The border-crossing behavior of eosinophils and neutrophils in the lung
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CHAPTER I

General Introduction
General Introduction

Asthma and its cellular participants

Allergic asthma

Asthma is defined as a clinical syndrome characterized by increased responsiveness of the tracheobronchial tree to certain external stimuli (1). Allergic asthma is, together with atopic dermatitis and allergic rhinitis, the major disease entity of the atopic syndrome, a hereditary disease that affects 20-30% of the population. The allergic asthma patient suffers from symptoms such as dyspnea, wheezing, coughing, and increased sputum production after inhalation of allergen; major allergens are house dust mite, grass pollen, or tree pollen. Clinically, the allergic asthmatic reaction can be subdivided in an early and a late phase. The early broncho-constrictive reaction is maximal at 15-30 minutes after allergen inhalation and resolves within 2-3 hours. During this early phase, specific IgE that is bound to Fce-receptors on mast cells and basophils binds allergen, and this induces release of mediators, such as histamine and leukotrienes, that promote smooth-muscle contraction, extensive vascular leakage, mucus production and influx of inflammatory cells. During the late broncho-constrictive reaction, starting several hours after allergen inhalation, the airways are initially infiltrated by neutrophils and eosinophils and later by lymphocytes and macrophages (2-11). Each of these cell types contributes to the pathophysiological changes that are characteristic for asthma.

Eosinophils:

Eosinophilic granulocytes are myeloid cells that comprise 2-5% of the blood leukocytes in healthy individuals. Eosinophils are present in the peripheral blood for about a day before infiltrating the tissues and are thought to play a role in immunity to helminth infections. These cells can directly harm invaders in two ways: 1) they can phagocytose bacteria and kill the ingested microorganisms or they can adhere to parasites and kill these organisms extracellularly; 2) appropriate stimuli can trigger the release of toxic bioactive mediators, such as eosinophil cationic protein, eosinophil-derived neurotoxin, eosinophil peroxidase and major basic protein, and the production of specific lipid mediators, such as LTC4 and PAF (12), and a large number of cytokines, including TNF-α, MIP-1α, IL-5, GM-CSF, IL-3, IL-1, IL-6 and IL-8 (15).

The presence of a marked eosinophil infiltration in the lungs of allergic asthmatic patients suggests a selective eosinophil extravasation into the airways. Until now, the precise mechanism behind this selectivity is obscure. It is likely that cytokines in the circulation are involved that prime the eosinophils. Priming is defined as an effect on cells that induces an increased reaction to a stimulus by an agent that, in itself, induces little or no response (16). The ongoing inflammatory reaction in the airways is presumably at the basis of this process. For instance, the presence of enhanced levels of the priming cytokines IL-3, IL-5 and GM-CSF in the circulation of allergic asthmatic patients gives rise to selective eosinophil growth, differentiation and prolonged survival, resulting in eosinophilia and a prolonged presence of eosinophils in the bronchial tissue (13). On the other hand, activated endothelial and epithelial cells at the inflamed site may also contribute to the eosinophil influx, e.g. by means of production of eosinophil chemoattractants and expression of adhesion molecules required for eosinophil diapedesis. Thus, the eosinophil infiltration is most probably the result of a complex interplay between inflammatory cells, including Th2 lymphocytes, mast cells and dendritic cells, and tissue-based cells at the inflamed site.
The role of eosinophils in the allergic asthmatic reaction is still a matter of debate. Most studies in guinea pig and mice have shown that eosinophils and IL-5 are required for the induction of airway hyperresponsiveness (17;18). These studies have also shown that the toxic and bioactive mediators secreted by eosinophils in the airways affect and damage the surrounding cells, e.g. the epithelium and inflammatory cells. However, other studies in mice have indicated that the mere presence of eosinophils in the airways does not always lead to hyperresponsiveness (19-22), and that release of IL-4 (19;23) and IL-13 (24;25) are sufficient to induce many of the pathophysiologic processes associated with asthma, such as hyperresponsiveness. In parallel, in man, reduced levels of eosinophils are not always accompanied by reduced bronchial hyperresponsiveness after allergen challenge (26). Thus, infiltrated eosinophils can cause pathological changes to the respiratory epithelium but may not always be involved in the induction of hyperresponsiveness after allergen challenge.

Neutrophils:
Neutrophils are myeloid cells that represent over 90% of the circulating granulocytes, and granulocytes represent about 60-70% of the total normal blood leukocytes. Neutrophils are an essential part of the innate immune response. Like eosinophils, neutrophils are not only capable of phagocytosing a variety of bacteria but can also degranulate after stimulation with appropriate stimuli.

