ICAM-1 AND CD44 EXPRESSION IN HUMAN BRONCHIAL EPITHELIUM AND THE ROLE OF CD44 ISOFORMS IN CELL ADHESION, MIGRATION, AND REPAIR

by

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ABSTRACT

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ICAM-1 and CD44 Expression in Human Bronchial Epithelium and the Role of CD44 Isoforms in Cell Adhesion, Migration, and Repair

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Supervisors: Professor Stephen T. Holgate and Dr. Peter M. Lackie

Airway epithelium plays a critical role in the defence against damaging airborne agents and regulates the migration of inflammatory cells into the subsmucosa by releasing cytokines. Several adhesion molecules are involved in this inflammatory response in airways. Human primary bronchial epithelial cells and four bronchial epithelial derived cell lines 16HBE 14o$^-$, 9HTE, BEAS-2B, and NCI-H292 were characterised and used to develop models for my study. I have studied the expression of ICAM-1 and CD44 using models of epithelial damage and repair with these cell lines. 16HBE 14o$^-$ cells showed high transepithelial electrical resistance (TER) and epithelial differentiation which was not seen in the other cell lines. Immunogold labelling indicated that the localisation of ICAM-1 and CD44 in 16HBE 14o$^-$ *in vitro* is similar to that of epithelial cells in bronchial tissue. By using flow cytometry and immunocytochemistry, the regulation of ICAM-1 and CD44 were studied including (a) their expression after mechanical damage (b) the influence of cell density, and (c) the effect of proinflammatory cytokine stimulation (IFNγ, TNFα, IL-1β and IL-4). Mechanical damage of confluent epithelial cells induced the expression of CD44 on the cells up to 500 μm from the wound edge and for up to 48 hours. Before cell confluence, the expression of CD44 at low cell densities was significantly higher than in confluent cultures, while ICAM-1 was lower. IFNγ and TNFα co-stimulation increased ICAM-1 expression significantly, while little change was seen in CD44. IL-1β and IL-4 induced the expression of CD44s, CD44v3 and CD44v9. Furthermore, using a message affinity paper (MAP) PCR-based technique, I demonstrated that individual primary epithelial cells expressed several CD44 isoforms, while CD44 isoforms were undetectable in columnar epithelial cells from human airway. The studies of splicing patterns of variant CD44 isoforms by PCR and sequencing showed the down-regulation of v8-v9-v10 isoforms and small decreases of v6-v7-v8-v9-v10 and a v3-containing isoforms were seen in the cells with cytokine treatments and mechanical damage. In addition, the function of CD44 was investigated in cell adhesion and migration. Cytokines induced a CD44s-dependent cell adhesion to hyaluronic acid (HA). IFNγ-induced cell binding to HA without increasing the level of cell surface CD44 indicated that other mechanisms are involved in the modulation of CD44/HA binding. CD44 antibodies inhibited cell migration and demonstrated that CD44 plays an important role in cell migration, and may be associated with the repair processes of bronchial epithelium. I have found increased CD44 protein expression or changes in the alternative splicing of CD44 isoforms during the repair of epithelial damage. This study describes the sub-cellular expression and regulation of ICAM-1 and CD44, and provides some indication of functions of these CAMs in human bronchial epithelial cells.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABBREVIATION

12-HETE	12-hydroxyeicosatetraenoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
AuTM	aurothiomalate
BAL	bronchoalveolar lavage
bp	base pairs
BSA	bovine serum albumin
CAM	cell adhesion molecule
CD	cluster of differentiation
CD44s	CD44 standard
CD44v	CD44 variant
cDNA	complimentary DNA
CK13	cytokeratin 13
CK14	cytokeratin 14
CK18	cytokeratin 18
COPD	chronic obstructive pulmonary disease
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FBV	Fibronectin/BSA/collagen (Vitrogen 100) mixture
FCS	foetal calf serum

FDA	fluorescein diacatate
FITC	fluorescein isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage - colony stimulating factor
HA	hyaluronic acid; hyaluronate
HBSS	Hanks' Balanced Salt Solution
HPBEC	human primary bronchial epithelial cells
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon-gamma
IgG	Immunoglobulin G
IL-1β	interleukin 1 beta
IL-3	interleukin 3
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IL-11	interleukin 11
IMEM	Iscove's Modified Dulbecco's medium
LFA-1	leukocyte function-associated antigen-1
LTC4	leukotriene C4
mAb	monoclonal antibody
MAP	message affinity paper
MBP	major basic protein
MEM	Minimum Essential Medium
MMP-9	matrix metalloproteinase-9
MMPs	matrix metalloproteinases
mRNA	message RNA

PAF	platelet activating factor
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PI	propidium iodide
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
RANTES	regulated on activation normal T cell expressed and secreted
RHAMM	receptor for hyaluronate-mediated molility
RNA	ribonucleic acid
RT-PCR	reverse transcription - polymerase chain reaction
SEM	scanning electron microscope
TEM	transmission electron microscope
TER	transepithelial electrical resistance
TGFβ	transforming growth factor beta
Th1	type 1 helper T cell
Th2	type 2 helper T cell
TNFα	tumour necrosis factor alpha
USG	Ultroser G (serum replacement supplement)
UV	ultraviolet

CHAPTER 1

General Introduction

1.1 Introduction

Airway is lined with epithelial cells with a film of mucus over them. The epithelium maintains the airways in a steady state by a number of physiologic processes including mucus secretion, ciliary motion, ion and fluid transport. These homeostatic processes control influx and efflux of inflammatory cells and small molecules support the functions of the airways. The airway epithelium is classically viewed as a simple interface between the host and its environment. In fact, it is an active, well-developed structure, which comprises several types of cells with specialised functions. The adhesion molecules expressed on the surface of epithelial cells are critical for maintaining normal epithelial function. Cell adhesion and interaction between epithelial cells and epithelial-leukocyte are mediated through cell adhesion molecules (CAMs) (Montefort and Holgate, 1997). Recognition of the underlying matrix substrate by epithelial cells also relies on CAMs (Polito and Proud, 1997). CAMs are crucial in retaining epithelial integrity and maintaining its normal functions.

Several adhesion molecules have been shown to have a role in supporting the integrity of airway epithelium. The occurrence of epithelial damage and restitution is important in the progression of the airway inflammatory disease, such as seen in asthma. The cascade of airway inflammation leading to epithelial damage is correlated with several physiological processes, including structural changes in the epithelium. Infiltrated cells in this process may produce disruption of cell adhesion mechanisms and structural changes in basement membrane (White and Leff, 1998). Epithelial shedding and denudation directly involve the disruption of cell adhesion. In the last few years, the roles of CAMs have generated great interest since many studies have demonstrated that these molecules play a vital role in the organisation and maintenance of tissue architecture. Intercellular adhesion molecule-1 (ICAM-1) (Manolitsas *et al.*, 1994; Gosset *et al.*, 1994) and CD44 (Peroni *et al.*, 1996; Lackie *et al.*, 1997) have been reported to be expressed at a high level during inflammatory responses in asthma. The aim of this study is to address the possible roles of ICAM-1 and CD44 on bronchial epithelial cells in maintaining the function of airway epithelium. Since asthma research is the major interest in the department where these

studies were carried out, I have discussed the role of ICAM-1 and CD44 in the context of the bronchial epithelial damage and repair processes which are believed to occur in asthma.

1.2 The Airways

The respiratory tract comprises of upper airways and lower airways. The lower airways can be functionally divided into conducting, transitional and respiratory zones. The conducting zone includes trachea, main bronchi, lobar and segmental bronchi, and the small bronchioles. Terminal and respiratory bronchioles are included in the transition zone. The respiratory zone is made up of the alveoli (Bannister, 1995). Epithelial cells from the epithelium in the bronchi are increasingly recognised to the major airway cell type involved in asthma (Montefort *et al.*, 1992) and are the main subject of my study.

1.3 Epithelium and Asthma

The pathological features of asthma may be characterised as increased thickness of bronchial smooth muscle, occlusion of the airway lumen by mucous plugs, enlarged seromucinous glands, predominantly eosinophil and T cell infiltration, as well as up to 60% of the epithelial area may have an altered structure associated with damage and repair processes (Roisman *et al.*, 1995; Peroni *et al.*, 1996). In the analysis of general concept of disease, asthma refers to the condition of individuals with symptoms arising from pathological features mentioned above. The definition of asthma used here is based on the summary of categories by Scadding (Scadding, 1993), which is widely used as in research and diagnosis.

1.3.1 Evidence of epithelial damage in asthma

Studies from the biopsies of severe asthmatics have indicated loss and destruction of bronchial epithelium as a characteristic of asthma and the epithelial shedding in the airways has long been regarded as a key feature of the asthmatic (Naylor, 1962). Other bronchial structural changes include denuded basement membrane and

spreading of epithelial cells, possibly derived from basal cells. Covering the denuded areas with flat, transitional cells may provide a new barrier in the damage area (Persson *et al.*, 1996). Epithelial damage and inflammatory responses occur not only in patients who died in severe asthma, but also in patients in the mild asthma (Laitinen *et al.*, 1996).

1.3.2 Epithelial repair in asthma

Following injury, repair of the airway epithelium occurs and restores the normal function of airways. Simple stratified non-ciliated epithelium or goblet cells are generated in the wound areas rather than columnar or cuboidal epithelial cells (Craige, 1941). Within 12 hours the damaged area is covered again by flattened epithelial cells, and nearly all the cells in the areas of regeneration show a high mitotic activity at 24 hours after damage (Lane and Gordon, 1974; Keenan *et al.*, 1982). During the repair process epithelial cells are recruited into the wound and cover the area, proliferate and eventually acquire features of differentiation. Epithelial repair has been demonstrated to increase cell proliferation not only in epithelial cells but also in subepithelial cells such as fibroblasts and smooth muscle (Erjefält *et al.*, 1997). The processes of allergen-induced epithelial repair may induce remodelling effects in the airways.

The increase in ciliated cell detachment is seen in patients with mild to severe asthma (Laitinen *et al.*, 1985). Ciliated cells appear to be the most damaged cell type in the epithelium, while basal cells remain attached to the basement membrane. Selective disruption of the columnar-basal cell attachments suggests the regulation of junctional adhesion between cells is the crucial process in the epithelial damage. On the other hand, the increase of non-junctional adhesion molecules in the airways of asthmatic including ICAM-1 (Wegner *et al.*, 1990; Milne and Piper, 1994), VCAM-1 (Ohkawara *et al.*, 1995), E-selectin (Bentley *et al.*, 1993) and CD44 (Lackie *et al.*, 1997) suggest that these molecules might be involved in cell-cell interaction and cell migration during inflammation (Ohkawara *et al.*, 1995). In the migration stage, the leading edges of the cells spread and attach to the extracellular matrix (ECM). Adhesion molecules on airway epithelial cells may play a key role in the modulation

of communication between neighbouring cells and cell migration on ECM. In addition, several cytokines, including interferon-gamma (IFNγ), tumour necrosis factor alpha (TNFα), transforming growth factor alpha (TGFα), and transforming growth factor beta (TGFβ), are able to regulate various aspects of epithelial repair and tissue remodelling.

1.4 Bronchial Epithelial Cells

Epithelia line and form the surfaces of cavities and tubes of the body. They consist of tightly knit cells bound laterally to each other by cell junctions. The apical surface of bronchial epithelium is exposed to the air; the basal surface of the epithelium is in contact with connective tissue from which the epithelium is supported and nourished. The normal airway epithelium is a pseudo-stratified, ciliated cell lining (*Figure 1-1*). Columnar and basal cells are the predominant cell types in the upper airways. The columnar cells are composed of both ciliated and non-ciliated cells, of which the latter comprise the goblet, serous, Clara and pre-secretory cell types. These are involved primarily in the production of airway secretions. These cells are also thought to be the progenitor cells for the ciliated cells, which are terminally differentiated epithelial cells (Evans *et al.*, 1986). The basal epithelial cells, attached to basement membrane, play an integral role in anchoring the epithelium to the extracellular matrix of basement membrane.

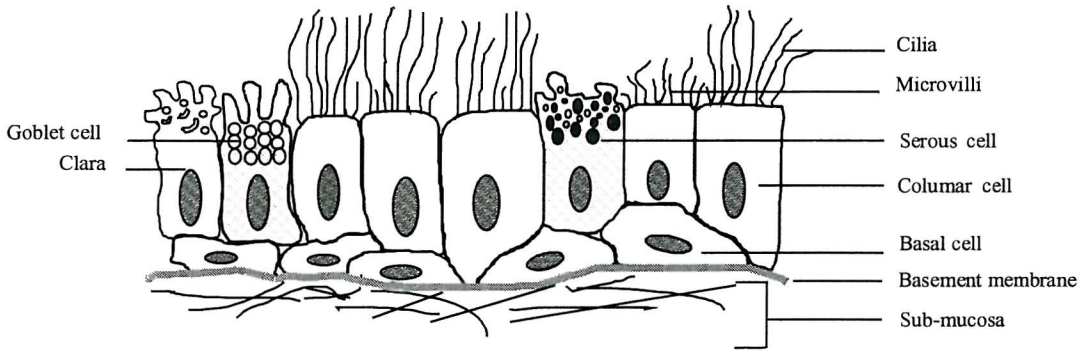


Figure 1-1. Diagram showing the architecture of bronchial epithelium.

The integrity of the bronchial epithelium, like that of other epithelia, is dependent on various adhesive mechanisms that serve to hold the composite structure together and control the influx and efflux of small molecules. The airway epithelium has been regarded as a physical barrier, processing the mechanical clearance of airborne agents and preventing entry of noxious agents into the underlying tissues. The airway epithelium also secrets several mediators and cytokines (see below) which may mediate interacting with other inflammatory cells, and/or activates cells in the inflammatory response. Once the epithelium is damaged, the equilibrium in the epithelial-immune cells network is disrupted, which may lead to fibroblast activation, smooth muscle contraction, and result in asthmatic attack (*Figure 1-2*). Combined with their cellular responses and the modulation of cell adhesion mechanisms, evidence suggests that airway epithelium is not only a physical barrier, but also plays a critical role in the immunological response in the airways (White and Leff, 1998).

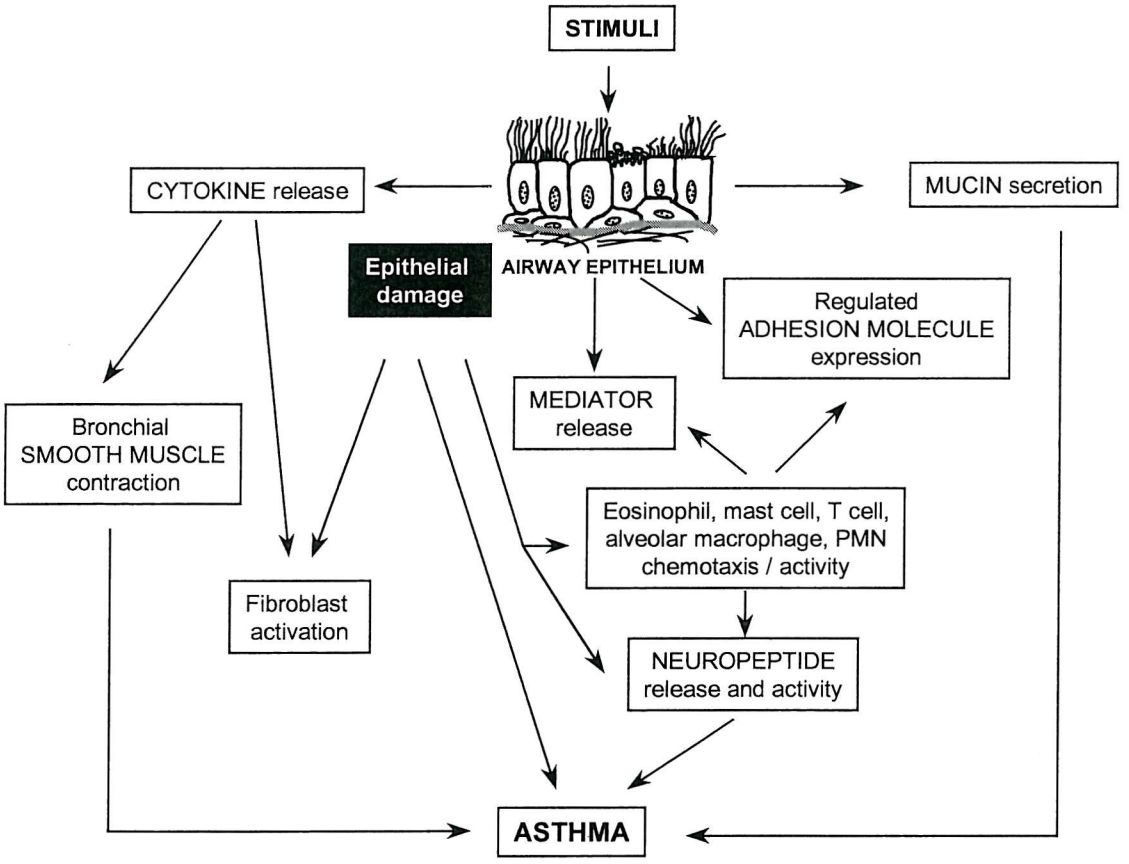


Figure 1-2. Airway epithelium in asthma network.

1.4.1 Cytokine and Mediator release

Cytokines play an important role in the inflammatory processes in the airways. For instance, interleukin 5 (IL-5) together with interleukin 3 (IL-3), and granulocyte macrophage-colony stimulating factor (GM-CSF), enhances human eosinophil differentiation (Campbell *et al.*, 1987) and degranulation (Fujisawa *et al.*, 1990). The pre-inflammatory cytokines TNFα and interleukin 1 alpha and beta (IL-1α and β) can up-regulate adhesion molecules in endothelium (Pober *et al.*, 1986), and co-stimulation of T cell activation (Hackett *et al.*, 1988). RANTES (Regulated on Activation Normal T cell expressed and Secreted) is a member of the C-C chemokine family and is a potent chemoattractant for eosinophils, lymphocytes, and monocytes and is increased in the epithelium of mild asthmatics (Davies *et al.*, 1995). Interleukin-8 (IL-8) is a potent chemotaxin and is increased in epithelial cells after TNFα and IL-1β stimulation (Nakamura *et al.*, 1991). Epithelial cells are known to produce a wide variety of cytokine (*Tabel 1-1*) which could regulate the functions of airways.

Airway epithelial cells also produce a variety of low molecular weight mediators allow the epithelial cells to communicate with other tissue in the airway, such as nerve, smooth muscle, gland cells, and inflammatory cells. The lipid mediators generated by airway epithelial cells include 15-hydroxyeicosatetraenoic acid (15-HETE), 14,15-dihydroxyeicosatetraeonic acid (14,15-diHETE), 8,15-diHETE (Hunter *et al.*, 1985), Prostaglandin E2 (PGE2) (Orehek *et al.*, 1975; Steel *et al.*, 1979), $PGF_{2\alpha}$ (Churchill *et al.*, 1989), and platelet-activating factor, PAF (Salari and Wong, 1990).

Table 1-1. Bronchial epithelial cytokine production.

cytokine	Functions	Reference
IL-1β	Enhance the presence of other cytokines	(Mattoli *et al.*, 1991)
IL-2	T cell growth factor	(Aoki *et al.*, 1997)
IL-6	Activate T cells, augment Ig production by B cells	(Mattoli *et al.*, 1991; Cromwell *et al.*, 1992; Takizawa *et al.*, 1992)
IL-8	A potent chemotaxin	(Nakamura *et al.*, 1991)
IL-10	Suppresses cytokine production by Th1 helper cells	(Bonfield *et al.*, 1995)
IL-11	Mediate virus-induced airway hyperresponsiveness	(Elias *et al.*, 1994)
G-CSF	promotes neutrophil survival	(Cox *et al.*, 1992)
GM-CSF	Eosinophil/neutrophil activation and survival	(Churchill *et al.*, 1992; Cromwell *et al.*, 1992; Cox *et al.*, 1992)
RANTES	Chemokine for epsinophils, lymphocytes and monocytes	(Davies *et al.*, 1995)
TGF-β	Involved in inflammatory process and repair	(Sacco *et al.*, 1992)
TNF-α	Induction of adhesion glycoproteins in endothelial and epithelial cell	(Devalia *et al.*, 1993)

1.5 Immune responses in asthma

1.5.1 Cellular responses in airway inflammation of asthma

Leukocyte infiltration is observed in the submucosa and in the epithelium of asthmatic airways (Gleich *et al.*, 1987; Laitinen and Laitinen, 1988). Studies have shown that these eosinophil-derived proteins can be cytotoxic and may cause epithelial damage leading to desquamation occurs (Gleich *et al.*, 1979; Frigas *et al.*, 1980). Lymphocytes are the predominant inflammatory cell in the airways. Other cell types including mast cells, macrophages and neutrophils are present in the bronchoalveolar lavage (BAL) and are thought to be important in the airway inflammation in asthma (Liu and Calhoun, 1998). The increased numbers and activation of inflammatory cells in the airways may damage to airway epithelium and increase the severity of disease.

A. T lymphocytes

Increased numbers of T cells are found in the bronchial mucosa of asthmatic subjects (Bentley *et al.*, 1992). The activation of T cells also correlates with the severity of asthmatic symptoms (Corrigan *et al.*, 1988; Azzawi *et al.*, 1990). One subset of T lymphocytes, $CD4^+$ cells, produce an array of cytokines that regulate several target cells that respond in the allergic response in the airways. The major cytokines involved in this process include interkeulin-4 (IL-4), IL-5, interleulin-6 (IL-6), IL-9 and interleukin-13 (IL-13) which are secreted by type 2 helper T (Th2) cells. On the other hand, type 1 helper T (Th1) cells secrete interleukin 2 (IL-2), IFNγ, TNFα, and lymphotoxin. Several studies suggested that the imbalance of Th2 and Th1 cytokine production can be an important alteration in the early stage of asthma and allergic diseases which are characterised by a predominace of Th2 features (Humbert and Durham, 1998).

B. Mast Cells

In vitro and *in vivo* studies have demonstrated that mast cells and their mediators can play an important role in chronic inflammatory responses. The contribution of mast cells to the airway remodelling response in asthma, however, remains obscure. One mast cell component, β-tryptase, which is the major protein component of the mast cell secretory granule, is present at high levels in airway fluid (Broide *et al.*, 1991). *In vitro* biological activity of tryptase includes metabolism of fibrinogen and airway neuropeptides peptides (Tam and Caughey, 1990), the generation of C3a (Schwartz *et al.*, 1983), activation of matrix metalloproteinase-3 (Gruber *et al.*, 1989; Lees *et al.*, 1994), activation of proteinase-activated receptor-2 (PAR-2) on cell surfaces (Molino *et al.*, 1997), and the stimulation of fibroblast proliferation and collagen production (Cairns and Walls, 1996). Of particular relevance to my studies, mast cell tryptase acts as a mitogen for bronchial epithelial cell stimulation of IL-8 and expression of ICAM-1 (Cairns and Walls, 1996). This suggested that mast cells can be involved in the immune response of airway epithelial cells.

C. Eosinophils

Eosinophils secrete a number of lipid mediators and proteins that may be relevant to the pathophysiology of asthma. For examples, leukotriene C4 (LTC4) and PAF cause bronchoconstriction (Hanna *et al.*, 1981; Cuss *et al.*, 1986). Eosinophil-derived proteins, such as major basic protein (MBP) and eosinophil cationic protein (ECP), are toxic for respiratory epithelial cells (Gleich and Adolphson, 1986). MBP and ECP are of interest here because they also showed some physiologic actions in airway epithelium, including prostaglandin secretion and ion transport (Jacoby *et al.*, 1988; White *et al.*, 1993). The increase of eosinophil penetration across airway epithelium might be linked to MBP or ECP production regulated by eosinophil activation (Masuda *et al.*, 1995). The detection of MBP and ECP in the sub-epithelial tissues of mucosal ulceration in patients dying in *status asthmaticus* suggest that eosinophils interact with airway epithelium in the pathology of asthma (Filley *et al.*, 1982). Therefore, eosinophil infiltration and activation might contribute to the airway damage in asthma supported by the observation that the concentration of ECP in the serum of asthmatics correlates with the severity of clinical disease (Roquet *et al.*, 1996; Niimi *et al.*, 1998; Hedman *et al.*, 1999).

Cell surface adhesion molecules on eosinophils include (1) L-selectin, P-selectin glycoprotein ligand-1 (PSGL-1), and E-selectin ligand-1 (ESL-1) which mediate the initial weak tethering of cells to the endothelial wall; (2) β2-integrins such as leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18 or αLβ2) and Mac-1 (CD11b/CD18, αMβ2) mediate firm adhesion of eosinophils to cells expressing ICAM-1 and VCAM-1(Bochner *et al.*, 1991); (3) β1-integrins, very late antigen (VAL-4, α4β1) which binds to vascular cell adhesion molecule-1 (VCAM-1); and (4) α4β7, a counterligand for mucosal addressin cell-adhesion molecules (MadCAM) (Bochner and Schleimer, 1994; Wardlaw *et al.*, 1994; Walsh *et al.*, 1996).

D. Neutrophils

The neutrophil influx may increase within 24 hours after allergen challenge, and the increases occur earlier than eosinophils (Metzger *et al.*, 1986). However, there is little

evidence to support the idea that the neutrophil is important in asthma, although increased number of neutrophils have been demonstrated in some studies (Broide *et al.*, 1991; Walker *et al.*, 1991). Neutrophil also express L-selectin and ligand for P- and E-selectin, as well as two members of the β2-integrin family, LFA-1 and Mac-1 (Smith *et al.*, 1988; Lawrence and Springer, 1991).

1.5.2 Development of asthma

The role of mast cells and eosinophils in the early and acute phase of asthma is well-documented (Walls, 1988; Kumar and Busse, 1998; Costa *et al.*, 1998). Recently, a growing number of studies have concentrated on the role of T lymophocytes in orchestrating chronic inflammation in asthma. The release of chemokines and cytokines by activated mast cells contributes to sustained recuitment and activation of eosinophils. Both activated mast cells and eosinophils play key roles in the late phase of the allergic reaction and may produce damage in the airways in asthma.

1.6 Cell Adhesion Molecules on Bronchial Epithelial Cells

Cell adhesion molecules are cell surface proteins involved in cell-cell interaction, or cell-extracellular matrix adhesion. They are key molecules in various physiological and pathological phenomena such as cell differentiation and pattern formation, embryonic development, inflammatory responses, and tumour metastasis. The interactions and responses initiated by binding of these CAMs to their receptors/ligands play important roles in the mediation of the inflammatory and immune reactions that establish a line of the body's defence against these insults. Most of CAMs fall into four general families of proteins: the integrins, the immunoglobulin superfamily, the cadherins, and the selectins.

In bronchial epithelial cells, CAMs are important in (1) Cell-cell interaction, such as connection between columnar cell and basal cells; and (2) Cell-extracellular matrix adhesion, for instance basal epithelial cells interacts with the basal lamina. The integrity and function of the epithelial barrier of the lung are dependent on specific CAMs. Operationally, these adhesion-mediating proteins on bronchial epithelial cells

can be classified into three groups of adhesion molecules according to the functions of CAMs: (1) Junctional adhesion molecules; (2) Non-junctional adhesion molecules; (3) Cell-substratum adhesion receptors (*Table 1-2*). However, in this classification some CAMs have more than one function and belong to different groups.

Table 1-2. Key adhesion mechanisms in bronchial epithelial cells

Junctional adhesion mechanism		
Mechanisms	***Function***	***Key cell surface adhesion molecules***
Tight Junctions	Define apical/basolateral in the polarised cells. Provide cell-cell permeability barrier	Occludin, JAM , claudins
Desmosomes	Tightly attach between epithelial cells, and act as anchorage for intermediate filaments	Desmoglein and desmocollin
Intermediate junctions	A belt of cell-cell adhesion basal to tight junctions	E-cadherin
Gap Junction	Exchange small molecules between cells	connexins
Non-junctional proteins		
Protein	**Function**	
ICAM-1	Ligands for LFA-1 and Mac-1	
VACAM-1	Binds to VLA4 on lymphocytes and monocytes	
Cell Substratum adhesion		
Protein	**Function**	
Integrins	Mediate cell-substratum attachment	
Hemidesmosome	Form tight point of contact with the basal lamina	$\alpha 4\beta 6$
CD44	Binding to HA, Collagen I, IV, and fibronectin	

1.6.1 Junctional adhesion mechanisms

The junctional adhesion mechanism is an adhesion complex containing several molecules. The studies of CAMs epithelial differentiation of mouse preimplantation

embryo provide a great of idea about the biogenesis of the juction formation. The regulation of the assembly of intercellular junctions appears to be initiated by E-cadherin mediated formation of the adherens junction, and followed the assembly of tight junctions. Desmosomes first assemble when tight junction formation is complete (Collins and Fleming, 1995a).

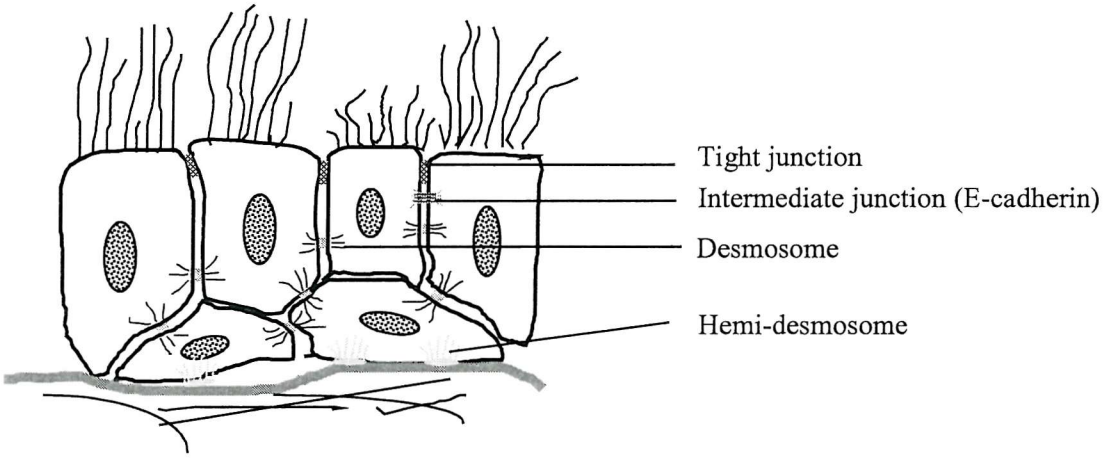


Figure 1-3. Schematic show the adhesion molecules on bronchial epithelial. Four major adhesion mechanisms on bronchial epithelial cells are shown in this diagram.

A. Tight junctions

One transmembrane protein, occludin (Furuse *et al.*, 1993), plays an important role in tight junctions, and regulates this important cell-to-cell adhesion with tight junctions-associated peripheral membrane proteins such as ZO-1 (Stevenson *et al.*, 1986), ZO-2 (Gumbiner *et al.*, 1991; Jesaitis and Goodenough, 1994), ZO-3 (Haskins *et al.*, 1998), cingulin (Citi *et al.*, 1988), the 7H6 antigen (Zhong *et al.*, 1993), symplekin (Keon *et al.*, 1996), Rab3B (Weber *et al.*, 1994), and AF-6 (Yamamoto *et al.*, 1997) have been identified which localise at tight junctions. Tranmembrane protein components of the tight junction include occludin (Furuse *et al.*, 1993), junctional adhesion molecule (JAM) (Martìn-Padura *et al.*, 1998), and the claudin family of proteins (Furuse *et al.*, 1998; Morita *et al.*, 1999; Simon *et al.*, 1999).

Tight junctions connect adjacent epithelial cells and prevent diffusion of proteins and lipids between the apical and basolateral regions (*Figure 1.3*). This structure also ensures the epithelial cells form a physical barrier protecting the organism from the harmful substances of the outside environment.

B. Desmosomes

The desmosome is a circular membrane domain of symmetrical disc-like plaque anchored on intermediate filaments. The two desmosomal halves form an electron-dense midline holding the cells securely together. The major components of desmosomes include several group of proteins: desmoglein, desmocollin, plakoglobin, desmoplakins, plakophilin (Garrod, 1993) and plakophilin-1 (Garrod, 1996; Smith and Fuchs, 1998). The desmosomal cadherins which include desmogleins and desmocollins, bind to the cytoplasmic protein plakoglobin (Garrod *et al.*, 1996; Witcher *et al.*, 1996; Kowalczyk *et al.*, 1999). Desmoplakin binds to both plakoglobin and intermediate filaments, and forms from the linkage between the demosomal cadherin-plakoglobin complex and intermediate filaments (Bornslaeger *et al.*, 1996; Gallicano *et al.*, 1998). Both desmogleins and desmocollins is expressed from three genes derived to different isoforms. The alternative splicing of desmocollins might regulate desmosomal plaque formation (Collins *et al.*, 1991). Desmosomes are present in most types of the epithelia (Cowin and Garrod, 1983; Cowin *et al.*, 1984) and could be thought of as a universal epithelial constituent. In airways, numerous desmosomes are found between cells within the epithelium (*Figure 1-3*).

C. Intermediate junctions

Intermediate junctions, also called adherens junctions or *Zonula adherens*, form a belt-like adhesion mechanism, which links the plasma membranes of adjacent epithelial cells mainly through E-cadherin-catenin complex. Cadherins comprise a family of transmembrane, cell surface glycoproteins that mediate Ca2+-dependent cell-cell adhesion in a mainly homotypic manner. E-cadherin, a 120kDa transmembrane glycoprotein, interacts with the cytoskeleton via associated cytoplasmic molecules, the catenins. E-cadherin is an important molecule for

maintaining the differentiation of normal epithelial cells. Loss of the expression or function of E-cadherin in transformed epithelial cells appears to be a key step in the progression of the cells to a malignant phenotype (Birchmeier *et al.*, 1993).

1.6.2 Non-Junctional proteins

The immunoglobulin superfamily includes a larger number of related proteins, most of which function in cell-cell recognition or adhesion (Williams and Barclay, 1988). Two of the family members present at low level on bronthelial epithelium under condictions, and might be involved in the inflammatory cell adhesion.

A. ICAM-1 (CD54)

ICAM-1 (CD54), is a 90 kD, single chain glycoprotein which contains five unpaired extracellular immunoglobulin domains and has a polypeptide core of 55 kD (Figure 1-4). ICAM-1 belongs to the C2 subset of the immunoglobulin (Ig) superfamily, and it is one of the native ligands for LFA-1 (Rothlein *et al.*, 1986) and Mac-1 (Diamond *et al.*, 1990). ICAM-1 is up-regulated by a variety of inflammatory stimui, such as IL-1β, IFNγ and TNFα (Rothlein *et al.*, 1988; Wawryk *et al.*, 1991; Lassalle *et al.*, 1993). ICAM-1 is present at a low level on airway epithelium, and increased its expression with IFNγ treatment (Look *et al.*, 1992). The complementary receptors of ICAM-1, LFA-1 and Mac-1 integrins, are expressed on several types of infiltrating cells. LFA-1 is found on the surface of eosinophils, neutrophils, and lymphocytes; and Mac-1 is expressed on granulocytes (Wegner *et al.*, 1990; Springer, 1990).

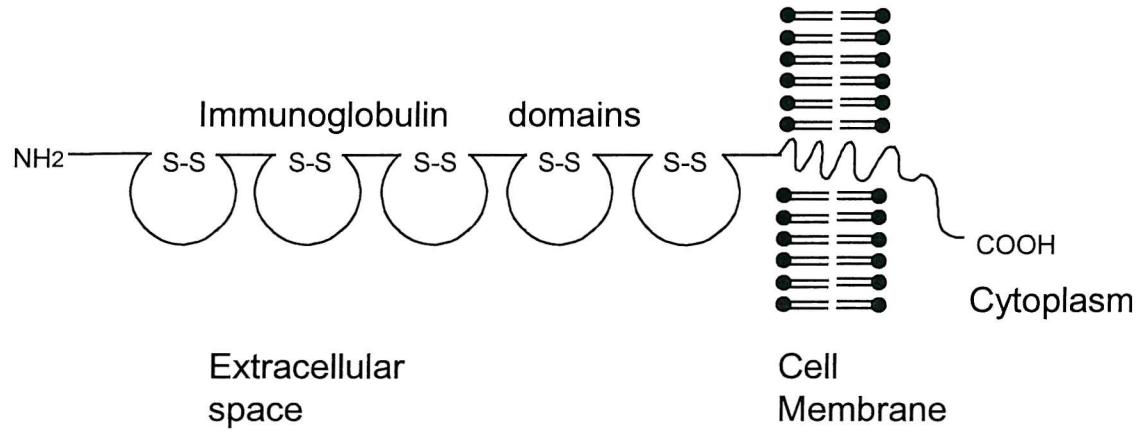


Figure 1-4. Structure of ICAM-1.

B. VCAM-1 (vascular cell adhesion molecule)

Like ICAM-1, VCAM-1 is an immunoglobulin superfamily adhesion protein present on the membrane of endothelial and epithelial cells. It binds to the integrin VLA4 ($\alpha 4\beta 1$) on lymphocytic and monocytic cells. In bronchial biopsies from allergic patients, an increased expression of VCAM-1 on epithelium has been reported, it is otherwise reported to be infrequently expressed and always at a low level (Gosset *et al.*, 1995) or absent on normal epithelium (Jagels *et al.*, 1999).

1.6.3 Cell-substratum adhesion receptor

In addition to an anchoring cell to substratum, cell-substratum adhesion molecules are also important for cell survival. With loss of extracellular matrix contacts, cells undergo programmed cell death or apoptosis. Integrin-mediated adhesion to extracellular matrix proteins is required for the survival of many cell types (Meredith, Jr. *et al.*, 1993).

A. Integrins

Integrins are transmembrane receptors mediating cell-substratum attachment or cell-cell adhesion. It contains one α-chain and one β-chain (*Figure 1-5*), which both are

single-pass transmembrane proteins. They are divided into subclasses according to the identity of the β-chain. Each β-chain can associate with several different α-chains, and some α-chains can interact with different β-chains. Eight β and sixteen α subunits have been identified, and more than 21 integrins identified.

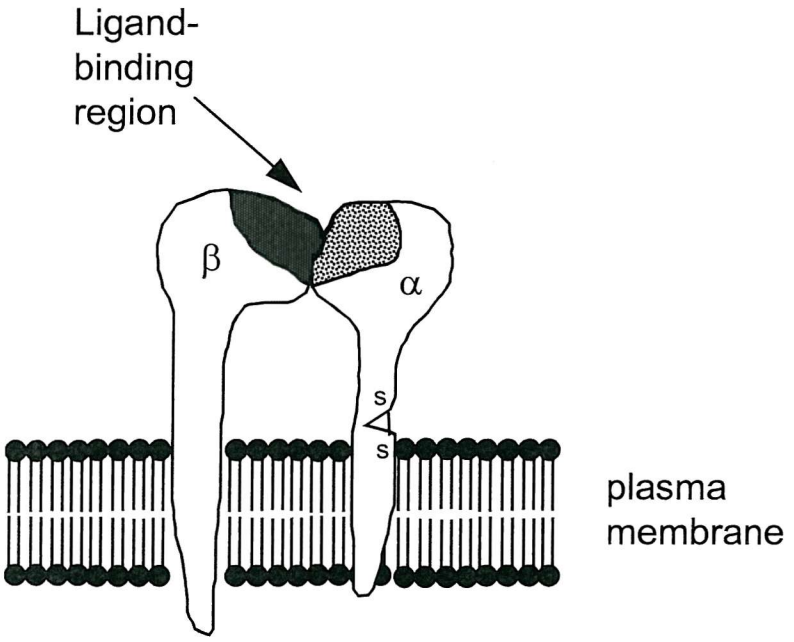


Figure 1-5. General structure of the integrin family of cell surface receptors.

In bronchial epithelium, the α_2-integrin is distributed at the basal cell-basement membrane interface, the junction between basal cells and columnar cells and along the lateral surface of columnar cells. The β_6 subunit is expressed exclusively in epithelial cells, and only in a single integrin heterodimer αvβ6, a receptor for the extracellular matrix proteins fibronectin (Busk *et al.*, 1992) and tenascin (Prieto *et al.*, 1993). The α9β1 integrin has been shown to be a tenascin-binding protein and expressed through the airway epithelium (but not on alveolar epithelium) (Weinacker *et al.*, 1995). Accordingly, the functions of integrins, Albelda (Albelda, 1991) have developed a simplified schema (*Table 1-3*) that categorises epithelial integrins to two groups: (1) binding to normal components of basement membrane (e.g. collagen, laminin); (2)

binding to the matrix proteins which are increased during inflammation, wound repair, and development (e.g. fibronectin, tenascin, fibrinogen, vitronectin, thrombospondin).

In many cases, more than one integrin is capable to recognise same ligand, and may provide redundant binding capacity. In bronchial epithelial cells, integrins may regulate apoptosis through signal transduction in cell-matrix response as well as in cell-cell interaction (Aoshiba *et al.*, 1997).

Table 1-3. Integrins associated with respiratory epithelium

Integrins binding to normal components of basement membrane

Integrin	Ligand
α2β1	Collagens, laminin, fibronectin
α3β1	Fibronectin, laminin, collagens
α6β1	Laminin
α6β4	Laminin

Integrins binding to ligands not normally present in basement membrane

Integrin	Ligand
α5β1	Fibronectin
αvβ1	Vitronectin, fibronectin
αvβ5	Vitronectin
α9β1	Tenascin
αvβ6	Fibronectin, tenascin

After (Albelda, 1991)

B. Hemidesmosome

Hemidesmosomes, are specialized, integrin-mediated adherens junctions characteristic of stratified epithelia (Garrod, 1993). Thee junctions are composed of α6β4 intergrin heterodimer (Stepp *et al.*, 1990; Garrod, 1993), which use laminin 5 anchoring filaments to attach an epithelium to the underlying basal lamina (Stepp *et al.*, 1990; Rousselle *et al.*, 1991; Garrod, 1993). The hemidesmosomes not only forms part of

the link that integrates the extracellular matrix and the cytoskeleton of cells, but also act to transduce signals (Clark and Brugge, 1995).

C. CD44

CD44 is an acidic, sulfated integral membrane glycoprotein ranging in molecular weight from 80 kDa to 200 kDa (Haynes *et al.*, 1989). Prior to being given a cluster of differentiation (CD) name, CD44 has also been termed H-CAM, GP90HERMES, Pgp-1, HCRM III, Ly-24, p85, and HUTCH-1. The CD44 molecules can be separated into three main regions: (1) The cytoplasmic domain which mediates the interaction with the cytoskeleton, (2) The middle domain which is responsible for the lymphocyte homing, and (3) the amino-terminal domain which binds to hyaluronate (HA) (Underhill, 1992). The smallest CD44 protein, known as the CD44 standard or CD44 standard (CD44s) lacks any of the 10 variant exons inserted into its middle domain. The larger CD44 variant (CD44v) is composed of CD44s protein and sequences selected from exons v2-v10 (in human) in the middle domain (*Figure 1-6*).

CD44 is abundant in many tissues and can bind the extracellular glycosaminoglycan hyaluronate (Miyake *et al.*, 1990; Aruffo *et al.*, 1990; Stamenkovic *et al.*, 1991), as well as collagen I, IV and fibronectin (Jalkanen and Jalkanen, 1992; Faassen *et al.*, 1992; Knutson *et al.*, 1996). HA is a major component of the extracellular matrix, and both bronchial and alveolar epithelial cells interact with HA. The regions of epithelia that express the highest levels of the variant CD44 isoforms are the generative cells, particularly the basal cells of stratified squamous epithelium, and of glandular epithelium (Mackay *et al.*, 1994). CD44 has been shown to be involved in cell locomotion in the presence of HA (Thomas *et al.*, 1992; Thomas *et al.*, 1993; Koochekpour *et al.*, 1995). The ligand binding and distribution suggest CD44 might play an important role in cell adhesion and migration. This may be by modifying the migratory ability of cells.

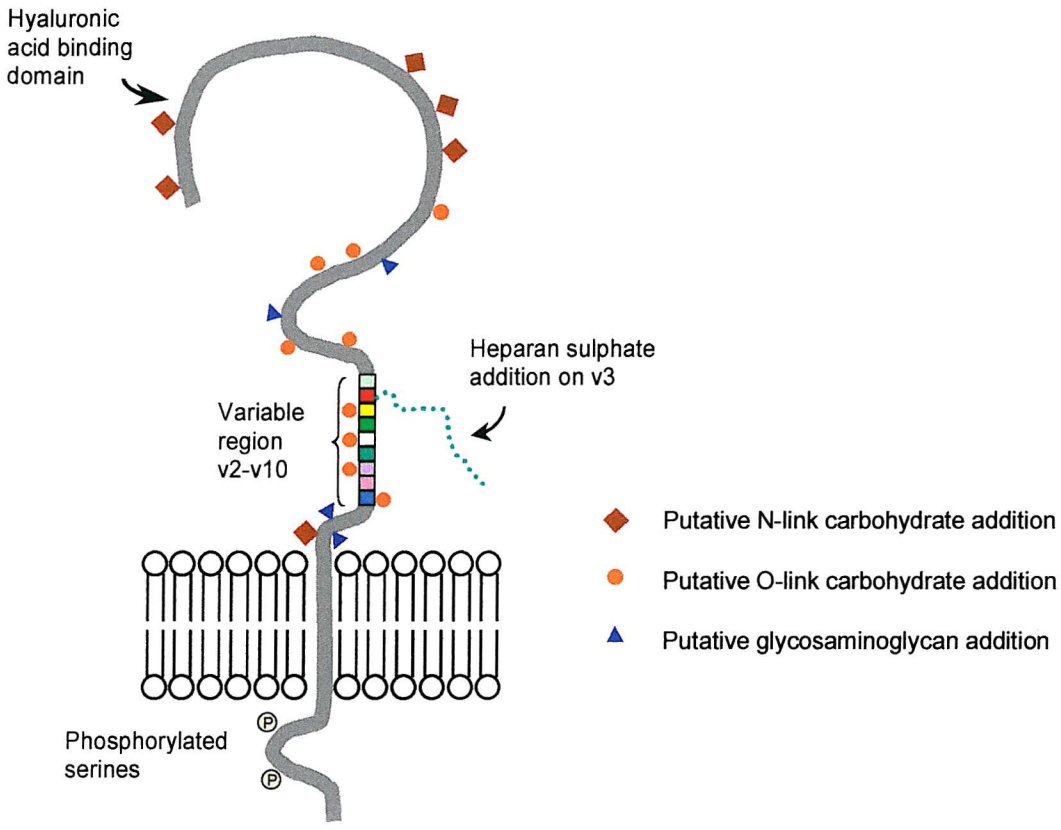


Figure 1-6. Schematic drawing of CD44 protein (after (Ponta *et al.*, 1998).

1.7 Extracellular Matrix in the Lung

ECM is a highly dynamic structure. The cells responsible for producing the different ECM components are highly active, so that some elements, such as collagen and proteoglycans, turn over extremely rapidly. The normal biosynthesis and turn over of ECM determines the architecture of the lung. The ECM also plays a critical role in the regulation of cell differentiation, proliferation, and migration (Schnaper *et al.*, 1993; Werb *et al.*, 1996). In the bronchus the basement membrane is composed of collagens, elastin, laminin, proteoglycans, and fibronectin. Increased thickness of the basement membrane has been observed in asthma as well as in other inflammatory step of bronchiectasis and tuberculosis (Callerame *et al.*, 1971; Cutz *et al.*, 1978).

HA, [d-glucuronic acid ($\beta1\rightarrow3$)N-acetyl-d-glucosamine ($\beta1\rightarrow4$)]$_n$, is also an important component of ECM. Because of its charged residues, it can accommodate many water molecules and occupy a space of several magnitudes greater than expected from its molecular mass of several million Daltons (Laurent and Fraser, 1992). It is a virtually ubiquitous component in the tissue. HA also associates with other components and carries out macro structural functions, forming the basic structural unit of cartilage and serve as a lubricant in synovial fluid. HA was the only glycosaminoglycan found in the BAL of severe asthmatics (Sahu and Lynn, 1978) and its levels have been correlated with severity of asthma (Bousquet *et al.*, 1991).

Several factors have been reported to regulate the synthesis of ECM. TGF-β1 is a potent modulator of ECM. It enhances the synthesis of ECM by fibroblasts, including collagens, fibronectin (Ignotz and Massague, 1986; Ignotz *et al.*, 1987; Varga *et al.*, 1987; Heckmann *et al.*, 1992), and proteoglycans (Bassols and Massague, 1988; Romaris *et al.*, 1995). In airways, eosinophil-derived TGF-β1 has been demonstrated to increase tenascin, fibronectin and laminin production by airway epithelial cells (Kumar *et al.*, 1995; Linnala *et al.*, 1995). In lung, matrix metalloproteinases (MMPs) released by macrophages and neutrophils are involved in the degradation of the ECM (Shapiro and Senior, 1999). MMPs and their inhibitors are also involved in the processes of matrix production and degradation associated with in the inflammatory reaction in asthma (Mautino *et al.*, 1999).

Cytokines such as TNFα and MIP-1 may bind specifically to components of the extracellular matrix (Tanaka *et al.*, 1993; Alon *et al.*, 1994). The binding of cytokine to ECM may retain the cytokine in certain areas and augment the immune response (Staunton *et al.*, 1989; Gailit and Clark, 1994). In addition, cytokine might stimulate other cells to produce MMPs and regulate the synthesis and degradation of ECM. Inflammatory cytokines, such as IL-1β and TNFα, increase matrix metalloproteinase-9 (MMP-9, 92-kD gelatinase) expression in human bronchial epithelial cells (Laurent and Fraser, 1992; Yao *et al.*, 1998).

1.8 The Regulation of ICAM-1 and CD44

1.8.1 ICAM-1 and CD44 in disease

ICAM-1 is expressed in human bronchial epithelium (Tosi *et al.*, 1992a) and has been reported to be up-regulated in asthma (Vignola *et al.*, 1993a; Gosset *et al.*, 1995). Expression of ICAM-1 is highly responsive to IFNγ and TNFα treatment (Look *et al.*, 1992). Numerous reports show that ICAM-1 expression can be induced by inflammatory cytokines in a wide variety of non-haematopoetic cell types, including endothelial cells, monocytes, fibroblasts, or epithelial cells from tissues such as the kidney, intestine, and airway (Dustin *et al.*, 1986; Jevnikar *et al.*, 1990; Kaiserlian *et al.*, 1991; Look *et al.*, 1994). In normal lung, ICAM-1 expression is high on type I alveolar epithelial cells, low on type II alveolar epithelial cells (Kang *et al.*, 1993; Paine, III *et al.*, 1994), and not detectable on bronchial epithelium (Kang *et al.*, 1996). *In vitro* type II cells increase ICAM-1 expression, and undergo transition toward a type I cells-like phenotype. With detergent extraction, ICAM-1 is associated with cytoskeleton in alveoli, but not in endothelial cells and alveolar macrophages (Barton *et al.*, 1996) .

CD44 was originally described in brain and mature T cells, granulocytes and cortical thymocytes (Dalchau *et al.*, 1980), and has been subsequently shown to be identical with the human lymphocyte homing-receptor that involves lymphocyte recognition of high endothelial venules (Jalkanen *et al.*, 1986; Jalkanen *et al.*, 1987; Picker *et al.*, 1989). Other studies suggested that CD44 is involved in lymphocyte activation (Haynes *et al.*, 1989; Shimizu *et al.*, 1989), hematopoiesis (Miyake *et al.*, 1990), organogensis (Wheatley and Isacke, 1995), and homing of lymphocyte to Peyer's patches (Williams and Butcher, 1997). CD44 is also involved in the activation of natural killer cells leading to the lysis of target cells (Tan *et al.*, 1993). All these studies demonstrate that activation via CD44 can trigger effector functions in human T lymphocytes, and enhance the natural killer cells mediated cytotoxicity. These results suggest CD44 may play an important role in signal transduction and cell activation. The role of CD44 in cell-cell interaction in epithelial cells remains unclear. Recent studies have shown that CD44v10-transfected cells have enhanced cell adhesion to

other CD44 bearing cells, which is through the recognition of chondroitin sulfate (Chiu *et al.*, 1999).

As described in *section 1.6.3.C*, CD44 may be involved in cell-cell interaction and cell-extracellular matrix adhesion in respiratory epithelium. It is also considered that bronchial and alveolar epithelial cells interact via CD44 with HA, the major component of extracellular matrix in the airways. Some studies have reported CD44 variants in a wide range of normal epithelial tissues including the skin, digestive tract and lung (Mackay *et al.*, 1994). Many studies have reported that CD44 isoforms are involved in metastasis. It is likely that expression of CD44 variants contribute to the enhanced metastatic properties of some lymphomas and melanomas (Günthert *et al.*, 1995). The CD44v6 and CD44v9 isoforms have been reported as the major forms expression in bronchial epithelial cells from normal adult (Kasper *et al.*, 1995). In airways, increased expression of CD44 has been found in areas of repair in bronchial epithelium and is also increased in asthmatic subjects (Lackie *et al.*, 1997).

1.8.2 CD44 isoforms

In human, various transcripts of CD44 are encoded by one gene locus on chromosome 11, which contains 20 exons. The smallest isoform, CD44 standard (CD44s), is derived from ten different exons of which seven encode the N-terminal extracellular part of the protein, one accounts for the transmembrane region, and two code for a short cytoplasmic tail. The larger CD44 isoforms, are formed with differential splicing of variant exons (v2 to v10 in human; v1 to v10 in mouse) in a multitude of combinations for the occurrence of large CD44 proteins with extend extracellular proteins (*Figure 1-7*). In human CD44 gene contains a stop codon with exon 6 (v1) and so does not code for a protein product as it does in the mouse.

