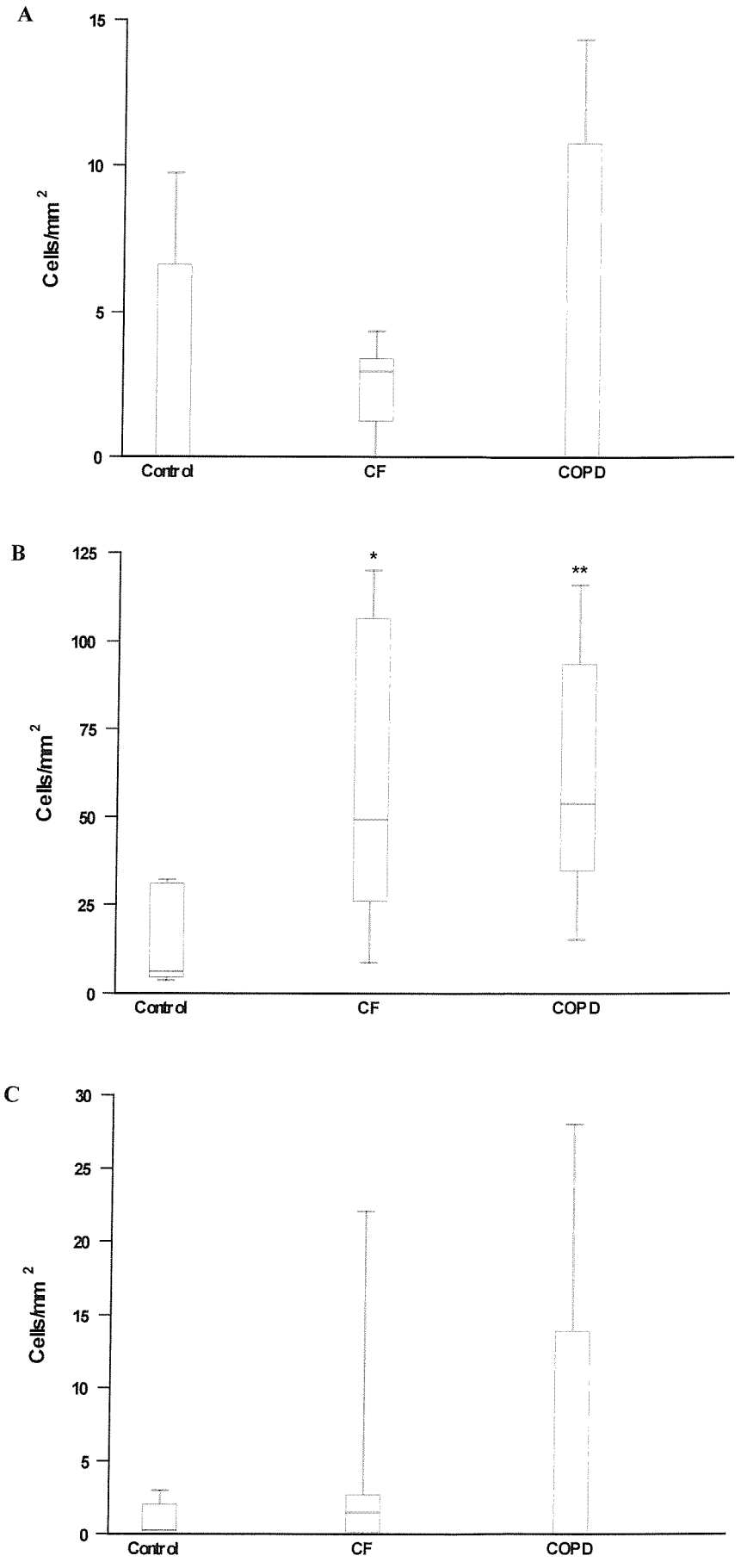

A
15
10
5
0
Cells/mm 2
Control
CF
COPD
B
125
100
75
50
25
0
Cells/mm 2
*
**
Control
CF
COPD
C
30
25
20
15
10
5
0
Cells/mm 2
Control
CF
COPD

Figure 3.10 Numbers of eosinophils in (A) the epithelial layer, (B) the submucosal layer, and (C) the vicinity of subepithelial glands of control, CF, and COPD tissues (same numbers as in Fig 3.1). Data are displayed as a boxplot showing the median value, interquartile range, and the tenth and ninetieth percentiles ($*$: $P \leq 0.05$; $**$: $P \leq 0.01$).

Chapter 4

Chymase as a Stimulus for Cytokine Release and Epithelial Cell Mitogenesis

4 Chymase as a Stimulus for Cytokine Release and Epithelial Cell Mitogenesis

4.1 Introduction

As an interface between the environment and the underlying tissue, the airway epithelium can respond rapidly to environmental insults such as injury and infection by releasing mediators capable of inducing a local inflammatory response (Holgate, 2000). A number of proteases have been reported to induce cytokine expression from the airway epithelium. The allergen Der p1 can induce NF-κB activation in primary epithelial cells, which can in turn increase mRNA expression of cytokines such as IL-8, GM-CSF, TNFα and RANTES (Stacey et al., 1997). Previous observations that mast cell tryptase can stimulate cell proliferation and secretion of IL-8 from the H292 epithelial cell line (Cairns and Walls, 1996), and induce IL-6, IL-8 and GM-CSF release from the 16HBEo- epithelial cell line (Perng et al., in press) have provided evidence that mast cell proteases may play key roles as modulators of epithelial cell function. However the actions of chymase on the epithelium have not been investigated.

In the series of experiments described in this chapter, the potential for chymase to induce mitogenesis and the synthesis and release of a range of inflammatory cytokines from 16HBEo- airway epithelial cells has been investigated. In parallel studies, the response to chymase of primary epithelial cells was compared with that of the 16HBEo- cells. We report that chymase may be an important proinflammatory mediator, which can induce increased expression of mRNA for several key cytokines, as well as their release from epithelial cells.

4.2 Materials and Methods

Details of materials and methods used for the studies in this section are provided below. Other material and methods used, particularly relating to the extraction and purification of human native chymase, as well as the production and purification of recombinant human prochymase are described in Chapter 2.

4.2.1 Materials

Enzyme-linked immunosorbant assay (ELISA) kits for IL-6, IL-8, and GM-CSF, containing capture antibodies, biotinylated detection antibodies, horseradish peroxidase (HRP) conjugated streptavidin, and lyophilised cytokines for standard were purchased from Biosource (Belgium). Maxisorp 96 well plates from Nalge Nunc International (Denmark), potassium phosphate, di-sodium hydrogen phosphate, potassium chloride, 3,5,3',5'-tetramethylbenzidine (TMB), dimethylsulphoxide (DMSO) and sulphuric acid from Sigma; phosphate buffered saline 10x from GibcoBRL (Life Technologies). The CytoTox 96 non-radioactive cytotoxicity assay was purchased from Promega (Madison, WI).

4.2.2 Methods

4.2.2.1 Cytokine release

Cells were seeded in 6-well plates at a density of 2 x 10^5 cells/ml and grown in full growth medium as described in section 2.2.1.1. At confluence, cells were transferred to serum-free medium and incubated for

24 hours before addition of chymase (10-40 mU/ml) or TNFα (100 U/ml) for 3h, 6h, and 24h. Supernatants were stored at -80°C until assaying for cytokines. Cell viability was determined by dye exclusion with trypan blue and the activity of lactate dehydrogenase in supernatants as described below.

4.2.2.2 Reverse transcription-polymerase chain reaction (RT-PCR)

Following removal of supernatants as described above, cells were washed with DMEM and lysed by the addition of Trizol (1ml/well). Cell lysates were mixed with chloroform (200μl), and RNA was extracted from the organic phase after being centrifuged at 12,000 g for 15 min. The RNA contained in the aqueous phase was precipitated by the addition of 500μl isopropanol, incubated at 4°C for 1-2 hours, and centrifuged at 12,000 g for 15 min. The pure RNA was washed twice by the addition of ice-cold 80% ethanol and centrifugation at 12,000 g for 15 min. RNA was then air-dried and resuspended in DEPC-treated water (Promega, UK). Following incubation at 60°C for 10 min to destroy secondary structures, the concentration of RNA was measured by absorption at 260nm, and purity assessed by determining the OD_{260}/OD_{280} ratio in quartz semi-micro cuvettes using a UVIKON 930 spectrophotometer (Kontron Instruments, Watford, UK).

The RNA (1μg) was reverse-transcribed into cDNA using AmV reverse transcriptase and oligo d(T) as a primer in a buffer containing $MgCl_2$, dNTP, and RNAse inhibitor, at 42°C for 60 min followed by heating at 95°C for 5 min. The cDNA was then amplified by PCR using *Taq* DNA polymerase in the presence of a master mix containing specific primer pairs (Table 4.1), *Taq* buffer, dNTP, and $MgCl_2$. PCR was performed in a programmable thermal cycler under the following

conditions: denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 90 sec. This was repeated for a total of 40 cycles, and followed by terminal polymerisation at 72°C for 5 min.

Amplified PCR-products were electrophoresed on a 2% agarose gel containing 0.5µl of ethidium bromide per 100ml TBE buffer, and compared with a DNA reference marker. The gel was visualized under a UV illuminator and photographed, and this was analysed using a software. Each band was quantified by calculating the ratio of the IL-6, IL-8, and GM-CSF cDNA signal to the adenine phosphoribosyltransferase (APRT) control, and mRNA expression presented as a percentage of APRT signal. The APRT, IL-6, IL-8, and GM-CSF specific primer pairs amplified a 246 bp, 628 bp, 186 bp, and a 215 bp PCR product, respectively. The sequences of specific primer pairs are summarised in Table 4.1 .

4.2.2.3 ELISA

Following challenge of monolayers, supernatants were harvested at various time points, aliquoted and stored at -80°C until measurement. To avoid possible catalysis of some components of the antibody kits used, residual chymotryptic activity in the samples was inhibited by adding the chymase inhibitor compound Y-40018 (Yoshitomi Pharmaceutical; Osaka, Japan) at 50µM. Preliminary experiments using cytokine standards with the inhibitor present in the dilution buffer showed that it did not interfere with the sensitivity of the assay. Sandwich-type ELISAs were performed according to the manufacturer's protocol. Briefly, 96 well plates were coated with 100µl per well of the capture antibody diluted at 1µg/ml in coating buffer (0.8% NaCl, 1.42g/L Na_2HPO_4, 0.2g/L KH_2PO_4, 0.2g/L KCl pH 7.4) and incubated at 4°C for 18 hours. All washes were performed with PBS (Sigma) + 0.1% Tween 20, and incubations were carried out at

room temperature with continual shaking (except for the blocking stage) for the time indicated. After one wash to remove non-bound capture antibodies, non-specific binding was prevented by incubating 300µl per well of blocking solution (coating buffer + 0.5% BSA) for 2h. After 4 washes, standards and samples were added (100µl/well) followed immediately by addition of 50µl of biotinylated antibody and incubated for 2 hours. After 4 washes, 100µl/well of streptavidin-HRP (1/1250 or 1/5000 in dilution buffer, according to manufacturer's recommendation) was added and incubated for 30 min. Following 4 washes, 100µl/well of the chromogen TMB was added and incubated until a blue colour developed. The reaction was stopped by the addition of 100µl/well H_2SO_4 2M and optical density at 450nm was read on a plate reader, and data analyzed using the Softmax sofware.

4.2.2.4 Inhibition of chymase

We studied the effect of enzymatically inactive chymase on the cell monolayers. Chymase activity was abolished by preincubation for 30 mins with chymostatin (10µg/ml), or by adding the compound Y-40018 to a final concentration of 50µM. Alternatively, chymase was heat-treated at 70°C for 5mins in a thermocycler. Activity was checked prior to addition to challenge medium, and inhibition with chymostatin, Y-40018 or heat treatment usually decreased chymotryptic activity by at least 95%.

4.2.2.5 Inhibition of cell signalling

Various studies have implicated p38α mitogen-activated protein kinase (p38α MAPK) in the regulation of cytokine production in a number of cell types, including bronchial epithelial cells (Griego et al., 2000; Guo

et al., 2003). To investigate the potential involvement of p38α MAPK in chymase-induced cytokine production, SB 203580 (an inhibitor of p38α MAPK) was first added to the challenge buffer (to a final concentration of 10μM) and cells were preincubated for 30mins prior to addition of chymase at 20mU/ml.

4.2.2.6 Lactate dehydrogenase (LDH) assay

LDH is a stable cytosolic enzyme that is released upon cell lysis. Cell viability was determined using a colorimetric assay kit to measure LDH activity, following the manufacturer's protocol. Briefly, aliquots (50μl) of supernatants were collected from the culture wells after incubation with agents of interest for 24h, transferred into 96 well plates and incubated for 30 minutes with 50μl of LDH substrate mix, protected from light. After the addition of 50μl of "stop solution", absorbance at 490nm was recorded and results expressed as a percentage of LDH in supernatant of cell lysate.

4.2.2.7 Cell proliferation assay

Cell proliferation was determined using a non-radioactive colorimetric assay (Promega), according to the manufacturer's protocol. The assay mix was composed of the compound 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (inner salt; MTS) and an electron coupling reagent (phenazine methosulphate; PMS). MTS is bioreduced by dehydrogenase enzymes found in metabolically active cells into a formazan product that is soluble in tissue culture medium. The quantity of formazan product is proportional to the

number of living cells in culture, and can be measured directly by absorbance at 490nm in the 96 well plates.

Cells (16HBEo-) were seeded at a density of 10^5 cells/ml into 96 well plates, incubated for 24h at 37°C, 5% CO_2, before addition of various concentrations of chymase (10-80 mU/ml), or EGF (10ng/ml) as a positive control. Chymostatin (10µg/ml) was used as an inhibitor of chymase. After 24h or 48 h incubation (37°C, 5% CO_2), 20µl of assay mix (MTS+PMS) were added to each well containing cells in culture with 100µl culture medium (final concentrations of reagents were 333µg/ml MTS and 25µM PMS). Plates were incubated for 1-4h at 37°C, 5% CO_2, and absorbance at 490nm was recorded. Basal values of OD_{490} were determined for wells without cells, and results were expressed as corrected absorbance at 490nm.

4.2.2.8 Statistics

Results are expressed as mean ± standard error (SEM). When analysis of variance (ANOVA) indicated significant differences, Student's *t* test was used to compare results between treated samples and controls. Values of $P \leq 0.05$ were considered significant.

4.3 Results

4.3.1 Purification of Recombinant Prochymase

Four to five days after High Five insect cells were transfected by the recombinant baculovirus containing the gene for human prochymase, a relatively high amount of the activable proenzyme was found to be present in the supernatant, according to the chymotryptic activity measured following activation of a 10µl sample incubated with 10µg/ml DPPI at 37°C for 4h (Fig. 4.1). As prochymase levels increased, cell viability, assessed by trypan blue exclusion, started to decrease. When levels of activatable prochymase started to reach a plateau, the preparation was harvested and stored at -20°C until purification of the prochymase.

After adjusting the salt concentration to 0.2mM, all the prochymase seemed to bind to a heparin-agarose affinity column, since no activity was measured in the run-through following activation of a sample with DPPI. Elution of that column using a NaCl gradient yielded two protein peaks, the second coinciding with prochymase-containing fractions (Fig. 4.2; A). Fractions from this second peak were concentrated to less than 1ml for gel filtration, and loaded onto a S-200 sepharose column. Elution of that column yielded to a single peak of prochymase (Fig. 4.2; B).

The purified prochymase had a molecular weight of about 28kDa (Fig. 4.3; A), which corresponded to values reported before (McEuen et al., 1998b), and the identity was confirmed by Western blotting (Fig. 4.3; B). The specific activity of the recombinant chymase preparation after activation with DPPI was between 23 and 24 U/mg. Specific activity was monitored regularly and did not vary during the whole study. Endotoxin levels in the preparation assessed by the E-toxate assay were less than 0.01 EU / 1U chymase.

4.3.2 Purification of Chymase from Skin Tissue

After the grinding procedure, skin tissue was resuspended in a buffered saline solution and centrifuged to separate cellular constituents and extract chymase. Following lysis of red blood cells by two successive centrifugations in "low salt" buffer (NaCl 0.2M), chymase was extracted from the skin preparation by three centrifugations in "high salt" buffer (NaCl 2M). Two thirds of the total chymotryptic activity was found in the first extract in "high salt" buffer, 22% was detected in the second one and 8.3% in the third one. After readjusting the salt concentration of the pool of chymase-containing extracts to 0.4M, chymase was loaded on a heparin-agarose affinity column, and eluted with a NaCl gradient. Two distinct peaks of chymase were obtained (Fig. 4.4;A), suggesting the presence of two different chymases with different affinity for heparin, as previously reported (McEuen et al., 1998b). Some skin tryptase also eluted as a single peak, which partly contaminated the first peak of chymase-containing fractions. The second peak of chymase, which eluted later, did not contain any tryptase, but corresponded to a relatively small amount of enzyme. Fractions containing the higher concentrations of chymase were chosen for further purification, concentrated to less than 1ml, and loaded on a S-200 Sephacryl column to separate tryptase from chymase. The two enzymes were well separated (Fig. 4.4; B), and were concentrated and stored separately at -80°C.

It appeared that most chymotryptic activity was still present in the run-through buffer following loading of the heparin-agarose column, indicating that most of the chymase did not bind to heparin. Since all the tryptase appeared to have bound and no contamination was detected in the run-though buffer, we decided to carry out another dialysis step to reduce the salt concentration to 0.2 M prior to a second attempt to bind the chymase to a separate heparin-agarose affinity column. The same result was obtained, with 90 % of the activity found in the run-through fractions.

The elution profile of chymase from that second heparin-agarose column was similar to the first one, with two distinct peaks in the same position (data not shown). Using a SBTI-agarose affinity column, as described by Schechter et al. (1986), all the chymase appeared to have bound since no activity was found in the run-though buffer. Fractions eluted with chlorydric acid, and neutralized with a Tris-HCl buffer, contained a single peak of protein (as indicated by UV spectrophotometry), corresponding to fractions that presented chymotryptic activity, suggesting that the chymase was pure, but this method allowed us to recover only about 20 % of the total amount of chymase initially loaded on the SBTI-agarose column. Chymase-containing fractions were diafiltered directly with the storage buffer, concentrated and stored at -80°C. The product size was similar to that of the recombinant enzyme, approximately 28kDa, and the identity was confirmed by Western blotting.

The specific activity of the native chymase preparation employed was between 3.0 and 3.1 U/mg throughout the whole study, and endotoxin levels assessed by the E-toxate assay were less than 0.01 EU / 1U chymase.