Several studies have shown that neutrophils infiltrate the airways of asthmatic patients during the late-phase reaction of asthma (2-11). The neutrophils are most probably attracted to the allergic inflammation site by chemoattractants, e.g. the neutrophil-chemotactic factors C5a and IL-8 have been detected in the BAL of asthmatic individuals (27). The exact role of neutrophils in asthmatic inflammation is not clear yet. The cells presumably alter the airway function in various ways. For instance, neutrophils are a potential source of toxic mediators, such as proteases and reactive oxygen metabolites, and of a wide variety of bioactive mediators, including potent lipid mediators, such as prostaglandins, thromboxanes, LTB₄ and PAF (28). Supernatants prepared from phagocytosing neutrophils in vitro have indeed been shown to induce hyperresponsiveness in the airways of rabbits (29). Thus, the presence of neutrophils in pathological sections of asthmatic airways and the ability of human neutrophils to alter airway function suggest that neutrophils do participate in the functional alterations associated with asthma.

Endothelial cells:
Endothelial cells line blood vessels in every organ system and regulate the flow of nutrients and bioactive molecules. Endothelial cells play an active regulatory role in the recruitment of leukocytes into allergic inflammatory tissues through various mechanisms (30). After stimulation with cytokines, such as IL-1β, IL-4, IL-13 and TNF-α, endothelial cells synthesize and release a wide range of proinflammatory mediators (31), including eotaxin (32;33), IL-6 (34), IL-8 (35;36), PAF (35;36) and RANTES (37) and express increased numbers of adhesion molecules required for leukocyte transendothelial migration (31), such as E-selectin, P-selectin, ICAM-1, ICAM-2, VCAM-1, and PECAM-1 (38-42). The response of the endothelial cells, e.g. which adhesion molecules are expressed and which cytokines are secreted, depends on the type and site of inflammation, and promotes in this way specific leukocyte transendothelial migration and accumulation in tissues.
Epithelial cells

The epithelium covers the exterior of the body and forms a physical barrier to protect the tissues from invasive pathogens. Epithelial cells not only form a passive barrier but also play an active role in the allergic immune response, which takes place at epithelial surfaces such as the skin, lungs, airways and gut (43-48). For instance, epithelial cells express adhesion molecules that are involved in leukocyte recruitment, e.g. ICAM-1, VCAM-1 (36;49) and CD47 (50). Beyond that, the epithelial cells can be stimulated to produce diverse proinflammatory mediators (48), including eotaxin (32;51-53), RANTES(54), IL-8 (36), IL-6 (55) and GM-CSF(56). Last but not least, asthma-associated factors, such as PAF, induce changes in lung epithelial monolayers in vitro, resulting in augmented passage of eosinophils (57). The mechanism behind this phenomenon is as yet unclear. Taken together, these findings indicate that epithelial cells also actively participate in the allergic immune response.

Inflammatory mediators

Cytokines:

Cytokines are secreted soluble molecules involved in the intercellular communication network of the immune system. The modulating effects of cytokines play a crucial role in every aspect of immunologic reactions, including cellular differentiation, activation and recruitment of leukocytes, antigen presentation and adhesion molecule expression. The mode of action of cytokines is quite complex, i.e. the molecules do not act in isolation but instead act in various sets of interacting sequences or cascades, resulting in targeted cellular responses. The cytokines have been organized into the following categories, according to their major functional activities (13):

1) Acute-phase reactants, promoting and mediating natural immunity (e.g. IFN-α, IFN-β, IL-1, IL-6, IL-8, TNF-α);
2) Cytokines that mediate cellular growth and differentiation (e.g. IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13);
3) Cytokines that act as hematopoietic growth factors (e.g. GM-CSF, IL-3, IL-9, IL-11, SDF);
4) Chemoattractive cytokines or chemokines (e.g. CXC, CC, C and CX3C chemokines)
5) Cytokines that exert lymphocyte regulatory activity (e.g. IFN-γ, TGF-β)

We are particularly interested in the cytokines involved in allergic inflammation. Evidence has been provided for enhanced levels of Th2-lymphocyte-derived cytokines, including IL-4, IL-5, IL-6, IL-10 and IL-13, in allergic inflammation (13). These cytokines induce, prolong, and amplify the inflammatory response by enhancing the production of specific IgE (IL-4), by enhancing the recruitment, growth, and differentiation of eosinophils (IL-5) and by directly causing airway hyperresponsiveness (IL-4, IL-5 and IL-13).

