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ARTICLE

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# The strength of integrin binding between neutrophils and endothelial cells

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**Abstract** The firm adhesion of activated polymorphonuclear neutrophils to endothelial cells in blood vessels is achieved through binding of the integrin intercellular adhesion molecule. To contribute to the better understanding of this adhesion step, our investigation is aimed at the relationship between integrin expression and the strength of neutrophil binding to endothelial cells. Flow cytometry and 3D scanning microscopy are used to study integrin expression and distribution, respectively. It is found that CD11b/CD18 integrin expression is localized in clusters distributed irregularly over the neutrophil surface. After cell activation, the cluster distribution polarizes, increasing the local CD11b/CD18 density concurrently with nearly doubled integrin expression. The neutrophil adhesion efficiency is measured in a flow chamber coated successively by various substrates, including endothelial cells in an activated state. Analysis of the flow dependence of the number of attached cells reveals the prevailing number of neutrophils with stronger binding to the endothelium when both cells are in the activated state in comparison with non-activated cells.

**Keywords** Endothelium · Integrins · Neutrophil adhesion · Scanning microscopy

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## Introduction

The adherence of polymorphonuclear neutrophils (PMNs) to the endothelium is a crucial early event in the inflammatory reaction. The PMN–endothelial cell (EC) interactions are mediated by adhesion molecules, which are expressed on both types of cells. The initial neutrophil rolling along the vessel wall during an inflammatory response consists of transient adhesive contacts mediated by the selectin family of adhesion receptors (e.g. Dunon et al. 1996; Ley and Tedder 1995). The rolling enables neutrophils to survey the endothelium for signs of inflammation, including chemoattractants that can activate PMNs and initiate their firm adhesion to the endothelium, especially in postcapillary venules. Among the adhesion molecules expressed on the activated neutrophils, CD11/CD18 integrins, which comprise an immunologically distinct $\alpha$-subunit (CD11a, CD11b, CD11c) and a common $\beta$-subunit (CD18), play a major role (Clark and Brugge 1995; Diacovo et al. 1996). The CD11b/CD18 (Mac-1) integrins are stored intracellularly in a rapidly mobilizable granule pool recruited to the plasma membrane when activated. The two most extensively characterized endothelial cell adhesion molecules are intercellular adhesion molecule-1 (ICAM-1) and E-selectin. ICAM-1 is constitutively expressed on ECs and acts as a ligand for CD11b/CD18.

The adhesion mechanism between the activated PMNs and the endothelium has been extensively investigated (e.g. Arnaout 1990; Clark and Brugge 1995; Diamond and Springer 1994; Rochon et al. 2000). To contribute to understanding of the integrin binding, a model study was carried out with living PMNs using the flow chamber technique. For modifying the adhesion properties, PMNs were activated by a synthetic pro-inflammatory activator, *N*-formylmethionyl-leucyl-phenylalanine (FMLP), which mimics bacterial membranes. The adhesive surface was a glass coated either by the inactivated or activated ECs by the

tumor necrosis factor α (TNF-α) as well as by bovine fibrinogen.

## Materials and methods

Human peripheral blood PMNs were isolated from the venous blood of healthy medication-free volunteers at room temperature (22 °C) using a standard three-step procedure. The citrated blood was treated with an equal volume of phosphate buffered saline (PBS)–6% (v/v) Dextran T250 solution for 45 min to sediment the red blood cells (RBCs). After washing, the leukocyte-rich pellet was exposed to hypotonic conditions to lyse the RBCs. Then the neutrophils were resuspended in the PBS until used. To activate the PMNs, the cells were incubated with FMLP (Sigma, St. Quentin Fallavier, France) for 30 min at 37 °C. The PMNs were used in the corresponding experiments within 3 h after isolation.

Preparation of the glass cover of the flow chamber coated with the substrate was as follows. The glass slides (70×44 mm) were cleaned for 20 min in a KOH/ethanol solution, abundantly rinsed with demineralized water and dried by air. In the case of the fibrinogen coating, fibrinogen diluted in a 100 mM NaCl solution at a concentration of 0.01% was deposited on the glass plate during 3 h. Afterwards the slide was rinsed over 12 h in a 100 mM NaCl solution. In the case of the glass coating by human ECs (line ECV304, 5ECACC no. 92091712, CERDICS, Sophia Antipolis, France), the ECs were grown in M199 medium (GibcoBRL, France) with 10% fetal calf serum for 48 h and cultured on the glass slide in a humidified atmosphere (5% $CO_2$, 95% air) at 37 °C. The EC activation was done over 4 h with TNF-α (100 U, 37 °C).

