Department of Medicine, Division of Respiratory Medicine
Karolinska Institutet, Stockholm, Sweden

Platelets and Eosinophils in Lung Tissue Remodelling

Ulrika Zagai

ABSTRACT

Tissue injury and inflammation followed by effective repair restores normal function, but defective repair with associated tissue remodelling and fibrosis can lead to loss of organ function. Thus, interactions between inflammatory and mesenchymal cells in connection with the remodelling processes are of considerable importance with regards to the development of pulmonary disorders, since this interplay determines the outcome of the disease. At present, no clinical treatment for fibrosis is available and it is of considerable importance to improve our knowledge concerning the mechanisms that lead to tissue remodelling and fibrosis in order to develop effective therapeutic strategies. The present thesis was designed to explore the impact of two important inflammatory cells, platelets and eosinophils, on remodelling of lung tissue employing *in vitro* systems.

Data are based on experimental models such as fibroblast-mediated contraction of collagen gels, which reflects the contractile process typical of tissue remodelling. In this model human lung fibroblasts are cultured in an artificial lung tissue consisting of type I collagen. The advantages of such a system are that the cells are allowed to spread in three dimensions as fibroblasts *in vivo* and, furthermore, demonstrate more *in vivo*-like functional properties. To monitor fibroblast recruitment, which is also an important step in the remodelling process, we employed Boyden chambers with type I collagen coated filters.

Platelets and eosinophils were cultured together with fibroblasts in collagen gels to explore the influence of these cells on gel contraction. Both platelets and lysate thereof stimulated fibroblast-mediated contraction of collagen gels and both PDGF and TGF-β contributed partially to this effect.

Furthermore, both peripheral blood eosinophils and eosinophil-like differentiated HL-60 clone 15 cells stimulated the fibroblast-mediated collagen gel contraction. ECP was one of the proteins produced by eosinophils involved in this interaction between eosinophils and lung fibroblasts. Moreover, ECP stimulated the release of TGF-β_1 by both monolayer and three-dimensional cultures of lung fibroblasts. ECP also enhanced the level of TGF-β_1 mRNA in these lung fibroblasts.

Both media from cultures of peripheral blood eosinophils and ECP (native and recombinant) alone stimulated the migration of lung fibroblasts, effects that were attenuated by neutralising antibodies directed towards ECP.

The present thesis highlights the ways in which platelets and eosinophils can influence fibroblasts and the extracellular matrix *in vitro*. Based on our findings, we propose that platelets and eosinophils participate in tissue remodelling *in vivo*. The results documented here offer some possible explanations and mechanisms with regards to how these inflammatory cells may contribute to defective tissue repair, fibrosis and impaired pulmonary function.

LIST OF PUBLICATIONS

I. **Zagai U**, Fredriksson K, Rennard SI, Lundahl J, Sköld CM. Platelets stimulate fibroblast-mediated contraction of collagen gels. *Respir Res* 2003; 4: 13.

II. **Zagai U**, Sköld CM, Trulson A, Venge P, Lundahl J. The effect of eosinophils on collagen gel contraction and implications for tissue remodelling. *Clin Exp Immunol* 2004; 135: 427-433.

III. **Zagai U,** Dadfar E, Lundahl J, Venge P, Sköld CM. Eosinophil Cationic Protein stimulates TGF-β_1 release by human lung fibroblasts *in vitro. Inflammation 2006, in press*.

IV. **Zagai U,** Lundahl J, Klominek J, Venge P, Sköld CM. Eosinophil cationic protein stimulates migration of human lung fibroblasts *in vitro. Manuscript in preparation*.

These papers will be referred to in the text by their Roman numerals.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
CM	Conditioned medium
COPD	Chronic obstructive pulmonary disease
CTAB	Cetyldimethylethyl ammonium bromide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophil peroxidase
EPX/EDN	Eosinophil protein X/Eosinophil-derived neurotoxin
FEIA	Fluoroenzymatic immunoassay
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyante
GMA	Glycolmethacrylate
GM-CSF	Granulocyte/macrophage-colony stimulating factor
HFL1	Human foetal lung fibroblasts
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
LAP	Latency associated peptide
LPA	Lysophosphatidic acid
MBP	Major basic protein
MCP	Monocyte chemotactic protein
MHC	Major histocompability complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
NK	Natural killer
PAF	Platelet activating factor
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PMD	Piecemeal degranulation
RANTES	Regulated upon activation normal T-cell expressed and secreted
SMA	Smooth muscle actin
SPARC	Secreted protein acid and rich in cysteine
TGF	Transforming growth factor
TIMP	Tissue inhibitors of matrix metalloproteinases
TNF	Tumor necrosis factor
TSP	Thrombospondin
TX	Thrombaxane
UIP	Usual interstitial pneumonia
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

1 INTRODUCTION

Tissue injury initiates a process of wound repair, which normally leads to healing and re-generation of the tissue. However, an impaired repair can result in remodelling of the extracellular matrix (ECM) and loss of tissue function (Figure 1). One consequence of such remodelling is fibrosis, which results in the tissue becoming thick and inelastic due to excessive deposition of matrix components. In the case of the lung, fibrosis may impair pulmonary function. A number of diseases of the lung are characterised by varying degrees of fibrosis, e.g., acute respiratory distress syndrome (ARDS), asthma, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF), which are all diseases with a various degree of inflammation.
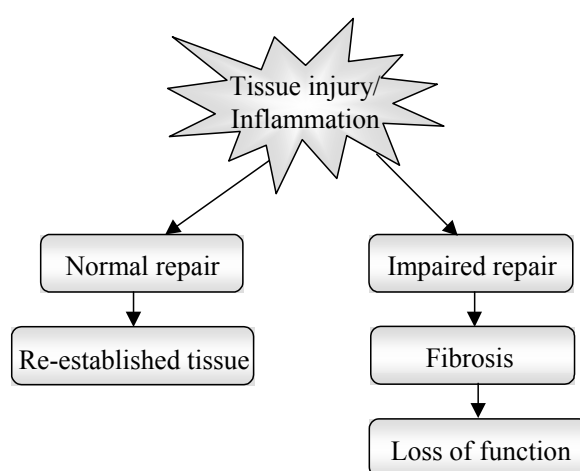
Figure 1. *Tissue injury initiates wound repair. Normal repair leads to re-generation of tissue architecture whereas a defective repair may result in fibrosis and loss of organ function.*

In the case of ARDS, which is characterised by leakiness of capillary membranes in the alveoli, platelets are initially sequestered in the pulmonary microvasculature, where they release substances that promote vaso- and bronchoconstriction. Histologically, the initial phase of ARDS is associated with an intense inflammation in the lung that may subsequently develop into pulmonary fibrosis. These observations suggest that platelets may be involved in the fibrotic response.

In connection with asthma, which is characterised by reversible obstruction of airways and chronic eosinophilic inflammation, subepithelial fibrosis is present in the airways. Eosinophils, which remain after the acute inflammatory response has subsided, produce a number of cytotoxic substances and cytokines which, when released, can promote inflammation and cause tissue injury. Therefore, these cells may play a role in the tissue remodelling and fibrosis associated with asthma.

At present, no clinical treatment for fibrosis is available and it is of considerable importance to improve our knowledge concerning the mechanisms that lead to tissue remodelling and fibrosis in order to develop effective therapeutic strategies.

1.1   Inflammation and inflammatory cells

Inflammation is a defensive response to, e.g., microbes, toxins or other foreign material. The clinical criteria for inflammation are; *rubor* (redness), *calor* (heat), *tumor* (swelling), *dolor* (pain) and *functio laesa* (loss of function). This non-specific response prevents the causative agent from spreading to other organs and prepares the wound for healing and tissue repair. The activated phagocytes which engulf microorganisms also secrete proteins called cytokines that exert a broad range of effects on other cells, which may enhance the inflammatory response (Janeway, 1999).

1.1.1   GRANULOCYTES

Granulocytes exhibit a nucleus with several lobes and cytoplasmic granules. These cells are approximately 10 μm in diameter and remain in the circulation from a few hours to a few days.

1.1.1.1   *Eosinophils*

Eosinophils normally represent only 2-4% of the total number of leukocytes in the circulation (Ganong, 1997). These cells circulate in the blood with a half-life of 18 hours before entering various tissues, which serve as their primary site of localisation. Eosinophils are most abundant in the mucosa of the respiratory system, lower urinary tract and gut.

Migration of eosinophils into extravascular compartments is initiated by the expression of the integrin very-late-antigen (VLA)-4, which binds to vascular cell adhesion molecule (VCAM)-1 on the surface of endothelial cells (Seminario, 1997). In addition, the eosinophil-specific CC chemokines eotaxins 1 and 2 play an important role in recruiting these cells to sites of inflammation (Wilson, 2001). The proliferation and maturation of eosinophils is regulated by the cytokines granulocyte/macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5. Moreover, these cells are activated by GM-CSF, IL-5 and platelet-activating factor (PAF).