Allergy-associated cytokines

IL-4 and IL-13

IL-4 and IL-13 are secreted by activated Th2 lymphocytes and can be detected in increased amounts in the BAL fluid of allergic asthmatic individuals (58). These cytokines enhance survival and activation of eosinophils (58) and promote proliferation, activation and IgE switching of B cells (13). Moreover, IL-4 and IL-13 selectively induce VCAM-1 expression on endothelial cells (40;41), whereas they hardly affect the expression of E-selectin (59). IL-4 and IL-13 are related, because they share a receptor component and signaling pathways. Generally speaking, any cellular response to IL-13 can also be mediated by IL-4. However, with regard to allergic inflammation, IL-4 and IL-13 are different in that: 1) only IL-4 can
prompt immature T cells to develop into Th2 cells; 2) IL-13, but not IL-4, induces hyperresponsiveness in an IgE and eosinophil-independent manner in an *in vivo* experimental model (25); 3) IL-13 is a more potent inducer of eotaxin production in the airways of mice than IL-4 (52). Thus, IL-13 is critical to allergen-induced asthma but operates through mechanisms other than those that are classically implicated in allergic responses.

**IL-5**

IL-5, a well-recognized mediator of eosinophil recruitment, is secreted primarily by activated Th2 cells, but also by mast cells and eosinophils. This cytokine is essential for the maturation of eosinophils in the bone marrow (17;26). *In vitro*, IL-5 stimulates eosinophil growth and differentiation, it primes eosinophils and it prolongs eosinophil survival (17). The precise mechanism behind this priming effect is not clear yet, but we do know that intracellular signaling via phosphotyrosine-associated PI3-kinase, ERK and p38 MAP kinase (60) is involved.

After priming, eosinophils in animal models become more responsive to eotaxin, an eosinophil chemoattractant, while the expression of the eotaxin receptor CCR-3 is not increased. This has been shown in *in vivo* experimental models such as mice (61) and guinea pigs (63). However, whether or not this also holds true for human eosinophils has not been established yet. In fact, conflicting data on this issue have been reported. It has been claimed that IL-5 priming of human eosinophils enhances the eosinophil chemotactic response to chemoattractants such as RANTES or eotaxin (64), but the opposite, i.e. no effect on eotaxin-induced chemotaxis, has been reported as well (65). Nevertheless, the ability of two cytokines (IL-5 and eotaxin) that are relatively eosinophil-selective to cooperate in promoting tissue eosinophilia would offer a molecular explanation for the occurrence of selective tissue eosinophilia in human allergic disease.

**IL-6**

IL-6 is a multifunctional Th2 cytokine produced by many different cell types, including macrophages, dendritic cells, T cells, endothelial cells, epithelial cells (66) and hepatocytes. It has been classified as an anti-inflammatory cytokine, e.g. it promotes IL-10 production by Th1 and Th2 cells and appears to be necessary for the suppression of the Th1 response (67). IL-6 is involved in the terminal differentiation of B cells, in proliferation of lymphocytes and endothelial cells, in promotion of IL-4 production by precursor Th2 cells and in the regulation of Th1-associated cytokine production.

**IL-10**

Although IL-10 belongs to the Th2 cytokines, it is considered to be a cytokine with potent anti-inflammatory and with immunoregulatory activity. IL-10 is produced by monocytes, T cells and B cells, and is thought to be implicated in the prevention of asthma (68). For instance, IL-10 can inhibit airway eosinophilic inflammation by reducing inflammatory Th2 cytokine production (69) and by inhibiting antigen presentation to T cells (70). Consistent with this assumption is the observation that subnormal amounts of IL-10 are found in lungs of patients with asthma (70) and that IL-10-deficient mice suffer from IL-5 overproduction and eosinophilic inflammation after allergen challenge (22;71). Thus, the normal production of IL-10 in the lungs of non-asthmatic subjects may inhibit the inflammatory Th2-like processes.
Next to this inhibitory function of IL-10, there are also indications that IL-10 is necessary for airway hyperresponsiveness, i.e. IL-10-deficient mice develop a pulmonary inflammatory response accompanied by eosinophil accumulation and IL-5 overproduction, but fail to exhibit airway responsiveness (22). This is another indication that eosinophilic inflammation and hyperresponsiveness are differentially regulated in allergic asthma, i.e. eosinophil influx is accompanied by overproduction of IL-4 and IL-5, whereas hyperresponsiveness appears to be dependent on IL-13 and IL-10.

**TNF-α**

TNF-α is produced by alveolar macrophages, mast cells and eosinophils and is found in BAL fluid of patients with asthma (72). It has multifunctional properties, including eosinophil and neutrophil activation *in vitro* (73;74), induction of neutrophil apoptosis (74), and the regulation of ICAM-1, VCAM-1 and PECAM-1 expression on endothelial cells (31;38;40-42).

