Neutrophil and Monocyte Adherence to and Migration Across Monolayers of Cytokine-Activated Endothelial Cells: The Contribution of CD18, ELAM-1, and VLA-4

By B.C. Hakkert, T.W. Kuijpers, J.F.M. Leeuwenberg, J.A. van Mourik, and D. Roos

Pretreatment of endothelial cells with cytokines enhances the adherence of leukocytes, a process that is mediated by surface proteins expressed on both cell types. A three-dimensional model system for the simultaneous determination of leukocyte adherence and migration was used to study the contribution of CD11/CD18, endothelial leukocyte-adhesion molecule-1 (ELAM-1) and VLA-4 in neutrophil and monocyte adherence to and migration through cytokine-activated endothelial cells. Pretreatment of endothelial cells for 4 hours with recombinant interleukin-1β (rIL-1β) was found to enhance neutrophil adherence and migration to a much greater extent than monocyte adherence and migration. Neutrophil adherence was almost completely prevented by the combined use of monoclonal antibodies (MoAbs) against ELAM-1 and CD18. Although ELAM-1 has been designated an endothelial cell-specific cytokine-inducible receptor for neutrophils, we observed that ENA2, an anti-ELAM-1 MoAb, significantly reduced monocyte adherence about 30%. MoAbs against VLA-4, the ligand of the cytokine-inducible receptor VCAM-1, did not affect monocyte adherence. However, the combined use of the MoAbs against CD18, ELAM-1, and VLA-4 had a very strong and additive inhibitory effect on rIL-1β-induced monocyte adherence. The anti-CD18 MoAb reduced both rIL-1β-induced neutrophil and monocyte migration far below the level of the unstimulated controls, whereas neither the anti-ELAM-1 nor the anti-VLA-4 MoAbs significantly affected the process of migration. Our results indicate that neutrophils and monocytes initially adhere to cytokine-activated endothelial cells by CD18-independent and (to a lesser extent) by CD18-dependent mechanisms and subsequently change gears to a completely CD18-dependent migratory mechanism.

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the previously used calf skin collagen (type I, Sigma Chemical Co, St Louis, MO). The collagen solution (0.25 mL), consisting of eight parts of bovine collagen, one part 10X phosphate-buffered saline (PBS), and one part 0.1 N NaOH, was allowed to form a gel at 37°C for 60 minutes in 48-well microtiter plates (Costar, Cambridge, MA). The gels were incubated overnight with culture medium and subsequently treated with human fibronectin (Fn; 10 μg/mL) for 30 minutes. The excess of Fn was removed by two washes with culture medium. Endothelial cells (4 x 10⁴/cm²) were seeded on top of the gel and grown to confluence. Confluency was determined by May-Grünwald/Giemsa staining.

Leukocytes. Blood was obtained from healthy volunteers. Monocytes and neutrophils were purified from buffy coats of 500 mL of blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) as described. In short, blood cells were centrifuged over isotonic Percoll (specific gravity at room temperature, 1.077/cm³). The interphase, containing the mononuclear cells, was taken for isolation of the monocytes by countercurrent centrifugal elutriation. Neutrophils were isolated from the pellet fraction of the Percoll gradient. Purified leukocytes (1 x 10⁶/mL) were resuspended in labeling medium (RPMI 1640/M199, 0.1% human albumin [HA]). Monocytes and neutrophils were greater than 95% pure, and viability was more than 95%, as determined by lactate dehydrogenase (LDH) release.

Monoclonal antibodies (MoAbs). The following MoAbs were used. CLB-LFA-1/1 (IgG1) recognizes the common β chain of CD18. MoAB ENA2 (IgG2a) recognizes ELAM-1. MoAbs HP1/3 (IgG1) and HP2/1 (IgG1) were a gift from Dr F. Sanchez-Madrid (University of Madrid, Spain). These MoAbs recognize the α chain of VLA-4 (CD49d). Isotype-matched antibodies were used as control. Studies were performed with F(ab')₂ fragments to prevent Fc receptor-mediated binding of leukocytes to endothelial cells. Fragments were used in saturating concentrations (15 to 20 μg/mL). Leukocytes and endothelial cells were preincubated with MoAbs for 20 minutes and the MoAbs were not removed during the adhesion/migration assays. MoAbs against ELAM-1 (ENA2), VCAM-1 (4B9 [IgG1], a kind gift from Dr J.M. Harlan, Harbour Medical Center, Seattle, WA), and ICAM-1 (RR1/1 [IgG3], obtained from the Fourth Leukocyte Typing Workshop) were used for immunofluorescence flow cytometry.

