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

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CHAPTER VI

PECAM-1 is Implicated in Transendothelial Migration of Neutrophils, but not of Eosinophils

Submitted for publication
AM-1 is implicated in the transmigration of neutrophils.
PECAM-1 is implicated in Transendothelial Migration of Neutrophils, but not of Eosinophils

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PECAM-1 is involved in leukocyte transendothelial migration. We have studied the role of PECAM-1 in the in-vitro migration of neutrophils and eosinophils across endothelial cell monolayers. Pre-treatment of neutrophils with an antibody to PECAM-1 (HEC170) blocked transendothelial migration of neutrophils in response to PAF, C5a and IL-8, but the migration was hardly affected when endothelial cells were pre-treated. Moreover, this mAb did not impair migration across epithelial monolayers. In contrast to neutrophils, eosinophil transendothelial migration was not blocked by HEC170 mAb, irrespective of the eosinophil activation state or the chemoattractants used, although eosinophils express PECAM-1 to the same level as neutrophils. Thus, neutrophil, but not endothelial, PECAM-1 is implicated in transendothelial migration in vitro, suggesting that an interaction between PECAM-1 and an endothelial ligand plays a role in the migration process. This interaction is not involved in eosinophil migration, pointing to a role for neutrophil PECAM-1 in selective neutrophil extravasation.

Introduction

In lung inflammation and asthmatic reactions, circulating leukocytes, primarily neutrophils and eosinophils, migrate across the lung endothelium and the lung epithelium into the airway lumen. The process of transmigration initially involves selectin-mediated rolling of the granulocytes on the endothelial cell surface and is followed by firm adhesion to and passage across the endothelial cell layer (1;2). Subsequently, the granulocytes migrate through the interstitial matrix and across the epithelium into the lung lumen.

The endothelium plays an important role in the recruitment of granulocytes. It is the first barrier that has to be crossed and acts therefore as a gate to the underlying tissue. Furthermore, endothelial cells can be activated by inflammatory stimuli, resulting in release of a wide range of proinflammatory mediators (3;4) and increased cell-surface expression of adhesion molecules required for leukocyte transmigration (3), such as E-selectin, P-selectin, intercellular adhesion molecule (ICAM)-1, vascular cellular adhesion molecule (VCAM)-1, and platelet/endothelial cell adhesion molecule (PECAM)-1 (5;6).

The role of the selectins, ICAM-1 and VCAM-1 has been extensively studied (5), but the precise role of PECAM-1 in transendothelial migration is less well defined. PECAM-1 or CD31 is a 130-kD single-chain molecule containing six Ig-like domains, a transmembrane region and a cytoplasmic tail (7;8). It is expressed on the surface of neutrophils (8-10), eosinophils(11), platelets(8), monocytes (8), NK cells, subsets of T cells(8), and on continuous endothelia where it is concentrated at the borders between opposing cells (7;12). Depending on the cell type, PECAM-1 is capable of mediating homophilic interactions, i.e. PECAM-1 on one cell binds to PECAM-1 on another cell, or heterophilic interactions, i.e. the ligand for PECAM-1 on the opposing cell is another...
structure (7:8). For instance, it has been shown that $\alpha_\beta_3$ integrin, which is expressed by endothelial cells and subsets of T cells, is a heterophilic ligand for PECAM-1 (13:14). Other described PECAM-1 ligands are an uncharacterized 120-kD molecule on activated T lymphocytes (15) and CD34 (16). Ligation of PECAM-1 stimulates tyrosine phosphorylation of its cytoplasmic tail, leading to association with a number of signaling proteins, including SHP-2 (7) and activation of the small GTPase Rap1 (17).

The role of PECAM-1 in transendothelial migration might be to act as a homophilic adhesion molecule and to direct migration of the cells through the endothelial cell junctions by the formation of a haptotactic gradient of PECAM-1 on the endothelial cell surface. This hypothesis is attractive, because there is indeed a gradient of PECAM-1 expression from the apical endothelial surface to the junctions. Within the junction, it is more concentrated at the basal side of the cleft (12). However, PECAM-1 may also act indirectly through activation of integrins by intracellular signaling (7:9;17). Stimulation of leukocyte PECAM-1 has been shown to cause up-regulation and activation of $\beta_2$ integrins on monocytes, neutrophils (9;18) and natural killer cells (19) and to increase adhesive functions of $\beta_2$ integrins on eosinophils (11), T cells (20) and CD34+ hematopoietic progenitor cells (21).