4.3.3 Expression of mRNA for Cytokines

In preliminary experiments, it was found that relative amounts of mRNA for the cytokines studied had increased significantly after 3h incubation, but the levels were little different from baseline at the 6h or 24h time points (data not shown), which were associated with increases in cytokine release in the medium (see below). All mRNA extractions were subsequently performed at the 3h time point. Incubation with chymase up to 40mU/ml for 3h provoked a dose-related increase in expression of mRNA for IL-6, IL-8, and GM-CSF mRNA (Fig. 4.6). Increases were also observed after 3h incubation with TNFα (Fig. 4.7). When chymase activity was inhibited with chymostatin (10μg/ml), there was no alteration in

expression of mRNA for any of the cytokines investigated. Densitometry readings from PCR gels and semiquantitative analysis of mRNA are shown (Fig. 4.8).

4.3.4 Cytokine Release from 16HBEo- Cells

After 3h incubation, only chymase applied at 10mU/ml or the positive control (TNFα at 100U/ml) stimulated increased release of IL-6 into the supernatants of 16HBEo- cells, when compared with supernatants from non-treated cells (Fig. 4.9). Maximum chymase-induced IL-6 release was obtained at the 6h time point, with chymase at 10mU/ml. At that time point, release of IL-6 was also increased from cells treated with chymase at 5mU/ml. After 24h, chymase at 20mU/ml also induced an increase in IL-6 release. Maximal release of IL-6 induced by chymase appeared to have occurred at 6h, whereas levels of IL-6 in supernatants still increased at 24h in the presence of TNFα. Interestingly, following 6h incubation with chymase at 40mU/ml, levels of IL-6 were less than those in supernatants from cells incubated with buffer only, and reached approximately 75% of control values after 24h. According to the standard curve prepared for each assay, basal levels of IL-6 in control supernatants corresponded approximately to 100pg/ml after 3h, and reached 200 to 300pg/ml after 24h.

Increases in IL-8 release from 16HBEo- cells in response to challenge with chymase appeared later than those for IL-6. There was no significant change in IL-8 secretion at 3h. After 6h incubation, only TNFα induced some increase in IL-8 release, which was sustained until 24h. In contrast, 6h incubation with chymase at 10mU/ml induced a temporary decrease in IL-8 concentration in the supernatants. After 24h incubation, levels of IL-8 in supernatants from cells treated with TNFα had markedly

risen, and maximum increase in IL-8 release induced by chymase was observed at that time only, with chymase applied at 20mU/ml. Basal levels of IL-8 in control supernatants were approximately 50 to 100pg/ml at 3h, and reached 100 to 150pg at 24h.

Increased release of GM-CSF from 16HBEo- cells challenged with chymase was detected from the 3h time point, though it was relatively limited, sometimes below the sensitivity of the assay, and GM-CSF levels never exceeded a maximum of 10pg/ml. At 3h, chymase at 10mU/ml and TNFα at 100U/ml induced a significant increase in GM-CSF release compared with non-treated controls, which was sustained until the end of the experiments. Release of GM-CSF also increased after 6h incubation with chymase at 20mU/ml, and that increase was more marked after 24h incubation. Interestingly, chymase at 40mU/ml resulted in increased concentrations of GM-CSF in the supernatants at 3h, but these levels had dropped back to control values at the 6 and 24h time points.

4.3.5 Cytokine Release from Primary Epithelial Cells

In preliminary experiments, no cytokine release was detected in the supernatants of primary epithelial cells at the 3 or 6h time points (data not shown). Primary epithelial cells incubated for 24h with buffer alone or various concentrations of chymase did not stimulate the release of detectable levels of IL-6 or GM-CSF (the sensitivity of the assays was approximately 4pg/ml), though incubation with TNFα for 24h induced an increase in release of IL-6 (Fig. 4.10; A). The maximum concentration of IL-6 obtained after incubation with TNFα was about 10 times lower than that obtained from the 16HBEo- cell line under the same conditions, as assessed from the standard curve.

Primary epithelial cells secreted IL-8 following 24h incubation with chymase at 10 to 80mU/ml, though no differences in IL-8 concentrations were observed in the supernatants of chymase-treated monolayers when compared with non-treated control, and only TNFα induced significant cytokine release (Fig. 4.10; B).

4.3.6 Cytokine Degradation Experiments

Since we observed a decrease in concentrations of IL-6 following incubation with chymase at 40mU/ml, and a temporary reduction in concentrations of IL-8 in the presence of chymase at 10mU/ml, we investigated the potential of chymase to degrade IL-6, IL-8, and GM-CSF. Standards of each cytokines were incubated for 24h at 37°C in buffer alone, or in the presence of various concentrations of chymase, or with chymase and the specific inhibitor Y-40018 (10μM). Concentrations of immunoreactive IL-6 or GM-CSF in samples did not appear to be affected by chymase at any of the concentrations tested. However when chymase was present at a concentration of 40mU/ml there was a significant reduction in concentrations of IL-8. This decrease was abolished by addition of the chymase inhibitor Y-40018 (Fig. 4.11).

4.3.7 Inhibition of Chymase Activity

Heat inactivation of the enzyme prior to challenge, or addition of Y-40018 at 50μM in challenge buffer resulted in 90 to 95% inhibition and completely inhibited the release of IL-6 induced by chymase at 20mU/ml (Fig. 4.12; A). Y-40018 alone also induced some increase in IL-6 release, though this corresponded to only about 50% of the increase observed with chymase.

Chymase-induced release of IL-8, which was more important than that of IL-6, was only inhibited at approximately 75% after inactivation of the chymase, and levels of IL-8 in these supernatants were still significantly higher than those obtained from supernatants of cells incubated with buffer alone (Fig. 4.12; B). The compound Y-40018 alone did not induce changes in IL-8 production.

GM-CSF levels were below the sensitivity of the ELISA kit (which detected the lower standard concentration of 3.9pg/ml), so comparison with the positive control was not possible.

4.3.8 Inhibition of p38 MAP Kinase

SB 203580 induced a drop in concentrations of IL-6 measured at 24h, which then represented approximately 20% of control values. SB 203580 also induced a dramatic decrease in IL-8 release, to approximately 25% of control values.

Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in supernatants and cell lysates. LDH release from cells exposed to chymase for 24h did not differ from that from monolayers incubated with buffer alone (Fig. 4.13), though some cytotoxicity was observed following incubation with chymostatin at 10μg/ml.

4.3.9 Cell Proliferation Assay

The lowest concentrations of chymase employed in these experiments (10 and 20 mU/ml) did not induce significant changes in cell numbers after 24h incubation (Fig. 4.14). Chymase at 40mU/ml actually induced a decrease in numbers of viable cells, which was more marked when cells were challenged with a mix containing the same amount of

inhibited chymase and chymostatin in challenge buffer. However, heat inactivation of the chymase prior to challenge prevented a decrease in cell numbers when compared with cells incubated with buffer alone. Epithelial growth factor (EGF) used as a positive control induced an increase in cell numbers after 24h incubation.

At 48h, cell proliferation was reduced in the presence of chymase at any of the concentrations employed. At that time the presence of chymostatin significantly reduced the action of chymase. Heat inactivation completely prevented the effect observed with the same amount of active chymase, and relative cell numbers were similar to those from wells containing cells with buffer alone. Cells incubated with EGF did not show a significant increase in proliferation after 48h incubation.

Table 4.1 Oligonucleotides for amplification of specific cDNA

mRNA	5' sense primer	3' antisense primer	Product size
APRT	GCTGCGTGCTCATCCGAAAG	AGTCAGTGTTGAGATGATGC	246bp
IL-6	ATGAACTCCTTCTCCACAAGCGC	CCGTCGAGGATGTACCGAAT	628bp
IL-8	GCAGCTCTGTGTGAAGGTGCA	CAGACAGAGCTCTCTTCCAT	186bp
GM-CSF	GCATGTGAATGCCATCCAGG	GCTTGTAGTGGCTGGCCATC	215bp

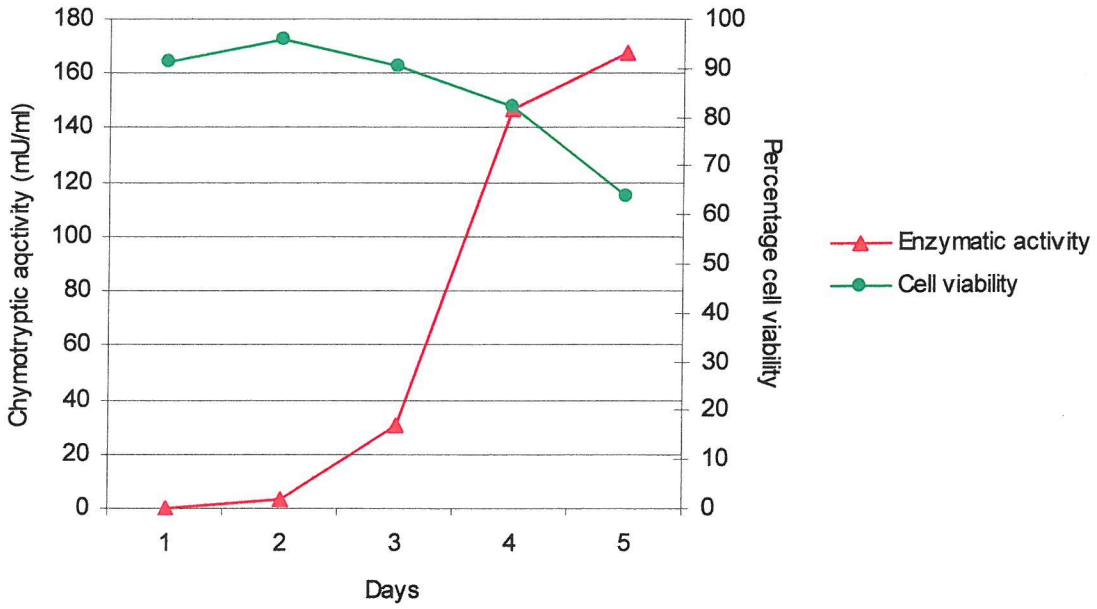


Figure 4.1 Expression of recombinant human prochymase in supernatant from transfected High Five cells. Data is shown for a representative batch, cell viability assessed by trypan blue exclusion and chymotryptic activity following activation with DPPI measured using AAPFpNA as a substrate.

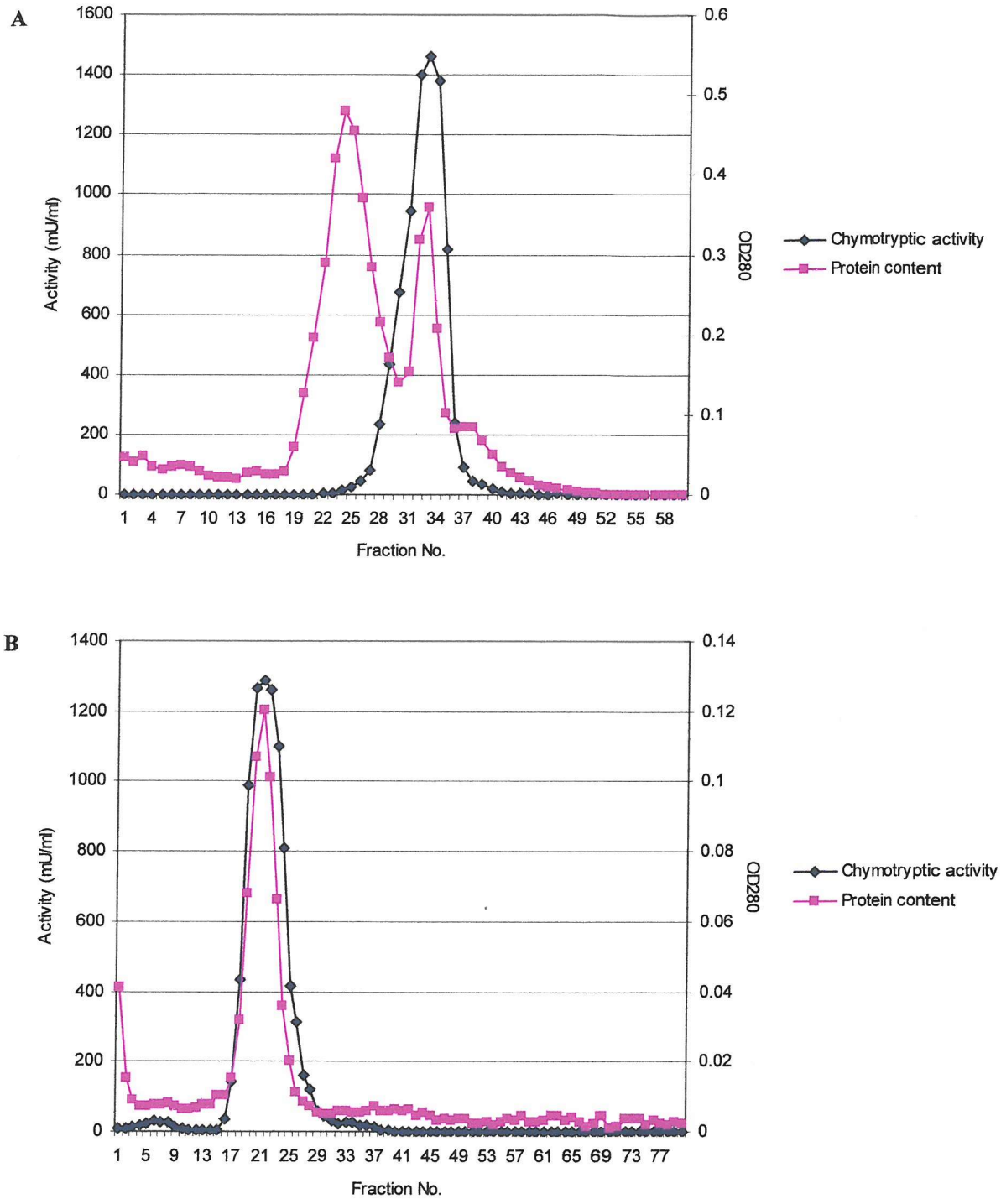


Figure 4.2 Elution profiles of recombinant prochymase from a heparin-agarose affinity column (A) and after gel filtration through a Sephacryl S-200 column (B). Chymotryptic activity (following incubation with DPPI) and protein content (assessed by absorbance at 280nm) of fractions collected are presented.

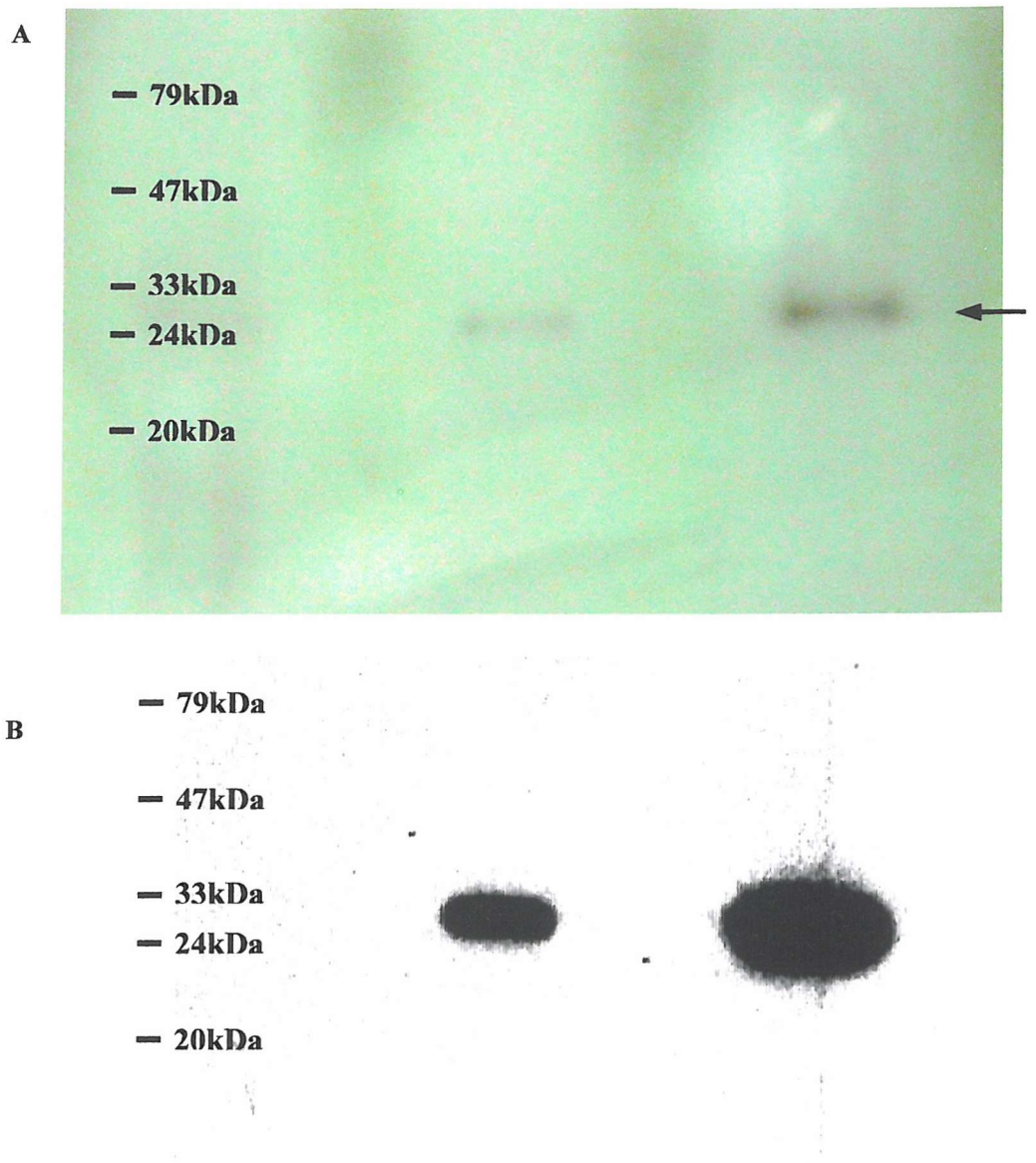


Figure 4.3 Recombinant prochymase (0.2µg and 1µg of protein respectively) bands on a silver stained gel (A) and on a Western blot (B) using chymase specific antibody CC1.