Immunofluorescence flow cytometry. To investigate whether the nature of the culture substrate influences the ELAM-1, VCAM-1, and ICAM-1 expression by endothelial cells, indirect immunofluorescence flow cytometry analysis was performed. In short, endothelial cells were cultured on either collagen matrices or fibronectin in 6-well culture dishes. After the endothelial cells had reached confluency, the monolayers were incubated with recombinant interleukin-1β (rIL-1β) (10 μg/mL, a gift from Dr P.T. Lomedico, Hoffmann-La Roche, Nutley, NJ) or culture medium (control) for 4 or 24 hours. Thereafter, the endothelial cells were washed several times with PBS and nonenzymatically detached (1.5 mmol/L EDTA, 20 minutes). The adhering cells were washed twice with ice-cold PBS, containing 0.5% (vol/vol) bovine serum albumin (BSA) and 7.5 mmol/L sodium azide. Thereafter, fluoresceinated goat-antimouse-Ig was added and left with the cells for another 30 minutes at 4°C. After two washes, MoAb binding was quantitated for 10,000 cells with a FACScan (Becton Dickinson, Mountain View, CA) and was expressed as mean fluorescence intensity (MFI).

Adhesion/migration assay. The adhesion/migration assay was performed as described. Briefly, leukocytes were labeled with 51Cr and resuspended (1 x 10⁶/mL) in incubation medium (RPMI 1640/M199, 0.5% HA). Endothelial cell monolayers pre-treated for 4 hours with rIL-1β (10 U/mL) or culture medium (control) were washed twice with incubation medium. MoAb-containing incubation medium (0.125 mL) was added to the endothelial cells. Radiolabeled leukocytes (2 x 10⁶/mL; ie, five leukocytes per endothelial cell) were added to the endothelial cells. Culture plates were gently agitated for 1 minute and incubated for 20 minutes at 37°C, 5% CO₂. All incubations were run in quadruplicate. After incubation, the medium with unattached cells was collected, and the endothelial monolayers were washed twice with 0.25 mL of warm (37°C) incubation medium. These fractions were pooled (luminal medium). The complete and intact endothelial cell monolayer together with the adhering leukocytes was harvested by incubation with 0.1 mL of collagenase (100 U/mL; Worthington Bioch Corp, Freehold, NJ) for 5 to 10 minutes at 37°C (endothelial cell fraction). The collagenase-induced detachment of the endothelial cell monolayer was followed by light microscopy. After the removal of the intact endothelial cell monolayers, the subendothelial matrices were carefully checked for remaining endothelial cells by microscopy. This method requires very tight endothelial cell monolayers, because the slightest gap or disruption inevitably results in the formation of several small endothelial cell fragments. After the harvesting of the intact endothelial cell monolayers, the collagen matrices were digested by collagenase (100 U/mL, 2 hours, 37°C), incubated with 0.2 mL of 1% (wt/vol) Triton X-100 (30 minutes), and collected (subendothelial fraction). Radioactivity was measured in the three fractions. The recovery of radioactivity was more than 92%.

In this assay, leukocyte adherence and migration is defined as previously described, ie, leukocytes that stick to the endothelium are defined as adhering leukocytes and those that are located in the collagen matrix as migrated leukocytes. Percentages adherence and migration were calculated as follows: cpm in the endothelial cell fraction (adherence) or in the subendothelial fraction (migration)/total cpm added x 100%.

Statistical analysis. For statistical analysis paired Student's t-tests with Bonferroni adaptation were used. P values exceeding .05 were not considered significant.

RESULTS

Effect of growth substrate on ELAM-1, VCAM-1, and ICAM-1 expression by endothelial cells. The extent and the kinetics of ELAM-1, VCAM-1, and ICAM-1 expression by endothelial cells grown on either collagen matrices or fibronectin were essentially identical (Table 1). ELAM-1 and VCAM-1 were not expressed by resting endothelial cells. ELAM-1 expression was strongly induced by cytokine treatment of endothelial cells, reaching a maximum 4 hours after induction. rIL-1β-treatment of endothelial cells for 4 hours also induced the expression of VCAM-1 on endothelial cells. Unlike ELAM-1 and VCAM-1, ICAM-1 was already expressed by resting endothelial cells. Cytokine-activation of endothelial cells markedly enhanced the expression of ICAM-1, which did not decline within 24 hours.

