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Research Article

Surface molecules regulating rolling and adhesion to endothelium of neutrophil granulocytes and MDA-MB-468 breast carcinoma cells and their interaction

C. Strell, K. Lang, B. Niggemann, K. S. Zaenker and F. Entschladen*

Institute of Immunology, Witten/Herdecke University, Stockumer Strasse 10, 58448 Witten (Germany),
Fax: +49 2302 926 158; e-mail: entschladen@uni-wh.de

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Abstract. The extravasation of leukocytes and tumor cells is a multi-step process with the involvement of various adhesion molecules depending on the three steps rolling, adhesion, and diapedesis. We have developed an *in vitro* model, by which we investigated the rolling and adhesion of neutrophil granulocytes and MDA-MB-468 human breast carcinoma cells to lung endothelial cells under physiological flow-conditions. We found that norepinephrine had an inhibitory function on the fMLP-promoted adhesion of neutrophil granulocytes due to a down-regulation of β2-integrin. Furthermore, neutrophil granulocytes serve as linking cells for the interaction of the MDA-MB-468 cells with the endothelium, which are both β2-integrin negative, but express the β2-integrin ligand ICAM-1. In addition, we show here that N-cadherin is up-regulated on the endothelial cells and on neutrophil granulocytes in response to fMLP. This up-regulation resulted in a significant increase of adherent MDA-MB-468 cells, which are also N-cadherin positive.

Keywords. Extravasation, tumor cells, neutrophil granulocytes, N-cadherin, β2-integrin, fMLP, norepinephrine.

Introduction

The extravasation of cells from the blood stream into surrounding tissues is an important step in both physiological and pathological processes. On the one hand, leukocytes need to leave the blood stream to reach tissue sites of injury or inflammation. On the other hand, tumor cells extravasate at certain places to form distant metastases during tumor progression. Regardless of the extravasating cell type, the process seems to be in principle the same, consisting of three sequential steps. In the first step, the cells loosely adhere to the cells of the vascular endothelium. As this attachment is not very strong, the cells are dragged along with the blood stream, which results in a rolling on the endothelial surface. In the second step, the cells tightly attach to the endothelial cells, and in the third step, the cells transmigrate through the endothelium (diapedesis).

The extravasation process is strongly regulated and depends on the cell type as well as on the type of endothelium. The ability of cells to adhere to endothelium is a complex network between various adhesion molecules expressed by different cell types, diversely regulated by chemokines, cytokines and their receptors. For leukocytes, the process is described and accepted so far with selectins/CD44 involved

* Corresponding author.

in the rolling procedure, integrins - mainly β2-integrin - participating in the adhesion process, and platelet/endothelial cell adhesion molecule-1 (PECAM-1) and CD99 in diapedesis [1–3]. Since most of the involved molecules are specific for blood cells, tumor cells must adhere to endothelial cells *via* other mechanisms. In the past decade the knowledge of adhesion molecules on tumor cells has tremendously increased. For example, Thomsen-Friedenreich-antigen and galectin-3 have been identified as an adhesion couple in some prostate and breast cancers [4, 5]. Additionally, CD24 expressed on many carcinomas was identified as a further ligand for P-selectin on activated endothelium [6, 7]. Cell adhesion molecules of the cadherin superfamily are also important in the regulation of cell-cell contact *via* homophilic interactions. While epithelial cadherins such as E-cadherin are mainly involved in the formation and maintenance of epithelial structures, mesenchymal cadherins such as N-cadherin are mostly expressed in migratory cells and connective tissue [8]. The loss of E-cadherin and *de novo* expression of N-cadherin in a number of human cancers is correlated with a bad prognosis, since N-cadherin is a promoter for motility, migration and invasion [9]. However, there are still many open questions as to how exactly N-cadherin contributes to an invasive phenotype, which form part of our investigations presented here.