Eosinophils perform two major functions: first, upon activation the cells can release a number of potent substances, including free radicals, highly toxic granule proteins, cytokines and proteases. Indeed, the most characteristic morphological feature of eosinophils is perhaps their membrane-bound specific granules containing the eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil protein X/eosinophil-derived neurotoxin (EPX/EDN), all of which effectively kill parasites and microorganisms. However, when activated in connection with other inflammatory disorders, such as allergic reactions, the release of these proteins may cause tissue damage. Secondly, eosinophils also produce and secrete prostaglandins, leukotrienes and cytokines (IL-3, IL-5, IL-6, IL-8 and GM-CSF) which facilitate the recruitment of additional cells and amplify the inflammatory response (Wilson, 2001).

There are several different patterns of degranulation of eosinophils. In the airway tissue cytolysis and piecemeal degranulation (PMD) of eosinophils have been observed *in vivo* (Erjefalt, 2000). Eosinophil cytolysis is characterised by chromatolysis, loss of plasma membrane integrity and release of membrane-bound specific granules that in turn release their protein contents. Cytolytic eosinophils and granule protein are found among intact eosinophils in inflamed airway mucosa and are not only a part of accidentally induced cell damage or tissue necrosis. Without losing viability, tissue eosinophils may undergo PMD resulting in partially or

completely empty specific granules. Investigations have revealed that eosinophil cytolysis occur in eosinophils that have exhibit a relatively low degree of PMD. This support the notion that eosinophil cytolysis and PMD are two distinct mechanisms for granule protein release in the airways (Erjefalt, 1998).

In association with parasitic disorders, as well as in allergic and asthmatic patients, the numbers of eosinophils are elevated. In the case of asthmatics enhanced numbers of detected eosinophils are in the peripheral blood, bronchoalveolar lavage (BAL) fluid, airway biopsies and in sputum (Kirby, 1987; Wardlaw, 1988; Beasley, 1989; Djukanovic, 1990; Griffin, 1991; Janson, 1992; Hargreave, 1993). This increase is due to enhanced recruitment of eosinophils from the blood into extravascular compartments in combination with attenuated apoptosis in the tissues (Simon, 1995; Woolley, 1996; Walsh, 1997). The decreased frequency of apoptosis among eosinophils from allergic patients may be due to the presence of high levels of survival factors for these cells, such as IL-5 and GM-CSF, (Simon, 1995; Woolley, 1996; Kankaanranta, 2000).

Elevated numbers of viable inflammatory cells aggravate tissue injury and impaired resolution of the inflammatory reaction and eosinophils have been proposed to play a role in the development of pulmonary fibrosis (Gharaee-Kermani, 1998). Interestingly, large numbers of eosinophils have been observed at the edges of wound healing and, furthermore, these cells produce the immunomodulatory and fibrogenic cytokine insulin-like growth factor (IGF)-1 (Todd, 1991; Wilson, 2001) and release transforming growth factor (TGF)-β and monocyte chemotactic protein (MCP), which are involved in the recruitment of fibroblasts (Gharaee-Kermani, 1998). Thus, eosinophils represent a linkage between acute inflammation and tissue repair.

1.1.1.2   Neutrophils

Neutrophils are the most numerous of the circulating granulocytes, accounting for 50-70% of all the white blood cells. With an average half-life of six hours, these cells are produced and lost in large numbers every day (Ganong, 1997). The essential role of neutrophils is to recognise and phagocytise both opsonise (bacteria coated with antibodies) and other foreign material. Accordingly, integrins present on the surface of these cells recognise several microbial substances, including bacterial lipopolysaccharide.

1.1.1.3   Mast cells

Mast cells, which are found in regions rich in connective tissue and abundantly below epithelial surfaces play a major role in allergic reactions (Ganong, 1997). The granules of these cells contain proteases, cytokines, histamine and other active amines, which are released when antigens recognised by type E immunoglobulins (IgE) bind to their surfaces (Hart, 2001; Boyce, 2003). For example, mast cells secrete IL-4, which up-regulates expression of VCAM-1, inhibits apoptosis in eosinophils and promotes eosinophilic inflammation by inducing enhanced expression of eotaxin (Steinke, 2001).

Mast cells can also be important effector cells in tissue repair/fibrosis and increased numbers of mast cells are found in several fibrotic diseases (Puxeddu, 2004). In the lung, mast cells have been suggested to play a regulatory role in idiopathic lung fibrosis, radiation-induced fibrosis and asthma.

1.1.1.4 Basophils

Accounting for approximately 0.5-1 % of the leukocytes in the circulation basophils, play a role in allergic responses by releasing active substances from their cytoplasmic granules. For instance, in connection with a type I hypersensitivity reaction, plasma cells secrete IgE antibodies which bind with high affinity to receptors on both tissue mast cells and blood basophils. This binding causes the basophils and mast cells to degranulate, giving rise to effects such as vasodilation and smooth muscle contraction.

1.1.2 MONONUCLEAR PHAGOCYTES

Monocytes normally constitute 3-10% of all circulating white blood cells, but their numbers may temporarily be elevated 2-3-fold during an acute inflammation (van Furth, 1988). Following their continuous production by the maturation of circulating monocytes, macrophages migrate into tissues throughout the body (Janeway, 1999), where their primary task is to remove from foreign material and invading bacteria. Large numbers of macrophages are present in the lungs and in the gastro-intestinal tract. Alveolar macrophages, which are the most accessible macrophages, being easily recovered by BAL demonstrate a life-span of approximately 80 days (du Bois, 1985; Marques, 1997). In addition to eliminating foreign substances, the alveolar macrophages contribute inflammatory reactions caused by inhaled exogenous material (e.g. in connection with smoking) as well as to a variety of other pathological processes, including alveolitis (Agostini, 1990; Sibille, 1990).

1.1.3 LYMPHOCYTES

The phagocytes of the innate immune system described above can recognise and eliminate many infectious organisms. The cells of the adaptive immune system, which alone can recognise a pathogen from an earlier infection, render our immunological defences even more effective (Janeway, 1999).

1.1.3.1 T lymphocytes

T lymphocytes are categorised as either CD4 (helper) or CD8 (cytotoxic) T cells. The CD4+ T cells, further subdivided into Th1 and Th2 cells, recognise extracellular pathogens that are presented by the class II major histocompability complex (MHC II). T helper cells also activate other cells by secreting cytokines. Both Th1 and Th2 cells secrete IL-3 and GM-CSF. In addition, Th1 cells secrete IL-2, interferon (IFN) -γ and tumour necrosis factor (TNF) -α whereas Th2 produce interleukins 4, 5, 6, 10 and 13. CD8 cells primarily recognise antigen presented by MHC I on the surface of virus-infected cells, thereby becoming stimulated to kill the infected cell (Romagnani, 1995). In patients with COPD the total numbers of T lymphocytes in the lung parenchyma and the peripheral and central airways are elevated, with the elevation in the numbers of CD8+ T cells being most pronounced (O'Shaughnessy, 1997; Saetta, 1999).

1.1.3.2 B lymphocytes

Although B lymphocytes can bind antigens directly, this ability is dependent on their stimulation by activated Th2 cells. Activated B cells proliferate and differentiate into plasma cells, which secrete large numbers and amounts of antibodies directed specifically towards various antigens into circulation (Ganong, 1997).

1.1.3.3 NK cells

The class of lymphocytes known as natural killer (NK) cells respond to intracellular microbes by killing the infected cells and producing the macrophage-activating cytokine IFN-γ. NK cells constitute approximately 10% of the lymphocytes present in the blood and the peripheral lymphoid organs (Janeway, 1999).

1.1.4 RED BLOOD CELLS

In addition to carrying oxygen and carbon dioxide erythrocytes act as scavengers binding inflammatory mediators such as IL-8, MCP-1 and RANTES (regulated upon activation normal T-cell expressed and secreted). Furthermore, certain evidence suggests that red blood cells may participate in the processes of inflammation and tissue repair (Fredriksson, 2004).

1.1.5 PLATELETS

Platelets help maintain homeostasis by releasing coagulation factors that to control bleeding. However, platelets may also participated in general inflammatory reactions by releasing cytokines and other mediators and by interacting with leukocytes and vascular endothelial cells (Weksler, 1988).

Disorders of the lungs or other organs may be associated with activation of platelets and their subsequent accumulation in the pulmonary microvasculature, where cellular interactions can occur (Heffner, 1997). In this context, there are indications that activated platelets adhere to leukocytes, at least *in vitro* (Jungi, 1986; de Bruijne-Admiraal, 1992; Fernvik, 1999). Their adherence to eosinophils is dependent on divalent cations and the responsible receptor on platelets involved in cell-cell interactions has been suggested to be P-selectin (de Bruijne-Admiraal, 1992).

Endothelial cells and platelets interact in a bidirectional fashion (Heffner, 1997). The endothelial cells release von Willebrand's factor, which promotes adherence of platelets to injured vascular membranes, as well as thrombaxane (TX) A_2 and PAF, which stimulate the platelets. Activated platelets, on the other hand, can cause endothelial injury by generating toxic species of oxygen in the extracellular fluid and releasing elastases.