Effects of MoAbs against CD18, ELAM-1, and VCAM-1. ELAM-1 and VLA-4 on rIL-1β-induced neutrophil adherence and migration. The pretreatment of endothelial cells for 4 hours with rIL-1β dramatically enhanced both neutrophil adherence and migration (165% ± 13% and 210% ± 17%, mean ± SEM, respectively) (Fig 1). The adherence of neutrophils to 4-hour rIL-1β-stimulated endothelial cells was only partly inhibited (29% ± 4%; P < .05) by the anti-CD18 MoAb CLB-LFA-1/1 (Fig 1). In
Table 1. Effect of the Nature of the Endothelial Growth Substrate on the Expression of Cytokine-Inducible Adhesion Molecules on Endothelial Cells

<table>
<thead>
<tr>
<th>ELAM-1</th>
<th>VCAM-1</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>68 ± 22</td>
<td>653 ± 93</td>
</tr>
<tr>
<td>4</td>
<td>62 ± 17</td>
<td>634 ± 22</td>
</tr>
<tr>
<td>24</td>
<td>ND</td>
<td>201 ± 65</td>
</tr>
</tbody>
</table>

Endothelial cells grown on collagen matrices

<table>
<thead>
<tr>
<th>ELAM-1</th>
<th>VCAM-1</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22 ± 4</td>
<td>638 ± 111</td>
</tr>
<tr>
<td>4</td>
<td>22 ± 4</td>
<td>638 ± 111</td>
</tr>
<tr>
<td>24</td>
<td>ND</td>
<td>188 ± 44</td>
</tr>
</tbody>
</table>

Endothelial cells grown on fibronectin

Abbreviation: ND, not determined.

*Time is expressed in hours and represents the period of rIL-1β activation of endothelial cell monolayers.

The expression of endothelial cell surface adhesion molecules is given as MFI (see Materials and Methods). Data are mean ± SEM of three independent experiments.

Binding of irrelevant control MoAb to endothelial cells.

In contrast, neutrophil migration across cytokine-activated endothelial cells was reduced below the level of the unstimulated control (baseline level) by the anti-CD18 MoAb.

The neutrophil adherence to rIL-1β-activated endothelial cells was remarkably sensitive to inhibition by the anti-ELAM-1 MoAb ENA2 (decrease, 55% ± 5%; P < .01). However, the anti-ELAM-1 MoAb was much less effective than the CD18 MoAb in blocking neutrophil migration across monolayers of rIL-1β-pretreated endothelial cells (inhibition, 28% ± 6%; NS; and 65% ± 3%; P < .01; respectively). The anti-ELAM-1 MoAb had no effect on neutrophil adherence to untreated or 24-hour rIL-1β-treated endothelial cells (data not shown). When the anti-ELAM-1 MoAb was used in combination with the anti-CD18 MoAb, an additive inhibitory effect was observed, resulting in a reduction of the rIL-1β-induced neutrophil adherence and migration below baseline levels.

The anti-VLA-4 MoAbs HP1/3 and HP2/1, either used alone or in combination with CD18 and ELAM-1 antibodies, had no effect on neutrophil adherence to or migration across cytokine-activated endothelial cells (data not shown). This finding is in line with the observation that VLA-4 is absent on neutrophils, as investigated with FACS analysis.

Effects of MoAbs against CD18, ELAM-1, and VLA-4-a on rIL-1β-induced monocyte adherence and migration. Pretreatment of endothelial cells for 4 hours with rIL-1β also enhanced monocyte adherence and migration (38% ± 5% and 94% ± 10%, respectively; Fig 2), although to a lesser extent than observed for neutrophils. The adherence of monocytes to rIL-1β-pretreated endothelial cells was only partly blocked (28% ± 4%; P < .05) by the anti-CD18 MoAb (Fig 2). In contrast, the anti-CD18 MoAb markedly reduced (51% ± 4%; P < .01) monocyte migration across monolayers of cytokine-activated endothelial cells.
The anti–ELAM-1 MoAb reduced monocyte adherence to rIL-1β-pretreated endothelial cells to a similar extent as was observed for the anti-CD18 MoAb (29% ± 5%; P < .05 and 28% ± 4%; P < .05, respectively). However, the anti–ELAM-1 MoAb was less effective in inhibiting monocyte migration across rIL-1β-stimulated endothelial cells (26% ± 4%). The anti–ELAM-1 MoAb had no effect on monocyte adherence to untreated or 24-hour rIL-1β-pretreated endothelial cells (data not shown). Combined use of these anti-CD18 and anti–ELAM-1 antibodies had an additive inhibitory effect (49% ± 6%; P < .01) on monocyte adherence.