Lesley and colleague (Lesley and Hyman, 1992; Kincade *et al.*, 1997) have defined three cell activation states of CD44 to identify cell lines and normal cell populations: (1) Active cells, expressing CD44 in an active state, can bind HA constitutively; (2) Cells in inducible state, do not bind HA constitutively, but can bind HA immediately upon binding of certain CD44 specific inducing monoclonal antibodies (mAbs), or can

be induced to bind HA after a period of hours by stimuli such as cytokines, or phorbol ester; (3) Cells in an inactive state cannot bind HA even in the presence of inducing mAb. This classification helps to understand the potential regulatory mechanisms of CD44, but not the mechanism of CD44-ligand binding. The different functions of CD44 binding may result from the differential splicing of variant exons in the isoforms. Immunostaining indicates which variants are expressed on the cells. However this does not unambiguously identify the combination of CD44 variant regions present in individual molecules. It also cannot provide information about the function attributable to the different CD44 variants.

Abnormal CD44 isoform patterns associated with malignancy indicate that precise regulation of its isoform expression is important. CD44v6 has been demonstrated the metastatic potential in a rat pancreatic carcinoma cell line (Günthert *et al.*, 1991), while is not necessarily affiliated with metastasis in other tumours (Lesley *et al.*, 1997; Kincade *et al.*, 1997). The variant 3 heparan sulfate side chains have been shown to bind heparin-binding growth factors (Bennett *et al.*, 1995). Most of the studies in the functions of CD44 variants have been focused on tumour metastasis, while the difference of CD44 isoform expression on other cells may have different functions.

I have summarised below the results of some studies in *Table 1-4* as a comparison for further investigating the function of CD44 variants.

Table 1-4. The reported functions of CD44 variants.

Exon	Functions	Cell type /species		**Reference**
S	Blocking of CD44s prevents progression of highly invasive tumour	glioblastoma	*r*	(Breyer *et al.*, 2000)
v2	Can be use as a prognostic factor for diagnosis	oesophageal sequamous cell carcinoma	*h*	(Gotoda *et al.*, 2000)
v3	Containing heparan sulfate addition site responsible for growth factor binding	COS cells; transfected CD44	*M*	(Bennett *et al.*, 1995)
		B cell lymphoma	*h*	(Jackson *et al.*, 1995)
v5	overexpression in advanced cancer	gastric cancer	*h*	(Stachura *et al.*, 1999)
v6	correlate with metastasis	pancreatic carcinoma	*r*	(Günthert *et al.*, 1991; Rudy *et al.*, 1993)
	Transiently express v6 during antigenic activation	lymphocytes	*h*	(Koopman *et al.*, 1993)
	v6 antibody inhibits generation of cytotoxic T cells and T help cells	*In vivo* lymphocyte activation	*r*	(Arch *et al.*, 1992)
v7	v7-antibody prevented experimentally induced lethal colitis	*In vivo*	*m*	(Wittig *et al.*, 1998)
	v7-antibody decreased IL-12, increases IL-10 production	*In vivo*	*m*	(Seiter *et al.*, 2000)
v10	Mediate signal inducing cell proliferation	Endothelial cells	*h*	(Lokeshwar *et al.*, 1996)
	Cell activation	B-cells	*m*	(Rosel *et al.*, 1998)
	Promote cell –cell adhesion through the recognition of chondroitin sulfate-associated CD44 on other cells	Transfected COS7 cells	*M*	(Chiu *et al.*, 1999)

h: human *M: monkey*

r: rat *m: mouse*

Even some certain variants have been shown as above to be important in different functions (*Table 1-4*). Whether the regulation of CD44 isoforms by alternative splicing might alter the binding ability to their ligands, and give a new function remain to be discovered. Recent study, an isoform of CD44 containing v3, 8-10 has been reported to be involved in cytoskeleton-mediated cell migration (Bourguignon *et al.*, 1998). Studies using engineered combination of variant proteins have helped to reveal the functions of CD44 isoforms in disease. Developing assays that can identify these CD44 expression patterns by molecular weight sizing with PCR may lead to a better understanding of the functions of CD44.

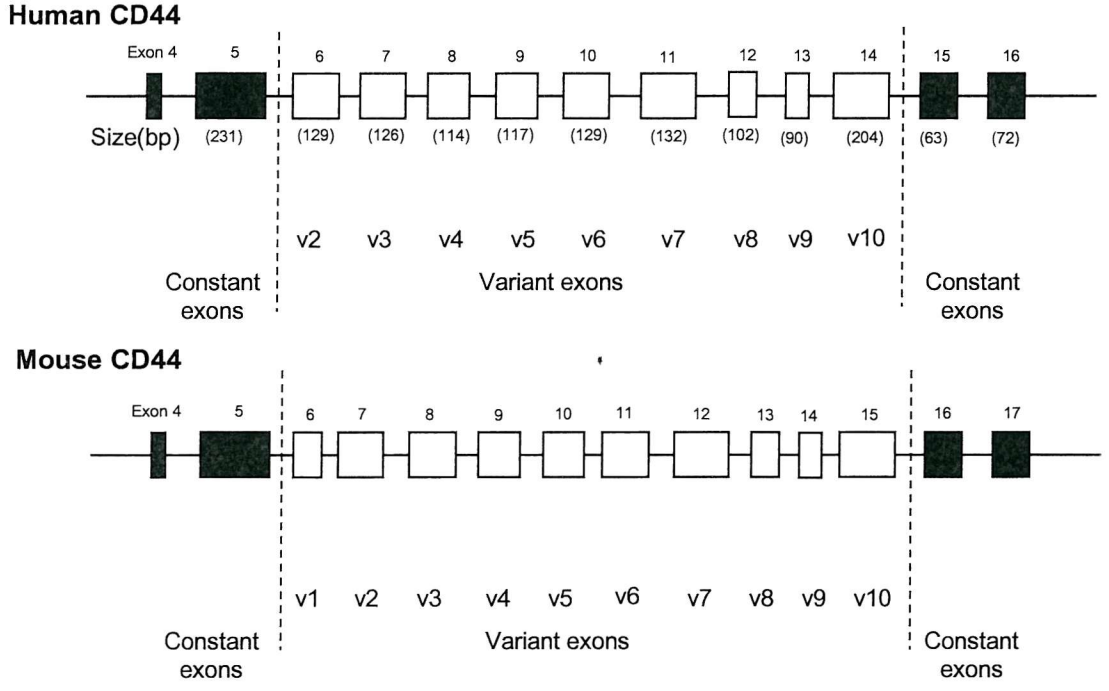


Figure 1-7. Schematic exon map of various CD44 isoforms in human and mouse. Diagrams show all 9 (10 in mouse) exon insertion, e.g. 6-14 (6-15 in mouse) between exon 5 and exon 15 (16 in mouse).

1.9 Aims of This Study

ICAM-1 and CD44 are up-regulated on the bronchial epithelium in asthma (see *section 1.7*), where airway inflammation, epithelial damage, and repair are increased and correlated. To further understand whether or how ICAM-1 and CD44 are functionally involved in airway inflammation, their roles in airway epithelial damage and repair were therefore examined here in my study. Based on the literature, ICAM-1 might play a critical role in the communication of infiltrating cells and epithelial cells; whereas, CD44 may be involved in the interaction of epithelial cell to extracellular matrix and cell signalling. These two molecules might act as receptors during airway inflammation, or only be involved in the later stages in these events and are up-regulated by other cells.

The aim of this study is to investigate the roles of ICAM-1 and CD44 on bronchial epithelial cells in epithelial damage and repair, based on the following hypotheses:

ICAM-1

(1) The localisation of ICAM-1 is correlated to its function and is up-regulated by cytokines in airway inflammation.
(2) ICAM-1 expression is cell density-dependent, and expression of ICAM-1 increases with epithelial cell damage.

CD44

(1) CD44 is functionally involved in epithelial response to damage, and it is required for epithelial repair.
(2) CD44 isoforms are involved in cell-cell adhesion and cell migration.

(3) CD44 expression is up-regulated by proinflammatory cytokines.
(4) CD44 expression is cell density-dependent, and alters its expression with epithelial cell damage.
(5) Individual airway epithelial cells express several CD44 isoforms.

(e) Proinflammatory cytokines and mechanicial cell damage can alter CD44 mRNA splicing in airway epithelial cells.

1.9.1 Outline of approach

In this study the cell lines to be used in models were first selected by comparing the localisation and expression of ICAM-1 and CD44 between bronchial epithelial cell lines and epithelium in normal tissue. By using the cell lines and primary culture, *in vitro* models were established. By treating the cells *in vitro* with proinflammatory cytokines or using mechanical damage, the expression of ICAM-1 and CD44 during inflammation and cell damage was studied. CD44 isoform expression was investigated by using a PCR technique to study CD44 mRNA splicing to identify the combinations of CD44 variant exons used during inflammation and damage. Finally, functional studies were performed in the models of cell adhesion and migration to provide more information about ICAM-1 and CD44 in epithelial cell damage and repair.

The detailed objectives to complete my studies are give below:

1.9.2 Objectives

1. Identifying suitable cell lines for *in vitro* study model. In chapter 3, the characteristics of bronchial epithelial cell lines and primary epithelial cells were compared with epithelium in airway tissue by using immunocytochemistry and the electron microscope. These studies include characterising:

(a) The expression of cytokeratins and adhesion molecules: Some cytokeratins (e.g. cytokeratin 18) and adhesion molecules (eg. E-cadherin) lineage specific in epithelial cells.

(b) Polarity: An essential feature of most epithelial cells which separates epithelium to apical and basolateral by tight junctions can be represented by measuring transepithelial electrical resistance.

(c) Ultrastructure: compare the morphology (e.g. Cilia and microvilli) and junctional mechanisms (e.g. Tight junctions, and desmosomes) of epithelium in cell line and tissues.

(d) Localisation: Examine the localisation of ICAM-1 and CD44 on cells from culture and tissue.

The main objective of this chapter is to establish an *in vitro* model for study the function and regulation of ICAM-1 and CD44 by using the cell lines with similar characteristics to the bronchial epithelial cell in tissue and the same localisation of these two molecules within the epithelium.

2. In Chapter 4 hypotheses about the regulation of ICAM-1 and CD44 were tested. By using flow cytometry, the expression of ICAM-1 and CD44 on bronchial epithelial cells is examined to established:

(a) If the expression of ICAM-1 or CD44 is up-regulated by inflammatory cytokines (IFNγ, TNFα, IL-1β, IL-4) in cells treated with cytokine for 24 hours the expression of ICAM-1 or CD44 should increase.

(b) If the expression of ICAM-1 or CD44 is cell density-dependent, the expression of ICAM-1 or CD44 will be different in confluent and sub-confluent culture. I used cells seeded in petri dishes in serial cell density and compared sub-confluent (<60% confluence) to confluent for 48 hours.

(c) If cell damage can regulate the expression of ICAM-1 or CD44, their expression level will change after mechanical damage. This will be monitored by the expression of ICAM-1 or CD44 from cell damage to wound closure.

3. With immunocytochemistry, it is impossible to examine the CD44 isoform combination expressed. In Chapter 5, I used an RT-PCR method, which can show the pattern of CD44 mRNA splicing to approach this study. A message affinity paper (MAP) PCR technique has been introduced. With this technique it is possible to compare the CD44 mRNA splice pattern in single or small numbers of epithelial cells.

(a) If single cells express only one CD44 isoform, individual cells from bronchial brushings or primary culture will show only one CD44 isoform mRNA species and different cells might show different CD44 isoforms. This will provide more information about the *in vivo* aspects of CD44.

(b) If IFNγ or TNFα can alter CD44 isoform expression, following IFNγ or TNFα treatment, cells will show different patterns of CD44 mRNA splicing to those from untreated cells.

(c) It is possible that cell damage can alter the expression of CD44 isoforms. The pattern of CD44 mRNA splicing in cells after mechanical damaged was compared the splicing pattern in undamaged cells.

4. In Chapter 6 I have investigated the function of CD44 in epithelial adhesion and migration. With cytokine induction, antibody blocking and different matrix-coated culture surfaces the roles of CD44 were investigated including:

(a) Is CD44 important for cell adhesion to its ligands? How do cytokines regulate CD44-dependent cell adhesion?

(b) Does CD44 participate in the process of cell migration? Is CD44 important in cell migration?

The objective of this chapter is to study whether CD44-dependent cell adhesion and migration is important in bronchial epithelial cells, and how is CD44 function involved in cell adhesion to matrix substrates and cell migration during repair.

CHAPTER 2

Materials and Methods

2.1.1Reagents and solutions

See appendix 1 and 2.

2.1.2 Cell lines

The bronchial carcinoma derived cell line NCI-H292 (Banks-Schlegel *et al.*, 1985) was obtained from the American Type Culture Collection (Maryland, USA) and cultured in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum (FCS) and 2 mM L-glutamine. The SV40-transformed bronchial epithelial cell line, 16HBE 14o$^-$ (Cozens *et al.*, 1994), was a gift from Dr D. Gruenert (Cardiovascular Research Institute, University of California, San Francisco, CA) and was cultured in Minimum Essential Medium (MEM, with Eagle's salts) supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. The SV40-transformed human tracheal epithelial cell line, 9HTE (Gruenert *et al.*, 1988) was grown in a mixture of F10 (HAM) and RPMI-1640 (1:1) media supplemented with 10% heat-inactivated FCS, and 2 mM L-glutamine. The Ad12-SV40-transformed epithelial derived cell line, BEAS-2B (Reddel *et al.*, 1988), was grown in LHC-9 medium supplemented with 2% serum substitute (USG), 2.5 ng/ml epidermal growth factor (EGF), and 2 mM L-glutamine. All the cell lines were grown in their media at 37 ^{o}C, in a humidified incubator with 5% CO_2. The cell density was maintained between $2x10^4$ and $1x10^5$ cells/cm^2 and cell viability was >95%.

2.1.3 Antibodies and purification

The antibody concentrations used in the immunocytochemistry and flow cytometry are listed in *Table 2-1.*

2.1.3.1 Antibody production from hybridomas

The 25-32 CD44s hybridoma was grown in Dulbecco's Modified Eagle medium (DMEM) with 10% FCS. CD44v6 and CD44v9 hybridomas were grown in Iscove's Modified Dulbecco's medium (IMEM) with 10% FCS. The hybridoma cells were then cultured in a miniPERM bioreactor (Heraeus Instruments GmbH, Germany) in high cell density culture conditions, and half of the culture medium was changed every 2 days according to the

manufacturer's suggestions. Culture supernatants were collected and antibodies were purified by protein A affinity column with MAPSII buffer system (BIO-RAD Laboratories, Hertfordshire, UK) as detailed in the method below.

Table 2-1. Antibodies for immunocytochemistry and flow cytometry

Name (antibodies to:)	*Label*	*Dilution or Conc.*	*Note*
Direct conjugated antibodies or Primary antibodies			
Human ICAM-1 (MAC532)	FITC	1/30	Serotec
Human CD44 s (25-32)	none	15 μg/ml	hybridoma
Human CD44 v3 (BBA 11)	none	1/500; 2 μg/ml	R&D
Human CD44 v6 (FW11.9.2.2)	none	15 μg/ml	hybridoma
Human CD44 v9 (FW11.24.7.36)	none	15 μg/ml	hybridoma
Cytokertin 13	none	1/500	Sigma
Cytokertin 18	none	1/500	Sigma
E-Cadherin (DECMA-1)	none	1/100	Sigma
Anti-ICAM-1 (RR1)	none	1/80	†
Secondary antibodies			
Anti-mouse IgG (H+L) $F(ab')_2$	FITC	1/80	Dako
Goat anti-Mouse IgG,	Gold (10mm)	1/25	Sigma

† Gift from Dr. R. Rothlein, Albany Medical College, USA.

2.1.3.2 Protein A purification

MAPSII binding buffer salts was added at 31.4g buffer salts/100 ml culture supernatant to enhance the affinity of antibody to protein A. After passing through a 0.45 μm filter, the culture supernatant was loaded onto a protein A cartridge (Econo-Pac Protein A cartridge, Bio-Rad) pre-equilibrated with MAPSII binding buffer. The flow rate of the cartridge was set to 1 ml/ minute. Following washing with 4-fold the cartridge volume of MAPSII binding buffer, the bound immunoglobulin G (IgG) was eluted with 6-fold cartridge volume of MAPSII elution buffer. The eluted fractions were collected at 1 ml/tube. The fractions were then dialysed against PBS (pH 7.2), and the concentration of purified

antibody calculated by measuring absorbance (OD_{280} and OD_{260}) with an UV-spectrophotometer. Binding activity in each fraction was determined by FACS analysis (see *section 2.3.3*).

2.2 Cell culture

2.2.1 Cell line maintenance

Cells were grown in their culture media in 75 cm^2 flasks and the media was changed every 3-4 days. When 80-90% confluent, cells were detached and passaged by using trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin/0.02% EDTA in Ca^{2+}- and Mg^{2+}-free Hanks' Balanced Salt Solution, HBSS). Briefly, monolayer cells in flask were washed twice with 10 ml Ca^{2+}- and Mg^{2+}-free HBSS. After the HBSS was removed, 1 ml of trypsin/EDTA was added and incubated at 37°C for 10-15 min until cells were detached. Cells were disperse by pipetting 2-3 times in the flask, and 10 ml of HBSS (without Ca^{2+} and Mg^{2+}) with 5% FCS was added to stop the activity of trypsin. Cells were collected and centrifuged at 150 x g for 10 minutes at 4°C, then cell pellets were resuspended in their culture medium. Cells were counted with a haemocytometer and viability was determined by trypan blue dye exclusion. The cell number for the culture was adjusted and cells were seeded in 75 cm^2 flasks (at 1-2x10^4 cells/cm^2 for NCI-H292 and 2-4 x10^4 cells/cm^2 for 9HTE, BEAS-2B, and 16HBE 14o$^-$ cells). Cells were cultured at 37°C with 5% CO_2 and plastics were obtained from Falcon (Becton Dickinson, Oxford, UK). All the cell lines were adherent. For maintenance, no culture was left more than two days after they were confluent. All cell lines in this study were used within 30 passages from same frozen cell stock to minimise the difference after long term culture.

2.2.2 Cell line storage

The single cell suspensions were prepared by using trypsin/EDTA detachment. The concentration of cells was adjusted to 2-5x10^6 cells/ml in culture medium with 20% FCS in a 50ml centrifuge tube and kept on ice. Dimethyl sulfoxide (DMSO) medium was made by adding 20% DMSO to the serum-free medium and kept at 4°C. An equal volume of DMSO medium was added to the cell suspension drop by drop with gently shaking. Thus, final concentration of the FCS and DMSO in the medium was both 10%. The cells were

transferred to 1.8 ml cryotubes and moved into a pre-cooled (0-4°C) box. The box contained a protective and insulating layer of cottonwool. The box was placed at -70°C, allowing the slow freezing at a rate of 0.5-1°C/minute. After 24 hours in -70°C, cells in cryotubes were transferred to liquid nitrogen or a -135°C freezer.

For thawing the cells, the cryotube was taken out of the liquid nitrogen storage container and rapidly thawed to 37°C within 2 minutes. The cell suspension was then added 10 ml culture medium and centrifuged at 150 x g for 10 min at 4°C. The cell pellet was resuspended in 8ml culture medium and seeded in 25 cm^2 culture flask. When the cells were 80-90 % confluent, the cells were detached and grown in 75 cm^2 flasks.

2.2.3 Primary culture

Bronchial tissue was obtained from surgical resection material. Normal areas of bronchial tissue had been selected and were collected by a pathologist. Normal bronchial tissue was transferred to Leibovitz's L-15 medium immediately and kept at 4°C before preparation for culture. The epithelial layer was used for primary culture within 24 hours of tissue collection. With a dissection microscope most of the submucousal layer and the other tissues below the epithelium, such as cartilage, were removed. Then the human bronchial epithelial cells were cultured by cutting this epithelial layer into 1- to 2-mm squares with a sterile scalpel. Individual explants were placed upside down in the middle of a well in a 24 well plate (Falcon Primaria, Becton Dickinson, UK) in LHC-9 medium with 2% USG, 2mM L-glutamine, penicillin-streptomycin (100U/ml and 100μg/ml) and amphotericin B (0.25 μg/ml). The epithelial cells from the explants were allowed to grow to cover at least 50% of each well. Culture media were replaced every 3-4 days. The explants were passaged by moving to a new well and continued to grow. The confluent monolayers within 3 passages from each explant were used for experiment. Each well was carefully checked for the cell morphology with an inverted phase-contract microscope, and the wells with fibroblast contamination were discarded. In addition, immunocytochemistry for cytokeratin 18 (CK18), cytokeratin 13 (CK13), and CD44 was also performed to characterise the epithelial cells.

2.2.4 Cell preparation for immunocytochemistry

Cells ($2x10^5$cells/ml, 150µl) were placed on 13-mm sterile glass coverslips in petri dishes. Four hours after seeding, cells were added to coverslips, and culture medium was added to cover the petri dishes. When the cell cultures reached the confluent level desired, the cells on coverslips were rinsed twice briefly in warm serum free medium. Cells were fixed with cold methanol (-20°C) for 5 minutes and then washed twice in PBS before immunostaining.

Coverslips were incubated in 1% bovine serum albumin/PBS (1% BSA in PBS) for 10 minutes, followed by primary antibody incubation at room temperature for 60 minutes (diluted in 0.1% BSA/PBS). After 3 washes in PBS, the coverslips were incubated with fluorescein isothiocyanate (FITC) conjugated secondary antibody (at a dilution of 1/80 in 0.1% BSA/PBS) at room temperature for 60 minutes. Then the cells on coverslips were mounted in mowiol with Citifluor anti-fading agent (Citifluor, London, UK). Immunostaining was examined using a Leica DMRB fluorescence microscope.

2.3 Flow cytometry

2.3.1 Cell preparation for FACS analysis

For FACS analysis, the cells were washed with HBSS without Ca^{2+} and Mg^{2+} twice and detached by cell dissociation solution (Sigma). The cells were washed with FACS buffer (HBSS with 2% FCS, and 0.1 % NaN_3) and adjusted to $5x10^6$ cells/ml. All procedures were carried out at 4°C. For direct staining, 30 µl diluted antibodies (e.g. anti-ICAM-1 FITC-conjugated antibody or isotype control antibody with FITC-conjugated) were added to 0.1 ml cell suspension and then incubated for 45 min, on ice, in the dark. For indirect staining, 30 µl of diluted antibodies or control (e.g. anti-CD44v3 or IgG_{2b} isotype control) was added to 0.1 ml cell suspension. The cells with antibody were then vortexed and incubated for 45 minutes on ice. The cells were washed then resuspended in 100 µl buffer. 30 µl of diluted FITC-labelled F(ab') fragments of goat anti-mouse IgG (H+L) antibody were added to the cells. The cell suspension was vortexed and incubated for 45 min, on ice, in the dark. After final incubation with antibodies, the cells were washed with

buffer and resuspended in 450 µl FACS buffer. Cell suspensions were analysed using a FACScan equipped with lysisII software (Becton Dickinson, NJ, USA).

2.3.2 FACS Scatter parameters and selective gating

A threshold which excluded most of the debris (see *Figure 2-1*) was set when acquiring data. A Gating technique was further used to obtain accurate results during analysis. The dot plots of forward scatter (FSC) and side scatter (SSC) were used to identify the cell population of bronchial epithelial cell lines (*Figure 2-1*). Since dead cells have a smaller FSC, data were collected for 10,000 events without setting any live gating. Gating was used in dot plots (FSC vs. SSC) for excluding dead cells and cell debris as in *Figure 2-1*. Fluorescence intensity data was processed into histograms.

2.3.3 Antibody titration

For antibody titration, $2x10^5$ cells (100 µl) were incubated with 30µl antibody at the following dilutions: 1/1024, 1/256, 1/64, 1/16, 1/4, and 1/1. The point at which saturation of antibody binding was defined on the flow cytometry was when increasing antibody concentration produces less than 5% increase in fluorescence intensity (*Figure 2-2*). The antibody was then used routinely at twice the saturating concentration to compensate for errors in cell number and for increased antigen expression.

2.3.4 FACS data analysis and presentation

In general, frequency histograms are used to present flow cytometry data. This display shows the relative cell number against fluorescence intensity. When comparing the change of fluorescence intensity in different samples, overlapping frequency histogram displays can show the change(s). However, it is difficult clearly to present more than three frequency histograms in the same chart in this format and small changes are difficult to see. In order to compare the expression levels of ICAM-1 and CD44 in the experiments with multiple time points, an alternative presentation method was applied by using cumulative histograms to show the results. Particularly when the frequency histogram data are not normally distributed, cumulative histograms can more clearly represent the original information from the flow cytometry. Therefore, some the data were transferred

from frequency histograms to cumulative histograms (*Figure 2-3*). For those data with adequate samples, the data were expressed as median of fluorescence or the percentage of positive cells calculated by using the number of cells having a fluorescence intensity of more than two standard deviations above the mean value for the control.

2.4 Electron microscopy and sample preparation

2.4.1 Sample preparation for TEM and SEM

Cells on filter inserts (*section 2.5.7*) were washed twice with PBS and fixed with freshly made 3% paraformaldehyde and 0.1% glutaraldehyde in PBS for 2 hours. The cells on the filter insert were then rinsed twice with PBS and treated with 50 mM NH_4Cl in PBS to quench free aldehyde groups. Filters were then cut out of their inserts. For ultrastructural studies, filters were embedded in resin. After embedding, sectioning and contrasting with lead citrate and uranyl acetate (saturated in water), the specimens were examined under a Hitachi 7000 electron microscope. For scanning EM, after fixation the cells on filters were rinsed in 0.1 M cacodylated buffer, pH 7.4, containing 6.8% sucrose overnight, and postfixed with 1% osmium tetroxide in cacodylate buffer for 2 hours at 4°C. After washing with distilled water, inserts were dehydrated in a graded ethanol series. Inserts were then dried in a freeze dryer and coated with platinum using a sputter coater. The morphology of cells on the filter inserts was observed with a scanning electron microscope (Hitachi S800).

2.4.2 Cryosectioning for Immunoelectron microscopy

For cryosectioning, the fixed tissue or cells on filters (fixed as *section 2.4.1*) were transferred into 2.3 M sucrose (in 1.1M Na_2CO_3, with 10% 10K polyvinyl pyprolidone in PBS) and stored overnight at 4 °C. The epithelial samples were mounted on aluminium holders, with approximately 1 mm of sample projecting. The samples were frozen by plunging them into liquid nitrogen and stored under liquid nitrogen until cutting ultrathin cryosectionings (70-90 nm). Sectioning was carried out using a glass or diamond knife at a temperature of -110°C in a liquid nitrogen chamber with a Leica EM FCS Cryomicrotome. The sections were transferred by using a small drop of 2.3M sucrose onto formvar-coated grids and stored inverted on 2% gelatine plates.

2.4.3 Localisation by immunoelectron microscopy

After the gelatine had been melted at 37 °C for 10 min, ultrathin cryosections were washed and labelled with anti-ICAM-1 mAb or anti-CD44 s mAb, then goat anti-mouse IgG with 10 nm gold conjugated antibody (at OD_{525} = 0.1 in PBS containing 1%BSA, 0.05% Triton X100, and 0.05% Tween 20). After contrasting and embedding with uranyl acetate (0.2%) in methyl cellulose, the specimens were examined for the localisation of ICAM-1 or CD44 with a Hitachi 7000 electron microscope.

2.5 Analysis of CD44 expression by flowcytometry

2.5.1 Basal expression of ICAM-1 and CD44 in the cell lines

9HTE, 16HBE 14o$^-$, BEAS-2B, and NCI-H292 cells were grown in 60 mm petri dishes. When 90% confluent, cells were collected by non-enzyme cell dissociation solution for flow cytometry analysis as in 2.3.1.

2.5.2 Shedding adhesion molecules after trypsin treatment

16HBE 14o$^-$ ($2.0x10^6$ cells/petri dish) and NCI-H292 ($1x10^6$ cells/petri dish) cells from trypsin digested culture were plated in 60 mm petri dishes. The cells were collected at different time points from 1-48 hours (1, 3, 6, 9, 12, 24, 36, and 48 hours) after plating. Up to 3-4 hours after seeding, some of the cells might still not have attached to the culture plastic surface. The suspended cells were collected and the adherent cells were collected by non-enzyme cell dissociate solution. Then cells were stained with antibodies for ICAM-1 and CD44 for FACS analysis as described in *section 2.3*. The fluorescence intensities of ICAM-1 or CD44 on the cells at different time points were compared.

2.5.3 Cytokines and the expression of ICAM-1 and CD44

(A) The effect of cytokines on ICAM-1 and CD44 expression

NCI-H292, 16HBE 14o$^-$, 9HTE, and BEASE-2B cells were detached by trypsin/EDTA and cultured in 24-well plates at $2-5x10^5$ cells/ml, 0.5 ml/well. When 60-70% confluent,

cells were serum deprived by incubation of cells in complete culture medium without serum for 16-18 hour, then changed into their medium containing 2% USG serum substitute instead of FCS. Cells were incubated with IFNγ and TNFα concentrations from 10 U/ml to 400 U/ml for 24 hours. Cells from 3 wells were collected by non-enzyme cell dissociation solution and stained with ICAM-1 or CD44 antibodies for FACS analysis. The fluorescence intensities of the cells after different treatments were compared. The concentration of 100-200 U/ml IFNγ or TNFα 100-200 U/ml were selected for the cytokine studies, because they gave maximal ICAM-1 responses, and also had been used in other studies (Mackay *et al.*, 1994) to examine up-regulation of CD44.

For some experiments, 16HBE 14o$^-$ cells were also prepared in the same way and treated with IL-1β (30ng/ml), IL-4 (30ng/ml), or phorbol myristate acetate (PMA) (250ng/ml) for 18 hours.

(B) The effect of gold thiomalate on ICAM-1 and CD44 expression

Cells were grown to 70% confluence in 24-well plates. Aurothiomalate (AuTM, May&Baker Limited) was added at concentrations from 10^{-5} to 10^{-2} M, and incubated for 24 hours. For some experiments 10^{-3} and 10^{-2} M of AuTM were added accompanied with 100 U/ml IFNγ or TNFα. The cells were harvested and analysed for ICAM-1/CD44 expression by flow cytometry.

2.5.4 Cell density and the expression of ICAM-1 and CD44

In order to understand the relationship of cell density and the expression of cell adhesion molecule, cells in different sub-confluent and confluent cultures were compared. However, in different cultures, soluble factors, such as cytokines, might be able to regulate the expression of cell adhesion molecules. To minimise the possible effects of nutrient depletion and soluble factors produced by cells at higher densities, low and high density cell cultures were grown in culture plates and shared the same medium. This model cultured the cells in 90mm petri dishes which were initially divided into two parts by a plastic spacer. The gap between the plastic spacer and petri dish was sealed by sterile petroleum jelly (Vaseline). Cell suspensions for high and low cell densities were plated on either side of the divider. High density cultures ($1.0x10^5$ cells /cm^2 to $0.3x10^4$

cells/cm^2) were plated on one side of petri dish, and low density cultures (0.125x high cell density) on the other. The cells were allowed to settle and adhere for 12 hours, after which the spacer was removed and cells were cultured in the same medium until the highest cell density culture in the series had been confluent for 3 days. The cells in two parts of the petri dishes were washed twice with Ca^{2+}- and Mg^{2+}-free HBSS, and the cells were carefully detached without mixing the high and low cell density culture. FACS analysis was performed as detailed in *section 2.3*.

To examine the effect of the period of time in culture on CD44 expression following plating, 1x10^5 cells/cm^2 were plated in one side of a dish and 1.25x10^4 cells/cm^2 in the other. After 12 hours, the spacers were removed and cells from each dish were collected as above but after different times (24, 48, 72, 96, 120 hours).

Cell confluence was defined as the point when the surface of the culture dish was covered in a layer of cells with no gaps visible by phase contrast light microscopy. Sub-confluent cultures were expressed by percentage of confluence culture using cell number counts in the sub-confluent culture divided by the cell number at confluence. Cell number was established using a haemocytometer.

2.5.5 Cell proliferation at different cell densities

Single cell suspensions at 3x10^6 cells/ml were prepared by non-enzymatic cell dissociation solution. One ml of cell suspension was transferred into a 15x75-mm centrifuge tube. After centrifugation, the cell pellet was fixed with 1ml of 70 % ice-cold ethanol (in PBS) overnight at 4°C. Following incubation with 1 ml of 50 μg/ml PI with 100 U/ml RNAse I for 30 minutes, cells were analysed for cell cycle analysis in a flow cytometry.

2.5.6 Cell damage and the expression of ICAM-1 and CD44

Cells were cultured in 90 mm diameter petri dishes. Approximately 48hrs after confluence, the cell layers were damaged using a fine plastic pipette tip by scribing 150 lines on the monolayer cells. The cells were washed twice with HBSS and changed into fresh medium. The damage was carried out from 120 to 1 hours (120, 96, 72, 48, 36, 24, 12, 6, 3, and 1 hour) before harvesting the cells. Cells were harvested as above and analysed by flow cytometry.

2.5.7 Epithelial integrity- TER measurement

The transepithelial electrical resistance (TER) was measured to monitor monolayer tightness, and indicates if tight junctions are formed between cells in various cell lines. Cells were grown in 24-well polyethylene terephthalate membrane inserts (PET, pore size 3 μm, Falcon 3096) at $2.5x10^4$ cells/cm^2 with 0.45 ml media in the upper chamber (insert) and 0.8 ml of culture media in the lower chamber (well). After day 3, the cell integrity was monitored daily by measuring the TER using an epithelial tissue voltohmmeter (EVOM, World Precision Instrument, FL, USA) every day. After measurement, the culture media was changed for fresh culture media.

2.5.8 Localisation of ICAM-1 and CD44 by FACS

When the resistance value, TER, was higher than 1000 ohms-cm^2, the cells were used for localisation studies. For flow cytometry, ICAM-1 or CD44 antibody was added from the upper chamber, which allowed antibodies to bind on the apical surface of the cells. after the antibody incubation of 30 minutes, the TER was checked to confirm that the epithelial layer is left intact and antibody can only bind to apical surface. Cells were washed with HBSS (Ca^{2+}- and Mg^{2+}-free) with 0.1% NaN_3 twice and detached with cell dissociation solution contained 0.1% NaN_3. The cells from each culture insert were derived to two tubes. Cells in one of the tubes were stained with ICAM-1 or CD44 again. For CD44 staining, cells in both tubes with or without secondary CD44 antibody incubation, were stained with secondary antibody for 30 minutes. After washing, the cells were subjected to flow cytometry analysis.

2.6 Image analysis

For each time point, duplicate samples were stained as above and measured at the same time. All the samples were kept in the dark and examined for the same short period of time to minimise fading. At 3 random points along the damaged edge in each sample, 7 neighbouring images at x200 magnification (field size 75x135 μm, image size 765x512 pixels) were taken. To do this, a cooled CCD camera (Digital Pixel, Brighton, UK) connected to a Leica DMRB fluorescence microscope was used and images were then transferred to TIF format and staining intensity assessed using Scion Image software

(http://www.scioncorp.com; Scion corporation, Maryland, USA). with a Pentium II-233 MHz based microcomputer. Fluorescence intensity was measured as the mean of pixel intensity in each rectangle.

2.7 Analysis of CD44 isoforms

2.7.1 Cells for PCR

Mouse embryos at 2-cell to blastocyst (32/64-cell or more than 32/64-cell stage, day 3.5-4.5) were provided by Doctor T Fleming (Biological Sciences, University of Southampton). Human primary bronchial epithelial cells less than 3 passages were used for the experiments. Single cell suspensions were prepared by trypsin digestion. Cells were resuspended in carbonate-free medium, Leibovitz's L-15, and prepared for message affinity paper (MAP) PCR. Cells from 3 wells (24 well plate) of the same tissue source were used for total RNA extraction as described below. One microgram of total RNA extracted from primary epithelial cells carried on conventional RT-PCR was used to compare with the results from MAP-PCR.

2.7.2 Total RNA extraction from cells

Monolayer cultures in 30 mm diameter petri dishes were washed with serum-fee medium and then lysed with 0.8 ml TRIZOL reagent. After pipetting several times through a pipette tip, the cell lysate was collected in a 1.5 ml polypropylene microcentrifuge tube (Eppendorf) and kept at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes. To each microcentrifuge tube, 0.2 ml of chloroform was added and mixed vigorously by vortexing. Following centrifugation at 12,000 x g for 15 minutes at 4°C, the upper aqueous phase in which RNA remained was collected to a new tube. An equal volume of isopropyl alcohol was added to each tube and the mixture was incubated at room temperature for 10 min. After centrifuging at 10,000 x g for 10 min at 4°C, the supernatant was removed and the RNA pellet was washed with 1 ml 75% ethanol/water. Following centrifugation at 7,500 x g for 5 minutes at 4°C, the RNA was briefly dried (air-dry for 5-10 minutes, without letting the RNA completely dry) and dissolved in diethyl pyrocarbonate (DEPC) water for RNA concentration measurement with an UV

spectrophotometer. The total RNA (1 μg) was used for comparing the results with the MAP-PCR.

2.7.3 Conventional RT-PCR

Total RNA (1 μg) was reverse-transcribed in a volume of 20 μl by 3' primer (*i*) of polymerase chain reaction (PCR) with Moloney Murine leukaemia virus (M-MLV) reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions. The complimentary DNA (cDNA) fragment was nested PCR-amplified by $Vent_R$ DNA polymerase (New England Biolabs, Inc) with 2mM $MgSO_4$ in a volume of 50 μl during 35 cycles of PCR with *h* and *i* primers (*Table 2-2*) in the first PCR. PCR cycles were as follows: denaturing for 30 seconds at 94°C, annealing for 45 seconds at 60°C, 1.5 minutes at 72°C, and, after 40 cycles, a final extension step for 3 minutes at 72 °C. Ten μl of the first PCR product was PCR-amplified a second time with primers *j2-k2* or exon v3 or v6 specific primers (*Table 2-2*) under the same conditions.

Table 2-2. Oligonucleotides used for the amplification or detection of CD44 variant

	Sequence
CD44	
5' Standard (*h*)	5'-CTA TTG TTA ACC GTG ATG G-3'
3' Standard (*i*)	5'-CAC AAC CTC TGG TCC TAT A-3'
5' Standard (*j*)	5'-CAT TAC GAA TTC GAT GTC AGC AGC GG-3'
3' Standard (*k*)	5'-CAT TAC GAA TTC ATT CGG ATC CAT GAG T-3'
5' Standard (*j2*)	5'-CCT ACT GAT GAT GAC GTG AGC AGC GG-3'
3' Standard (*k2*)	5'-ACT CAT GGA TCT GAA TCA GAT GGA CAC T-3'
pv3	5'-TAC GTC TTC AAA TAC CAT CTC AGC-3'
pv6	5'-CAG GCA ACT CCT AGT AGT AC-3'
rv6	5'-CAG CTG TCC CTG TTG-3'
rv10	5'-TTC CTT CGT GTG TGG GTA ATG AGA-3'
5' actin	5'-GACTACCTCATGAAGATCCT-3'
3' actin	5'-ATCCACATCTGCTGGAAGGT'-3'

2.7.4 PCR with small numbers of cells using messenger affinity paper

This technique was based on a previously described technique (Sheardown, 1992; Collins and Fleming, 1995b). Mouse embryos or single cell suspensions (3T3 cells or primary bronchial epithelial cells) were resuspended in Leibovitz's L-15 medium. Cells were picked up by using a fine capillary with a micromanipulator under the inverted microscope. All solutions used for RNA preparation, cDNA synthesis, and amplification, were pre-treated with 4000 mJ /cm^2 UV-irradiation (254 nm). MAP (Amersham International) was cut into small squares (2 mm^2), which were wetted with 0.5 M NaCl and allowed to air dry on a pad of filter paper. Single cells were placed onto MAP in a minimal volume (<5 µl) of medium. The cell was lysed *in situ* with 10 µl of lysis buffer (pH 7.5, 0.1 M, Tris-buffered, 4M guanidinium thiocyanate, 0.1 % β-mercaptoethanol) in 1µl drops, ensuring that the solution soaked through the membrane and did not overflow onto the filter pad. Binding of mRNA was similarly accomplished by dropwise application of 20 µl of 0.5 M NaCl to the membrane.

After loading, the membrane squares were immediately washed by vortexing in two changes of 0.5 M NaCl (1 ml) followed by two changes of 70% ethanol/ DEPC water (1 ml). After air drying, the membranes were reverse-transcribed with M-MLV reverse transcriptase in a volume of 20 µl. After reverse transcription, membrane squares were transferred directly to the first nested PCR mix without further dilution, and the PCR carried out with the membrane in a 50 µl mixture of PCR reagents as described in *section 2.7.3*.

Oligonucleotide primers for CD44 amplification are listed in *Table 2-2*. The PCR products were analysed on 1.5% agarose gel. Gels were run in TAE buffer for 1.5 hours at 100 volts, and stained with 2 µg/ml ethidium bromide solution before visualisation by UV irradiation of the gel.

2.7.5 Cloning of CD44 isoforms

A. Preparation of DNA fragments for ligation

Following electrophoresis the CD44 cDNA fragments were isolated from agarose gels using a QIAquick PCR purification kit (Qiagen Ltd) as described by the manufacturer. The cDNA was phosphorylated by T4 polynucleotide kinase at 37 °C for 60 minutes, then inactivated at 90 °C for 15 minutes (Appendix 3-A).

B. Preparing the CD44-pGFP vector for cloning

Plasmid pGFP (CLONTECH Laboratories) was digested by enzyme *Hpa I* (Promega) and dephosphorylated with Calf Intestinal Alkaline Phosphatase (Promega) to prevent self-ligation of the vector (Appendix 3-B, C). Insert DNA and plasmid vector were ligated at the ratio of 1:1 at 14 °C overnight (Appendix 3-D).

C. Preparation of competent cells

A single colony of *Escherichia coli* (*E. coli*) JM101 was inoculated and cultured in 10 ml of Luria-Bertani (LB) broth at 37°C overnight. One ml of the overnight *E. coli* culture was used to inoculate 25 ml LB broth in a flask at 37°C with shaking at 180 rpm. *E. coli* was grown until the OD_{550} reached 0.4-0.6 (approximately 1 hour 40 minutes). Cells were cooled down on ice for 20 minutes and then pelletted by centrifugation at 4,500 x g for 10 minutes at 4°C. The cell pellet was gently resuspended in 10 ml of ice-cold 0.1 M $MgCl_2$. After centrifugation, the cell pellet was resuspended in 1 ml of 0.1 M $CaCl_2$ and kept on ice for at least one hour.

D. Transformation of competent cells

Ten µl of CD44-pGFP was mixed with 100 µl of competent cells, and incubated on ice for 30 minutes. Following heating at 42 °C for 2 minutes, the transformation competent cells were placed on ice for 30 minutes. To select for transformants, 10 µl of each transformation mix was plated onto an ampicillin selection LB plate; and another 90 µl was plated on another.

E. Analysis of transformants

The successful transformants were detected by the eye when LB plates were exposed to standard long-wave UV light. The green fluorescent clones were transfered into 10 μl of H_2O in Eppendorf tubes. One μl of the cells was grown on a LB plate at 37°C overnight and stored at 4°C. To the rest of the cells in the tube was added 10 μl of 0.25M KOH, and this was then boiled at 100°C for 5 minutes. Ten μl of 0.5M tris-HCl (pH 0.75) and 270 μl of H_2O were added to each tube. Following centrifugation at 13,000 rpm for 1 minute, cDNA was amplified with *taq* DNA polymerase (Progema) in a reaction volume of 25 μl according to the manufacturer's instructions, namely 25 cycles of 94°C for 15 seconds, 50°C for 15 seconds, and 72 °C for 15 seconds.

2.7.6 Sequencing of CD44 isoforms

Following purification using a by QIAquick PCR purification kit, cDNA samples were analysed with an Applied Biosystems model 373A automated sequencer using Taq cycle dideoxy terminator chemistry (Amersham Life Science Ltd., UK). Computer analyses of the sequence data were performed using Lasergene sofware (DNASTAR Inc., Madison, Wisconsin, USA)

2.8 Functional assays of CD44

2.8.1 Adhesion assay

96-well microplates (Costar #3590) were coated with 50 μl of 1mg/ml HA; 30 μl of 100 μg/ml collagen I, collagenII, or collagen IV (equal to 10 μg/cm^2); 30 μl of 20 μg/ml fibronectin, or laminin (equal to 2 μg/cm^2) (all the matrix substrates from Sigma) or 30 μl of 500 μg/ml BSA overnight at 4 °C. Collagen coated surfaces were left to air dry, and excess of HA solution was removed before air-drying. After blocking with 500 μg/ml heat inactived-BSA for 30 min at room temperature, the plates were washed with 100 μg/ml BSA/PBS three times in 30 minutes before using for adhesion assay.

16HBE 14o$^-$ or primary bronchial epithelial cell suspension were prepared by trypsin/EDTA, and grown at 5x10^5 cells/cm^2 in serum substitute medium (with 2% USG)

for at least 24 hours. For cytokine treatments, cells were cultured in medium with cytokines for 16 hours. The cells were then detached by non-enzyme cell dissociation solution. The cell concentration was adjusted to $2.5x10^6$ cells /ml and incubated with CD44 or control antibodies.

After pre-incubation cells with anti-CD44(s, v6, or v9) or isotype control antibodies for 45 minutes at 4°C, the cells were allowed to adhere to the wells of matrix-coated or BSA-coated plates (5×10^4 cells in 100 µl USG-medium) for 1.5 hours at 37°C in a CO_2 incubator. Non-adherent cells were gently but consistently removed by titurating 8-channel electronic pipette with warm medium containing 5 % FCS three times. Adherent cells were quantitated using CellTiter 96® AQueous MTS assay (Promega) by measuring the enzymatic conversion of a tetrazolium dye during a 2 hour incubation at 37°C. Plate absorbtion at 490 nm was determined using a microplate reader.

2.8.2 Cell Migration on matrix substrates

Six well culture plates were coated with with 100µl/well of 100 µg/ml (collagen I, collagen IV or fibronectin) or 1mg/ml (HA) extracellular matrix or 1% BSA in PBS overnight at 4 °C. Plates were then washed with 0.5% BSA/PBS 3 times, and 4 ml of cell suspension ($2x10^5$ cells/ml) prepared as described in adhesion assay (*section 2.8.2*) was added in each well. After cells had been seeded for 4 hours, the culture medium was changed with or without anti-CD44 antibodies (s, v6, and v9). Cells were grown for a further 4 hours to allow for cell migration. The cells were fixed with cold methanol for 5 minutes and stained with 1 % toluidine blue/ 1% Borax for 1 minute. Cell migration was determined by counting cell number/clump.

2.8.3 Cell migration after mechanical damage

16HBE $14o^-$ cells were cultured in 40-mm petri dishes with a 2x2 mm grid (Nunc, Life technologies, Paisley, UK). Two days after cell confluence, the epithelial monolayer was damaged by a pipette tip. Following washing twice with warm HBSS, fresh culture media was added with or without CD44s antibody (25-32). The cell migration was measured by measuring the damaged area immediately, and then after 3, 6 and 9 hours with a cooled

CCD camera connected to a Leica DM IRB inverted microscope. The decrease in the damaged area in the image was analysed using Scion Image software.

2.8.4 Localisation of CD44 blocking antibody

Cells incubated with CD44s antibody after mechanical damage (*section 2.8.3*) were washed twice with warm serum-free medium and fixed with cold methanol (-20°C) for 5 minutes. After 3 washes in PBS, the cells were incubated with FITC-conjugated goat anti-mouse antibody (at a dilution of 1/80 in 0.1% BSA/PBS) at room temperature for 60 minutes. Followed 3 washes in PBS, the cells on petri dishes were mounted in mowiol with Citifluor anti-fading agent. CD44 antibody staining was examined using a Leica DMRB fluorescence microscope.

2.9 Statistical analyses

All flow cytometry data were expressed as median fluorescence values. Statistical significance was assessed with a non-paired two-group t test, paired two-group t test (cell migration experiments), or the ANOVA test (cytokine treatments) with Sigmaplot 5.0 (SPSS inc.) or Statview 4.0 (SAS Institute).

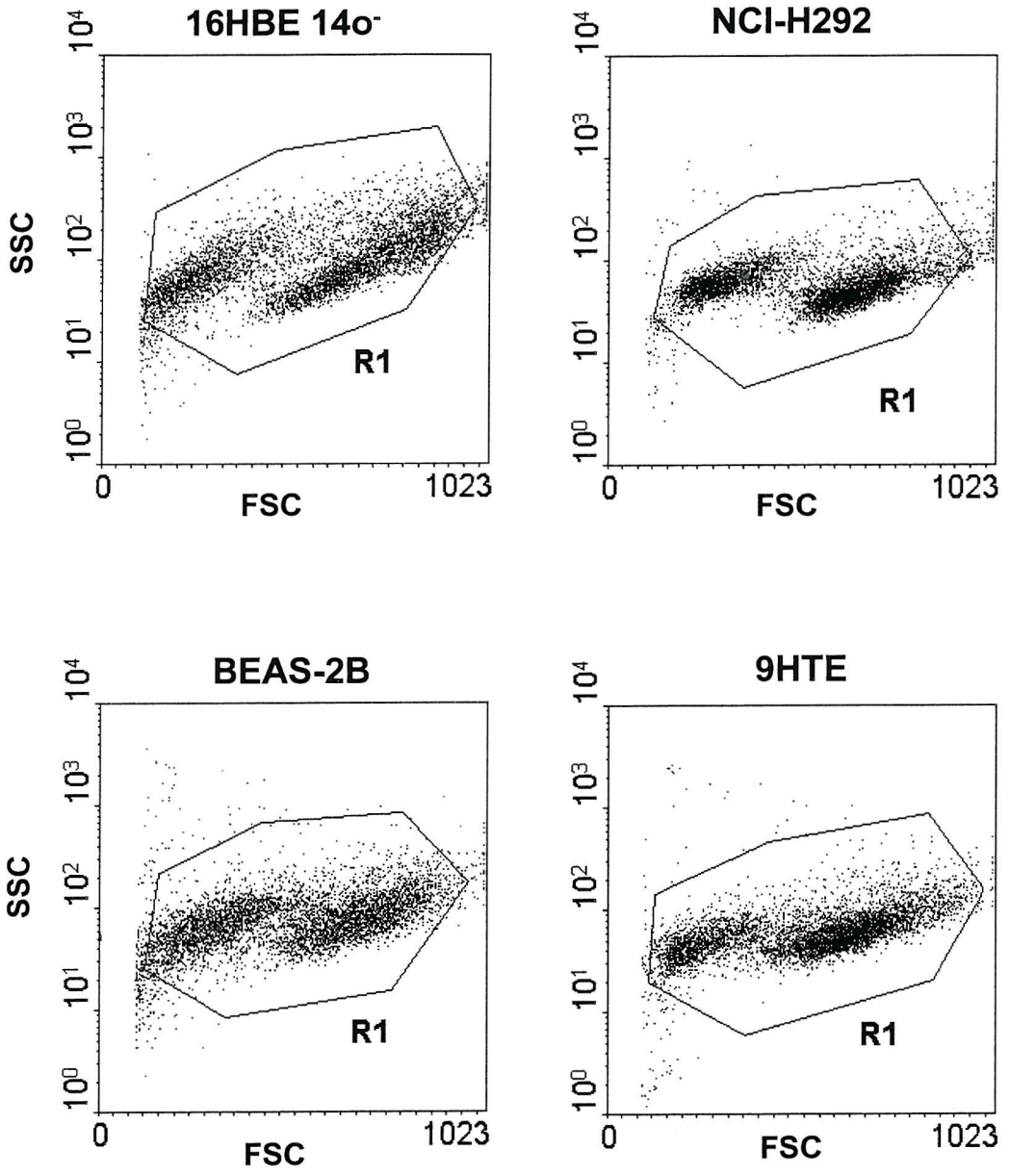


Figure 2-1. The dot plots (FSC vs. SSC) of the cell population of bronchial epithelial cell lines. Polygons gates were used for excluding debris in the left lower corner.

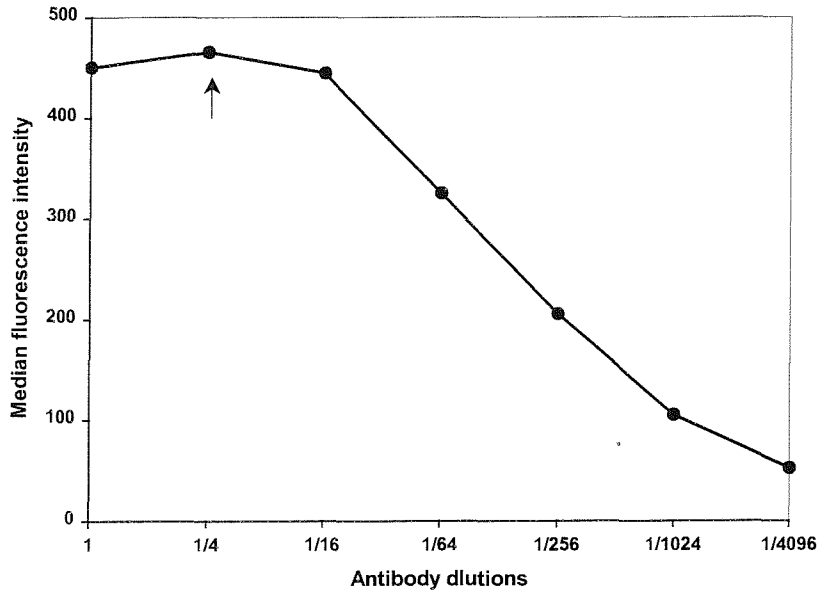


Figure 2-2. Titration of CD44 antibody.