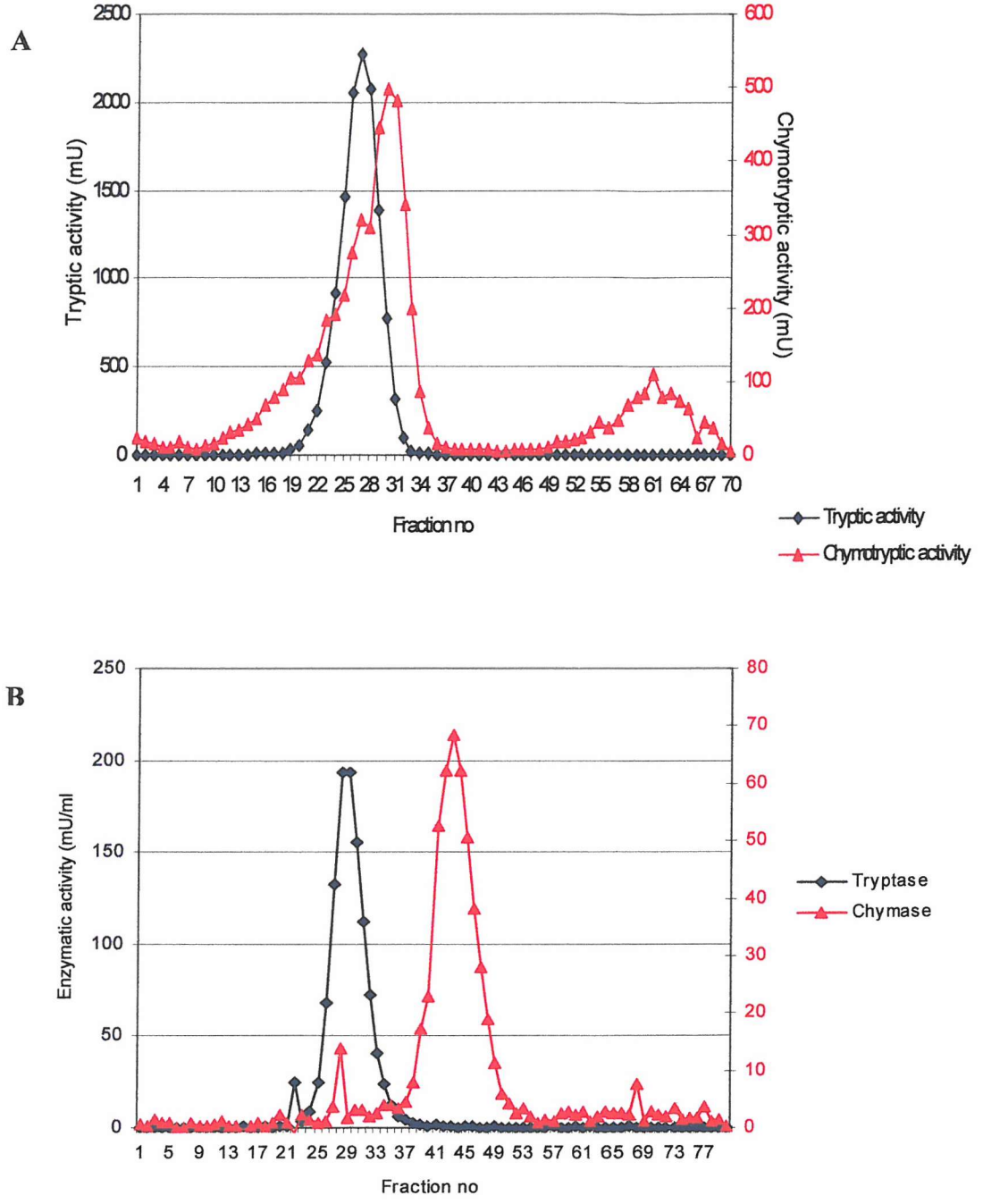


Figure 4.4 Elution profiles of human skin chymase (red) and tryptase (blue) from a heparin-agarose affinity column (A) and after gel filtration through a Sephacryl S-200 column (B). Chymotryptic activity (following incubation with DPPI) and protein content (assessed by absorbance at 280nm) of fractions collected are presented.

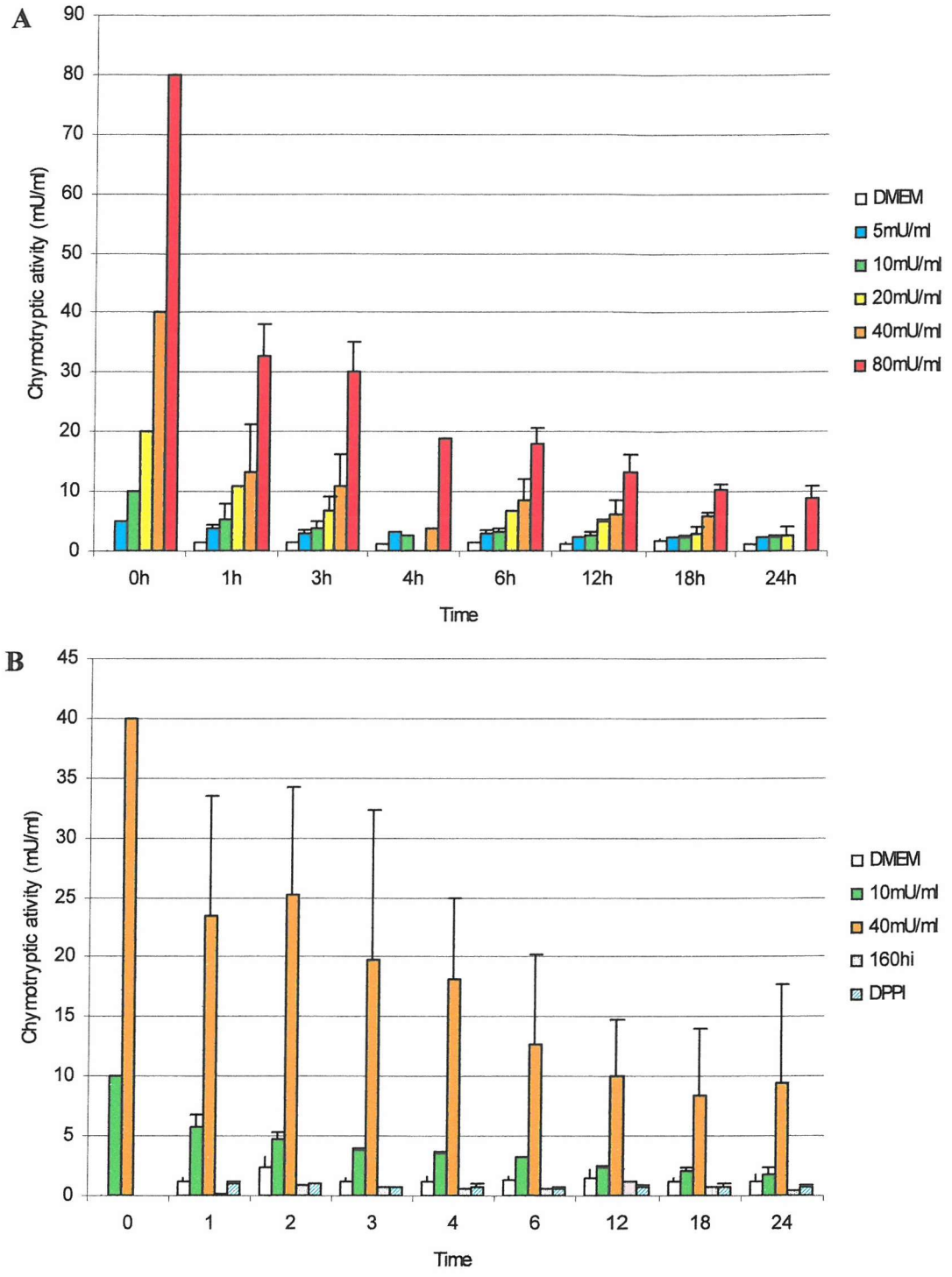


Figure 4.5 Chymotryptic activity in culture medium during challenge of 16HBEo- monolayers with various concentrations of (A) native and (B) recombinant chymase. Activity was expressed as mU/ml using AAPFpNA as a substrate (160hi: same amount as 160mU/ml chymase, after heat inactivation; DPPI: dipeptidyl peptidase I).

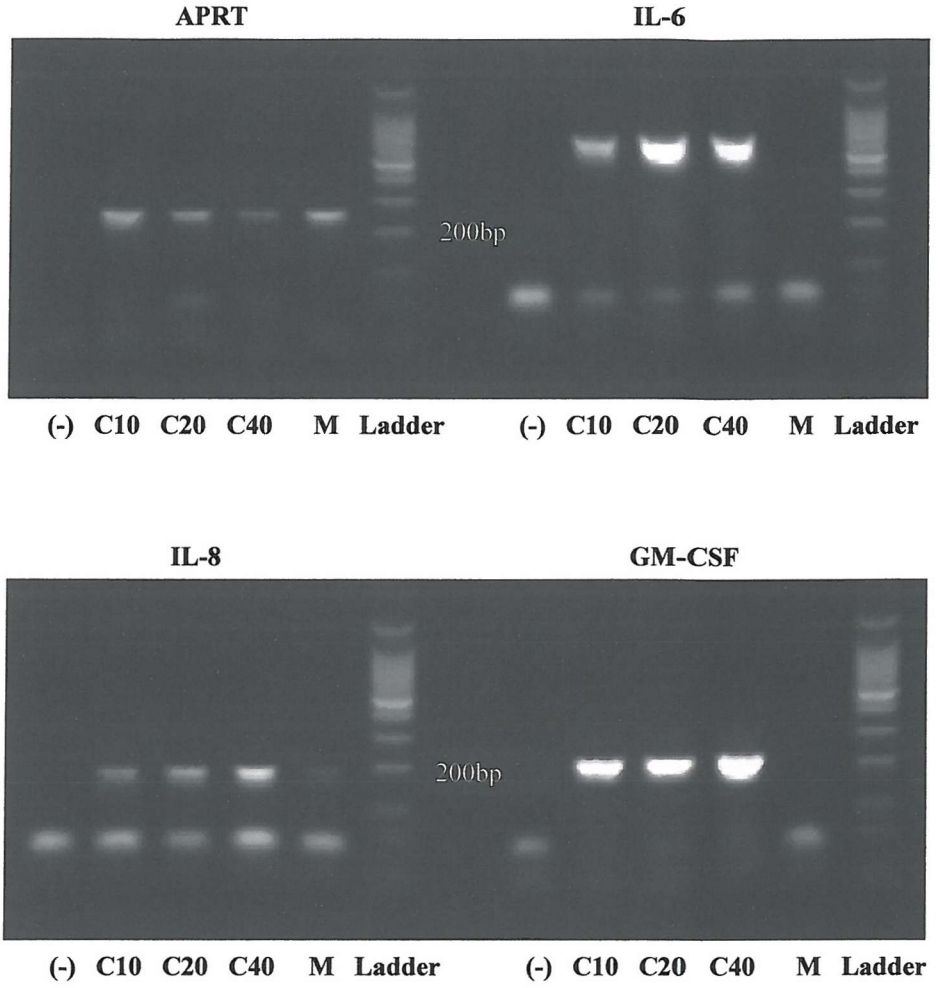


Figure 4.6 Semiquantitative RT-PCR analysis of mRNA for the housekeeping gene APRT, IL-6, IL-8 and GM-CSF following incubation of 16HBEo- cells for 3h with various concentrations of chymase. A representative ethidium bromide-stained gel is shown for negative control (no RNA), cells incubated with, native chymase (C) at 10mU/ml, 20mU/ml, 40mU/ml, or medium alone (M), aside with a ladder to confirm products size (APRT: 246bp; IL-6: 628bp; IL-8: 186bp; GM-CSF: 215bp).

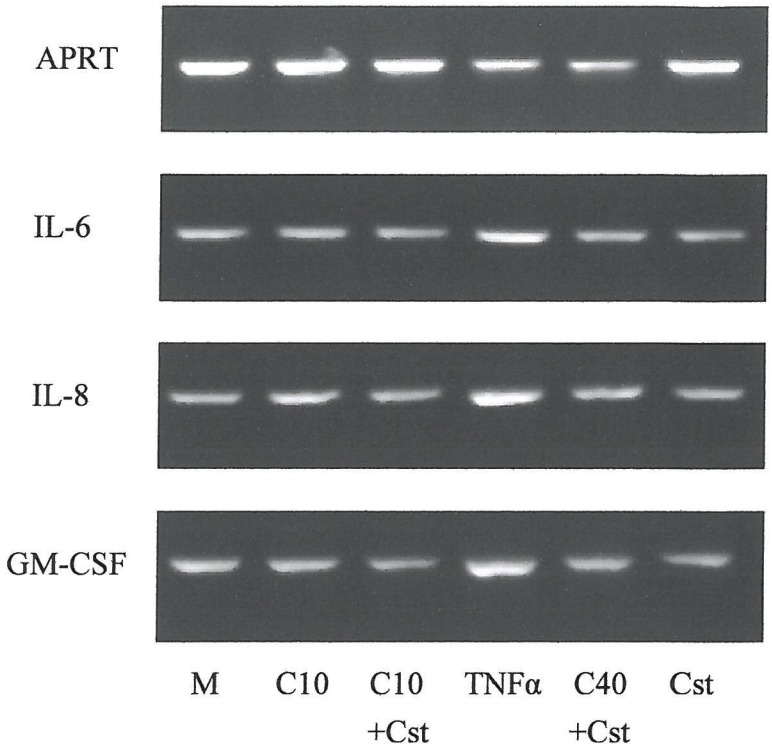
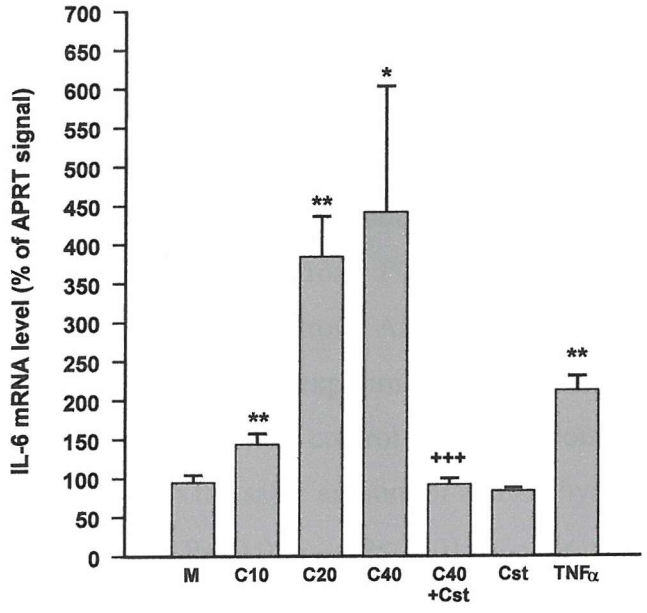
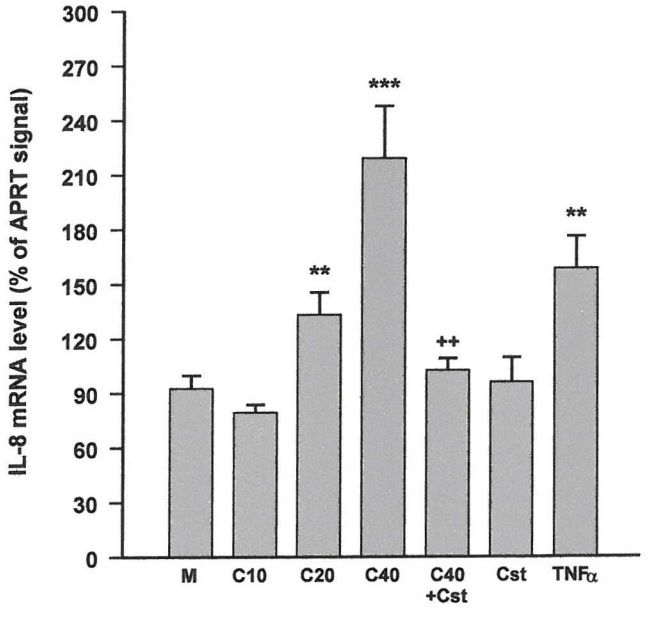


Figure 4.7 RT-PCR analysis of mRNA for IL-6, IL-8, and GM-CSF following incubation of 16HBEo- cells for 3h with medium alone (M), chymase (C) at 10mU/ml or 40mU/ml, TNFα, chymase (10mU/ml) with chymostatin (Cst; 10μM), or chymostatin alone.


IL-6 mRNA level (% of APRT signal)
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
*
**
**
+++
**
M
C10
C20
C40
C40 +Cst
Cst
TNFα


IL-8 mRNA level (% of APRT signal)
300
270
240
210
180
150
120
90
60
30
0
***
**
++
**
M
C10
C20
C40
C40 +Cst
Cst
TNFα

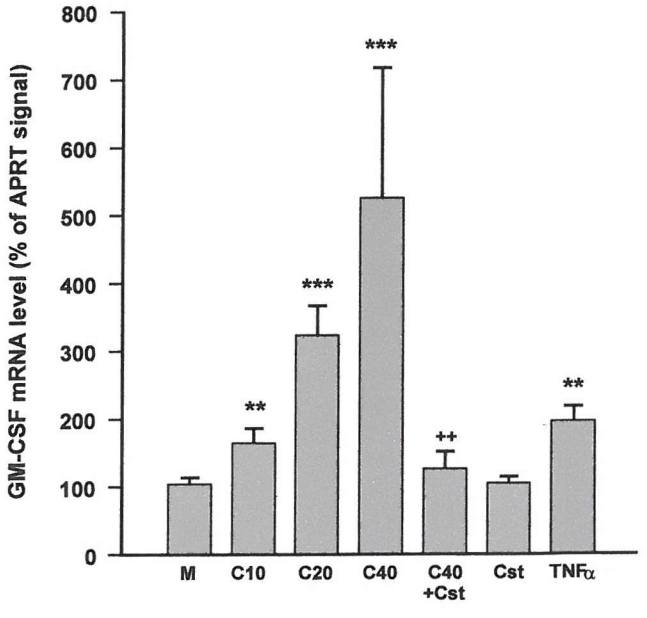

GM-CSF mRNA level (% of APRT signal)
800
700
600
500
400
300
200
100
0
***
**
***
++
**
M
C10
C20
C40
C40 +Cst
Cst
TNFα

Figure 4.8 Semiquantitative RT-PCR analysis of mRNA for IL-6, IL-8, GM-CSF, and the housekeeping gene APRT following incubation of epithelial cells for 3h with various concentrations of chymase or with TNFα. Densitometry readings from PCR gels were determined and expressed relative to the signal from APRT. The mean band density (±SEM) is shown for 5 separate experiments. Statistical significance of increases observed compared with control (medium alone) and significant inhibition (compared with the same amount of active chymase) is indicated (significant increases: *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; significant inhibitions: +++: $P \leq 0.001$).

