Kindlin-3 in hematopoietic integrin activation: Absence in leukocyte adhesion deficiency type III

van de Vijver, E.

Citation for published version (APA):
van de Vijver, E. (2013). Kindlin-3 in hematopoietic integrin activation: Absence in leukocyte adhesion deficiency type III.
CHAPTER 5

Kindlin-3-independent adhesion of neutrophils from Leukocyte Adhesion Deficiency type III (LAD-III) patients
Neutrophils are indispensable for host defense against bacterial and fungal infections. To get to sites of infection, circulating neutrophils need to extravasate and migrate into interstitial tissues. This involves interactions with endothelial cells and the extracellular matrix, mediated by adhesion molecules, in particular the β2 integrin CR3. Integrins are activated via complex signaling cascades including several adaptor proteins, among which kindlin-3. Deficiency of kindlin-3 results in a rare immunodeficiency presenting with recurrent nonpussing infections and a Glanzmann thrombastenia-like bleeding tendency, called Leukocyte Adhesion Deficiency type III (LAD-III) or LAD-I/v syndrome. Here we analyzed neutrophil functions in a series of six proven LAD-III patients. We observed completely defective zymosan-mediated NADPH-oxidase activation in all patients. Despite complete absence of kindlin-3, CR3-dependent neutrophil adhesion was still found under some (e.g. with fMLP and PMA) but not all (e.g. with C5a and TNF-α) conditions of activation. However, we also reveal that the adhesion of kindlin-3-deficient neutrophils was of lower avidity compared to control neutrophils. Our findings demonstrate, for the first time, kindlin-3-independent integrin-mediated neutrophil adhesion. This may explain why the leukocyte defect of LAD-III is clinically less severe than that of LAD-I, which is characterized by complete absence of functional β2 integrins.
INTRODUCTION

Neutrophils form the most abundant white blood cell type in mammals and perform a key role in innate immune responses. They are among the first responding inflammatory cells and maintain tissue homeostasis by elimination of pathogens and removal of damaged tissue. Neutrophils migrate through the blood vessel wall and interstitial tissue towards the site of inflammation, following a gradient of chemokines originating from the pathogen or from the infected tissue. Upon recruitment to a local vessel, the cells slow down due to transient interactions with the endothelial cells via selectins and their ligands. Subsequently, tight adhesion to the vessel wall occurs by binding of adhesion molecules, mostly integrins, to ligands upregulated on the vessel wall. Hereupon, the cells extravasate and continue their way into the affected tissue. Integrin-mediated adhesion is also involved in the binding of pathogens and their uptake through phagocytosis.

Integrins are transmembrane glycoprotein heterodimers consisting of an α and a β chain. On neutrophils, complement receptor 3 (CR3, Mac-1) and lymphocyte function-associated antigen-1 (LFA-1) are the most prominent integrins, consisting of β2 (CD18) and αM (CD11b, in CR3) or αL (CD11a, in LFA-1). The major ligands of CR3 are complement fragment iC3b, yeast structures, bacterial lipopolysaccharide (LPS), coagulation proteins such as fibrinogen, various extracellular matrix proteins and a surface-expressed cellular ligand known as intercellular adhesion molecule 1 (ICAM-1). LFA-1 is a specific receptor for cellular ligands only, i.e. ICAM-1 and ICAM-2. Integrins on resting cells are in an inactive conformation. To bind their ligands, these integrins need to switch into an active conformation. This activation process requires complex signaling cascades, comprising (de)phosphorylation of plasma membrane phosphoinositides and interaction with many cytoplasmic proteins, among which are the small GTPase Rap1 as well as structural proteins such as talin-1, filamin, α-actinin and the kindlins. The kindlin family consists of the fibroblast-specific kindlin-1, the ubiquitously expressed kindlin-2, and the hematopoietic cell-specific kindlin-3, which are highly homologous in sequence and structure. Talin-1 and kindlins contain a FERM domain (called after the homologous 4.1 protein, ezrin, radixin, moesin). FERM-domain containing proteins are involved in protein-protein and protein-lipid interactions, which implies that FERM domains link the plasma membrane with cytoskeletal structures. The FERM domains of talin-1 and the kindlins bear a phosphotyrosine-binding (PTB) site specific for integrin β chains. In addition to the FERM domains, the kindlin proteins also contain a PH domain, known as a domain involved in binding to the plasma membrane, often regulated by cellular activation.