Several studies have shown that PECAM-1 is required for transendothelial migration of neutrophils in vitro (6:8) and in vivo (8:10,22). However, little is known about the role of PECAM-1 in eosinophil transendothelial migration. In this paper we show that neutrophil, but not eosinophil transendothelial migration is mediated by PECAM-1, although both cell types express PECAM-1 to the same extent. Moreover, we show that neutrophil, but not endothelial, PECAM-1 is involved in in vitro transendothelial migration.

**Materials & Methods**

**Reagents**

Platelet-activating factor (PAF) and C5a were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human (rh) IL-5 was bought from Pepro Tech Inc. (Rocky Hill, NJ, USA), and rhIL-8 and bFGF from Boehringer Mannheim (Mannheim, Germany). Eotaxin was derived from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD31 monoclonal antibody (mAb) (IgG1; HEC170) (23) was isolated from hybridoma supernatant by precipitation with 50% saturated ammonium sulphate and subsequent protein-A affinity chromatography. CD14 mAb (IgG1; 8G3), human serum albumin (HSA) and fibronectin were obtained from the Central Laboratory of the Netherlands Blood Transfusion Service (CLB) (Amsterdam, The Netherlands). Fluorescently labeled secondary antibody (Ab) to mouse immunoglobulins was from Dako (Glostrup, Denmark). C5a, PAF, IL-5 and IL-8 were dissolved in phosphate-buffered saline, pH 7.4, (PBS) supplemented with 0.5% (w/v) human serum albumin (HSA) and were stored at -20°C. HEPES medium contained 132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl$_2$, 1.0 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) HSA (pH 7.4). Lysis buffer consisted of PBS supplemented with 0.1% (v/v) Tween-20 (Merck, Schuchardt, Germany), 0.2% (w/v) N-acetyl-N,N,N-trimethyl ammonium bromide (Sigma), 0.2% (w/v) bovine serum albumin (BSA) (Sigma) and 20 mM EDTA.
Cell culture
Freshly isolated, primary HUVECs (24) and HUVEC cell line cells (25) were cultured in HUVEC medium (RPMI 1640 (Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with 10% (v/v) heat-inactivated human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 1 ng/ml bFGF) in culture flasks coated with 1 mg/ml fibronectin. The 2\textsuperscript{nd}-4\textsuperscript{th} passages of the primary HUVECs and the 10\textsuperscript{th} to 30\textsuperscript{th} passage of HUVEC cell line cells were used for sub-culturing on fibronectin-coated polycarbonate Transwell membranes (3.0 μm pore size, 12 mm diameter; Costar, Cambridge, MA, USA).

The human lung epithelial adenocarcinoma-derived cell line H292 (American Type Culture Collection CRL 1848) (26) was grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated human serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine, in uncoated culture flasks. The 4\textsuperscript{th} to 30\textsuperscript{th} passages of H292 cells were used for the transmigration assay. To study the granulocyte migration across epithelial monolayers in the physiological direction, H292 cells epithelial cells were routinely cultured on the bottom side of Transwell polycarbonate membranes (3.0 μm pore size, 12 mm diameter) (Costar, Cambridge, MA), as described (27).

Granulocyte isolation
Blood was obtained from healthy volunteers. Granulocytes were purified from a buffy coat of 500 ml of blood by density gradient centrifugation over isotonic Percoll, as described (28). After lysis of the erythrocytes in the pellet fraction with a cold erythrocyte lysis buffer containing 155 mM NH\textsubscript{4}Cl, 10 mM KHCO\textsubscript{3} and 0.1 mM EDTA (pH 7.4), the granulocytes were washed twice in PBS. The granulocyte cell suspension consisted for more than 95% of neutrophils. This cell suspension was used for the isolation of eosinophils via the formyl-methionyl-leucyl-phenylalanine (fMLP) method (29). The purity and viability of the eosinophils were more than 95%. This procedure leads to the isolation of relatively unprimed eosinophils compared to conventionally used isolation procedures with immunomagnetic beads (30).

Transmigration assay
Fresh medium was added to the Transwells 4 hours prior to the start of the transmigration assay, and the Transwells were washed twice with HEPES medium just before starting the transmigration assay. The lower compartment was filled with pre-warmed HEPES medium with or without individual chemoattractants. The granulocytes (10\textsuperscript{7}/ml) were labeled with 4 μg/ml Calcein-AM (Molecular Probes) diluted in HEPES medium for 30 min at 37°C prior to the onset of the transmigration assay (31). After labeling, the cells were washed twice with HEPES medium. Where indicated, purified eosinophils (2x10\textsuperscript{7} cells/ml) were primed with 10\textsuperscript{-10} M IL-5 in HEPES medium for 30 min at 37°C, and were washed in incubation medium. Monoclonal Abs (10 μg/ml) were added to the granulocytes and endothelial cells 10 min before the start of the transmigration assay, or the cells were separately pre-incubated with mAbs for 15 min and washed before the onset of the transmigration assay.