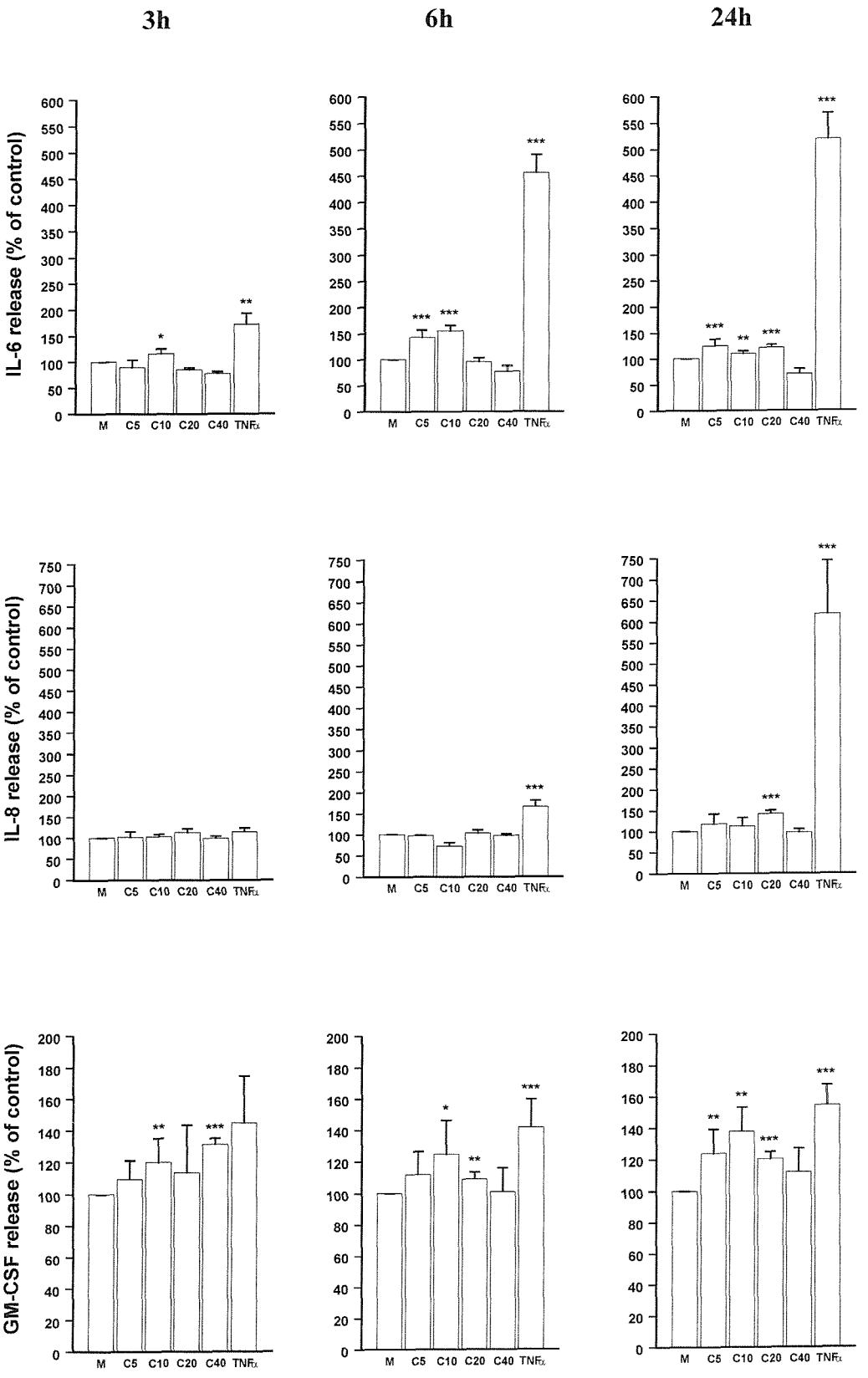

3h
6h
24h
IL-6 release (% of control)
IL-8 release (% of control)
GM-CSF release (% of control)
M
C5
C10
C20
C40
TNFα

Figure 4.9 IL-6, IL-8 and GM-CSF concentrations in supernatants of 16HBEo- epithelial cells incubated for 3, 6 or 24 h with buffer alone (M), chymase (C) at 5, 10, 20 or 40mU/ml, or with TNF-α at 100U/ml. Mean values (±SEM) relative to those of control with medium alone are shown for four experiments performed in duplicate (*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$).

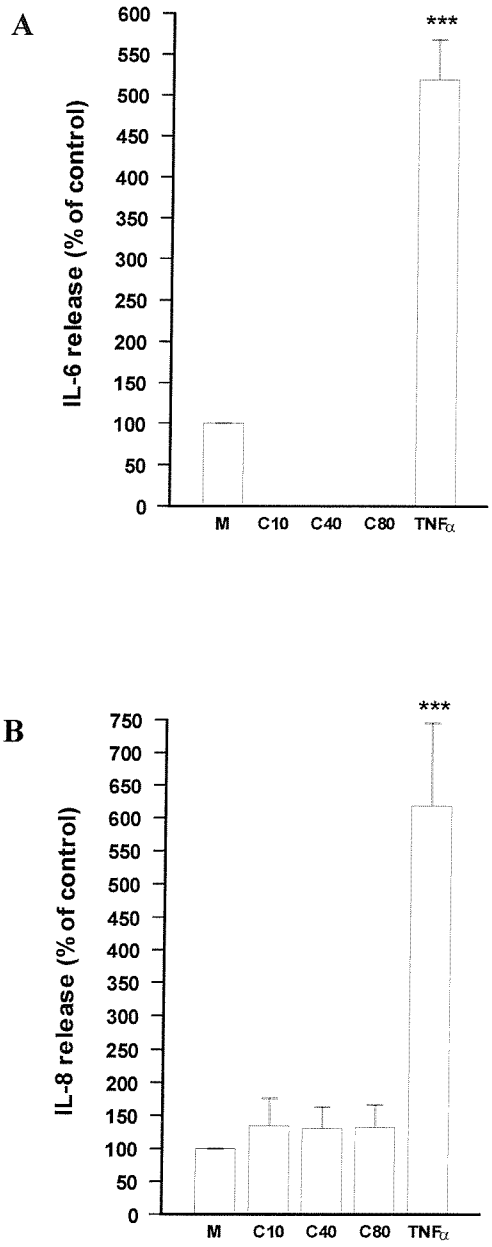
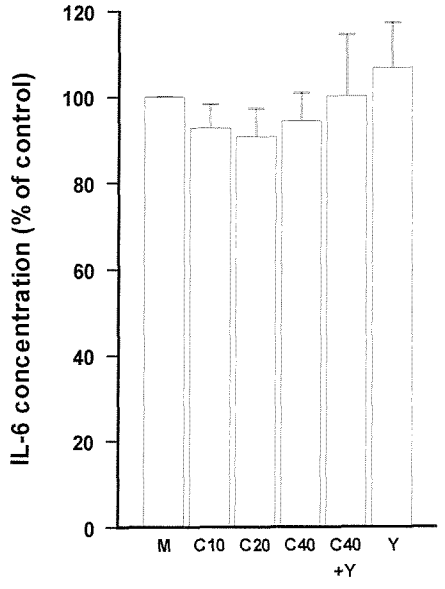
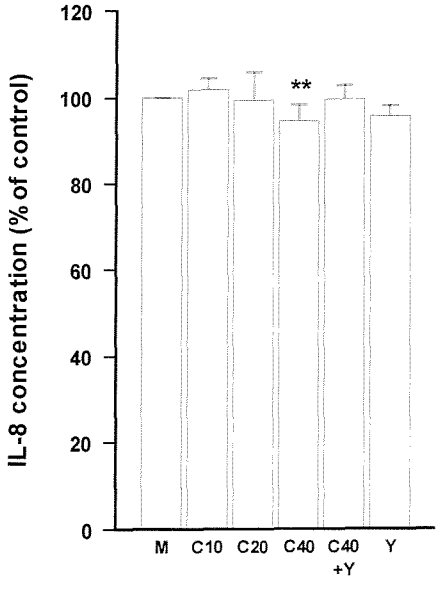


Figure 4.10 Concentration of (A) IL-6 and (B) IL-8 in supernatants from primary human epithelial cells incubated for 24 h with buffer alone (M), chymase (C) at 10, 40 or 80mU/ml, or with TNF-α at 100U/ml. Mean values (±SEM) relative to those of control with medium alone are shown for four experiments performed in duplicate (***: $P \leq 0.001$).

A


IL-6 concentration (% of control)
120
100
80
60
40
20
0
M
C10
C20
C40
C40 +Y
Y

B


IL-8 concentration (% of control)
120
100
80
60
40
20
0
**
M
C10
C20
C40
C40 +Y
Y

C

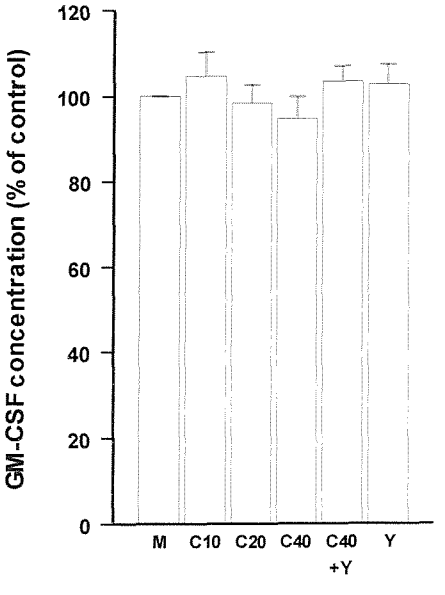

GM-CSF concentration (% of control)
120
100
80
60
40
20
0
M
C10
C20
C40
C40 +Y
Y

Figure 4.11 Concentrations of (A) IL-6, (B) IL-8, and (C) GM-CSF standards (100pg/ml) after 24h incubation with buffer only (M), chymase (C) at 10, 20, or 40mU/ml, chymase at 40mU/ml with the specific inhibitor Y-40018, or with Y-40018 alone. Mean values (±SEM) relative to that of control are shown for four experiments (**: $P \leq 0.01$).

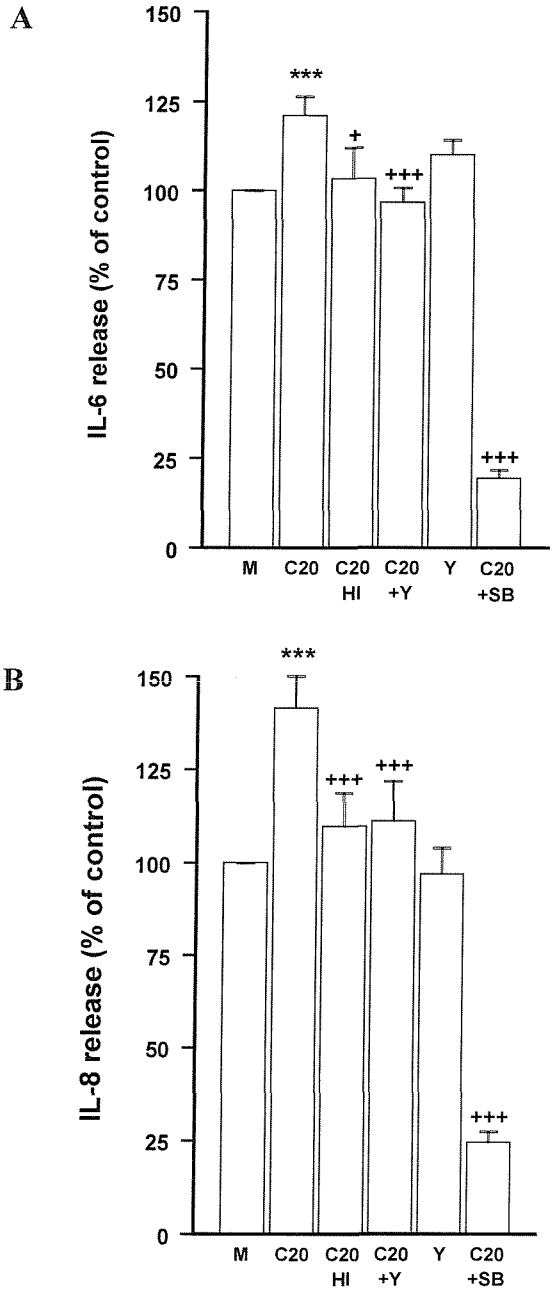


Figure 4.12 Concentration of (A) IL-6 and (B) IL-8 in supernatants from 16HBEo- cells incubated for 24 h with buffer alone (M), chymase at 20mU/ml (C), the same amount of heat-inactivated chymase (C20 HI), chymase with the specific inhibitor Y-40018 at 50μM (C20+Y), Y-40018 alone (Y), or chymase at 20mU/ml in the presence of the p38 kinase inhibitor SB203580 (C20+SB). Mean values (±SEM) relative to that of control shown for four experiments (***: $P \leq 0.001$ when compared with control; +: $P \leq 0.05$; +++: $P \leq 0.001$ when compared with chymase alone).

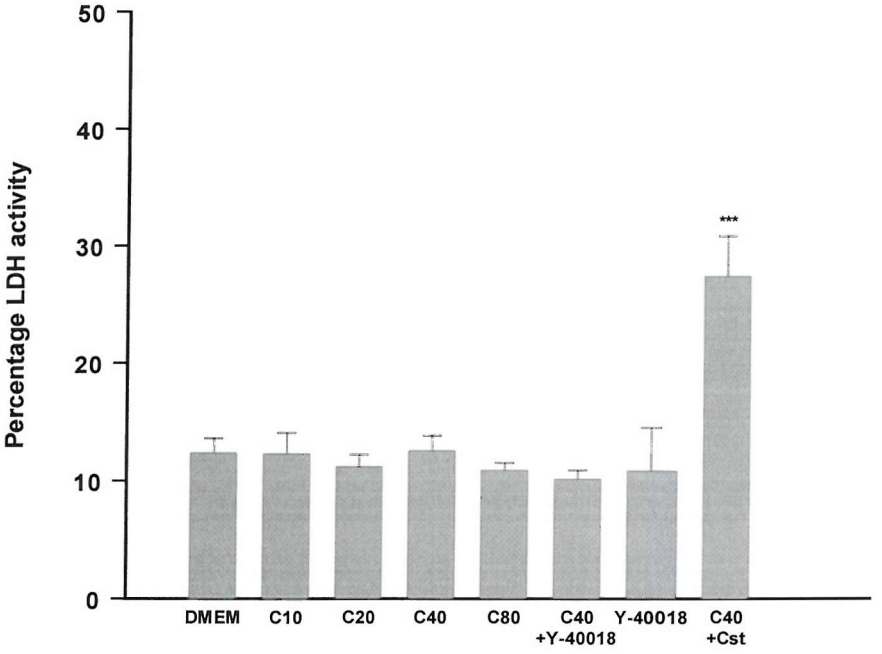


Figure 4.13 LDH activity in supernatants of epithelial cells incubated for 24h with buffer alone, chymase (C) at 10, 20, 40, or 80 mU/ml, chymase (40mU/ml) in presence of the specific inhibitor Y-40018 (10μM), Y-40018 alone, or the chymase with chymostatin at 10μg/ml (C40+Cst). Data are expressed as a percentage of the total levels in supernatants after cell lysis. Mean (±SEM) from 6 experiments are shown (***: P≤ 0.001 compared with buffer control).

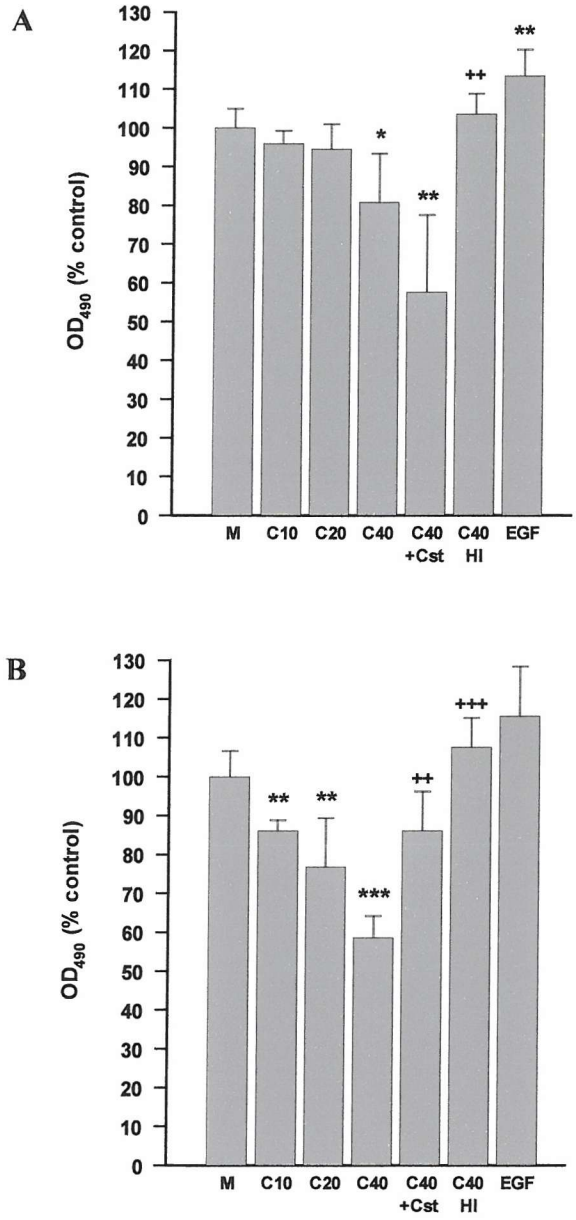


Figure 4.14 Action of various concentrations of chymase (C; 10-40mU/ml), or EGF on the proliferation of 16HBEo- cells after (A) 24h and (B) 48h incubation at 37°C, 5%CO_2. The highest concentration of chymase tested was also inhibited by preincubation with chymostatin at 10μg/ml (Cst). Data are presented as percentage of absorbance at 490nm read from wells with medium alone (M). (*: P≤ 0.05; **: P≤ 0.01; ***: P≤ 0.001; +++: P≤ 0.001 compared with the same amount of active chymase). Control values after 48h incubation corresponded to 118.2 ± 9.6 % of control values at 24h.

Chapter 5

Alterations in Bronchial Epithelium Permeability Induced by Mast Cell Chymase

5 Alterations in Bronchial Epithelium Permeability Induced by Mast Cell Chymase

5.1 Introduction

Epithelial permeability may be altered by a range of chemical irritants, bacterial compounds and inflammatory mediators (Lewis et al., 1995). In the lungs, epithelial permeability can be assessed *in vivo* by the levels of albumin measured in lavage fluids, and it has been noted that albumin concentrations may be closely correlated with levels of markers of mast cell activation such as tryptase and histamine (Salomonsson et al., 1992). Our findings that numbers of chymase-containing mast cells are elevated in the airways of CF patients, and that chymase can induce proinflammatory responses in the epithelial cells, call attention to the potential of this mast cell product to alter epithelial permeability.