The adhesion experiments were carried out in a chamber consisting of plane parallel Plexiglas walls (height and width of the flow chamber was 0.27 mm and 10 mm, respectively) with a window where the glass slide coated by the substrate was fastened. The flow in the cell was generated by a peristaltic pump. The neutrophil adhesion was performed at rest during 30 min. Then a slow flow (wall shear stress 0.03 Pa) was applied to wash away the non-attached cells before the first 50 pictures at the initial static conditions were taken. For studying the adhesion efficiency, the attached cells were then submitted to the flow. The flow rate was fixed for 10 min, then increased to a new, higher value again for 10 min, etc., until the largest flow rate was attained. The range of the corresponding calculated shear stress to the flow rate was in the range 0.01–0.15 Pa (PBS viscosity, 0.69 mPa s). The particular values are evident in the plot of the wall shear rate dependence on PMN attachment presented below. The detached cells did not re-absorb and thus are not included in the attached cell population.

An inverted optical microscope (IMT-2, Olympus, Rungis, France) was used for the observation of the attached PMNs. The pictures were taken by a charge-coupled device (CCD) camera (Princeton Instruments, Evry, France) and digitized with an 8-bit flash analogue-to-digital converter. They were stored in a 768×574 element pixels memory array of 8 bits installed in the computer equipped with image processing software. Fifty pictures were taken over the whole glass surface at the end of the 10-min period at the fixed flow rate. A typical picture is shown in Fig. 1.

The image processing software Visilog 5.1 (Noesis, France) allowed the counting of the number of adhered cells present. The first step of the image analysis was the separation of the attached cells from the background and from the cells that did not touch the surface. The selection procedure involved a single threshold level, which was determined manually by visually inspecting the images. The attached cells were like perfect white circles while the non-attached cells looked like deformed grey circles, smaller than the attached ones. We kept the perfect circles and eliminated the non-attached cells with the opening. Finally, the function "separate" permitted us to eliminate cell aggregates and count the number of attached cells. Moreover, each previous step passed through the visual check and not only through the computer analysis.

To control the activation state of the PMNs after isolation and activation by FMLP, the flow cytometry measurement of the PMN surface adhesive molecules (CD62L-selectin, CD11 and CD18 integrins) was done according to the indirect immunofluorescence technique using the calibration kit Qifikit (Dako, Trappes, France) (Poncelet and Carayon 1985; Poncelet et al. 1996). All monoclonal antibodies (MoABs) were from Dako (Trappes, France): CD62L MoAB (FMC46), CD11a MoAB (MHM24), CD11b MoAB (2LPM19c), CD11c MoAB (KB90) and CD18 MoAB (MHM23). MoABs were applied under previously determined saturating conditions. The unspecific labelling was blocked by the bovine serum albumin (0.4%) (Sigma). After addition of 10 μL of the respective MoAB to 100 μL of the PMN suspension, the mixture was kept in the dark room at room temperature (20–22 °C) for 45 min and then washed in PBS. Subsequently, the cells were suspended in 100 μL of a second coating solution (1/50 dilution) of the F(ab′)2 fraction of goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate and kept again for 45 min in the dark room at room temperature. After washing, the cells were resuspended in PBS. The number of receptors per cell was evaluated using the Facscan (Becton Dickinson, Pont-De-Claix, France) and PClysis software. Under saturating conditions, the number of antigenic sites per bead is proportional to the mean fluorescence intensity (MFI) (Poncelet and Carayon 1985). Thus for each sample tested, the corrected MFI, that is the specific MFI minus that of the isotypic negative control measured under the same conditions, can be converted into the mean number of molecules expressed on the cell surface.