Granulocytes were placed in the upper compartment. The Transwells were incubated for either 40 min (neutrophils) or 1.5 h (eosinophils) at 37°C with 5% CO\textsubscript{2} and maximal humidity. After the incubation, the upper and lower compartments were washed with HEPES medium. The fluids were collected and the membranes were excised. The cells in the fluids and the excised membranes were lysed in lysis buffer. The extent of transmigration was quantified by means of fluorescence measurement, i.e. the levels of Calcein-AM in the upper compartment, lower compartment and membrane were measured with a spectrofluorometer (Model RF-540, Shimadzu Corporation, Kyoto, Japan). The percentage of
labeled cells that had transmigrated was calculated from the amount of fluorescence detected in the lower compartment in relation to the fluorescence of the originally added Calcein-AM-labeled granulocytes.

**FACS analysis**

The expression of surface antigens on granulocytes was measured by indirect immunofluorescence with flow cytometry. The cells were incubated with the mAb CD31 HEC170 or with mAb CD14 8G3, as indicated in the text, for 30 min at 4°C. After the cells had been washed with a 30-fold excess of ice-cold PBS containing 1% (w/v) BSA and 14 μg/ml azide, binding of mAb was detected by incubation with PE-conjugated goat-anti-mouse-Ig for 30 min at 4°C. The fluorescence intensity of the cells was measured with a flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA).

**Statistical analysis**

The results were expressed as the mean ± SEM of the number of different experiments mentioned in the legends and analyzed with the Student's t-test. Two-sided p values were calculated, and p values exceeding 0.05 were considered not to be significant.

**Results**

To investigate the role of PECAM-1 in the transmigration of granulocytes across monolayers of endothelial cells, we performed a transmigration assay in the presence of mAb HEC170. As shown in Fig. 1, mAb HEC170 blocked migration of neutrophils across HUVEC monolayers in response to PAF by 47%, whereas neutrophil migration across H292 bronchial epithelial monolayers, which are PECAM-1-deficient, was not affected (Fig. 1). The inhibitory effect on migration seemed not to be restricted to PAF-driven migration, because transendothelial migration of neutrophils towards other chemoattractants, such as C5a and IL-8, was also reduced when HEC170 was added (Fig. 2).

Moreover, incubation with CD31 mAb HEC65, which is directed against domains 3-6 (32), did not impair neutrophil transendothelial migration (data not shown). Thus, these results suggest that the first two domains of CD31 are implicated in neutrophil transendothelial migration.

Both neutrophils and endothelial cells express PECAM-1, and the molecules on both cell types may play a role in transmigration. To unravel which molecular interaction is blocked by the HEC170 mAb, we determined whether endothelial or neutrophil-derived PECAM-1 was inhibited in its function after HEC170 mAb treatment. Endothelial monolayers and neutrophils were separately pre-treated with HEC170 mAb and washed. We found that the migration was hardly affected when HUVEC was pre-treated with HEC170, whereas neutrophil pre-treatment reduced migration by 39% (Fig. 3).

**Figure 1. Inhibition of neutrophil migration across monolayers of either H292 lung epithelial cells or HUVEC towards PAF (100 nM).** The cells were pre-incubated for 10 min with CD31 mAb HEC170 or with an irrelevant mAb (CD14), and the mAbs remained present during the transmigration assay. The results are shown as percentage inhibition of control transmigration, i.e. pre-incubated with mAb CD14. Data are means ± SEM of five independent experiments. Asterisks indicate significance of difference with control migration (** p< 0.01).
In contrast to neutrophils, eosinophil transmigration across endothelial monolayers was not blocked by HEC170 mAb (Fig. 4) irrespective of whether the endothelium or the eosinophils or both were pre-treated with the mAb (data not shown). The proportion of eosinophils associated with the HUVEC monolayer was not affected by HEC170 mAb either (data not shown). In addition, the lack of inhibition was not due to the fact that the transmigration was assessed through monolayers of endothelial cell-line cells, because HEC170 mAb did not affect eosinophil migration across monolayers of primary HUVEC either (data not shown). Moreover, priming of the eosinophils did not increase the sensitivity to HEC170 mAb, because migration of IL-5-primed eosinophils was not blocked by HEC170 mAb (Fig 5a & 5b). The type of chemoattractant also seemed not to matter, because CD31 mAb inhibited neither the migration towards PAF/C5a nor towards PAF or cotaxin (Fig. 5a & 5b). These results were unexpected, because eosinophils express PECAM-1 on their surface to the same extent as found on neutrophils (Fig 6).
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Figure 4. Effect of CD31 mAb HEC170 on neutrophil migration across monolayers of HUVEC towards PAF (100 nM) and on eosinophil migration in response to PAF (1 μM) combined with C5a (10 nM). The cells were pre-incubated for 10 min with mAb HEC170 or with an irrelevant mAb (CD14), and the mAbs remained present during the transmigration assay. The results are indicated as percentage inhibition of control transmigration, i.e. pre-incubated with mAb CD14. Data are means ± SEM of four independent experiments. Asterisks indicate significance of difference with control migration (**p<0.01).