The importance of kindlin-3 in hematopoietic cells is illustrated by Leukocyte Adhesion Deficiency type III (LAD-III), a rare autosomal recessive syndrome caused by mutations in FERMT3, the gene encoding kindlin-3. In 1997, we were the first to report on this syndrome of recurrent bacterial and fungal infections as well as a Glanzmann thrombastenia-like bleeding tendency, which was at that time designated LAD-I/variant syndrome. The expression levels of integrins on LAD-III leukocytes and platelets is normal, but integrin activation is defective due to the absence of kindlin-3. Upon stimulation with several cytokines, integrin activation epitopes are hardly detectable on all hematopoietic cells, and functional responses such as adhesion and aggregation are reduced or absent. Direct interaction between kindlin-3 and
β1, β2 and β3 integrins has been concluded from pull-down experiments with recombinant cytoplasmic tails of the various β chains.\textsuperscript{18,19}

Although the relevance of kindlin-3-induced activation of hematopoietic integrins is no longer disputed, it is still unclear how and when kindlin-3 contributes to the activation of the β2 integrins. In the current study, we demonstrate a clear kindlin-3-independent activation of CR3 in primary human neutrophils from LAD-III patients tested on various occasions. Although adhesion is completely absent in response to several stimuli on all occasions in all patients, significant levels of binding may still be induced by other stimuli. By titrating CR3-blocking monoclonal antibodies (mAbs), we show that kindlin-3-independent adhesion of LAD-III neutrophils is of lower avidity than kindlin-3-dependent CR3-mediated adhesion of normal neutrophils. Our results lead to a working model for CR3 activation in which kindlin-3 partially contributes to initial ligand-affinity and plays a key role in regulating optimal avidity.

**MATERIALS AND METHODS**

**Neutrophil isolation**

Heparinized venous blood was collected from healthy donors and from LAD-III patients after obtaining informed consent. The study was approved by the AMC Institutional Medical Ethics Committee in accordance with the 1964 Declaration of Helsinki.

Human neutrophils were isolated as described and resuspended in Hepes buffer (20 mM Hepes, 132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl\textsubscript{2}, 1.0 mM MgSO\textsubscript{4}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 5.5 mM glucose and 0.5% (w/v) human serum albumin, pH 7.4).\textsuperscript{20,21}

**NADPH oxidase activity**

NADPH oxidase activity was assessed as the release of hydrogen peroxide determined with an Amplex Red kit (Molecular Probes, Eugene, OR, USA). Neutrophils (0.25 x 10\textsuperscript{6}/ml) were stimulated with 1 mg/ml unopsonized zymosan (MP Biomedicals, Solon, OH, USA) or serum-treated zymosan (STZ, previously described\textsuperscript{22}) in the presence of Amplex Red (0.5 µM) and horseradish peroxidase (1 U/ml). Fluorescence was measured at 30-second intervals for 30 minutes with a Genios plate reader (Tecan, Mannedorf, Switzerland) at an excitation wavelength of 535 nm and an emission wavelength of 595 nm. Maximal slope of H\textsubscript{2}O\textsubscript{2} release was assessed per 2-minute interval.