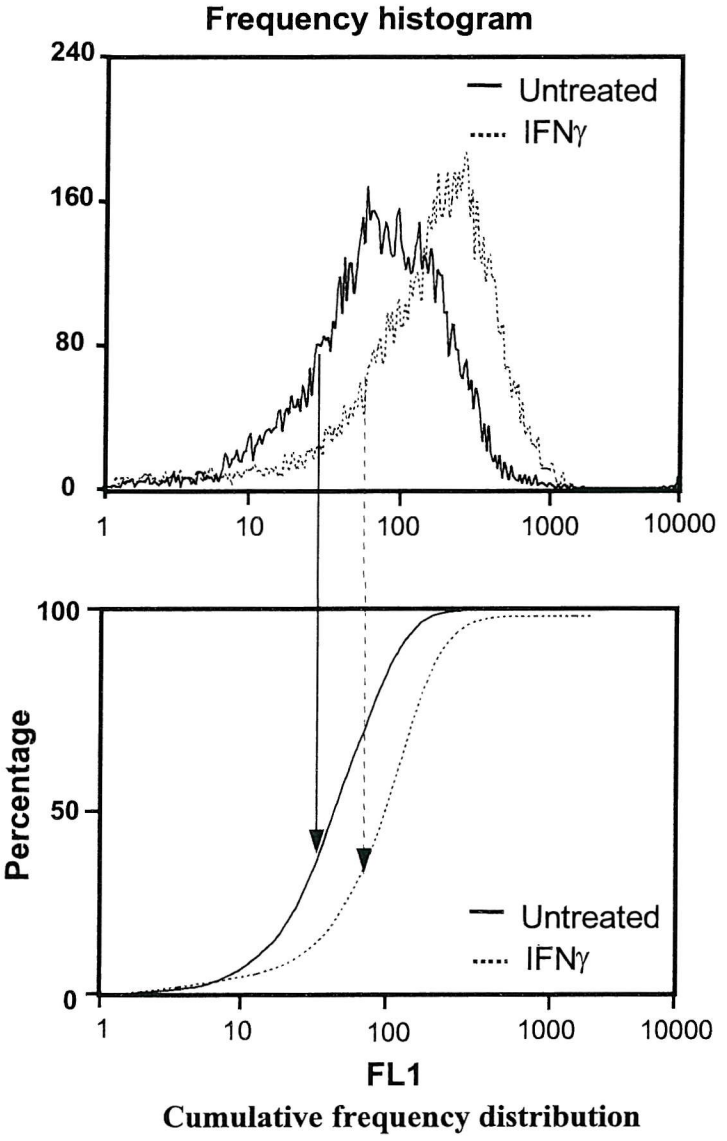


Figure 2-3. Frequency histogram to cumulative histograms

CHAPTER 3

Characteristics of Bronchial Epithelial Cells

3.1 Introduction

The normal differentiation and reparative re-epithelialisation of bronchial epithelium are not well understood. Of the three predominant cell types, basal and secretor cells are known to divide, while ciliated cells are considered terminally differentiated (McDowell *et al.*, 1985). *In vitro*, epithelial differentiation is usually lost. For instance, a decrease in the number of ciliated cells was seen in the primary culture of human nasal epithelial cells (Chevillard *et al.*, 1991), and loss of mucus secretion has also been reported in rabbit tracheal explants (Kennedy and Ranyard, 1983). Thus, investigation of the characteristics of epithelial cells and an understanding of the cell properties is important to help to select the best cell lines for study. These studies may also help to determine the culture requirements and monitor the identity of the cells under investigation.

Most epithelial cultures have a characteristic tight polygon-shape or a honeycomb-like appearance with cells growing in well-circumscribed patches. However, not all of the epithelia grow in this manner and other types of cells may have similar morphology to epithelia. For these reasons, more reliable methods for epithelial identification are necessary. This can be approached by using certain specific markers. One group of intermediate filament proteins, the cytokeratins, have been found predominately in epithelia. Of the cytokeratins, cytokeratin 13 (CK13) is considered as a marker of basal cells; cytokeratin 14 (CK14) is a marker of non-ciliated epithelial cells; cytokeratin 18 (CK18) is regarded as a marker of bronchial epithelium (basal, ciliated and non-ciliated cells) (Brezillon *et al.*, 1995); (Sun *et al.*, 1985; Schlage *et al.*, 1998)and J.E. Baker personal communication,.(Baker *et al.*, 1994). With the exception of Langerhans cells in skin, desmosome junctions are specific to epithelia (Moll *et al.*, 1982). Other adhesion molecules, such as E-cadherin, are important in maintaining the epithelial layer (Näthke *et al.*, 1993), although they are not exclusively found in epithelia.

The polarity of epithelial cells is an important feature and is essential for the barrier function of epithelium to work correctly. In bronchial epithelial cells, for example in columnar cells, the functions of the epithelium are dependent on specialised cellular adhesion mechanisms including tight junctions, and intermediate junctions (See *section 1.6*). These adhesion molecules and the junctional complexes are located at the top of the

lateral domain thereby dividing the cell surface into the apical surface, lateral domain, and the basal domain.

Among these molecules, CK13, CK14, CK18 and E-cadherin were used in this study for the identification of bronchial epithelial cells. With immunohistological techniques and electron microscopy, this provides an insight into differences in various features between transformed cell lines and normal epithelial cells. The transepithelial resistance (TER), which reflects the tight junction function, was investigated in cultures of different cell lines. In this chapter, I have compared different bronchial epithelial cell lines and primary cells in terms of their morphology, expression of cytokeratins, TER, and ultrastructure. Furthermore, by using immunogold labelling the localisation of ICAM-1 and CD44 was compared in the cell line and primary cell. The purpose of this chapter was to compare the characteristics of epithelial cells in different cell lines and primary cells to establish an *in vitro* culture model to study the regulation of ICAM-1 and CD44 in bronchial epithelial cells.

3.2 Results

3.2.1 Basic characteristics of bronchial epithelial cells

On plastic tissue culture surfaces, the cell lines, 9HTE, 16HBE 14o$^-$, BEAS-2B, and NCI-H292 showed strong attachments to the substratum. Under the inverted phase-contrast microscope, three cell lines, 9HTE, 16HBE 14o$^-$ and NCI-H292 cells displayed a polygon-shape or honeycomb-like morphology at confluence (*Figure 3-1A, B, D*). The cells generally grew without visible gaps with adjoining cells. However, BEAS-2B cells were spindle-like without well-formed cell-cell contacts (*Figure 3-1C*). This cell line was cultured in low calcium medium (0.11 mM), LHC-9 (Lechner and LaVeck, 1985), with 2% USG. Since many adhesion mechanisms are dependent on calcium concentration, higher concentration of Ca^{2+} (1-2.5 mM) was tested in the cultures. Even supplying sufficient nutrition or in high calcium medium (up to 2.5 mM), BEAS-2B cells were not able to fill the culture surface. Basically all the four cell lines grew as monolayers. Upon prolonged maintenance in the confluent state, some 16HBE14o$^-$ cells started to grow over the other cells. However, 9HTE and NCI-H292 cells did not show this growth pattern in a confluence. Through the microscope, cells at confluence looked smaller in profile, although the size of the cells remained similar to sub-confluent culture after cell dissociation.

In culturing the human primary bronchial epithelial cells (HPBEC), only when tissue explants attached to the bottom of the well, would cells grow outwards and form an epithelial layer. Normally HPBEC were seen to grow after 1-2 weeks in culture, and epithelial cell covered 50% of the wells after 4-10 weeks. After this time the tissue explants could be transferred to a new well and cells could grow from the tissue again. Most of the explants were kept more than 10 passages, and some of them were able to grow after 15 passages. Most of the HPBEC were clearly senescent 2 weeks after the tissues had been removed. The cells showed the typical appearance of bronchial epithelia in culture (*Figure 3-2A and B*) and beating cilia were seen on most of the cells around the tissue. HPBEC were able to be passaged by trypsin/EDTA and grew on FBV (a fibronectin, BSA, and collagen I mixture) coated plates. However, they became senescent after 2 weeks.

The indirect immunofluorescence staining for CK18, CK13, CK14 and E-cadherin in the four cell lines and HPBEC is summarised below (*Table 3.1*). All cell lines (*Figure 3-3A-D*) and HPBEC expressed strong CK18 staining. CK13 staining was only observed in HBPEC, 16HBE 14o$^-$ and NCI-H292 cells. CK14 was weak stained in NCI-H292 and HBPE cells. E-cadherin staining was strong in NCI-H292 and HBPE cells. Although weak immunostaining was seen in 9HTE, 16HBE 14o$^-$ and BEAS-2B cells, the staining pattern was similar to the background pattern in controls.

Table 3.1 Cytokeratins (CKs) and E-cadherin staining in bronchial epithelial cells

Cell	CK18	CK13	E-cadherin	CK14
9HTE	+++	-	+/-	-
16HBE 14o$^-$	+++	+	+	+/-
BEAS-2B	++	-	+	-
NCI-H292	++	++	++	+
Primary cells	+++	+++	++	+

+++ Very strong staining

++ Strong staining

+ Positive staining

+/- Positive/negative

- Negative

3.2.2 Transepithelial electrical resistance

In cultures of 9HTE, 16HBE 14o$^-$ and NCI-H292 cell lines all appeared to be in close cell-cell contact under the inverted phase-contrast microscope. Only 16HBE 14o$^-$ cells and not 9HTE or NCI-H292 cells showed high levels of TER (*Figure 3-4*). When seeded with $2.5x10^4$ cells/cm^2 on the filter inserts, the cultures were confluent after 5-10 days. The TER of 16HBE 14o$^-$ cells stayed at a level below 500 ohms-cm^2 for up to two weeks and then increased rapidly after 15-20 days (to more than 600 ohms-cm^2). The TER of 16HBE 14o$^-$ stayed at this high level for up to 3 weeks, and reached a maximum of 2650 ± 145

ohms-cm^2. Although cells cultured on the tissue culture plastics will enter crisis 5 days after confluence, the cells were able to grow on the filter inserts for more than 3 weeks after confluence. After that the, cells began to enter cell crisis. Lots of cell detachment was seen at this stage. By changing the medium more frequently than twice a day, the TER stayed around 300-600 ohms-cm^2.

3.2.3 Ultrastructure of epithelial cells and localisation of ICAM-1 and CD44

(A) Ultrastructure

To obtain more information about epithelial differentiation, the ultra structure of the epithelial cells was studied by growing cells to confluence on filter inserts (*Figure 3-5A*). Under the transmission electron microscope, 16HBE14o$^-$ cells showed microvilli, tight junctions and desmosomes (*Figure 3-5B, C and D*). By scanning electron microscope, microvilli were found on the apical surface of most 16HBE 14o$^-$ cells, and cilia were seen on some cells (*Fig 3-5E*). On the other hand, the cell surfaces of 9HTE and NCI-H292 cells were smooth, only few microvilli were seen occasionally on 9HTE cells.

(B) ICAM-1/CD44 localisation in 16HBE 14o$^-$ cells

The localisation of ICAM-1 and CD44 was examined by using cryosectioning and immunogold labelling techniques with electron microscopy. ICAM-1 only appeared on the apical surface of 16HBE 14o$^-$ cells (*Figure 3-6A and B*), no staining was found on lateral or basal surfaces. Most of the CD44 immunoreactivity was expressed on the surface of the lower layer of cells in the double layer culture (*Figure 3-7A*), or on the basolateral surface of these cells (*Figure 3-7B).* Little CD44 was found on the surface between the basal and the supra-basal cells.

Using 16HBE 14o$^-$ cells from high TER cultures incubation apically with anti-CD44 or anti-ICAM-1 antibody for 30min, the TER of the culture did not significantly change. After dissociation, and incubation with or without antibody, cells showed different localisation of ICAM-1 and CD44 by using flow cytometry (detail see *Figure 3-8*). ICAM-1 staining apically and further secondary incubation with antibody showed a little increase in fluorescence intensity (*Figure 3-9A*). Alternatively, apical staining with anti-

CD44 antibody showed a low expression of CD44, and with a strong staining when cell suspension incubated with the antibody (*Figure 3-9B*). These results compliment as the localisation by immunoelectron microscopy. The ICAM-1 was expressed on the apical surface and CD44 was expressed on the basolateral surface of the cells.

(C) ICAM-1/CD44 localisation in bronchial tissue

Using immunogold-labelling techniques, the ICAM-1 immunoreactivity was undetectable on epithelial cells of normal bronchial tissue from 7 individuals. Only when the epithelium was treated with IFNγ and TNFα was immunolabelled for ICAM-1 seen only on the apical surface (*Figure 3-10A, B*). The immunogold staining of ICAM-1 was higher on microvilli (*Figure 3-10A*), but less seen on the cilia (*Figure 3-10C*).

CD44 was not found on the columnar cells, but only expressed on basal cells. The distribution of CD44 molecules was not uniform on basal cells. No CD44 was seen on the cell membrane of closely apposed columnar-basal or basal-basal cells. Only the free cell membrane in areas where cells were not in direct contact between basal cells, referred to as the "free surface" of basal cells, showed CD44 staining (*Figure3-11B and C)*. No CD44 staining was found in the areas of cell-matrix contact.

3.3 Discussion

3.3.1 Comparisons of epithelial cultures by morphology and immunocytochemistry

The bronchial epithelial cell lines, 9HTE, 16HBE 14o$^-$, BEASE-2B and NCI-H292 cells showed a simple squamous structure when cultured on plastic culture surfaces. The cells appeared closely adjoin neighbouring cells in the 9HTE, 16HBE 14o$^-$, NCI-H292 cells, which kept the basic morphology of epithelial cells. However, the islet-like cell growth pattern was not seen in BEAS-2B cells, even those cells grown in low density culture. Reddel and co-workers (Reddel *et al.*, 1988) have reported that BEAS-2B cells shown a typical appearance of epithelial cell and were more tightly packed than normal primary epithelial cultures, and revealed desmosomes and tight junctions. This was not apparent in my studies and the loss of the honeycomb-like appearance and islet-like pattern in this BEAS-2B cell line might reflect changes which have occurred during the maintenance and storage of this cell line. In immunocytochemical studies, the positive staining for CK18 and E-cadherin demonstrated some epithelial characters still remain in the BEAS-2B cells. If this cell line was to be used, further examination might be required to analyse whether the cells still retain the properties of the original cell line, and if the morphological alteration was reversible.

Although immortalisation techniques can produce cells with a desired phenotype, the cells are transformed, and there is often some loss of the normal characteristics of the cells in the tissue. For example, the 9HTE, BEAS-2B and NCI-H292 cells have poor tight junctions and are poorly differentiated. Human airway epithelium consists of a heterogeneous population of cells including basal, columnar ciliated cells and goblet cells. A cell lines will at best only be presentable one of these cell types. Since most of the virus-transformed or carcinoma-derived cell lines may lose some characteristics and function of normal bronchial epithelia, the cell lines chosen in a study should take account of the characteristics of the cell line in relation to the similar responses *in vivo*.

Recently, several groups have successfully generated primary epithelium from different sites in the airways and studied their roles in inflammatory diseases, such as allergic rhinitis and asthma. For example, culture of human nasal epithelial cells grown from

nonatopic normal subjects without rhinitis, atopic patients without rhinitis and rhinitic individuals produce different cytokines (Calderon *et al.*, 1997). The nasal epithelial cells from atopic individuals release significantly greater amounts of IL-1β, IL-8, GM-CSF, TNFα, and RANTES than do non-atopic individuals. In addition Devalia and colleagues (Bayram *et al.*, 1999) have shown that pollutants, such as NO_2, increase the release of pre-inflammatory mediators from epithelial cells cultured from bronchial biopsies of atopic asthmatic patients. Bronchial epithelial cells from asthmatics showed an increased level of mRNA for the cytokines in (Takizawa, 1998). Primary cells retain most of the properties of airway epithelia, and may be a good model between *in vivo* normal tissue and *in vitro* models using immortalised cell lines. However, the short lifespan and difference between individuals are disadvantages of using primary cells. Further understanding the basic requirements component for the culture of HBPEC may improve the practicality of this technique.

3.3.2 Differentiation and tight junctions in the cell line

When 16HBE14o$^-$ cells are grown at an air/liquid interface, the cells have well differentiated cilia on their apical surface. In addition, they retain tight junction and vertical ion transport (Cozens *et al.*, 1994). In the present research, transwell permeable inserts were used for cell culture in which cells can obtain nutrition underneath. In this culture system, microvilli were seldom or not seen on the culture of the 9HTE or NCI-H292 cells, whereas, 16HBE 14o$^-$ cells are able to form stratified or pseudostratified structure; also, microvilli and cilia have been formed. Some studies have examined on the effect of growth factors on epithelial differentiation and proliferation (Nielsen *et al.*, 1997). However, the mechanism of epithelial differentiation is still unclear. This might involve the interaction of epithelial cells to extracellular matrix and other cell type in the sub-mucosa.

The 16HBE14o$^-$ cells were confluent on the filter inserts 7-10 days after the seeding of the cells. The dramatic increase of TER during day 15-20 might be caused by the migration and growth of the cells on the other side of the filter. After the epithelial layers on both sides form tight junctions, the TER can further increase. Meanwhile, the basal cells, which were covered by adjoining cells on the filter, could lack nutrition and go into crisis.

3.3.3 The localisation of ICAM-1 and CD44 in 16HBE 14o$^-$ and HPBEC

ICAM-1 and CD44 molecules were localised by using cyrosectioning and immunoelectron microscope techniques that can preserve good antigenicity for immunostaining, combined with the high resolution of the electron microscopy. These tools provided good resolution for the localisation of ICAM-1 and CD44 molecules. Comparing with the localisation of ICAM-1 and CD44 in bronchial tissue and 16HBE 14o$^-$ cells, these two molecules showed similar distribution patterns in 16HBE 14o$^-$ cells and epithelial cells in tissue. Although the 16HBE14o$^-$ cells on the filter inserts formed a pseudostratified cell layer, the cells on the top layer with microvilli or cilia were not columnar cell-like.

In reviewing of the attributes of these bronchial epithelial cell lines and primary cells, it is suggested that the 16HBE14o$^-$ cells retain the most morphological and functional characteristics of bronchial epithelial cells. The 16HBE14o$^-$ cell line was therefore chosen, meanwhile, NCI-H292, the carcinoma-derived cell, which expresses low ICAM-1, was used for comparisons in this research.

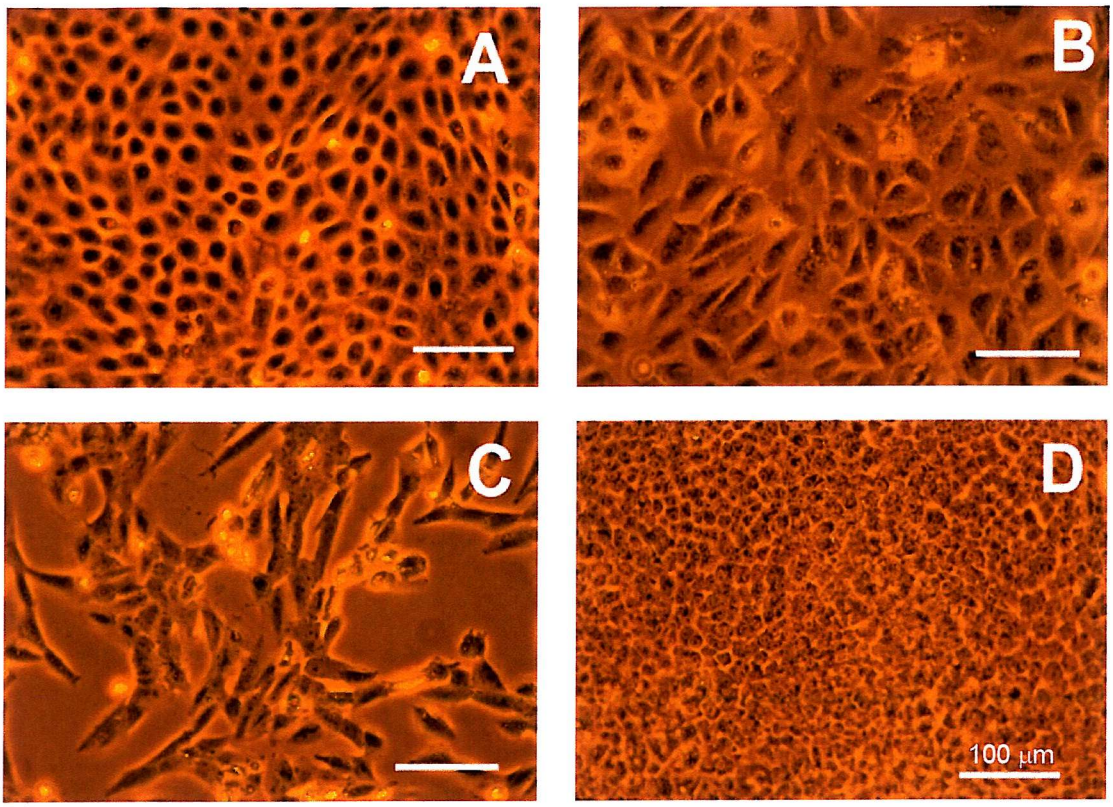


Figure 3-1. The morphology of the bronchial epithelial cell lines. 16HBE 14o$^-$ (A), 9THE (B), BEAS-2B(C) and NCI-H292 (D). Bars: 100 μm.

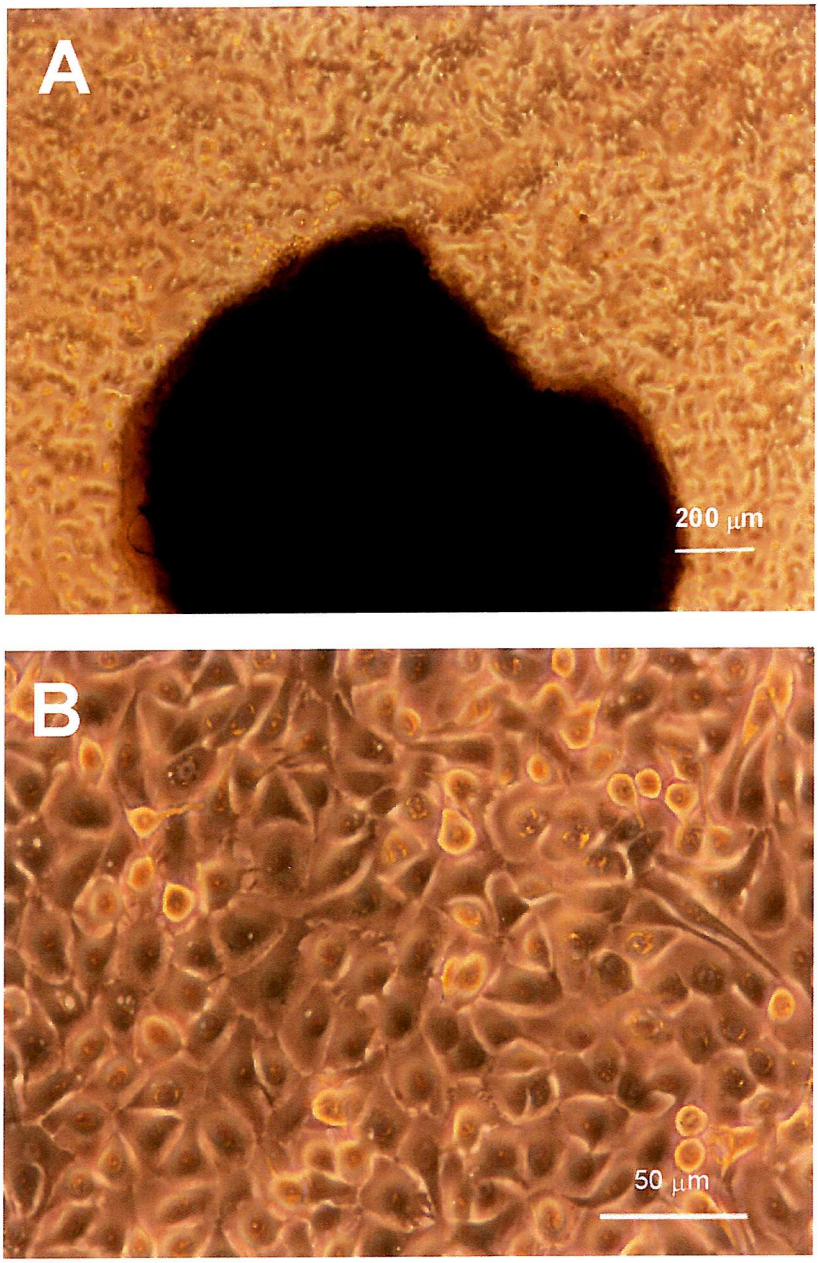


Figure 3-2. The morphology of primary bronchial epithelial cells. Cells grew out from bronchial explant (A) and giving large number of cells. High magnification view (B).

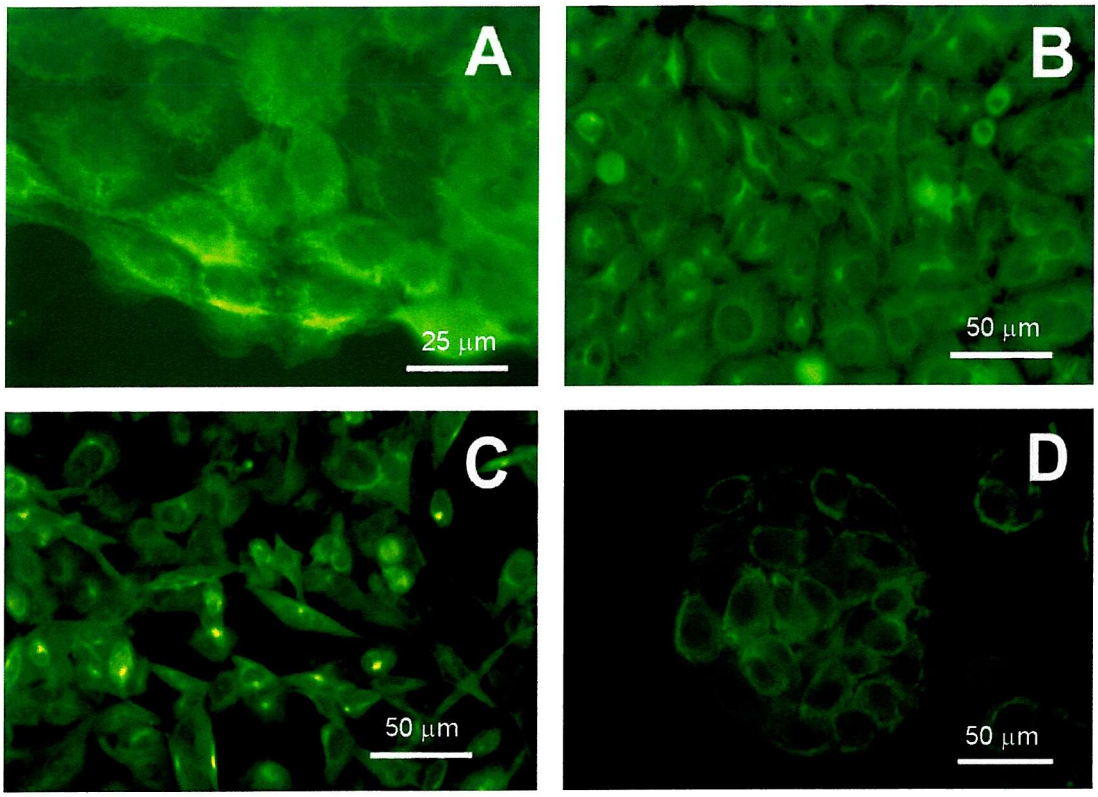


Figure 3-3. Indirect immunofluorescence staining of cytokeratin 18 in bronchial epithelial cells. 16HBE 14o$^-$(A), 9THE (B), BEAS-2B (C) and NCI-H292 (D).

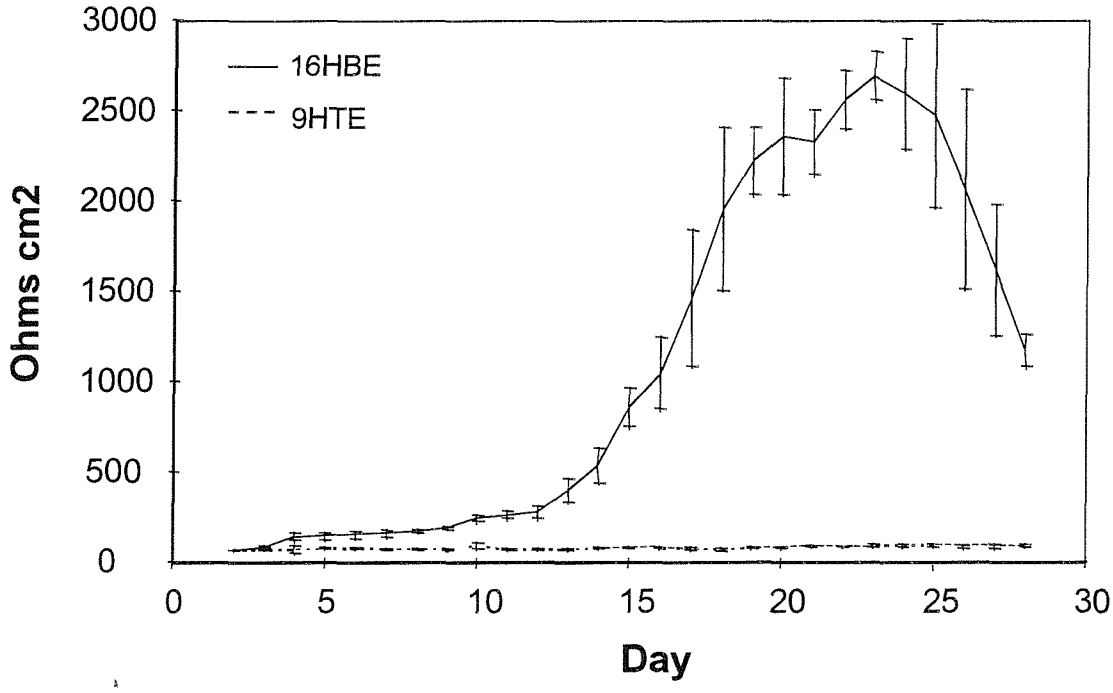


Figure 3-4. Transepithelial electrical resistance (TER) of 9HTE and 16HBE 14o$^-$ cells. Cells were cultured on filter inserts and the TER was measured every day. Results were from 8 (in 16HBE 14o- cells) or 4 insert filters (in 9THE cells). H292 cells (not shown in this figure) have the same pattern as 9HTE cells. The baseline TER of the filters was 61 ±6 ohms-cm^2, and all the TER values in the studies are expressed after subtraction of the baseline.

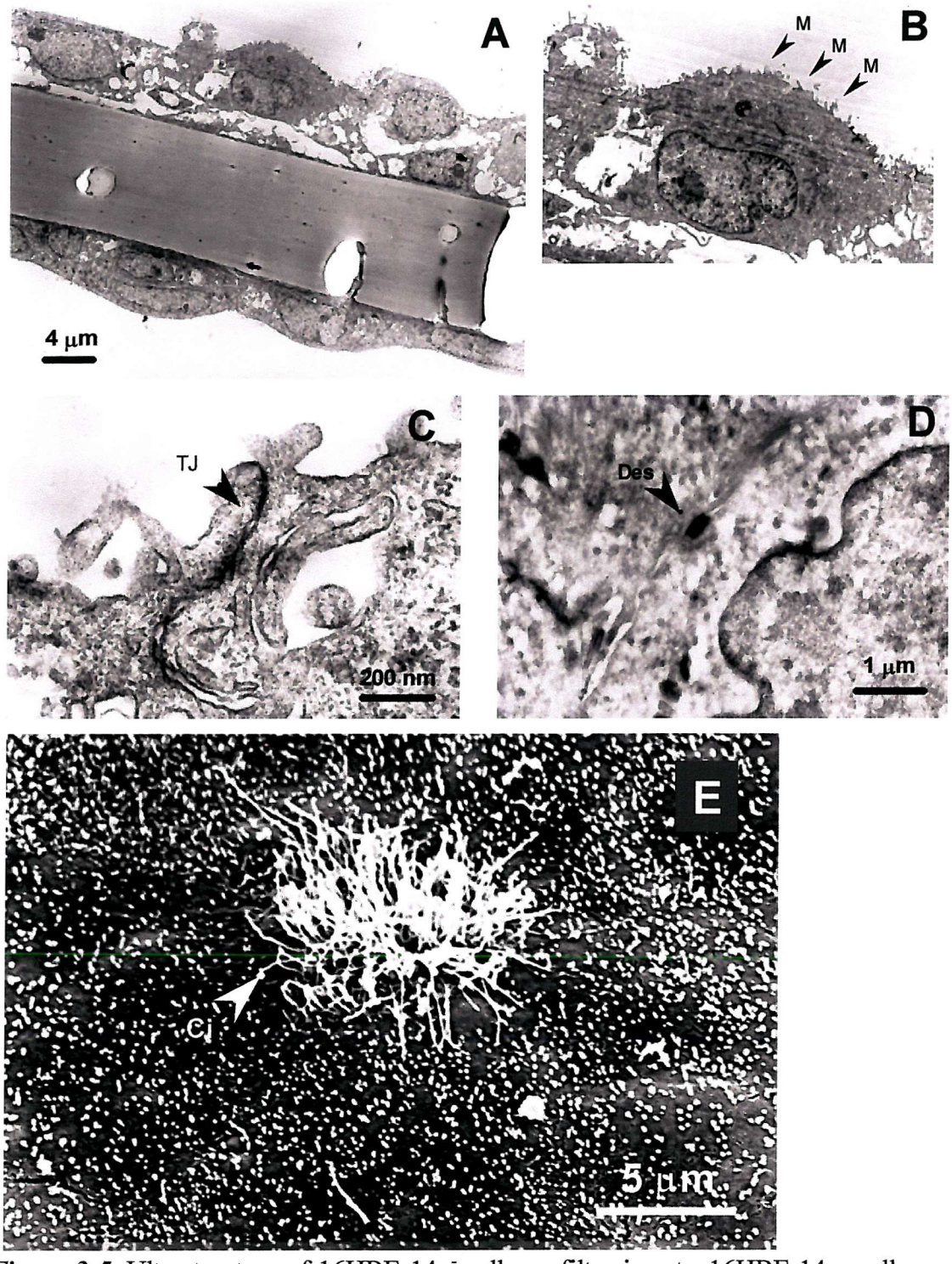


Figure 3-5. Ultrastructure of 16HBE 14o$^-$ cells on filter insert. 16HBE 14o- cells were cultured on filter insert to confluence for 3 weeks (A). Cells were fixed with 3% paraforaldehyde plus 0.1% glutaraldehyde and then embedding for TEM or SEM studies. Transmission electron microscopy showed cells on membrane with expression of microvilli (B, arrowheads) (B), tight junction (C, arrowhead), and desomosome (D, arrowhead). Cilia (arrowhead) and microvilli (E) on 16HBE 14o$^-$ cells by SEM. M: microvilli; TJ: tight junction; Des: desomosome; Ci: Cilia.

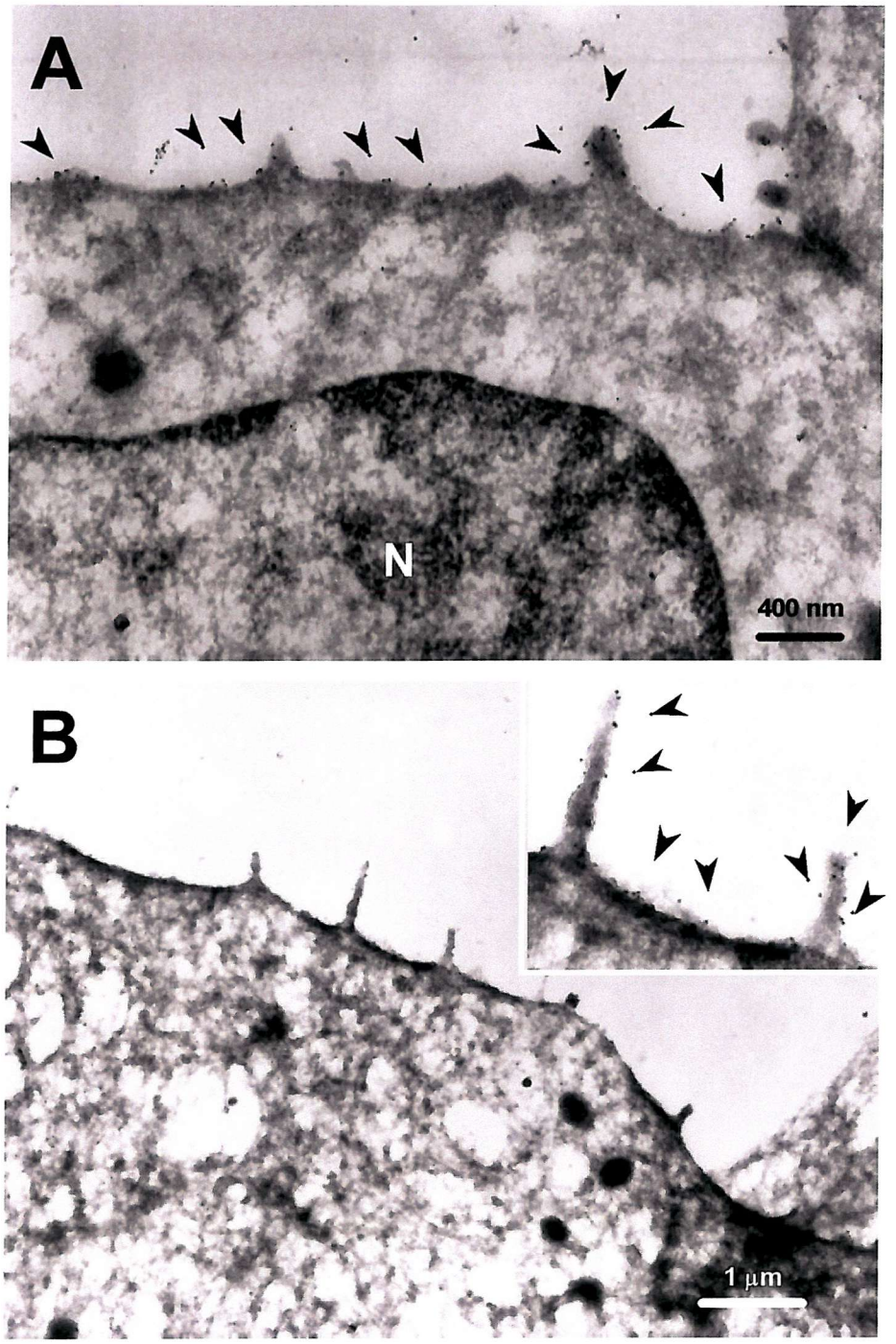


Figure 3-6. ICAM-1 only immunostaining on the apical surface of 16HBE 14o$^-$ cells. 16HBE 14o- cells were grown on filter insert. Two weeks after cell confluence, the cells on inserts were fixed and processed for cryo-sectioning and immunoelectron microscopy. The dots were the binding sites for anti-ICAM-1 antibody conjugated with gold particles (10nm) (arrowheads). Apical expression of ICAM-1 (A); ICAM-1 staining on microvillia (B). N: nucleus.

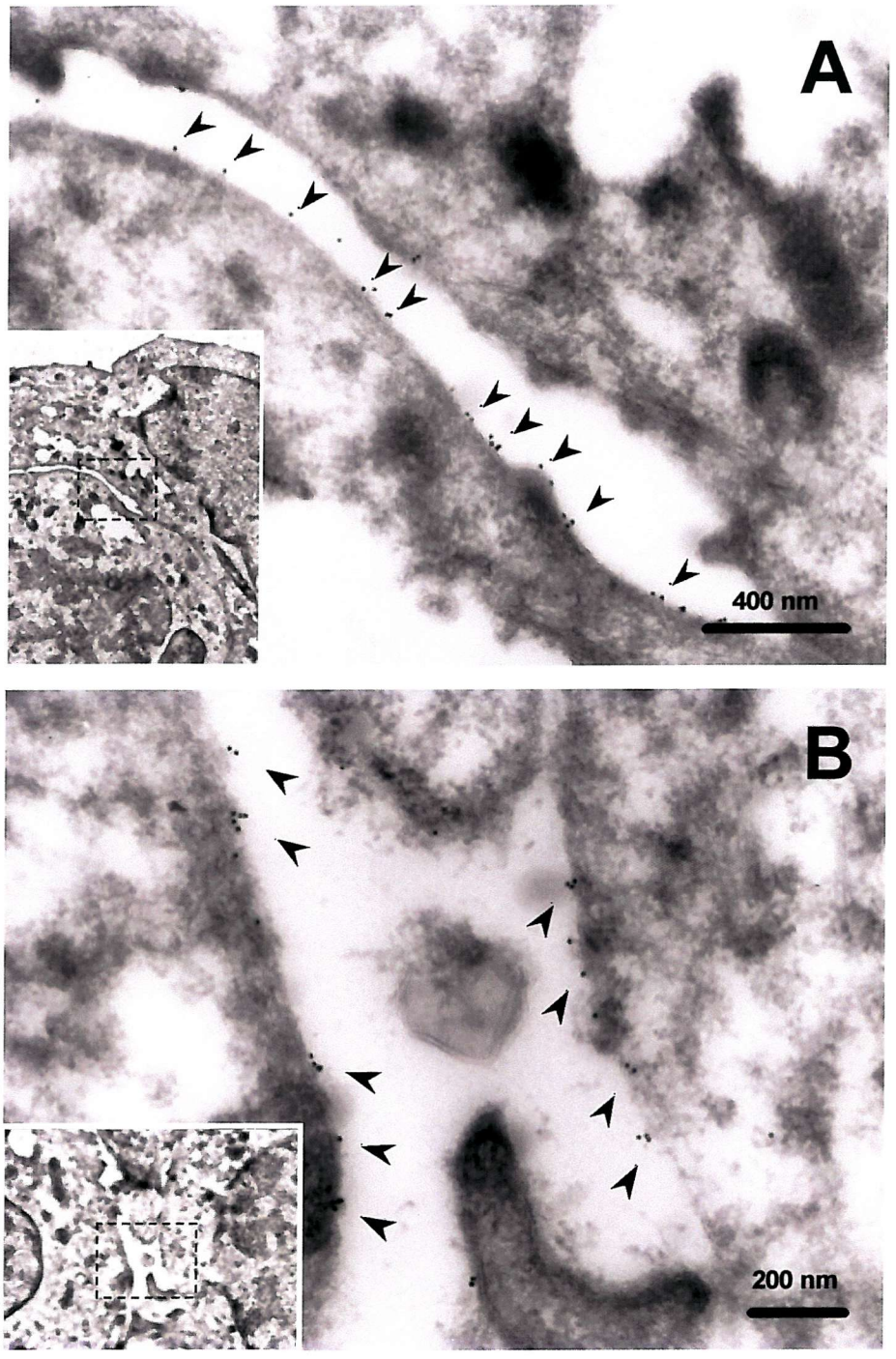


Figure 3-7. The localisation of CD44 in 16HBE 14o- cells. 16HBE 14o- cells were cultured and immunogold labelled for CD44 localisation. The dots (10nm gold particles, arrowheads) show most CD44 is expressed on the surface of lower layer cells of the double layer culture. Few CD44 was found on the basal surface of the top layer cells that closed the lower layer cells (A). CD44 was found on the basalateral surface of adjoint cells (B). High magnification images were taken from the area indicated by the dotted line in the insert.

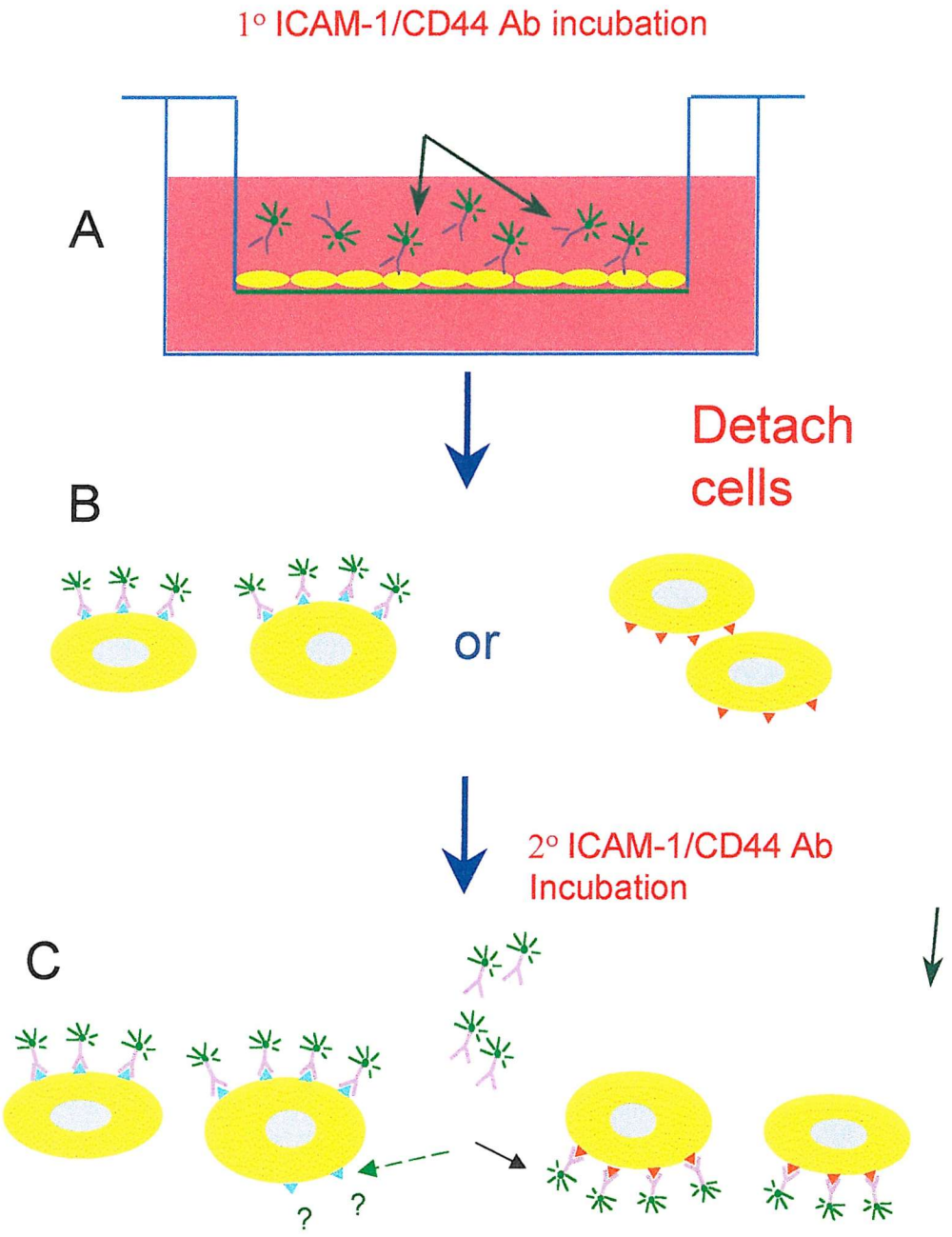


Figure 3-8. Diagram of localisation of ICAM/CD44 by flow cytometry. Cells on transwell filter insert were incubated with ICAM-1 or CD44 antibody (A). Cells were detached to be monodispersed cell suspension (B). Following second incubation with the antibody, cells are analysed by flow cytometry (C).

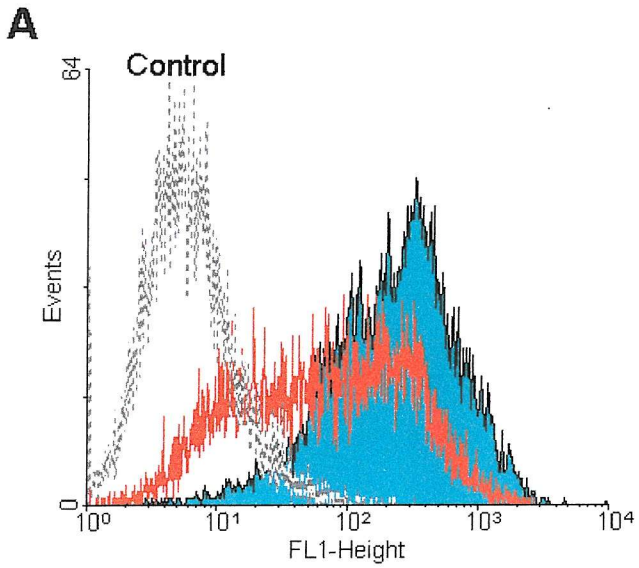


Figure 3-9A. Localisation of ICAM-1 by flow cytometry. Confluent cells on membrane inserts were incubated with anti-ICAM-1 antibody apically as described in materials and methods. After staining with anti-ICAM-1 antibody from the apical surface, single cell suspension was prepared by trypsin digestion and incubated with anti-ICAM-1 antibody. Fluorescence intensities of the cells were compared before (red) and after (blue, solid) secondary ICAM-1 antibody incubation. Isotype antibody control was shown in dotted line.

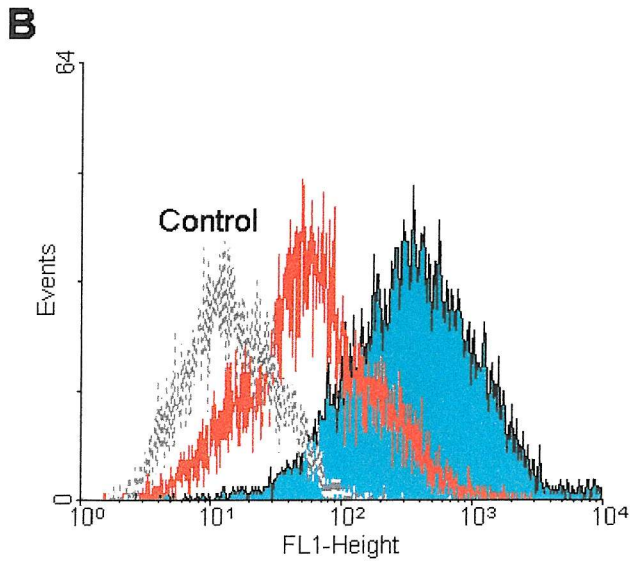


Figure 3-9B. Localisation of CD44 by flow cytometry. Cell with apical CD44 antibody incubation showed a low level of CD44 expression (red). When the cells were detached by non-enzymatic cell dissociation solution and stained with CD44 antibody, the level of CD44 was increased (blue, solid).

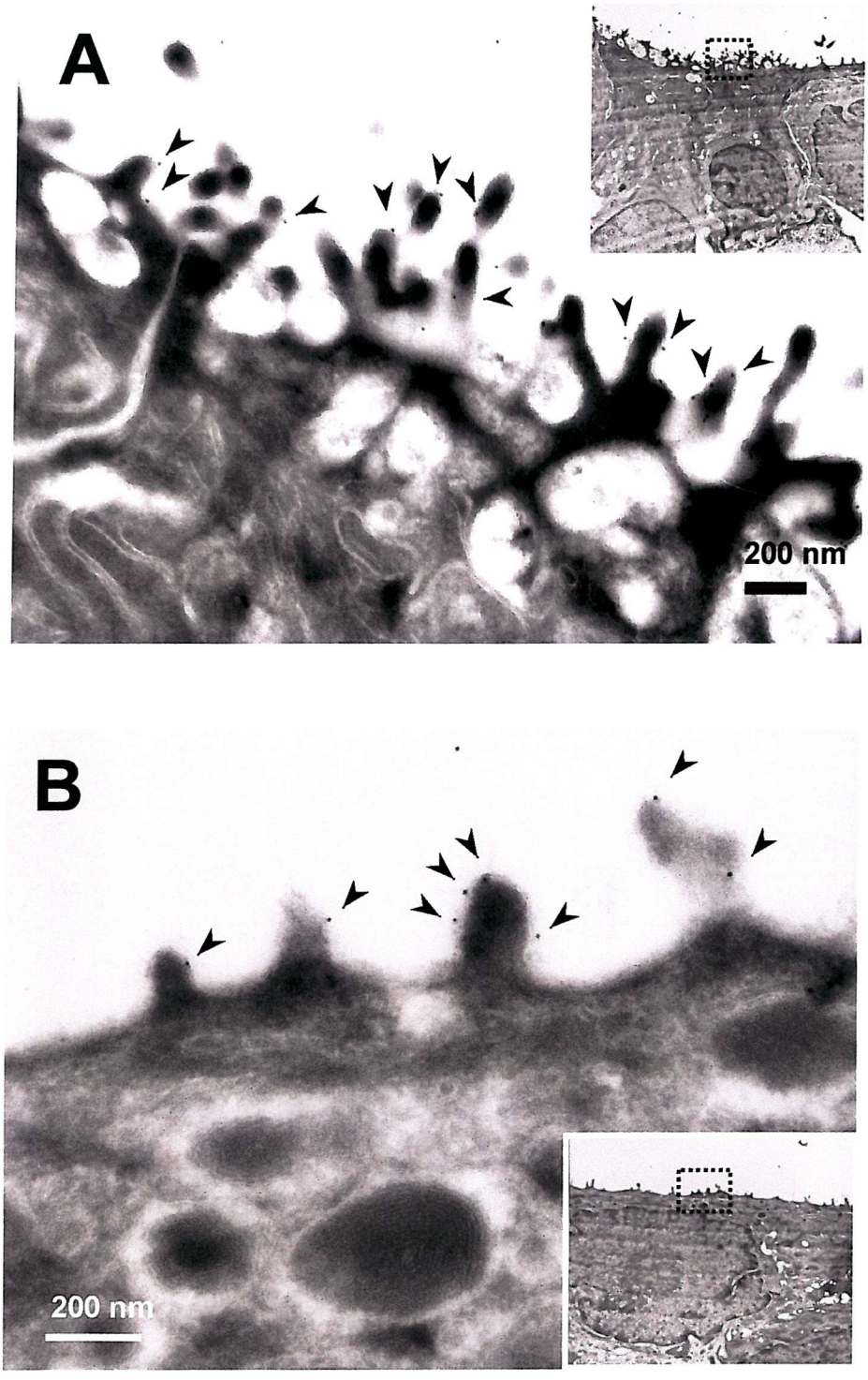


Figure 3-10. The localisation of ICAM-1 in epithelium of bronchial tissue treated with 200U/ml IFNγ and TNFα for 24 hours. Immunogold labelling (arrowheads) showed ICAM-1 is localised on the apical surface of microvilli (A). ICAM-1 staining also presents in short microvillia area (B). The high magnification image of highlight area is indicated by the dotted line in the insert.