Other proteases including rat mucosal mast cell protease II (RMCP II, a chymotryptic serine protease) (Scudamore et al., 1998), Der p 1 (a cysteine protease), and serine proteases found in house dust mite faecal pellets, can alter the permeability of cultured epithelium monolayers, apparently by disrupting the structure of tight junctions (Wan et al., 1999 & 2001). Tryptase, as well as the peptide SLIGKV (an agonist of PAR-2) are both capable of altering TER in monolayers of 16HBEo- cells (Perng et al., in press). The effect of chymase on the structure of the airway epithelium and epithelial permeability has not been investigated.

The aim of the present studies has been to grow monolayers of 16HBE 14o- cells on a porous support and to examine the effects of human mast cell chymase on epithelial structure and transepithelial electrical

resistance (TER). We report that chymase can alter TER through a catalytic process and induce dramatic changes in the structure of airway epithelium.

5.2 Materials and Methods

Details of methods relating to this particular chapter are described below. Description of methods employed, particularly relating to the production and purification of recombinant human prochymase, as well as the extraction and purification of human native chymase are provided in Chapter 2.

5.2.1 Materials

The 24 well culture plates and low density pore inserts were purchased from Falcon. Insulin-selenium-transferrin (ITS), soybean trypsin inhibitor (SBTI) were obtained from Sigma (Dorset, UK). The specific inhibitor of chymase, Z-Ile-Glu-Pro-Phe-CO_2Me (ZIGPFM; Bastos et al., 1995) was prepared by Ferring Research Institute (Chilworth, UK). Staurosporine, wortmannin, PD-98059, U-73122 were purchased from Calbiochem (La Jolla, CA).

5.2.2 Cell Preparation

The 16HBE 14o- cells were seeded onto Falcon culture inserts (0.4 μm pore size) at a density of 1 x 10^5 cells/insert. These inserts incorporate a polyethylene terephthalate (PET) track-etched membrane used as the growth matrix, and were free-standing in 24-well culture plates. Culture medium was added to each culture insert and insert well (0.3 and 0.8 ml respectively), and changed every other day until the cells had grown to confluence and developed a TER of 300-400 Ohm.cm^2. Cells normally became confluent by day 5 to 6, and developed TER by day 9 to 10.

5.2.3 Measurement of Transepithelial Electrical Resistance.

The TER of epithelial monolayers was measured using a micro volt-ohm-meter with a chopstick-style electrode (Fig. 5.1). TER was measured before seeding the cells to assess the contribution of the PET membrane and the bathing solution. For each insert, the value obtained was then subtracted from further readings at given time points, and TER was expressed as a percentage of the initial value.

5.2.4 Cell Challenge

When monolayers reached a TER of 300-400 Ω/cm^2, culture medium was replaced with serum free DMEM supplemented with 2% ITS (insulin-selenium-transferrin, Sigma, Dorset, UK), and monolayers were left to equilibrate for 1h at 37°C / 5% CO_2 prior to the addition of chymase. Concentrations used ranged from 5 to 80 mU/ml of native chymase, and up to 160mU/ml of recombinant chymase, applied inside the insert or inside the well (for apical or basolateral stimulation, respectively). Plates were then replaced at 37°C / 5% CO_2. In some experiments, a 10µl sample was taken at each time point to assess chymotryptic activity. After challenge, culture medium in each insert and well was harvested, aliquoted and stored at -80°C until further analysis.

5.2.5 Inhibition of Chymase

The effect of SBTI (10 µg/ml), chymostatin (10 µg/ml), and the specific inhibitor of chymase, Z-Ile-Glu-Pro-Phe-CO_2Me (ZIGPFM; 100nM) (Bastos et al., 1995) were tested. Inhibitors and chymase were mixed in DMEM+ITS, left to incubate in ice for 20 mins and then briefly

warmed up prior to challenge. Alternatively, incubation at 70°C for 5mins was employed to inactivate the enzyme.

5.2.6 Inhibition of Cell Signalling

To identify potential signalling pathways mediating the action of chymase, the cells of the monolayers were preincubated with various inhibitors of intracellular signalling molecules prior to addition of chymase, and alterations in TER were recorded and compared with those of monolayers treated with chymase alone. The minimum concentrations of chymase that consistently increased TER (20mU/ml) or induced cell detachment (80mU/ml) were added to the apical side of monolayers pretreated with either staurosporine at 1nM (an inhibitor of protein kinase C), wortmannin at 1nM (an inhibitor of PI3-kinase), or PD 98059 at 10μM (an inhibitor of extracellular signal-related kinase-activating kinase, MEK). In parallel experiments, the potential effect of these inhibitors on their own on the TER of monolayers was recorded during 24h incubation.

5.2.7 Immunostaining of Occludin

After monolayers were exposed to chymase for 24 h, some inserts were removed, washed and immediately fixed in cold ethanol for 30 min, followed by a blocking stage with PBS containing 3% BSA. The membranes were cut off the inserts and incubated with mouse monoclonal anti-occludin antibody (15 μg/ml) for 1h at room temperature. Mouse IgG specific for an irrelevant antigen was used as a negative control. After washing to remove unbound antibodies, goat anti-mouse IgG FITC conjugate was applied for 1h at room temperature, excess washed off, and membranes were mounted in mounting medium (Citifluor) on a slide. ZO-

1 was visualised and photographed using a Leica DMRBE fluorescent microscope with a MPS 60 photographic system.

Monolayers were also observed under an inverted light microscope to assess structural integrity and adherence to the membrane.

5.2.8 Measurement of intracellular calcium

The dye Fluo3 fluoresces in the presence of calcium. It is loaded into the cells as the cell permeant acetotoxymethyl ester (Fluo3-AM), but is de-esterified by the cells, limiting its ability to leave the cells by crossing the cell membrane passively. However, Fluo3 can be actively pumped out of the cells, so sulphinpyrazone is added to inhibit this process.

Following resuspension of 16HBEo- cells as described in section 2.2.1.1, cells were washed twice with DMEM and incubated in DMEM+ITS overnight at room temperature on a rotating mixer to recover from trypsinisation. Viability was assessed by trypan blue exclusion, cells were centrifuged at 120g for 5 min, and resuspended in 3ml HEPES-buffered medium containing sulphinpyrazone (0.25nM) and Fluo3-AM (25μg/ml). Cells were left to incorporate the dye by incubation at room temperature for 35 min on an orbital platform, protected from light. Cells were then diluted in challenge buffer and 3ml aliquots were loaded in quartz cuvettes, placed in a spectrofluorometer. The dye was excited at 488nm, and the fluorescence emitted at 530nm was recorded, using a 10nm bandwidth. Each recording lasted 10 min, during which cells were challenged with increasing concentrations of chymase (10-80mU/ml), or calcium ionophore (1mM), ATP (0.3nM), or trypsin (1mM) as controls. At the end of the recordings, total calcium levels were measured by lysing the cells with Triton X-100 (0.1%), and basal fluorescence was determined by chelating calcium with EDTA (20mM). The recordings were corrected for basal fluorescence and presented as percentage of total release.

5.2.9 Statistical Analysis

Variations in TER were analysed using a general linear model (two-way ANOVA), or using the Student's *t* test in the statistical package SPSS for Windows. A value of $P \leq 0.05$ was considered significant.

5.3 Results

5.3.1 Alterations in TER Induced by Recombinant Chymase

After monolayers of 16HBEo- cells were transferred to serum-free medium and left to recover for 1h, initial TER was recorded by means of chopstick electrodes (Fig. 5.1) before adding the compounds of interest. In all experiments, TER of controls (medium alone) slowly increased from 20 to 40% above initial value until the 4h time point, then TER kept stable at least until 24h.

Recombinant chymase activated with DPPI (10μg/ml) was applied to the apical or the basolateral side of monolayers, at concentrations ranging from 10mU/ml to 160mU/ml. When applied to the apical side of monolayers, recombinant chymase from 10 to 40mU/ml induced a dose-dependant increase in TER when compared with non-treated cells until 4h after challenge, after which TER remained above control values until at least 24h (Fig.5.2). However, from 4h, a marked drop in TER was observed for monolayers treated with chymase at 160mU/ml, which was also sustained until at least the 24h time point. This effect was abolished by heat treatment of the chymase. Cell viability did not seem affected by the concentrations of chymase employed, as assessed by trypan blue exclusion after 24h incubation. Interestingly, the same amount of DPPI as that which was present with 160mU/ml, applied alone to the apical side of monolayers, induced a slight increase in TER above control values, though not as marked as that induced by chymase, and which was sustained until 18h.

Applied to the basolateral side, recombinant chymase at 10 or 40 mU/ml failed to induce significant changes in TER when compared with control, but 160mU/ml chymase induced an increase in TER until 3h after

challenge, followed by a marked decrease until the end of the experiments, as seen with apical challenge (Fig. 5.3). Again, this was not observed when chymase had been heat-inactivated. Applying the highest concentration of DPPI used to the basolateral side of monolayers did not induce any changes.

5.3.2 Examination of Monolayers by Light Microscopy and Staining for Occludin

Observation of monolayers following exposure of the apical or basolateral side to concentrations of native and recombinant chymase up to 40mU/ml did not reveal any alterations in the structure of the cell layers. However, at 80mU/ml, gaps started to appear in the monolayers, and cells detached from the sides of the inserts (Fig. 5.4). Using 80 or 160mU/ml recombinant chymase, after 3 to 4h monolayers began to shrink towards the centre of the membranes, until they reached about 60% of their initial surface area. Eventually cells became completely detached from the inserts. This effect was not observed using heat-treated chymase. Cells still adherent were stained for occludin, but no differences in the intensity of fluorescence were observed between chymase-treated and control monolayers (Fig. 5.5).

5.3.3 Inhibition of Cell Signalling

To investigate which intracellular signalling processes may be involved in mediating increased TER and cell detachment, cells of the monolayers were pretreated with specific metabolic inhibitors.

Chymase applied at 20mU/ml as a positive control induced a moderate increase in TER values compared with those of buffer controls

until 6h, after which TER of monolayers treated with chymase kept increasing until at least 24h, while those of cells with buffer only did not vary (Fig. 5.6; A). Staurosporine did not affect the cellular response to this concentration of chymase at any time point. Wortmannin did not induce any differences during the first 6 hours, but from 12h after challenge and until the end of the experiments, there was an increase in TER values compared with those obtained with chymase alone. The TER of monolayers treated with both chymase at 20mU/ml and PD 98059 markedly increased until 4h. The TER remained above values obtained with any other treatment until the end of the experiment.

Chymase at 80mU/ml did not induce significant changes before 12h. Thereafter TER values decreased slowly compared with those of the buffer controls (Fig. 5.6; B), and this appeared to be linked to cell detachment from the side of the inserts. Interestingly, none of the inhibitors used prevented the shrinking of monolayers. The TER values of monolayers treated with chymase at 80mU/ml and staurosporine followed the same pattern as those recorded with the positive control (chymase alone), although TER was slightly increased by the presence of staurosporine until the 6h time point. Wortmannin induced a more pronounced increase in TER that was sustained until 12h, thus delaying the subsequent decrease which correlated with the TER of positive controls. Addition of the MEK inhibitor PD 98059 with chymase at 80mU/ml induced a similar increase in TER as that which was observed with PD 98059 and chymase at 20mU/ml. After reaching a peak at 4h, TER decreased following a pattern similar to that observed with the positive controls.

We also investigated the potential changes on the TER of monolayers, induced when these inhibitors were added alone (Fig. 5.7). Staurosporine induced a relatively small increase until at least 24h comparable to that observed in combination with chymase at 20mU/ml.

The effect of wortmannin was limited, although TER was significantly reduced at 3h, and then increased slightly until 24h. The TERs of monolayers incubated with PD 98059 increased markedly until the end of the experiments, reaching approximately 150% of control values at 24h. This corresponded to about half the increase observed when PD 98059 was added with chymase at 20mU/ml.

5.3.4 Alterations in TER Induced by Human Skin Chymase

Skin chymase applied at concentrations of up to 40mU/ml to the apical side of 16HBEo- monolayers induced a dose-dependent increase in TER when compared with control values. This increase was similar to that observed with the recombinant chymase (Fig. 5.8). Values of TER peaked at 4h, and after a relatively small decrease between 4h and 12h, TER values of monolayers treated with chymase rose again and the increase was sustained until the end of the experiments. When applied to the basolateral side, the same concentrations of skin chymase failed to alter significantly TER when compared with non-treated monolayers (Fig. 5.9).

The increase in TER observed when chymase was applied apically was abolished by heat inactivating the enzyme prior to challenge, or by adding the specific inhibitor ZIGPFM at 100nM (Fig. 5.10). Interestingly preincubating the cells with 15ng/ml pertussis toxin, an inhibitor of G-coupled protein receptors, did not prevent the response to chymase. The inhibitors incubated alone had little effect on the TER.

To confirm the results obtained with pertussis toxin, we measured the release of calcium from the intracellular stores of 16HBEo- cells in suspension using a spectrofluorometer. When cells were challenged with chymase from 10 to 80mU/ml, no calcium release was detected (Fig. 5.11).

However, cells were responsive to calcium ionophore (A23187; 1mM), ATP (0.3nM), or trypsin (1mM) used as positive controls.

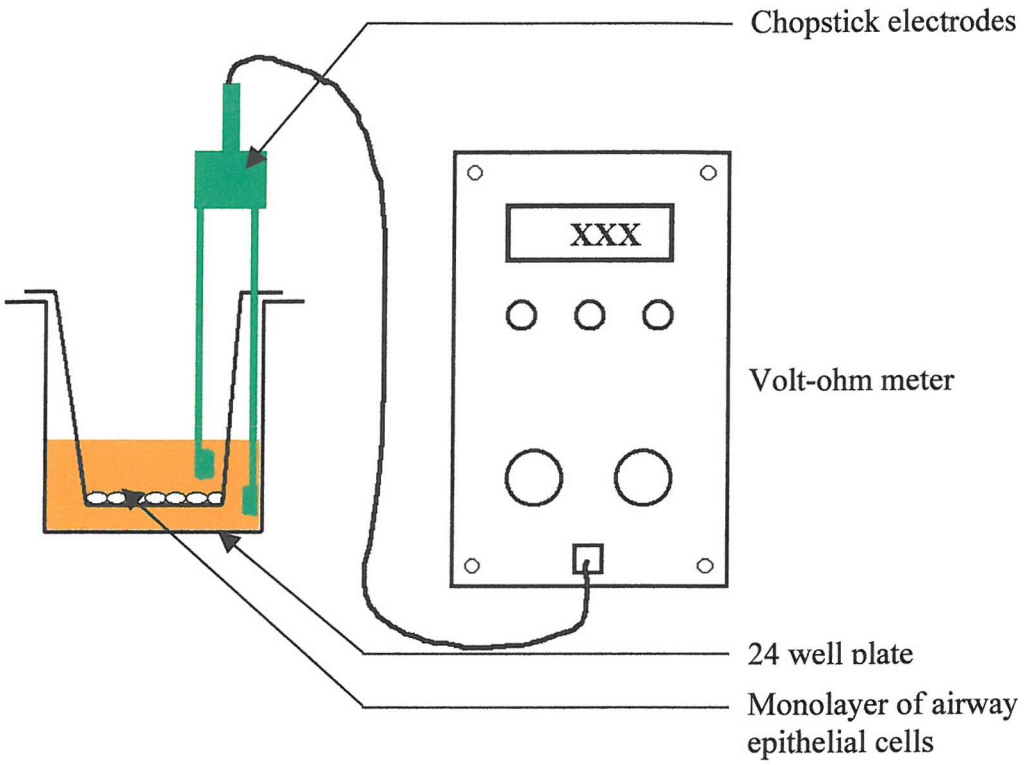


Figure 5.1 Assessment of epithelial permeability by measuring transepithelial electrical resistance (TER). Specially designed chopstick electrodes (represented in green) were inserted into culture medium of both sides (i.e. insert and well) of the epithelial monolayer grown on a porous membrane fitted to the insert, and instantaneous TER was read on the volt-ohm meter at desired time points.

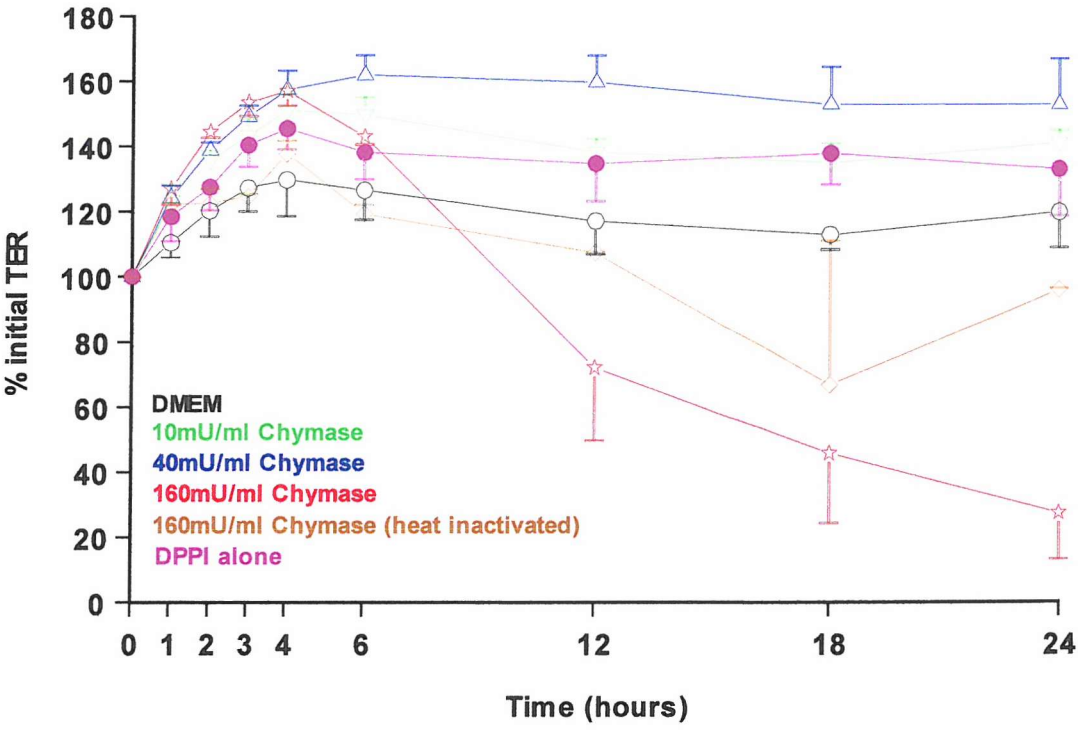


Figure 5.2 Time and dose dependent changes in TER following exposure of the apical side of bronchial epithelial monolayers to various concentrations of activated recombinant chymase. The highest amount of DPPI present in the system, corresponding to the quantity present with 160mU/ml chymase, was also applied on its own. The highest concentration of chymase used (160mU/ml) was also applied after heat inactivation (70°C, 10mins). Results are expressed as the percentage of initial TER at time zero. Mean ± SEM are shown (n=4). For clarity, TER data obtained with intermediate doses of 20mU/ml and 80mU/ml are not included.