Chemokines:

Chemokines (75) form a large family of chemotactic cytokines that activate leukocytes. Leukocytes express several chemokine receptors, which are G-protein-coupled, seven-transmembrane-domain molecules that can bind multiple chemokines. The chemokine receptors are constitutively expressed on some cells, whereas they can be up- or down-regulated on others. Binding of a chemokine to its receptor leads to a cascade of intracellular signaling events accompanied by rapid receptor internalization, and it results in cell activation.

Chemokines have been divided into four groups, designated CXC, CC, C and CX3C chemokines, depending on the spacing of conserved cysteines. CXC chemokines predominantly affect neutrophils, whereas CC chemokines react with a variety of cell types, including eosinophils, macrophages and basophils (Tables I and II). The concentrations of allergy-associated chemokines, such as the CC chemokines eotaxin (32;64), RANTES (76), MIP-1α (76) and MCPs (76) and the CXC chemokine IL-8 (15), are elevated in BAL fluid from patients with asthma and are produced by local endothelial cells (37), epithelial cells (52-54) and infiltrated granulocytes (15).

**Table I: Chemokine receptors on granulocytes**

<table>
<thead>
<tr>
<th>Chemokine receptor</th>
<th>Expressed on</th>
<th>Chemokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR-1</td>
<td>neutrophils</td>
<td>IL-8</td>
</tr>
<tr>
<td>CXCR-2</td>
<td>neutrophils; activated eosinophils</td>
<td>IL-8</td>
</tr>
<tr>
<td>CCR-1</td>
<td>eosinophils</td>
<td>RANTES; MCP-3; MIP-1α</td>
</tr>
<tr>
<td>CCR-3</td>
<td>eosinophils</td>
<td>Eotaxin-1,-2; RANTES; MCP-2,-3,-4</td>
</tr>
</tbody>
</table>

**Allergy-associated chemokines**

*RANTES and MCPs*

RANTES, a major eosinophil chemoattractant (77), is released by platelets (77). T lymphocytes, macrophages, fibroblasts (78), endothelial cells (37) and epithelial cells (54). It is important for eosinophil accumulation in the lungs of asthmatics (76;79;80). MCP-3 and MCP-4 are also potent eosinophil chemoattractants, and have also been implicated in the allergic asthmatic reaction (81).
**General Introduction**

**Eotaxin**

Eotaxin (82), eotaxin-2 (83) and eotaxin-3 (33) have been shown to participate in the eosinophil recruitment in allergic inflammation of the lungs of asthmatics. Increased eotaxin levels are found in the airways of asthmatic patients, and allergen challenge further induces eotaxin release in BAL fluid (64). Eotaxin is a potent eosinophil chemoattractant, and studies in mice models suggest that it is also involved in the mobilization of eosinophils from the bone marrow into the blood (84).

Eotaxin-3 is a novel eotaxin that differs from the other eotaxins in its expression profile, i.e. eotaxin-3 is produced by endothelial cells after IL-4 and IL-13 activation (33), whereas the others are induced after stimulation with TNF-α and IFN-γ (37). In general, eotaxins are produced by bronchial epithelial cells (32;51-53), endothelial cells (32;33) and lung fibroblasts (78).

**IL-8**

IL-8 is a CXC chemokine implicated in the allergic asthmatic reaction (79;85) e.g. increased IL-8 levels in the BAL fluid have been demonstrated in the lungs of asthmatic patients (15). IL-8 is produced by several inflammatory cells implicated in asthma, including neutrophils, eosinophils (15), endothelial cells (35;36), epithelial cells (36;66), monocytes, macrophages, mast cells and fibroblasts. This cytokine is predominantly chemotactic for neutrophils (86), but it has also been reported to attract eosinophils from atopic donors (79) (87) and long-term IL-5-primed eosinophils (65). Moreover, anti-IL-8 antibodies inhibit the chemotactic activity of eosinophils in BAL fluid from asthma patients (79).