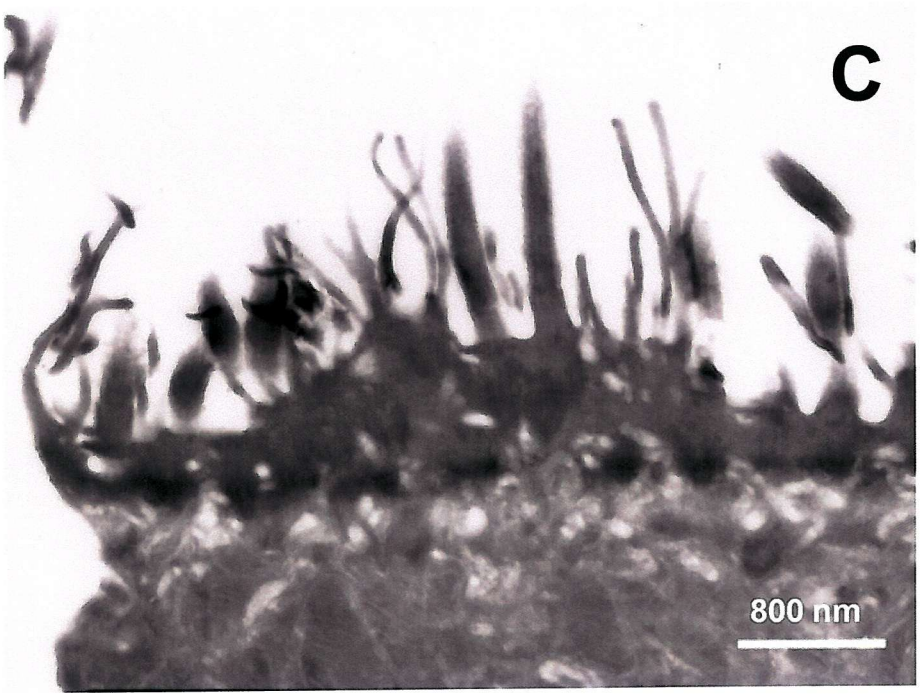


Figure 3-10 continue Less ICAM-1 is seen on the apical surface of ciliated cells (C).

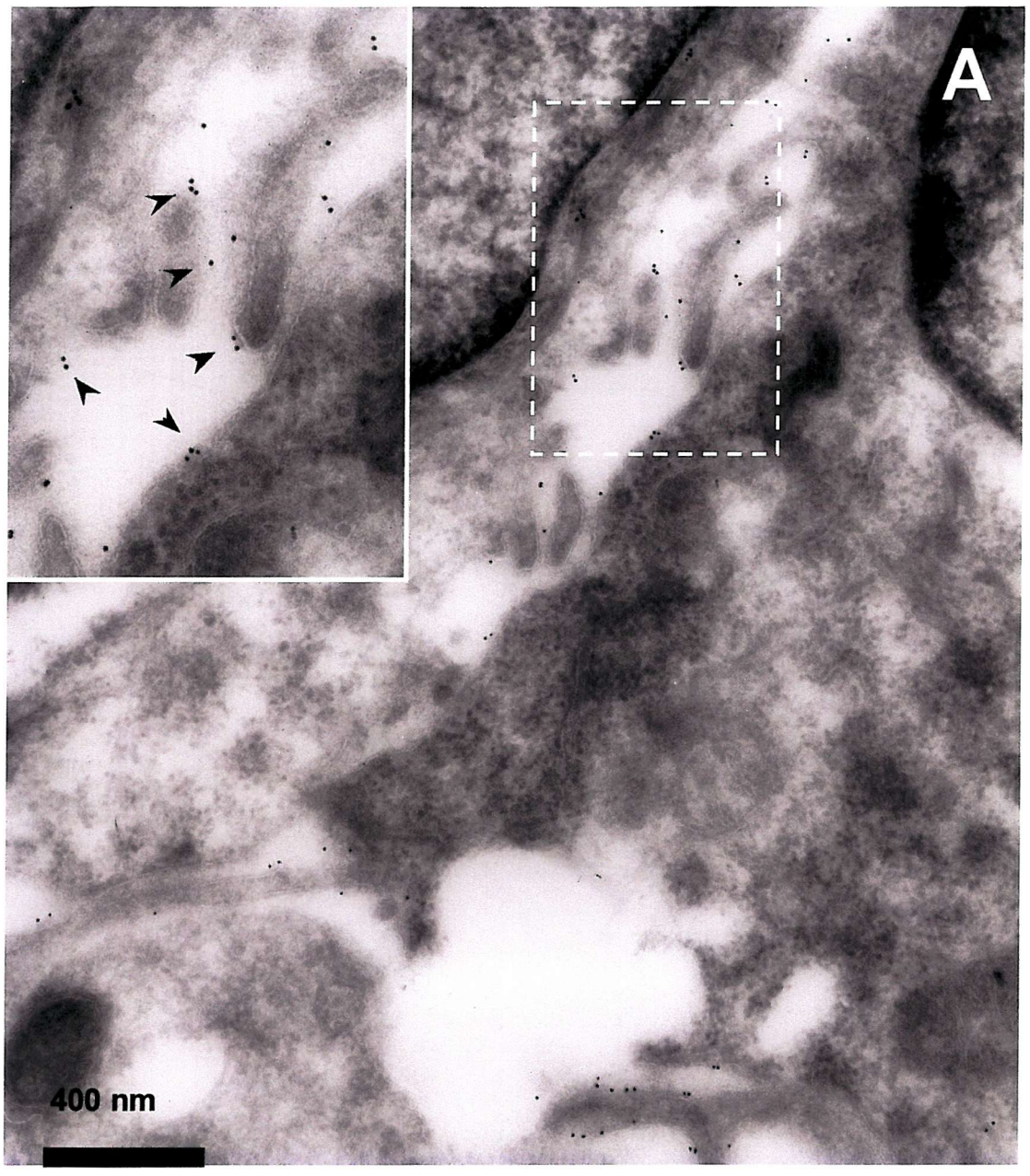


Figure 3-11. The localisation of CD44 in epithelium of bronchial tissue. CD44 was expressed on basal cells (A and B arrowheads). CD44 is highly expressed on the free surface of basal cells (B). Little CD44 staining was found on cell-extracellular matrix surface. High magnification images were taken from the area indicated by the dotted line in the insert.

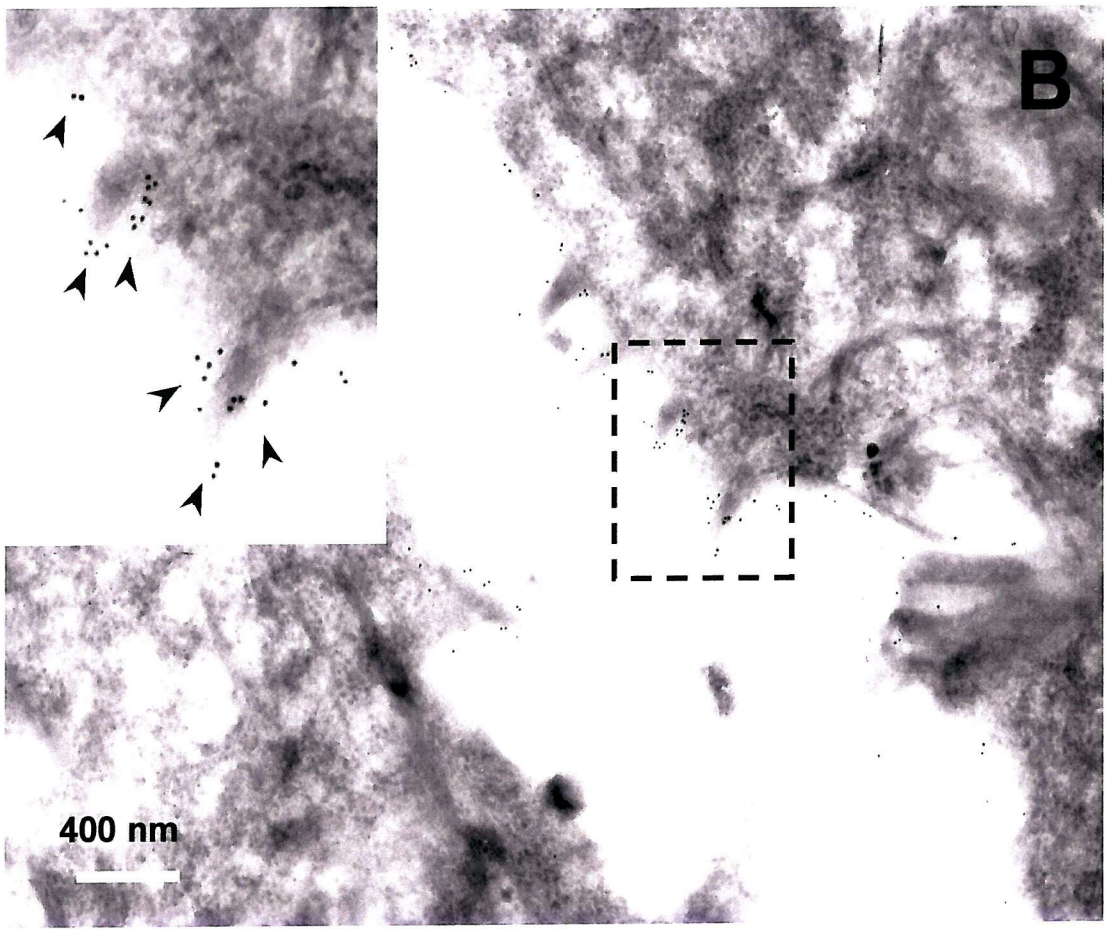


Figure 3-11. Continue.

CHAPTER 4

Expression And Regulation of ICAM-1 and CD44 on Bronchial Epithelial Cells In Vitro

4.1 Introduction

ICAM-1 is considered being a marker of allergic inflammation, and also thought to have a pivotal role in the immune response associated with allergic reactions (Canonica *et al.*, 1995) . It is now clearly established that ICAM-1 plays an important role in the interaction of inflammatory cells to other cell types (*see section 1.5.2A*). Bronchial biopsies from patients with allergic asthma expressed higher levels of ICAM-1 on the epithelium than non-allergic asthmatics and normal subjects. However, ICAM-1 expression on the epithelium from non-allergic asthmatics and normal subjects did not show any difference (Gosset *et al.*, 1995). Increased the expression of ICAM-1 was seen in human airway epithelium with cytokine treatment, including IL-1β, IL-4, IFNγ, and TNFα (Look *et al.*, 1992; Krunkosky *et al.*, 1996; Atsuta *et al.*, 1997; Striz *et al.*, 1999). The increase of ICAM-1 expression by proinflammatory cytokines *in vitro* suggests that a role for ICAM-1 during inflammation associated with allergic reaction is involved in the immune response of asthma.

Although the level of ICAM-1 expression in normal epithelium is low, most cell lines derived from normal epithelium express a high level of ICAM-1 and the expression of ICAM-1 also increases in primary bronchial epithelial cells. Aurothiomalate (AuTM), a anti-rheumatic gold compound, has been demonstrated to suppress cytokine (IFNγ)-stimulated expression of ICAM-1 in endothelial cells (Koike *et al.*, 1994). The mechanism of action of this compound in ICAM-1 expression is still unclear. Makino and colleagues (Makino *et al.*, 1993) suggested that aurothiomalate might modulate glucocorticoid receptor-mediated intracellular signals. A means of down-regulating ICAM-1 in culture was sought. AuTM was therefore tested in my experiments.

Epithelial expression of CD44 variants has been described in a wide range of normal epithelial tissues including skin, digestive tract and lung (Mackay *et al.*, 1994). Increased expression of CD44 has been found in areas of repair in bronchial epithelium and is increased in asthmatic subjects (Lackie *et al.*, 1997). Compared with ICAM-1 the effects of cytokines on CD44 expression seem to be varied in different cell types. Mackay and colleagues (Mackay *et al.*, 1994) have shown that

the expression of CD44 variants on leukocytes and epithelial cell line was up-regulated by IFNγ and TNFα. In contrast, Koopman and co-workers (Koopman *et al.*, 1998) reported that the expression of CD44 was unaffected by IL-1β, IL-8, TNFα, IFNγ, or IL-4 in endothelial cells. As mentioned in *section 1.5*, expression of CD44 in growth and metastasis of tumours has been well documented (Matsumura and Tarin, 1992). Although several studies have reported that airway epithelial cells express CD44 variants (Bloemen *et al.*, 1993; Mackay *et al.*, 1994), little is known about their regulation and function. Due to their ability to bind to multiple ligands (*see section 1.5.3*) relevant to epithelial cell function in the airways, the regulation of CD44 in bronchial epithelium was considered worthy of investigation.

In asthma, up to 60% of the epithelial area (Roisman *et al.*, 1995; Peroni *et al.*, 1996) may have an altered structure associated with damage and repair processes reflecting increased epithelial fragility in asthma. The increase of ICAM-1 and CD44 expression in bronchial epithelium of asthma suggests that these two molecules may be involved in the immune processes and response to epithelial damage. To further understand the roles of ICAM-1 and CD44 in bronchial epithelial cells, immunostaining techniques with fluorescence microscopy and flow cytometry were used to study the regulation of ICAM-1 and CD44 in bronchial epithelial cells. My interest in ICAM-1 and CD44 centres around their putative roles as proteins that are important for epithelial repair after cell damage. In order to understand how these two molecules are expressed during the response to damage *in vitro,* I have investigated their expression during epithelial damage and proinflammatory cytokine treatment. Firstly, I have investigated (a) The baseline of ICAM-1 and CD44 expression in four cell lines and human primary bronchial epithelial cells (HPBEC), and (b) the regulation of ICAM-1 and CD44 expression after mechanical damage of confluent cultures. Secondly, more studies were prompted by the results from regulation of ICAM-1 and CD44 after epithelial damage including the effect on expression of ICAM-1 and CD44 induced by: (a) different cell densities and (b) cytokine-induction, which may reflect the condition of cells in damage and inflammation area. These studies provide further insights into the functions of ICAM-1 and CD44 during epithelial repair.

4.2 Results

4.2.1 Trypsin and ICAM-1/CD44

Differences in time of detachment were observed between the cultures after treatment with trypsin/EDTA and non-enzymatic cell dissociation solution. Detachment and dissociation of 16HBE 14o$^-$ cells with trypsin/EDTA required 10-15min, whereas, more than 30 minutes was needed with non-enzymatic cell dissociation solution. Cells viability was much higher after trypsin/EDTA treatment (>95%) compared to non-enzymatic cell dissociation solution (<70%).

Trypsin/EDTA removed surface CD44 from 16HBE 14o$^-$ cells reducing the fluorescence intensity down to 1/6 of that for the cells detached by non-enzymatic cell dissociation solution (*Figure 4-1A*). This decrease of CD44 staining was also seen in NCI-H292 cell. Since NCI-H292 cells expressed a low level of ICAM-1, the effect of trypsin on ICAM-1 was not tested in this cell line. 16HBE 14o$^-$ cells, which express a high level of ICAM-1, were used to test the effect of trypsinisation. Result showed the shedding effect by trypsinisation was not pronounced on ICAM-1. Less surface ICAM-1 was removed, and the fluorescence intensity of ICAM-1 staining fell by less than 10% (*Figure 4-1B*).

Additional experiments were also performed to study the time course of regeneration of CD44 after trypsinisation. The restoration of cell surface CD44 was studied by plating the trypsinised cells on culture plates and harvesting using non-enzymatic cell dissociation solution at different time. *Figure 4-2* shows that at 6 hours less than 50% of CD44 was re-expressed, 9 hours after trypsinisation the levels of CD44 had returned to 90% of the original.

4.2.2 The baseline level of ICAM-1 and CD44 in different cell lines and primary cells.

(A) ICAM-1 was expressed at a high level in SV40-transformed cell lines and primary bronchial epithelial cells

Normal epithelial cells in bronchial tissue did not express ICAM-1 (see *section 3.2.3.C*). However, after separating with basement membrane and growing on the plastic culture surface, the expression of ICAM-1 on primary bronchial epithelial cells was increased. About 60-70% HPBEC expressed ICAM-1 in a 6-weeks culture (*Figure 4-3A*). In the four cell lines, three SV-40-transformed cell lines, 16HBE 14o$^-$, 9HTE, and BEAS-2B, had high ICAM-1 expression, and more than 75-90% cells were ICAM-1 positive (*Figure 4-3B~D*); NCI-H292, a carcinoma derived cell line, expressed a lower level of ICAM-1 (*Figure 4-3E*).

(B) All four cell lines and primary cells express CD44s

CD44 is constitutively expressed *in vitro* in bronchial epithelial cells. Primary cells, 9HTE, 16HBE 14o$^-$, BEAS-2B, and NCI-H292 all expressed CD44 by immunocytochemistry. Most of the CD44s staining was preferentially in regions of cell-cell apposition and at the free edge of the cells (*Figure 4-4*). By flow cytometry, CD44 had a different level of expression in confluent and sub-confluent cultures. Cells in the confluent state showed a lower immunoreactivity for CD44 and its isoform staining (*Figure 4-5*).

4.2.3 Epithelial damage and the expression of CD44 and ICAM-1

By flow cytometry, after 16HBE 14o$^-$ or NCI-H292 cell layers were mechanically damaged, the median value of cell fluorescence intensity for CD44s was decreased 6 hours after damage. After 12 hours, the level was increased and this was maintained until at least 48 hours after damage (*Figure 4-6*). Other CD44 isoforms showed similar changes after cell damage (*Figure 4-6*), while no significant change was seen on ICAM-1.

Altered expression of CD44 after cell damage was also seen using indirect immunofluorescence staining. The increase in the mean pixel intensity towards the wound edge in digitised images indicated an increase in CD44 immunoreactivity associated with cells in this area. After an initial small drop in intensity at 3 hours, there was an overall increase in CD44 immunoreactivity up to 48 hours, even up to 450 μm from the wound edge in the culture (*Figure 4-7D and E*). The CD44

immunostaining was stronger closer to the wound edge, particularly in the first 12 hours (*Figure 4-7 A and C*). At 36 (*Figure 4-7 D and E*) and 48 hours (*Figure 4-7E*) staining intensity was increased over a wider band around the track of the wound. Wound closure in these samples was seen in some areas of the wound by 12 hours and was complete at 24 hours. From 24 hours it was not possible to distinguish increased CD44 expression in the damaged cultures. When using fluorescein diacatate (FDA) combined with propidium iodide (PI) to examine of the cell viability and permeability, 10 minutes after cell damage, permeabilised cells which showed only red nuclear PI staining without green fluorescence were found (Figure 4-8A). These were damaged cells. At 1 hour, most of the cells were FDA-positive which were viable cells without damage, less than 5% of PI single positive cells was seen (Figure 4-8B). At 3 hours, no difference in the percentage of PI single positive cells between damaged and undamaged cultures was seen.

4.2.4 Cell density effect on the expression of CD44 and ICAM-1

(A) CD44 isoform expression is increased at lower cell densities.

By flow cytometry, the immunoreactivity of antibodies to ICAM-1, CD44 and CD44 isoforms was significantly above control level in 16HBE 14o$^-$ cells (*Figure 4-9*). ICAM-1 fluorescence was increased in high density 16HBE 14o$^-$ cultures (*Figure 4-9A*), while the level of CD44 immunoreactivity for all isoforms was greater in cells cultured at lower cell density (*Figure 4-9 C-F*). Before achieving confluence, more than 90% (CD44s, CD44v3, and CD44v9) or 80% (CD44v6) of cells were CD44 positive, while the percentage of CD44v3$^+$ and CD44v6$^+$ cells decreased (*Figure 4-10*). Using high and low cell density co-cultures, cells were seeded at densities from 130 to 0.5 x 10^3 cells/cm^2 in high cell density, and 1/8 of the high-density cells were seeded in the low-density culture. Before achieving confluence, the cells at lower density in the co-cultured preparations in all cases expressed more CD44 than those at high density (*Figure 4-11A*). A similar pattern was seen in NCI-H292 cells, although CD44 immunoreactivity was generally less in the NCI-H292 cells. The total CD44 expression decreased with increased cell density until the cells were confluent in both 16HBE 14o$^-$ and NCI-H292 cell lines. There was a good correlation of the CD44

expression between the lower densities of the "high density" series and the higher densities of the "low density" cultures (overlapping lines in *Figure 4-11*). At confluence, cells reached a density of approximately 1.5 x 10^5 cells/cm^2 in 16HBE 14o$^-$ and NCI-H292 cells. Maximum cell densities were 2.5 x 10^5 cells/cm^2 in 16HBE 14o$^-$ cells, and 5.0 x 10^5 cells/cm^2 in NCI-H292 cells. Little change in CD44 expression was seen after confluence (*Figure 4-11*).

(B) CD44 expression on cell lines is not affected by length of culture.

Cells collected at the same final cell density, but cultured for different time, showed the same relationship of final cell density to CD44s expression (*Figure 4-12*) as seen for cells seeded at different densities but cultured for a consistent period of time (*Figure 4-11*). There was a relative reduction in CD44 expression on cells collected after 1 day which probably reflected the time taken to recover in the passage by trypsin. Other CD44 isoforms showed the same pattern as CD44s. Thus, according to the results given in *Figure 4-11* and *Figure 4-12*, CD44 expression was dependant on final cell density and not the time of culture before collection (*Figure 4-12*).

(C) Cell proliferation is unaffected at the levels of confluence

To determine the effect cell density on proliferation, cell cycle analyses was carried out for different densities. Compared to fully confluent cells (18.3 ± 2.4), there was no significant difference in the percentage of cells in the G2/M-phase cell population at 10% sub-confluent (23.7 ± 2.6; p=0.642), 50% sub-confluent (17.1 ± 3.2; p=0.058).

4.2.5 Cytokine-regulation of ICAM-1/CD44 expression in cell lines

(A) ICAM-1 is up-regulated by IFNγ and TNFα in bronchial epithelial cells

16HBE 14o$^-$ and NCI-H292 cells were treated with IFNγ, TNFα, or IFNγ and TNFα together at 0, 25, 100, 200, and 400 U/ml for 24 hours (*Figure 4-13*). IFNγ treatment showed greater increases in ICAM-1 expression in NCI-H292 than 16HBE 14o$^-$ (*Figure 4-12A and B*). The large increase in ICAM-1 on NCI-H292 cells (5.1-

fold at 200U/ml) reflects a relatively low baseline ICAM-1 expression. In 16HBE 14o$^-$, IFNγ did not show a great increase in ICAM-1 expression (1.5-fold at 200U/ml). TNFα alone had no significant effect on either cell line. However, when cells were treated with TNFα and IFNγ together, the ICAM-1 expression was much increased (2.0-fold in 16HBE 14o$^-$ and 10-fold in NCI-H292 with 200U/ml IFNγ and TNFα) compared to IFNγ treatment alone (*Figure 4-13*).

(B) TNFα can enhance CD44 isoform expression

IFNγ and TNFα at 200 U/ml were chosen for treatment in this study as these concentrations gave maximal ICAM-1 responses and have also been used in other studies (Mackay *et al.*, 1994). IFNγ and TNFα together strongly increased the expression of ICAM-1 in both cell lines (see *section 4.2.5A*). In contrast to the ICAM-1 response, the effect of IFNγ and TNFα on CD44 and its isoforms was less marked. No significance was seen with IFNγ treatment, whereas the change of CD44 induced by TNFα in NCI-H292 was generally greater than by IFNγ (*Figure 4-14*). Only the increased CD44 expression of CD44v9 due to TNFα on 16HBE 14o$^-$ cells reached statistical significance (*Figure 4-14A*, $p < 0.05$). On NCI-H292 cells the most marked effect was on CD44v3, CD44v6 and CD44v9 when treated with TNFα (*Figure 4-14B*, $p < 0.01$). Treatment of TNFα and IFNγ together did not increase the expression of CD44 isoforms in either cell lines.

Since the effect of IFNγ and TNFα on the expression of CD44 might be mediated by a change in the cell density induced by these cytokines, the cell density after IFNγ and TNFα treatment was compared to untreated cells at 24 hours. No changes in cell density were seen, which might account for the changes in CD44 with these treatments.

(C) IL-1β and IL-4 increase CD44 expression

Treatment with IL-1β and IL-4 increased of CD44s, CD44v3, and CD44v9 (*Figure 4-15*). At the concentration of 20ng/ml, IL-1β showed marked increase of CD44

expression than IL-4. At the concentration of IFNγ or TNFα at 400U/ml, TNFα showed 20% increase of CD44s, but no effect was seen with IFNγ. All four cytokines increased CD44v9 in 16HBE 14o$^-$ cells. The up-regulation of CD44v9 by IL-1β and IL4 was more than 50% increased, but only 20% increase was seen with TNFα and IFNγ.

4.2.6 Effect of Gold compound

(A) AuTM has no effect on the baseline of ICAM-1 expression

Most of the bronchial cells, such as 16HBE 14o$^-$, 9HTE, BEAS-2B, and primary cells, expressed a high level of ICAM-1 (*section 4.2.2.A*). Cells were treated with AuTM from 10^{-5} M to 10^{-2} M (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M) for 24 hr. No difference was seen in baseline of ICAM-1 expression between treated and untreated 16HBE 14o$^-$ or NCI-H292 cells. Even in cells treated up to 10^{-2} M of AuTM, no cytototoxicity was seen in either cell line by using a trypan blue exclusion assay.

(B) Cytokine-induced ICAM-1 expression is inhibited by gold compound.

Although AuTM had no significant effect on basal expression of ICAM-1 on 16HBE 14o$^-$ and NCI-H292 cells, inhibition of AuTM on IFNγ-induced ICAM-1 expression was seen on NCI-H292 and 16HBE 14o$^-$ cells. AuTM showed the down-regulation on IFNγ-induced ICAM-1 expression in NCI-H292 and 16HBE 14o$^-$ in a dose-dependent manner (*Figure 4-16*).

4.3 Discussion

4.3.1 ICAM-1 and CD44 after tryptic cell dissociation

In passaging of cells in culture, trypsin/EDTA is routinely used to detach adherent cells. However, the proteolytic activity of trypsin may affect antigenic determinants of cell surface antigens. Trypsin/EDTA can be used for rapid monolayer cell detachment, but may cause loss of antigenic determinants. My studies show that ICAM-1 immunoreactivity is resistant to tryptic digestion, whereas CD44 immunoreactivity is decreased after trypsin/EDTA treatment. The results are similar to those of Gardner (Gardner *et al.*, 1995) which showed that CD44 immunoreactivity was decreased by trypsin treatment in ovarian tumour cell lines. Although Corver and colleagues (Corver *et al.*, 1995) showed that ICAM-1 was only decreased in certain carcinoma cell lines after trypsin treatment, the resistance of ICAM-1 to trypsin is probably caused by a small number of proteolytic sensitive sites or because antigenic determinant(s) detected by the antibody is(are) not sensitive. Whereas, CD44 may be more proteolytic sensitive or antigenic determinants for CD44 antibodies are sensitive to trypsin.

4.3.2 Regulation of ICAM-1 and CD44 after epithelial damage

In the present study, I found that all four cell lines expressed CD44 and its isoforms. The expression of ICAM-1 is higher on 16HBE 14o$^-$, 9HTE, and BEAS-2B than on NCI-H292 cells. Among them 16HBE 14o$^-$ and NCI-H292 were used for the following studies, since the characteristics of 16HBE 14o$^-$ are similar to normal airway epithelial cells, excluding high ICAM-1 expression, and NCI-H292 cells is similar to normal bronchial epithelial cells in having a low ICAM-1 expression.

Results show two cell lines derived from bronchial epithelium both expressed CD44 (total) and isoforms containing CD44v3, CD44v6 and CD44v9. In a mechanical damage model, CD44 expression was increased after cell damage and these changes showed a predictable time course. An increase of CD44 was seen at the wound

margin by immunofluorescence, particularly in the 150 μm proximal to the wound track. The locally increased CD44 expression in the cells correspond to those previously shown to be involved in repair processes (Zahm *et al.*, 1997). Quantification by image analysis also revealed a general increase in CD44 up to 500 μm from the track of the wound and up to 48 hours after wounding. Fluorescence intensity of CD44 measured by flow cytometry also confirmed this pattern. Both showed a similar general increase from 12 hours after cell damage. This expression of CD44 was increasing until wound closure. Cells in epithelial layer after damage were more spread out than in confluent cultures before damage, suggests that there was a decrease of the average cell density in the damaged area (*Figure 4-7A and C*).

In the mechanically wounded epithelium, the small decrease in CD44 expression shortly after damage seen by flow cytometry may reflect reduced expression on damaged cells. FDA with PI staining shows that the decreasing percentage of PI single positive cells suggests that the cells along the mechanical damage area were not reversibly damaged. By immunostaining, ICAM-1 and CD44 immunoreactivity was increased at 10 minutes and 1 hour, but this pattern was not seen for ICAM-1 by flowcytometry. This difference may be caused by the permeabilised cells along the damaged edge absorbing antibody. Another possibility is that the low percent of increased ICAM-1 expressing cells, might be fragile and was destroyed during sample preparation, and was therefore not detectable by flow cytometry.

Studies in other cell types showed that CD44v6 was up-regulated in smooth muscle cells in injured arteries *in vivo* (Jain *et al.*, 1996). CD44 enhanced cell migration in repair also has been shown in dendritic cells (Weiss *et al.*, 1997) and melanoma cells (Goebeler *et al.*, 1996) and implicated in epithelial cells (Ito *et al.*, 1997). In my study of bronchial epithelial cells, the CD44 increase after damage suggests that CD44 was involved in the repair response. The later increase away from the damaged edge indicates that as expected, the response is propagated away from the wound margin to undamaged cells

4.3.3 ICAM-1/CD44 expression and cell density

Following damage, a rapid response to restore airway epithelial structure is often critical to prevent infection and ingress of harmful environmental agents. Local loss of epithelial cells such as is frequently seen in asthma results in locally reduced cell density that is only restored relatively late in the repair response. In my study, the level of expression of CD44 on cells was significantly reduced as cell density increased. CD44 immunoreactivity showed changes of approximately 3-4 fold between low and high density (confluent) cultures. Although all cells continued to express some CD44 at high densities, the percentage expressing CD44 isoforms containing CD44v3 or CD44v6 fell to 65%, however, CD44s and CD44v9 expression in the cells was unchanged. These results indicate that different CD44 isoforms are differentially regulated in relation to cell density. Although the pattern of CD44 expression with cell density is similar between the two cell lines, the absolute cell density at which the reduction in CD44 was seen was higher in NCI-H292 cells. The level of expression for all CD44 isoforms was lower in NCI-H292 than 16HBE 14o$^-$ cells and the maximum cell density achieved in NCI-H292 in cultures was also higher. This may due to the differences in cell size or the way in which the cell grew. At confluence, NCI-H292 cells increased cell-cell contact by "packing" tightly together. However, 16HBE 14o$^-$ cells did not show this growth pattern.

4.3.4 The basis of CD44 reduction with increasing cell density

While the relationship between CD44 expression and cell density could be due to altered regulation controlled by cell-cell contact or soluble factors, it is also possible that nutrient depletion at high cell densities could contribute. Since cells were grown at different cell densities, but in the same culture dish, and sharing the same medium showed this density dependent expression of CD44, it is unlikely that this effect was mediated by nutrient depletion or soluble factors produced by the cells themselves. The observation of the same density-related CD44 expression trends irrespective of the previous period of growth in culture again suggests that cell-cell contact at higher cell densities regulated CD44 expression. The contrasting increase in ICAM-1 expression at higher cell densities further reduced the likelihood that nutrient depletion was responsible. Taken together these results indicate that CD44 expression

is inversely correlated with cell density and that the effect is likely to be mediated through cell-cell contact. These results reflect the pattern seen in tissue samples in which more differentiated cells, particularly columnar epithelial cells, do not express CD44 (Lackie *et al.*, 1997).

4.3.5 Cell adhesion and cell density

Although the regulation of cell adhesion molecules has been extensively studied, most of the work has been devoted to the effects of cytokines (Collins *et al.*, 1995), growth factors (Wang *et al.*, 1996), and extracellular matrix (Rubin *et al.*, 1996). Little is known about the direct effects of cell density on adhesion molecule expression. Up-regulation of NCAM in neuronal cells at high density has been observed (Breen and Ronayne, 1994) and is suggested to enhance aggregate formation. Studies in bladder and colonic carcinoma cell lines (Stanley *et al.*, 1995) have also shown that integrin expressions changed with cell density, and that these changes varied between cell types and different integrin molecules.

The 2-4-fold change in CD44 expression with cell density also indicates the importance of controlling for cell density in experiments looking at the expression and regulation of CD44 and other adhesion molecules in epithelial cells.

4.3.6 ICAM-1/CD44 expression and cytokines

It has been reported that the TNFα levels are increased in the bronchoalveolar lavage fluid of symptomatic asthmatics (Broide *et al.*, 1992). Several studies have been reported that TNFα enhanced the expression of ICAM-1 in primary bronchial epithelial cells and BEAS-2B cells (Krunkosky *et al.*, 1996; Atsuta *et al.*, 1997). My studies showed TNFα itself does not show significant effect on the ICAM-1 expression in these two cell lines. This might be due to the different cell type having varied levels of response to TNFα. Treating the cells with IFNγ and TNFα together induced a greater increase in ICAM-1 expression is induced than when treated by IFNγ alone. This result suggests that the mechanism of IFNγ- or TNFα-induced ICAM-1 expression is through different pathways.

Gold compounds are widely used in the treatment of rheumatoid arthritis, but the detailed mechanism of their action is still unclear. Some of the studies in lymphocytes might provide a clue. AuTM has been shown as an inhibitor of protein kinase C (Hashimoto *et al.*, 1992). The inhibition of AuTM on IFNγ-induced ICAM-1 increase suggests that the IFNγ-induced ICAM-1 expression might be through the activation of protein kinase C. In addition, it has been reported that AuTM inhibited the activity of CD45 which is involved in antigen-receptor-mediated lymphocyte signalling (Wang *et al.*, 1997). AuTM may interact with the cysteine residue in the active site of CD45, which is essential for its catalytic activity, further inhibiting cell signalling molecules. Makino and co-workers demonstrated that AuTM inhibited glucocorticoid receptor-mediated intracellular signals (Makino *et al.*, 1993). My studies showed that treatment with AuTM can inhibit the IFNγ-induced ICAM-1 expression, whereas it has no effect on reducing basal levels of ICAM-1 in the bronchial epithelial cell lines.

The up-regulation of ICAM-1 by inflammatory cytokines is well documented. However, less is known of the regulation of cytokines in CD44 expression. I demonstrated that total CD44 and some of the CD44 isoforms were less affected by IFNγ and TNFα. This confirms some of previously published studies reporting CD44 expression in bronchial cell lines which have also shown no effect with IFNγ and TNFα treatment (Bloemen *et al.*, 1993). In other systems, TNFα up-regulates CD44 on endothelial cells (Mackay *et al.*, 1993), and TNFα increases CD44v9 and IFNγ induces CD44v6 expression in myelomonocytic cell lines. Furthermore, IFNγ down-regulated total CD44 and CD44v9 and up-regulated CD44v6 in some carcinoma and keratinocyte epithelial cell lines (Mackay *et al.*, 1994). In melanoma cells little change in CD44 expression was seen with IFNγ and TNFα treatment (Creyghton *et al.*, 1995). Because the CD44 isoform expressed in different cell types might not be the same, this could account for the differences in cytokine-regulation of CD44 expression.

Recent studies indicate a predominance of Th2 cells and Th2 cell-derived cytokines in the pathogenesis of bronchial asthma (Anderson and Coyle, 1994). Increased levels of IL-4 have been found in BAL (Walker *et al.*, 1994), and IL-4 receptors are expressed on human bronchial epithelial cells (van der Velden *et al.*, 1998). The up-regulation

of CD44 by IL-4 was reported in human colonic epithelial cells (Trejdosiewicz *et al.*, 1998). Other cytokines, such as IL-1β, are also considered to be important cytokines in proinflammatory responses. IL-1β and IL-4 are present during inflammatory responses in the human airways (Bittleman and Casale, 1994). Therefore, it is of interest to know the effect of IL-1β and IL-4 on CD44 expression in bronchial epithelial cells. When 16HBE 14o$^-$ cells were treated with IL-1β and IL-4, up-regulation of CD44s, CD44v3 and CD44v9 were seen.

This suggests that the increased expression of CD44 seen in airway epithelium in asthma (Peroni *et al.*, 1996; Lackie *et al.*, 1997) is likely to be associated with repair processes and directly induced CD44 expression by certain cytokines, such as IL-1β and IL-4.

4.3.7 ICAM-1 expression in airways

ICAM-1 is found on the surface of airway epithelial cells, such as alveolar epithelial cells. However, it is undetectable in normal bronchial epithelial cells. In alveolar cells, only type I alveolar cells consistently express ICAM-1. Type II alveolar cells do not express ICAM-1. However, type II cells grown *in vitro* had an altered phenotype similar to type I cells and expressed ICAM-1 (Christensen *et al.*, 1993). In the cells with low ICAM-1 expression, ICAM-1 may be induced when the cells change their phenotype. Thus Piedboeuf (Piedboeuf *et al.*, 1996) concluded that ICAM-1 synthesis on type II cells may be important in attracting inflammatory cells to the site of the injury. The increase of ICAM-1 expression in human primary bronchial epithelial cells might reflect a similar situation. The primary epithelial cells in the environment of culture system were not able to maintain the same morphology as *in vivo*. As mentioned in Chapter 3, ICAM-1 is localised on the microvilli, but less express on ciliated cells. Primary cells are gradually de-differentiated and loss their cilia, and more and more cells have only microvilli. These de-differentiated processes might increase the expression of ICAM-1. Altered the structure of the cells might increase the expression of ICAM-1.

The physiological role of epithelial ICAM-1 in the airways remains to be determined. In contrast to CD44, increase of ICAM-1 was parallel to cell density before cell confluence. The increase of ICAM-1 following damage but before restoring the epithelial monolayer, may improve the binding of inflammatory cells to the damaged area.

4.3.8 Putative functions of CD44 variation with cell density

Changes in cell-matrix adhesion might be expected to result from increased CD44 expression and could be further affected by the differing extra cellular matrix affinities of the variants of CD44 expressed and the relative importance of HA as a ligand. However, since *in vivo* CD44 does not appear to be in areas of close cell-matrix contact (Lackie *et al.*, 1997), it is possible that CD44 is involved in the early stages of cell processes which include cell adhesion, spreading and migration. In normal epithelium, decreased cell motility may down-regulate CD44 expression. The CD44v3 variant, which contains the predominant CD44 glycosaminoglycan modification site, was expressed on fewer cells than total CD44 or CD44v9 at high cell densities. This is of particular interest as CD44v3 is implicated in modulating the effects of heparin binding growth factors on cells (Bennett *et al.*, 1995).

Increased CD44 has previously been associated with cell proliferation in normal and neoplastic human colorectal epithelial cells (Abbasi *et al.*, 1993), proliferation being increased during repair and reduced at cell confluence. However, the *in vitro* systems used in this study differ significantly from the intestinal system that is more stratified and has morphologically defined areas of cell proliferation. Previous studies of airway epithelial repair after damage show that proliferation is maximal in the region 160-400 μm from the wound edge at 48 hours (Zahm *et al.*, 1997). Studies of the repair of human airway epithelial also showed that cell proliferation is not important until wound closure (Shimizu *et al.*, 1994; Zahm *et al.*, 1997; Kim *et al.*, 1998), which supported proliferation only occurred in the later stage of repair processes. In the current study, CD44 was increased in this area at this time, however the marked increase closer to the wound edge and at earlier times suggests that the increased CD44 expression is related to other processes. In the current model at different cell

density cultures showed no significant difference in the percentage of cells in the G2/M phase cell population (range 6%) while CD44 expression varied by 2-3 fold suggesting that the expression of CD44 is cell proliferation-independent. In common with CD44v3, the number of cells expressing CD44v6 was significantly reduced at confluence. CD44v6 variant expression in tumours has been correlated with malignancy (Culty *et al.*, 1994), and could reflect its involvement in cell migration.

Taken together the localisation and regulation of ICAM-1 and CD44 and the distribution of CD44 in the area of free surface of basal epithelial cells might show CD44 has the potential to act as a signalling receptor in tissue. In addition, its variant isoforms and multiple ligands might lead to varied and complex biological activities. The expression of CD44 correlated with epithelial repair processes and cell-density dependence suggests that CD44 plays an important role in cell migration. Increased ICAM-1 might have an important role in the binding to some of the airborne pollutants, such as virus and pollen in the airways, and may regulate the binding between neutrophil-epithelial cells in the damage sites.

The roles of adhesion molecules in the mechanisms of airway inflammation are only beginning to be defined. This study using mechanical damage, with cytokines and cell density provides a model for studying the regulation of ICAM-1 and CD44 on bronchial epithelial cells. These results suggest that ICAM-1 and CD44 are involved in the inflammatory mechanism of airway disease. Further understanding of their importance in the airway inflammation and the study of their functional roles in binding and migration will be the next step to be investigated.

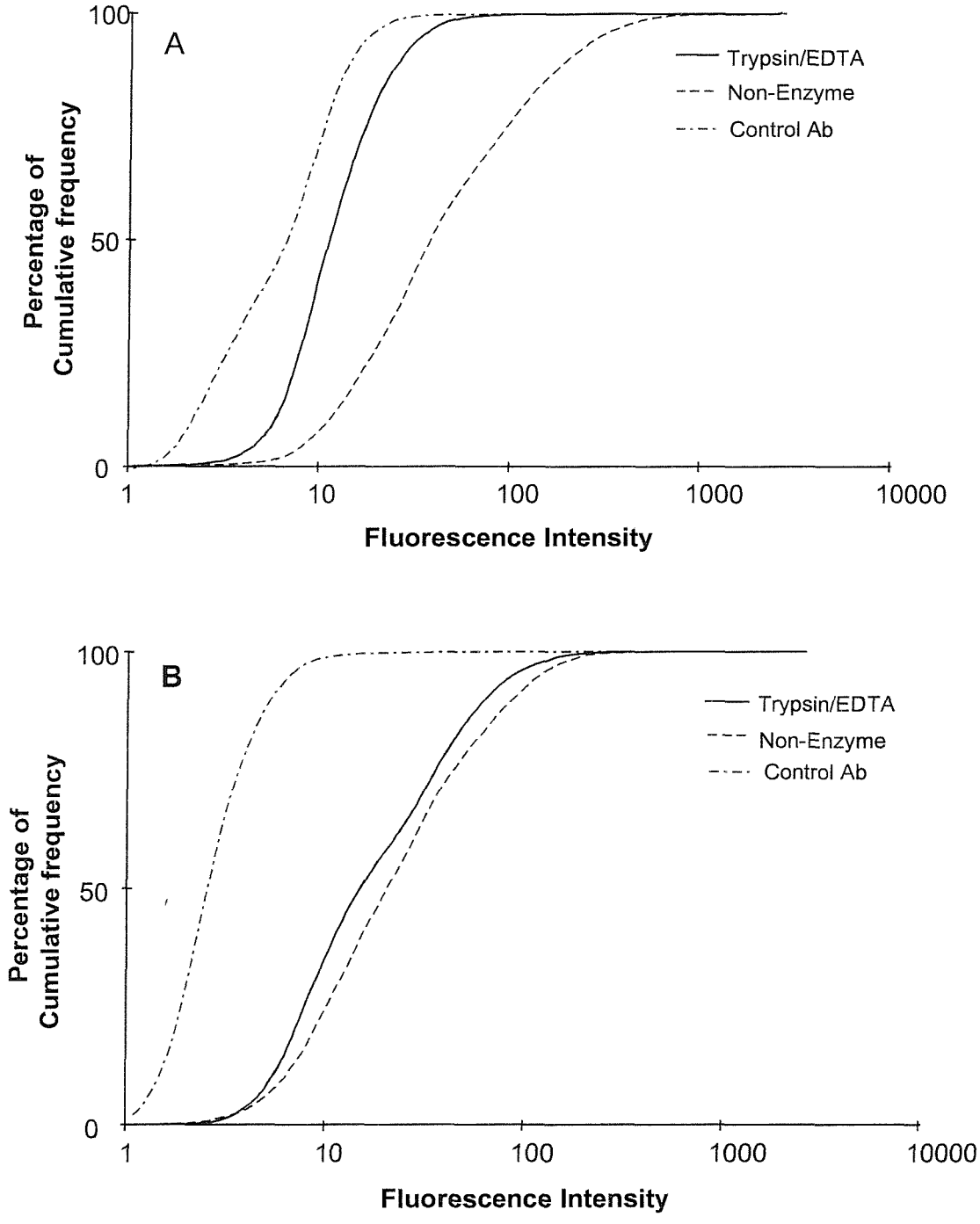


Figure 4-1. Comparison of CD44 (A) and ICAM-1 (B) expression on cell surface after detaching using trypsin or non-enzyme cell dissociation solution. After trypsin treatment, CD44 immunoreactivity was removed from the surface of 16HBE 14o$^-$ cells. Even in 16HBE 14o$^-$ cells, which express a high level of ICAM-1, the shedding effect by trypsinisation was not marked (<10%).

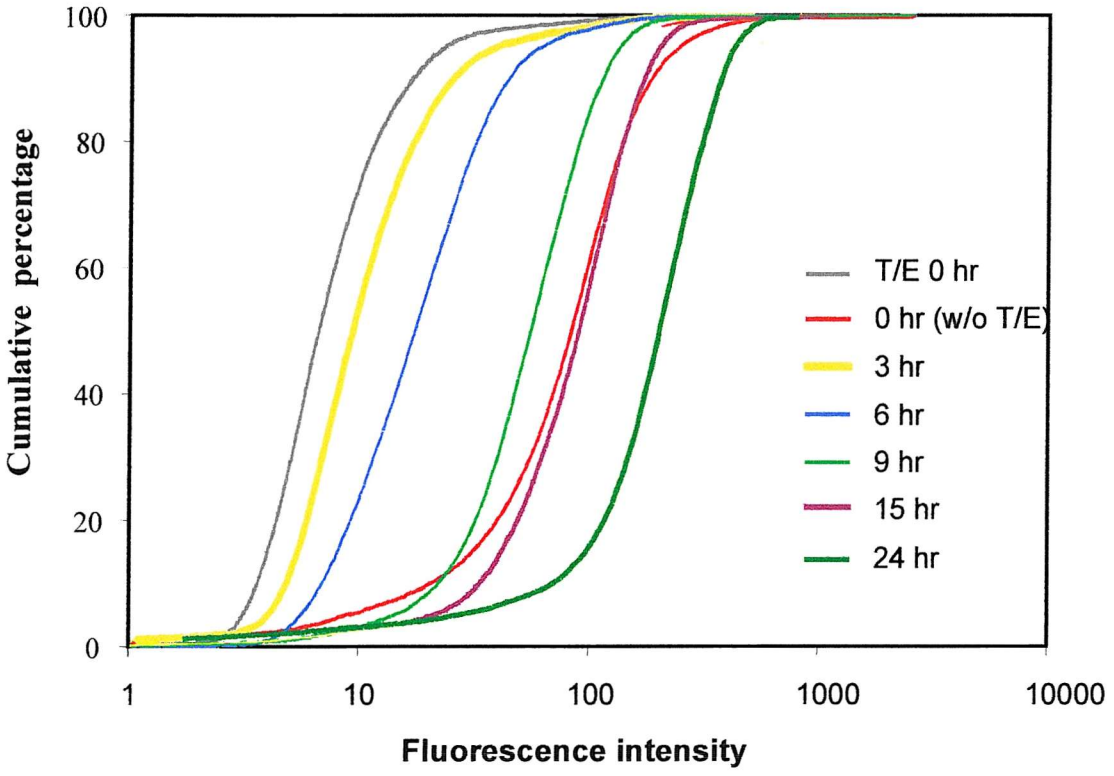


Figure 4-2. Time course of CD44s expression after trypsin/EDTA(T/E) treatment. 16HBE 14o$^-$ cells were detached by trypsin/EDTA. After different time point cells were harvest by non-enzymatic cell dissociation solution and CD44 expression was analysed by flow cytometry as described in *section 2.3.*

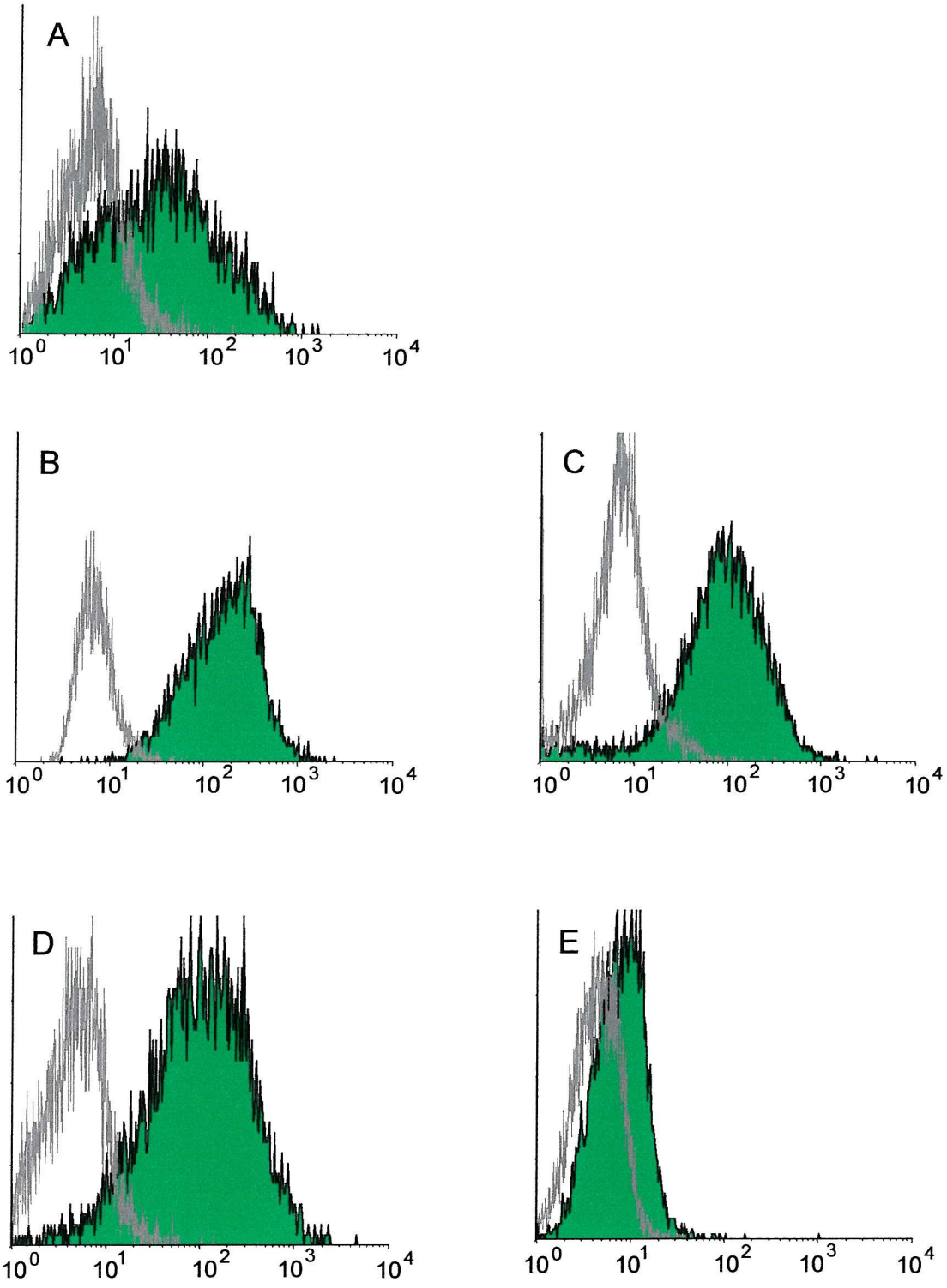


Figure 4-3. The expressed levels of ICAM-1 on human bronchial primary epithelial cells (A); 16HBE $14o^-$ (B); 9THE (C); BEAS-2B (D); H292 (E). Cells were prepared for flow cytometry analysis as described in ***Materials and Methods*** (*Section 2.3.1*). Grey line represented the isotype control for individual cell line.