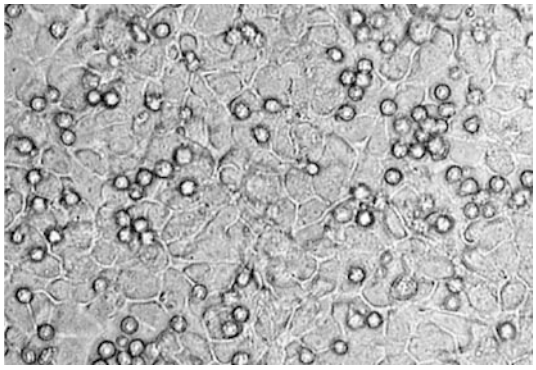

**Fig. 1** Typical distribution of attached PMNs on endothelial cells

Three-dimensional (3D) scanning microscopy (COSM) is a technique recently developed as an alternative to confocal microscopy to study cells in the living state (Scalletar et al. 1996; Shaw 1995). This technique enables observation of the distribution of labelled L-selectin as well as integrin receptors on the PMN surface. For each experiment, 100 μL of the PMN suspension were deposited on a glass plate (code 79726, Polylabo, Strasbourg, France) and immediately analysed. The optical cell cuts were obtained with an Olympus IX-70 epifluorescence inverted microscope equipped with the CELLscan optical sectioning acquisition system (EPR System CELLscan, Scanalytics, Fairfax, Va., USA) and using a 60×PSF/1.2-NA water immersion apochromatic objective (Olympus, Rungis, France). The scanning along the optical axis was performed at a $z$ spacing of 0.25 μm by a piezo $z$-axis focus device. The images of the biological samples were collected by the cooled 12-bit CCD camera. The filter set selected the fluorescence excitation and the integral part of the emission spectrum (Olympus WIB Cube, 460-490, BA515). The blurring function of the optical system (the point spread function) was characterized by imaging through the focus a series of optical sections of a 0.17-μm diameter green fluorescent bead (Microscope Point Source Kit, Molecular Probes, Eugene, Ore., USA).

ICAM-1 expression on the EC surface was observed using the indirect immunofluorescence labelling technique. The monoclonal anti-ICAM-1 (IgG1, mouse anti-human) (Immunotech, France) was used as the primary antibody and FITC-conjugated goat anti-mouse (Dako) was used as the secondary antibody.

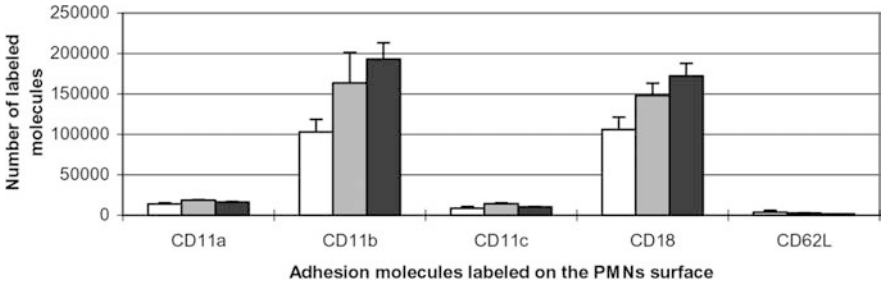


**Fig. 2** The expression of PMN molecular receptors under different conditions: *white areas*, resting PMNs; *grey areas*, PMNs activated by FMPL (15 min at room temperature); *black areas*, PMNs activated by FMPL (30 min at 37 °C)

## Results

The results of the cytometry measurements are summarized in Fig. 2. From this figure follows that expression of CD11a, CD11c and CD62L was low ($<20{,}000$ mol/cell), whereas expression of CD11b and CD18 was five times higher ($>100{,}000$ mol/cell). The effect of the pro-inflammatory factor FMLP was tested under different conditions (incubation 15 min at room temperature, incubation 30 min at 37 °C) and each time a strong increase of CD11b and CD18 expression is observed. On the other hand, the expression of CD11a and CD11c did not change significantly after activation.

The COSM technique used for evaluation of the receptor distribution on the PMN surface revealed localized expression of CD11b and CD18 receptors to clusters (see Fig. 3). After PMN activation by FMLP, a polarized distribution of receptors on the surface of all cells was observed.

The distribution of ICAM-1 receptors on the EC surface is shown in Fig. 4. The localization is peripheral at rest. When the ECs were submitted to a shear flow, the distribution of ICAM-1 molecules was uniform over the surface. A similar distribution was induced also after EC activation by TNF-$\alpha$.