Stimulated platelets release a wide variety of inflammatory mediators, including PAF, IL-1 and histamine. The two most important of these are platelet-derived growth factor (PDGF) and TGF-β (described in more detail in the section on *1.4 Cytokines and other mediators*). Another mediator released by platelets, i.e. epidermal growth factor (EGF), acts as a mesenchymal mitogen, stimulating excess deposition of collagen (Heffner, 1997; Hasleton, 1999).

1.2 Structural cells in the lungs

The trachea extends from the larynx to about the middle of the thorax, where it divides into two primary bronchi (Figure 2). The C-shaped rings of hyaline cartilage surrounding the trachea stabilise this passage and keep it open. Located between these rings are fibroelastic tissue and smooth muscle (trachealis muscle). The trachea and bronchi consist of four layers of cells: a mucous membrane (consisting of a ciliated pseudostratified columnar epithelium and a lamina propria), a submucosa, a layer of cartilage and smooth muscle, and an adventitia. Located furtherst out in this branching structure, the alveoli are small air sacs with thin walls that allow

diffusion of gases between the air and the blood. The area of the airways increases as they branch, with the area of the alveoli constituting the largest area (800 000 cm^2).

1.2.1 AIRWAY EPITHELIAL CELLS

Epithelial cells line the entire airways and constitute a first barrier against foreign agents such as microorganisms and pollutants. The mucous cells in the epithelia produce an acidic mucin that captures such agents and the ciliated cells move this mucous layer upward to the esophagus. The tracheobronchial epithelium mainly contains ciliated cells, goblet cells and basal cells but also two less common cell types: brush cells and dense core granule cells (Ross, 1989). As the bronchi branch into smaller tubes, bronchioles (Figure 2), the cartilage and submucosal glands are no longer present. Just before reaching the respiratory portion of the tract, terminal bronchioles, the epithelium consists mainly of ciliated cells and clara cells. The respiratory bronchiole is first the part of the respiratory tract in which gas exchange occurs, and here, clara cells are the dominant cell type. The alveolar wall consists of three types of epithelial cells: type I cells, type II cells and brush cells. The type II cells produce and secrete pulmonary surfactant, which is crucial in maintaining surface tension and preventing collapse of the alveoli.
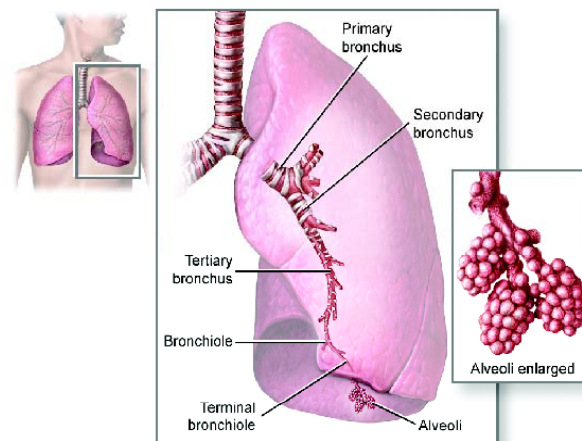
Figure 2. *The human bronchial tree.*

The epithelial barrier is also a potent regulator of inflammation in the lung (Chapman, 2004). Epithelial cells can release chemotactic factors and cytokines and, together with macrophages, control the types of inflammatory cells recruited to the lungs. These cells secrete TGF-β, fibronectin and other growth factors, which, in addition to activating other epithelial cells, may modulate the production of extracellular matrix and functions of mesenchymal cells (Rennard, 1997). For instance, TGF-β_1 stimulates the migration of fibroblasts and accumulation of collagen. Moreover, enhanced death of alveolar epithelial cells primarily by apoptosis, is consistently observed at early stages of fibrotic lung disorders (Chapman, 2004). Thus, it has become appeared that epithelial cells of the lung are an important site of initial injury and also play a role in tissue repair.

1.2.2 FIBROBLASTS

Fibroblasts are the major type of mesenchymal cell present in the matrix of connective tissue. In addition to their structural role, fibroblasts make important contributions to the inflammatory and remodelling processes by producing various cytokines, matrix components and matrix-degrading enzymes. (Sime, 1997).

In response to activation by the pro-inflammatory cytokines IL-1α, IL-1β and TNF-α, fibroblasts synthesise and secrete a wide range of inflammatory mediators, including IL-1, IL-8, IL-6, MCP-1, PDGF, TGF-β, macrophage inflammatory protein (MIP)-1α, fibroblast growth factor (FGF), and prostaglandin (PG) E2. Furthermore, fibroblasts produce a number of matrix proteins, including collagen, proteoglycans and fibronectin. Secretion of these mediators and matrix components plays an important role in the amplification of the inflammatory response, partially leading to the remodelling of fibrotic tissue. Fibroblasts also have the capacity to affect the extracellular matrix turnover by producing matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). Imbalance in the production of MMPs and TIMPs is important in both diseases associated with excessive extracellular matrix deposition such as IPF, and in diseases with increased matrix degradation such as pulmonary emphysema (Fujita, 1990; Beeh, 2003; Belvisi, 2003).

A number of mediators present at sites of inflammation act as chemoattractants for fibroblasts. Thus, both PDGF and fibronectin have been demonstrated to recruit fibroblasts to inflammatory sites, as well as to activate these cells (Postlethwaite, 1981; Rennard, 1981; Osornio-Vargas, 1996; Kohyama, 2002). Moreover, the proliferation of fibroblasts is stimulated by a variety of mediators, including cytokines, components of the extracellular matrix and granule proteins of eosinophils and mast cells (Sime, 1997; Puxeddu, 2004). Accordingly, FGF, IL-1, PDGF and TGF-β, which are produced by mesenchymal cells and activated macrophages, have all been shown to act as mitogens for fibroblasts *in vitro*. Furthermore, the mast cell products histamine, heparin and tryptase are also mitogens for fibroblasts and have been detected in lung biopsies from patients with fibrotic lung disorders and in BAL fluid from patients with interstitial lung diseases.

Different tissues, and even one and the same tissue contain fibroblasts of varying phenotypes (Sime, 1997). For instance, fibroblast cell lines derived from chronically inflamed tissue and tissues undergoing repair differ in significant ways from fibroblasts obtained from normal tissues. These differences include the rate of cell proliferation, pattern of gene expression of matrix proteins and integrins, and cytokine production.

Of particular interest in connection with asthma are myofibroblasts, which are contractile, smooth muscle-like fibroblasts often classified on the basis of the filaments they contain, i.e., vimentin, desmin and α-smooth muscle actin (SMA) (Powell, 1999a; Powell, 1999b). Myofibroblasts are present in enhanced numbers beneath asthmatic bronchial epithelium (Sime, 1997). Investigations on rats have revealed that TGF-β and GM-CSF can induce the differentiation to myofibroblasts *in vivo*. In association with other fibrotic diseases of the lung, such as usual interstitial pneumonia (UIP), clusters of activated fibroblasts (fibroblastic foci) are detected within the extracellular matrix (Pardo, 2002).

Fibrocytes are progenitor cells in peripheral blood, which may migrate into the tissue and differentiate into fibroblast-like cells. The presence of circulating fibrocytes in peripheral blood was first reported in 1994 (Bucala, 1994). These cells, which comprise less than 1% of the total

number of leukocytes, characterised antigen-presenting cells, that express a specific pattern of both mesenchymal (e.g. procollagen I and α-SMA) and haematopoietic markers (including CD34) (Quan, 2004; Nihlberg, 2006). Investigations concerning the clinical significance of fibrocytes focused initially on their potential role in wound repair, but more recent data indicate the participation of these cells in disorders such as tumours, scleroderma and pulmonary fibrosis. For example, in mild asthmatics an increased number of fibrocytes in tissue have been correlated to a thicker basement membrane (Nihlberg, 2006). Further, fibrocytes can contract collagen gels and, upon activation by TGF-β, express α-SMA and develop to a myofibroblastic phenotype (Abe, 2001).

1.2.3 AIRWAY SMOOTH MUSCLE CELLS

Alterations in the contractile and proliferative properties of the smooth muscle cells in airways are associated with several pulmonary disorders (Black, 2003; Hirst, 2003). These cells play the major role in regulating bronchoconstriction and relaxation in asthmatics. In these same patients, these cells may proliferate, thereby increasing the total mass of smooth muscle in airway walls. At the same time, smooth muscle cells are an important source of pro-inflammatory cytokines, chemokines and other growth factors and, moreover, they produce components of the extracellular matrix, including, matrix metalloproteinases and their inhibitors in tissues. Smooth muscle cells also express adhesion molecules and integrins, which act as receptors for most extracellular matrix proteins.

1.3 Cytokines

Originally, cytokines were categorised as growth factors on the basis of their primary known function. Later, as additional information was obtained it became apparent that several of these proteins are involved in various aspects of inflammation (Diegelmann, 2004), e.g., cell migration, production of matrix, changes in enzyme expression and differentiation. Even when present at low levels, cytokines, which are also in general highly stable, regulate cellular activities. However, in a chronic inflammatory environment, proteolytic enzymes, such as neutrophil elastase can cleave and modify cytokines. Cytokines also exert important effects on adhesion molecules, thereby causing circulating leukocytes (initially neutrophils, and later on monocytes) to migrate to the site of inflammation in a tissue.