**Table II: CC chemokines**

<table>
<thead>
<tr>
<th>CC chemokines</th>
<th>Receptor</th>
<th>Chemotactic for</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES</td>
<td>CCR1; CCR3</td>
<td>eosinophils, basophils, monocytes, lymphocytes</td>
</tr>
<tr>
<td>MCP-3</td>
<td>CCR1; CCR2; CCR3</td>
<td>eosinophils, basophils, monocytes, lymphocytes</td>
</tr>
<tr>
<td>MCP-4</td>
<td>CCR2; CCR3</td>
<td>eosinophils, basophils, monocytes, lymphocytes</td>
</tr>
<tr>
<td>eotaxin</td>
<td>CCR3</td>
<td>eosinophils, basophils, basophils, Th2 lymphocytes</td>
</tr>
<tr>
<td>eotaxin-2</td>
<td>CCR3</td>
<td>eosinophils, basophils, Th2 lymphocytes</td>
</tr>
<tr>
<td>eotaxin-3</td>
<td>CCR3</td>
<td>eosinophils, basophils, Th2 lymphocytes</td>
</tr>
</tbody>
</table>

**Other chemoattractants**

Besides the chemokines and cytokines described above, there are also other mediators that attract granulocytes. For instance, C5a, the cleavage product of complement component C5, is a potent chemoattractant and activator of neutrophils and eosinophils, and greatly increases capillary permeability. Another example is the bacterial product fMLP, a potent chemoattractant and activator of neutrophils. However, fMLP attracts neutrophils in vivo to sites that have been invaded by bacteria, and the role of fMLP in allergic inflammation is therefore expected to be limited. In contrast, the acetylphospholipid PAF, produced by endothelial cells, neutrophils and eosinophils, is thought to play an important role in asthma (88). It primes and attracts neutrophils and eosinophils (57), stimulates airway mucus production, increases vascular permeability and causes bronchoconstriction (89) and hyperresponsiveness (90).
Integrins and adhesion molecules

Integrins

Integrins form a superfamily of widely expressed cell surface adhesion molecules. These heterodimeric transmembrane glycoproteins consist of an $\alpha$ subunit, non-covalently associated with a $\beta$ subunit. Subfamilies exist of certain $\alpha$ chains linked to a common $\beta$ chain. The extracellular domain can specifically bind to one of many different ligands and the cytoplasmic domain is associated with the actin cytoskeleton and associated proteins, including vinculin, talin and $\alpha$-actinin. Thus, as their name implies, integrins create an “integrated” link between the outside and the inside of the cell. Currently, eight different $\beta$ subunits and more than sixteen different $\alpha$ subunits been recognized on human cells. Certain heterodimers are found exclusively on one cell type, whereas others are widely expressed. Integrins permit cell adhesion to extracellular matrix (ECM) proteins and to other cells and are important transducers of signals that are vital to cell functions. For instance, signals from the extracellular environment initiate general responses such as cell motility, growth, survival and differentiation, and other signals induce specialized functions such as degranulation and oxidant production in granulocytes.

In resting leukocytes, integrins are maintained in an inactive conformation, i.e. they are unable to bind their ligand. Integrins are primed for subsequent cell activation by a mechanism called inside-out signaling, which induces a conformational change in the integrin molecule that allows subsequent ligand binding. In addition, integrin clustering on the cell surface further primes the cells for enhanced reaction after ligand binding to the integrins. This inside-out signaling can be induced by cytokine or adhesion molecule binding to the integrin molecule binding to the cells. Direct activation of integrins is also possible, e.g. by divalent cations or activating antibodies that bind to the extracellular regions of the integrins. Outside-in signaling takes place when the ligand has bound to the activated integrin, resulting in the above-mentioned cellular responses.

Integrins on granulocytes

Integrins are important to virtually all responses of granulocytes to infection and injury (91), but they are particularly vital in mediating cell adhesion, migration, phagocytosis, and oxidant production. Neutrophils and eosinophils possess only integrins belonging to the $\beta_1$ and $\beta_2$ integrin subfamilies (Table III). The three members of the $\beta_2$ subfamily are expressed on both neutrophils and eosinophils, but $\beta_1$ integrins are more abundantly expressed on eosinophils than on neutrophils and are thought to play a key role in the extravasation of eosinophils during allergic inflammation (92,93). In vivo studies in mice treated with blocking antibodies against $\alpha_4\beta_1$ and/or its ligand VCAM-1 on endothelial cells have demonstrated the participation of the $\alpha_4\beta_1$/VCAM-1 pathway in allergen-induced eosinophil recruitment into the airways (93). Moreover, the expression of VCAM-1 on endothelial cells in bronchial mucosa biopsies from asthmatic individuals has been correlated with eosinophil migration into the airways (94).