The results summarizing the effect of flow on the adhesion efficiency of PMNs on different substrates are shown in Fig. 5. The number of cells ($N$) is expressed as the percentage of the attached cells at a particular flow related to the initial static case. The absolute initial PMN number at no flow condition ranges from 100 to 140 cells in each picture. The effect of flow is expressed in terms of the wall shear stress corresponding to the controlled flow rate in the chamber. The data are shown as the average of the number ($n$) of measurements. The statistical analysis was carried out using an unpaired $t$-test. A $P$ value of less than 0.05 was considered to be significant. The curves represent the least-squares fitting of the data by an exponential function, $N = 100\exp[-(t/\tau)^b]$, where $\tau$ and $b$ are parameters.

The data in Fig. 5 show nearly homogenous PMN binding to fibrinogen. The majority of cells are resistant to flow up to a wall shear stress of about 0.1 Pa or 0.17 Pa when the neutrophils are non-activated or activated, respectively. If this limit is exceeded, then nearly

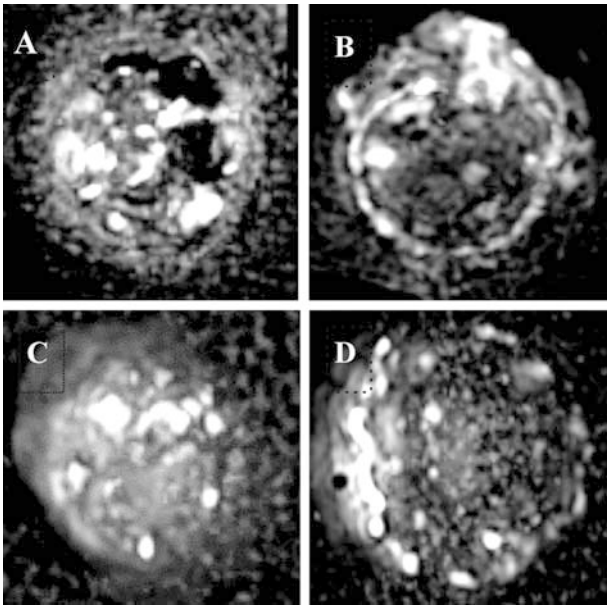


**Fig. 3** CD11b and CD18 molecular distribution on PMNs localized in clusters (*bright spots*) at rest (**A**, **C**) and after activation by FMLP (**B**, **D**), respectively

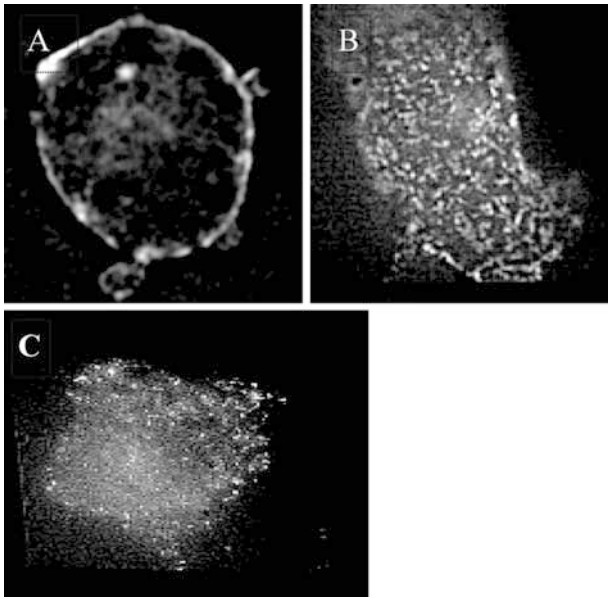


**Fig. 4** ICAM-1 distribution on the surface of the (**A**) resting ECs, (**B**) activated ECs by wall shear stress (0.2 Pa, 4 h) and (**C**) activated ECs by TNF-$\alpha$ (100 U, 4 h)