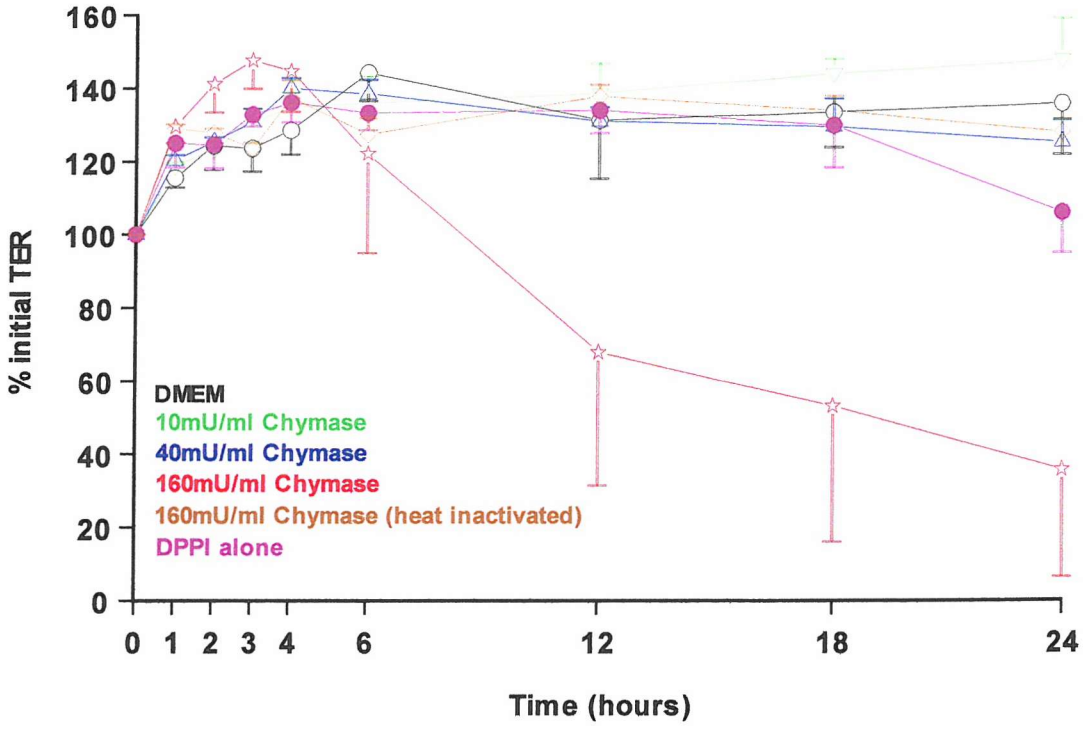


Figure 5.3 Time and dose dependent changes in TER following exposure of the basolateral side of bronchial epithelial monolayers to various concentrations of activated recombinant chymase. The highest amount of DPPI present in the system, corresponding to the quantity present with 160mU/ml chymase, was also applied on its own. The highest concentration of chymase used (160mU/ml) was also applied after heat inactivation (70°C, 10mins). Results are expressed as the percentage of initial TER at time zero. Mean ± SEM are shown (n=4). For clarity, TER data obtained with intermediate doses of 20mU/ml and 80mU/ml are not included.

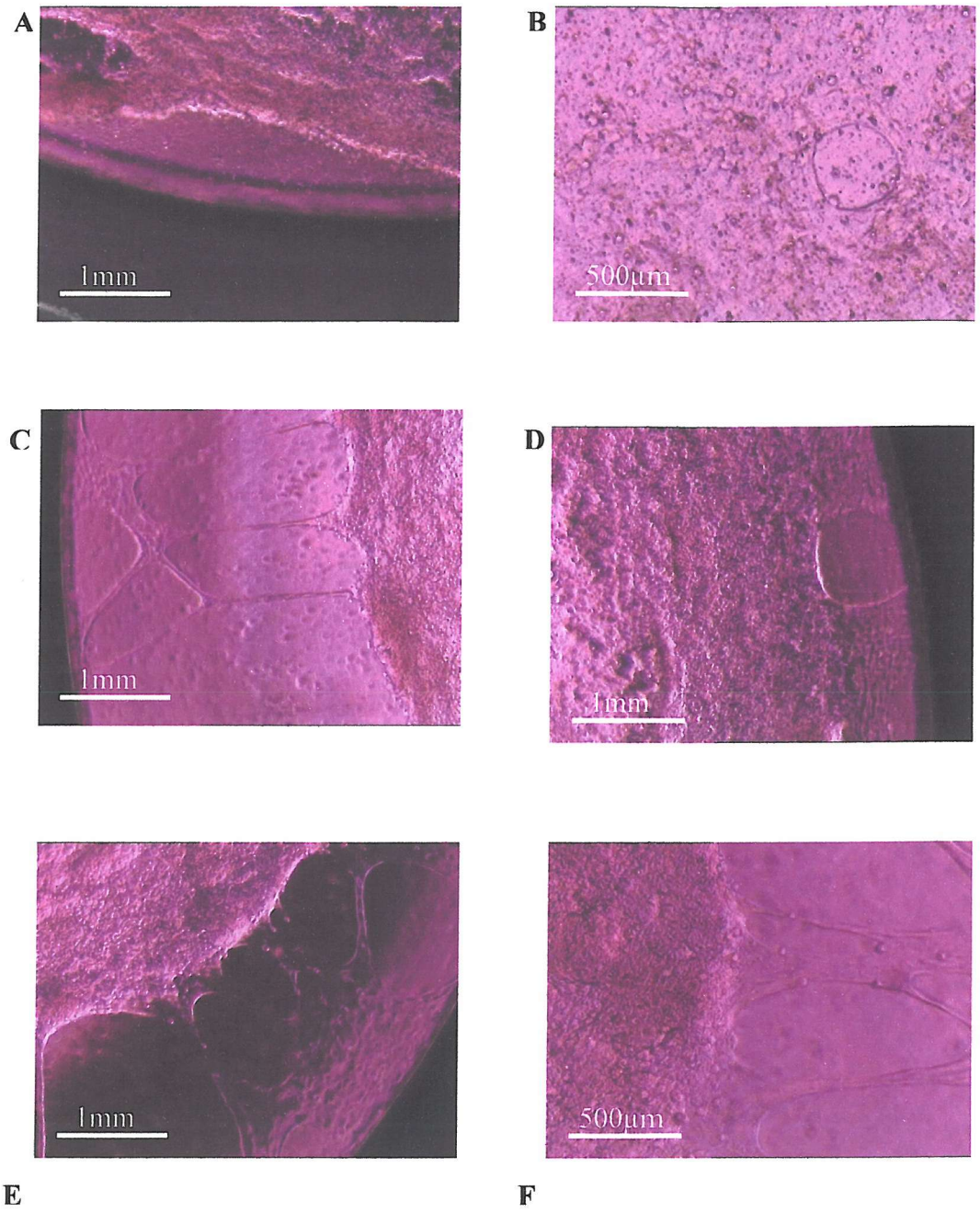


Figure 5.4 Apparent contraction of 16HBEo- monolayers after 24h incubation with recombinant human chymase (A: 40mU/ml on the basolateral side; B: 80mU/ml on the apical side; C-F: 80mU/ml on the basolateral side).

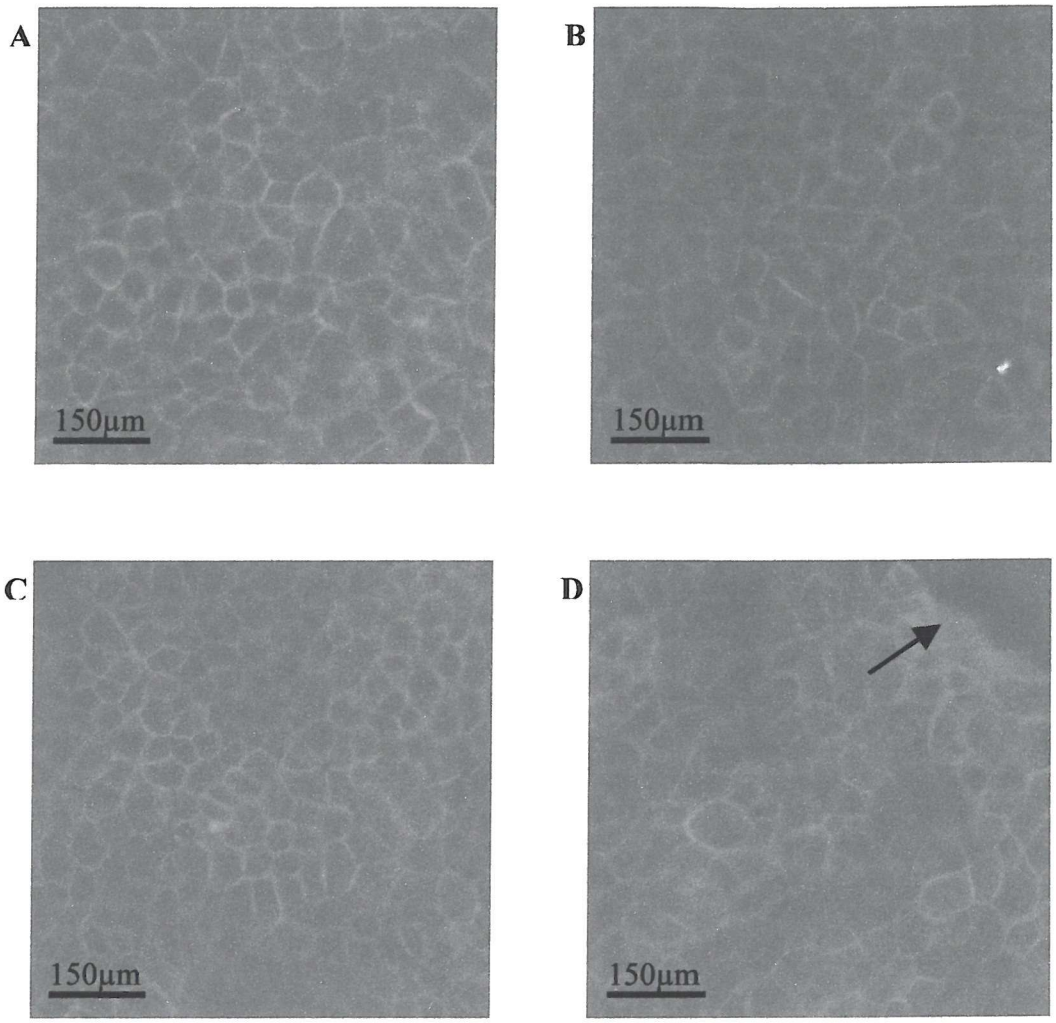


Figure 5.5 Immunostaining of 16HBEo- monolayers using a fluorescent labelled antibody against occludin, after 24h incubation with (A) buffer alone, (B) chymase at 10mU/ml, (C) chymase at 20mU/ml, and (D) chymase at 40mU/ml. Note cell detachment from the side of the insert following incubation with chymase at 40mU/ml.

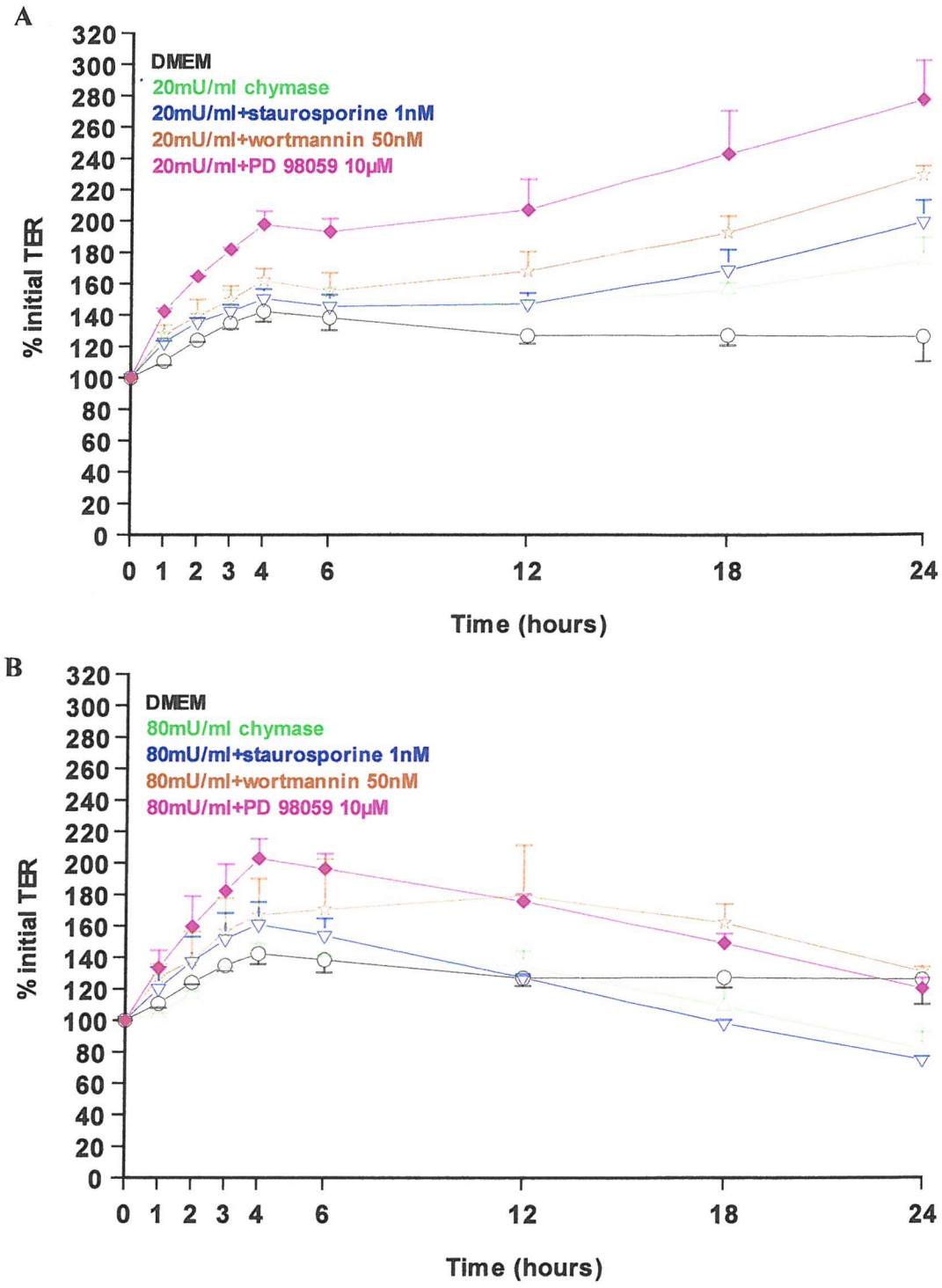


Figure 5.6 Effect of inhibitors of some intracellular signalling molecules on the alterations in TER induced by 20mU/ml (A) or 80mU/ml (B) of recombinant chymase applied on the apical side of 16HBEo- monolayers. Values are given as percentage of initial TER (mean ± SEM from 4 experiments).

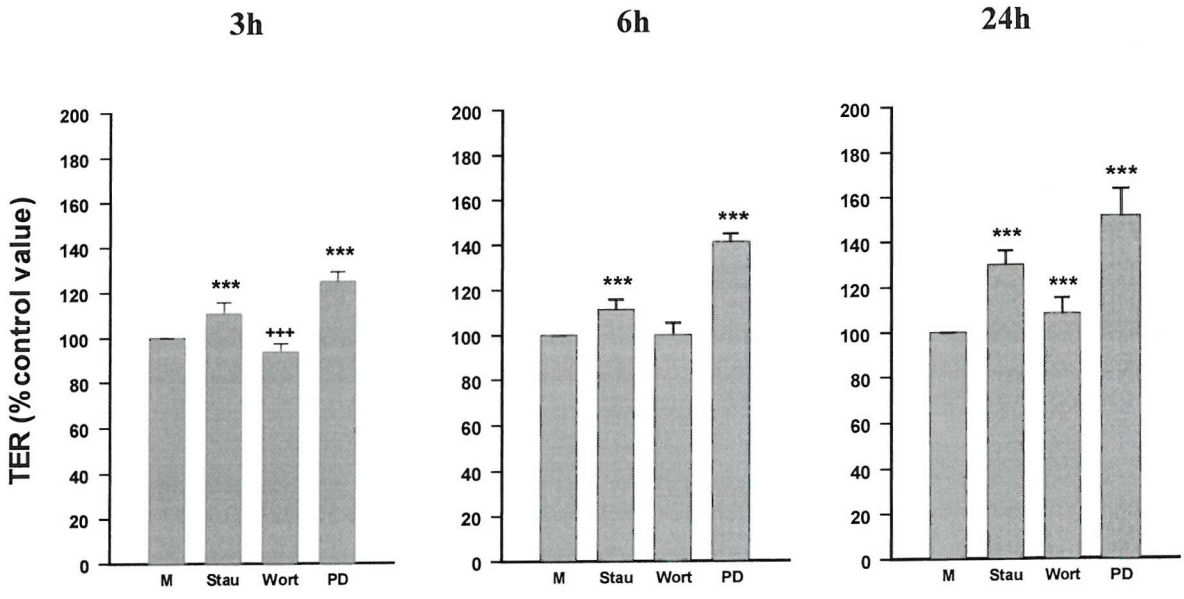


Figure 5.7 Alterations in the TER of 16HBEo- monolayers induced by addition of staurosporine at 1nM (Stau), wortmannin at 50nM (Wort), or PD98059 at 10μM (PD) for 3h, 6h, and 24h. Values are given as percentage of initial TER (mean ± SEM from 4 experiments). Significant increases (***: P≤ 0.001) or decreases (+++: P≤ 0.001) when compared with the medium control are indicated.