1.3.1 PDGF

PDGF exist in three dimeric isoforms; AA, BB and AB. PDGF-AA is reported to be the most abundant form released from platelets (Soma, 1992). The mediator is secreted from α-granules of platelets and can for example, stimulate the chemotaxis of neutrophils and fibroblasts, and promote mitogenesis of fibroblasts and smooth muscle cells (Sime, 1997; Diegelmann, 2004).

The effects of PDGF is mediated through two distinct receptors, termed α (binds AA and AB) and β (binds BB and AB). The regulation of these receptors is important in fibroblasts, i.e. TGF-β has been demonstrated to down regulate the α-receptors on human fibroblasts (Bonner, 1995; Soma, 2002) and the expression of the β-receptors in human airway fibroblasts are increased in severe asthmatics (Lewis, 2005).

1.3.2 TGF-β

TGF-β is another important mediator that attracts macrophages and stimulates these cells to secrete additional cytokines, including TNF-α, FGF, IL-1 and PDGF. Moreover, TGF-β enhances the chemotaxis of fibroblasts and smooth muscle cells, differentiation of myofibroblasts and production of matrix proteins (Krein, 2002; Diegelmann, 2004). There are three structurally related isoforms (β_1, β_2 and β_3) of this protein that share 60-80 % sequence homology (Bartram, 2004).

The cytokine most closely linked to tissue fibrosis is TGF-β_1 (Chapman, 2004), which is synthesised as an inactive precursor and subsequently activated via acomplicated process. Initial cleavage of pro-TGF-β_1 yields TGF-β_1 together with a peptide referred to as LAP (latency-associated peptide), which must then be removed for full activation. There are at least three mechanisms for this removal that are all potentially active in the lung: binding of LAP/TGF-β_1 complex to an epithelial integrin, presumably followed by a conformational change; binding of the LAP/TGF-β_1 to the matrix protein thrombospondin (TSP)-1; and proteolytic cleavage of LAP by plasmin and certain metalloproteases.

The micro-localisation of TGF-β_1 is regulated by a set of so-called latent TGF-binding proteins, which are capable of binding both TGF-β_1 and components of the extracellular matrix. For this reason, samples must be activated prior to analysis of this cytokine by an enzyme-linked immunosorbent assay (ELISA) procedure, e.g., by acidification. The total amount of TGF-β detected after activation should not be less than the amount of bioactive TGF-β, which can also be determined employing a biological assay based on an epithelial cell line derived from mink lung (Abe, 1994; Haagmans, 2003).

1.4 Eosinophil Cationic Protein

Eosinophil cationic protein (ECP) was first purified in 1971 from the granules of myeloid cells, but it was not until 1975 it became clear that this protein was of eosinophil origin (Venge, 1999).

ECP is a single-chain, zinc-containing, protein with a molecular weight ranging from 16 to 22 kDa. The heterogeneity is partly due to differences in glycosylation of the molecule, since ECP has three potential glycosylation sites. This protein has also been named ribonuclease 3 (RNase 3) because of homologies with both human ribonucleases and RNases. An 8-9 amino acid loop found in ECP, however, seems to distinguish this protein from other RNases. Further, ECP has a very high-calculated pI of 10.8, which is due to the high arginine content. The extreme isoelectric point makes the protein very sticky and prone to bind to negatively charged molecules such as those found on cell membranes. ECP can be secreted from specific granules via a degranulation process regulated by various types of interactions, e.g., receptor interactions with IgG (Wardlaw, 1988). This protein is highly cytotoxic and this characteristic renders ECP to make pores in cell membranes that allows the passage of water and other small molecules into the cell resulting in osmotic cell lysis.

ECP participates not only in specific defences against parasites, but also in inflammatory reactions in general (Hernnas, 1992). Accordingly, elevated levels of this protein are detected in association with a variety of inflammatory and fibrotic disorders. For instance, ECP is present in the sputum of asthmatics and is employed as a clinical marker for eosinophilic inflammation. Furthermore, this protein inhibits both degradation of proteoglycans by fibroblasts as well as

fibroblast proliferation (Hernnas, 1992; Birkland, 1994). Polymorphisms of ECP have also been detected and correlated to allergy and ECP-content of eosinophils (Jonsson, 2002; Jonsson, 2006).

1.5 Components of the extracellular matrix

The extracellular matrix in different tissues and organs consists of complex mixtures of collagens, glycoproteins and proteoglycans in unique proportions (Table 1) (Fernandes, 2006). Type I collagen, the most common form of this protein, accounts for, e.g., approximately 84% of the collagen synthesised by fibroblasts (Cutroneo, 2003). The essential functions of extracellular matrix include structural support, adhesion of cells and provision of a barrier towards the movement of fluid and macromolecules (Branton, 1999). Moreover, this matrix is also a source of inflammatory mediators and growth factors that promote cell migration, and localisation, activation, proliferation and differentiation (Sime, 1997; Powell, 1999a; Black, 2003).

Table 1. *Examples of matrix proteins in the airways.*

Matrix proteins
Collagens: -   Type I-V, VII, IX, XII
Laminins
Fibronectin

Glycoproteins:
   -   Tenascin
   -   Thrombospondin
   -   Vitronectin
   -   Sparc

Proteoglycans:
small*:
   -   Decorin
   -   Biglycan
   -   Lumican
   -   Fibromodulin
large**:
   -   Perlecan
   -   Agrecan
   -   Versican

* 1-2 glycosaminoglycan chains
** 10-100 glycosaminoglycan chains

The extracellular matrix in the airways is constantly undergoing turnover, also in dense fibrotic areas (Crystal, 1997; Fernandes, 2006). For example, total collagen turnover in the lung has been estimated to occur at a rate of greater than 10% per day. Turnover of extracellular matrix is regulated primarily in three ways: regulation of *de novo* synthesis of matrix proteins by TGF-β (Branton, 1999), degradation of matrix proteins by MMPs; and finally, attenuation of MMP activity by TIMPs (Powell, 1999a; Black, 2003). The mechanisms underlying this turnover may thus be targets for therapeutic interventions.

Matrix metalloproteinases form a family of zinc-dependent endoproteases that share structural domains and are capable of degrading protein components of the extracellular matrix (Birkedal-Hansen, 1993). MMPs are secreted as inactive pro-enzymes that can be activated by mediators in the extracellular space. The 23 presently known members of the human MMP family can be divided on the basis of their substrate specificity into several subgroups, including collagenases, gelatinases, stromelysins and membrane-type MMPs.

1.6 Wound repair, tissue remodelling and fibrosis

The repair of wounds is a complex process involving the release of pro-inflammatory mediators, recruitment and proliferation of mesenchymal cells (mainly fibroblasts), production of components of the extracellular matrix and, finally, tissue remodelling (Figure 3). During the rearrangement of mesenchymal cells and matrix components that occurs in connection with the remodelling process, an imbalance between metalloproteinases and their inhibitors may result in accumulation of an excessive amount of extracellular matrix in the interstitial space (Geiser, 2003). Such remodelling, which also involves contraction of tissue, participates not only in scar formation, but also in most fibrotic conditions.
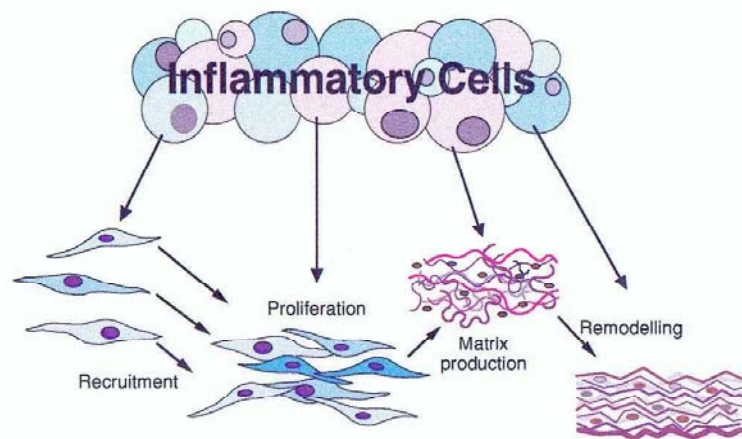
Figure 3. *The complex process of tissue repair includes influx of inflammatory cells to tissue site, recruitment and proliferation of mesenchymal cells, matrix production and remodelling.*

In connection with normal tissue repair, the appropriate architecture is re-established via an equilibrium between scar formation and tissue remodelling (Diegelmann, 2004). In sharp contrast, a tissue injury followed by defective repair is characterised by deposition of collagen and other matrix components in a manner that results in an abnormal tissue structure, fibrosis, and, ultimately, loss of organ function. Tissue fibrosis is thus associated with a pronounced fibroproliferation and excessive and disordered deposition of matrix proteins by these cells, leading to aggregation and the formation of scar tissue (Chambers, 2002). In the case of the lung this may lead to dense fibrosis and permanent impairment of pulmonary function.