As mentioned above, the whole extravasation process - regardless of the cell type - is regulated and modified by many soluble factors such as cytokines and chemokines, which can act on the extravasating cells or on the endothelium [10]. The bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP) is known to promote the adhesion of neutrophil granulocytes by up-regulating the surface β2-integrin in a calcium-dependent manner [11], and IL-1, tumor necrosis factor (TNF)-α and lipopolysaccharides modulate the surface expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on endothelial cells depending of the type of endothelium [12]. We have shown previously that neurotransmitters are potent regulators for the migration of both cell types – leukocytes and tumor cells, whereas the nature of action varies [13]. Among the neurotransmitters, the so-called stress hormone norepinephrine is the strongest inducer of the migratory activity of tumor cells of cell lines derived from prostate, colon and breast carcinomas [14–17], and norepinephrine enhances the lymph node metastasis formation of human prostate carcinoma cells injected in mice thighs [18]. In contrast, norepinephrine reduces the fMLP-induced migration of neutrophil granulocytes [19]. Since migratory activity is essential for the third step of the extravasation process, it is reasonable to assume that neurotransmitters may influence extravasation in a cell type-specific manner, too. Furthermore, the adhesion of tumor cells to endothelium can also be modulated by interaction with blood cells. Platelets can contribute to the adhesion of tumor cells to endothelium [20, 21], and a pro-adhesive function has been shown for lymphocytes [22]. Much time has been spent on the investigation of these cells and their tumor promoting function, whereas neutrophil granulocytes - the most abundant cells in the blood stream - and their interaction with tumor cells have only been at the edge of the focus of interest [23]. Thus, we addressed here the question of whether the first two steps of extravasation – rolling and adhesion – of neutrophil granulocytes and tumor cells are influenced by soluble signaling molecules such as norepinephrine or fMLP, or by an interaction with each other.

A major problem in the investigation of extravasation is that it has to be performed under flow conditions. Otherwise, at least the first and the second steps of extravasation mentioned above (rolling and tight attachment) cannot be separated from each other with regard to the investigation of the involved receptors or signaling pathways. However, in contrast, *in vivo* assays (*e.g.*, intravital microscopy) provide flow conditions, but do not allow the investigation of pharmacological effects under defined conditions, as discussed previously [24, 25]. Therefore, we have developed a flow-through adhesion assay on the basis of our well-established migration system. In this assay, tumor cells or leukocytes run over a monolayer of endothelial cells with a flow and shear rate similar to *in vivo* conditions. The interaction of the floating cells with the endothelium is recorded by digital time-lapse video microscopy. Here we provide results on the distinct regulation of rolling and adhesion of neutrophil granulocytes and tumor cells during extravasation as well as the interaction of these two cell types under the influence of soluble substances such as norepinephrine and fMLP.

Materials and methods

Leukocyte isolation and cell culture. Neutrophil granulocytes were isolated from human peripheral blood in a two-step process as described previously [26]. The neutrophil granulocytes and erythrocytes were separated from the peripheral blood mononuclear cell fraction by density-gradient centrifugation using lymphocyte separation medium (PAA, Pasching, Germany). The neutrophil granulocytes/erythrocytes fraction was mixed with platelet-depleted serum

of the same blood donor and diluted (1:1.3) with a high molecular weight dextran solution (Macrodex, Fresenius, Bad Homburg, Germany) containing 0.01 mol/l EDTA. After 3 h, the supernatant containing the neutrophil granulocytes was separated from the erythrocytes-containing pellet. Residual erythrocytes in the neutrophil granulocyte population were removed by hypotonic lysis with 0.3% NaCl for 3 min on ice. The purified neutrophil granulocytes were used for the experiments immediately after isolation. More than 98% of the cells were viable as assessed by Trypan blue exclusion.

The MDA-MB-468 human breast carcinoma cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM (PAA) containing 10% fetal calf serum (PAA) at 37°C humidified atmosphere with 5% CO_2 [19]. Cells were harvested and used for experiments at the subconfluent (90% confluency) stage of growth.