Several studies have suggested cooperation in neutrophil $\beta_1$ and $\beta_2$ integrin functioning. For instance, it has been found that activating antibodies to $\beta_1$ integrins activate $\beta_2$ integrins on human neutrophils (95), and vice-versa that anti-$\beta_2$ integrin mAbs enhance $\alpha_4\beta_1$-mediated neutrophil adhesion to fibronectin (96). In parallel, $\beta_2$ integrin-mediated transendothelial migration has been reported to increase $\alpha_4\beta_1$ expression (97). This regulation of $\beta_1$ integrin expression by $\beta_2$ integrins may serve to prepare the cell for interaction with the subendothelial matrix (primarily mediated by $\beta_1$ integrins) while it is crossing the endothelium (primarily mediated by $\beta_2$ integrins).
General Introduction

Table III: Integrins expressed on granulocytes

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Other name</th>
<th>CD number</th>
<th>Ligand(s)</th>
<th>Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β1</td>
<td>VLA-4</td>
<td>CD49d/CD29</td>
<td>VCAM-1, fibronectin</td>
<td>Adhesion; chemotaxis</td>
</tr>
<tr>
<td>α5β1</td>
<td>VLA-5</td>
<td>CD49e/CD29</td>
<td>Fibronectin</td>
<td>Adhesion; chemotaxis; respiratory burst</td>
</tr>
<tr>
<td>α6β1</td>
<td>VLA-6</td>
<td>CD49f/CD29</td>
<td>Laminin</td>
<td>Adhesion; chemotaxis</td>
</tr>
<tr>
<td>α4β2</td>
<td>LFA-1</td>
<td>CD11a/CD18</td>
<td>ICAM-1, ICAM-2, ICAM-3</td>
<td>Transmigration; adhesion</td>
</tr>
<tr>
<td>α4β2</td>
<td>Mac-1, CR3</td>
<td>CD11b/CD18</td>
<td>ICAM-1; fibrinogen, C3bi</td>
<td>Transmigration; adhesion; chemotaxis; respiratory burst; phagocytosis; degranulation</td>
</tr>
<tr>
<td>α4β2</td>
<td>P150,95, CR4</td>
<td>CD11c/CD18</td>
<td>Fibrinogen, C3bi</td>
<td>Adhesion</td>
</tr>
</tbody>
</table>

Adhesion molecules

The first step in extravasation of granulocytes is tethering to and rolling over the endothelial surface (Fig. 1). This step is mediated by selectins. Selectins are inducible transmembrane glycoproteins that bind cell-surface carbohydrate ligands, such as sialyl Lewis X on granulocyte and endothelial-cell surface proteins. The family of selectins is composed of three members: L-selectin, E-selectin and P-selectin. L-selectin is expressed on granulocytes and is shed from the cell surface after activation. E-selectin is expressed on endothelial cells after de novo protein synthesis upon activation by IL-1β, TNF-α or LPS. Binding of E-selectin on endothelial cells to sialyl Lewis-X glycoproteins on granulocytes leads to activation of β2 integrins on the granulocytes through inside-out signaling (98). P-selectin is stored in Weibel-Palade bodies in endothelial cells and is transiently expressed at the cell surface immediately after activation by thrombin or histamine.

The next step in extravasation is adhesion of the granulocytes to the endothelium (Fig. 1). This step is mediated by adhesion molecules on endothelial cells such as ICAM-1, ICAM-2 and VCAM-1, which bind to integrins expressed on granulocytes (30;39;99;100). The β2-integrins LFA-1 and Mac-1 bind to ICAM-1 and ICAM-2, members of the immunoglobulin gene superfamily. ICAM-1 is constitutively expressed on endothelial cells and epithelial cells, and its expression can be up-regulated by inflammatory agents such as IL-1β, TNF-α and LPS (31). ICAM-2 is expressed at the junctions and on the apical surface of endothelial cells (101) and can be down-regulated at the junctions by the combination
of TNF-α and IL-1β (102). VCAM-1, also a member of the immunoglobulin gene superfamily, binds to the integrin α4β1 expressed on eosinophils (93;94). In contrast to ICAM-1, VCAM-1 is expressed on activated endothelial cells at sites of inflammation only (31). Inflammatory mediators, including IL-4, TNF-α and IL-13, stimulate its expression (31;40;41).

A molecule that is thought to be involved in the actual migration of granulocytes across the endothelium is PECAM-1 (CD31), again a member of the immunoglobulin gene superfamily (103). Several studies have shown that PECAM-1 is required for transendothelial migration of neutrophils in vitro (42;104;105) and in vivo (104;106). PECAM-1 is expressed on the surface of platelets, monocytes, neutrophils, NK cells and subsets of T cells, but most profoundly on continuous endothelia where it is concentrated at the borders between opposing cells (Fig. 2). Depending on the cell type, PECAM-1 is capable of mediating homophylic interactions, i.e. PECAM-1 on one cell binds to PECAM-1 on another cell, or heterophylic interactions, i.e. the ligand for PECAM-1 on the other cells is another structure, e.g. αvβ3 (107). The role of PECAM-1 in transendothelial migration is not yet fully understood. It might acts as a homophilic adhesion molecule and direct migration of the cells through the endothelial cell junctions by the formation of a haptotactic gradient on the endothelial cell surface. However, it has also been speculated that PECAM-1 acts indirectly through activation of integrins via inside-out signaling (108-111).