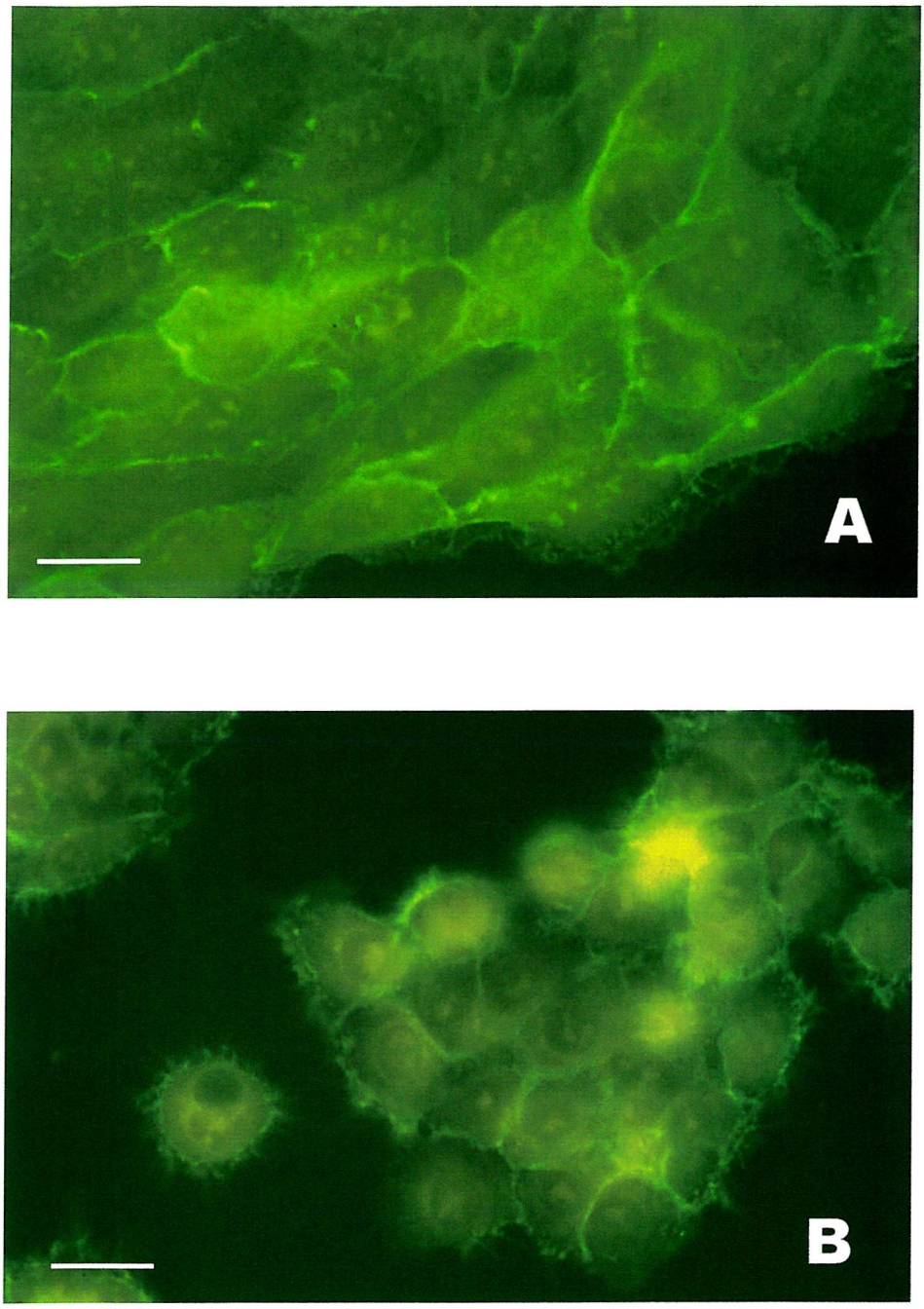


Figure 4-4. Pattern of CD44 expression in bronchial epithelial cells. By immunostaining with CD44 antibody (25-32), CD44 was expressed in 16HBE 14o- (A) and NCI-H292 (B) cells. Bars: 25 μm.

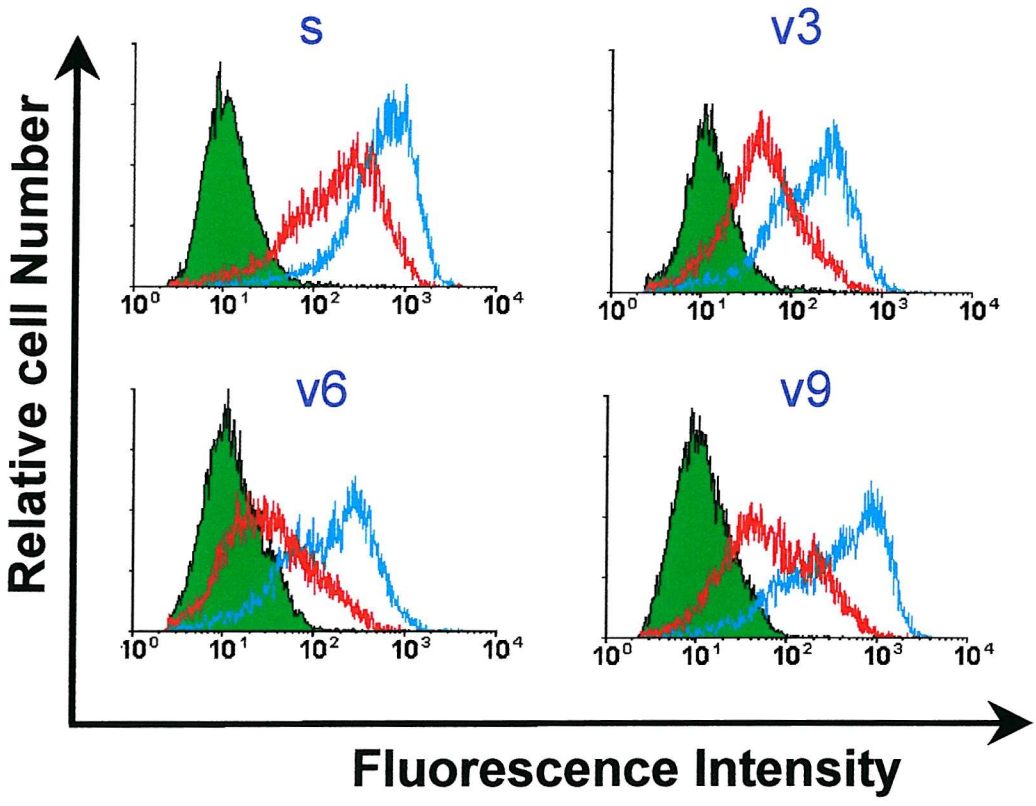


Figure 4-5. Flow cytometry analysis of CD44 and its isoforms on 16HBE 14o$^-$ cells. Cells from sub-confluent (blue) and confluent (red) cultures were stained with CD44 antibodies using a second antibody conjugated to FITC. Flow cytometry results show the fluorescence intensity histogram on a log scale for 10,000 cells from each sample. An isotype control (solid green) for CD44. Result shows no difference between confluent and sub-confluent cells while CD44s (s), CD44v3 (v3), CD44v6 (v6) and CD44v9 (v9) show different levels of staining under these conditions.

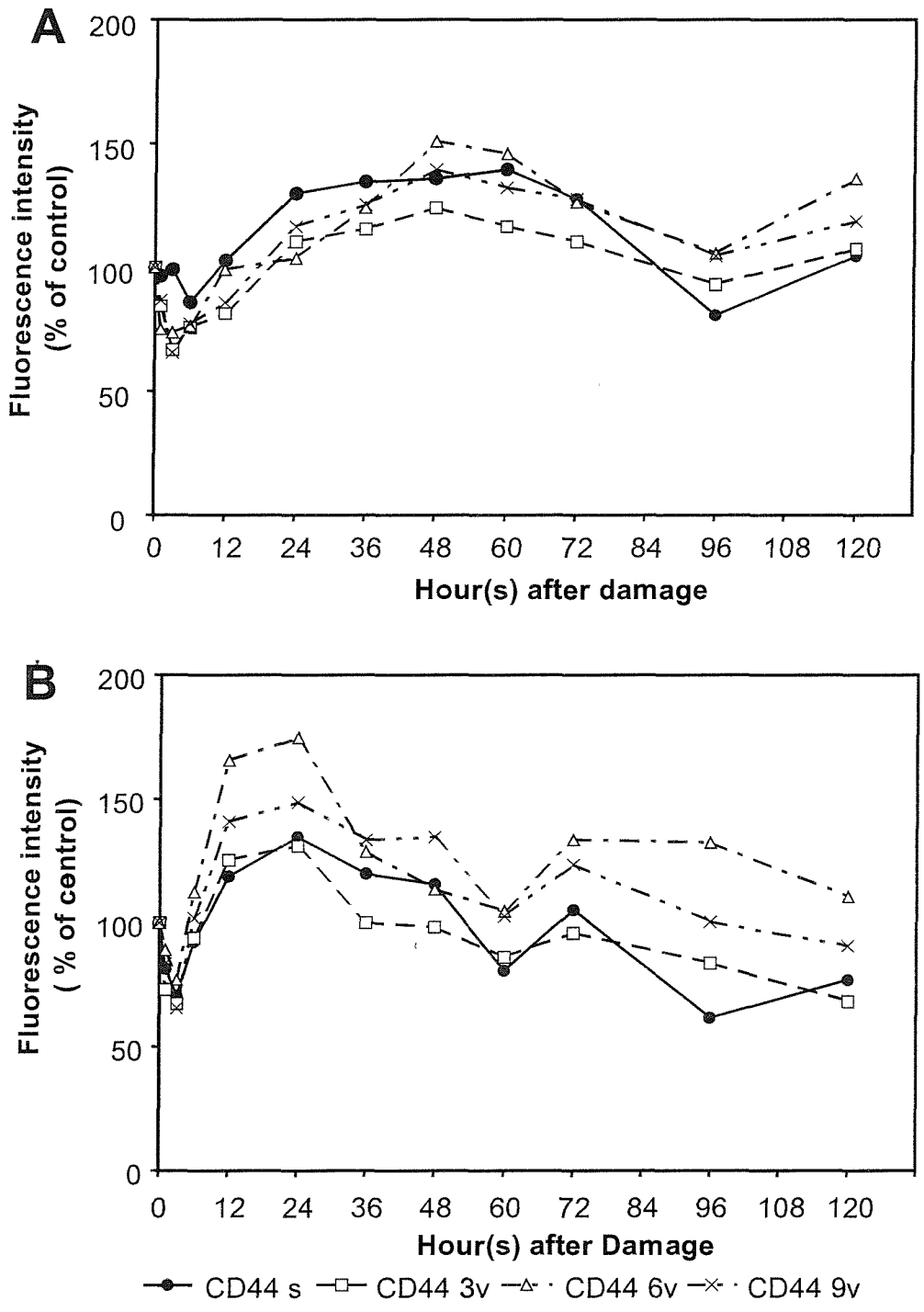
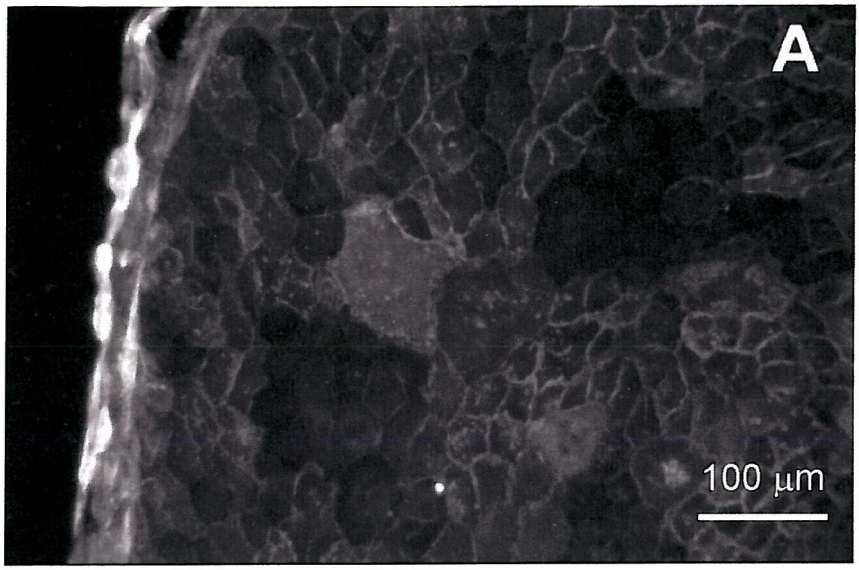
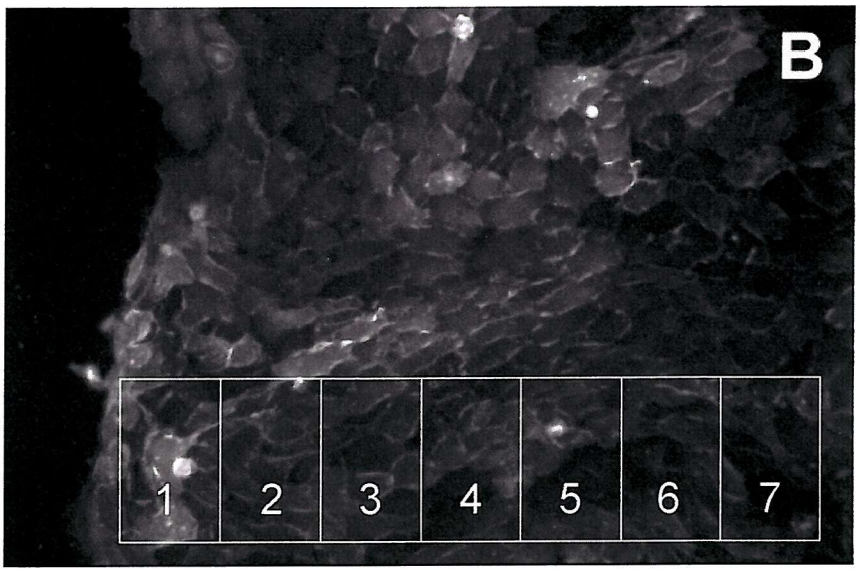


Figure 4-6. CD44 isoform immunoreactivity measured by flow cytometry in 16HBE 14o$^-$ and NCI-H292 cells after mechanical damage. Fluorescence intensity of CD44 isoform immunoreactivity was determined by flow cytometry as described in ***Materials and Methods*** (*section 2.3*). Data are presented as mean percent of fluorescence intensity normalized to undamaged cells. The results are presented as 16HBE 14o$^-$ cells (A); NCI-H292 cells (B).


A
100 μm


B
1
2
3
4
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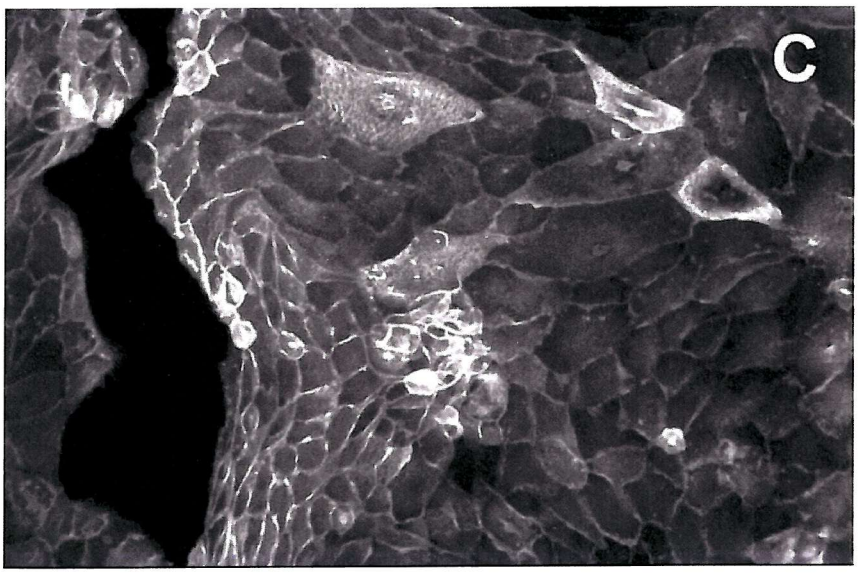
7


C

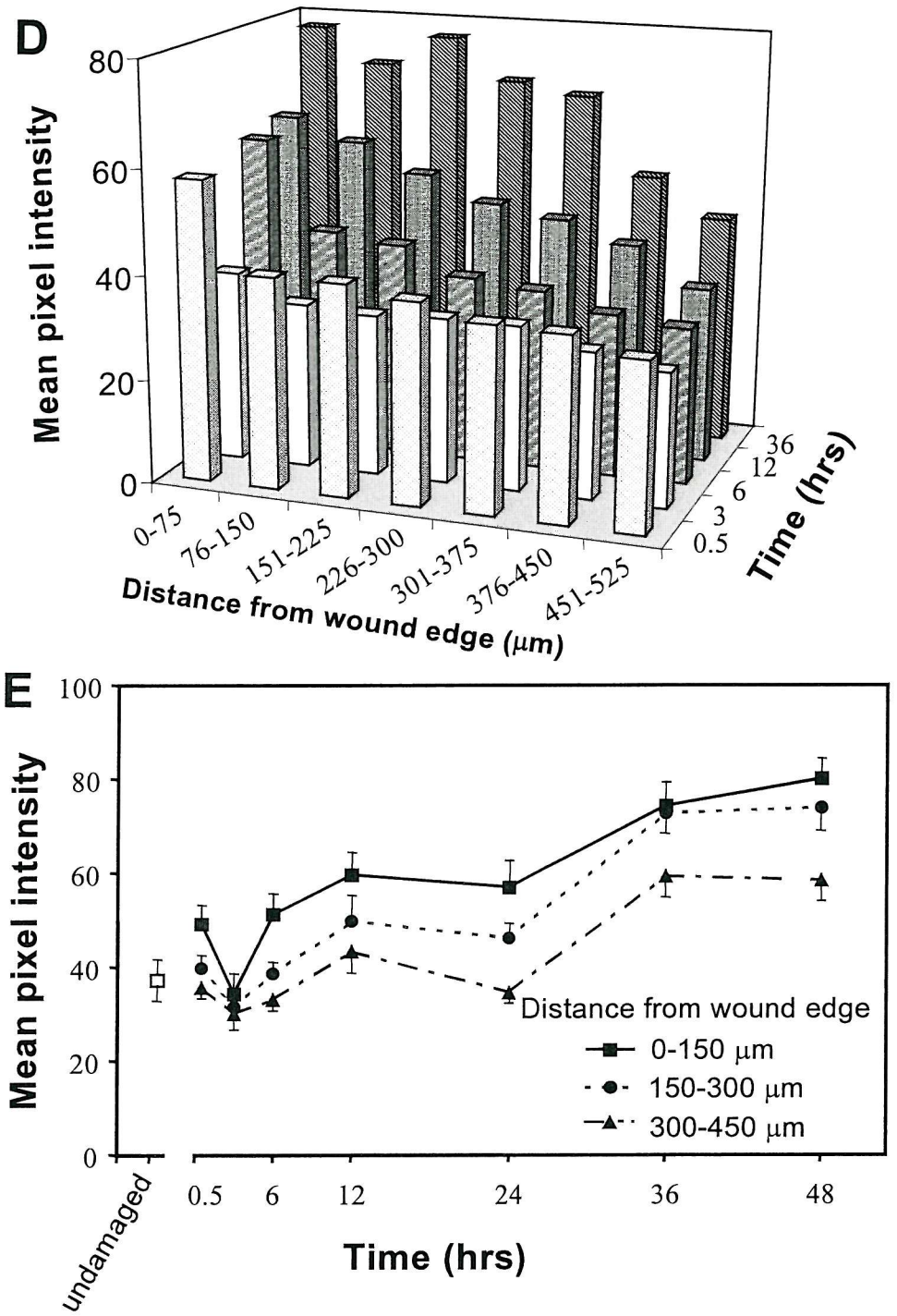


Figure 4-7. Quantitative evaluation of CD44s immunoreactivity in damaged areas. CD44s immunoreactivity on 16HBE 14^{-} cells was measured by analysis of the pixel intensity of 7 adjoining 75x135 μm rectangular areas along the wound edge as illustrated (1-7 in B). Examples of the pattern of CD44s staining at 30 minutes (A), 3 hours (B), and 12 hours (C) after damage are shown. The change of mean pixel intensity of CD44 in wounded cultures at different times and distances from the wound margin are shown (D). The change in mean pixel intensity, against time, of areas grouped and averaged is shown (E). Each value represents the mean pixel intensity ± SEM.

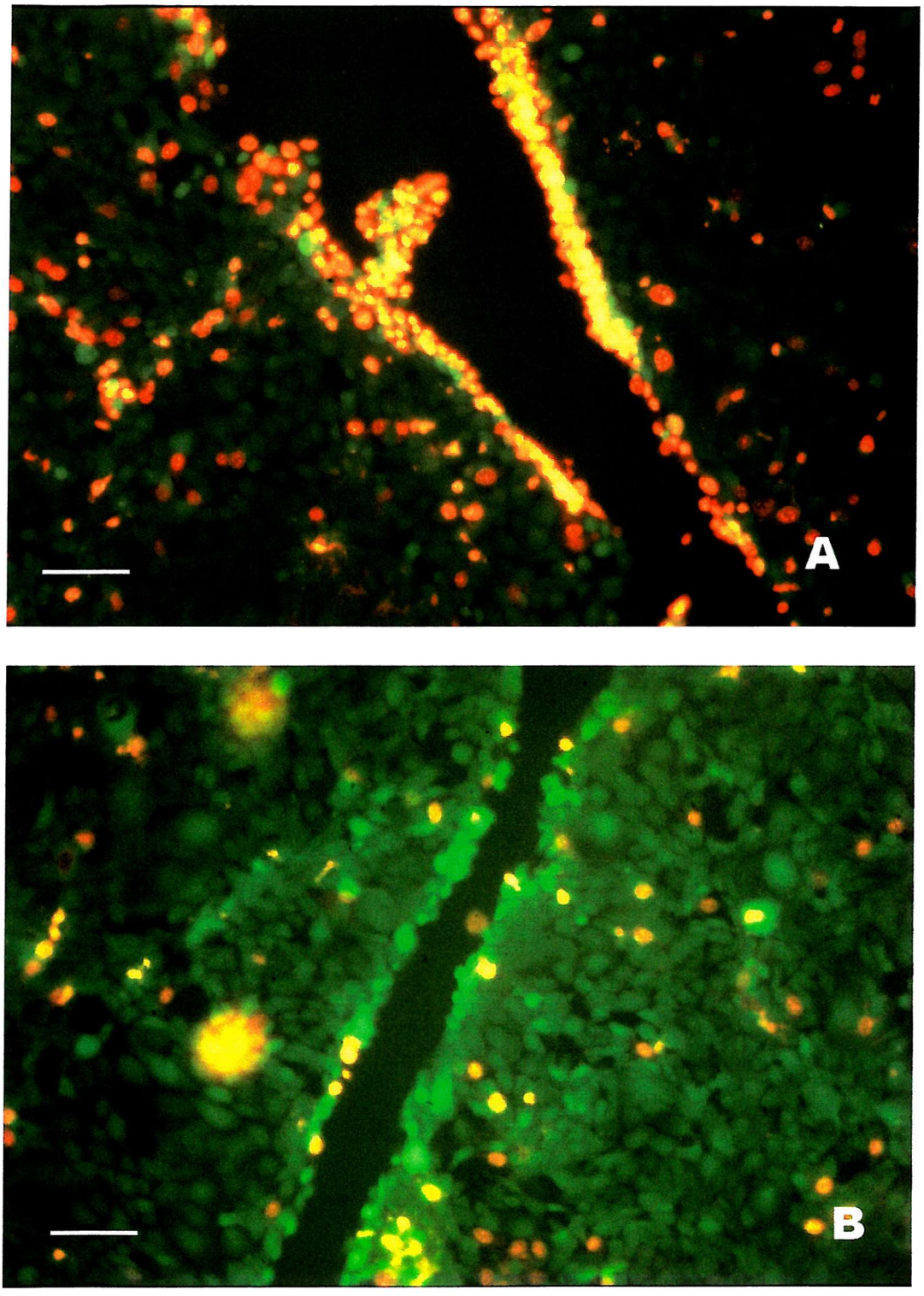


Figure 4-8. Determination of cell damage with fluorescein (FDA) and propidium (PI). 16HBE 14o$^-$ cells were mechanical damage and stain with FDA and PI at 10 minutes (A) and 1 hour (B) after damage. Cells in green colour were viable. Those with PI staining were permeable cells which were damaged cells. Bars: 100 μm.

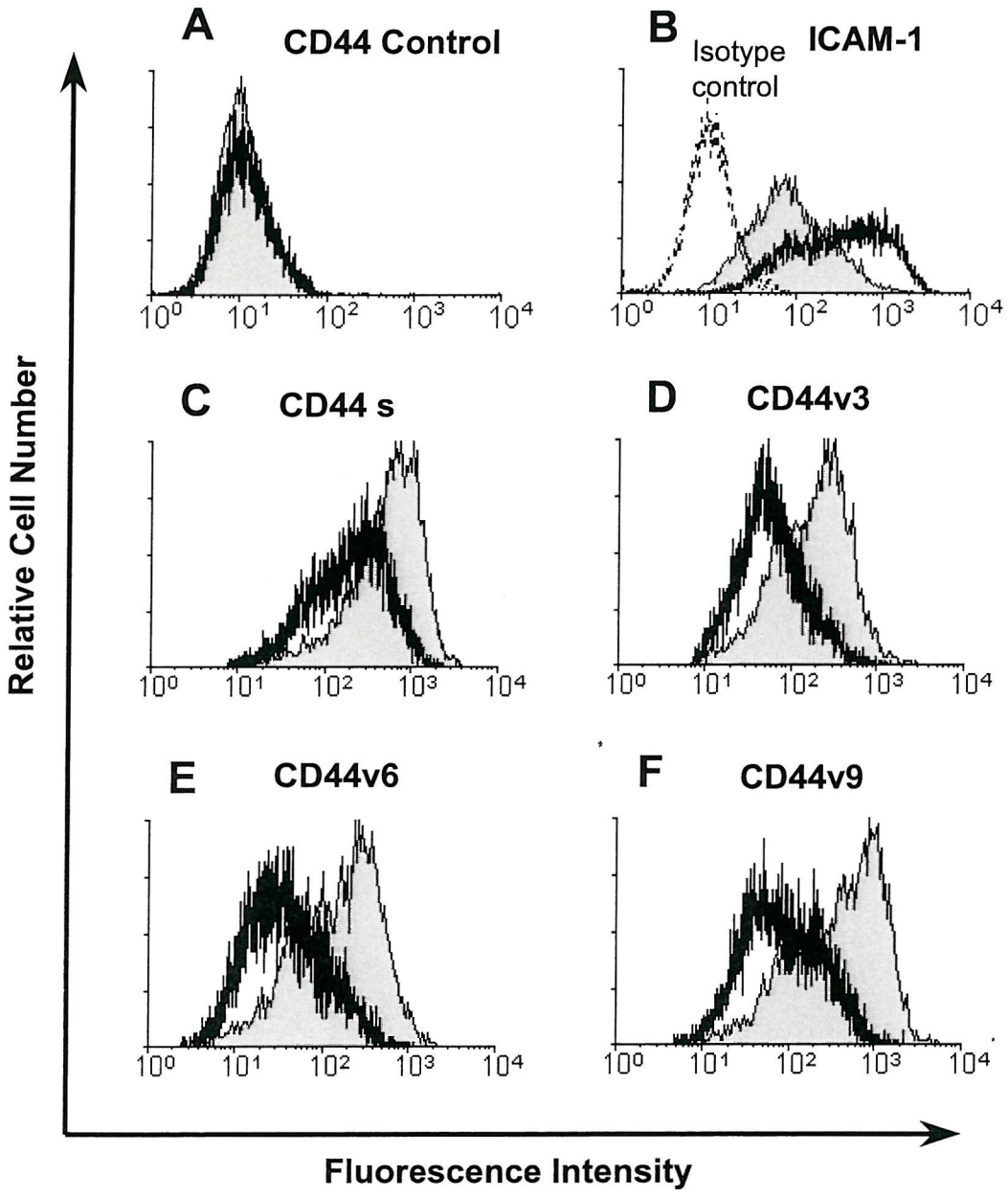


Figure 4-9. Flow cytometry analysis of CD44 and its isoforms on 16HBE 14o$^-$ cells. Cells from sub-confluent (grey shading) and confluent (solid line) cultures were stained with CD44 antibodies using a second antibody conjugated to FITC or ICAM-1 antibodies directly labelled with FITC. Flow cytometry results show the fluorescence intensity histogram on a log scale for 10,000 cells from each sample. A secondary antibody control for CD44 (A) shows no difference between confluent and sub-confluent cells while ICAM-1(B), CD44s (C), CD44v3 (D), CD44v6 (E) and CD44v9 (F) show different levels of staining under these conditions. For ICAM-1 another directly FITC labelled antibody of the same isotype was used as a control (B, dotted line).

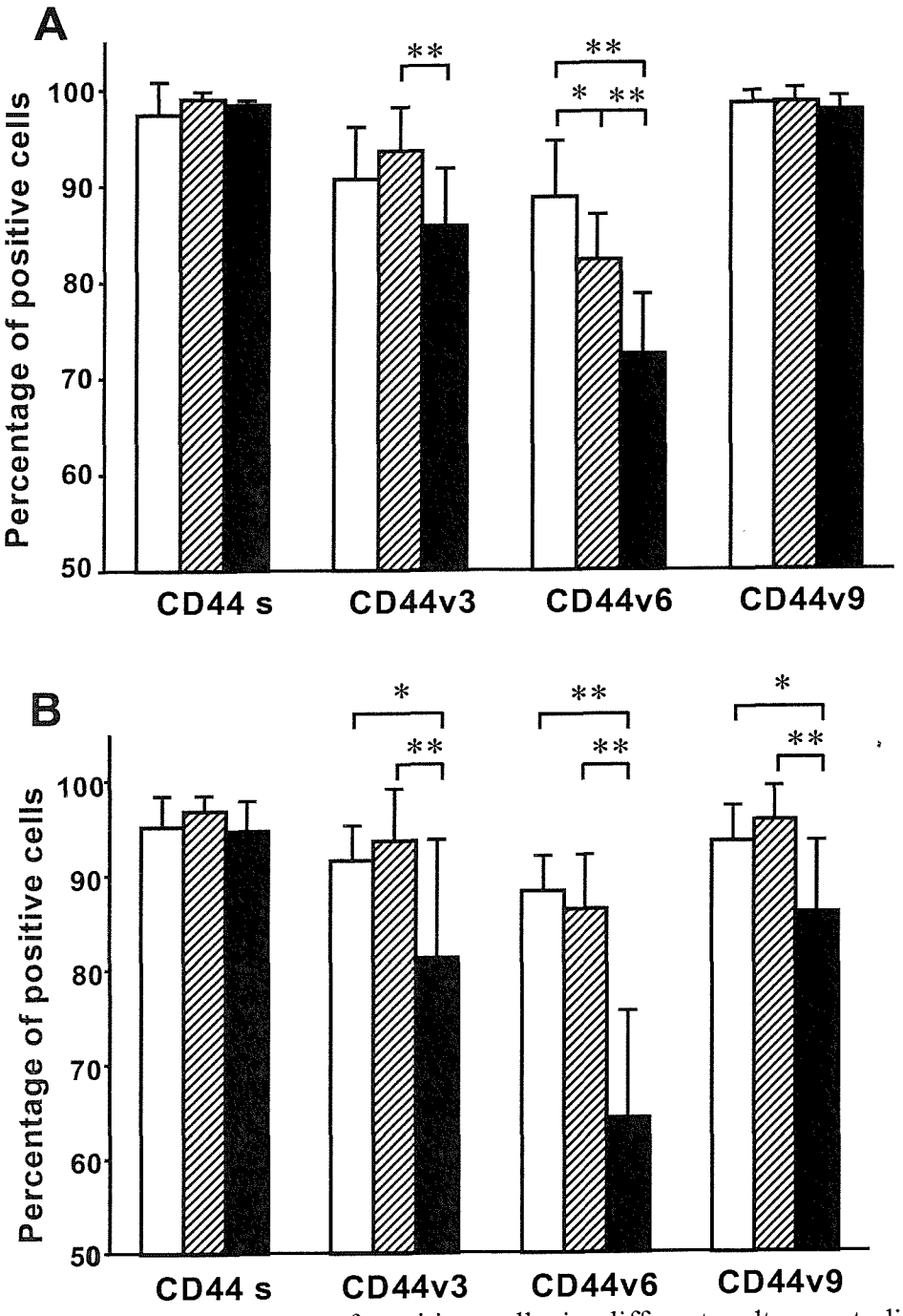


Figure 4-10. Percentage of positive cells in different cultures at different levels of confluence. Flow cytometry analysis for CD44s, CD44v3, CD44v6 and CD44v9 was performed on cultured bronchial cells grown to less than 60% confluent (open bars), 80-90% confluent (shaded bars), and confluence (for more than 24 hours; solid bars). The results are presented as the number of cells having a fluorescence intensity of more than two standard deviations above the mean value for the control (no primary). (A) 16HBE $14o^{-}$ cells. (B) NCI-H292 cells. Samples differing significantly are marked (* = $p< 0.05$, ** = $p< 0.01$), error bars show standard error of the mean.

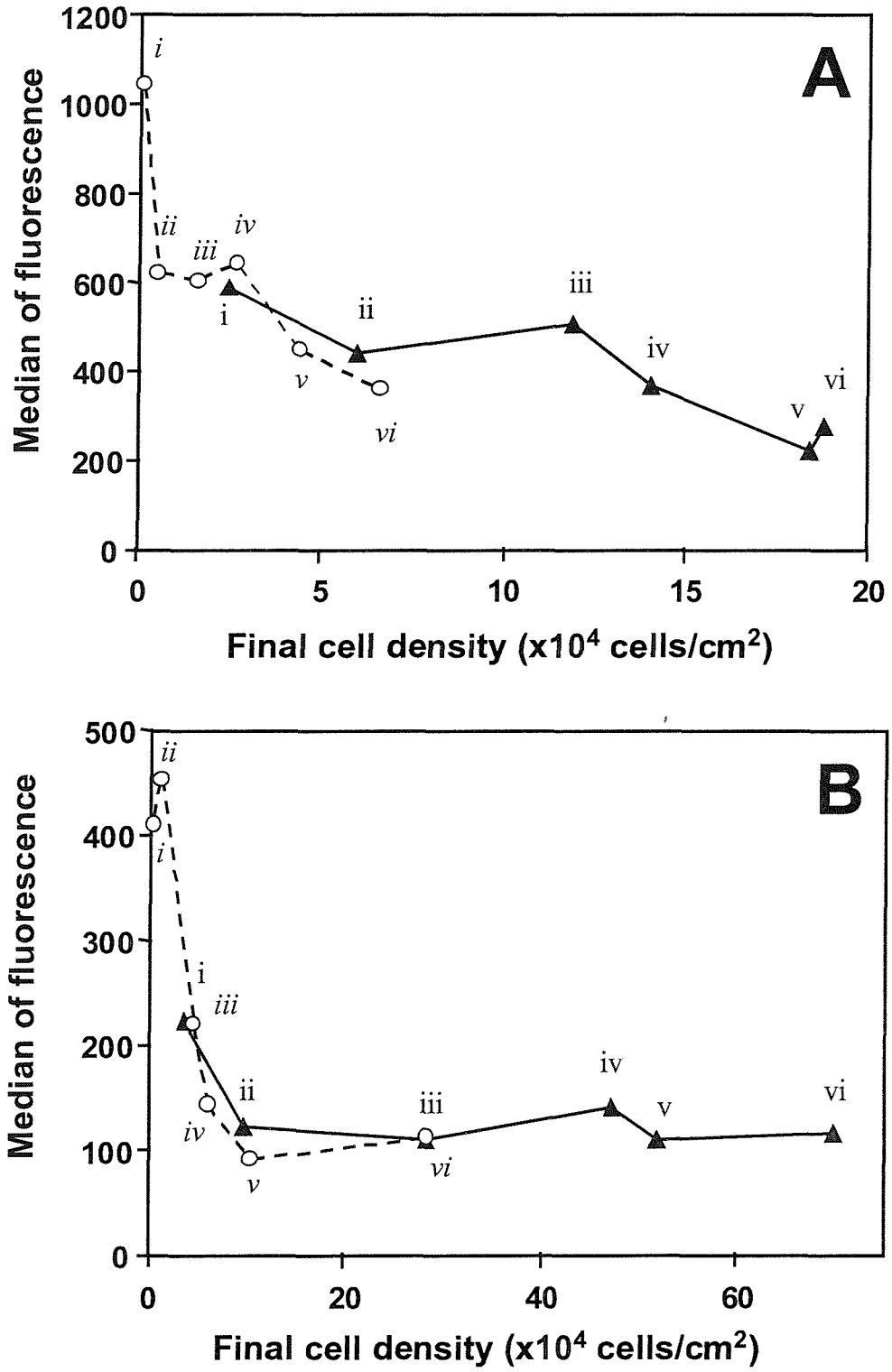
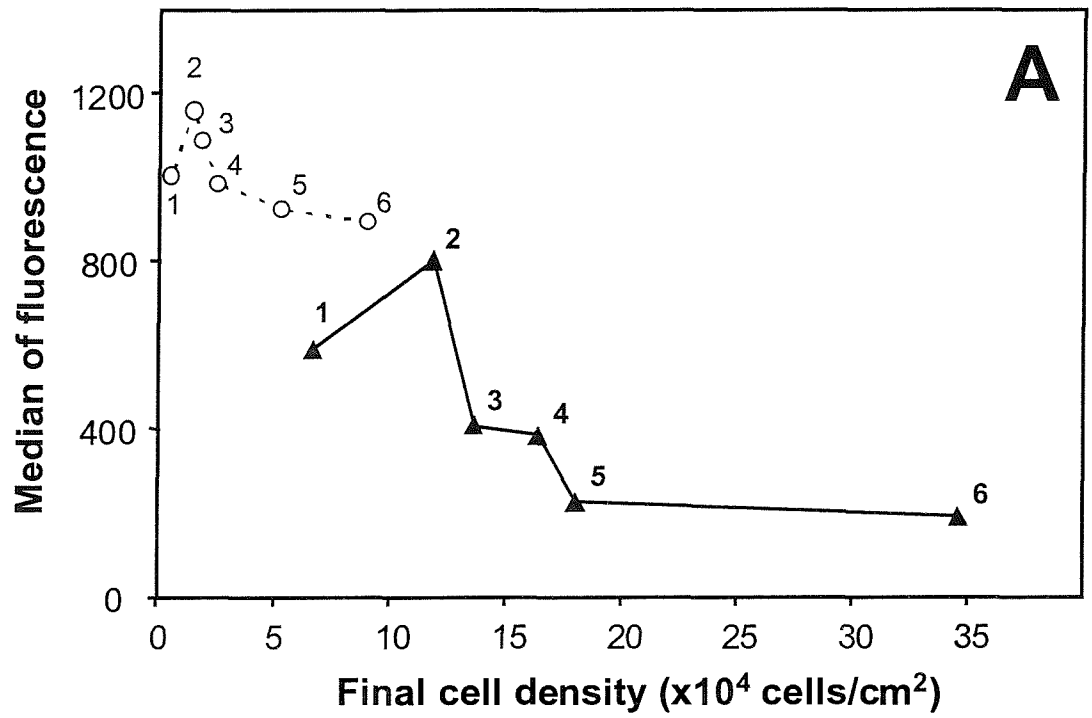


Figure 4-11. Cell density and CD44 expression. Cells were seeded at serial cell densities in pairs at "high" and "low" cell density (1/8 of "high" cell density) in the two halves of a petri dish. The points labelled with the same roman numerals in each plot (e.g. ii and *ii*) were high and low cell density cultures from the same petri dish. Since the maximum "low" density was higher than the minimum "high" density there is some overlap. Cells were harvested for FACS analysis and the final cell density calculated as in methods section. CD44 expression on 16HBE 14o$^-$ cells (A) and NCI-H292 cells (B).



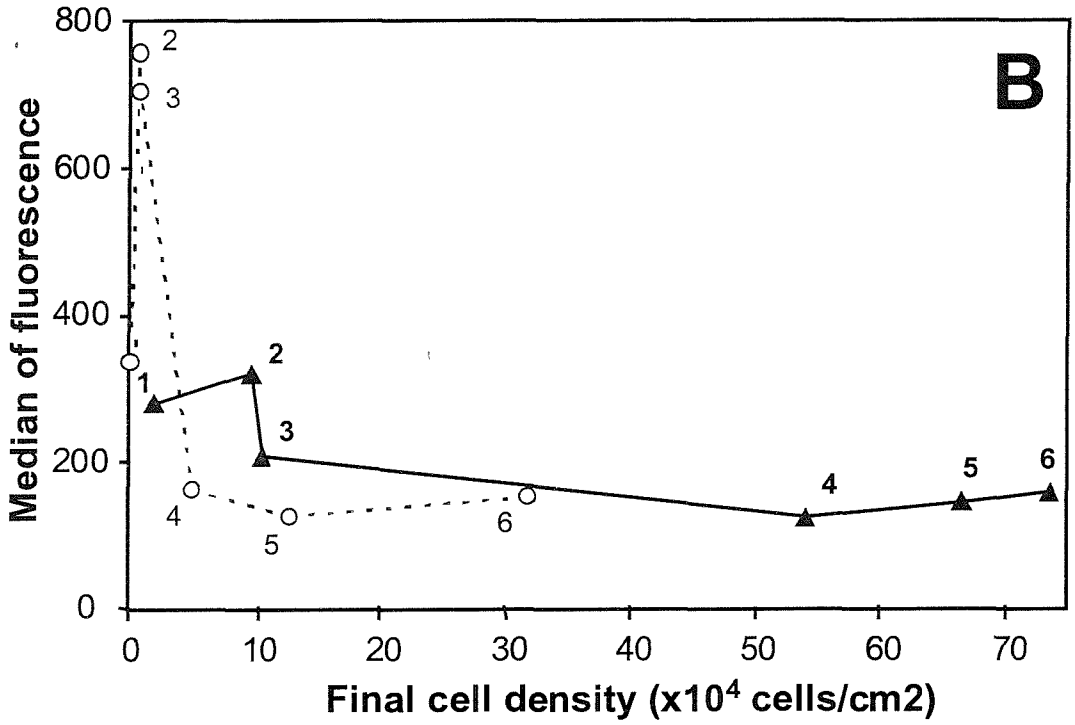


Figure 4-12. Plating time and CD44 expression. Cells were plated at constant "high" (solid line) and "low" (dotted line- 1/8 of "high" cell density) cell densities in the two halves of a petri dish. The labels indicate the period of culture for the sample in days and the median fluorescence is plotted against the final cell density at the time when the cell were collected for flow cytometry. CD44 expression on 16HBE 14o⁻ cells (A) and NCI-H292 cells (B).

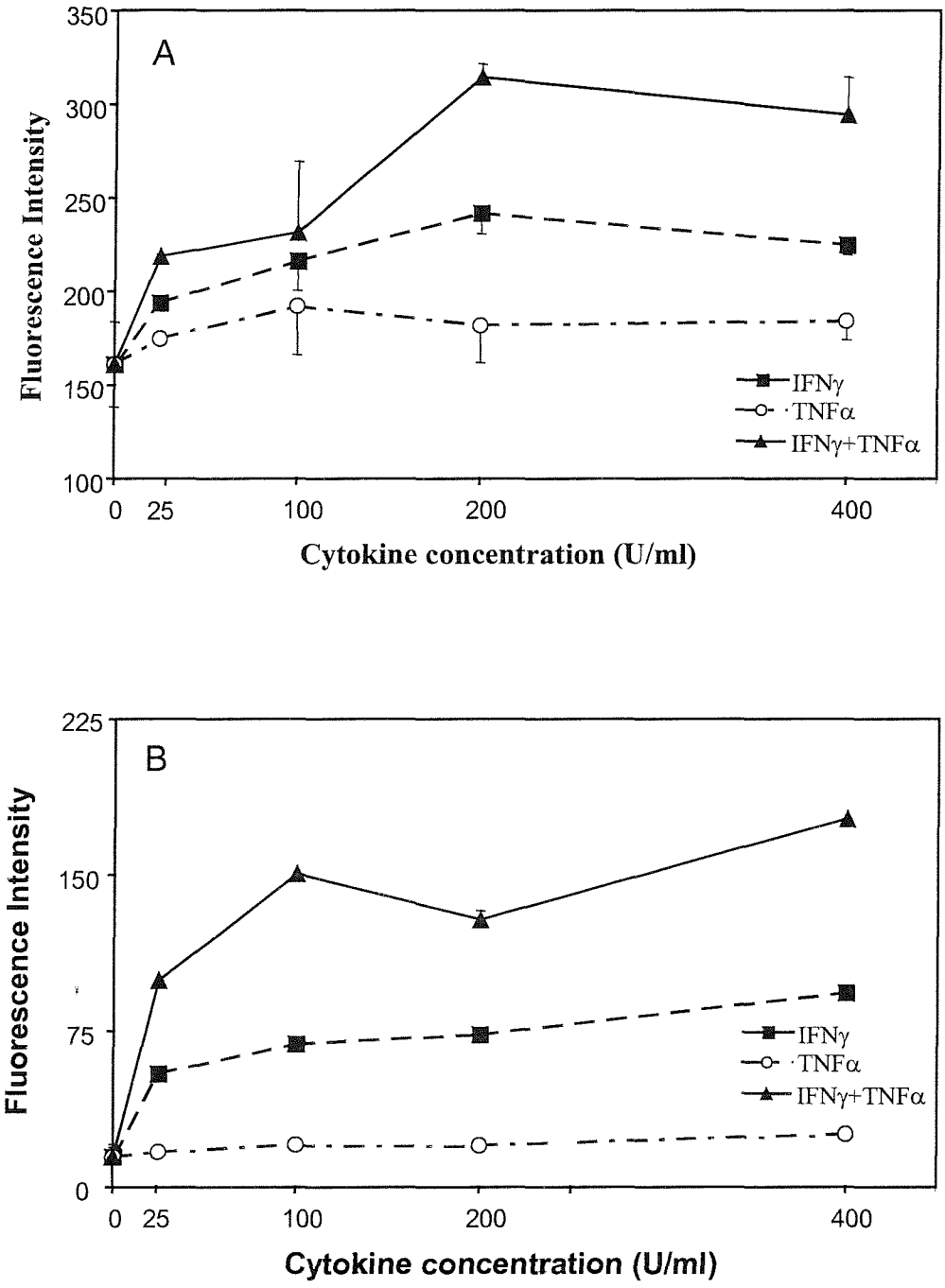


Figure 4-13. Dose response of IFNγ and TNFα on ICAM-1 expression in 16HBE 14o- and H292 cell. IFNγ and TNFα at 200 U/ml increased the expression of ICAM-1 relative to control values on confluent cells by up to 2.0 fold (16HBE 14o-, A) or 10.0 fold (H292, B). When cells were treated with TNFα and IFNγ together, the ICAM-1 expression was increase significantly than IFNγ treated only.

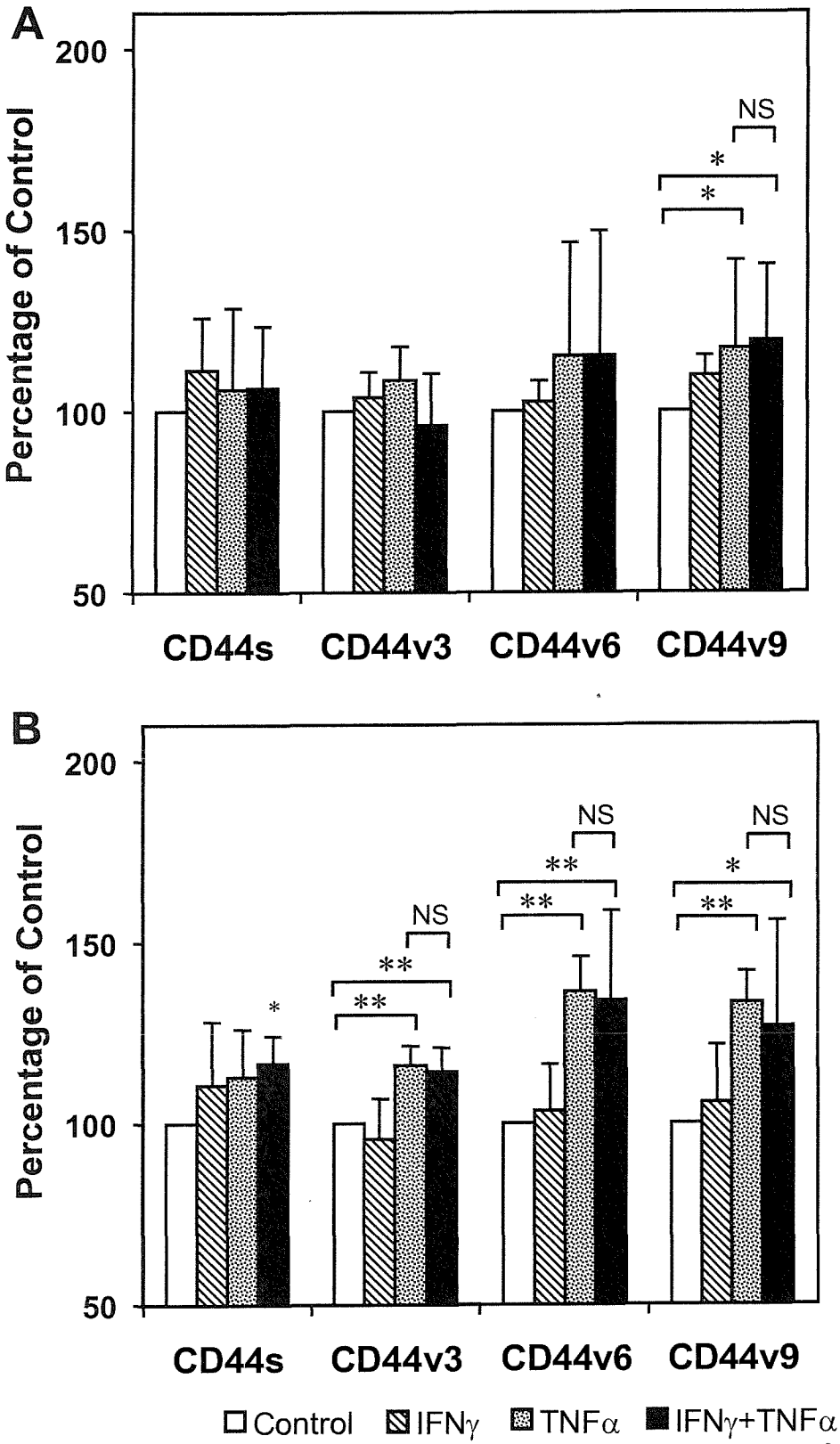


Figure 4-14. IFNγ and TNFα at 200 U/ml increased the expression of CD44 relative to control values on confluent. Values for each antibody were normalised to 100% for untreated cells. Samples differing significantly are marked (* = $p< 0.05$, ** = $p< 0.01$), error bars show standard error of the mean. Results are shown for 16HBE 14o- cells (A) and NCI-H292 cells (B).

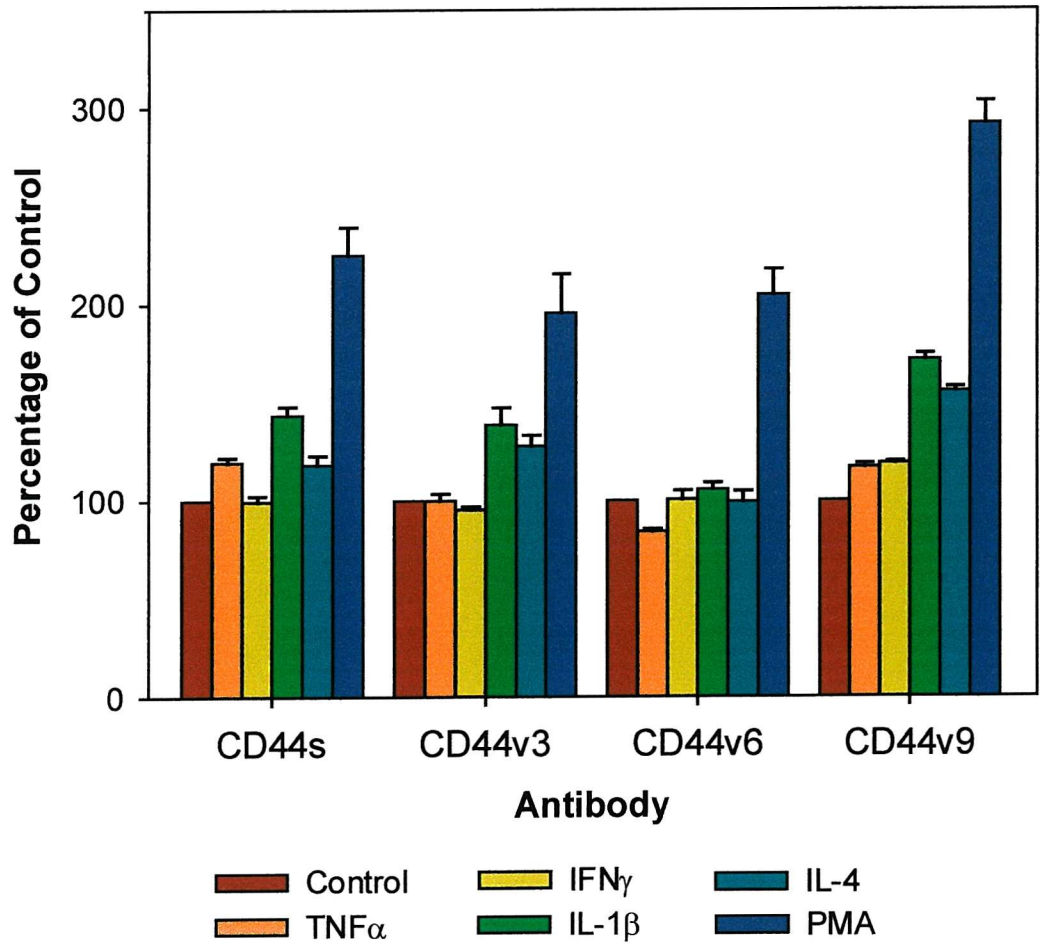


Figure 4-15. Effect of cytokines on CD44 expression. 16HBE 14o$^-$ cells were treated with IFNγ, TNFα (400 U/ml), IL1β or IL-4 (20ng/ml) for 18 hours and stained with antibodies for CD44. Values from flow cytometry analysis for each antibody were normalised to 100% for untreated cells.