Human endothelial cells from the pulmonary arteries (HPAEC) and pulmonary microvasculature (HMVEC) were used as endothelium in all experiments. We have observed no differences between these two primary cells with regard to the interaction of neutrophil granulocytes and MDA-MB-468 human breast carcinoma cells. Thus, throughout, only results with HMVEC are shown in the graphs. HPAECs (Promocell, Heidelberg, Germany) were cultured in endothelial cell growth medium with supplements (Promocell) and HMVECs (Cambrex, Verviers, Belgium) were cultured in EBM-2 media with supplements (Cambrex) in a humidified incubator with 5% CO_2. Endothelial cells were only used within six passages and routinely screened for mycoplasmal infection (MycoAlert, Cambrex).

Flow-through adhesion assay. The endothelial cells were seeded on collagen IV-coated flow chambers (μ-chamber I, IBIDI, Munich, Germany) in normal culture medium and incubated to confluency for 2 days. Before each experiment the endothelium was activated by treatment with 10 ng/ml IL-1β (Invitrogene, Nivelles, Belgium) for 4 h. During the flow-through assay the chambers were placed in a temperature-regulated box (37°C) under a microscope (Fig. 1). Tumor cells or leukocytes were stored in a reservoir and kept in suspension by a stirrer. The suspension was also heated to 37°C. The cell suspension was drawn through the flow chamber by a perfusion pump (Perfusor IV, B. Braun Melsungen AG, Melsungen, Germany), which worked in a reverse mode at the exit of the flow chamber. The volume flow was 12.2 ml/h, which results in a shear stress of 0.25 dyn/cm^2 and represents physiological blood flow conditions in small vessels. The cell number was adjusted to 150 000 cells/ml for tumor cells and to 300 000 cells/ml for leukocytes. In co-culture experiments first only 3 ml of leukocytes were drawn over the monolayer, and a mixture of tumor cells and leucocytes at the ratio of 1:2 was then added. For the experiments endothelial basal media (PAA) without supplements but containing 2% fetal calf serum (PAA) was used. The bacterial peptide fMLP, propranolol and norepinephrine (all from Sigma Aldrich, Taufkirchen, Germany) were added to the cell suspension immediately before the start of the experiments at the concentrations described in the results section. None of the used substances affected the cell's viability, as confirmed using the Annexin V-FITC Apoptosis Detection Kit from MBL International (Woburn, MA, USA).

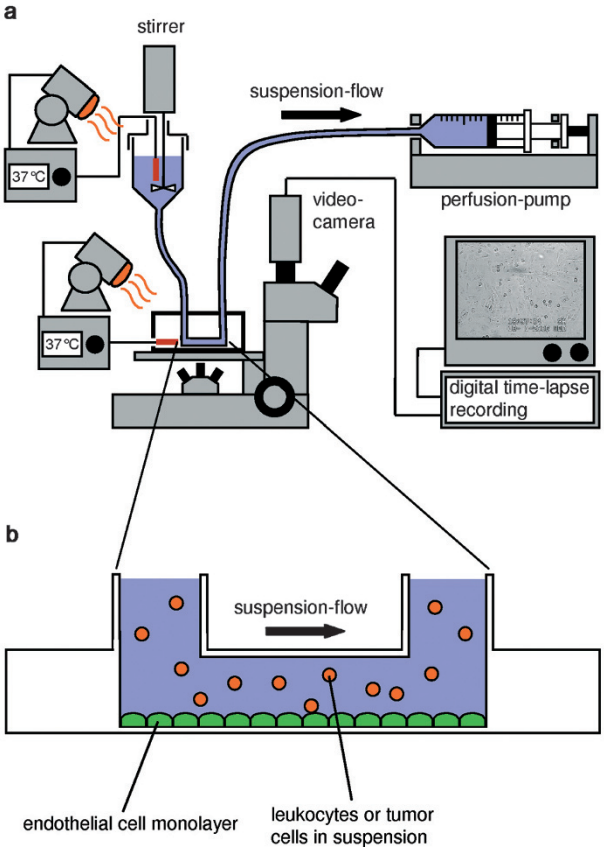

Figure 1. Experimental setup for the analysis of extravasation under flow conditions. (*a*) *In vitro* flow-through adhesion assay. (*b*) Flow chamber.