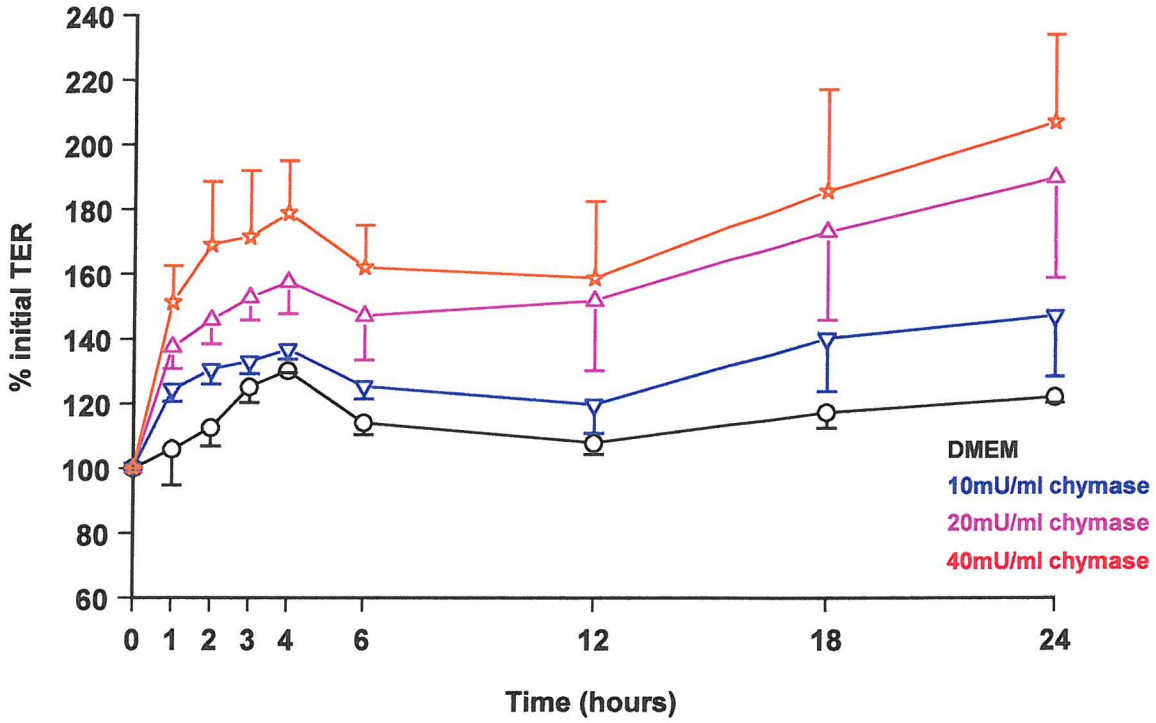


Figure 5.8 Time and dose dependent changes in TER following exposure of the apical side of bronchial epithelial monolayers to various concentrations of native chymase. Results are expressed as percentage of initial TER at time zero. Mean ± SEM are shown (n=4).

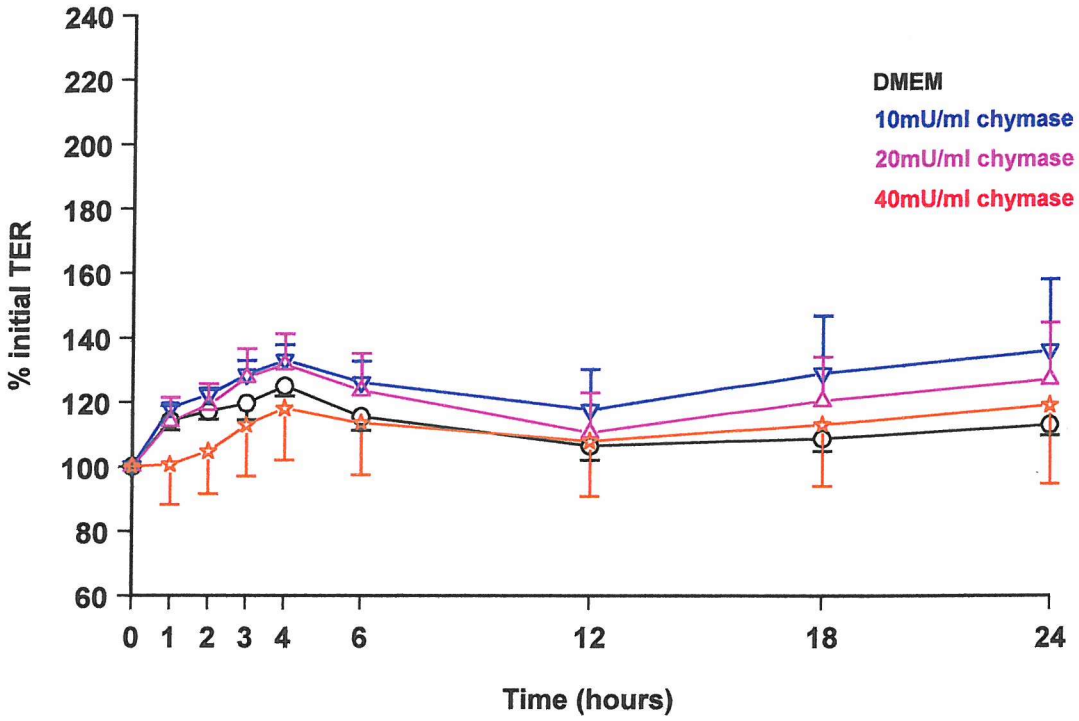


Figure 5.9 Time and dose dependent changes in TER following exposure of the basolateral side of bronchial epithelial monolayers to various concentrations of native chymase. Results are expressed as percentage of initial TER at time zero. Mean ± SEM are shown (n=4).

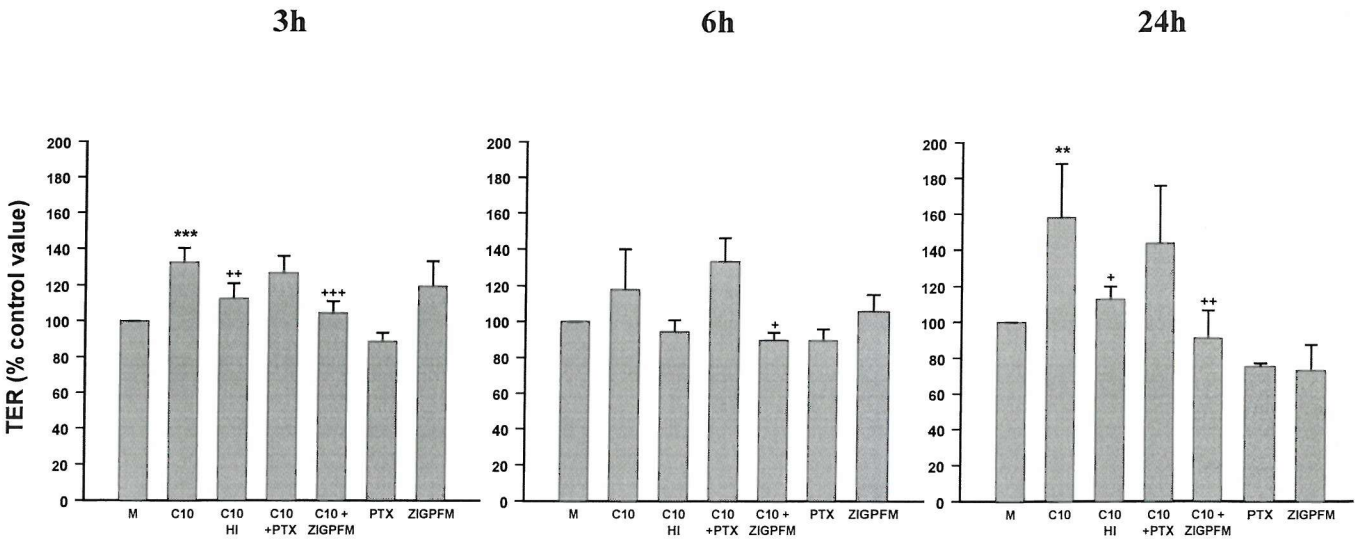
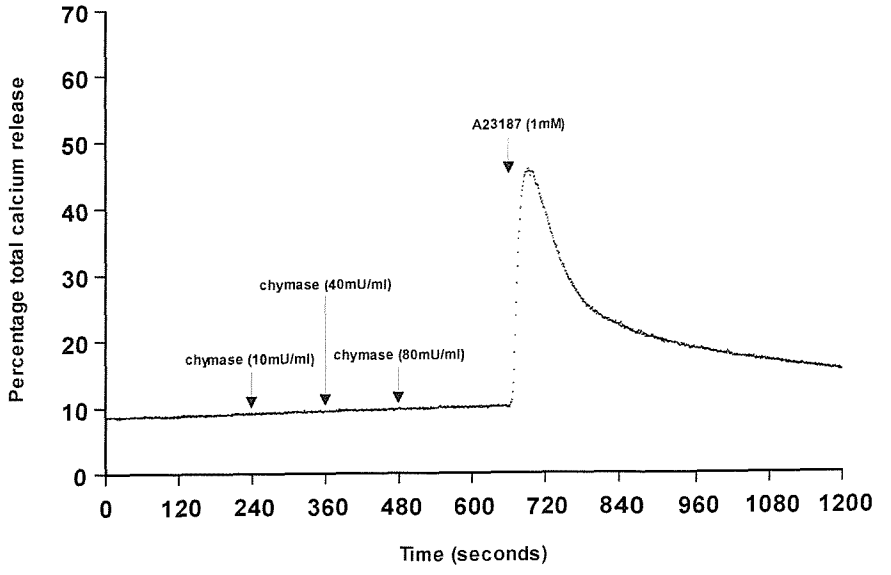
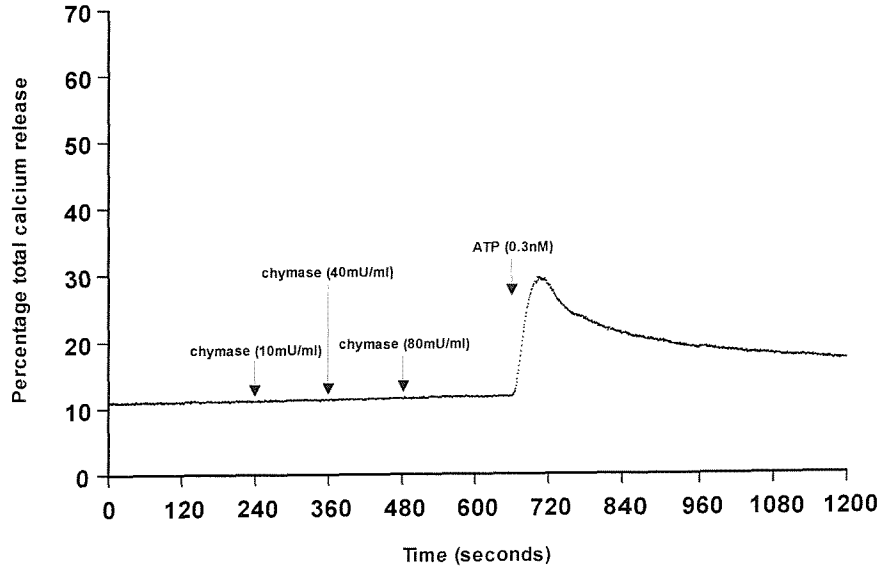


Figure 5.10 Alterations in the TER of 16HBEo- monolayers induced by skin chymase at 10mU/ml (C10) as a positive control, the same amount of enzyme heat-inactivated (C10 HI), chymase at 10mU/ml applied on monolayers preincubated with pertussis toxin (C10 + PTX), or chymase mixed with the specific inhibitor ZIGPFM (100nM; C10 + ZIGPFM) at 3h, 6h, and 24h. The response to pertussis toxin (PTX) or ZIGPFM applied alone is also shown. Values are given as a percentage of the initial TER (mean ± SEM from 4 experiments). Significant increase induced by chymase compared with buffer control (**: $P \leq 0.01$; ***: $P \leq 0.001$) and significant inhibition compared with positive control (+: $P \leq 0.05$; ++: $P \leq 0.01$; +++: $P \leq 0.001$) are indicated.


Percentage total calcium release
70
60
50
40
30
20
10
0
A23187 (1mM)
chymase (40mU/ml)
chymase (10mU/ml)
chymase (80mU/ml)
0
120
240
360
480
600
720
840
960
1080
1200
Time (seconds)


Percentage total calcium release
70
60
50
40
30
20
10
0
ATP (0.3nM)
chymase (40mU/ml)
chymase (10mU/ml)
chymase (80mU/ml)
0
120
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360
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600
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840
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1080
1200
Time (seconds)

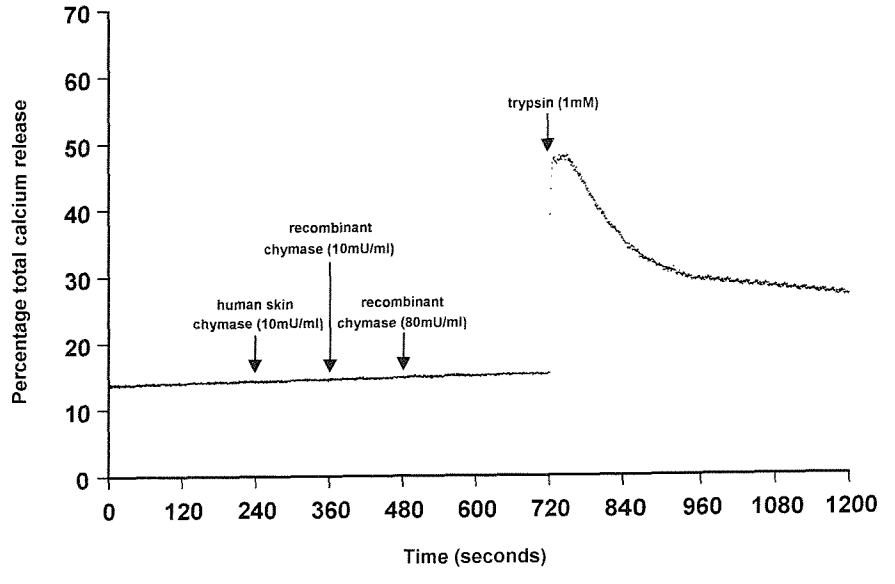
Percentage total calcium release
70
60
50
40
30
20
10
0
trypsin (1mM)
recombinant
chymase (10mU/ml)
human skin
chymase (10mU/ml)
recombinant
chymase (80mU/ml)
0
120
240
360
480
600
720
840
960
1080
1200
Time (seconds)

Figure 5.11 Recordings of intracellular calcium release from 16HBEo- cells in suspension, challenged with various concentrations of chymase, or calcium ionophore (A23187), ATP, or trypsin as controls. Data show percentage of total calcium release, from three representative experiments.

Chapter 6

General Discussion

6 General Discussion

These studies provide compelling evidence that mast cell chymase may be a key mediator of inflammatory processes in the airway epithelium. Substantial numbers of chymase containing mast cells were found in the airways, even of control subjects. Numbers were increased in the airways of CF patients, particularly in the epithelial layers. Chymase was demonstrated to provoke marked increases in expression of mRNA for IL-6, IL-8, and GM-CSF, and to stimulate the release of these cytokines from the epithelial cell line investigated (16HBE 14o-), and to some extent from primary epithelial cells. This major mast cell enzyme was found to reduce epithelial cell proliferation in a dose dependent manner, though it did not appear to be cytotoxic at the concentrations employed. Moreover, both recombinant and native human skin chymase were able to induce alterations in the permeability of the epithelial monolayers, and at higher doses induced dramatic changes in epithelial structure by non-cytotoxic processes.

The observations that (1) the numbers and distribution of mast cell subtypes may be altered in the airways in disease, (2) chymase is capable of stimulating cytokine release and (3) chymase can induce major alterations in the structure of the airway epithelium, will be considered separately.

6.1 Distribution of Mast Cell Subtypes in the Airways of CF and COPD Patients

Tissue sections employed in these studies were cut with a thickness of 2μm to increase likelihood of the same cells being present on sequential sections, allowing for good colocalisation of chymase- and tryptase-

positive cells. This approach also reduced the number of stages during the staining procedure, limiting the risk of antigen degradation linked to the application of successive antigen detection methods. In addition, this approach is likely to have limited the extent of antigen masking, and allowed each monoclonal antibody to be used in optimal conditions. Virtually all mast cells observed contained tryptase, therefore for the purpose of analysis we attributed mast cells to either the MC_T or the MC_{TC} subtype.

We observed that mast cell numbers were elevated in the airways of CF patients, but not in the airways of COPD patients. The mast cell hyperplasia was related to tissue compartment, and statistically significant changes were found only in the epithelial layers. These findings strengthen the idea that mast cells are likely to play a role in inflammatory processes of the lung of patients with CF. The products of mast cell degranulation may have a particular pathological importance in the airway epithelium of these patients. Interestingly, a significant increase in the numbers of chymase-containing cells was also observed in the epithelium of tissues from CF patients when compared with that of control tissues, and increases in mast cell numbers affected both the MC_T and the MC_{TC} subtypes. This also suggests that chymase may be a key molecule in inflammatory processes in the airway epithelium of patients with CF. The presence of elevated numbers of chymase-containing cells in the epithelium of these tissues could be in keeping with the preliminary report of increased numbers of MC_{TC} cells in BAL fluid from CF patients (Irani et al., 1986b). It is likely that alterations in the cellular constituents of BAL fluid reflect similar alterations in the airway epithelium of these patients.

Chymase actions as a potent secretagogue of bovine serous glands has been demonstrated *in vitro* (Sommerhoff et al., 1989), and mucus hypersecretion is a hallmark of CF airways. We did not observe significant

alterations between the tissues from CF or COPD patients and the control tissues, though there was a trend for increased numbers of chymase-containing cells in the vicinity of submucosal glands in CF tissues. If chymase has a similar action on human serous glands *in vivo* as that which has been reported on bovine glands *in vitro*, it may contribute to some extent to the mucus hypersecretion observed in CF airways. We also observed signs of fibrosis in the submucosa of some COPD tissues. Mast cell tryptase has been reported to induce collagen synthesis in human lung fibroblasts (Cairns and Walls, 1997), but the effect of chymase on these cells remains to be investigated.

Another finding from the present study is that the ratio of MC_{TC} cells within different areas of the human airways may be altered in disease. There was a trend for the MC_{TC} subtype to be present in higher numbers in the vicinity of submucosal glands, although differences between compartments failed to reach significance. A previous report based on a small number of non-diseased tissues indicated that MC_{TC} cells were present predominantly in that area (Matin et al., 1992). According to the new data presented here, this observation can be extended to tissues from CF and COPD patients. However, the ratio of MC_{TC} cells was increased in the epithelial layer of tissues from CF patients, and to a lesser extent in the submucosa of tissues from CF and COPD patients. Although the proportion of MC_{TC} cells was still higher in the vicinity of submucosal glands, the trend observed in CF and COPD also suggests that chymase-containing mast cells could contribute to inflammatory processes in the submucosa and epithelial layer of these tissues.