Figure 5. Transendothelial migration of IL-5-primed eosinophils. The cells were pre-incubated for 10 min with an irrelevant mAb (CD14) (open bars) or with CD31 mAb HEC170 (black bars) and the Abs remained present during the transmigration assay. (a) Migration in response to the combination of PAF (1 μM) and C5a (10 nM). Data are means ± SEM of three independent experiments. (b) Migration in response to PAF (1 μM) and eotaxin (100 ng/ml). Data are means of two independent experiments.

Figure 6. PECAM-1 surface expression measured with CD31 mAb HEC170 (thick line) was compared to a control mAb (thin line) for neutrophils and for eosinophils by flow cytometry. The mean fluorescence intensities are depicted in the figure. Results are representative of three independent experiments.
Discussion

The role of PECAM-1 in neutrophil transendothelial migration was investigated in an in vitro transmigration model, consisting of confluent primary HUVEC monolayers grown on Transwell membranes. To study the role of PECAM-1, either neutrophils or endothelial cells were pre-treated with a blocking monoclonal antibody to PECAM-1 (HEC170) prior to the transmigration assay. Our results suggest that the first two domains of CD31 are implicated in neutrophil transendothelial migration. The fraction of neutrophils associated with the HUVEC monolayer, i.e. the number of neutrophils bound to the endothelial cells or the Transwell membrane, was not affected by blocking HEC170 mAb (data not shown); thus, the neutrophils were not accumulating above the Transwell membrane. These results suggest that the reduced neutrophil migration was not due to impaired capability of the neutrophils to cross the basement membrane deposited by endothelial cells on the Transwell membrane. Because HEC170 binds to the first two domains of PECAM-1, it can be concluded that these domains are not implicated in neutrophil passage across this basement membrane. This notion has been confirmed by other investigators, who showed that the first two domains of PECAM-1 are implicated in the passage of leukocytes through endothelial junctions and not in passage through the basement membrane (8,10).

HEC170 mAb did not block neutrophil migration across H292 bronchial epithelial monolayers, which are PECAM-1-deficient. This indicates that HEC170 mAb does not induce signaling in neutrophils leading to reduced motility, and that the HEC170 mAb-sensitive molecular interaction is absent or plays a less prominent role in transepithelial migration. Furthermore, preliminary results obtained with stable H292 transfectants expressing CD31 at the intercellular junctions suggest that neutrophil transepithelial migration is unaffected by CD31 transfection (data not shown). Taken together, these results indicate that CD31 plays a prominent role only in endothelial transmigration, and that the process of transepithelial migration differs from transendothelial migration even when CD31 is present at the intercellular junctions of the epithelial cells. Comparable experiments have been performed by Zocchi et al (33) but with different results. These investigators reported that lymphocyte migration is greatly stimulated when CD31 is transfected in NIH 3T3 fibroblasts. This difference might be due to the fact that other cell types were used. The molecular basis underlying lymphocyte transfibroblast migration and neutrophil transepithelial migration surely differs and this may also apply to the role of CD31 in these processes. It would be instructive to investigate whether or not lymphocyte migration across CD31-expressing epithelial cell monolayers is augmented as well, in that case CD31 on endothelial cells would be involved in lymphocyte, but not in neutrophil, transmigration. Vice versa, we do not expect that neutrophil migration across CD31-expressing fibroblast monolayers would be affected because we show in this report that neutrophil CD31, but not CD31 expressed on cellular monolayers, is involved in neutrophil transmigration. We found that only pre-treatment of neutrophils, and not of endothelial cells, with blocking CD31 mAb inhibited migration. This lack of effect of endothelial cell pre-treatment cannot be due to detachment of mAb during washing of the endothelial cells, because this mAb remained bound to endothelial cells after washing for several days (data not shown). Such a lack of effect has been observed before by Muller et al (34), but these investigators attributed this to presumed difficulty in delivering the mAb to the intercellular junctions where the lion share of the CD31 molecules are expressed. However, we presume that HEC170 mAb can readily reach the extracellular molecules in the endothelial intercellular junctions, because this has been shown for other mAbs (35). Moreover, in theory one would expect that in this experimental set-up (with the mAb present during the transmigration assay), CD31 mAb is able to reach those CD31 molecules that interact initially with neutrophils.
because these molecules must be reached by neutrophils too. In addition, the finding that transfection of CD31 into epithelial cells did not affect neutrophil transepithelial migration also points to a modest, if any, contribution of CD31 expressed on cellular monolayers to neutrophil transmigration. We therefore conclude that domains 1 and/or 2 of neutrophil, but not endothelial, PECAM-1 are implicated in neutrophil transendothelial migration \textit{in vitro}.