The suspension flow was digitally recorded by a video camera mounted on the microscope and a connected computer. The number of adhesive cells and of rolling cells were analyzed as well as their rolling velocity. The adhesive cells were counted as the absolute number during the entire recording period of 20 min. The

rolling cells are calculated per minute, and the rolling velocity is the distance in μm per second. Statistical significance was calculated using the Student's *t*-test (two-tailed and unpaired).

Flow cytometry. The expression of adhesion receptors on the surface of the cells was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Cells were incubated with the referred substances for 20 min followed by fixation with 0.4% paraformaldehyde (Sigma Aldrich) for 10 min on ice. Cells were stained with the primary antibody for 1 h on ice at a dilution of 3 : 100 in PBS. After a washing step, the cell-antibody complexes were fixed with 1% paraformaldehyde for 10 min on ice. The staining with the secondary antibody was performed for 20 min at room temperature at a dilution of 1 : 100 in PBS.

The functional mouse anti-N-cadherin monoclonal antibody (mAb GC-4) was obtained from Sigma Aldrich. The functional mouse β2-integrin mAb (7E4), the mouse CD54 (ICAM-1) mAb and the mouse IgG1 isotype control antibody were obtained from Beckman Coulter (Nyon, Switzerland). The same antibodies were used for blocking experiments (10 μg/ml), with the exception of the isotype control. In the blocking experiments, the IgG1 isotype control from R&D Systems (Wiesbaden, Germany) was used. Mouse fMLP receptor mAb (5F1) and mouse IgG1κ isotype control were obtained from BD Biosciences (Heidelberg, Germany). Goat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody was from Dianova (Hamburg, Germany). Every flow cytometric analysis was repeated at least three times, and changes of N-cadherin or β2-integrin expression are displayed as % changes ± SD. The significance of changes was calculated from the original geometrical mean values using the Student's *t*-test (two-tailed and paired).

Intracellular calcium. Changes of the cytosolic calcium concentration were measured by flow cytometric analysis on the basis of a protocol described by Gergely et al. [27]. Briefly, cells were stained with the calcium dye fluo-3/AM (Invitrogene) at a concentration of 2.5 μM in PBS for 30 min at 37 °C. Cells were washed with PBS and then resuspended in HBSS flux buffer (HBSS with or without $CaCl_2$/$MgSO_4$/phenol red, 10 mM HEPES, 5% fetal calf serum, 1.5 mM $CaCl_2$, pH 7.2) to a final concentration of 50 000 cells/ml. Changes in the fluorescence intensity of fluo-3 due to changes in intracellular calcium were plotted logarithmically against the time. After 30 s of baseline determination, the referred substances were added and the measurement proceeded to a final time of 3 min. Ionomycin (500 ng/ml; Sigma Aldrich) served as a positive control, the addition of PBS as a negative control.

Transfection with siICAM-1 RNA. MDA-MB 468 cells were transfected with 0.07 pmol siICAM-1 RNA or control siRNA (Dharmacon; ON-TARGET plus smart pool) using an Amaxa Nucletransfector II (Cologne, Germany) on program T-32 and Amaxa Transfection Solution R. Transfection was performed according to the manufacturer's manual. Success of transfection was controlled by immunoblotting against ICAM-1.