An injury may also result in deficient healing and occurs when there is increased turnover and breakdown of a connective tissue matrix. The tissue affected may be weakened and in the case of the lung this gives rise to emphysema.

2 AIMS OF THE PRESENT THESIS

The aim of the present investigation was to characterise the roles played by platelets and eosinophils in the remodelling of lung tissue employing *in vitro* systems. More specifically aims were as follows:

1. to study the influence of platelets on fibroblast-mediated contraction of collagen gels and to evaluate the contribution of PDGF and TGF-β;

2. to evaluate the effect of eosinophils on fibroblast-mediated contraction of collagen gels and to investigate the role of the eosinophil granule protein ECP in mediating this effect;

3. to determine the effect of ECP on the release of TGF-β by fibroblasts cultured both as monolayer and in collagen gels; and

4. to characterise the involvement of eosinophils and, in particular, ECP on fibroblast migration.

3 METHODOLOGICAL CONSIDERATIONS

3.1 Cell cultures

In these studies we employed a cell line derived from human foetal lung fibroblasts (HFL1) originating from a healthy donor and with a normal phenotype. This well-established cell line is commonly utilized for *in vitro* investigations of lung fibroblasts. The cells were cultured in medium supplemented with 10% foetal bovine serum and used in passages 15 to 20.

In addition, a commercially available promyelocytic cell line (HL-60 clone 15) was examined in Paper II. Under the proper conditions (i.e. in sodium butyrate buffer at pH 7.8), these cells can be stimulated by cytokines (GM-CSF, IL-5 and IL-3) to differentiate into eosinophil-like cells (Fischkoff, 1985; Hutt-Taylor, 1988; Lundahl, 2000). The presence of an eosinophilic phenotype was confirmed on the basis of expression of CD9 (determined by flow cytometry) and morphological examination of cells stained with May-Grünewald Giemsa dye.

3.2 Purification and preparation of human platelets and eosinophils

Blood cells from healthy volunteer donors were obtained from the blood bank at Karolinska University Hospital (Stockholm, Sweden). Previous studies have shown that serum, neutrophils, monocytes, mast cells and red blood cells can all exert effects in our *in vitro* model system (see also below) (Skold, 1999a; Lijnen, 2001; Zhu, 2001; Fredriksson, 2002; Garbuzenko, 2002). It was therefore essential to use highly pure cell populations in our experiments.

3.2.1 PLATELETS (I)

Platelets were isolated employing a blood cell separator and a closed-system apheresis kit, which yields concentrated platelets depleted of leukocytes ($<1\text{x }10^6$ leukocytes/L). Prior to use, the platelets were washed four times with sterile phosphate-buffered saline in order to remove the plasma. Fixed platelets were prepared by resuspending platelets washed in this manner in paraformaldehyde for 10 minutes.

To obtain a lysate of these cells, isolated platelets were frozen and thawed three times, followed by centrifugation to remove cell debris. The resulting lysate contained significant levels of both PDGF and TGF-β_1 (i.e. approximately 500 ng for each per mL).

3.2.2 EOSINOPHILS (II, III, IV)

Since it is difficult to remove contaminating blood cells, and especially erythrocytes, from preparations of eosinophils, we developed a procedure designed especially to deplete red blood cells. First, a solution of dextran was added to achieve sedimentation of the majority of the erythrocytes (Cramer, 1992). Subsequently, the leukocyte-rich plasma thus obtained was washed and layered onto a solution of Percoll in order to isolate the granulocytes (Moshfegh, 1999). The cell pellet obtained by centrifugation, contained mostly granulocytes and a few remaining erythrocytes, which were finally hemolysed by suspension in NH_4Cl-EDTA.

Thereafter, eosinophils were separated from neutrophils by negative selection. First, magnetic beads coated with anti-CD16 antibodies, which bind neutrophils specifically, were added to the cell suspension and, following incubation, this mixture was placed on top of a

column in a magnetic field. Neutrophils bound via the anti-CD16 antibodies to the magnetic beads were trapped in this column, from which the unbound eosinophils could be selectively eluted. The purity of the eosinophil preparations thus obtained was assessed by immunostaining with FITC-conjugated monoclonal antibodies directed towards platelets (CD61), red blood cells (glycophorin) and neutrophils (CD16), followed by flow cytometric analysis. This revealed that the number of each of these different types of contaminating cells present was well below the number required to influence on fibroblast-mediated contraction of a collagen gel.

To obtain conditioned medium (CM) of eosinophils purified peripheral blood eosinophils purified in this manner were cultured in serum-free medium for 2-24 hours. Cell viability was always > 95%, even after the longest period of culture. The media from these cell cultures were collected and stored at -20° C until use in studies on cell migration.

3.3   Assay of the contraction of collagen gels (I, II and III)

Fibroblasts are considered to be the key cell type involved in tissue remodelling and the development of fibrosis. Here, we employed an *in vitro* model for tissue remodelling referred to as fibroblast-mediated contraction of collagen gels, developed originally by Bell and co-workers (Bell, 1979). Even though this system is obviously a simplified version of the *in vivo* process, exploring only one aspect of tissue remodelling, it has proven useful in elucidating the effects of potential fibrotic mediators (Fukamizu, 1990; Adachi, 1998; Ohga, 2000; Mio, 2001).

The ability of fibroblasts to contract the three-dimensional collagen gel is dependent on a variety of factors, including the strain, and density of the fibroblasts themselves, the concentration of collagen and the presence of various soluble agents (Montesano, 1988; Gullberg, 1990). For instance, fibronectin, TGF-β and PDGF stimulate gel contraction; whereas PGE_2, heparin and β-agonists have inhibitory effects (Ehrlich, 1983; Montesano, 1988; Fukamizu, 1990; Gullberg, 1990; Imaizumi, 1996; Adachi, 1998; Skold, 1999b; Carnevali, 2003).

Culturing human lung fibroblasts in a three-dimensional gel composed of type I collagen causes these cells to acquire a more tissue-like phenotype than when they are cultured in monolayer (Figure 4). Upon stimulation, these fibroblasts will rearrange the surrounding matrix so that the gels contract. In addition to characterising the influences of various types of cells and mediators, this model system can be employed to test drugs of potential use in treatment of fibrotic conditions.

3.3.1   EXTRACTION OF TYPE I COLLAGEN

The type I collagen utilized in the gel contraction assay was isolated from rat tail tendons (Elsdale, 1972). Briefly, after excising the tendons from the tails, the sheath and other connective tissue were carefully removed. Following repeated washing with Tris-buffered saline, the tendons were then washed with increasing concentrations of ethanol. Finally, the type I collagen was extracted from these washed tendons with acetic acid. In order to maintain these preparations of collagen in a fluid state, they were stored at 4° C until use.

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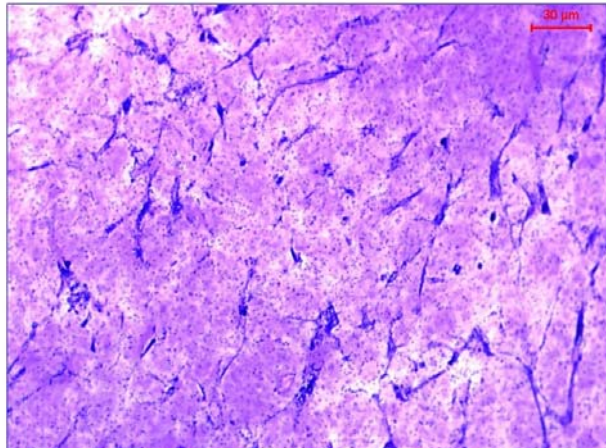
Figure 4. *Section of a fibroblast-populated collagen gel embedded in glycolmethacrylate (GMA) resin (Blomberg 1997). Advantages with the GMA embedding is the thin (2 μm) sections that can be cut, which makes it possible to get several sections from a single cell.*

3.3.2 PREPARATION OF GELS

The collagen gels were prepared by mixing distilled water, type I collagen, cell culture medium and the human foetal lung fibroblasts (Mio, 1996). Thereafter, suspensions of platelets, eosinophils, HL-60 cells or ECP (Table 2) were added to this mixture, which was then poured onto wells of tissue culture plates. After allowing gel formation in the cell incubator (at 37° C), the resulting gels were removed from the surface of the culture well and transferred into tissue culture dishes containing serum-free medium (Figure 5).
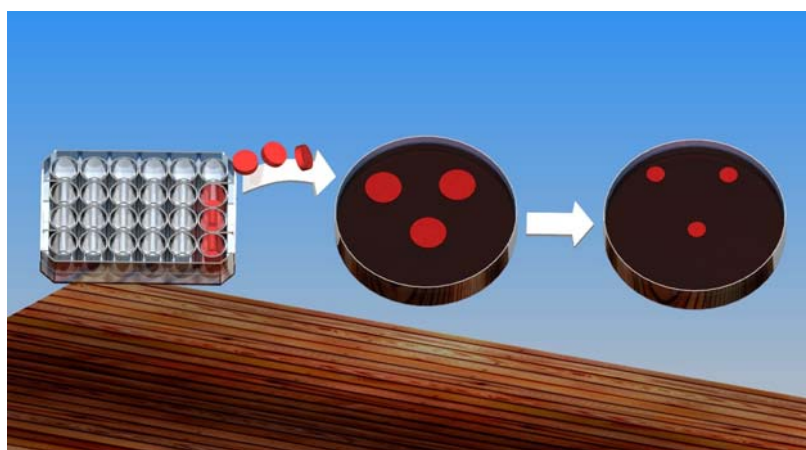
Figure 5. *Collagen gels were cast in 24-well tissue plates and then transferred into dishes containing serum free culture medium. The floating gels were cultured and gel areas measured for four days.*

Table 2. Cells in co-culture with fibroblasts.