Anti–VLA-4 MoAb HP1/3 had little effect on monocyte adherence to, as well as migration across, cytokine-activated endothelial cells (17% ± 6% and 17% ± 4% inhibition, respectively). Similar results were obtained with anti–VLA-4 MoAb HP2/1 (data not shown). Anti-CD18 and anti–VLA-4 MoAbs used in combination had an additive inhibitory effect on monocyte adherence to rIL-1β-pretreated endothelial cells (41% ± 6%; P < .01). The anti–VLA-4 MoAbs had no effect on monocyte adherence to untreated endothelial cells (data not shown).

Both rIL-1β-induced monocyte adhesion and migration were reduced to far below baseline levels (61% ± 7%; P < .01 and 68% ± 4%; P < .01, respectively) when the MoAbs against CD18, ELAM-1, and VLA-4 were used in combination.

**DISCUSSION**

**Leukocyte adherence to and migration across monolayers of cytokine-activated endothelial cells.** Several studies have shown that cytokine activation of endothelial cells increases the adherence of neutrophils to a much greater extent than monocyte adherence.4,25,26 Although cytokine-pretreatment of endothelial cells enhanced both neutrophil and monocyte migration, clear differences were observed in the migration properties of these cells. First, neutrophil migration was to a much greater extent increased by rIL-1β-activation of endothelial cells than monocyte migration (Figs 1 and 2). Secondly, visual evaluation of the number of migrated leukocytes under these conditions showed that neutrophils penetrated deeper into the collagen matrices than monocytes (not shown). In the present study we observed that both neutrophil and monocyte migration across monolayers of rIL-1β-stimulated endothelial cells was enhanced to a much greater extent than we have observed before.4 The differences in our findings were due to the two types of collagen used in these studies, indicating that the migratory properties of leukocytes into the subendothelial culture matrix are directly influenced by the matrix itself.

**Contribution of CD18, ELAM-1, and VLA-4 to leukocyte adherence.** The adherence of nonstimulated neutrophils to endothelial cells pretreated for 4 hours with rIL-1β is in part CD18-dependent and in part CD18-independent.14,27 Our studies confirm the importance of ELAM-1 in neutrophil adherence to rIL-1β-pretreated endothelial cells.7,14 Neutrophil adherence was almost completely prevented when the anti–ELAM-1 MoAb was used in combination with the anti-CD18 MoAb (Fig 1). The anti–ELAM-1 MoAb had no effect on the adherence of neutrophils to 24-hour rIL-1β-pretreated endothelial cells, which was due to the rapid decline of ELAM-1 expression in vitro (Table 1).

Previously, ELAM-1 has been identified as a cytokine-inducible endothelial receptor for neutrophils.9 Here, we show that the anti–ELAM-1 MoAb ENA2 also significantly blocked (29% ± 5%) monocyte adherence to rIL-1β-stimulated endothelial cells (Fig 2). Similarly, in a separate study, Leeuwenberg et al26 showed that at 4°C monocyte adherence to cytokine-activated endothelium was strongly inhibited by MoAb ENA2. The finding that ELAM-1 is not only involved in neutrophil adherence to cytokine-activated endothelium but in monocyte adherence as well is in agreement with the recent observations that the carbohydrate ligand of ELAM-1, the sialated Lewis-X antigen, is expressed on both neutrophils and monocytes.29 The inhibitory effects of the anti–ELAM-1 and anti-CD18 MoAbs on monocyte adherence were similar when used alone and additive when used in combination. However, under these latter conditions, a substantial number of monocytes still adhered to the rIL-1β-treated endothelial cells (Fig 2). This finding indicates that additional, CD18- and ELAM-1–independent mechanisms are involved in monocyte adherence to cytokine-activated endothelial cells.