Another molecule implicated in granulocyte migration across the endothelium and epithelium is the immunoglobulin family member CD47 (50). CD47 is a multi-span membrane protein that is associated with integrins (112) and which may be implicated in transmigration via its influence on the activity of integrins participating in the migration process (113). However, neutrophils as well as epithelia and endothelia express CD47, and it is not clear which molecules are playing a role in transmigration, i.e. the neutrophil, epithelial and/or endothelial CD47.

**Intercellular junctions of endothelial and epithelial cells**

Cell-cell junctions in a cellular monolayer are composed of gap junctions, desmosomes, adherens junctions and tight junctions (Fig. 2). Even though the presence and arrangement of these different types of intercellular junctions varies in epithelia (114) and endothelia (115), many of the basic components of epithelial and endothelial junctions are the same. Gap junctions contain channels that allow physical passage of low-molecular-weight molecules from one cell to another. Desmosomes, adherens junctions and tight junctions are formed by specific transmembrane proteins that mediate homophilic adhesion between cells through their extracellular domains, and anchor to a complex network of intracellular cytoskeletal proteins and actin microfilaments through their cytoplasmic regions.

The adhesion receptors of desmosomes and adherens junctions are composed of proteins belonging to the cadherin family members, e.g. VE-cadherin on endothelial cells and E-cadherin on epithelial cells. Cadherins are single-chain transmembrane polypeptides that mediate homophilic binding. The short cytoplasmic domain of cadherins is linked to cytoplasmic proteins called catenins, which provide the linkage to the actin cytoskeleton. These desmosomes and adherens junctions differ in their localization and function. Adherens junctions are found at the apical end of the lateral membrane and are implicated in the signal transduction between neighboring cells, whereas desmosomes are spread over the entire lateral membrane and lack signaling capacity (115).

Tight junctions or zonula occludens, which are the most apical intercellular junctions, are composed of transmembrane and cytoplasmic plaque proteins (e.g. occludin and claudin). These
juncti  ons represent an apparent fusion of the outer leaflets of the plasma membranes of two adjacent cells. They seal the intercellular space and control the paracellular permeability and cell polarity, functions that most epithelia and many endothelia display. Endothelial and epithelial tight junctions are similarly organized but differ in their localization. In contrast to epithelial tight junctions, endothelial tight junctions can be less strictly localized to the apical cell membrane and can be spatially intermingled with adherens junctions. This is possibly due to the far more flat aspect of the endothelium.

Next to the structures described above, endothelial junctions contain PECAM-1, which is not only implicated in endothelial cell-to-cell adhesion but also in angiogenesis, vascular injury repair, and control of leukocyte extravasation (105). Moreover, integrins are found at cellular junctions and in the endothelial and epithelial basal plasma membrane, where they are concentrated in focal contacts and promote cell attachment to extracellular matrix proteins (116)(Fig. 2).

The mechanisms by which leukocytes trigger the reversible opening of endothelial and epithelial cell junctions during diapedesis are still obscure. The possibility that leukocyte passage requires protease digestion of membrane proteins seems unlikely in view of the very rapid closure and reorganization of the junctions after granulocyte diapedesis (117), the lack of effect on the barrier

**Figure 2. Schematic representation of junctions between endothelial cells.**
function (114) and the fact that neither matrix metalloproteinase nor serine proteinase digestion is required for successful neutrophil transendothelial migration (118:119). Even at granulocyte concentrations that cause a transient increase in permeability, the monolayer reseals quickly behind the passing granulocytes (120). An attractive hypothesis is that leukocyte adhesion causes a cascade of events within the endothelial and epithelial cells that eventually leads to opening of their junctions. In particular, leukocyte ligation to adhesion molecules, such as selectins, VCAM-1, ICAM-1 or PECAM-1, may generate such intracellular signals (121-123). According to this hypothesis, endothelial and epithelial cells would not only play an important role in regulating leukocyte attachment to their surface but also actively modulate leukocyte diapedesis.