	Number of cells/mL collagen gel
Platelets	10^6-10^8
Eosinophils	10^4-10^6
HL-60 clone 15	10^4-10^6
Platelets/Eosinophils	10^8/10^6

3.3.3 MEASUREMENT OF THE AREA OF GELS

The floating gels were incubated at 37° C for 4 days, during which time their areas were determined daily employing an image analyser system. The images were recorded with a video camera; the area of the gels captured and processed by computer software; and contraction of the collagen gels in the horizontal direction thereby determined. With this described procedure, the area of each gel could be measured while preserving sterility.

3.3.4 NEUTRALISATION OF PDGF AND TGF-β (I)

Neutralising antibodies directed towards PDGF or TGF-β were utilized to evaluate the relative contributions of these two cytokines to the fibroblast-mediated contraction of collagen gels induced by platelets. For this purpose, platelets were pre-incubated with the antibodies prior to addition of these cells to the gels. Antibodies were also added to the media surrounding the gels, which were then cultured and analysed as described above.

3.3.5 PRESENTATION OF THE DATA CONCERNING GEL AREAS

The areas of gels are presented as percentages of the initial area (on day 0), which is a well-established and highly illustrative way to document gel contraction. Due to variations in, for example, the number of cell passages and different batches of collagen, we have chosen to present representative experiments. However, experiments were, repeated on multiple occasions. Pooled data from different individual experiments were also pooled and the values thus obtained are presented in the tables as well.

3.4 Monitoring cell proliferation

3.4.1 DETERMINATION OF DNA CONTENT (I, II)

Previous studies has revealed that an increase in the number of fibroblasts present in the collagen gels enhances contraction (Bell, 1979). Therefore, in order to assure that the numbers of fibroblasts remained constant in our experiments, cell proliferation was monitored on the basis of DNA content (Labarca, 1980). Briefly, after dissolving the gels with collagenase, the cells were collected and sonicated. This sonicate was then stained with Hoechst dye 33258, a fluorochrome that binds DNA, and the resulting fluorescence measured with a fluorescence spectrometer.

3.4.2 MITOCHONDRIAL ACTIVITY (II)

Cell proliferation was also monitored with a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells. An increase in the number of viable cells is associated with an increase in the total activity of mitochondrial

dehydrogenase. In brief, fibroblasts were cultured together with eosinophils in culture medium (containing 1% foetal bovine serum) on 96-well culture plates for two days. Subsequently, in order to remove the eosinophils, these cultures were washed three times with serum-free medium, followed by incubation with WST-1 for two hours and the absorbance then determined in a spectrophotometer.

3.5 Assay of various mediators

3.5.1 PDGF (I)

In the studies presented in Paper I, the medium surrounding the gels was collected after culture and analysed for PDGF-AA and -AB employing an ELISA.

3.5.2 TGF-β_1 (I, III)

Since TGF-β_1 is first synthesised in inactive precursor form (see Introduction), the samples had to be activated prior to analysis by ELISA. This can be accomplished in several different ways, including incubation under acidic conditions for 10 minutes, which was the procedure employed here. In order to measure the total amount of TGF-β_1 released.

The TGF-β_1 in the medium surrounding the collagen gels and in the media from monolayer cultures, was assayed either with a commercially available ELISA kit or as previously described by Kim et al. (Kim, 2003), both with and without prior activation.

3.5.3 ECP (II, III AND IV)

In these investigations the amounts of ECP released by co-cultures of fibroblasts and eosinophils were determined as follows: the collagen gels (in II, III) were dissolved with collagenase (Liu, 1998), the resulting supernatants collected and cetyldimethylethyl ammonium bromide (CTAB) added in order to prevent ECP from sticking to plastic. The levels of this mediator in various samples were measured employing a fluoroenzymatic immunoassay ECP-CAP-FEIA.

3.6 Monitoring cell transmigration (IV)

To monitor fibroblast recruitment, which is also an important step in the remodelling process, we employed 48-well Boyden chambers (Boyden, 1962) with type I collagen coated filters. This procedure allows determination of the migration of cells through a membrane with 8-μm pores (Figure 6).

Medium collected from cultures of eosinophils, ECP and antibodies were placed in the lower compartment of the Boyden chambers and lung fibroblasts were placed in the upper compartment, followed by incubation for 5 hours in a humidified incubator at 37° C, 5% CO_2. At the end of this incubation period the filters were removed and attached cells fixed in methanol, followed by Giemsa staining. The filters were then placed onto glass slides and cells on the surface, which had been in added to the upper compartment, were wiped away with cotton swabs. The number of fibroblasts that had migrated through the pores of the filter was then quantitated by counting the cells on the other surface under a light microscope.

Two neutralising antibodies to ECP were used to evaluate specificity of ECP-induced fibroblast migration. Either a polyclonal rabbit antibody or a monoclonal mouse antibody was

mixed together with ECP or CM before added to the lower wells of Boyden chambers. Cell migration assay was performed as described above.
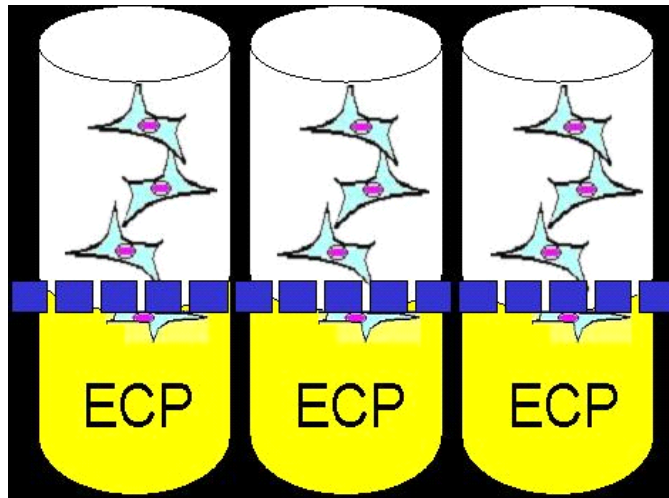
Figure 6. *Lung fibroblasts were added to the upper wells and chemoattractant i.e. ECP in the lower wells. The compartments were separated by a filter with 8-μm pores, which through the fibroblasts could migrate.*

4 RESULTS AND DISCUSSION

4.1 Platelets stimulate tissue remodelling *in vitro* (I)

4.1.1 EFFECTS OF PLATELETS ON FIBROBLAST-MEDIATED CONTRACTION OF COLLAGEN GELS

In Paper I we demonstrate that platelets in co-culture with fibroblasts can augment contraction of three-dimensional collagen gels in a time- and concentration-dependent manner with 10^8 platelets/mL giving the most pronounced stimulation. Two observations indicate that this effect may be mediated by soluble factors. First, fixed platelets caused no augmentation, i.e. the presence of the platelet structure *per se* did not stimulate contraction and secondly, a soluble platelet lysate caused a pronounced enhancement in contraction indicating that platelets release stimulatory factors.

4.1.2 RELEASE OF PDGF AND TGF-β_1

Platelets are known to produce PDGF and TGF-β, both of which stimulate fibroblasts, and elevated levels of these mediators have been detected in association with inflammatory and fibrotic conditions *in vivo* (Denis, 1994; Bergmann, 1998; Haque, 1998; Hoyle, 1999). Since these cytokines are known to enhance fibroblast-mediated contraction of collagen gels (Montesano, 1988; Gullberg, 1990), we assayed their concentrations in our culture media employing an ELISA procedure. PDGF-AA, -AB and TGF-β_1 were also released in significant amounts into the media of cultures containing platelets.

The level of PDGF was highest during the initial 24 hours of culture and declined significantly thereafter. In contrast, the level of TGF-β_1 increased continuously during the initial 48-hour culture period. One plausible explanation for this difference is that PDGF is only released by platelets, whereas TGF-β_1 can also be produced by stimulated fibroblasts.

4.1.3 NEUTRALISATION OF THE BIOACTIVITIES OF PDGF AND TGF-β

The involvement of PDGF and TGF-β in the stimulation of fibroblast-mediated contraction of collagen gels by platelets was confirmed by the demonstration that addition of these two mediators at the same concentrations as those detected in the media of fibroblast/platelet co-cultures, resulted in a small increase in the contraction. Furthermore, both this enhancement and the platelet-induced augmentation of contraction could be prevented (although only partially in the case of platelets) by excess amounts of neutralising antibodies towards PDGF and TGF-β. Together, these findings suggest that PDGF and TGF-β mediate part of the stimulation of contraction by platelets, but that additional platelet-derived mediators such as lysophosphatidic acid (LPA) (Mio, 2002), are likely to contribute to this effect as well.