Immunoblotting. Cell lysates were prepared by lysis of 100 000 cells in 25 μl Laemmli buffer [28] and 5 min of heating at 95 °C. Samples were run on a 10% SDS-polyacrylamide gel and transferred by semi-dry Western blot to Immobilon-P membrane (Millipore, Bedford, MA, USA). Nonspecific sites were blocked with 5% (w/v) bovine serum albumin (BSA, Sigma Aldrich) solution. ICAM-1 was detected using the polyclonal rabbit ICAM-1 antibody at a dilution of 1 : 1000 in 5% BSA at 4 °C overnight. To standardize the applied amounts of protein a rabbit β-actin antibody was added simultaneously at a dilution of 1 : 2000. Both antibodies were detected with horseradish peroxidase-linked secondary antibody against rabbit IgG used at a dilution of 1 : 1000 in non-fat dry milk solution. Staining intensity was quantified with a 300 dpi 8-bit flat-bed scanner and analyzed using NHI image software version 1.62 (National Institute of Health, Bethesda, MD, USA).

The rabbit polyclonal ICAM-1 antibody and the rabbit β-actin mAb were obtained from Cell Signalling (Frankfurt am Main, Germany). Goat anti-Rabbit IgG linked to horseradish peroxidase was from Biozol (Echingen, Germany).

Results

Rolling and adhesion of neutrophils to endothelium. Neutrophil granulocytes constitutively adhered to a layer of HMVECs without additional stimulation, *e.g.*, by proinflammatory substances (all experiments were also performed with HPAECs with the same results). Nevertheless, adhesion significantly ($p < 0.001$) increased by 58% (from 148 ± 16 to 234 ± 4 cells) after stimulation with fMLP (Fig. 2a). This result reflects the normal (patho-) physiological response to inflammation and bacterial contamination and, furthermore, delivers proof of function for our *in vitro* flow assay used here. After addition of 10 μM norepinephrine, the fMLP-induced adhesion of

the neutrophil granulocytes to the endothelial cells significantly ($p = 0.015$) decreased by 20 % (from 234 ± 4 to 188 ± 19 cells; Fig. 2a). Equimolar amounts of the beta-blocker propranolol alone had no effect but abolished the effect of norepinephrine (229 ± 11 and 237 ± 14 cells, respectively, Fig. 2a). In contrast to these changes in adhesion, neither the number of rolling cells nor the rolling velocity were significantly influenced by treatment with fMLP, norepinephrine or both (Fig. 2b, c). In summary, norepinephrine has a significant inhibitory effect on the adhesion of neutrophil granulocytes during the second step of extravasation, in addition to the significant inhibition of migration, which we have investigated and published previously [19].

The adhesion of the neutrophil granulocytes to the vascular endothelium is mediated by β2-integrins, as shown using a blocking antibody (Fig. 3a). The number of adhesive cells significantly ($p = 0.002$) decreased by 31 % from 234 ± 4 to 161 ± 13 cells, whereas an isotypic control antibody did not cause any changes (not shown). Interestingly, inhibition of N-cadherin by a blocking antibody led to a stronger reduction of adhesion (130 ± 4 cells; $p < 0.001$) than inhibition of β2-integrin; simultaneous blockade of both receptors did not further reduce adhesion (133 ± 10 cells; $p < 0.001$; Fig. 3a). However, the number of rolling cells significantly ($p = 0.001$) increased by 31 % from 64 ± 7 to 84 ± 17 cells/min after inhibition of β2-integrin function (Fig. 3b). In contrast, blockade of N-cadherin alone or in combination with β2-integrin inhibition led to a slight, but significant ($p = 0.030$) reduction of rolling cells by 14 % to 55 ± 11 cells/min (Fig. 3b). These results led us to the conclusion that N-cadherin, besides other molecules such as selectins (since N-cadherin blockade did not cause a full inhibition of rolling), contributes to the rolling of the neutrophil granulocytes on the endothelium. This explains why a blockade of N-cadherin reduced the number of adhesive cells, because rolling is an essential step before adhesion can take place. Without slowing down during the rolling process, the cells are not able to tightly adhere. Blockade of β2-integrins reduced the adhesion, but increased rolling, because the rolling process is extended since tight adhesion cannot take place. Notably, none of the receptor inhibitions had a significant effect on the rolling velocity (Fig. 3c) and none of the observed inhibitory effects was due to cell death, as confirmed by propidium iodide/annexin staining and flow cytometry.