**Adhesion**

Neutrophils (5 x 10\textsuperscript{6}/ml) were labeled with calcein-AM (1 µM final concentration; Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C, washed twice, and resuspended in Hepes buffer at a concentration of 2 x 10\textsuperscript{6}/ml. Adhesion was determined in an uncoated 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany), which has high affinity for proteins. Calcein-labeled cells (2 x 10\textsuperscript{4}/well, final volume 125 µl) were stimulated with 10 mM of the reducing agent dithiothreitol (DTT; Sigma Aldrich, St. Louis, MO, USA), 100 ng/ml PMA (Sigma Aldrich), 20 µg/ml synthetic bacterial-like TLR-2/TLR-1 ligand lipopeptide Pam3Cys (EMC Microcollections, Tübingen, Germany), 20 ng/ml bacterial TLR-4 ligand lipopolysaccharide (LPS; isolated from \textit{E. coli} strain O55:B5, Sigma Aldrich) in the presence of 50 ng/ml lipopolysaccharide-binding protein (LBP; R&D Systems, Minneapolis, MN, USA), 1 µM synthetic bacterial-like GPCR ligand formyl-methionyl-leucyl-phenylalanine (fMLP;
Sigma Aldrich), 1 µM GPCR ligand platelet-activation factor (PAF; Sigma Aldrich), 10 nM GPCR ligand complement factor C5a (Sigma Aldrich) or 10 ng/ml tumor necrosis factor-α (TNF-α; Peprotech EC, London, UK). Plates were incubated for 30 minutes at 37°C and washed 3 times with PBS. Adherent cells were lysed in 0.5% (w/v) Triton X-100 in H₂O for 10 minutes at room temperature. Fluorescence was measured with a Genios plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Adhesion was determined as a percentage of total input of calcein-labeled cells and normalized to the average of 17 healthy controls when indicated.

When indicated, cells were preincubated for 10 minutes at 37°C with 10 µg/ml mouse anti-β2 integrin monoclonal antibody (mAb) clone IB4 or anti-αM integrin mAb clone 44a, both isolated from the supernatant of hybridoma clones obtained from the American Type Culture Collection (Rockville, MD, USA), to test the contribution of each integrin subunit. Dose-response curves of fMLP-, PMA- and DTT-induced adhesion were obtained with concentration series of 0-10 µg/ml IB4 or 44a. Data were analyzed and IC50 values were determined with GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA).

Spreading
Neutrophils (1x10^5 cells) were incubated for 20 minutes at 37°C in 400 µl of Hepes buffer with or without 1 µM fMLP in a 24-well culture plate. After washing once with PBS, images were taken at x600 magnification with an EVOS microscope (AMG, Bothell, WA, USA).

Statistics
Graphs were drawn and statistical analysis was performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Data were evaluated by student’s t-test. The criterion for significance was p < 0.05 for all comparisons.

RESULTS
Patient characteristics
We report on six different LAD-III patients assessed over the last 15 years, all bearing a mutation in their FERM T3 gene (Table 1). Apart from the mutations reported before, we found one novel mutation, c.1173delT, p.Asp393ThrfsX29, which was homozygously expressed.10-12

<table>
<thead>
<tr>
<th>Family*</th>
<th>Mutation</th>
<th>Kindlin-3 protein expression</th>
<th>Zymosan-induced NADPH oxidase</th>
<th>Adhesion assays (# tests, over time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p.Arg509X</td>
<td>None</td>
<td>Strongly decreased</td>
<td>2x</td>
</tr>
<tr>
<td>6 (1)</td>
<td>p.Arg509X</td>
<td>None</td>
<td>Strongly decreased</td>
<td>4x</td>
</tr>
<tr>
<td>8</td>
<td>p.Arg573X</td>
<td>None</td>
<td>Strongly decreased</td>
<td>3x</td>
</tr>
<tr>
<td>10</td>
<td>p.Lys82ThrfsX67</td>
<td>None</td>
<td>Strongly decreased</td>
<td>1x</td>
</tr>
<tr>
<td>11</td>
<td>p.Arg509X</td>
<td>None</td>
<td>Strongly decreased</td>
<td>1x</td>
</tr>
<tr>
<td>12</td>
<td>p.Asp393ThrfsX29</td>
<td>None</td>
<td>Strongly decreased</td>
<td>6x</td>
</tr>
</tbody>
</table>

* Numbers according to our previous publications where family 1, 6 and 8 have been described in more detail elsewhere.11-14
As demonstrated previously, neutrophil NADPH oxidase activity induced by unopsonized versus serum-treated zymosan is a strong diagnostic tool to suggest the diagnosis of LAD-III. The neutrophils from all patients tested in the present study showed hardly any NADPH oxidase activity in response to unopsonized zymosan, in contrast to control neutrophils (Figure 1). Western blot analysis revealed that the full-length kindlin-3 was completely absent in the patients' neutrophils (data not shown). We used a polyclonal antibody directed against a peptide located in the F1 subdomain (amino acids 156-170), which recognizes the first 25% of the 667 amino acids-containing full-length protein. Based on the mutated amino acids, this antibody would recognize shorter functional proteins, if expressed, in five of the six patients. By mass spectrometry we also did not observe shorter kindlin-3 proteins in any of the patients tested – even in the presence of missense mutations, in-frame deletions or splice site variants (data not shown).