Up to date, only the homophilic interaction between endothelial PECAM-1 and neutrophil PECAM-1, has been considered to be involved in transendothelial migration \textit{in vitro} and \textit{in vivo} (8,34). However, our results indicate that this interaction plays a minor role in \textit{in-vitro} neutrophil transendothelial migration. Instead, an interaction between neutrophil PECAM-1 and an, as yet unknown, ligand on endothelial cells seems to play a role in the migration process. This ligand seems to be absent on epithelial cells, because transepithelial migration was not affected by HEC170 mAb. Interaction with this ligand on endothelial cells could lead to activation of the neutrophil and result in enhanced migration. The recent publications that ligation of PECAM-1 on neutrophils results in down-regulation of L-selectin and up-regulation and activation of \(\beta_2\)-integrins (18,36) support this hypothesis.

A potential candidate for such a ligand is \(\alpha_v\beta_3\). This integrin binds to PECAM-1 via its second Ig-like domain (13) and is expressed by endothelial cells and not by epithelial cells. However, whether or not HEC170 mAb affects the interaction between PECAM-1 and \(\alpha_v\beta_3\) is unclear. Moreover, it has recently been reported that, in rat, PECAM-1 and \(\alpha_v\beta_3\) integrin play different and independent roles in neutrophil migration (37). Thus, it cannot be deduced from our data whether or not neutrophil PECAM-1 interaction with endothelial \(\alpha_v\beta_3\) leads to activation of neutrophil integrins, resulting in stimulated transendothelial migration.

In contrast to neutrophils, eosinophil transmigration across endothelial monolayers was not blocked by HEC170 mAb. These results were unexpected, because eosinophils express PECAM-1 on their surface to the same extent as found on neutrophils. In eosinophils, the interaction between eosinophil PECAM-1 and endothelial \(\alpha_v\beta_3\) has been shown to cause activation of eosinophil \(\beta_1\)-integrins, leading to firm adhesion of eosinophils to endothelial cells through \(\alpha_v\beta_3/VCAM-1\) interaction (11). In theory, treatment of eosinophils with HEC170 mAb could affect the eosinophil adhesion and migration in two ways: a) it could mimic the interaction with a ligand by cross-linking PECAM-1; b) it could block the interaction of PECAM-1 with a ligand. In practice, we did not observe any effect of HEC170 mAb on eosinophil adhesion and migration, although HEC170 mAb bound to eosinophils to the same extent as to neutrophils. Thus, either PECAM-1 is not involved in eosinophil transendothelial migration or HEC170 mAb does not affect the molecular interaction involved in a PECAM-1-dependent migration pathway.

The reason why eosinophil adhesion and transendothelial migration is not sensitive to HEC170 treatment is obscure. One major difference between eosinophils and neutrophils is that neutrophils migrate more rapidly and more efficiently across endothelial monolayers. Eosinophil migration may be less sensitive to activation by means of PECAM-1 ligation and thus, the PECAM-1-mediated interaction may be of less importance to the migration process of eosinophils. The observation that PECAM-1 plays different roles in neutrophil and eosinophil migration confirms the idea that each cell type is differently equipped to reach its destination.
On the basis of these results it can be concluded that leukocyte PECAM-1 could be implicated in the specificity of leukocyte recruitment, i.e. endothelial cells may express a ligand that interacts with PECAM-1 and selectively activates the migratory machinery of neutrophils, but not of eosinophils, resulting in selective neutrophil extravasation. This finding may be of importance for the understanding of neutrophil-mediated inflammation, such as inflammation caused by bacteria and chronic inflammation such as chronic obstructive lung disease (COPD).

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References

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