4.1.4 PLATELETS INDUCE CELL PROLIFERATION

Proliferation of fibroblasts could also lead to enhanced collagen gel contraction and, indeed, there was a significant increase in the DNA content of the fibroblast/platelet co-cultures during the culture period. However, no more than 10-15% of the platelet-induced augmentation of fibroblast-mediated collagen gel contraction can be explained by this increase in the number of fibroblasts. Since, the combined effects of PDGF, TGF-β and fibroblast proliferation cannot fully explain the stimulation caused by platelets, other mechanisms, such as up-regulation of

integrin receptors, an alteration in fibroblast phenotype and/or production/secretion of additional stimulatory mediators must be considered.

4.2   Eosinophils stimulate tissue remodelling *in vitro* (II)

4.2.1   EFFECTS OF EOSINOPHILS ON FIBROBLAST-MEDIATED CONTRACTION OF COLLAGEN GELS

In Paper II we demonstrate that both human peripheral blood eosinophils and differentiated eosinophil-like HL-60 clone 15-cells stimulate fibroblast-mediated contraction of collagen gels, whereas undifferentiated HL-60 clone 15 -cells have no such effect. Although it has been shown previously that a lysate of eosinophils can influence gel contraction in this model system (Levi-Schaffer, 1999). However, our findings provide new insights regarding the influence of eosinophils on tissue remodelling *in vitro*. In the first place, our three-dimensional collagen gels contained intact and viable eosinophils from two different sources together with lung fibroblasts. In addition, our culture system was completely free of serum, which is known to augment collagen gel contraction and to stimulate fibroblast proliferation (Shock, 1991), whereas Levi Shaffer and co-workers (Levi-Schaffer, 1999) supplemented their medium with serum. Furthermore, the eosinophils employed by Levi-Schaffer and colleagues (Levi-Schaffer, 1999) were obtained from atopic individuals, whereas we have investigated cells from non-allergic donors.

4.2.2   EOSINOPHILS DO NOT STIMULATE FIBROBLAST PROLIFERATION

Following two days of culture the amount of DNA (in terms of optical density) in collagen gels containing fibroblasts alone, blood eosinophils alone and both cell types together were 350, 100 and 450 respectively. This indicates that the eosinophils did not induce proliferation of fibroblasts. This conclusion was confirmed by demonstrating that the rate of mitochondrial activity by monolayer cultures containing fibroblasts alone or fibroblasts and eosinophils together were not significantly different.

### 4.2.3   RELEASE OF ECP IN THREE-DIMENSIONAL COLLAGEN GEL CULTURE

Infiltration of eosinophils into tissues is commonly associated with the release of granule proteins from these cells (Bousquet, 1990). Indeed, we found that more ECP was released when fibroblasts and eosinophils were co-cultured in three-dimensional collagen gels than when eosinophils were cultured alone in gels. Moreover, addition of ECP itself to fibroblast-containing collagen gels augmented gel contraction. These observations are consistent with Takafuji and co-workers (Takafuji, 2000), who showed that cell-cell contact between eosinophils and fibroblasts enhances the release of ECP and that this process is integrin-dependent. Furthermore, ECP stimulates several other fibroblast activities as well, including proteoglycan metabolism and DNA synthesis (Hernnas, 1992; Noguchi, 1992).

However, the levels of ECP we detected in the media from our gels were lower than those required to augment fibroblast-mediated gel contraction. Thus, eosinophils may release other factors that, alone or in combination with ECP, stimulate gel contraction. In addition to ECP, a number of factors that may potentially be involved in tissue eosinophilia associated with the fibrotic processes have been identified, including growth factors such as TGF-β and the Th2-like cytokines IL-4 and IL-13. Eosinophils are the major source of TGF-β, which stimulates proliferation of fibroblasts in airways that are severely asthmatic (Birkland, 1994; Ohno, 1996).

Moreover, TGF-β, IL-4 and IL-13 can cause fibroblasts to assume a myofibroblastic phenotype and can stimulate the production of matrix proteins such as pro-collagen I and tenascin (Ohno, 1996; Phipps, 2002).

4.3 Platelets and eosinophils have an additive effect on fibroblast-mediated collagen gel contraction

In paper I and II we demonstrated that both platelets and eosinophils can augment fibroblast-mediated contraction of collagen gels. It is also known that platelets can attach on eosinophil cell surface and thereby stimulate eosinophil adhesion (de Bruijne-Admiraal, 1992; Fernvik, 1999). The two cell types can also be involved in inflammation and tissue repair. We therefore aimed to examine what influence the both cell types together had in the collagen gel system. Platelets (10^8 cells/mL) and eosinophils (10^6 cells/mL) were co-cultured together with lung fibroblasts in collagen gels. We found that gels with all the three cell types gave a pronounced collagen gel contraction compared to platelets and eosinophils cultured alone with fibroblasts (Figure 7). The increased gel contraction was, however, additive and no synergistic effects could be determined.
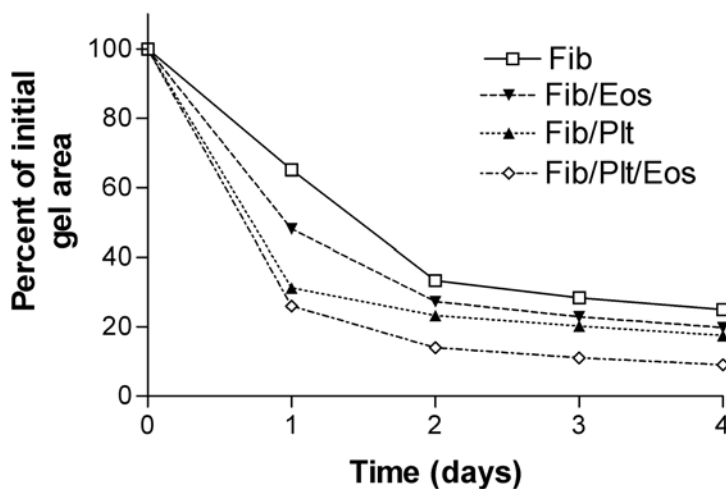
Figure 7. *Platelets (Plt) and eosinophils (Eos) have an additive effect on fibroblast-mediated collagen gel contraction.*

4.4 ECP stimulates the release of TGF-β₁ by human lung fibroblasts (III)

4.4.1 EFFECTS OF ECP ON THE RELEASE OF TGF-β₁ BY FIBROBLASTS CULTURED IN MONOLAYER

In order to we investigate whether the ECP-induced effect observed in Paper II might be mediated by the pro-fibrotic cytokine TGF-β_1, we first, added ECP to monolayer cultures of lung fibroblasts, and found that this resulted in a dose-dependent enhancement of the release of TGF-β_1 by these cells. A time course study further showed the most pronounced effect of ECP after 48 hours of culture. In addition, we demonstrated that ECP induces an almost two-fold increase in the level of TGF-β_1 mRNA in these same cells.

4.4.2 Effects of ECP on the release of TGF-β_1 by fibroblasts cultured in three-dimensional collagen gels

Subsequently, we performed similar experiments employing fibroblasts cultured in a collagen matrix. The advantages of such a system are that the cells are allowed to spread in three dimensions as fibroblasts *in vivo* and, furthermore, demonstrate more *in vivo*-like functional properties. When ECP was added to fibroblast-containing collagen gels, elevated levels of TGF-β_1 were observed both in the collagen gels themselves and in the surrounding medium. In addition, ECP augmented fibroblast-mediated contraction of these gels in a time-dependent fashion. Since TGF-β_1 is known to exert a similar effect on contraction in this system (Fukamizu, 1990), we propose that enhanced release of TGF-β_1 by fibroblasts is at least partially responsible for the ECP-induced stimulation of contraction.

4.4.3 ECP had no effect on fibroblast proliferation

Addition of ECP had no significant effect on mitochondrial activity in fibroblasts in monolayers or on the DNA content of fibroblast-containing collagen gels. These observations provide a strong indication that the increase in release of TGF-β_1 elicited by ECP was not due to proliferation of fibroblasts.

4.4.4 Two-way interactions between eosinophils and fibroblasts in collagen gels

Finally, we demonstrated that three-dimensional co-cultures of lung fibroblasts and peripheral blood eosinophils released increased levels of TGF-β_1 compared to fibroblasts cultured alone. A correlation between these factors has also been observed in tissue (Buron, 1999; Lee, 2003).This data further strengthen a role for eosinophils in tissue remodelling. We also demonstrated that there is an extensive interplay between the cell types, where fibroblasts also enhanced the ECP release by eosinophils.

4.5 ECP stimulates fibroblast migration (IV)

In paper IV we studied the effect of eosinophils and ECP in another important step of tissue remodelling namely fibroblast recruitment and migration. We used the 48-well Boyden chambers to study cell migration.