**Figure 1. Neutrophil NADPH oxidase activity.** NADPH oxidase activity induced by bare versus opsonized zymosan (STZ) of control (□) (n=18) and LAD-III (■) neutrophils (n=6). Mean ± SEM. * p<0.05.

*Kindlin-3-independent adhesion through CR3*

To test integrin function, adhesion of LAD-III patient neutrophils was assessed upon stimulation with various stimuli (Figure 2A). Blocking mAb directed against either the common integrin β2 chain (clone IB4) or the αM chain (clone 44a) of CR3, prevented essentially all neutrophil adhesion (Figure 2B-C). In line with this observation, no adhesion was detected in this assay with neutrophils of classical LAD-I patients, characterized by the complete lack of functional β2 integrin (data not shown). On the other hand, antibodies against LFA-1, the only other known integrin to be significantly expressed by neutrophils, did not prevent neutrophil adhesion (data
Collectively these data demonstrate that all of the observed neutrophil adhesion is essentially mediated by CR3 (Figure 2C).

In the absence of a stimulus, less than 5% of control and less than 3% of LAD-III neutrophils were adherent. DTT is a non-physiological reducing agent that forces the integrin conformation into the high-affinity state by disrupting disulfide bonds in cysteine-rich repeats in the extracellular EGF domain, independent of intracellular signaling. All LAD-III patients showed adhesion upon stimulation with DTT, demonstrating that the integrins are fully capable of undergoing the conformational change to the high-affinity state (Figure 2A). DTT-induced adhesion was similar for LAD-III and control neutrophils, as could be anticipated from the comparable CR3 expression levels (Figure 2A).

Adhesion induced with the phorbol ester PMA, which is known to trigger integrin-mediated adhesion intracellularly, was hardly impaired with LAD-III neutrophils (Figure 2A).

Adhesion of LAD-III neutrophils was strongly decreased when induced with TLR ligands Pam3Cys (mean 5.1% adhesion for LAD-III compared to 40.3% for control neutrophils) and LBP/LPS (mean 9.8% compared to 40.9%). Most remarkably, we repeatedly observed the induction of neutrophil adhesion with the GPCR ligand fMLP, being on average 29.7% with patient cells compared to 37.1% with control neutrophils. Hence, it seems possible that LAD-III neutrophils are able to adhere through CR3 under these in vitro conditions in the complete absence of kindlin-3. Adhesion induced with other GPCR ligands, i.e. PAF (mean 99% compared to 36.1% in control neutrophils) and C5a (mean 10.8% compared to 33.7%) was strongly decreased. Adhesion was almost absent when induced with TNF-α (mean 7.6% compared to 36.1%). Collectively, these findings demonstrate that neutrophil adhesion induced by certain stimuli, in particular fMLP and PMA, occurs in a CR3-dependent but kindlin-3-independent manner.

Several patients were tested on multiple occasions, up to six times in total. As indicated by the different symbols used per patient, the proportion of fMLP-induced adherent cells varied considerably not only among different patients, but also when tested on different occasions for the same patient, varying from 15% to up to 120% of the mean of the 16 healthy controls used in these diagnostic tests (Figure 2D). This suggests that differences are not only due to patient intrinsic factors, such as e.g. additional genetic differences, but are also subject to other regulatory influences (e.g. variation in priming).