Besides ELAM-1 induction, cytokines also induce the expression of VCAM-1 on endothelial cells (Table 1). The leukocytic receptor for VCAM-1 is VLA-4, which also functions as a receptor for fibronectin.12,13 The anti–VLA-4 MoAb HP1/3, which potently blocks VCAM-1/VLA-4 interactions without interfering in the binding to fibronectin,12 did not significantly inhibit monocyte adherence, unless used in combination with the anti-CD18 MoAb.

The reduction in monocyte adherence to rIL-1β-prestimulated endothelial cells was even more pronounced when MoAbs against CD18, ELAM-1, and VLA-4 were combined (Fig 2). Yet, even the combination of these three MoAbs did not completely prevent monocyte adherence to endothelial cells, indicating that other adhesive mechanisms are also involved.

Endothelial cells grown in monolayers are presumably covered with several matrix proteins. Preliminary studies, using inhibitory MoAbs against the α chains of several VLA integrins, indicate that this "remaining" monocyte adherence is at least in part mediated by the binding of monocytes to fibronectin and laminin through the specific receptors for extracellular matrix proteins15 VLA-5 and VLA-6 (B.C. Hakkert, T.W. Kuijpers, unpublished results).

**Contribution of CD18, ELAM-1, and VLA-4 to leukocyte migration.** We4 and others3a have shown that anti-CD18 MoAbs reduce neutrophil migration across monolayers of cytokine-activated endothelial cells to baseline levels. We show here that the anti–ELAM-1 MoAb is much less effective in inhibiting the rIL-1β–induced neutrophil migra-
tion than the anti-CD18 MoAb (Fig 1).* Taken together, our data strongly indicate that neutrophil adherence largely proceeds in a CD18-independent way, subsequently switching to a CD18-dependent mechanism of migration. In a way, this concept perfectly matches with a recent report by Lawrence et al., who noted that under flow conditions the neutrophil adherence to cytokine-activated endothelial cells was completely CD18-independent, whereas migration was strongly reduced by anti-CD18 MoAbs. The investigators suggested that CD18-independent mechanisms function to initially "catch" the circulating phagocyte. In line with this idea, we recently found that neutrophil contact with ELAM-1 on rIL-1β-stimulated endothelial cells leads to enhanced adherence through CD18.27

Monocyte migration across cytokine-pretreated endothelial cell monolayers was also strongly reduced by the anti-CD18 MoAb, whereas MoAbs against ELAM-1 and VLA-4 had no significant effect (Fig 2). These results are in accordance with the findings that neutrophils and monocytes from patients with a genetic defect in the β2 integrin CD18 (leukocyte adhesion deficiency [LAD] patients) fail to accumulate at inflammatory sites.2 In contrast, the migration of lymphocytes from LAD patients is essentially normal, and it has been suggested that VCAM-1/VLA-4 interactions play an important role in the recruitment of lymphocytes.12,16 MoAbs against VCAM-1 or VLA-4 only affected cytokine-induced monocyte adherence17 and migration (our study) when used in combination with MoAbs against CD18, indicating that the VCAM-1/VLA-4 interactions alone are not sufficient, either for monocyte adherence or for migration.

Conclusions. We have shown that ELAM-1 is not only involved in neutrophil adherence to rIL-1β-prestimulated endothelial cells, but in monocyte adherence as well. The reduction in monocyte adherence was most pronounced when MoAbs against CD18, ELAM-1, and VLA-4 were used in combination. Although both neutrophil and monocyte adherence to cytokine-activated endothelial cells is mediated by CD18-independent and (to a lesser extent) by CD18-dependent mechanisms, migration across monolayers of cytokine-activated endothelial cells is predominantly CD18-dependent.

REFERENCES


*While this report was under review, a study by Luscinskas et al. was published in which greater than 90% inhibition of neutrophil migration across 4-hour rIL-1β-activated endothelial cells by MoAb H18/7 against ELAM-1 is described.

28. Leeuwenberg JFM, Jeunhomme TMAA, Buurman WA: ELAM-1 mediated adhesion of monocytes to endothelium. (submitted)


Neutrophil and monocyte adherence to and migration across monolayers of cytokine-activated endothelial cells: the contribution of CD18, ELAM-1, and VLA-4

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