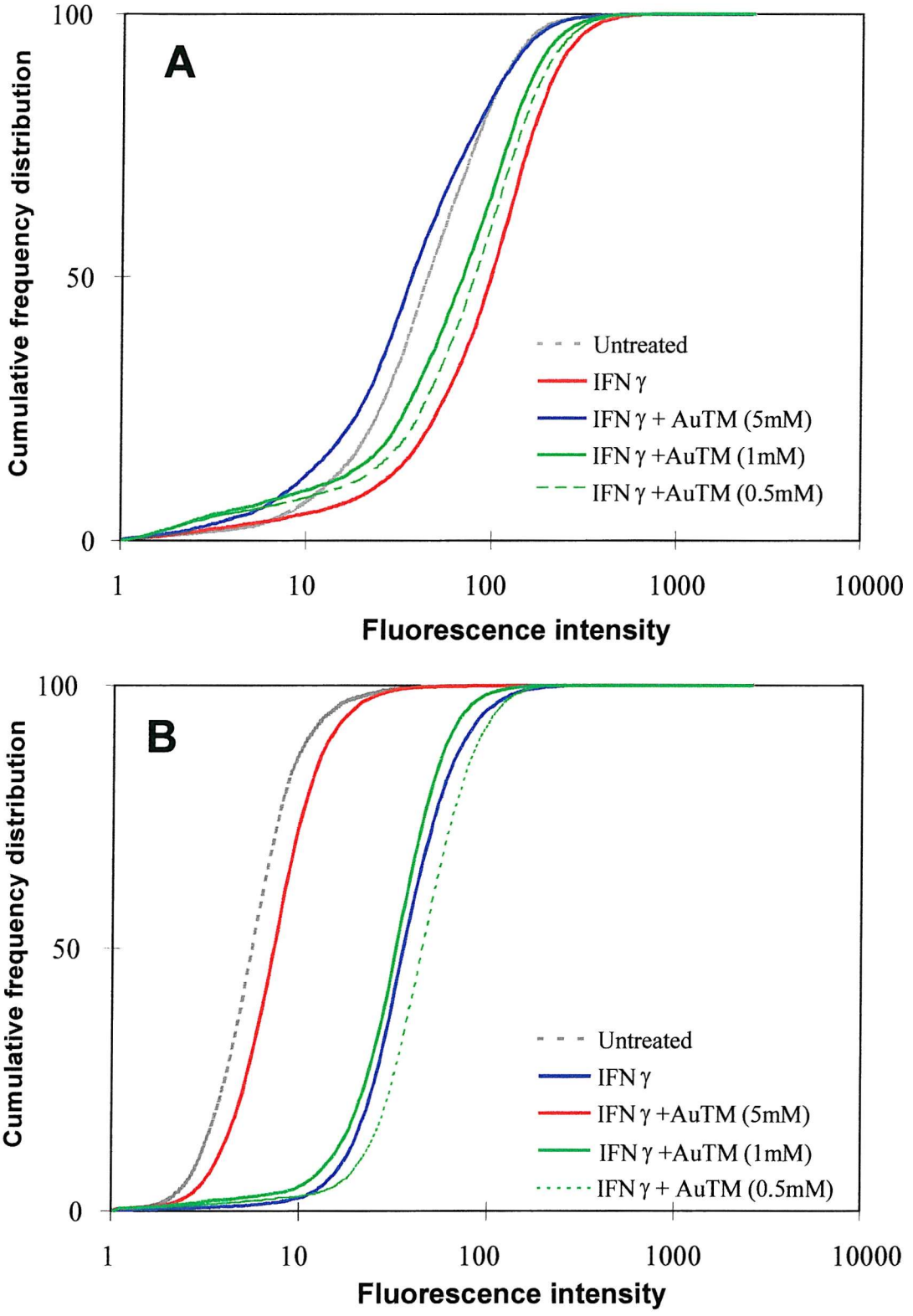


Figure 4-16. AuTM down-regulated IFNγ-induced ICAM-1 expression on 16HBE 14o$^-$ (A) and H292 (B). Cells treated with IFNγ and AuTM for 24 hours. The levels of cell surface ICAM-1 were compared by using flow cytometry.

Chapter 5

CD44 Isoform Expression in Epithelial Cells

5.1 Introduction

CD44 isoform diversity is generated by the incorporation of amino acid sequences encoded by 10 alternatively spliced exons into a membrane proximal position of the extracellular portion (*see section 1.8.2*). Other changes in the extracellular domain of CD44 by post-translational modification, dimerisation in the transmembrane domain, and phosphorlyation in the cytoplasmic domain also contribute to the regulation of the multiple functions of CD44 (Lesley and Hyman, 1998). Alternative splicing of variant exons is thought to be an important pathway in the modification of CD44 functions, since theoretically hundreds of different isoforms can arise from the alternative splicing of 10 variant exons. In most cell types, the most common and widely expressed CD44 isoform is CD44 standard (CD44s) that is coded only by CD44 constant exons. Other CD44 isoforms containing sequences encoded by the variant exons are strictly regulated and restricted in their expression to a limited selection of epithelia and leukocytes (Lesley *et al.*, 1993a; Mackay *et al.*, 1994). Any of the variant exons v2-v10 can be inserted into the human CD44 mRNA by alternative splicing (see *Figure 5-1*), which creates the large family of CD44 isoforms with splice variants which may serve multiple functions (See *Table 1-4*). The complex patterns of tissue-specific expression of CD44 isoforms makes functional studies difficult.

Their varied functions in different cell types reflect the complex patterns of tissue-specific expression of CD44 isoforms, and shows the difficulty in the investigation of their functions.

Much interest and investigative effort has revealed the possible involvement of CD44 variants in tumour metastasis and other disease (Günthert *et al.*, 1995; Naot *et al.*, 1997). Antibody against CD44 v6-encoded epitope mitigated metastasis of an adenocarcinoma cell line in rat (Günthert *et al.*, 1991). Furthermore, transfecting exon v6-containing CD44 into the cells of a non-metastatic clone from the same pancreatic cancer, induced the cells to metastasise. Additionally, the relevance of CD44 variant expression in other disease has been demonstrated in animal models. For instance, antibodies to CD44s interfered with collagen-induced arthritis (Verdrengh *et al.*, 1995;

Brennan *et al.*, 1997); antibody to CD44v7 can be curative in preventing lethal colitis in mice (Wittig *et al.*, 1998); while antibodies to CD44s, CD44v6 and CD44v10 inhibited delayed-type hypersensitivity reaction (Camp *et al.*, 1993; Weiss *et al.*, 1997; Rosel *et al.*, 1997). Apart from the studies in tumour metastasis and lymphocyte activation, the functions of CD44 variants are less well defined in other cell types. The role of CD44 isoforms in physiological processes and diseases of other epithelial cells is therefore interesting to investigate.

Early embryo differentiation during blastocyst formation provides a model for investigating epithelium development, such as epithelial transport and polarisation. Previous studies on mouse and human embryos by RT-PCR showed that CD44 isoforms encoded by numerous variants were expressed during embryo development (Behzad *et al.*, 1994; Ruiz *et al.*, 1995). However, the expression of CD44 isoforms was unclear in epithelial cells of early embryos. Studying CD44 isoform expression in embryonic epithelial cells may help us to understand the importance of CD44 in epithelial differentiation and pattern formation.

CD44 is expressed in both embryonic and extraembryonic tissue (Terpe *et al.*, 1994). The formation of epithelial tissues is a major aspect of embryonic morphogensis. In embryogenesis, the expression of CD44 variants is restricted and is especially seen in instructive epithelia which control outgrowth of tissue of mesenchymal origin (Wainwright *et al.*, 1996; Yu *et al.*, 1996; Yu and Toole, 1997; Sherman *et al.*, 1998). The importance of CD44 variants has been demonstrated in the transplantation of the apical ectodermal ridge of the embryo, in which tissue treated with CD44v3 or CD44v6 specific antibody inhibited bud formation (Wainwright *et al.*, 1996; Sherman *et al.*, 1998) suggesting that CD44 is required during embryo development.

Studies of CD44 variants have shown that the presence of certain variant exons can be associated with different cell functions (*Table 1-4*). Immunohistochemistry only shows whether a certain variant protein was expressed in a specific cell type. It is not able to indentify how many isoforms are expressed by one cell. My interest in CD44 as a protein that might be important in the functions of airway epithelial cells was prompted by the following: Firstly, individual cells might express different

combinations of CD44 isoforms. Since CD44 mediates cell-cell and cell-substrate interactions, the expression of CD44 isoforms in one cell type may be different in another subset of cells. Thus, the expression patterns of CD44 isoforms might be different between columnar and basal bronchial epithelial cells. Secondly, cell detachment and CD44 up-regulation are found in the bronchial epithelium of asthmatic (Lackie *et al.*, 1997). The splicing patterns of CD44 in normal and damaged/stimulated cells might be different.

Using the MAP-PCR technique which can detect the mRNA expression in a small number of cells (Collins and Fleming, 1995b), the expression of CD44 isoforms in pre-implantation mouse embryos and human bronchial epithelial cells was studied. With this technique, I have demonstrated that the CD44 isoforms expressed at different stages of the early mouse embryo was altered. My results indicate that CD44 isoform expression and regulation may be important in the epithelia of early embryos. The human columnar and basal epithelial cells from bronchial brushings also showed different CD44 isoform expression. By using techniques based on RT-PCR, cloning and sequencing, I have investigated the change of CD44 isoforms after cell damage and proinflammatory cytokine treatment. This study may help us to understand which CD44 isoforms are expressed in bronchial epithelial cells, and how are they regulated during epithelial repair. These results may provide another approach for further studies of their function.

5.2 Results

5.2.1 Optimising the PCR conditions

In this study, when the primers- CD44*h* and CD44*i* were used, the expected smallest product size of PCR product (for CD44s cDNA) was 347 base pairs (bp). On the other hand, the expected smallest product size of PCR product of CD44 cDNA was 220bp when the primers- CD44*j* and CD44*k* were used (*Figure 5-2*). For MAP-PCR, as a small amount mRNA was harvested on the MAP, it was necessary to increase the amplification by using the nested PCR protocol. Using primer CD44*h* and CD44*i* followed by primer CD44*j* and CD44*k*, the 347bp cDNA product was not detectable in first PCR amplification, and only the 220bp cDNA product of the secondary PCR amplification was seen.

The optimum conditions for the conventional PCR reaction was tested by using 1 μg of total mRNA extracted from mouse 3T3 fibroblasts. For each pair of primer, the annealing temperatures of 50, 55, 60 and 65°C and the Mg^{2+} concentration of 1.0, 1.25, 1.5 and 2.0 mM were compared for maxium amount of PCR products. The final annealing temperature and Mg^{2+} concentration were selected described in *Materials and Methods* (*Section 2.7.3*).

5.2.2 CD44 isoform expression in mouse lung tissue and fibroblasts

The RT-PCR results showed that there were at least four CD44 isoforms expressed in mouse 3T3 fibroblasts (*Figure 5-3*). Based on the calculation of the product size, the 220 bp PCR product corresponded to CD44 standard (CD44s) encoded by constant exons of CD44 without other variant exons inserted. Other products at the sizes of ~280, 360, and 640 bp were isoforms with variant exon(s) encoded sequences inserted. In addition, I examined the expression of CD44 isoforms in mouse lung tissue, which showed a similar pattern to 3T3 cells (*Figure 5-3*).

5.2.3 Sensitivity of MAP PCR

Small numbers of mouse fibroblast 3T3 cells were placed on MAP and processed for RT-PCR to test the sensitivity of MAP-PCR for CD44. mRNA prepared by using 5-

20 cells on MAP was compared with 1μg total RNA by conventional PCR, which was extracted from the same cell line. The results shown in *Figure 5-4* demonstrated that the signal from 5-20 cells on MAP was comparable with conventional PCR. Most of the samples with MAP-PCR distinctly showed 4 major CD44 isoforms (220, 280, 360, and 640 bp) in the same pattern as 1 μg total RNA by conventional PCR.

5.2.4 CD44 isoform expression in mouse embryos

To understand how CD44 alternative splicing is regulated in embryogenesis, early mouse embryos at the blastocyst stage were selected to compare their expression and alternative splicing of CD44 variants. When single pre-implantation mouse embryos at the blastocyst stage (32/64 cells or more, day 3.5 ~ 4.5) were used, no CD44 cDNA product was found in any of the 15 samples with single embryos. The possibility that the PCR conditions were incorrect was ruled out, because the positive control with β-actin primers showed the predicted cDNA product (*Figure 5-5*). By placing 5 blastocysts on a MAP, 4 of 8 samples showed weak CD44 cDNA products on the gel. The signal was not significantly increased by using 10 embryos at the blastocyst stage, and only 4 of 7 samples showed positive result (*Table 5-1*). In the samples with CD44 isoform expression, the 220bp CD44s was the major band, however, the expression was very weak. Two faint bands running at ~ 320bp and 500bp were also seen (*Figure 5-6*).

Campbell and co-workers (Campbell *et al.*, 1995a) demonstrated that CD44 protein was highly expressed at the 1-8 cell stage in human pre-implantation embryos, and down-regulated during trophoblast differentiation. I therefore examined the alternative splicing of CD44 in the embryo stages earlier than the blastocyst stage. The patterns of splicing were also examined by using embryos at different stages. Results showed that increase of CD44 expression and specific CD44 splice variants were observed at the 2-cell (*Figure 5-7*) and 8-cell embryo stage. *Table 5-1* summarises the results of CD44 PCR from the 2-cell, 8-cell and blastocyst embryo stages. These results showed that embryos at the 2-cell stage expressed more CD44 mRNA, and the expression of specific CD44 splice variants was more variable. Larger sizes (from 300 to 850bp) of CD44 cDNA products were seen and the signal

was strong. Unlike embryos in the 2-cell stage which expressed more larger CD44 (CD44 isoforms contain variants) mRNA, the major CD44 product in the blastocyst and 8-cell stage was 220bp (CD44s). Some minor products at 300, 500 and 550bp were seen occasionally. Interestingly, in the early embyros some samples only expressed the large products of CD44 isoforms without the 220bp (CD44s) product.

Table 5-1. The ratio of positive results of CD44 MAP PCR in mouse embryos at different stages.

Embryo stage	Blastocyst	8-cell	2-cell
Number of embyro(s)			
1	0/15 (0 %)	--	1/2 (50%)
5	4/8 (50%)	2/10 (20%)	4/5 (80%)
10	4/7 (57%)	2/8 (25%)	3/4(75%)

-- no data

Samples were prepared by immobilized embryo mRNA on MAP. PCR was processed as described in *section 2.7.4* with primers CD44*j* and CD44*k* for the second PCR.

5.2.5 Analysis of CD44 present in human bronchial epithelial cell lines

The primers (CD44*j* and CD44*k*) used for the second PCR of mouse samples had a 2-3 bp mismatch for human CD44 mRNA. For best results with human CD44, human optimised primers (CD44*j2* and CD44*k2*) were used for human samples.

Using primary bronchial epithelial cells and two human bronchial epithelial cell lines, 16HBE 14o$^-$ and NCI-H292, expression of CD44 in human bronchial cells were analysed using the techniques developed in the previous section. Total RNA was isolated from 16HBE 14o$^-$, NCI-H292 and primary bronchial epithelial cells. The RNA was reverse transcribed, followed by PCR with the same nested PCR method used for mouse RT-PCR using human optimised primers. The results showed that NCI-H292 expressed three major bands between 200-300bp, whereas 16HBE 14o$^-$ expressed another two bands between the sizes of 500 and 600bp (*Figure 5-8*). The PCR products from 16HBE 14o$^-$ and NCI-H292 cells showed a similar size of CD44

PCR product between 200-500 bp, however, the predominant CD44 isoform in NCI-H292 is different from 16HBE 14o- bronchial epithelial cells.

5.2.6 CD44 mRNA in primary human bronchial epithelial cells

In primary bronchial epithelial cells, the 220-bp PCR product corresponding to the coding region of CD44s was the predominant mRNA isoform species (*Figure 5-9*). With 10 cells on the MAP, all the 8 MAP samples tested showed a positive CD44 band using PCR. Using only one cell, the CD44 mRNA was still detectable some samples, although some samples (50%) did not show any detectable signal (*Table 5-2*). There were also some minor bands seen at 300-400 and ~600bp in both 10 cells and 1 cell samples (*Figure 5-9*). No signal was seen in the control samples taken from the medium of cell suspensions (not shown) .

Table 5-2. Positive results for CD44 MAP PCR in primary bronchial epithelial cells

Number of cell(s) per sample	Positive ratio (Samples postive/total)
10	8/8 (100%)
1	4/8 (50%)

Samples were prepared by immobilizing epithelial mRNA on MAP. PCR was carried out as described in s*ection 2.7.4* with human optimised primers CD44*j2* and CD44*k2* in second PCR.

5.2.7 CD44 in bronchial epithelial cells from airway brushing

The predominant cell type in samples from airway bushing was columnar epithelial cell. Columnar cells could be easily identified under the microscope and picked out for MAP-PCR, greatly reducing the possibility that basal cells were included in the sample. With MAP-PCR only CD44s mRNA (220bp) were seen in the positive results (*Figure 5-10)*. Using one cell, no CD44 signal was found in any sample (n=7). Only 10% of samples with 5 cells expressed CD44s (*Table 5-3*).

Table 5-3. Positive result of CD44 MAP PCR in brushed bronchial columnar epithelial cells

Number of cell(s) per sample	Positive ratio (Samples postive/total)
15*	8/10 (80%)
5*	1/10 (10%)
1	0/7 (0%)

PCR was performed as described in *section 2.7.4* with human optimised primers CD44*j2* and CD44*k2* for the second PCR. * indicates samples may contain basal cells.

5.2.8 Cloning and sequencing of CD44 cDNA

CD44-pGFP was constructed by fusion of the pGFP vector with CD44 cDNA. pGFP vector was digested by *Hpa I* enzyme, followed by dephosphorylation with Calf Intestinal Alkaline phosphatase to remove the phosphate group from 5'-end to prevent self-ligation of the vector and increase the efficiency of CD44 cDNA fusion with pGFP vector. In this system, CD44 cDNA was inserted immediately upstream (788) of GFP fluorescent chromophore gene (805-813) in the pGFP vector. *E. coli* (JM101) was used as the host to grow up CD44-pGFP for cloning, and the ampicillin resistance gene in the pGFP vector allowed transformed *E. coli* cells to be selected on the basis of their resistance to ampicillin. Analysis of pGFP expression allowed retrieval of a higher number of CD44 insert positive clones compared with reliance on ampicillin resistance alone. 10-30% of resistant clones expressed green fluorescence and analysis of the GFP positive transformed clones showed more than 90% (52 of 55 clones) encoded CD44 cDNA sequences. CD44s was the major isoform presenting in 16HBE 14o$^-$ cell clones. *Table 5-4* shows the CD44 cDNA sequences from cloning. Results from sequencing these clones showed that the predominant isoforms were CD44s, v8-v9-v10 and v8-v9 isoforms (*Figure 5-11*). Other variant isoforms, v6-v7-v8, v6-v7-v8-v9, v3 and v8, were less frequently seen in the CD44 PCR product (*Table 5-4*). No large isoforms (more than 700 bp) were cloned in this system.

Table 5-4. Cloned CD44 cDNA sequence.

Product	Start-end (variant exon only)	Combination	Size (bp)	Percentage of match	Frequency (n=52)
1		nil (CD44s)	217	85-95%	51.9%
2	1420-1789	v8-v9-v10	613	89-98%	26.9%
3	1414-1606	v8-v9	409	93-98%	11.5%
4	1184-1478	v6-v7-v8	580	97%	3.8%
5	1187-1569	v6-v7-v8-v9	670	93%	1.9%
6	813-922	v3	343	94%	1.9%
7	1414-1515	v8	319	99%	1.9%

Sequences were aligned and analysed the match by "Blast 2 Sequences" (http://www.ncbi.nlm.nih.gov/gorf/bl2.html)

5.2.9 Isoform identification using exon-specific PCR primers

An alternative to sequencing to help identify which CD44 exons were transcribed was to use exon-specific primers. With this method, isoforms containing v3 and v6 variant exons can be identified using exon specific primers and comparing with the anticipated size of CD44 cDNA products. *Table 5-5* shows the size of CD44 PCR products which were amplified by exon specific primers and their size measured from their different mobility in agarose gels. The sizes of the possible CD44 isoforms calculated from the variant exon sequences are listed in *Table 5-6*. In this method, four isoforms were found, including isoforms containing v3-v4-v5-v6-v7-v8-v9-v10, v6-v7-v8-v9-v10 and v3-v4-v5-v6 (*Table 5-6*). There was also a ~420 bp isoform contain v3 and v10. However, this method can only partially identify the variant composition of the isoforms. If there were two products encoded with different variant exons but of an identical (*Table 5-6*, markers) or similar size, then this method can not deduce their sequences. For example, by using v3-v10 primers the variant composition of the 420 bp CD44 product was difficult to identify (compare *Table 5-5* and *Table 5-6*).

5.2.10 Epithelial damage and CD44 expression

The alternative spliced pattern of CD44 mRNA in normal and damaged epithelial cells was compared by using total RNA extracted from undamaged confluent 16HBE 14o$^-$ cells or mechanical damaged cells (*section 2.5.6*). By using exon specific primers, there was a small decrease of CD44v3 and v6 containing isoforms in damaged cells. Nested PCR with human optimised primers (CD44*j2*-CD44*k2*) from constant exons 5 and 15 showed there was a decrease in the CD44 product band at ~600 bp. Based on previous sequencing of 600 bp product, this is likely to represent isoforms v8-v9-v10 or v6-v7-v8 (*Figure 5-12*). The two larger isoforms between 600-900 bp were also decreased. Interestingly, there is *de novo* synthesis or increased expression of an isoform with a product size between 220-300 bp (*Figure 5-12*).

Table 5-5. Sizes (bp) of CD44 cDNA with exon-specific primers

Primers	Calculated size	Possible composition
v3-v6		
	~ 450	v3-v4-v5-v6
v3-v10		
	~ 900	v3-v4-v5-v6-v7-v8-v9-v10
	~ 420	v3-?-v10
v6-v10		
	~550	v6-v7-v8-v9-v10

Primers CD44*h* and CD44*i* were used in the first PCR. In the second PCR, exon-specific primers (*Table 2-2*) were used to amplify the CD44 cDNA. The possible composition of CD44 isoforms was obtained from calculation and comparison with *Table 5-6*.

5.2.11 Cytokines alter the expression of CD44 isoforms

As seen in epithelial damage, CD44v8-v9-v10 isoform (~ 600 bp) mRNA amplicons were also decreased in TNFα treated 16HBE 14o$^-$ cells (*Figure 5-13*). In addition, in a preliminary experiment by using exon-specific primers, isoforms containing v3 variant but not v6 variant was little decreased after cytokine treatment (not shown).

Table. 5-6. Examples of some possible CD44 isoforms by exon specific primers and their calculated sizes

Primers / Possible composition	calculated sizes	Primers / Possible composition	calculated sizes
v3-v10			
v3-v4-v5-v6-v7-v8-v9-v10	869	v3-v4-v5-v10	416¶
v3-v5-v6-v7-v8-v9-v10	755	v3-v4-v6-v10	428
v3-v4-v6-v7-v8-v9-v10	752	v3-v4-v7-v10	431
v3-v4-v5-v7-v8-v9-v10	740	v3-v4-v8-v10	401
v3-v4-v5-v6-v8-v9-v10	737	v3-v4-v9-v10	389
v3-v4-v5-v6-v7-v9-v10	767	v3-v5-v6-v10	431
v3-v4-v5-v6-v7-v8-v10	779	v3-v5-v7-v10	434
v3-v6-v7-v8-v9-v10	638*	v3-v5-v8-v10	404@
v3-v4-v7-v8-v9-v10	632	v3-v5-v9-v10	392
v3-v4-v5-v8-v9-v10	608	v3-v6-v7-v10	446
v3-v4-v5-v6-v9-v10	635	v3-v6-v8-v10	416¶
v3-v4-v5-v6-v7-v10	677	v3-v6-v9-v10	404@
v3-v5-v7-v8-v9-v10	626	v3-v7-v8-v10	419
v3-v5-v6-v8-v9-v10	623	v3-v7-v8-v10	407
v3-v5-v6-v7-v9-v10	653	v3-v7-v8-v10	377
v3-v5-v6-v7-v8-v10	665		
v3-v4-v6-v8-v9-v10	620	**v3-v6**	
v3-v4-v6-v7-v9-v10	650**	v3-v4-v5-v6	445
v3-v4-v6-v7-v8-v10	662	v3-v4-v6	328
v3-v4-v5-v7-v9-v10	638*	v3-v5-v6	231
v3-v4-v5-v7-v8-v10	650**		
v3-v4-v5-v6-v8-v10	647	**v6-v10**	
v3-v4-v5-v6-v10	545	v6-v10	
v3-v5-v6-v7-v10	563	v6-v7-v8-v9-v10	512
v3-v6-v7-v8-v10	548†	v6-v7-v8-v10	422
v3-v7-v8-v9-v10	509	v6-v7-v9-v10	410
v3-v6-v8-v9-v10	506	v6-v8-v9-v10	380
v3-v6-v7-v9-v10	536	v6-v7-v10	320
v3-v6-v7-v8-v10	548†	v6-v8-v10	280
v3-v4-v7-v9-v10	521•	v6-v9-v10	278
v3-v4-v7-v8-v10	533		
v3-v4-v5-v8-v10	518§		
v3-v5-v8-v9-v10	494		
v3-v4-v5-v8-v10	518§		
v3-v5-v6-v9-v10	521•		
v3-v4-v6-v9-v10	518§		
v3-v5-v6-v7-v10	563		

Markers (*, **, •, §, †, ¶, @) identify the same size of different isoforms with the same predicated product size.

5.3 Discussion

5.3.1 CD44 isoform expression in early embryo

Many studies have indicated that during embryonic development the extra cellular matrix surrounding migratory and proliferating cells is enriched in HA. Since the concentration of HA decreases during subsequent differentiation, HA has been thought to be involved in pattern formation and the differentiation of embryonic cavities (Toole *et al.*, 1989; Brown and Papaioannou, 1993; Fenderson *et al.*, 1993). The removal of HA has been indicated as a critical stage of morphogensis, and CD44 appears to mediate HA endocytosis (Underhill *et al.*, 1993; Underhill, 1993; Pavasant *et al.*, 1994). Most of these studies of CD44 in embryogenesis have focused on investigating this role in the embryo after implantation. Less is known about the expression of CD44 isoforms in the pre-implantation embryo. Campbell and co-workers (Campbell *et al.*, 1995a; Campbell *et al.*, 1995b) have reported that several integrins (α3, αV, β1, β3, β4 and β5) were consistently expressed throughout pre-implantation development, whereas CD44, E-cadherin, ICAM-1, NCAM, VCAM-1, and L-selectin were decreased after the 8-cell or morular stage of the embryo. The decrease of these surface adhesion molecules suggested that their regulation might play a role in pre-implantation embryo development.

The study of CD44 in pre-implantation embryos showed that CD44 immunoreactivity was highly expressed in mouse embryos during the 1 to 8 cells and morula stage, but only weakly in mouse blastocysts by immunostaining (Campbell *et al.*, 1995a). However, it remains unclear whether CD44 variant isoforms are expressed and/or how they are regulated during this stage. In my study, results from MAP-PCR indicate that the expression of CD44 isoforms was not consistent between the morula and blastocyst stage. In agreement with the results of Cambell's study, my PCR results showed that there is a corresponding change in the expression of CD44 depending on the stage of the embryos. Not all pre-implantation embryos expressed CD44 after the morula stage, whereas, embryos in the 2-cell to 8-cell stage highly expressed CD44. The amount of CD44 mRNA from down to one embryo in the 2-cells stage can be detected, which revealed that this technique is very sensitive, and can helped to

overcome problems of the detection for determination of CD44 isoform expression pattern of single cells.

More large CD44 isoforms were expressed in the 2-cells stage embryo. In addition, some embryos only expressed the large isoforms without CD44s isoform expression. Three of these larger isoforms (850 bp, 750 bp, and 650 bp) were not detected in the embryos of later stages. This stage-dependent large CD44 isoform expression might indicate that these isoforms are important in this period. The physiological significance of these larger CD44 isoforms still remains undefined, and confirmation that these mRNA species are translated into protein is also needed.

CD44 is expressed in both embryonic and extraembryonic tissue from the onset of embryogenesis (Terpe *et al.*, 1994), and is restricted to instructive epithelia (Wainwright *et al.*, 1996; Yu *et al.*, 1996; Yu and Toole, 1997; Sherman *et al.*, 1998). The re-expressed CD44 splice variant in the post implantation embryo has been shown to be crucial for the outgrowth of limbs and the proliferation of mesenchymal cells (Wainwright *et al.*, 1996; Sherman *et al.*, 1998). During oocyte maturation, HA is present in the area of cumulus cell-oocyte complex (Dandekar *et al.*, 1992). Tirone and co-workers reported that the synthesis of HA by cumulus cells is regulated by soluble factor(s) produced by oocytes (Tirone *et al.*, 1993). Ohta and co-workers also reported that expression of CD44 on cumulus cells is correlated to oocyte maturation (Ohta *et al.*, 1999). Whether regulation of oocyte maturation is related to interaction with HA synthesised by cumulus cells and CD44 on both cumulus cells and oocyte or not remains to be defined.

Campbell has demonstrated that CD44 was highly expressed in embryo before the 8-cell stage (Campbell *et al.*, 1995a) and suggested that CD44 may be involved in homotypic or heteotypic adhesion before implantation. My results suggest that several alternatively spliced CD44 isoforms may play a role in adhesion as the mRNA for large CD44 isoforms appeared to be highly expressed in this stage. To my knowledge, no other cell type has been shown to express only larger CD44 isoforms which contain variant exons. Further information of the function of CD44 variants

may be forthcoming from investigation of the roles of these large CD44 isoforms in early embryos.

5.3.2 Major CD44 isoforms in bronchial epithelial cells

The low efficiency (10-30%) of GFP transformation as compared to the maximum of 60% suggested by manufacture may be due to: (1) some G-C rich cDNA can induce deletion of inserted cDNA and thus loss of the adjacent GFP gene, whilst still retaining the ampicillin resistance gene; (2) some clones may express low copy numbers of pGFP or exhibit slow formation of the GFP chromophore which is below the limit of detection; or (3) down-regulation of GFP gene that leads to a low GFP positive transformation. My results showed that approximately 95% of GFP positive clones contain CD44 products. From sequencing, the two major PCR products at 220bp and 600bp are CD44s and CD44v8-v9-v10. The minor products were CD44v8-v9, CD44v6-v7-v8, CD44v6-v7-v8-v9, CD44v3 and CD44v8 which were identified by cloning and sequence analysis.

With v3- and v6-specific primers, 3 more isoforms were also seen. There were less than 10 different sizes of CD44 PCR products seen on the gel and no two isoforms of the same size were found in the size range of 200-600 bp. Recently Lockhart (Lockhart *et al.*, 1999) has demonstrated a method using alternative exon-specific PCR amplification without radioactive primers, which showed similar sensitivity but required less time to perform. With this technique, it might be possible to determine which of the CD44 isoforms bronchial epithelial cells expressed. Perhaps, this method can help to sequence these large CD44 isoforms. However, it would be even more important to know whether these isoforms have any physiological function. I have studied CD44 isoform function during bronchial epithelial cell damage and repair. This is described in the next chapter.

5.3.3 Some columnar epithelial cells do not express CD44 as detected by MAP-PCR

CD44 expression between individual cells might differ depending on environmental factors. Using this single cell PCR technique, it was shown to be possible to study the exon composition of CD44 between different subsets of airway epithelial cells. When carefully selected single columnar cell were processed on CD44 MAP-PCR, CD44 product was not seen in any sample. However, samples containing basal cells always expressed a CD44 product. This is in agreement with my study of the expression of CD44 variant protein in 16HBE $14o^-$ cells on filter inserts and epithelium of human bronchial tissue, which showed that CD44 is preferentially expressed basally in epithelium (See *chapter 3*).

The possibility of false-positive results due to the pipette picking up more than one cell can be eliminated by picking up the cell in very dilute cell suspension, and also by comparing negative control samples taken from the nearby media. The positive control, reactions in single cell with primers for β-actin gave positive results. It is unlikely that the epithelial cells were lost during the PCR processes, and more likely that the columnar and some primary bronchial epithelial cells do not express CD44. In addition, MAP-PCR results for mRNA from one cell showed individual cells expressed both CD44s and also several variant-containing isoforms. This indicates that individual cell can express several CD44 isoforms.

5.3.4 The CD44 isoform expression are changed after epithelial damage and cytokine treatment

The decrease of v8-v9-v10 isoform after epithelial damage and inflammatory cytokine treatment suggests that this isoform may be not involved in the epithelial repair process. The small decrease of v6-v7-v8-v9-v10, and a v3-containing isoforms seen in cells with TNFα treatment suggest these cytokines do not affect alternative splicing of CD44. However, the change in mRNA level might be not correlated to its protein expression.

The result in the previous chapter showed that CD44 expression is altered and CD44 v3 and v6 isoforms are up-regulated after cytokine stimulation and cell damage. However, antibody staining of CD44 proteins fails to disclose the actual exon combinations in the CD44 isoforms. It is possible that certain variant exons combination have specific and different functions. RT-PCR followed by hybridisation gives reliable results, but is time-consuming. In my study, RT-PCR followed by exon-specific amplification and direct sequence gave an alternative method to determine what variant exons are contained in the isoform sequence. Furthermore, with the study in single cell MAP-PCR I have shown that (1) most of columnar bronchial epithelial cells do not express CD44, (2) single cells can express more than one CD44 isoforms, (3) v8-v9-v10 is the predominate alternatively spliced isoform following cell damage.

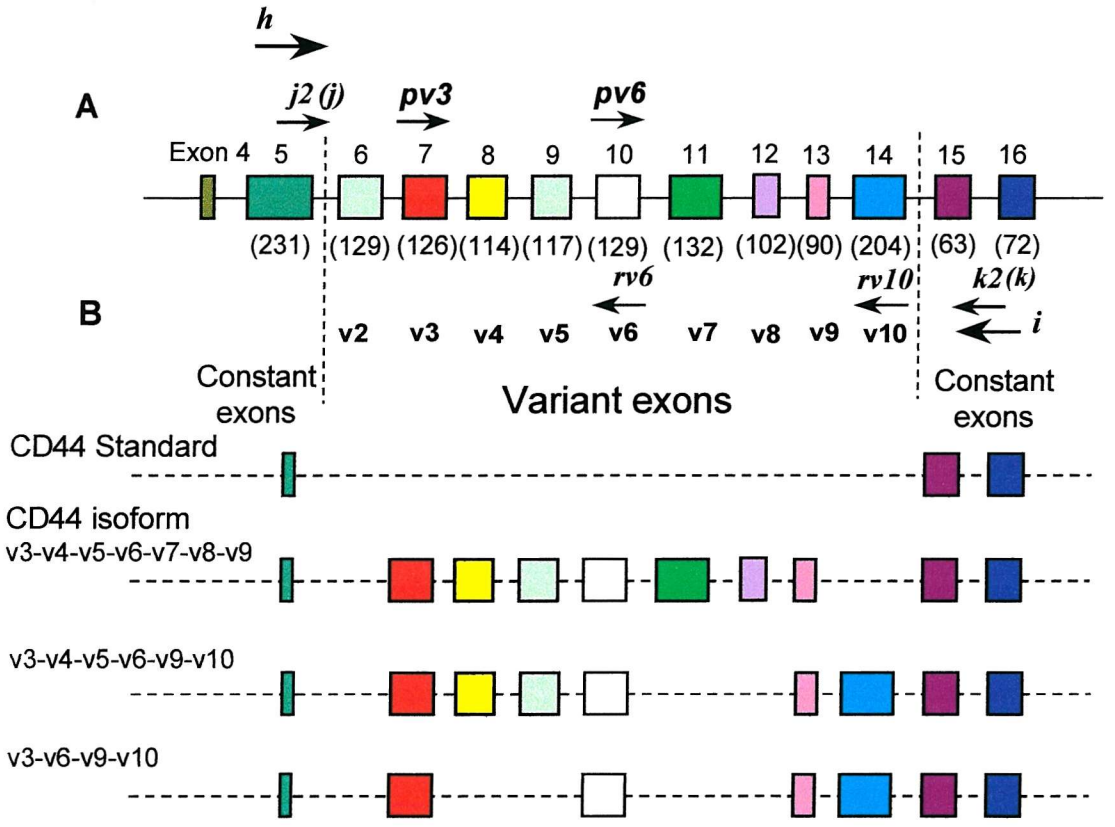


Figure 5-1. The expression patterns of standard and variant human CD44 molecules. The primers used in this exon-specific RT-PCR for detection the combination of CD44 isoforms are shown their binding sites in the CD44 variant exons (A). Primers *h* and *i* were used for the first PCR in both mouse and human samples. In the second PCR primers *j* and *k* were used in mouse samples, and the human optimised primers *j2* and *k2* were used for human samples for best results. Examples of the different combinations of CD44 isoforms which only contain certain variant exons inserted (B).

Figure 5-2. Sequence of human CD44 exons 1-16. Characters labelled with different colours corresponding to the start of exons labelled in the same colour. Primers used in first and second PCR are coloured green or red respectively and underlined.

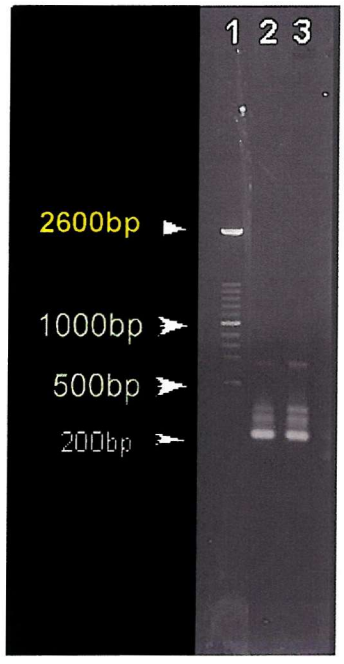


Figure 5-3. CD44*j-k* primers amplified products of the mouse CD44 mRNA. CD44 PCR products in agarose gel were stained with ethidium bromide and visibilized on UV transilluminator showing the mouse lung total RNA (lane 2) and 3T3 fibroblast cell line total RNA (lane 3). Lane 1 show molecular mass marker (DNA molecular weight marker XIV, Boeheringer Mannheim) which contains 100 bp ladder in the range of 100-1500bp with high intensity bands at 500 and 1000 and an addition band at 2600 bp. The major amplification product of the CD44 mRNA species in mouse lung and 3T3 cell is at 220bp corresponding to CD44s in which no variant exons are included in the CD44 mRNA. Both samples also show the products of three minor splicing variants at ~280, 360 and 640bp which are isoforms containing sequences from variant exons. Samples were prepared and carried out conventional RT-PCR as described in ***Materials and Methods (section 2.7.3)***.

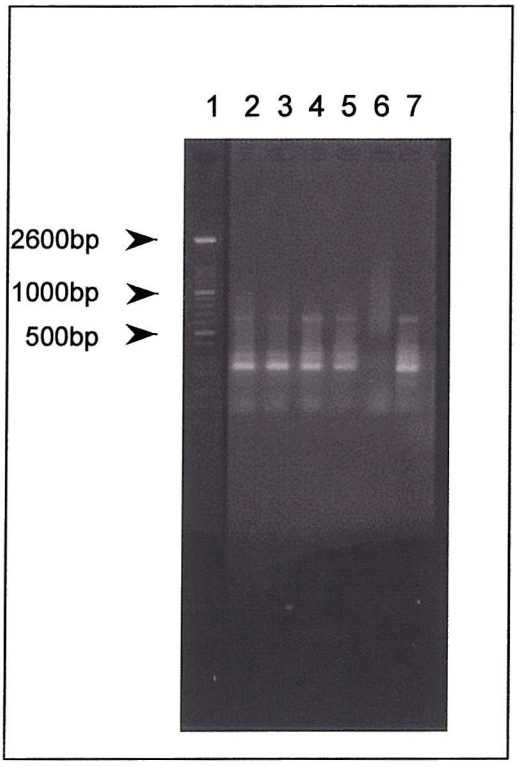


Figure 5-4. Sensitivity test for MAP PCR. The 3T3 mouse fibroblasts (~5-20 cells) were placed on MAP. Following lysis and washing as described in ***Materials and Methods*** (***section 2.7.4)***, the mRNA of 3T3 fibroblasts bound on poly(U)-rich MAP was reverse transcribed and nested PCR performed. One microgram total RNA extracted from the same cell line was used for comparing the sensitivity of PCR amplification. The mRNA from 5-20 fibroblast cells (lane 2-5) shows similar CD44 PCR amplification products to 1μg total RNA by conventional PCR (lane 7). Lane 1 shows the molecular marker as describe in *Figure 5-3*. Lane 6 shows the negative control in which MAP without cells was processed using the same lysis, wash and RT-PCR procedures as other samples. PCR was performed as describe in ***Materials and Methods*** (***2.7.3 and 2.7.4***) with primers *j* and *k* for the second PCR. This shows products at 220 bp the same size as products confirmed to be CD44s by sequencing (*section 5.2.8*). Other larger products are presumed to be CD44 isoforms containing sequences from variant exons.

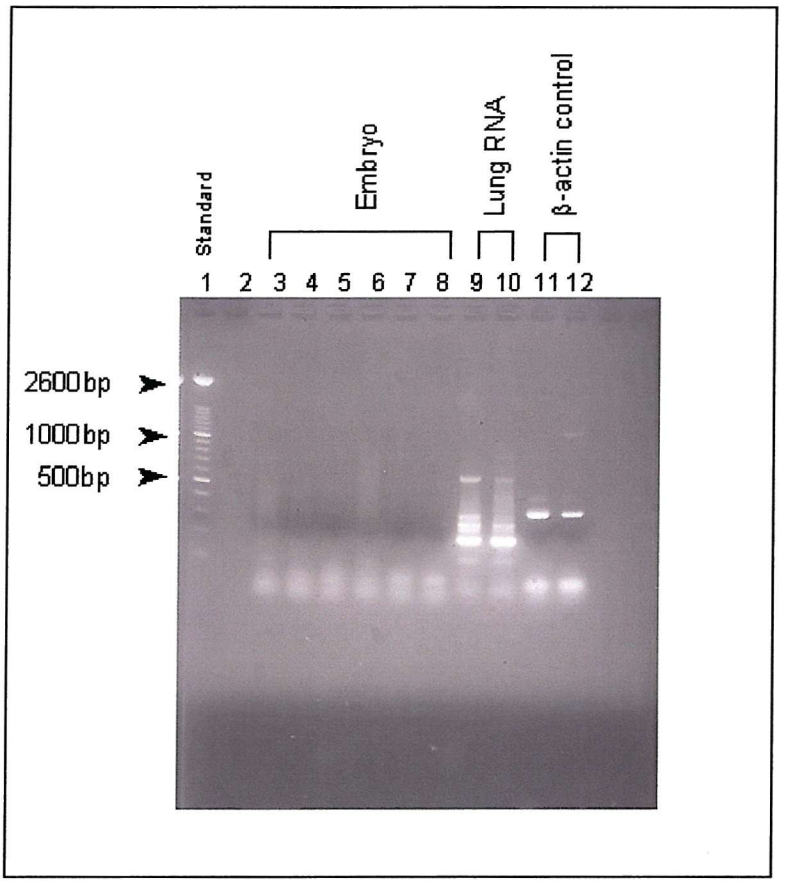


Figure 5-5. The CD44 PCR amplification of mRNA from single mouse embryos on MAP. The MAP with a single blastocyst (32/64 cells or > 32/64 cells, day 3.5-4.5) (lane 3-8) was processed and the RT-PCR reaction performed as described in ***Material and Methods (2.7.4)***. None of the six samples from embryos showed a positive result. When the MAP with mRNA from a single embryo in the same stage was processed the PCR with house keeping gene (β-actin) primers, the samples (lane 11-12) showed the predicted cDNA products. Total mouse lung RNA (lane 9-10) was used as a positive control for the RT-RCR reaction. This shows products at 220 bp the same size as products confirmed to be CD44s by sequencing (*section 5.2.8*). Other larger products are presumed to be CD44 isoforms containing sequences from variant exons. Lane 1 shows the molecular markers as described in *Figure 5-3*.

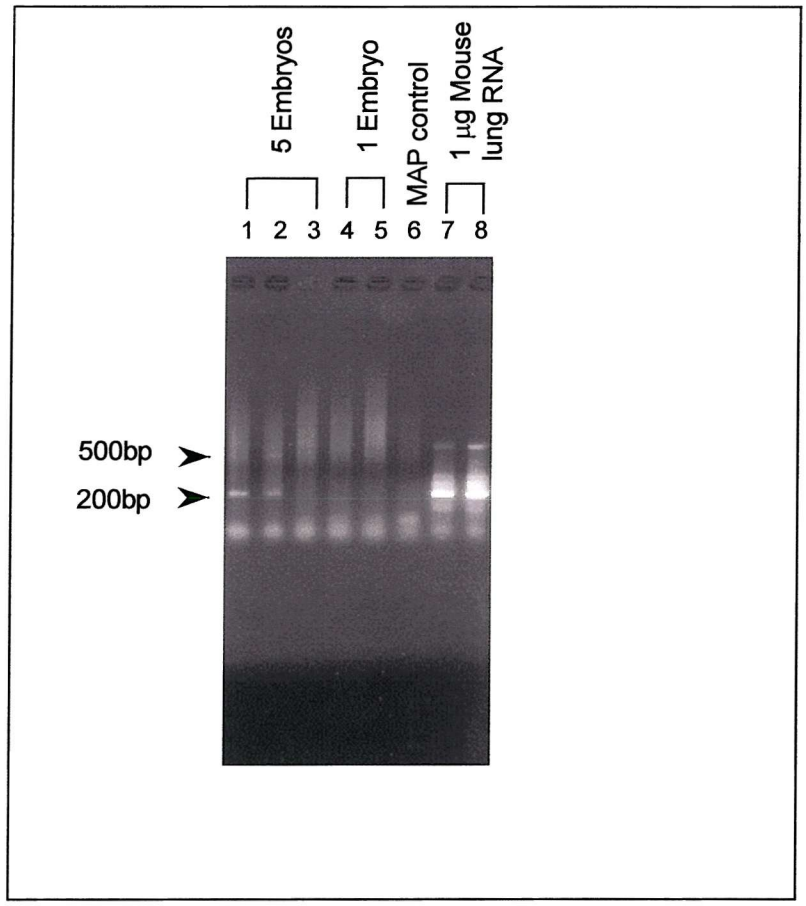


Figure 5-6. CD44 PCR products from 5 mouse embryos on MAP. In this experiment 2 of 3 samples from 5 embryos at blastocyst stage on each MAP (lane 1-3) showed the PCR products one running at ~ 220bp and the two faint bands at ~320bp and 500bp. Because the low intensity of the bands on the original film, they are not visible on the reproduction. One embryo on the MAP still showed no CD44 PCR product (lanes 4 and 5). Lane 6 shows the MAP without embryo as negative control. In lanes 7 and 8, 1 µg total mouse lung RNA processed by conventional PCR was used as a positive control for the PCR reaction. From sequencing of 220 bp products using the same primer these products are likely to be CD44s, and the other larger products isoforms containing sequences from variant exons.

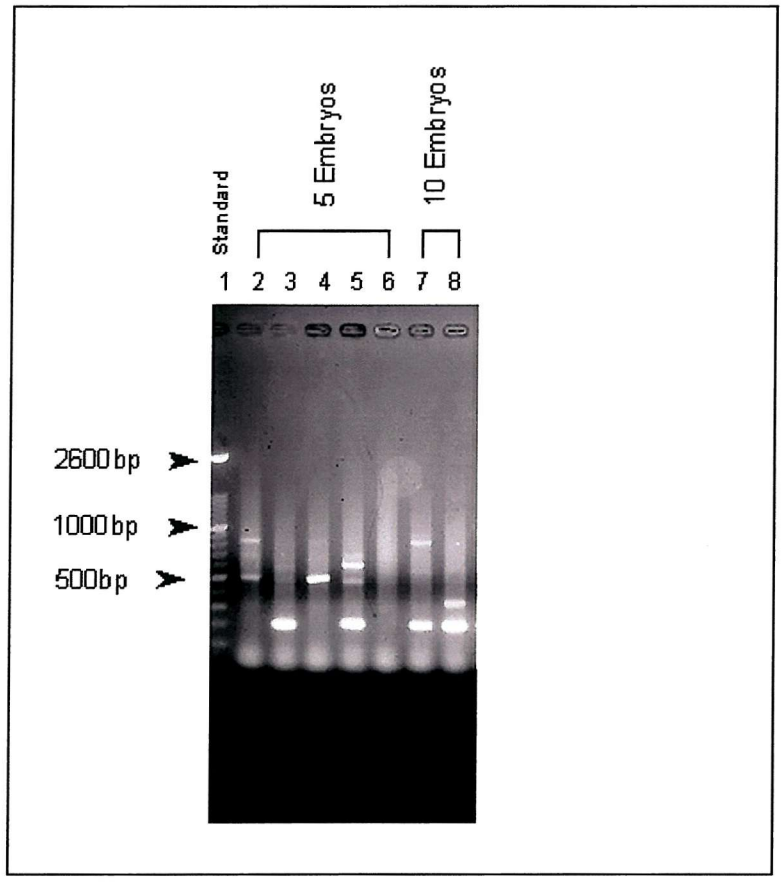


Figure 5-7. RT-PCR product analysis of mouse embryo at the 2-cell stage. mRNA from 5 embryos (lane 2-6) or 10 embryos (lane 7-8) was prepared on MAP and the RT-PCR reaction performed. Using embryos at the 2-cell stage, some of the samples (lanes 3, 5,7 and8) still expressed the 220 bp PCR product corresponding to CD44s, while most of the samples expressed large CD44 products between 300-800 bp which presumably contain sequences from variant exons (lanes 2,3,4,5,7 and 8). Lane 1 shows the molecular markers as describe in *Figure 5-3*.

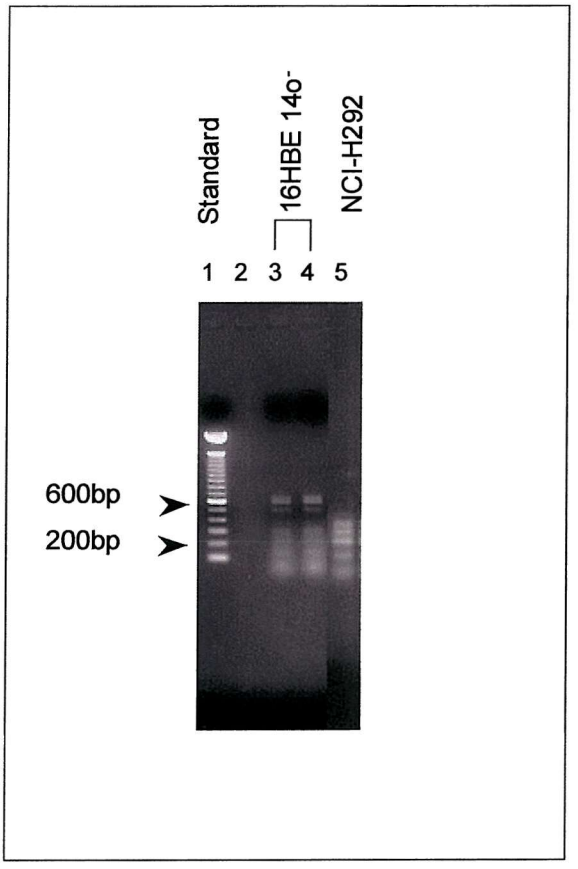


Figure 5-8. RT-PCR product analysis of 16HBE 14o$^-$ and NCI-H292 cells. Total RNA extracted from16HBE 14o$^-$ (lane 3 and 4) and NCI-H292 cells (lane 5) were used at 1 μg per CD44 PCR reaction. Lane 1 shows the 100 bp molecular marker ladder (Gibco) with high intensity bands at 100, 600, 1500 and 2072 bp (arrows at 200 and 600 bp). This shows the smallest CD44 product at 220 bp which is believed from sequencing the products to be CD44s. Based on sequencing of 600bp products (*see 5.2.8*), this is likely to be CD44v8-v9-v10 or CD44v6-v7-v8, and the products between 220-500 bp could be CD44 v8-v9, CD44v3 or CD44v8.