**Kindlin-3 is essential for optimal avidity**

The strength of fMLP-, PMA- and DTT-induced adhesion via CR3 was assessed by a dose-response analysis with blocking mAbs 1B4 and 44a against β2 and αM, respectively (Figure 3A). In this set of three experiments, the induced adhesion was comparable between control and patient neutrophils. Determining the IC50 values revealed that 4-5-fold less 1B4 and 44a is required to block fMLP- or PMA-induced adhesion of LAD-III neutrophils compared to control cells, suggesting that the overall avidity of the interaction with LAD-III cells is lower (Figure 3B-C). In contrast, when DTT was used to activate the integrins, pre-incubation with 1B4 had equally potent effects on LAD-III and control cells, demonstrating that when intracellular signaling is bypassed, the integrins in both cell suspensions can bind to the substrate with equal avidity (Figure 3D). To investigate whether reduced integrin avidity was
Figure 2. Neutrophil adhesion. Adhesion of control (□) and LAD-III (■) neutrophils in response to reducing agent DTT, phorbol ester PMA, bacterial stimuli (Pam3Cys, LBP/LPS, fMLP) or endogenous stimuli (PAF, C5a, TNF-α). (A) Neutrophil adhesion (control n=15, LAD-III n=6). (B) Adhesion in the presence of
C

Adherent cells (% of total)

-  +  -  +  -  +  -  +

44a  DTT  FMLP  PMA

β₂ blocking antibody IB4 (n=3-6). (C) Adhesion in the presence of α₅ blocking antibody 44a (n=3). (D) Variation among patients and among samples of patients tested on different occasions (patient 1 (＊), 6.1 (♦), 8 (▼), 10 (●), 11 (△) and 12 (▲)). Mean, * p<0.05.
KINDLIN-3-INDEPENDENT ADHESION OF NEUTROPHILS FROM LEUKOCYTE ADHESION DEFICIENCY TYPE III (LAD-III) PATIENTS

Figure 3. Inhibition of neutrophil adhesion with blocking antibodies. (A) Dose-response curve of IB4 pre-incubation of fMLP-stimulated control (■) and LAD-III (▲) neutrophils. Representative of 3 independent experiments. IC50 values of IB4 and 44a pre-incubated control (□) and LAD-III (▲) neutrophils stimulated with fMLP (B), PMA (C) or DTT (D). Mean ± SEM, n=3. * p<0.05. (E) Adhesion and spreading of control and LAD-III neutrophils stimulated with fMLP. Representative of 3 independent experiments. (F) Spreading of control and LAD-III neutrophils stimulated with fMLP. Original magnification x600. Mean ± SEM, n=3. * p<0.001.
correlated with defective downstream integrin-mediated processes in the neutrophils, we evaluated their spreading. However, even when fMLP-induced adhesion was comparable to control neutrophils, fMLP-induced spreading of neutrophils was less abundant in LAD-III than control neutrophils (3E-F).

DISCUSSION

Previous studies on LAD-III neutrophils and platelets have suggested a major role of kindlin-3 in integrin activation. Here, we present our findings on neutrophil adhesion based on several LAD-III patients tested over the past few years, of which most patients have been tested more than once over several years apart. Our present findings show that fMLP- or PMA-induced adhesion can occur, to a variable extent, in a kindlin-3-independent manner. Our data suggest that the importance of kindlin-3 for integrin activation is dependent on the stimulus used. Thus, as yet undefined priming signals seem to exist, as suggested by the variation in kindlin-3-dependent fMLP-induced adhesion in individual patients analyzed at different time points. The adhesive phenotype of LAD-III neutrophils may be less absolute than previously thought, which may relate to the leukocyte defect of LAD-III being less severe in clinical terms as compared to that in LAD-I, caused by the complete absence of β2 integrins.

GPCR-stimulation is generally known to induce the generation of specific phosphoinositol phosphates (PIPs) and to activate guanine exchange factors (GEFs) and their downstream small GTPases. Small GTPases like Rap-1, together with PIPs, may cooperatively act to recruit integrin-binding molecules such as talin-1 and kindlin-3, which contribute to conformational changes that enhance ligand binding affinity. Interestingly, the kindlin-3-independent adhesion triggered by fMLP in LAD-III neutrophils contrasted with kindlin-3-dependent PAF or C5a activation, indicating that the requirements for CR3 activation may vary even among different GPCR receptors. The exact basis for these differences is as yet unknown.