Chymase-containing cells were scarce in the epithelium from tissues of COPD patients that could be examined, though a relatively high proportion of these cells were found in the submucosa of the same tissues. Interestingly, chymase has been reported to induce epithelial detachment in

the skin (Briggaman et al., 1984). It is possible that chymase release from MC_{TC} cells close to the basement membrane could contribute to epithelial cell detachment in the airways. This phenomenon could be exacerbated by the potential of MC_{TC} cells to release a wider range of neutral proteases than MC_T cells. However, the elevated proportions of MC_{TC} cells found in the submucosa of some CF and COPD tissues did not appear to be associated with epithelial detachment. Epithelial shedding has been well documented in asthma, but not in CF or COPD. In the tissues from CF and COPD patients we investigated, there were similar proportions of tissues without an intact epithelium, although mast cell numbers were elevated only in tissues from CF patients. This suggests that increases in the numbers of cells present in the tissue are not directly associated with the extent of tissue damage and remodelling.

Differences in the distribution and proportion of chymase-containing mast cells between the different groups suggest that mast cells, and more particularly MC_{TC} cells are recruited towards the submucosa and epithelial layer in the lungs of CF patients. If both mast cell subtypes come from a common progenitor, fully differentiated MC_{TC} cells could be attracted towards the submucosa and airway epithelium of CF patients at an early stage of the disease process. Indeed the airways of foetuses with CF have been reported to have increased numbers of mast cells and macrophages when compared with those of non-CF foetuses (Hubeau et al., 2001). Alternatively, mast cells of the MC_T subtype could be present in elevated numbers in the airways of CF patients, and elevated proportion of MC_{TC} cells could reflect transient increases in chymase expression within the same cells, concomitant with inflammatory episodes. Differentiation of MC_T cells towards the MC_{TC} subtype could be driven by cytokines such as IL-4, as suggested by *in vitro* studies (Toru et al., 1998; Ahn et al., 2000). Tissue from one of the control subjects investigated was found to have chymase-positive/tryptase-negative cells in the vicinity of glands. This

single case suggests that occasionally, chymase-positive/tryptase-negative mast cells may be present in human lung.

Neutrophils, which like mast cells contain a number of neutral proteases (Walls, 2000a), have been implicated in inflammatory processes of the airways. We observed elevated numbers of these cells in the tissues from CF and COPD patients, and inflammatory processes mediated in part by neutrophils are likely to take place in the airways of these patients. In contrast with mast cells, there were no differences between groups in the numbers of neutrophils in the vicinity of submucosal glands. The presence of neutrophils in clusters, close to blood vessels, and the observation that numbers were almost as numerous in the epithelium as within the submucosa suggests that neutrophil recruitment into the lung tissues is dependent on mechanisms different from those of mast cells. Being present in relatively high numbers, neutrophils may contribute a great deal to local inflammatory processes. Eosinophilia, which was observed in the subepithelial layers of some tissues from CF and COPD patients, did not seem to be a general feature and showed great variability within each group of patients examined. This suggests that eosinophils may participate in some cases of lung inflammation, but their contribution, compared to that of mast cells or neutrophils, may be variable or sporadic in nature.

Our findings highlight the potential involvement of mast cells in CF, as well as in COPD, and suggest that mast cells are key effector cells in inflammatory processes in the human lung. The specific increase in numbers of chymase-containing cells in tissues from CF patients, and more particularly in the epithelium, calls attention to this neutral protease as having an important role in the pathophysiology of CF.

6.2 Chymase as a Stimulus of Cytokine Release

Chymase was found to provoke marked increases in the expression of mRNA for IL-6, IL-8, and GM-CSF and stimulated the release of these cytokines from epithelial cells. Specific inhibitors of chymase or heat treatment of the enzyme abolished these effects. These findings indicate the potential for chymase to mediate the release of proinflammatory cytokines from epithelial cells via a mechanism requiring an active catalytic site.

The activity of chymase relies mostly on the presence of an intact catalytic site. Chymotryptic activity, which was monitored during the experiments using AAPFpNA as a substrate, decreased relatively rapidly during incubation in serum-free medium at 37°C, with a half-life of approximately 1 to 2h for both the recombinant and the skin chymase. This could be due to autocatalysis, denaturation of the protein during incubation, competitive interaction with other substrates present in the supernatant, adhesion to the plastic surfaces, or a combination of these different factors. The use of PEG 4000 at 0.01% was found to reduce significantly the adhesion of chymase to plastic surfaces during purification, and was added at that concentration in the storage buffer. However, the final concentration of PEG 4000 in challenge buffer after dilution to working concentrations was negligible, and not sufficient to prevent adhesion of chymase to the plates or inserts. No PEG was added during challenge to avoid potential interference with the cell monolayers.

After 24h incubation at 37°C, chymotryptic activity detected in supernatants remained only in wells containing initial concentrations of active chymase above 40mU/ml. Therefore, it would seem that only chymase used at 40mU/ml or more would be able to elicit a relatively long-lasting cellular response after 24h challenge, or be able to degrade

substrates present in the supernatant after several hours. Activated mast cells may release inflammatory mediators capable of inducing long-term effects. Tissue degradation has been observed in the skin following incubation with chymase (Briggaman et al., 1984) and tryptase (Kaminska et al., 1999). The recent finding that tryptase may alter the permeability of the airway epithelium (Perng et al., in press) suggests that airway epithelial cells may be affected by mast cell products to the same extent as what can be observed in the skin tissue. The cellular response could rely on either a short pulse of chymase or on the presence of chymase over several hours, or both. Our observation that mast cells are present in substantial numbers in lung tissues indicate the potential of cells of the airways to be exposed constantly to high local levels of mast cell proteases.

From 6h after addition of chymase to epithelial cells, IL-6, IL-8, and to a lesser extent GM-CSF were released into the supernatants. The increases in expression measured for the corresponding mRNA molecules were more marked. The pattern of cytokine release in response to chymase appeared bell-shaped, with a maximum release measured after incubation with 10 or 20mU/ml. Epithelial cells were grown to confluency before being challenged with chymase, and concentrations of chymase employed did not induce cell proliferation, suggesting that increases in cytokine release were not related to increases in cell numbers. Chymase at 40mU/ml actually inhibited cell proliferation. However, the proliferation assays were carried out on sub-confluent cells, to reduce the baseline rate of proliferation, and it is possible that non-confluent cells were more susceptible to the proteolytic action of chymase, though no cell detachment from the surface of the membrane was observed when cells were treated with up to 40mU/ml. In addition, the same concentrations of chymase as those employed to induce cytokine release did not induce cytotoxicity, as assessed by trypan blue dye exclusion and measurements of LDH activity in supernatants after 24h incubation. This suggests that the decrease in

cytokine release observed when cells were incubated with concentrations of chymase of 40mU/ml was not caused by cell death.

The chymotryptic activity remaining in the supernatant at 6h and 24h was probably not responsible for degrading the secreted cytokines, since incubation of standards with various concentrations of active chymase did not result in a major decrease in the amounts of detectable cytokines. However, levels of immunoreactive IL-8 were slightly reduced following incubation with 40mU/ml chymase. Since for this series of experiments standards were used in relatively high concentration (100pg/ml), this could indicate that IL-8 is a very low affinity substrate for chymase, or that chymase interfered with the assay employed. All ELISAs were performed in the presence of compound Y-40018 at 50μM, in order to prevent possible degradation of the assay components by active chymase remaining in samples.

The addition of SB 203580, an inhibitor of p38α MAP kinase, dramatically reduced cytokine release from the 16HBEo- epithelial cells. The p38α MAP kinase has been implicated in the downstream activation of multiple transcription factors including transcription factor 2, Elk-1, C/EBP homologous protein, and cAMP response element binding protein (reviewed in Lee et al., 1999). This kinase has been implicated in the regulation of cytokine release in a variety of cells (Guo et al., 2003). Rhinovirus infection has been shown to induce the activation of the p38 MAP kinase pathway in BEAS-2B airway epithelial cells (Griego et al., 2000). Although we were not able to study in detail the activation of intracellular signalling molecules, the results we obtained by challenging 16HBEo- cells with chymase in the presence of SB 203580 suggest that p38α MAP kinase is involved in the induction of cytokine release from these cells. In addition, the chymase-induced release of IL-6 and IL-8 from

16HBE cells is likely to involve p38α MAP kinase. Further studies will be required to elucidate the processes involved in greater detail.

The cytokines released in response to chymase have a range of potent proinflammatory actions. As indicated previously (section 1.4.4.1) a major function of IL-6 is to augment immunoglobulin production by B lymphocytes. It can also induce B cell and cytotoxic T cell proliferation and differentiation, and stimulate haematopoietic stem cell growth (reviewed in Akira et al., 1990). Il-8 is a potent granulocyte chemoattractant that has been implicated in airway inflammation (Adler et al., 1994). GM-CSF is a key cytokine involved in modulating the growth and differentiation of haematopoietic cells and can play a central role in mediating cellular responses in inflammation (Tarr, 1996). Interestingly, neutral proteases found in house dust mite faecal pellets can also induce the release of proinflammatory cytokines from airway epithelial cells (King et al., 1998). A similar response can be induced by mast cell tryptase (Perng et al., in press). This suggests that the airway epithelium can react to various proteases by developing an inflammatory response. Endogenous proteases can undoubtedly contribute a great deal in airway inflammation. Chymase could provide an important stimulus for the production of these cytokines in the epithelium of CF patients or in allergic inflammation.

6.3 Alterations in Transepithelial Electrical Resistance and Epithelial Structure

We have found that chymase can have potent actions on the transepithelial electrical resistance (TER) of epithelial monolayers. Recombinant and skin chymase induced similar responses, indicating that the recombinant enzyme was functionally similar to the native one. Although no changes were observed in the integrity of the tight junctions

or cell viability, elevated concentrations resulted in visible epithelial detachment, and this effect appeared to be mediated in part by intracellular signalling molecules. Interestingly, while the monolayers appeared to be contracted and torn apart, leading to the appearance of gaps and cellular detachment from the side of the inserts, intercellular adhesion seemed to be preserved. The processes involved in mediating the actions of chymase were not clear, although the response of epithelial cells to chymase did not appear to involve intracellular calcium flux.

Although cells were left to stabilize in challenge buffer for at least 1h before the beginning of experiments, the TER of control monolayers incubated with buffer alone increased to some extent at early time points. This suggests that monolayers were sensitive to being transferred into serum-free challenge buffer. This increase in TER may indicate that monolayers require several hours to readjust to different environmental conditions. In preliminary experiments, some monolayers were preincubated for 24h before the 24h challenge, however viability started to decrease after two days in serum-free conditions, although medium was supplemented with ITS, and TER dropped to 50% of the initial values before the end of the experiments, regardless of the treatment. This narrowed the window for gathering reliable data to less than 48h, before cells showed signs of serum deprivation. Although control TER was still evolving at early time points, differences in TER values between treated and control monolayers were apparent even during the first hours of challenge, so we decided to carry on this study without extending further the period of equilibration.

The relation between the concentrations of active enzyme applied and the magnitude of the alterations in TER and tissue structure we observed, as well as the fact that inactivated chymase failed to induce these changes, suggest that chymase acted on epithelial cells via a catalytic

process. After a rapid drop during the first hour, the chymotryptic activity of the recombinant chymase appeared to last longer than that of the skin chymase. This may have been caused by an increased stability of the recombinant material, perhaps related to glycosylation. For each experiment involving the recombinant enzyme, a concentrated sample was activated for 4h with DPPI, and at this time no further increase in activity was detected using AAPFpNA as a substrate. It is unlikely that DPPI activated any remaining prochymase after being further diluted in challenge buffer. Increases in TER were observed from the 1h time point, and reached a maximum at 4h for both preparations of chymase tested. At that time, chymotryptic activity of the native and recombinant enzymes had declined to about 25 to 50% of initial values, respectively. Higher concentrations of chymase initially resulted in a faster increase in TER, as well as a more elevated peak value.

Interestingly, the increases in TER observed during the first four hours of challenge with low doses of chymase applied on the apical side were transient using the native enzyme, but were sustained for at least 24h using recombinant chymase. The dose-related response observed for these low concentrations of active enzyme, as well as the fact that this response was sustained using an enzyme which was found to be active for a longer time, suggest that epithelial monolayers are capable of increasing TER, i.e. reducing epithelial permeability, according to the intensity of a noxious stimulus acting on the apical side such as active chymase. This fine adjustment of epithelial permeability was not observed when monolayers were challenged with similar concentrations on their basolateral side. Such a mechanism could have important significance *in vivo*, where the airway epithelium is known to act as a protective barrier against noxious compounds.

In these studies, the initial concentration of active chymase determined the intensity of the insult during the first hour of challenge, as well as the duration of that insult. With initial concentrations of native chymase of 80mU/ml, or recombinant chymase at 40mU/ml, there was still some significant chymotryptic activity after 24h incubation. These concentrations of chymase applied to the apical side of cells induced a visible shrinking of the monolayers after 4h. This was preceded by a steep increase in TER, until cells began to detach from the sides of the inserts, resulting in a TER drop to nearly basal values. At that time, the chymotryptic activity of skin chymase or recombinant chymase was approximately 20mU/ml. Although cell structure was dramatically altered, viability did not decrease as assessed by trypan blue dye exclusion.

Fluorescent staining for occludin, one of the major transmembrane proteins involved in the building up of tight junctions, did not reveal differences between monolayers treated with buffer alone or various concentrations of chymase. These findings suggest that the permeability of the airway epithelium may be reduced when epithelial cells are stimulated by moderate concentrations of chymase. Higher concentrations applied to any side of the epithelium may result in profound alterations in cell structure and function, leading to cellular detachment from the basement membrane. Interestingly, mast cell tryptase at 10 to 40mU/ml induces similar effects when it is applied to the apical side of epithelial cells (Perng et al., in press). These effects could be interpreted as a protective mechanism by which epithelial cells seek to prevent the entry of noxious molecules. However, higher concentrations of chymase resulted in epithelial detachment from the side of the inserts and the membrane, and this has not been reported with tryptase. Chronic inflammation and frequent release of chymase in the lungs could be of clinical importance, and particularly in a condition such as CF, in which there are large

numbers of chymase-containing cells, notably in the vicinity of the epithelial layer.

The chymase-induced increase in TER was enhanced by the addition of PD98059, an inhibitor of MEK, a kinase responsible for the phosphorylation/activation of ERK1/2. The vehicle alone did not induce any response (data not shown). When monolayers were incubated with PD98059 on its own, it also induced a significant increase in TER, although not as pronounced as with chymase. This suggests that ERK1/2 may be involved in regulating TER. PD98059 has been reported to induce a decrease in basal TER of Madin-Darby canine kidney (MDCK) epithelial cell monolayers, and to prevent cyclosporin A-induced increases in TER in these monolayers (Kiely et al., 2003). ERK1/2 activation has also been reported to be a key event in PKC-induced endothelial cell barrier dysfunction (Verin et al., 2000). Staurosporine, an inhibitor of protein kinase C, had no effect on chymase-induced increases in TER at the concentration employed (1nM). However, when 16HBEo- monolayers were incubated with 1mM staurosporine alone, we observed a slow increase in TER. Wortmannin, an inhibitor of PI3 kinase, slightly enhanced the effect of chymase, but had relatively little effect on its own, suggesting that PI3 kinase may be involved in the regulating the TER of 16HBEo- monolayers. Further studies will be required to identify the mechanisms involved in alterations of the TER and cell structure mediated by chymase.

Cellular contraction and rearrangement of the cell cytoskeleton are likely to involve a number of intracellular mediators. The alterations in TER and cell structure we observed when epithelial cells were challenged with chymase suggest that this neutral protease can trigger a cascade of events leading to the contraction of the airway epithelium. Although it is not clear which intracellular signalling molecules are involved, our finding

that pertussis toxin did not alter the response to chymase indicate that a classic G protein-coupled receptor is unlikely to be involved. This idea was reinforced by lack of calcium flux observed spectrofluorometrically in cells treated with chymase. Although calcium ionophore and ATP induced calcium mobilization in suspended 16HBEo- cells, chymase did not elicit any response. The fact that trypsin also induced a cellular response indicates that surface receptors had recovered from the trypsinisation step the day before the experiment, and that they were intact and able to be activated. Chymase may thus act on a receptor other than one of the PARs that have been characterised to date, that is expressed by the 16HBEo- cell line and airway epithelial cells. Alternatively, the response of epithelial cell to chymase could involve a direct mechanism whereby a cell-associated substrate (paracellular or free in the medium) is cleaved, leading to production of a reactive metabolite.

6.4 Conclusions

The present studies indicate that chymase could have an important role in inflammatory processes in the human airways. Chymase-containing cells were found to be abundant in different tissue compartments. Moreover, increased numbers of these cells were present in the airways of patients with cystic fibrosis, where they were found to be in close contact with the epithelial cells. Following release from mast cells, chymase could have profound actions on the airway epithelium.

Chymase was able to stimulate the synthesis and release of potent proinflammatory cytokines including IL-6, IL-8, and GM-CSF. The potential for chymase to alter the permeability of airway epithelial cell monolayers, and to induce the contraction and detachment of epithelial cells suggests that this major mast cell product may play a key role in

tissue degradation and remodelling in the human airways. In these studies, the actions of chymase were dependent on an intact catalytic site, being inhibited by the presence of protease inhibitors or by heat-treating the enzyme.

The mechanisms that mediate the actions of chymase are not clear. SB 203580, an inhibitor of p38α kinase, reduced markedly the release of IL-6 and IL-8 from epithelial cells, even in the presence of chymase in a concentration that was found to induce cytokine release. This suggests that the chymase-induced cytokine release could be mediated by p38α kinase. The increase in TER was not prevented by any of the kinase inhibitor we tried. PD 98059, an inhibitor of the ERK1/2 kinase MEK, actually increased the TER when it was added alone, and had a synergistic effect when added with chymase. This suggests that chymase may act via the corresponding p44/42 pathway to induce cell contraction. Further investigation is required to identify the molecular targets of chymase. However, pertussis toxin did not prevent chymase-induced cytokine release from epithelial cell, and chymase did not induce the influx of calcium. These observations suggest that the actions of chymase do not involve one of the protease activated receptors that has been described to date, or any a G-coupled protein receptor.

Chymase could represent an important mediator of airway inflammation, and this mast cell protease must be considered a promising target for therapeutic intervention. There is a need for further investigations, but the large numbers of chymase-containing mast cells in the airways of patients with CF highlight the potential for strategies involving inhibition of chymase in this condition.

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