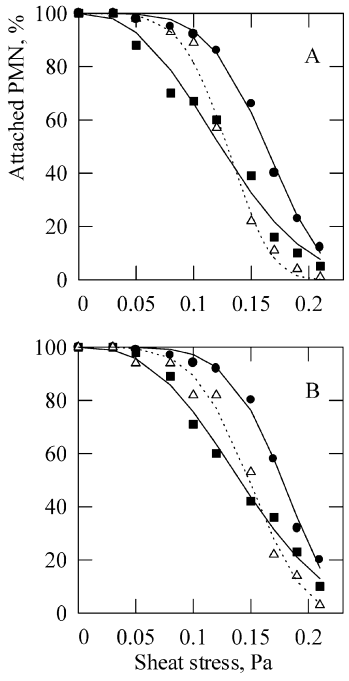


**Fig. 5** The effect of flow on the attachment of non-activated (**A**) and activated (**B**) neutrophils to: the fibrinogen layer [*open triangles*; average of $n$ (=7) measurements], the layer of non-activated ECs (*filled squares*, $n=7$), and the layer of activated ECs (*filled circles*, $n=5$). The *dashed line* (the fibrinogen coating) and the *solid lines* represent data fitting by the exponential function introduced in the text

all cells are washed away. On the other hand, the number of neutrophils attached to endothelial cells decreases gradually over the range of the acting shear stresses. The wall shear stress at which 50% of non-activated PMNs are attached to the non-activated and activated endothelial cells is 0.12 Pa and 0.17 Pa, respectively. Similar wall shear stresses in the case of activated PMNs are 0.14 Pa and 0.18 Pa. This shows that the binding is promoted above all by EC activation. Altogether, the activation of both cell types increases the binding by about 50% compared with the non-activated PMNs and ECs by comparing at the level of 50% attached cells.

The fluid flow with the intensity specified as the wall shear stress $\sigma_w$ in Fig. 5 acts on PMNs with a shear force $F^s = 32.05R^2\sigma_w$, where the numerical coefficient corresponds to the assumed cell spherical shape and $R$, $\sigma_w$ and $F^s$ are expressed in μm, Pa and pN, respectively (Shao et al. 1998). With the radius $R=4.25$ μm for PMNs, the binding bonds are loaded by a shear force of 578 pN per Pa wall shear stress. The force stretching a single bond, $F^b$, is related to the shear force as $F^s = F^b\cos\theta$, where $\theta$ is the angle of the bond with the substrate (Shao et al. 1998). This angle and consequently the stretching force may vary according to the bond geometry. For example, the distribution of Mac-1 molecules on the inactivated neutrophils was observed to be confined mainly to the cell body, that is, the cell membrane lying at the base of and between adjacent cell surface projections (microvilli and ruffles) (Erladsen et al. 1993). On the other hand, the distribution of Mac-1 on the activated neutrophils occurs on both the membrane of the cell body and microvilli. Moreover, the possible extension of the natural length of a microvillus (0.35 μm) by fluid shearing, or the formation of a long thin membrane cylinder (tether) from it, can affect the binding geometry (Shao et al. 1998). Thus at the maximum microvillus extension (length about 1 μm) and a bond angle $\theta = 62°$, the corresponding bond stretching force is about 760 pN per Pa wall shear stress. At the binding by a microvillus of natural length (0.35 μm) the stretching force is about 1240 pN per Pa wall shear stress (Alon et al. 1997). Thus under our maximum flow conditions at the wall shear stress of 0.22 Pa, the maximum stretching force ranged between 167 and 273 pN.

## Discussion

When PMNs and ECs are in their activated states, then according to our data there is increased expression of CD11b/CD18 integrins on PMNs and ICAM-1 molecules on ECs, which results in increased binding between both cells. In terms of bond formation and bond mechanics, the increased density of receptor and ligand molecules is favourable for the probability of molecular binding, which may increase the number of molecular complexes forming one bond (Zhu 2000), and thus an enhanced cell ability to resist the applied force. That, rather straightforward, consideration may be supported by the results of PMN attachment to the fibrinogen layer. Fibrinogen is a ligand specific to CD11b/CD18 integrins. Consequently, PMN binding to fibrinogen is uniquely through these integrins. When the expression of CD11b/CD18 integrin was increased, the binding to fibrinogen was enhanced. A similar proportionality between the increased density of either CD11b/CD18 integrins and/or ICAM-1 molecules and the enhanced attachment of PMNs was observed in all other experiments. However, despite the increased binding strength of neutrophils to endothelial cells after their activation, the binding strength is not strong enough to keep PMNs attached at higher shear stresses. The physiological wall shear stresses can be of one order higher than the experimental ones in the postcapillary venules, where the leukocyte homing is mostly evoked (Chen and Springer 1999). It shows that the model experiments probably do not correspond exactly to the pathological case when the firm adherence and subsequent PMN transmigration through the vascular endothelium to a site of inflammation is recruited.

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