Current models of hematopoietic integrin activation discriminate three affinity states, i.e. a low-affinity conformation with a closed headpiece, an extended but closed intermediate-affinity conformation and a fully unclasped high-affinity conformation. For LFA-1, it was recently suggested that kindlin-3 is required for the high-affinity but not for the intermediate-affinity conformation of LFA-1, which instead required talin-1. With murine kindlin-3-neutrophils, low levels of binding to ICAM-1 and to antibodies specific for the extended conformation were detected, but not to antibodies specific for the high-affinity conformation. Also for VLA-4 (α4β1) on T cells and monocytes, it was shown that kindlin-3 is primarily important for adhesion strengthening rather than for initial affinity upregulation. Our current results, which are in general consistent with this idea, provide the first evidence that for CR3 activation, initial integrin activation can occur both in a kindlin-3-dependent and in a kindlin-3-independent manner, and that the necessity for kindlin-3 varies depending on the stimulus and on as yet undefined priming signals. How kindlin-3 sterically contributes to β integrin activation is not known, although Qu et al. have suggested that kindlins, by interacting with both β integrins and the membrane, may help to tether additional sites of the β integrin tails to the membrane for optimal β integrin activation.
The findings on kindlin-3-independent adhesion raise questions about the in vivo relevance of kindlin-3. The fMLP-induced kindlin-3-independent adhesion of LAD-III neutrophils was less stringent than adhesion of control neutrophils, as was shown with suboptimal concentrations of blocking mAbs IB4 and 44a against the common $\beta_2$ chain and $\alpha$M, respectively. We conclude that, besides its role in the initial switch of CR3 towards a ligand-binding conformation, kindlin-3 participates to increase CR3 avidity. Accordingly, even when fMLP-induced adhesion was comparable to control neutrophils, the fMLP-induced spreading of neutrophils was less abundant in LAD-III than control neutrophils. As we have also reported previously, the ability to migrate towards a gradient of fMLP was absent in LAD-III patients. Neutrophil chemotaxis assays help to distinguish patient from control cells instantly. Thus, whereas kindlin-3-independent CR3 activation may allow initial adhesion under certain conditions, we conclude that the overall integrin avidity seems too weak to allow more complex adhesion-mediated processes, such as spreading or chemotaxis.

Avidity depends on the affinity of individual integrins as well as on the number of receptors per cell surface area. Kindlin-3 may increase this density of CR3 via clustering, i.e. local recruitment of receptors facilitated by dynamic interactions with the cytoskeleton. Interactions with the cytoskeleton may occur via kindlin-3 binding to RACK-1, as was suggested to occur downstream of LFA-1 activation, or via ILK or migfilin, as was reported for kindlin-1 and kindlin-2. Clustering of CR3 has previously described on macrophages. In addition, kindlin-3 was recently reported to induce LFA-1 clustering when over-expressed on human myeloid leukemia K562 cells, as was detected with a FRET-based assay. A role for kindlin-3 in integrin clustering would explain why spreading and migration are defective downstream of several hematopoietic integrins in kindlin-3-deficient cell types even when the high ligand-binding affinity conformation is induced extracellularly with Mn$^{2+}$ or activating antibodies.

Avidity may be further increased by upregulation of integrins at the plasma membrane via release of stored CR3 from intracellular granules. However, this process occurs independently of kindlin-3, as fMLP-induced upregulation of CR3 on neutrophils was shown to be unaffected in LAD-III patients.

In conclusion, our data reveal that stimulation of cytokine receptors in combination with neutrophil priming leads to either kindlin-3-dependent or kindlin-3-independent transformation of the integrin to a ligand-binding conformation. Kindlin-3 is essential to obtain maximal avidity of CR3, e.g. via integrin clustering, which is needed for optimal functioning and contributes to complex processes such as directional neutrophil migration.

**ACKNOWLEDGMENTS**

We gratefully acknowledge Dr. A.B. Meijer for mass spectrometry analysis on control and patient material and Prof. D. Roos for critically reading the manuscript. This work was supported by the Landsteiner Foundation for Blood Transfusion Research (LSBR 0619).
REFERENCE LIST