Transmigration

During inflammation, leukocytes tether to and roll over the endothelial cell surface via selectins expressed on both cell types (Fig. 1 & 3). Leukocytes usually attach to the endothelial cells where shear stress is lowest, i.e. in the post-capillary venules. The slow velocities of rolling granulocytes favor encounters with chemoattractants or cell activators bound to the apical surface of the endothelium. These mediators activate the $\beta_1$ and $\beta_2$ integrins on leukocytes to bind firmly to their ligands VCAM-1 and ICAM-1 on the endothelial surface (Fig. 1 & 3). The cells then arrest, spread, and finally emigrate between endothelial cells to reach the underlying tissues (Fig. 3). For subsequent passage of the leukocytes through the extracellular matrix proteins of the endothelial cells, the basement membrane and tissue cells, protein degradation by metalloproteinases is probably required.

**Figure 3. Schematic representation of granulocyte recruitment.** Granulocytes in the circulation (I) are activated when they pass an inflamed site, and as a result start to roll on the endothelium (II). This is followed by adhesion to the endothelium and flattening of the granulocytes (III). The cells subsequently migrate through the junctions between the endothelial cells and cross the basal lamina (IV). The chemotactic gradient guides the granulocytes through the interstitial tissue towards the site of inflammation, where they encounter the next barrier, the epithelium. The granulocytes cross the basal lamina and migrate between the epithelial cells to reach the luminal side of the inflamed organ (IV).
When the inflammation site is located in the lumen of organs, like in the gut, kidney or lungs, the granulocytes will find the epithelium on their way. The granulocytes presumably interact with epithelial adhesion molecules and subsequently crawl through the cell junction and finally reach the site of inflammation. We know little about the adhesion molecules involved in transepithelial migration. For instance, the β2 integrin Mac-1 has been shown to mediate adhesion of neutrophils to epithelia, but the epithelial counter-receptor has not yet been identified. Moreover, although it has been demonstrated that CD47 plays a role (50), its precise function and which molecules are involved, i.e. the neutrophil, epithelial and/or endothelial CD47, is not clear yet. In brief, the granulocyte migration across the endothelium and epithelium is regulated by mechanisms that are not completely understood but are affected by gradients of chemoattractants, leukocyte integrins, adhesion molecules and opening of the cell junctions.

Scope of this thesis
In the late phase of allergic asthmatic reactions, massive amounts of eosinophils and neutrophils infiltrate the lungs and cause damage to the tissue. In this study, we have tried to contribute to the elucidation of the mechanism that lies at the basis of this infiltration. To investigate this mechanism we have used an in vitro transmigration model, in which the granulocyte migration across cellular monolayers can be analyzed under different conditions. Granulocyte migration across endothelial monolayers in vitro has already been studied extensively, but less is known about migration across lung epithelial monolayers. Therefore, we have determined the effect of putative participants in allergic asthmatic inflammation, such as PAF (Chapter 2) and neutrophils (Chapter 3), on the migration of eosinophils across epithelial monolayers.

Another potentially important aspect of allergic asthmatic inflammation that needed further investigation in our view, is the cellular environment in which the inflammatory reaction is taking place in vivo. To study this aspect, we have developed a bi-layer transmigration model comprised of primary human endothelial cells and lung epithelial cells, simultaneously cultured on opposite sides of Transwell filters (Chapter 4). We have investigated the migration of neutrophils and eosinophils across these bi-layers and the production of cytokines and chemokines by these bi-layers (Chapter 4). Moreover, we have further analyzed the effect of the paracrine interaction between endothelial and epithelial cells that we demonstrated by means of an in vitro co-culture model. Endothelial and epithelial cells were cultured in the same wells but on different surfaces, i.e. on the Transwell filter and on the bottom of the Transwell, to prevent any physical contact. The effects of the paracrine interaction on the migration of granulocytes and the expression of adhesion molecules on endothelial and epithelial cells were studied in this co-culture model (Chapter 5).

In the process of the characterization of “trans-bi-layer” migration of neutrophils and eosinophils, we came across the observation that neutrophil, but not eosinophil, PECAM-1 is implicated in transendothelial migration in vitro. This is an unexpected and, as yet unpublished observation, which might be of importance for the regulation of selectivity in leukocyte recruitment. Therefore, we further analyzed the role of PECAM-1 in neutrophil and eosinophil transendothelial migration (Chapter 6). Last but not least, we intended to investigate the migration across lung microvascular endothelial cells (LMVEC) as compared to migration across endothelial cells derived from umbilical veins (HUVEC) that are routinely used for the study of transendothelial migration in vitro. We figured that specific features of
LMVEC might contribute to the eosinophil and neutrophil recruitment as seen in allergic inflammation. Despite a great deal of effort, we unfortunately did not succeed in isolating and cultivating human LMVEC (Chapter 7). Nevertheless, we were eventually able to study the expression of adhesion molecules on these cells, because they have recently become commercially available (Chapter 7). Our results are summarized and discussed in Chapter 8.

References

Introduction


General Introduction