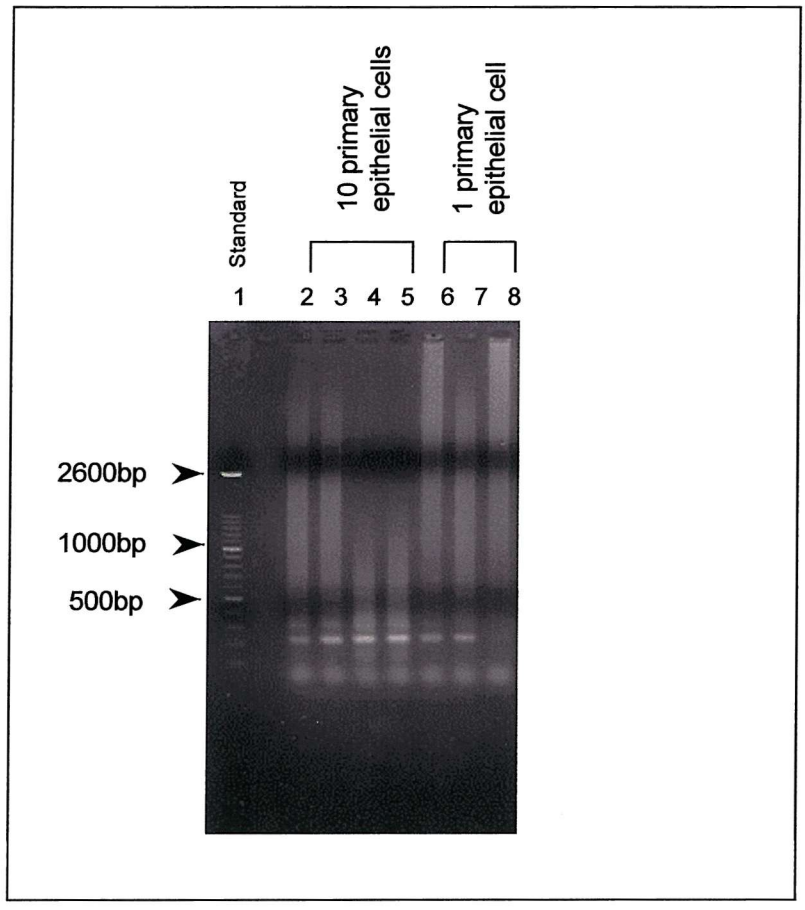


Figure 5-9. The CD44 PCR amplification of human primary epithelial cells. MAP with 10 cells (lane 2-5) and only one cell (lane 6-8) were processed for RT and CD44 nested PCR amplification as described in *Materials and Methods (2.7.4)*. Lane 1 shows the 100 bp molecular markers ladder as described in *Figure 5-3*. From sequencing results (*section 5.2.8*), the predominant CD44 products (220bp) are likely to be CD44s, and the 300 bp product could be CD44v8.

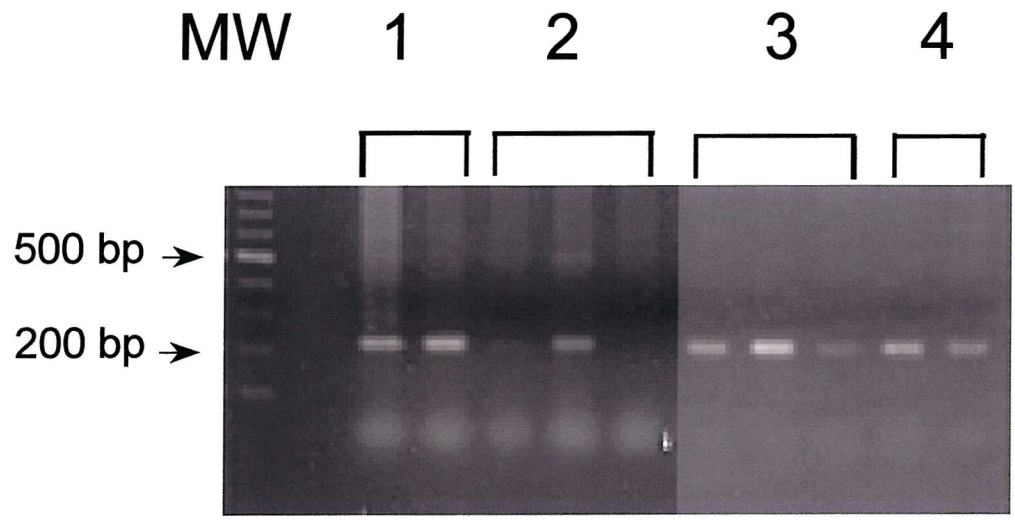


Figure 5-10. The CD44 PCR amplification of human bronchial epithelial cells from airway brushing. MAP with 5 cells (group 3) and 15 cells (group 4) were carried on RT and CD44 nested PCR amplification as described in ***Materials and Methods (section 2.7.4)***. Human primary bronchial epithelial cells were also compared (Group 1: ten cells, and Group 2: one cell). Compared with sequencing results (*Section 5.2.8*), the 220 bp CD44 products are believed to be CD44s.

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Query: 7    tgacgtgagcagcggntcntccagtgaaaggagcagcacttcaggaggttacatcttttacacctttctact 80
            ||||||||||||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 528  tgacgtgagcagcggctcctccagtgaaaggagcagcacttcaggaggttacatcttttacacctttctact 600

Query: 81   gnnnnnnnnatcccagacgaagacagtccctggatcaccgacagcacagacagaatccctgctacca 147
            |        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 601  gtacaccccatcccagacgaagacagtccctggatcaccgacagcacagacagaatccctgctacca 667


Query: 132  gagaccaagacacattccaccccngtgggggtcccataccactcatggatctgaat 188
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 1810 gagaccaagacacattccaccccagtgggggtcccataccactcatggatctgaat 1866
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v8-v9-v10 (variant exons only)

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Query: 29   ttagagttggaatctccaacagtaactgcagtaactccaaaggacccagtcttagctgaggtcactggga 98
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1789 ttagagttggaatctccaacagtaactgcagtaactccaaaggacccagtcttagctgaggtcactggga 1720


Query: 99   tgaaggtcctgctttcctncgtgtgtgggtaatgagaggtataaccttccagtaaagtagttgagcctt 167
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1719 tgaaggtcctgctttccttcgtgtgtgggtaatgagaggtataaccttccagtaaagtagttgagcctt 1651


Query: 168  cagaatgatttgggtctcttcttccacctgtgacatcattcctattgcttgatgtcagagtagaagtt 235
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1649 cagaatgatttgggtctcttcttccacctgtgacatcattcctattgcttgatgtcagagtagaagtt 1583


Query: 236  gttggatggtctttatcttcttccaagccttcatgtgatgtagagaagctctgagaattactctgctgc 304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1612 gttggatggtctttatcttcttccaagccttcatgtgatgtagagaagctctgagaattactctgctgc 1514


Query: 305  gttgtcattgaaagaggtcctgtcctgtccaaatcttccaccaaacctgtgtttggatttgcagtaggc 373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1513 gttgtcattgaaagaggtcctgtcctgtccaaatcttccaccaaacctgtgtttggatttgcagtaggc 1445

Query: 374  tgaagcgttgtactatgactggagtncat 402
            |||||||||||||||||||||||| |||
Sbjct: 1444 tgaagcgttgtactatgactggagtccat 1416
```

v8-v9 (variant exons only)

```
Query: 12   ttgcttga-gtcagattagaa-ttgttggatggtctttatcttcttccaagccttcatgtgatgtagaga 79
            |||||||| |||||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1606 ttgcttgatgtcagagtagaagttgttggatggtctttatcttcttccaagccttcatgtgatgtagaga 1537

Query: 80   agctctgagaattactctgctgcgttgtcattgaaagaggtcctgtcctgtccaaatcttccaccaaacc 149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1536 agctctgagaattactctgctgcgttgtcattgaaagaggtcctgtcctgtccaaatcttccaccaaacc 1467