4.5.1 Effects of conditioned medium from eosinophils on fibroblast migration

The eosinophil conditioned medium (CM) significantly stimulated fibroblast migration. The effect was concentration dependent, where undiluted CM gave the most stimulatory effect. This result suggests that stimulatory factors are released by eosinophils over time. Therefore eosinophils were cultured for various time points ranging from 2-24 hours and supernatants were collected and added to the lower wells of the Boyden chambers. The longer time eosinophils were cultured the more enhanced effect on fibroblast migration was. The increased fibroblast migration to CM harvested after longer culture periods also corresponded to elevated ECP-release noted in CM.

4.5.2 Effects of ECP on fibroblast migration

To evaluate if ECP *per se* could affect fibroblast migration, the protein was extracted from eosinophils and tested in the Boyden chambers. ECP extracted from eosinophil granules

stimulated fibroblast migration in a dose dependent manner. The ECP concentration used to stimulate cell migration was higher than that found in the conditioned media. One explanation to this could be that conditioned media constitute other stimulatory proteins than ECP. However, due to the sticky characteristics of ECP (Sime, 1997) another explanation may be that measured levels of the protein are underestimated. To confirm the specificity of ECP a recombinant form of the protein was tested. This protein showed a similar effect as native ECP on fibroblast migration.

4.5.3 INHIBITION OF ECP-INDUCED FIBROBLAST MIGRATION

By adding blocking ECP-antibodies to ECP and CM we were able to block the stimulating effect on fibroblast migration. The antibodies *per se* showed a minor stimulating effect. Importantly, the antibodies inhibited the stimulating effect of both native and recombinant ECP as well as CM.

5 CONCLUSIONS

In the present thesis we demonstrate that platelets and eosinophils constitute important actors in the interplay between fibroblasts and extracellular matrix, thus may impact the process of tissue remodelling.

➢ Both platelets and lysate thereof stimulate fibroblast-mediated contraction of collagen gels. Both PDGF and TGF-β contribute partially to this effect.

➢ Both peripheral blood eosinophils and eosinophil-like differentiated HL-60 clone 15 cells stimulate the fibroblast-mediated collagen gel contraction. ECP is one eosinophil-derived proteins implicated in this interaction between eosinophils and lung fibroblasts.

➢ ECP stimulates the release of TGF-β_1 by both monolayer and three-dimensional cultures of lung fibroblasts. ECP also enhances the level of TGF-β_1 mRNA in these lung fibroblasts.

➢ Both media from cultures of peripheral blood eosinophils and ECP alone stimulate the migration of lung fibroblasts, effects that are attenuated by neutralising antibodies directed towards ECP.

6 CONCLUDING REMARKS

Tissue injury and inflammation followed by effective repair restores normal organ function, but defective repair with associated tissue remodelling and fibrosis can lead to loss of function. Thus, interactions between inflammatory and mesenchymal cells in connection with the remodelling processes are of considerable importance with regards to the development of lung disorders, since this interplay determines the outcome of the disease for the patient. The present thesis was designed to explore the impact of two important inflammatory cells, platelets and eosinophils, on remodelling of lung tissue employing *in vitro* systems.

Data from the present thesis are based on experimental models. As always, interpretation of *in vitro* data to the clinical situation is difficult. However, three-dimensional culture of fibroblasts in collagen gels offers advantages including a more tissue-like morphology. On the basis of the findings presented here we conclude that both platelets and eosinophils have the potential to influence functions of fibroblasts important for tissue remodelling. For example, the capacities of these cells to stimulate fibroblast-mediated contraction of collagen gels, as well as secretion of TGF-β indicate that they may have pro-fibrotic effects. However, many questions remain unanswered.

For instance, is the phenotype of fibroblasts affected by platelets and/or eosinophils in connection with fibrotic lung disorders i.e., do platelets and eosinophils promote the formation of myofibroblasts? TGF-β is potently pro-fibrotic and contributes also to the differentiation of myofibroblasts. Could this protein provide a useful target for the prevention and perhaps reversal of the inflammatory and remodelling processes associated with chronic asthma? In this context Leung and co-workers (Leung, 2006) have demonstrated that inhibition of the TGF-β receptor I prevents both eosinophilia in the airways and the proliferation of airway smooth muscle cells in rats.

The ever-intriguing question concerns the role that eosinophils play in asthma. Our findings support that these cells can influence several fibroblast functions. Since, the characteristics of eosinophils from different groups of individuals may differ, it would be of interest to compare our observations on peripheral blood eosinophils from healthy donors with eosinophils from, e.g., patients with atopic and non-atopic asthma, applying our experimental models.

What is the consequence for patients if eosinophils are completely depleted? Investigations from the late 90s hypothesised that this strategy would be beneficial for the asthmatic patient. In a study published by Leckie and co-workers (Leckie, 2000) it was demonstrated that depletion of peripheral eosinophils in asthmatics using anti-IL-5 antibodies, did not reduce any symptoms, such as hyperreactivity or late asthmatic reaction. Three years later, Flood-Page and colleagues (Flood-Page, 2003) reported that depletion of eosinophils in mild asthmatic patients with antibodies directed towards IL-5 results in attenuated deposition of collagen and reduced levels of TGF-β_1. In addition, when challenged with OVA, IL-5-deficient mice develop a significantly lower degree of peribronchial fibrosis (Cho, 2004).

Another strategy for treatment of asthma presently being tested includes humanised anti-IgE antibodies. Interestingly, such-antibodies also cause a reduction in the number of eosinophils both in peripheral blood and in the airway (Holgate, 2005). Together, these findings clearly indicate the contribution of the eosinophil in the development of asthma and warrant further research and development in the field of effective eosinophil-depleting agents for clinical use.

In summary, the present thesis highlights potential mechanisms by which platelets and eosinophils can influence fibroblasts and the extracellular matrix *in vitro*. Based on our findings, we propose that platelets and eosinophils participate in tissue remodelling *in vivo*. The results documented here offer some possible explanations and mechanisms with regards to how these inflammatory cells may contribute to defective tissue repair, fibrosis and impaired pulmonary function.

7 SUMMARY IN SWEDISH

När kroppen får ett sår eller en vävnadsskada ansamlas inflammatoriska celler. Samtidigt startar en läkningsprocess för att reparera och återställa vävnaden och dess funktion. När läkningen av någon anledning inte fungerar och är defekt, kan det medföra att ärrbildningar med tjock och oelastisk vävnad bildas. I lungan bildas det sådana fall så kallad fibrotisk vävnad som medför försämrad andningsförmåga. Vid fibrosbildning spelar samspelet mellan inflammatoriska celler och strukturella celler i lungvävnaden en betydande roll. Exempel på inflammatoriska lungsjukdomar där fibrosbildning sker är astma, lungfibros och kroniskt obstruktiv lungsjukdom (KOL).

I dagsläget saknas farmakologisk behandling av fibrostillstånd och det är därför av största vikt att öka kunskaperna inom detta område och kartlägga de mekanismer som resulterar i fibros så att effektiva läkemedel kan utvecklas.

I denna avhandling har vi undersökt hur två viktiga inflammatoriska celler, trombocyter (blodplättar) och eosinofila granulocyter (en typ av vita blodkroppar), kan påverka den vanligaste strukturella cellen i bindväven, nämligen fibroblasten.

Vi har använt oss av experimentella metoder, vilket innebär att vi har arbetat med humana celler från lunga och perifert blod i olika laborativa modeller. En metod som vi har använt kallas fibroblastmedierad kontraktion av kollagengeler, vilket avspeglar den kontraktila delen av bindväven, som är typisk vid fibrosutveckling. En fördel med modellen är att fibroblaster kan odlas i en gel bestående av kollagen, vilket är en huvudkomponent i lungans bindväv. Cellerna kan växa ut tredimensionellt precis som de gör i lungorna och fibroblasterna kommer att ha större likheter och på många sätt uppföra sig som dess motsvarighet i lungan, jämfört med vanlig konventionell odling på platta.

När vi samodlade trombocyter och eosinofiler med fibroblaster i kollagengeler kunde vi se en ökad kontraktion av gelerna. Vi har också visat att den trombocyt-inducerade stimuleringen delvis berodde på två mediatorer som finns i trombocyter, nämligen PDGF och TGF-β. Eosinofiler visade sig också kunna förstärka fibroblastmedierad kollagengelskontraktion. Här var det ett mycket basiskt och celltoxiskt protein, ECP, som var involverad i samspelet mellan eosinofiler och fibroblaster. Våra experiment visade även att detta ECP-protein kunde stimulera TGF-β-frisättning hos fibroblaster och även öka dessa cellers rörlighet, vilket är tecken på att ECP har en profibrotisk roll.

I denna avhandling har vi visat att trombocyter och eosinofila granulocyter kan påverka en viktig strukturell cell i lungan, fibroblasten. Detta kan ha betydelse vid utveckling av den fibros som ses vid ett flertal inflammatoriska lungsjukdomar och som kan ge nedsatt lungfunktion. Förhoppningen är att dessa data kan användas som underlag för vidare studier, vilka kan resultera i nya behandlingsmetoder för patienter med exempelvis astma och KOL.

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