Query: 150  tgtgtttggatttgcagtaggctgaagcgttgtactatgactggagtccatat 202
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1466 tgtgtttggatttgcagtaggctgaagcgttgtactatgactggagtccatat 1414
```

Figure 5-11. The sequences of three predominant CD44 isoforms from cloning. CD44 cDNA from RT-PCR was inserted into pGFP for cloning and sequencing as described in ***Materials and Methods (section 2.7.5)***. CD44s, $CD44_{v8\text{-}v9\text{-}v10}$ and $CD44_{v8\text{-}v9}$ were the predominant CD44 isoforms. The sequences here show cloned sequences (Query) which were aligned by "Blast 2 Sequences" (http://www.ncbi.nlm.nih.gov/gorf/bl2.html) with whole CD44 cDNA (Sbjct) from GeneBank.

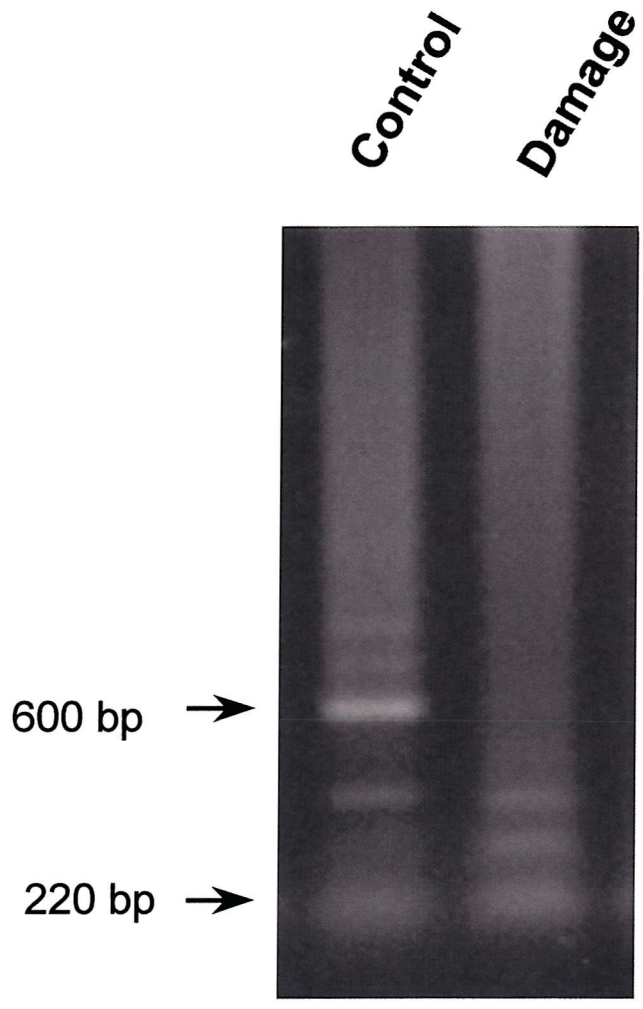


Figure 5-12. CD44 expression after cell damage. RNA extracted by Trizol as describe in *Materials and Methods* from confluent 16HBE 14o$^-$ cells (Control) or confluent cells 6 hours after mechanism damage (Damage) were processed RT-PCR with human optimised primers (*j2* and *k2*) for the detection of CD44 mRNA species. The pattern of CD44 PCR products was changed in the cells with mechanical damage. Compared with sequencing results the CD44 PCR product at ~600 may be v8-v9-v10 (613 bp) or CD44v6-v7-v8 (580 bp).

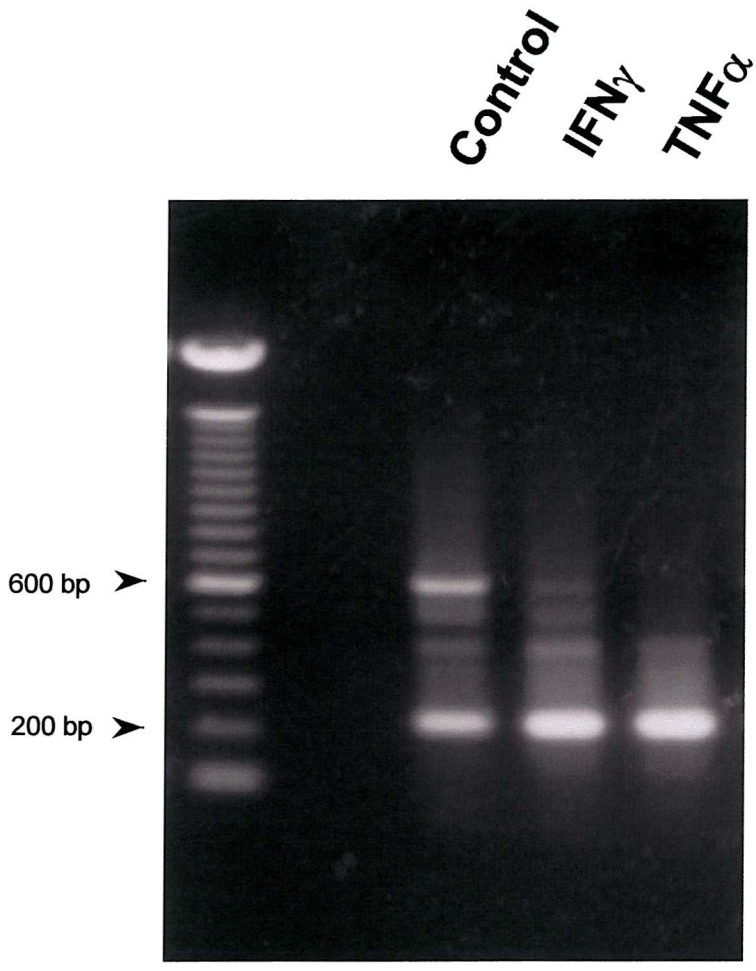


Figure 5-13. Effect of TNFα and IFNγ on CD44 isoform alternative splicing. 16HBE 14o$^-$ cells in sub-confluent culture (70-80% confluence) were treated with 200 U/ml of TNFα or IFNγ for 8 hours. The CD44 alternative splicing pattern in the cells with TNFα, IFNγ and untreated cells were compared by RT-PCR with human optimised primers (*j2* and *k2*). Standard was the 100 bp molecular marker ladder (Gibco) with highlight at 100, 600, 1500 and 2072 bp. Compared with sequencing of similar products (*section 5.2.8*), the predominant CD44 products (220bp) are CD44s; the 600 bp products are likely to be CD44v8-v9-v10 (613 bp) or CD44v6-v7-v8 (580 bp); and 400 bp products are likely to be CD44v8-v9. The products between 220-400 bp may include isoforms CD44v3 and/or CD44 v8.

Chapter 6

The Role of CD44 in Epithelial Cell Adhesion and Cell Migration on Matrix Substrates

6.1 Introduction

Epithelial shedding is regarded as a signal feature of asthma (Laitinen *et al.*, 1985; Jeffery *et al.*, 1989; Beasley *et al.*, 1989), and it might play an important role in the pathogenic mechanism in airway inflammatory diseases. Epithelial damage may result in increased epithelial permeability and easier access of allergens, and further induce airway inflammation and bronchial hyperresponsiveness. However, the airway epithelium possesses considerable capacity to repair following injury (Demoly *et al.*, 1994), which may help resolve an exacerbation of asthma with reparative processes. Both *in vivo* (Keenan *et al.*, 1982; Erjefält *et al.*, 1995; Erjefält *et al.*, 1997) and *in vitro* (Zahm *et al.*, 1991; Zahm *et al.*, 1997) studies have demonstrated that repair of the damaged airway epithelium occurs after disruption of small sections of epithelium. After full-thickness damage to the epithelium, this repair process starts with flattened cells of a basal phenotype covering the denuded area. Cell proliferation and differentiation mostly begins after cell migration is complete (Shimizu *et al.*, 1994; Zahm *et al.*, 1997; Kim *et al.*, 1998). This re-epithelialisation requires communications between epithelial cells, and ECM is also critical to regulate such processes in the repair. These events directly or indirectly involve the regulation of cell adhesion molecules (CAMs).

Of the adhesion molecules in bronchial epithelium, CD44 is a candidate to play an important role in the repair processes of bronchial epithelium. Not only is CD44 the ligand for several extracellular matrix molecules in the airways (Aruffo *et al.*, 1990; Faassen *et al.*, 1992; Knutson *et al.*, 1996), but it has also been reported to be up-regulated in the epithelium of airways of asthmatic subjects (Peroni *et al.*, 1996; Lackie, 1997). Airway structural changes and inflammation appear to be initiated in the early stage of asthma, and the changes of matrix protein expression also occurs in the basement membrane at this stage (Laitinen *et al.*, 1996). The interaction between cells and cell-extracellular matrix is important. In the early stages of airway inflammation, CAMs are likely to be key mediators of these interactions.

CD44 has been demonstrated to be involved in cell motility including leukocyte interaction/migration (Lesley *et al.*, 1993a; Mikecz *et al.*, 1995), tumour

migration/metastasis (Zahalka *et al.*, 1995; Knutson *et al.*, 1996; Henke *et al.*, 1996), and thymus colonisation by T-cells (Lesley *et al.*, 1993a; Zahalka *et al.*, 1995; Mikecz *et al.*, 1995; Patel *et al.*, 1995; Masellis-Smith *et al.*, 1996). Most of these studies of CD44 have concentrated on its role as a receptor for HA, but there are marked cell-specific differences associated with this function. It is still unclear if CD44-ligand binding is only regulated by the level of CD44 expression, or if, as seems likely, other modifications of CD44, such as N- and O-linked glycosylation and substitution with glycosaminoglycans influence the interaction of CD44 with extracellular matrix. For instance, the chondroitin sulphate-modified form of CD44 has been shown to regulate motility and invasiveness in collagen I gels (Faassen *et al.*, 1992; Faassen *et al.*, 1993); and blockade of O-linked glycosylation enhances CD44-mediated adhesion to HA (Dasgupta *et al.*, 1996). Alternatively, spliced variants may also possess different functional characteristics of CD44 (see *Table 1-3*), which may also regulate the functions of CD44.

In chapter 4 and 5, I demonstrated that expression of CD44 is associated with epithelial damage and may be regulated by inflammatory cytokines. The relationship between enhanced expression of CD44 and the motility of airway epithelial cells therefore needed to be investigated. Using cytokine treatment and antibodies against CD44 (standard, CD44v6, and CD44v9), I therefore examined the effect of CD44 antibodies in a cell adhesion assay, and an *in vitro* wounding model of epithelial culture. With these models I have investigated whether CD44 is involved in cell adhesion or cell migration in relation to (1) cell adhesion on extracellular matrix (2) cytokine-regulated cell adhesion (3) cell migration on matrix substrates (4) epithelial repair after mechanical damage.

6.2 Results

6.2.1 Cell adhesion to different extra cellular matrixes

In order to understand the functions of CD44 in bronchial epithelial cell adhesion, the baseline of cell binding to the ligands of CD44 was investigated. Cell adhesion to collagen I, collagen II, collagen IV, fibronectin, HA, and bovine serum albumin (BSA) was measured. As seen in *Figure 6-1*, The percentages of adherent cells were 91% (collagen I), 65% (collagen II), 75% (collagen IV) and fibronectin (65%). Less then 25 % of the cells had bound to HA. Only 4% of cells were bound to BSA control (*Figure 6-1 and Figure 6-2*).

6.2.2 The kinetics of cell binding to matrix substrates

Cell adhesion to different matrixes was compared after plating cells at different time intervals (30 min, 1, 1.5, 2, and 4 hour(s)). Increased cell binding to HA was seen with time, although no increase was seen in other matrix proteins (*Figure 6-2*) after 1.5 hours. For the other matrix proteins, more than 90 % of cells were bound on the matrix-coated surfaces at 1.5 hours, and there was no significant increase in cell binding up to 2 hours. Therefore, a 1.5-hour incubation time for cell adhesion was used in the following experiments.

6.2.3 Effect of CD44s antibody on epithelial adhesion

Serial dilutions of CD44 antibodies (6.25, 12.5, 25, 50, 100, and 200 μg/ml) were assayed for their ability to inhibit cell adhesion. High concentrations of CD44s antibody inhibited cell binding to collagen I, collagen IV, fibronectin, HA (CD44 ligands), but not the controls, collagen II or BSA. The maximum inhibition of CD44s antibody was noted at concentrations of 100 and 200 μg/ml, which gave 30-40% decreases in cell binding to collagen I, collagen IV and fibronectin, and 70% to HA (*Figure 6-3A and B*). Less inhibition was seen for cells on collagen IV or fibronectin with low CD44s antibody concentrations (*Figure 6-3A*), while at as low as 12.5

μg/ml, CD44s antibody still showed a significant inhibitory effect ($p<0.01$) on cell binding to collagen I and HA (*Figure 6-4A and B*). The decrease of cell adhesion by CD44 antibody was 40% (maximum) for cells binding to collagen I and 70% (maximum) to HA (*Figure 6-3A and B*).

6.2.4 Cytokine and epithelial adhesion to HA

Because TNFα, IFNγ, IL-1β, IL-4 and PMA had different effects on CD44 expression by bronchial epithelial cells (see *section 4.2.5*), it was therefore interesting to compare their effect on cell binding to HA. 16HBE 14o$^-$ cells treated with TNFα, IFNγ, IL-1β, IL-4 and PMA increased 16HBE 14o$^-$ cell binding to a HA-coated surface 2- to 5-fold (*Figure 6-4*). This increase of cell binding to HA was inhibited by CD44s antibody (*Figure 6-5*).

Human primary bronchial epithelial cells treated with IFNγ, TNFα, IL-1β and PMA also showed increased cell binding to HA, but no increase was seen with IL-4 treatment. In cytokine treatments, IL-1β-induced cell binding to HA was also inhibited by CD44v6 and CD44v9 antibodies (*Figure 6-6*).

6.2.5 Developing a model for epithelial cell migration studies

The "under-agarose migration assay" (Thorgeirsson *et al.*, 1979; Hoying and Williams, 1996) was developed for the study of endothelial migration by using agarose solidified on to a monolayer of endothelial cells in culture, and punching a hole in a layer of agarose. Endothelial cells were removed because they were attached in the bottom of the agarose plug, and cell migration could be measured at the increase in surface area of cell colonies from under the agarose gel. However, unlike endothelial cells, in my pilot experiments epithelial cells in 1% or 1.5% agarose were not detached from the culture dishes. Therefore, this model could not be adapted to study epithelial cell migration.

Other migration assays studied have been performed by measuring cells migrating through filter membrane, and this approach has been widely applied in the study of

PMN or tumour cells migration since the cell movement was invasion into tissue or infiltration through cell/matrix barriers in three-dimensional migration. Unlike PMN or tumour cells, bronchial epithelial cell migration is a two-dimensional migration. These models were consequently not suitable for studying the migration of bronchial epithelial cells. I have therefore used two models which I developed to study epithelial migration.

Model I --Counting cell numbers in clumps

When epithelial cultures were set up from a single monodispersed cell suspension, epithelial cells adhered and then clumped. In order to clump, cell must migrate and epithelial cells characteristically form an island-like pattern of 5-15 cells (cell numbers are dependent on the incubation period). Based on the above observation, I seeded single cell suspensions of 16HBE 14o$^-$ cells in 6-well plates with or without CD44 antibody treatment. After 2 and 8 hours, the cell numbers in each clump were counted. The advantage of this model is it can be use for comparing cell migration on different matrix substrates as well as assessing the effect of antibody blocking on cell migration.

6.2.6 Cell migration on CD44 ligands

Using model I, at 2 hours after plating cells on collagen I, collagen IV, fibronectin, HA, or BSA, no difference was seen between cells migrating on different matrix- and BSA-coated surfaces (*Figure 6-7*). Cells on collagen I, collagen IV and fibronectin were flat (but not grouped), but cells on HA and BSA remain round. However, the average cell numbers in each clump on collagen I, collagen IV, fibronectin and HA were increased compared with BSA control after 8 hours incubation (*Figure 6-7*). The total cell number in each field was similar for cells on collagen I, collagen IV, and fibronectin under a light microscope. However, less cells were bound to HA and BSA.

6.2.7 Effect of CD44 antibodies in epithelial migration

Single cell suspensions were seeded on different matrix-coated surfaces. To avoid the inhibitory effects of CD44 antibodies on cell adhesion, CD44 antibodies were added to the cultures 4 hours after cell adhesion. Four hours after antibody treatment, cells were fixed and the cell number in each clump was counted. The results showed that CD44s antibody inhibited cell clumping on fibronectin, while CD44v6 and CD44v9 antibodies did not (*Figure 6-8*). The total cell number in each field under the microscope remained similar indicating that the antibody inhibited cell migration, not binding.

Model II--Measuring the decrease of surface area after mechanical damage

Confluent bronchial epithelial cultures were mechanically damaged using a plastic pipette tip that removed a 120-150 μm wide strip of cells. In this system, epithelial cells covered the wound area by migrating and to fill the denuded area in 12-16 hours. With this model, cells were damaged and then treated with or without CD44 antibody. Cell migration was compared by measuring the decrease in surface area of the damaged area as cells covered it. I used petri dishes with a 2 mm grid on the underside which can be easy labelled permitting easy, consistent repositioning and allowing several different areas in a petri dish to be measured repeatedly.

6.2.8 CD44 in epithelial repair after mechanical damage

Using model II, CD44s antibody at the concentration of 50 μg/ml showed inhibition of epithelial cell migration at 3 hours after cell damage, but no significant effect at 6 and 9 hours (*Figure 6-9A*). Lower CD44 antibody concentrations did not affect cell migration at 3 and 6 hours after cell damage, while there was an increase of cell migration at 9 hours (*Figure 6-9B*).

6.2.9 Localisation of CD44 blocking antibody in the migrating cells

As described in chapter 4, CD44 protein was expressed on cells at a high level on cells close to areas of damage (*Section 4.2.3*). Here, I also examined the localisation of blocking antibody in the damage epithelial layer. Briefly, after 3- or 9-hour incubation with CD44s blocking antibody, the damaged monolayers were fixed with methanol and stained with secondary antibody conjugated with FITC. The result showed CD44 antibody was mainly binding to the cells along the border of the damaged areas (*Figure 6-10A and B).*

6.3 Discussion

The functions of cell adhesion molecules in the airway inflammatory reaction have received extensive attention especially in asthma. Expression of several adhesion molecules, including ICAM-1 (Wegner *et al.*, 1990; Vignola *et al.*, 1993b) and VCAM-1 (Gosset *et al.*, 1995) have been reported to be correlated to eosinophil and lymphocyte infiltration into airways and mediated in the inflammation response of asthma. CD44, has also been reported to be responsive to cell damage and has been suggested to be involved in the processes of airway epithelial remodelling in asthma (Peroni *et al.*, 1996; Lackie *et al.*, 1997). The up regulation of CD44 in the damaged areas of epithelium reveals a potential role of CD44 in the disease processes of asthma. In this chapter, 16HBE $14o^-$ and primary bronchial epithelial cells were employed to investigate the functions of CD44 in cell adhesion and migration. The results demonstrate that CD44 is important for bronchial epithelial cell adherence to collagen I, collagen IV, fibronectin and HA, and may also be involved in cell migration after mechanical damage.

Collagen I, collagen IV and fibronectin are ligands for CD44, as well as ligands for other CAMs, such as integrins. CD44 is thought to be the major receptor of HA which is widely distributed in tissue. Cell surface CD44 and HA was thought to be important in cell signalling in several cell types (Haegel-Kronenberger *et al.*, 1998; Beck-Schimmer *et al.*, 1998; Khaldoyanidi *et al.*, 1999; Bourguignon *et al.*, 2000). Most of our knowledge on the mechanism of CD44-HA interaction is derived from haematopoietic cells. Their interaction was described by Lesley and Hyman (Lesley and Hyman, 1992) reporting that cells in different activated states have different CD44 functions with respect to HA binding. However, unlike hematopoietic cells, which predominantly expressed only CD44s, epithelial cells mainly express several variant-containing isoforms. Since CD44 variant expression may be associated with the different functions of CD44 (see chapter 1, *Table 1-4*), the study in the role of CD44 should focus on not only CD44 total protein expression but also on CD44 variants and other process which might modify the function of CD44.

6.3.1 CD44s is important for cell adhesion

In addition to CD44, several β1 or β6 integrins (Hynes, 1987) are also able to bind collagens and fibronectin. For example α3β1 and α5β1 integrins expressed on bronchial epithelial cells are able to bind to fibronectin (Albelda, 1991). While the inhibition of cell binding to collagen I, IV, and fibronectin, CD44s antibody suggested that there is a CD44s-dependent mechanism involved in cell-matrix binding. There was 40% (of total added cells) of binding which CD44s antibody could not inhibit suggesting that other adhesion mechanism are also involved in cell binding to matrix proteins.

Cell binding to HA was less than on collagens and fibronectin, but 100% of the HA binding was inhibited by CD44s antibody. CD44 has been regarded as the major receptor for HA, although other adhesion molecules, such as RAMM and ICAM-1, have also been reported as a receptor of HA. An N-terminal link module and the membrane proximal region outside the link protein homologous domain in the CD44 constant area have been identified as important in HA binding (Goldstein *et al.*, 1989; Peach *et al.*, 1993; Yang *et al.*, 1994). Although several HA binding sites have been identified, some studies suggest that CD44 expression is insufficient to prime HA binding and the level of expression does not always correlate with binding of HA. This inference is based on accumulated evidence from studies in lymphocytes and CD44 negative cells transfected with different CD44 mutants. First, not all CD44 bearing cells can bind to HA. Normal lymphocytes express high levels of CD44 but do not bind soluble HA (Lesley *et al.*, 1990). Second, LPS and anti-IgD-dextran induce CD44 expression on murine B cells, whereas these cells do not bind to HA. In contrast, IL-5 stimulated B cells did not increase the expression of CD44, but the HA binding to cells was significantly boosted (Hathcock *et al.*, 1993).

6.3.2 Cytokine-induced cell adhesion to HA is CD44s dependent

The effect of cytokines on CD44 expression and CD44-HA interactions has been investigated in several cell types. GM-CSF, IL-3 and stem cell factor induce haematopoietic progenitor cell adhesion to HA, the increase was very rapid and no alteration of CD44 expression was seen (Legras *et al.*, 1997). IL-4 enhanced

expression of CD44 isoforms containing v3, v6 and v9, CD44-mediated cell adhesion to HA, and alterations in the CD44 splicing pattern in B cells (Kryworuchko *et al.*, 1999a). Furthermore, IFNγ and IL-4 inhibited PMA-induced HA adhesion, which may influence B cell migration through down-regulation of CD44-HA interactions (Kryworuchko *et al.*, 1999b). Other proinflammatory cytokines including TNFα increased cell binding to HA in monocytes (Levesque and Haynes, 1999), whereas, IFNγ and IL-4 did not (Levesque and Haynes, 1999; Kryworuchko *et al.*, 1999a).

The binding of CD44 to HA seems to be a complex and a dynamic process. Cytokines may regulate the expression of cell surface adhesion molecules and make further links to signal transduction pathways and cellular activation. In my studies, after cytokine treatment (see chapter 4), CD44v9 was up-regulated by all the cytokines (IFNγ, TNFα, IL-1β and IL-4); CD44v3 was only induced by IL-1β and IL-4; CD44s was up-regulated by most of the cytokines except IFNγ. These results suggest that different cytokine treatments induced different mechanisms that involve the change expression of CD44 variant (v3 or v9)-containing isoforms. On the other hand, significant increases in the level of CD44s were demonstrated following treatment with TNFα (19.5 ± 2.7), IL-1β (43.5 ± 4.4), IL-4 (18.2 ± 4.9), and PMA (125.1 ± 14.2) (*Figure 4-15*). However, this increase of CD44 expression after cytokine and PMA treatment is not parallel to the increase of CD44-HA binding in bronchial epithelial cells (compare *Figure 4-15* and *Figure 6-6*). For instance, TNFα only increased the median fluorescence intensity for CD44s by 20%, but enhanced cell binding to HA 2-fold. PMA dramatically increased CD44 expression by up to 2.5-fold, but only induced cell adhesion to HA 3-fold. In addition, IFNγ did not increase CD44 expression, but it still increased cell binding to HA by more than 30 %. These results suggest that there are other factors that can modulate the function of CD44, these will be discussed later in this chapter.

The monoclonal antibody 25-32, which recognizes an N-terminal epitope on all CD44 proteins had the ability to inhibit the myelopoiesis of human bone marrow culture (Moll *et al.*, 1998). The abrogation of cell adhesion on HA to basal levels suggests that the region on CD44 associated with mAb 25-32 is the major binding site for HA in bronchial epithelial cells. The cytokine-induced CD44 expression does not

correlate with cell adhesion to HA, which also supports the suggestion that other mechanisms may be involved in the modification of CD44 function.

The contribution of CD44 to cell adhesion in bronchial epithelial cells is demonstrated by three parts of my investigations: (1) CD44s is important for bronchial epithelial adhesion to collagen I, collagen IV, fibronectin and HA (*Figure 6-2*) (2) cytokine-induced cell adhesion to HA is CD44s-dependent (*Figure 6-3*) (3) with cytokine treatment the level of surface CD44 expression is not correlated to cell adhesion to HA (*Figure 4-15 and Figure 6-3*). These result indicate that CD44 is important for bronchial cell adhesion, and cytokine-regulated cell adhesion to HA is linked with mechanisms other than up-regulated CD44 protein expression.

6.3.3 Adhesion events in cell migration

Initiation of cell locomotion involves a directional protrusion of the leading edge of the cell, which forms a lamellipodium via polymerisation of actin networks (Small *et al.*, 1998; Sheetz *et al.*, 1998). After formation and stabilisation of the lamellipodium, cells use these adhesive interactions to generate the traction and force required for cell movement. There is a general agreement that adhesion molecules play a key role in cell locomotion. Studies have indicated that CD44 is important in adhesion (Faassen *et al.*, 1992; Lokeshwar *et al.*, 1994). The expression of CD44 has been implicated in the process of cell motility, including tumour formation (Sy *et al.*, 1991), and increased tumour cell motility (Thomas *et al.*, 1992). The highly expressed CD44 was found in the filopodia and lamellipodium that are highly motile structures at the leading edge of migrating cells that participate in cell motility in fibroblasts and T lymphocytes (Svee *et al.*, 1996; Schwarzman *et al.*, 1999).

Accumulated evidence suggests that CD44 participates in cell adhesion and migration, involving interaction with intracellular proteins associated with the cytoskeleton (Lokeshwar *et al.*, 1994; Tsukita *et al.*, 1994). One group of intracellular proteins linked with CD44, the ERM family members, ezrin, radixin, and moesin, are thought to work as molecular linkers between the cytoplasmic domain of CD44 and actin-based cytoskeletons (Mangeat *et al.*, 1999).

6.3.4 CD44 in cell migration

Several CD44 isoforms have been associated with cell motility (Thomas *et al.*, 1992; Weber *et al.*, 1996) and tumour growth (Bartolazzi *et al.*, 1994a). Results in chapter 3 shows that CD44 was highly expressed on the leading edge of bronchial epithelial cells in sub-confluent cultures and the damage area after mechanical damage. This supports a correlation between cell migration and CD44 expression.

The contribution of CD44 to cell migration in bronchial epithelial cells is supported by the following observations: (1) CD44s inhibited cell migration on ligands of CD44 and after damage (*section 6.2.7*) (2) the inhibitory effect of CD44s is at 3 hours; no difference was seen after 6 hours. The decrease of cell migration by CD44s antibody reveals that CD44-mediated effects are important in cell repair processes in bronchial epithelial cells. (3) Blocking antibody was bound to the areas of damage where cells have a high migratory activity.

As described in chapter 3, CD44 was only basally expressed on epithelial cells. Studies in normal bronchial epithelium have shown a negative expression of HA on columnar cells in normal pseudo-stratified bronchial epithelium but weak staining around the basal epithelial cells (Pirinen *et al.*, 1998). This indicates that CD44 could be important for basal epithelial cells to interact with matrix substrates. In this chapter, the functional involvement of CD44 was also investigated in epithelial adhesion and migration. The results suggest that the function of CD44 is regulated by cytokines, which is only partially through up-regulated expression of CD44. In addition, the migratory processes of bronchial epithelial cells after cell damage involved the expression of CD44. The net contribution of CD44 in cell adhesion and migration, may be regulated by several pathways which could modify the function of CD44 in a variety of ways as discussed in *section A to E* below.

(A) Via alternative splicing

Galluzzo (Galluzzo *et al.*, 1995) found CD44 isoform containing v6 and v9 are required for HA binding to lymphocytes. However, other studies have demonstrated that cells transfected with v9-containing CD44 isoforms could bind HA (He *et al.*,

1992; Dougherty *et al.*, 1994). Stamenkovic and co-workers (Stamenkovic *et al.*, 1991) also demonstrated that CD44s but not v9-containing isoform are important for cell binding to HA. Since 10 variant exons inserted into the CD44 core sequence can result in more than 700 CD44 isoforms (see chapter 5), it is very difficult to discuss the function of CD44 containing only certain variants. My results in chapter 5 demonstrated that CD44 isoforms were changed through alternative splicing of variant exons in cells after mechanical damage and inflammatory cytokine treatment. This indicates that CD44 isoforms containing v8-9-10 may be involved in the response to cytokine treatment and in the processes of epithelial damage.

(B) Modified Glycosylation

Studies in tumour metastases showed different alternatively spliced variants of CD44 possess different functional characteristics, such as their ability to bind to HA (Lesley *et al.*, 1993a; Culty *et al.*, 1994; van der Voort *et al.*, 1995). Other studies in N- and O-linked glycosylation and substitution with glycosaminoglycans showed that modifications of CD44 influenced the regulation of CD44 interaction with extracellular matrix (Dasgupta *et al.*, 1996). Blocking mouse melanoma cell production of surface chondroitin sulfate proteoglycan decreased melanoma cell motility and the invasive behaviour on type I collagen (Faassen *et al.*, 1992). In addition, inhibition of O-linked glycosylation decreases colonal carcinoma cells bind to HA (Dasgupta *et al.*, 1996). Studies have shown that the CD44s protein could not bind directly to chondroitin sulfate (Miyake *et al.*, 1990), or only showed a weak affinity to chondroitin sulfate (Underhill *et al.*, 1983; Aruffo *et al.*, 1990; Peach *et al.*, 1993). The modification of N-linked or O-linked glycosylation on the variant exons could be important in the regulation of CD44 binding.

(C) Relocalisation/clustering of CD44

Clustering of CD44 proteins seems to be important for their ability to bind to HA, because activating antibodies only worked when binding is multivalent. Binding of high levels of HA to CD44 has been reported to be required for the reorganisation of cytoskeletal proteins and clustering of CD44, and dimerisation of CD44 is not necessary for a low level of HA binding (Liu *et al.*, 1998). In the dimerisation of

CD44, a cysteine residue in the transmembrane domain is involved, which is mediated by the binding of high levels of HA (Liu and Sy, 1996; Liu *et al.*, 1996; Liu and Sy, 1997). Studies of PMA-induced cell binding showed that PMA stimulates the binding of CD44 to HA, which is also mediated by dimerisation of CD44 on the cell surface (Liu and Sy, 1997). CD44 has been reported to be physically associated with PKC (Kalomiris and Bourguignon, 1989), so up-regulation of CD44 binding to HA might involve PMA activation of PKC protein (Nishizuka, 1984).

(D) The cytoplasmic domain of CD44 in CD44-HA binding

The cytoplasmic domain of CD44 is encoded by exon 18 and 19. There are six serine residues in human CD44 cytoplasmic domain, of which Ser325 is the principle phosphorylation site and Ser323 is required as part of kinase consensus site (Peck and Isacke, 1998). Studies using a CD44 negative cell line transfected with a CD44 gene showed that potential for phosphorylation of CD44 on serines 325 and 327 within the cytoplasmic domain was required for CD44-mediated HA binding (Puré *et al.*, 1995). This may be involved direct or indirect interaction between CD44 and cytoskeletal proteins. Recent studies showed that peptides containing a phosphoserine at residue 325 can block CD44-transfected melanoma cell migration on HA (Peck and Isacke, 1998).

(E) Other factors

Bourguignon and co-workers (Bourguignon *et al.*, 1998) reported that CD44 isoform containing v3,8-10 is associated with the active form of MMP-9. The interaction of CD44 and MMP-9 might regulate several cell processes including matrix degradation and cell migration. Over expressing CD44 isoforms containing v3,7-10 in murine carcinomas increased collagen IV degradation and this increase could be blocked by antibodies to MMP-9 (Yu and Stamenkovic, 1999). The link between CD44 and MMP-9 might involve cell migration.

In conclusion taken together, it seems that increased expression of CD44 and alternative splicing of CD44 variants may have an important role in epithelial

adhesion and migration. The possible mechanisms for altering CD44 binding indicated that a diverse range of effects might be mediated by CD44.

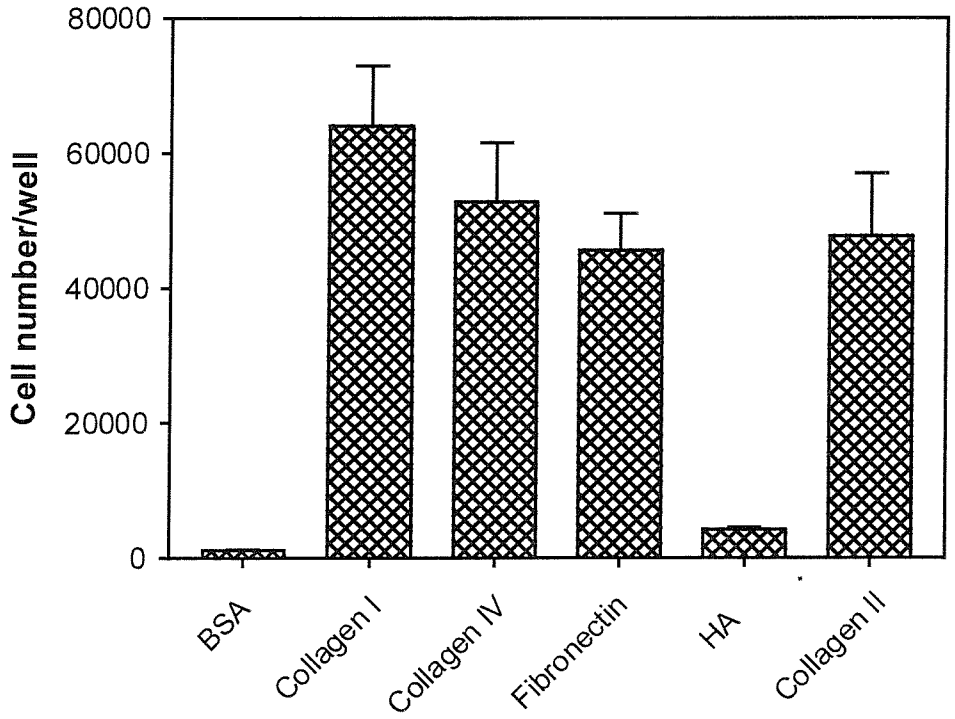


Figure 6-1. Cell adhesion to different extracellular matrix substrates. Monodispersed 16HBE 14o$^-$ cells were added to collagen I-, collagen II-, collagen IV- (10 μg/cm^2), Fibronectin- (5 μg/cm^2), HA- (100 μg/cm^2), and BSA- (200 μg/cm^2) coated 96-well ELISA plates. After 90 minutes incubation, plates were rinsed gently 3 times with Eagle's MEM with 5% FCS to wash away unbounded cells. Adherent cells were determined using a MTS assay as described in ***Materials and Methods*** *(Section 2.8.1)*.

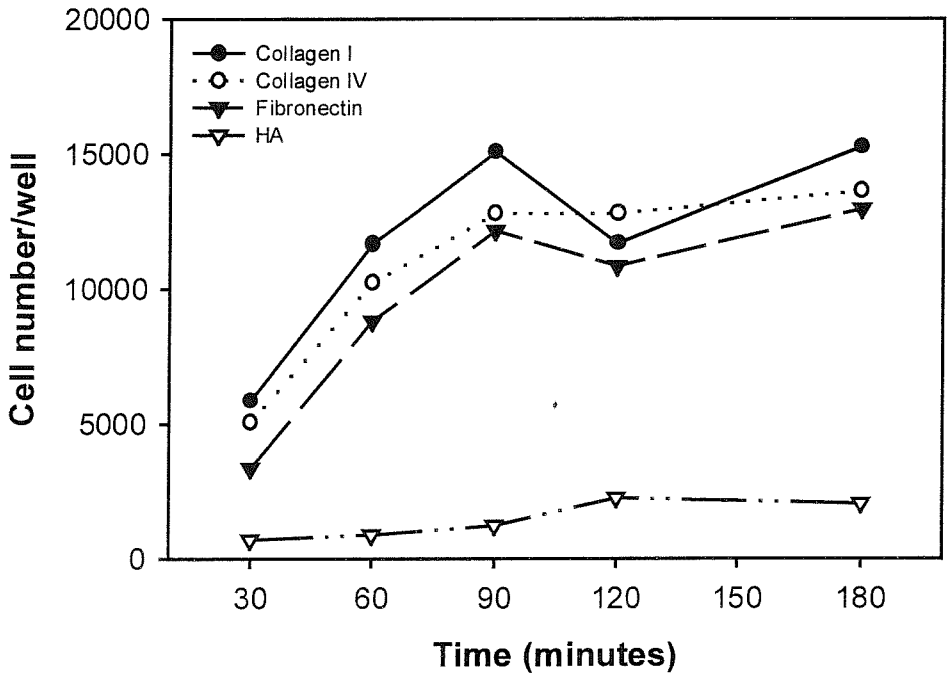
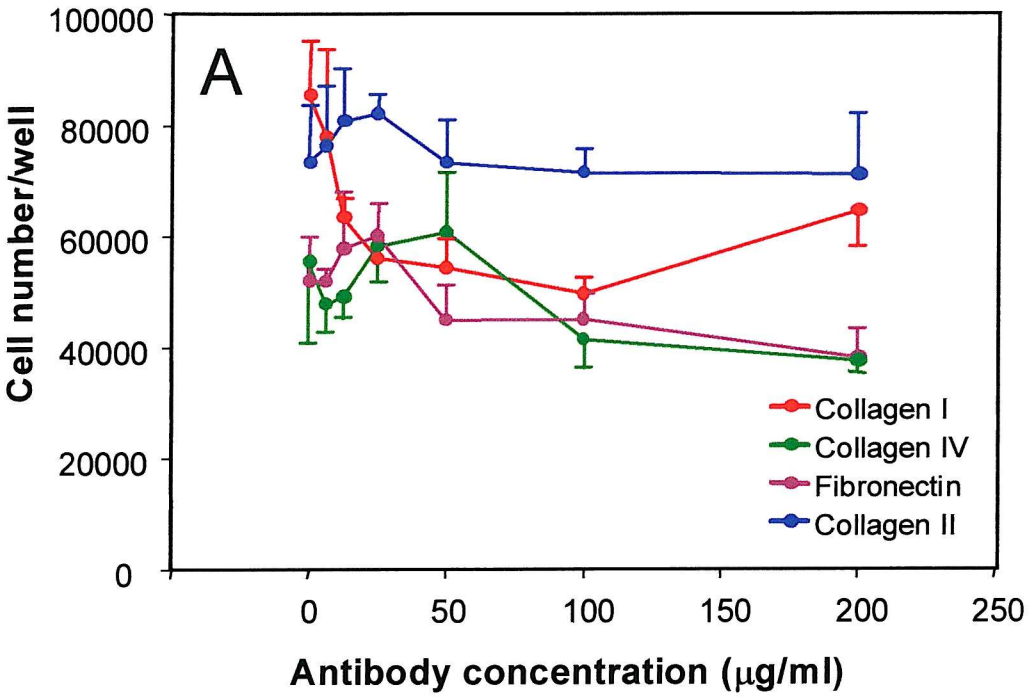


Figure 6-2 The kinetics of cell binding to matrix substrates. 16HBE 14o- cells were seeded in matrix coated Elisa plates and incubated for the time indicated. The binding of cells to the matrix substrates was determined using a MTS assay as described in ***Materials and Methods*** *(Section 2.8.1)*.



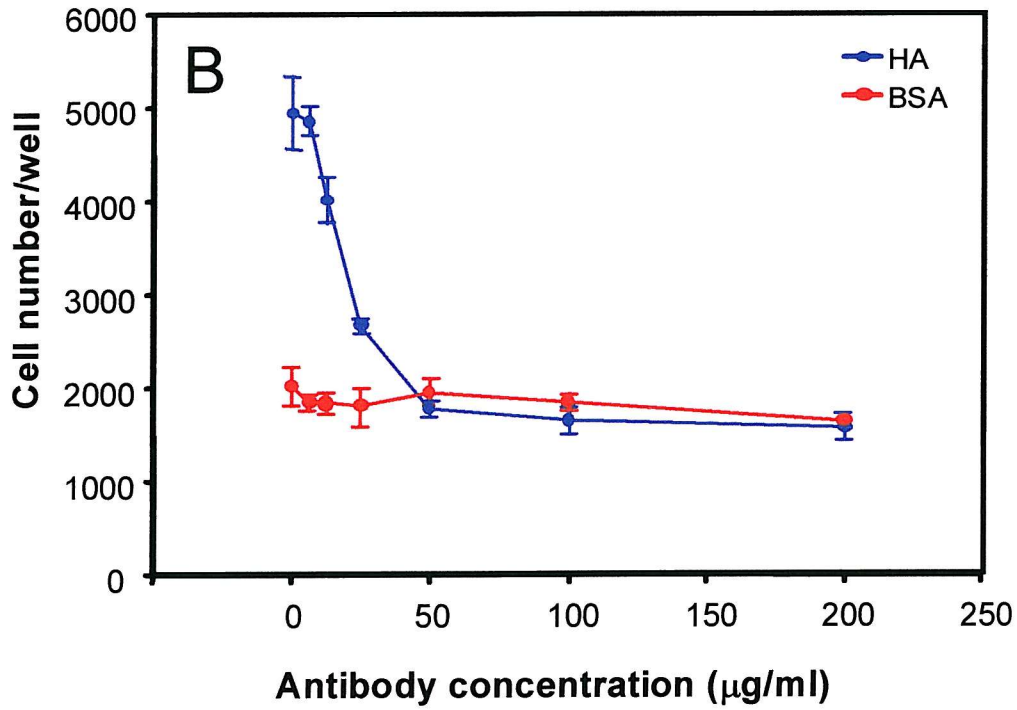


Figure 6-3. Effect of CD44s antibody on epithelial adhesion. Monodispersed 16HBE 14o$^-$ cells were pre-incubated with anti-CD44s antibody (25.32) for 45 min on ice before performing the cell adhesion assay. Data are presented in (A) for cell binding on collagen I, II, IV and fibronectin coated plate; and (B) on HA and BSA.

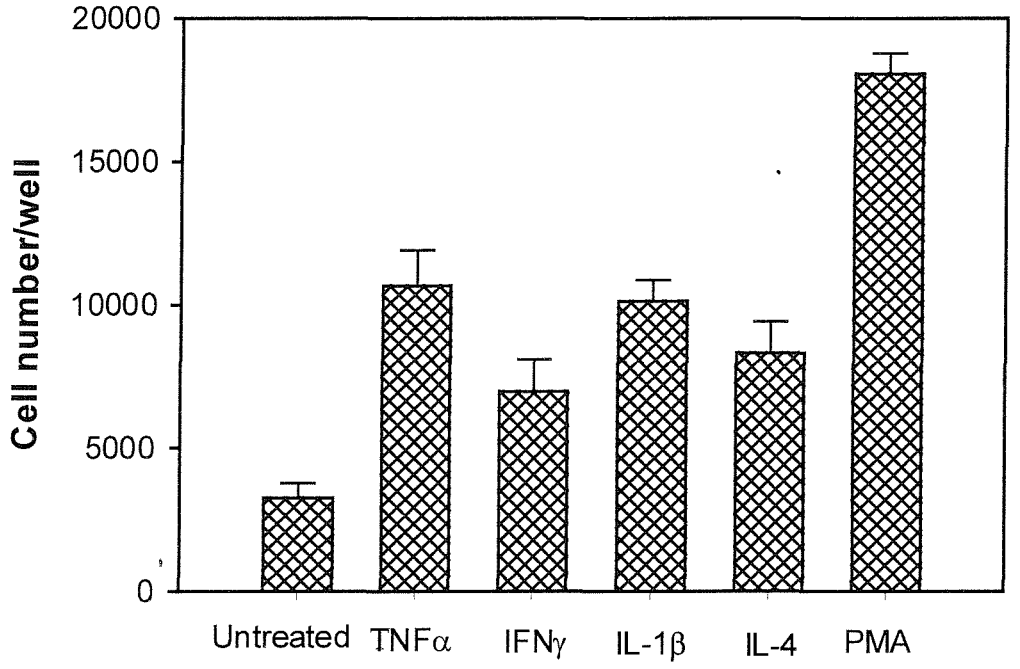


Figure 6-4. Cytokines and epithelial adhesion. 16HBE 14o$^-$ cells were incubated with TNFα (400 U/ml), IFNγ (400 U/ml), IL-1β (30 ng/ml), IL-4 (30 ng/ml) and PMA (250 ng/ml) for 24 hours and a cell adhesion assay performed on HA-coated ELISA plates.

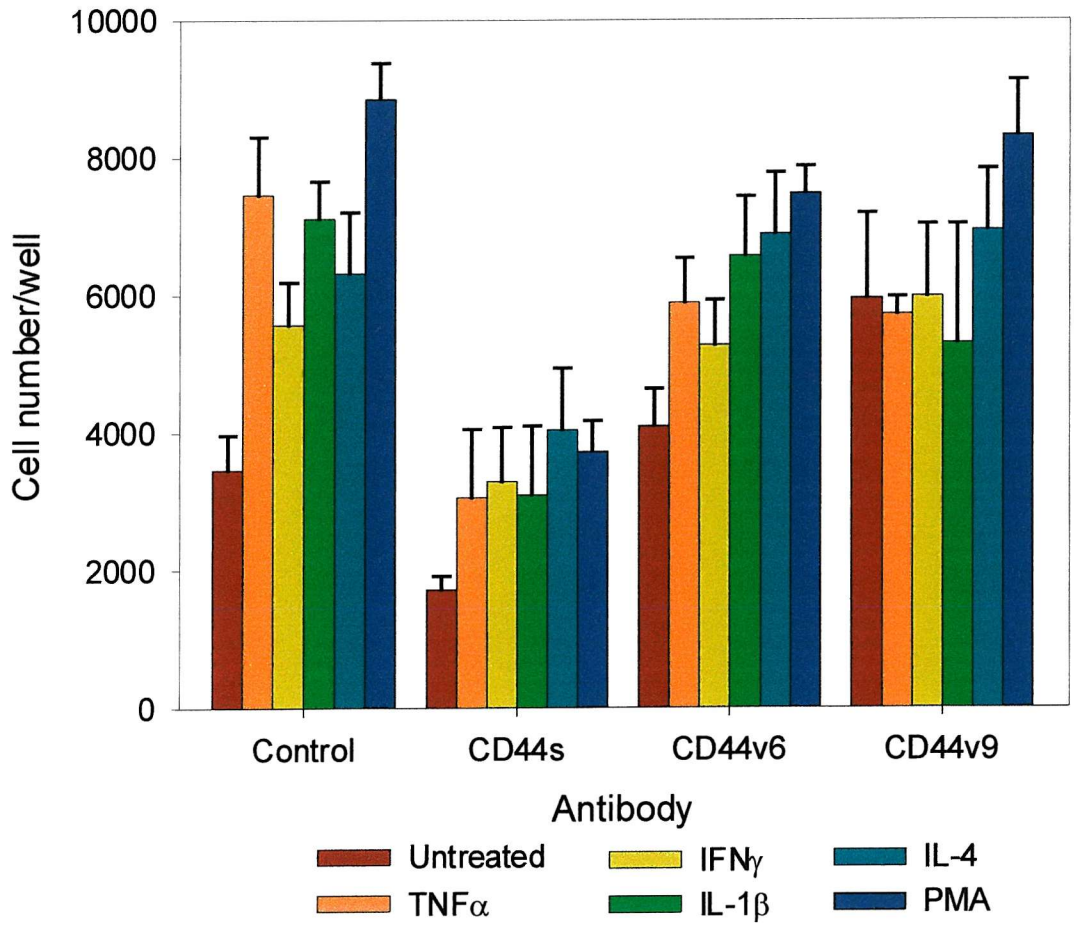


Figure 6-5. CD44 variants and epithelial adhesion to HA. 16HBE 14o$^-$ cells were incubated with cytokine for 24hours, monodispersed cells were then incubated with CD44 variant antibodies or control antibody for 45 min before performing a HA binding assay.

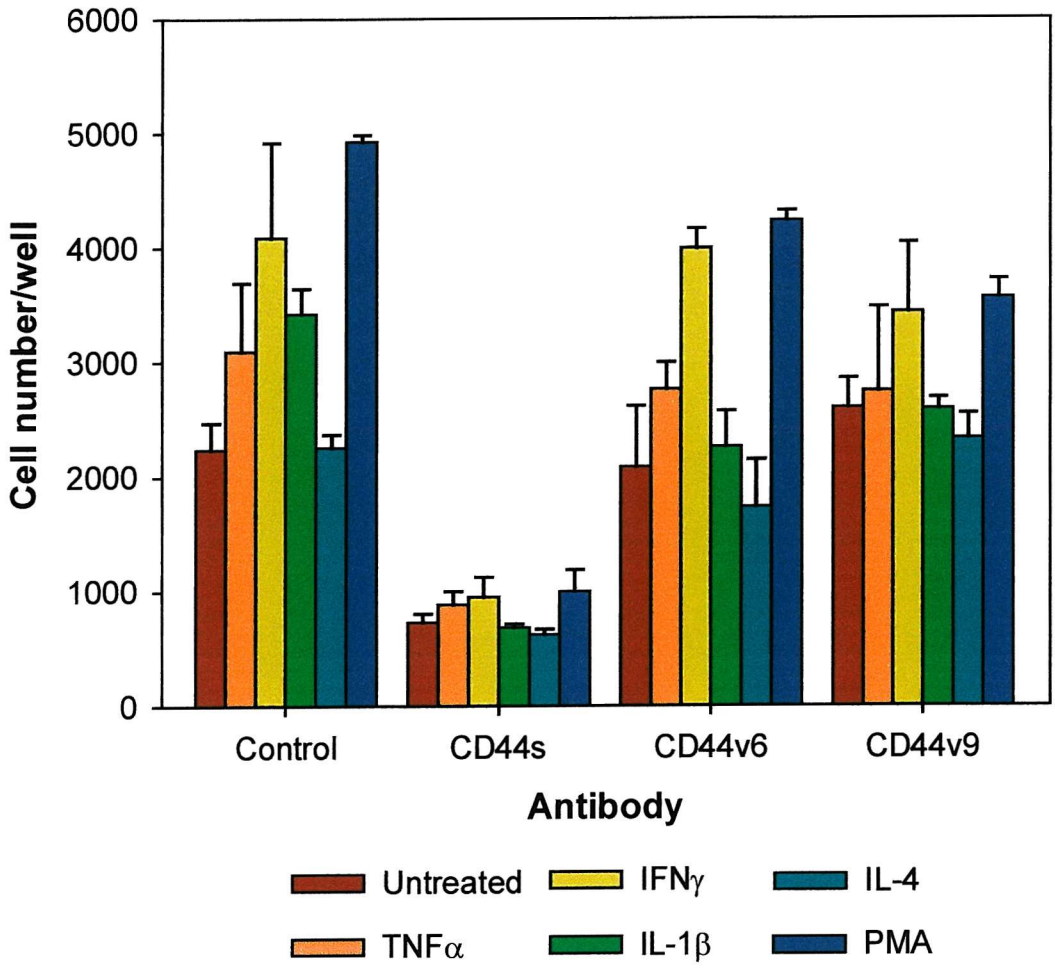


Figure 6-6. CD44 variant and primary bronchial epithelial cell adhesion to HA. Effect of CD44 variant antibodies (CD44s, CD44v6 and CD44v9) on human primary epithelial cell adhesion to HA.

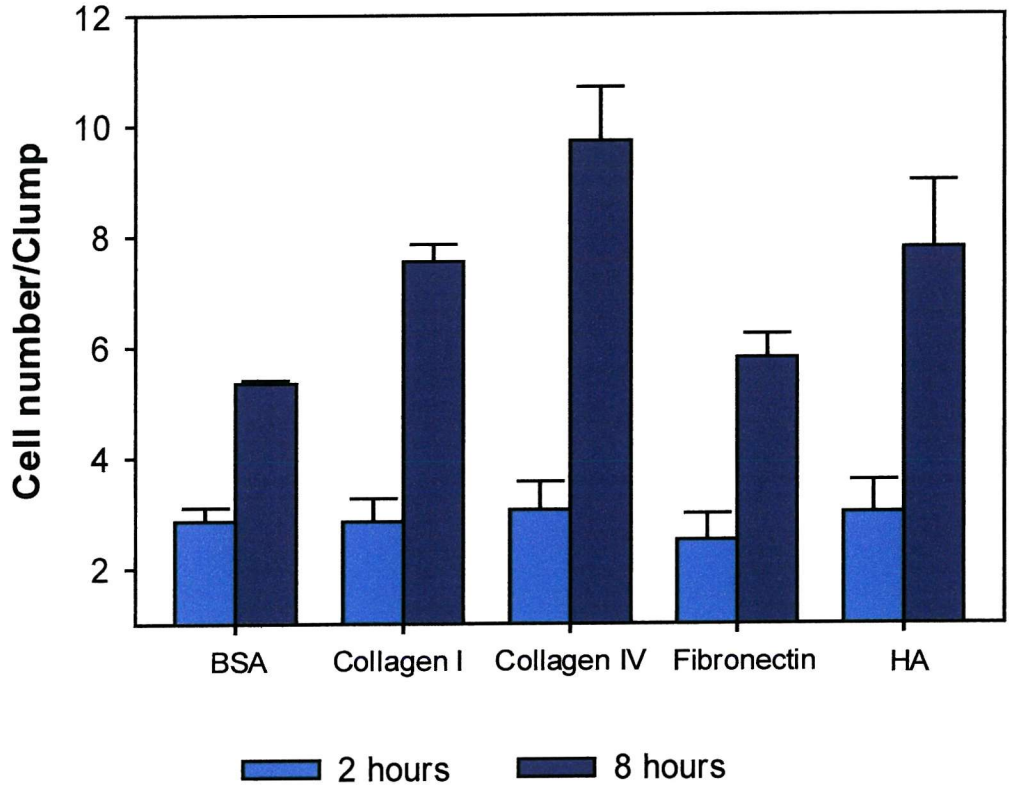


Figure 6-7 Cell migration on CD44 ligands. Monodispersed cells were seeded onto different matrix-coated petri dishes. At 2 and 8 hours, cell numbers in each clump were measured by counting the average cell number from 5 random fields.

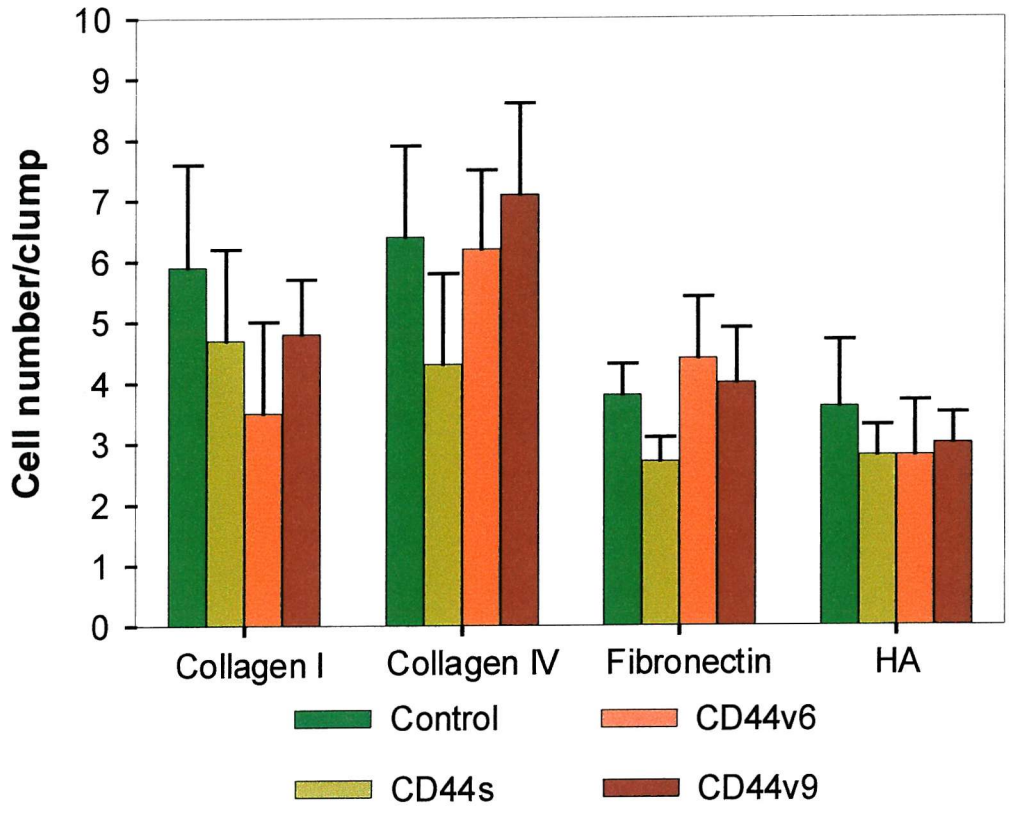


Figure 6-8 Effect of CD44s antibody in epithelial migration. CD44 antibodies (100μg/ml) were incubated with the cells plating on different matrix-coated wells. Average cell numbers in each clump were measured as used *in* ***Figure 6-7***.

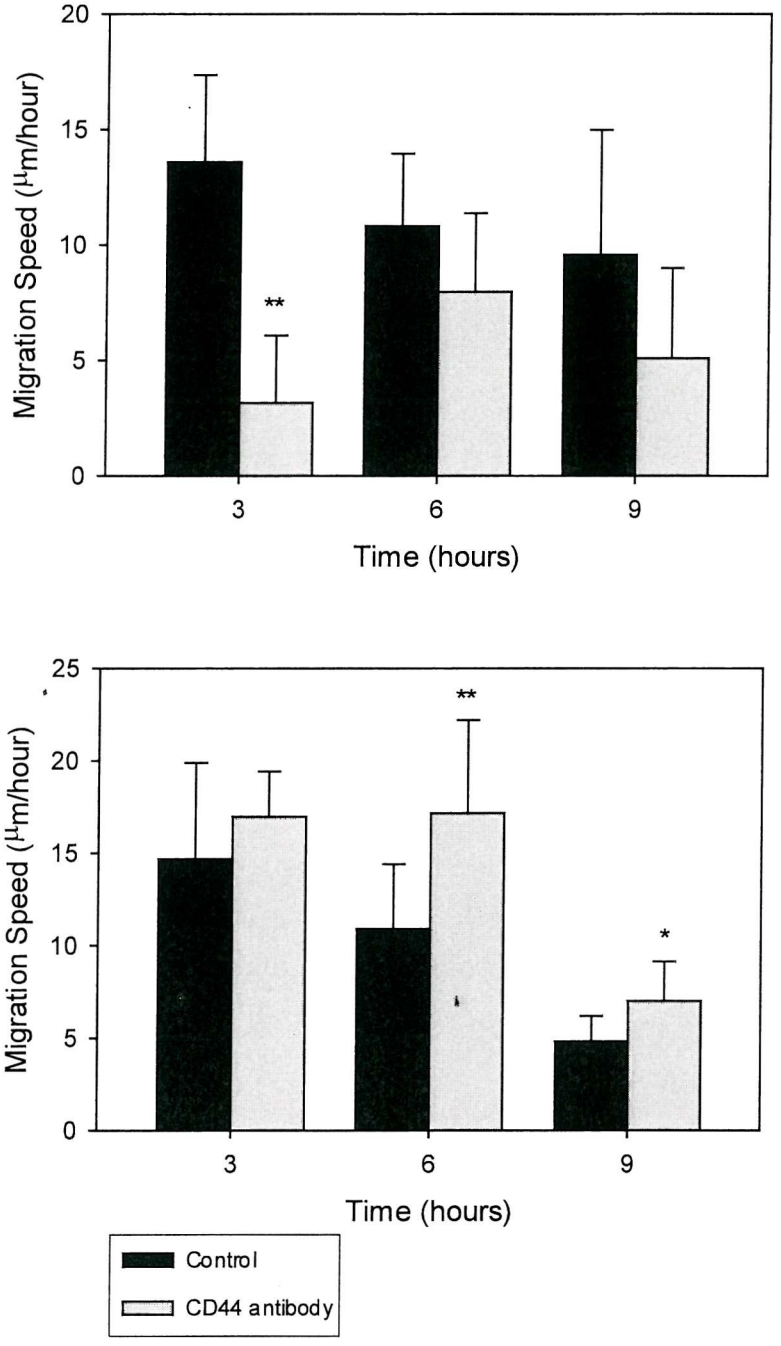


Figure 6-9. CD44s antibody inhibits cell migration. Confluent 16HBE 14o$^-$ cultures were mechanical damaged with a pipette tip. CD44s or isotype control antibody were added into the culture medium with the culture. Every 3 hours the decrease in surface area of the damage area was measured, and culture media were changed with fresh medium with CD44s antibody. CD44s antibody at a concentration of 50 μg/ml (A), 25 μg/ml (B).

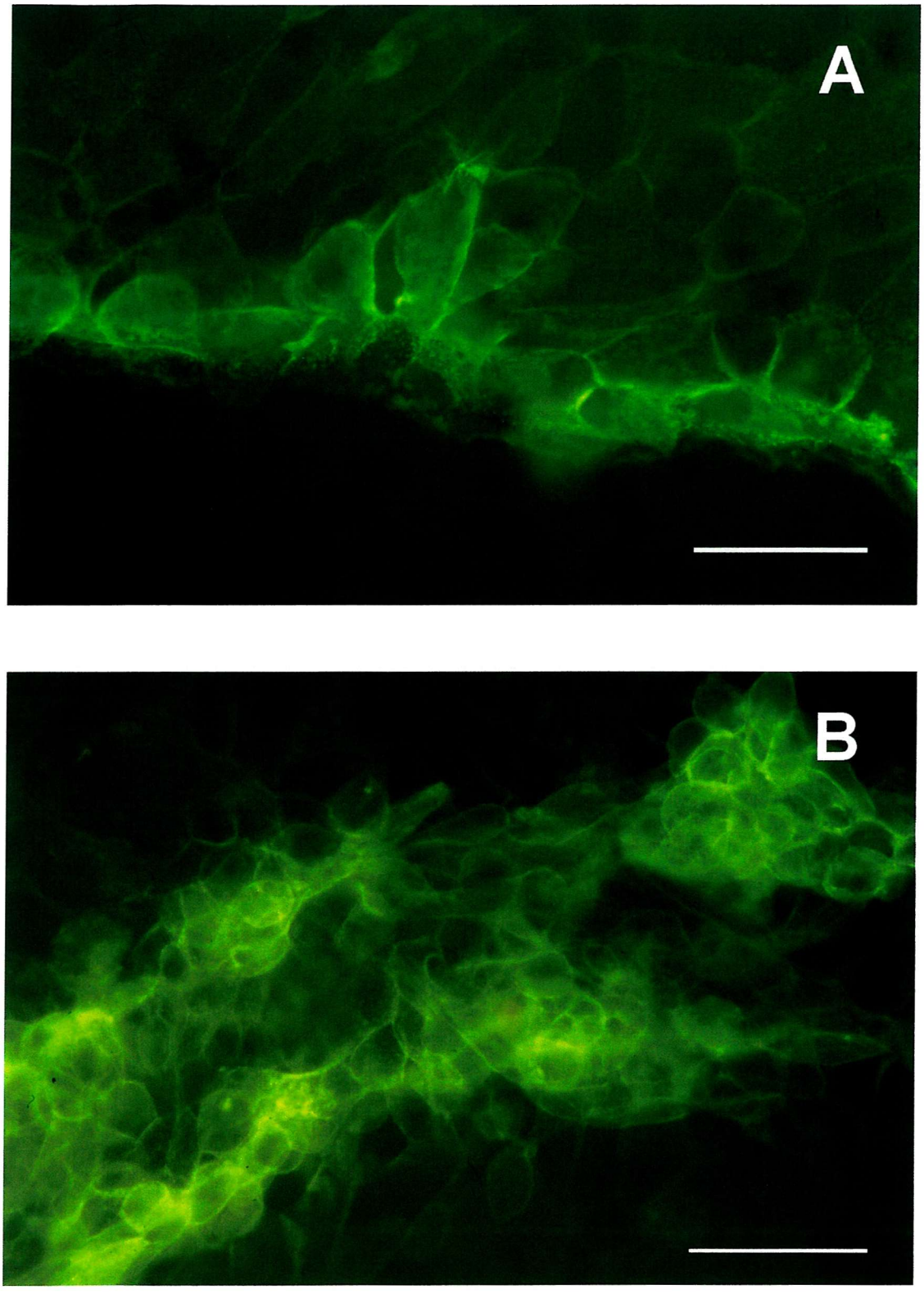


Figure 6-10. Localisation of CD44 blocking antibody to damage epithelial cells. Blocking CD44s antibody was binding to damage areas of epithelial culture. (A) 6 hours after damage (B) 12 hours after cell damage. Scale Bars, 50 μm.

Chapter 7
General Discussion

The lung is regularly challenged by infectious, and potentially life-threatening microorganisms. The immunocompetent individual usually has the capacity to fend off these pathogens. The remarkable effectiveness of the lung in resisting infection has been attributed to its triad of defensive systems. The first line of innate defence consists of the aerodynamic filtration system of the upper airways and the mucous lining of the lower airways, effectively transporting intruding microorganisms away from the lung. If, however, infectious organisms or pathogens break this defensive perimeter, then as the second line of defence, a large number of neutrophils are rapidly summoned to the site by cytokines secreted from lipopolysaccharide activated macrophages. If the pathogen still survives the onslaught of recruited neutrophils, the third line of defence, local macrophages and neutrophils, is activated. Meanwhile lymphocytes of the adaptive immune system develop pathogen specific immune responses to provide a more versatile means of defence. Since there is a delay of 4-7 days before the initial adaptive immune response takes effect, the innate immune response has a critical role in controlling infection during this period. Bacterial or viral molecules binding to the surface receptors of macrophages and neutrophils induce the cells to secrete an array of mediators and cytokine, including leukotrienes and interleukins, which also provide the further protection against infection.

Normal airway epithelium implements mucociliary clearance and serves as an efficient barrier to prevent infection. In many infections, there is little or no residual pathology after an effective cellular and humoral immune response. In some cases, however, the response or infection causes tissue damage. Columnar epithelial cell shedding and increased inflammatory cells are present in the airways as seen in the airway of even mild asthmatic subjects (Jeffery *et al.*, 1989; Beasley *et al.*, 1989; Laitinen *et al.*, 1996). The airway epithelium can express adhesion molecules that could bind inflammatory cells migrating in from airway capillaries, mediate epithelial cell-cell contact, or basal cell binding to matrix substrates. Among these cell adhesion molecules, ICAM-1 is associated with increased influx and binding of both neutrophils (Tosi *et al.*, 1992a) and eosinophils (Wegner *et al.*, 1990) into airways. Expression of ICAM-1 can be induced by proinflammatory cytokines such as IFNγ and TNFα (Look *et al.*, 1992; Tosi *et al.*, 1992b; Bloemen *et al.*, 1993), and parinfluenza type 2 virus infection (Tosi *et al.*, 1992a). Another molecule, CD44

expressed on basal cells has putative function of presenting heparin-binding growth factor or cytokines (Tanaka *et al.*, 1993; Bennett *et al.*, 1995). The high expression of CD44 which has been reported to be correlated with areas of epithelial damage (Lackie *et al.*, 1997), is likely to be involved in early repair processes following airway epithelial damage.

The molecular mechanisms of airway damage and repair is only partly understood. Airway inflammation processes are present even at a clinically early stage of asthma (Laitinen *et al.*, 1996; Warner *et al.*, 2000) suggesting that the up-regulation of ICAM-1 and CD44 might be not any only outcome of epithelial damage but that they are also involved in the inflammatory processes of the disease.

7.1 Models of the airways: In vitro models of the in vivo system

One of the areas of study which would greatly benefit from a good model of the airway is asthma research. Animal airway models are problematic since lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are unique to human (Hulsmann and de Jongste, 1993). Some of the clinical features of human asthma can be displayed with antigen challenge in the animal model. However, it is important to be aware that because of the fundamental differences which exist between species, animal models fail to mimic the complexity of asthma in humans. Some fundamental differences in pathophysiological, physiological and pharmacological responses compromise the value of animals as substitute for human (Coleman, 1999). For instance, although guinea-pigs, rats, rabbits, sheep and mice all can demonstrate an asthma-like syndrome, some of the mediators involved in the response in animal models are different from that in human. For example, guinea pigs show that histamine is a key mediator in "asthma models" (Santing *et al.*, 1994), while rabbit and sheep studies indicate that PAF is an important mediator (Abbaham and Baugh, 1995). However, for neither of these has this key role been borne out in clinical asthma (Finnerty *et al.*, 1990; Ruffin and Latimer, 1991). Most animal models of asthma can mimic certain aspects of asthma, however, it is now general

agreed that asthma is not a single disease but a syndrome of similar symptoms but with different etiologies. Using an animal model for the study of airway disease needs to be carefully considered, because of functional and structural differences between species. Therefore, human airway epithelial culture could be a valuable model for the investigation of the mechanisms of the disease.

Human cell lines are easy to maintain in culture and reproducible between experiments. However, the clonality and lack of other types of cells as in tissue can be disadvantages of cell lines. To study the roles of ICAM-1 and CD44 in bronchial epithelial, the human bronchial epithelial cell lines 16HBE 14o$^-$ and NCI-H292 and primary epithelial cells were used. The major advantages for these studies were their characteristics in term of protein expression and epithelial function. For examples, NCI-H292 cells had a low level of ICAM-1 expression, while 16HBE 14o$^-$ cells possessed cilia, tight junctions and similar ICAM-1/CD44 localisation to that seen in normal bronchial epithelium. One of the alternatives was to use primary cells by culturing airway tissue from surgical resection, to give an *in vitro* model using cells similar to normal human airway epithelium.

7.2 The role of ICAM-1 in bronchial epithelium

7.2.1 In cell adhesion

When pathogens cross epithelial barriers and establish a local infection, the host must activate its defences and direct inflammatory cells to the site of pathogen growth. Such local inflammatory responses need to recruit and activate several circulating inflammatory cells. In airway inflammation, eosinophil accumulation in the airways is thought to be a critical feature that is correlated to disease severity (Virchow, Jr. *et al.*, 1994; Leff, 1994; Bousquet *et al.*, 1994; Makino and Fukuda, 1995). Extravasation of eosinophils to a site of inflammation consists of several sequential steps. On receiving the signals by chemokines from inflammatory sites, circulating eosinophils are recruited to the bronchial mucosa. These processes can be divided into three important steps: eosinophil-endothelial interaction and transmigration,

eosinophil-extracellular matrix interaction, and eosinophil-epithelial interaction. The mechanisms of the initial step of the eosinophil-endothelial interaction and eosinophil migration through endothelium has been well documented (Williams and Hellewell, 1992; Carlos and Harlan, 1994; Malik and Lo, 1996). The second step involves the eosinophil surface matrix protein receptors, such as Mac-1, VAL-4, VAL-6 and $\alpha 4\beta 7$, adhering to fibronectin and laminin (Walsh *et al.*, 1996; Montefort and Holgate, 1997). However, little is known about the mechanisms that facilitate eosinophil recruitment in the airway sub-mucosa.

Most of the recognised potential counterligands of the eosinophil CAMs (VCAM-1, E-selectin and P-selectin), which are expressed on endothelial cells, are not detected on bronchial epithelial cells (Walsh and Wardlaw, 1997). ICAM-1 is the only CAM known to date to mediate eosinophil adhesion to bronchial epithelium. Inflammatory cell binding to ICAM-1 can be mediated by LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) which are also expressed on neutrophils, lymphocytes and monocytes (Wegner *et al.*, 1990; Look *et al.*, 1992; Tosi *et al.*, 1992b). As described in chapters 3 and 4, my results showed that ICAM-1 expression was undetectable in normal bronchial epithelial cells, but can be up-regulated by pro-inflammatory cytokines such as IFNγ, TNFα and IL-1β. The increase in ICAM-1 in response to inflammatory signals indicated that ICAM-1 is associated with the immune response in airway inflammation.

Although ICAM-1 is the ligand of several adhesion proteins on leukocytes, the physiological meaning of the up-regulation of ICAM-1 expression on bronchial epithelial cells is only partly understood. Recent studies showed the CD18/ICAM-1-dependent pathway is important in eosinophil adhesion to cytokine-activated bronchial epithelial cells, and neutrophil adhesion to bronchial epithelial cells is also ICAM-1 dependent (Jagels *et al.*, 1999). The binding also increases when eosinophils or neutrophils are stimulated by proinflammatory signals associated with airway inflammation (Burke-Gaffney and Hellewell, 1998). These results revealed the importance of ICAM-1-dependent pathways in the cell adhesion during leukocyte recruitment, and leukocyte-mediated injury of mucosal epithelia under inflammatory conditions. However, since ICAM-1 is expressed apically on bronchial epithelial

cells, it is not likely to be necessary for the penetration of leukocytes across airway epithelium. In the activated condition, airway epithelial cells are an abundant source of cytokines for attracting both eosinophils and neutrophils into the airway lumen (Bedard *et al.*, 1993; Davies *et al.*, 1995). These chemokines can increase and direct leukocyte infiltration into inflammatory sites. The up-regulation of ICAM-1 could then facilitate local emigration of ICAM-1 ligand-bearing leukocytes into the airway lumen at the site of damage to provide defence against invading pathogens.

7.2.2 Viral infection and ICAM-1

In addition to its important role in inflammatory cell recruitment, ICAM-1 also plays a crucial role in rhinovirus infection of airway epithelial cells, since it is also the surface receptor for the major group of rhinoviruses (Staunton *et al.*, 1989; Greve *et al.*, 1989; Uncapher *et al.*, 1991). Respiratory viral infections have been reported to be associated with the majority of asthma exacerbation (Nicholson *et al.*, 1993; Johnston *et al.*, 1995), of which the majority of viral infections are rhinovirus infections (Nicholson *et al.*, 1993; Johnston *et al.*, 1995; Johnston *et al.*, 1996). Unlike other respiratory viruses rhinovirus infection does not cause extensive epithelial destruction (Turner *et al.*, 1984), even though the airway epithelial cell is the host cell for rhinovirus replication. Increase of ICAM-1 expression is seen on airway epithelium by allergen challenge in both nasal and bronchial epithelial cells (Bentley *et al.*, 1993; Ciprandi *et al.*, 1994). It is not clear if rhinovirus infection is confined to upper airway and increased asthma symptoms through an indirect mechanism, or if the viral infection of lower airway directly induces local inflammation. *In vitro*, rhinovirus infection also up-regulated ICAM-1 expression in human primary bronchial epithelial cells (Papi and Johnston, 1999). In *in vivo* studies, rhinovirus has been detected from bronchial bushings in subjects with experimental rhinovirus infection (Halperin *et al.*, 1983). Thus, the allergen increased expression of ICAM-1 on airway epithelial cells may modulate the immune response in the disease, while rhinovirus use the newly expressed ICAM-1 to facilitate further infection of other epithelial cells in the airway.

When airways are exposed to parainfluenza virus, the columnar cells but not the lower basal cells in mucosal layer are infected (Massion *et al.*, 1993). Human primary

tracheal epithelial cells increased ICAM-1 expression during parainfluenza virus infection (Tosi *et al.*, 1992a), which suggested that the expression of ICAM-1 on columnar cells may be associated with viral infection.

7.3 The role of CD44 in epithelium

The embryo develops from the cleavage of one single cell into many cells, which perform a series of cell movements. Some cells move as free individuals, others are bound together. One of the crucial processes is the formation of polarized epithelium, involves the generation of cell-cell junctions. CAMs, especially, E-cadherin and cell-cell junction, such as tight junctions, and desmosomes are thought to play important roles in the spatial organisation of epithelial differentiation, since they are found during embryo development from as early as the 8-cell to 32-cell stage (Collins and Fleming, 1995a). CD44 is present on oocytes (Campbell *et al.*, 1995a), and CD44 v3 and v6 have been reported to be involved in bud formation (Wainwright *et al.*, 1996; Sherman *et al.*, 1998). My results have demonstrated that CD44 mRNA is expressed during the 2-cell to 8-cell stage, and CD44 isoforms with variant exon-encoded sequences inserted are the predominant isoforms in the 2-cell stage embryos. The expression of mRNA encoding large CD44 isoforms in the 2-cell stage suggests that CD44 plays a role in the early stage of preimplantation embryo development.

Cell adhesion is an important event in normal and pathological processes. For instance, lymphocyte migration and inflammatory cell infiltration rely on the regulation of the adhesion mechanisms between cells through the interaction of cell surface adhesion molecules and their ligand(s) on other cells (Leung and Picker, 1997). During the epithelial repair process, the adherence of the leading edges of migrating cells with the basement membrane is also associated with cell adhesion.

The epithelial lining of the airway lumen also relies on CAMs to maintain its architecture. During repair of epithelial damage, following columnar epithelial cell shedding, non-ciliated epithelial cells at the border of the damage de-differentiate into flattened cells to rapidly cover the naked basement membrane (Evans and Plooper, 1988; Erjefält *et al.*, 1995). These cells spread and migrate leading to a repair process

which involves epithelial restitution, proliferation and differentiation. My results have demonstrated that ICAM-1 is inducible and apically expressed on bronchial epithelial cells by proinflammatory cytokines, which may associate with inflammatory cells adhering to the airway lumen. I was therefore interested to investigate another adhesion molecule, CD44 which is up-regulated in the damaged airway epithelium and may be involved in cell adhesion in inflammatory conditions (Lackie *et al.*, 1997). Firstly, I focused on the function of CD44 in bronchial epithelial cell adhesion, since cell adhesion is involved in epithelial repair processes. I have shown that CD44 was highly expressed on the edge of cells in sub-confluent cultures, and was increased in low cell density cultures. The function of CD44 in cell adhesion could be associated with cell migration in epithelial repair.

7.3.1 CD44 in cell adhesion

I demonstrated that CD44 is involved in bronchial epithelial cell adhesion to collagen I, IV, fibronectin and HA which are ligands of CD44 (Miyake *et al.*, 1990; Aruffo *et al.*, 1990; Jalkanen and Jalkanen, 1992; Faassen *et al.*, 1992; Knutson *et al.*, 1996) and major matrix substrates in the airways. Cell binding to collagen I, IV and fibronectin is partly CD44-dependent, while cell binding to HA is mainly through a CD44-dependent pathway. The importance of CD44 in cell adhesion is therefore associated with the substrate the cell is binding to.

The physiological importance of CD44/HA interaction is based on several lines of evidence. CD44 has been referred to as a homing receptor for human lymphocytes binding to Peyer's patch high endothelial venules (Jalkanen *et al.*, 1986). Cell adhesion to endothelium at the site is thought to involve the binding of CD44 and its ligands. Although other studies showed that endothelial cells from Peyer's patches and lymph nodes increased synthesis of glycosaminoglycans, no CD44 ligand was reported on the endothelial cells in this site. Recently Mohamadzaheh and colleagues (Mohamadzadeh *et al.*, 1998) have demonstrated that the HA expression on endothelial cells derived from microvascular is inducible by the proinflammatory cytokines, TNFα and IL-1β. The up-regulation of HA on endothelial cells could enable the recruitment of cells to an inflamed site, which supports the possibility in

inflammatory conditions, cell motility can be up-regulated through the mechanism of CD44/HA regulation and CD44/HA interaction. *In vivo*, an animal model also supports the suggestion that CD44/HA interaction initiates T cell extravasation into sites of inflammation (Estess *et al.*, 1998).

7.3.2 Cytokines-induce CD44 expression and epithelial adhesion

My observations of the effect of proinflammatory cytokines in epithelial adhesion indicate that: (1) IL-1 β, and IL-4 up-regulated CD44 expression in 16HBE 14o$^-$ cells and HPBEC. (2) TNFα, IL-1β, IL-4 and IFNγ increase CD44-dependent cell binding to HA. (3) This involved alternative splicing of CD44 mRNA, the v8-v9-v10 containing CD44 isoform was especially decreased.

Interestingly, my results demonstrated that the cytokine-induced cell adhesion to HA is not necessarily mediated by increasing the level of CD44 expression on the cell surface. IFNγ significantly increased CD44-mediated cell adhesion to HA without any increase of CD44 protein expression. Although TNFα, IL-1 β, and IL-4 increase cell surface CD44 expression, the increased level of CD44 is not correlated with the increase of CD44-mediated cell adhesion. Therefore, there is a surface CD44 level-independent mechanism which is also involved in modulating the function of CD44. The alternative splicing of CD44 variants may be one of the mechanisms to modulate the function of CD44 in this cytokine-induce CD44/HA binding. It is suggested that cytokines can induce several mechanisms that affect the functions of CD44 at the same time.

Using cell transfected with tailess CD44 showed that the cytoplasmic domain of CD44 is necessary for binding of HA (Thomas *et al.*, 1992; Lesley *et al.*, 1992). The cell binding to HA in these transfected cells can be activated by CD44-specific antibody, which suggested that multivalent CD44 was required for CD44-HA binding (Lesley *et al.*, 1993b; Perschl *et al.*, 1995). Studies have demonstrated that phosphorylation in the cytoplasmic domain of CD44 is needed for cell binding to HA (Puré *et al.*, 1995; Uff *et al.*, 1995; Peck and Isacke, 1998). Several regions of the cytoplasmic domain may contribute to HA binding by promoting clustering or conformational change of

CD44, since dimerisation of CD44 with an antibody eliminates the requirement for the cytoplasmic domain. Other studies have showed a phosphorylation-independent mechanism is involved in CD44/HA binding (Uff *et al.*, 1995). In addition, experiments using functionally incompetent-CD44 cells or phosphopeptides indicated that phosphorylation of the CD44 cytoplasmic domain is required for HA-dependent cell migration (Peck and Isacke, 1996; Peck and Isacke, 1998).

In hematopoietic cells, elevated levels of CD44 do not necessarily correlate with increased HA-binding, and thus the ability of CD44 to bind HA is not constitutive; rather, CD44 may require the alteration of its structure to engage this ligand (Lesley *et al.*, 1993a; Katoh *et al.*, 1995; Lesley *et al.*, 1995). The proinflammatory cytokine-TNFα has been shown to induce monocyte CD44 to bind HA, which involved modified glycosylation of CD44 (Levesque and Haynes, 1996). Regulation of CD44/HA-dependent adhesion in endothelial cells also involved the up-regulation of HA expression (Mohamadzadeh *et al.*, 1998). These studies suggest that not only can the modification of CD44 itself alter its functions, but also that environmental factors can modulate CD44-ligand binding *in vivo*.

7.3.3 CD44 in tumour metastatsis

Several lines of evidence have suggested CD44 that variants are involved in tumour metastasis. Günthert and colleagues first reported that transfecting CD44v isoform into non-metastasis rat pancreatic carcinoma cells can induce the cells to establish metastatic behaviour in an athymic mouse model (Günthert *et al.*, 1991). Other groups subsequently provided evidence that CD44v6 is correlated with metastatic potential (Rudy *et al.*, 1993; Seiter *et al.*, 1993). Studies using an antisense mRNA approach showed that down-regulating CD44 isoforms containing CD44v6 in the cell line HT29 reduced metastasis in nude mice (Reeder *et al.*, 1998).

In a tumour formation models have demonstrated that tumour growth can be suppressed by adding a soluble CD44-immunoglobulin fusion protein which can bind to HA (Sy *et al.*, 1992). This revealed that the CD44/HA interaction may be a crucial event during cell adhesion. Other studies also showed that CD44s/HA binding is

related to metastasis in a human melanoma cell line transfected with CD44s (Sy *et al.*, 1992; Bartolazzi *et al.*, 1994b). Studies using rat pancreatic carcinoma cells transfected with CD44 variant sequences encodes by exon v6 and v7, which present increased metastatic ability, demonstrated that the increase of HA binding might be mediated by clustering of CD44 (Sleeman *et al.*, 1996). Alternative splicing of CD44 variant isoforms may also increase the CD44/HA interaction.

These results reveal that CD44v6 is associated with tumour metastasis in many tumour models, and also that alternative splicing of CD44 variants into CD44 isoform may modulate the functions of CD44. My results indicated that the percentage of CD44 v3 and CD44v6 positive cells was increased in low cell density cultures. This cell density-dependent CD44v3 and CD44v6 expression in the cells suggest that CD44 variants might be associated with cell locomotion. This cell locomotion facilitated by CD44 might enhance the metastatic ability of cells.

7.4 Cell Motility

Cell locomotion is powered by actin polymerisation (Condeelis, 1993) when rapid actin filament turnover is co-ordinated to generate cell movement. The interaction between cytoskeletal proteins and ECM-binding proteins on the cell surface regulate cell pathfinding and motility (Sheetz *et al.*, 1998). The actin polymerisation at the leading edge and depolymerisation at the rear of lamella helps in determining the direction of cell movement. The motile response is mediated by a highly integrated signalling cascade that transduces external signals to the actin cytoskeleton to induce actin polymerisation in appropriate parts of the cell. Many molecules, such as small GTPases of the Rho family, act as molecular regulators of actin assembly and control the formation of focal adhesion, lamellipodia, and filopodia (Kozma *et al.*, 1995; Nobes and Hall, 1995).

When the lamellipodia, and filopodia adhere to basement membrane, CAMs in their plasma membrane, such as integrins create a robust anchorage for the actin filaments

inside the motile cells (Huttenlocher *et al.*, 1995). CD44 is also highly expressed on lamellipodia, and filopodia in fibroblasts (Svee *et al.*, 1996) and murine carcinoma cells (Ladeda *et al.*, 1998). It has been reported that CD44 associates with ankyrin (Kalomiris and Bourguignon, 1988; Kalomiris and Bourguignon, 1989) or ERM proteins (Tsukita *et al.*, 1994) both of which are thought to function as general cross-linkers between plasma membrane proteins and actin filaments. Cross-linking of CD44 by antibody can induce actin polymerisation in the cell (Fujisaki *et al.*, 1999), which could be consistent with an association of such cross-linkers with CD44.

7.4.1 CD44 and cell migration in repair

In the airway inflammatory diseases, such as asthma, bronchial epithelial cell shedding reduces cell density and the increase of inflammatory cytokine production may up-regulate the expression of CD44 or modulate it function. There are several lines of evidence from my results that suggest CD44 is involved in epithelial processes: (1) CD44 was increase in the epithelial cells after mechanical damage; (2) after damage, CD44 was highly expressed on the cells along the damaged areas; (3) antibody against CD44s was able to inhibit cell migration, and the blocking antibody was binding to the highly migrating cells. My results suggest that CD44 was also involved cell migration after mechanical damage. HA levels in BAL fluids are also increased in persistent asthmatic patients (Vignola *et al.*, 1998). I propose that the CD44/HA interaction participates in bronchial epithelial cell repair through: (a) increase of both HA and CD44 binding in damaged areas (b) induction of these high CD44 expressing epithelial cells which migrate and cover the damaged area; (c) initiating the interaction of CD44 and other proteins (eg. MMPs) in airway repair (see below). The abundance of HA and collagen IV in the basement membrane of airways suggested that CD44 interaction with HA could regulate cell motility.

7.4.2 Cell density

My results showed that the expression of CD44 in bronchial epithelial cells is inversely correlated with cell density, suggesting that cell-cell contact at high cell density down-regulates CD44 expression. The cells at the edges of sub-confluent

cultures which also have a free surface have high CD44 expression which also supports the suggestion that CD44 down-regulation is associated with cell-cell contact. The loose contact of cells at low cell density reflects high cell motility, in which cells increase the expression of CD44 on their surface. Once cells are confluent, they are in a sedentary state which is associated with the down-regulation of CD44 expression.

7.4.3 CD44 and MMP-9

Tumour invasion into tissue is associated with increased MMPs expression (Stetler-Stevenson *et al.*, 1993). Studies have reported that CD44 associates with MMP-9 on mouse and human tumour cells (Bourguignon *et al.*, 1998; Yu and Stamenkovic, 1999). The colocalisation of CD44 and MMP-9 and the decrease of MMP-9 activity by addition of soluble CD44 suggested that there are functional interactions between CD44 and MMP-9. A recent study also showed that CD44-MMP-9 complex can activate latent TGF-β (Yu and Stamenkovic, 2000). This finding suggested that interactions of these proteins may provide physiological mechanism regulates tissue remodelling, and changes in these are associated with tumour growth and invasion. Thus a CD44-dependent mechanism may allow tumour cells to invade tissue and survive in a new environment. It is possible that MMP-9-dependent ECM degradation may be mediated by CD44, which is required for cell migration.

7.5 The functions of CD44

It is possible that the stringent regulation of CD44/HA interaction in the epithelial repair processes might be controlled by levels of both CD44 and HA. To maintain homeostasis, it would be expected that to retain airways in a non-inflamed state would result in resetting of HA and CD44 expression levels back to baseline. High and low molecular weight HA can induce conflicting inflammatory responses, such as HA fragment can induce IL-8 expression in macrophages and MCP-1 expression in renal epithelial cells (McKee *et al.*, 1996; Beck-Schimmer *et al.*, 1998; Neumann *et al.*, 1999). The accumulation of HA in angiogensis (Trochon *et al.*, 1996), wounds

healing (Jain *et al.*, 1996), and embryonic cell migration (Wainwright *et al.*, 1996; Sherman *et al.*, 1996) reveal that the involvement of CD44 in cell motility, tissue structure, and pattern formation might be through the regulation and binding of HA.

My proposal that CD44 has an important role in bronchial epithelial damage and repair, are based on these characteristics:

(1) In situations where epithelial cells migrate, like low cell density and after epithelial damage, CD44 is increased. Its up-regulation is associated with cell adhesion and migration on the ligands of CD44. This also includes the up-regulation of CD44 by proinflammatory cytokines which induce CD44-mediated cell binding.

(2) In sedentary cells, as seen in normal epithelium of bronchial tissue, the expression of CD44 is low.

In epithelial cells, the existence of large number of CD44 isoforms increases the difficulty of studying their functions. The increase of surface CD44 protein in low cell density and ligand binding affinity induced by proinflammatory cytokine, and the association with cell migration, reveal that the role of CD44 in cell adhesion and migration may be related to the status of the cells.

In these studies I have investigated the role of ICAM-1 and CD44 in bronchial epithelium by studying their localisation, regulation and function. The results suggest that the up-regulation of ICAM-1 and CD44 is involved in airway inflammation. The cell density-dependent expression of CD44, the ability of CD44 antibodies to inhibit cytokine-induced cell binding to HA, and inhibit cell migration after mechanical damage demonstrate that CD44 is functionally involved in epithelial repair in airways. I have found increased CD44 protein expression or changes in the alternative splicing of CD44 isoforms during the repair of epithelial damage. Questions remain at the biochemical level about how the altered CD44-ligand interaction and function of CD44 may occur, and if it is through altered variant exon inserted, modified glycosylation, or phosphorylation of the cytoplasmic domain. This study has begun to systematically describe the sub-cellular expression and regulation of ICAM-1 and CD44, and to thereby provide some indication of the functions of these CAMs in human bronchial epithelial cells.

APPENDICES

Appendix 1

Reagents

Supplier / Reagent (abbreviation)	Catalogue number
Boehringer Mannheim, East Sussex, UK	
DNA molecular weight marker XIV(100bp)	1 721 933
CLONTECH Laboratories, UK	
pGFP Plasmid	6097-1
DAKO Ltd., Bucks, UK	
Mouse IgG1	X0931
Mouse IgG2a	X0943
Mouse IgG2b	X0944
Rabbit anti-mouse IgG (H+L) FITC-conjugated $F(ab')_2$ fragment	F0313
The European Collection of Cell Culture, Porton Down, UK	
Anti-CD44 standard (CD44s) hybridoma	25.32
Anti-CD44v6 hybridoma	FW11.9.2.2
Anti-CD44v9 hybridoma	FW11.24.7.36
Genzyme, Kent England	
Human IFNγ (4.75×10^4U/μg)	80-3348-01
Human TNFα (2.0×10^4U/μg)	TNF-H
Gibco BRL, Paisley, UK	
LHC-9 medium	
Medium 199	21181-029
Minimum Essential Medium (MEM)	32360-026
Nutrient mixture Ham's F-12 (F12)	11760-022
RPMI-1640 medium	21875-034
Foetal calf serum, inactive (FCS)	

Sera substitute Ultroser® G (USG)	091-25950H
10X Hank's balanced salt solution (HBSS)	14180-046
10X Ca^{2+} and Mg^{2+}-free HBSS	14060-040
10X Trypsin-EDTA	35400-027
Moloney Murine leukaemia virus reverse	28025-013
TRIzol® Reagent	15596
100 bp standard	

Harlow Chemical Company Ltd, Harlow, Essex, UK

Mowiol	4-88A

May&Baker Limited, UK

AuTM (Myocrisin)	AUST R 27518

MWG BIOTECH AG, Ebersberg, Germany

Primers (see *section 2.7.3, Table 2-2*)	

New England Biolabs, Inc, UK

$Vent_R$ DNA polymerase	254S
T4 DNA polymerase	203S

Pharmacia Biotech, UK

Ultrapure dNTP set 2'-Deoxynucleoside 5'-	27-2035-01

Promega, UK

Calf Intestinal Alkaline Phosphatase (CIAP)	M183A
CellTiter 96® AQ_{ueous} assay	G5434
Hpa I	R630A
Recombinant Rnasin® Ribonuclease Inibitor	N2511
T4 DNA ligase	M180A
T4 Polynucleotide Kinase (10u/μl)	M4101
taq DNA polymerase	M1665

Qiagen Ltd, Crawley, UK

QIAquick gel extraction kit	28704
QIAquick PCR purification kit	28160

R & D Systems Europe Ltd., Abingdon, UK

Anti human CD44 3v antibody, clone 3G5	BBA11
Human IFNγ (2.0×10^4 U/μg)	285-IF
Human IL-1β (2.0×10^5 U/μg)	201-LB

Human IL-4 (2.9 x 10^4 U/μg)	204-IL
Human TNFα (1.1 x 10^5 U/μg)	210-TA

Serotec Ltd ,Oxford, UK

anti-Human CD54 (ICAM-1), FITC labelled	MCA532F
anti-Human CD54 (ICAM-1)	MCA532

Sigma, Dorset UK

Bovine serum albumin (BSA)	A-7906
Cell dissociation solution, non-enzymatic	C-5789
Epidermal growth factor (EGF)	E-4127
Fibronectin	F-0895
Gold conjugated goat anti-mouse IgG	G-7777
Guanidine Thiocyanate	G-9277
Hyaluronic acid	H-1751
Laminin	L-6274
Monoclonal anti-cytokeratin 13 antibody	C-0791
Monoclonal anti-cytokeratin 18 antibody	C-8541

Appendix 2

Solution

Fixation Solution

3g paraformaldehyde (electron microscopy-grade)

100 ml PBS

Heat to 60°C in a fume hood. Cool to RT and adjust pH to 7.2 with 0.1M NaOH

Aliquot and stock at -20°C

2% gelatin plates

Gelatin	2.0 g
PBS	100ml

Heat to dissolve gelatin in a microwave oven. Cool down to ~50 °C

Pull to 40 mm petri dishes at 3 ml/dish. Keep at 4 °C

Phosphate-buffered saline (PBS)

0.23 g NaH_2PO_4 (anhydrous) (1.9mM)

1.15 g Na_2HPO_4 (anhydrous) (8.1mM)

9.00 g NaCl (154mM)

Add H_2O to 900 ml. Adjust pH to 7.2 using 1M NaOH or 1M HCl

Add H_2O to 1 liter

Loading buffer (for PCR)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Ficoll 400	15 ml
H_20	100 ml

Luria-Bertani (LB) broth

Trypton (Difco)	5.0 g
Yeast Extract (Difco)	2.5 g
NaCl	2.5 g
H_20	500 ml

1. Adjust to pH 7.5 with 1M NaOH
2. Autoclave at 15 psi for 15 minutes

Luria-Bertani (LB) Plates with ampicillin

Trypton (Difco)	5.0 g
Yeast Extract (Difco)	2.5 g
NaCl	2.5 g
Agar	7.5 g
H_20	500 ml

1. Adjust to pH 7.5 with 1M NaOH
2. Autoclave at 15 psi for 15 minutes
3. Cool down to 60 ^{o}C in water both
4. Add ampicillin to final concentration 100µg/ml
5. Pull to 90 mm petri dishes

TAE buffer (50X)

Tris base	242 g
EDTA	18.6 g
H_20	1000

Adjust with glacial acetic acid to pH 7.2

Trypan blue solution

0.25 % trypan blue in medium (with 2%FCS and 0.1%NaN_3)

0.45 µm filtered sterile

Appendix 3

A. Phosphorlation of DNA.

The insert DNA was phosphorylated by folling protocal:

cDNA (from gel electrophoresis)	5 µl
ATP/Buffer (6.1 mg/ml)	1 µl
BSA (1mg/ml)	1 µl
DTT (15.8 mg/ml)	1 µl
T4 Polynucleotide Kinase (10u/µl)	1 µl
H_20	1 µl
final volume	10 µl

Incubate at 37 °C for 60 minutes, then 90 °C for 15 minutes.

B. Vector Digest

Plasmid (pGFP), 1 µg	2 µl
Hpa I, 10 u/µl	1 µl
Buffer (J)	2 µl
Nuclease-Free Water	15 µl
final volume	20 µl

Incubate 37 °C for 60 minutes

C. Dephosphorylation of vector

With a single enzyme digestion, the ends of the prepared vector are identical. It is advantageous to treat the vector with Calf Intestinal Alkaline Phosphatase (CIAP) to remove the phosphate groups from the 5'-end to prevent self-ligation of the vector.

Digested vector from ***B***	20 µl
CIAP	2 µl
CIAP buffer	5 µl
DEPC Water	13 µl
final volume	50 µl

Incubate at 37 °C for 30 min. To stop the reaction, heat at 55 °C for 15 min

D. Ligation of plasmid vector and insert DNA

Insert DNA	3 µl
Vector	2 µl
ATP/Buffer (6.1 mg/ml)	1 µl
BSA (1mg/ml)	1 µl
DTT (15.8 mg/ml)	1 µl
T4 DNA ligase	1 µl
DEPC Water	1µl
final volume	10 µl

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