Inhibition of fMLP-induced adhesion by norepinephrine is mediated by a reduction of β2-integrin present on the surface of the neutrophil granulocytes, as measured by flow cytometry (Fig. 4a, b). The mean fluorescence intensity (MFI) of β2-integrin staining decreased after norepinephrine treatment, regardless whether the cells were stimulated with fMLP (Fig. 4b)

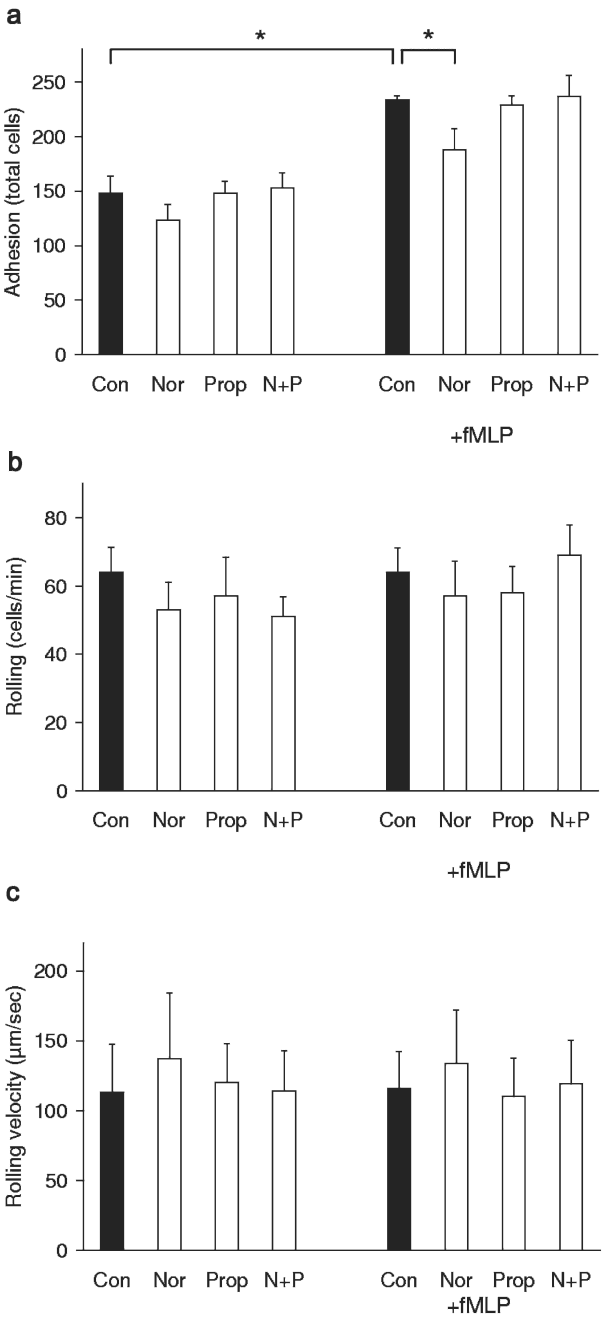

Figure 2. Interaction of neutrophil granulocytes with endothelial cells under the influence of formyl-methionyl-leucyl-phenylalanine (fMLP) and norepinephrine. (*a*) Total adherent cells during the 20-min observation period. (*b*) Number of rolling cells analyzed in 1-min intervals. (*c*) Velocity of the rolling cells. All graphs show mean values and SD of three independent experiments with neutrophil granulocytes from different donors. Con, control; Nor, norepinephrine; Prop, propranolol; N + P, norepinephrine and propranolol. fMLP was added at a concentration of 10 nM where indicated. Asterisks indicate statistically different changes ($p < 0.05$).

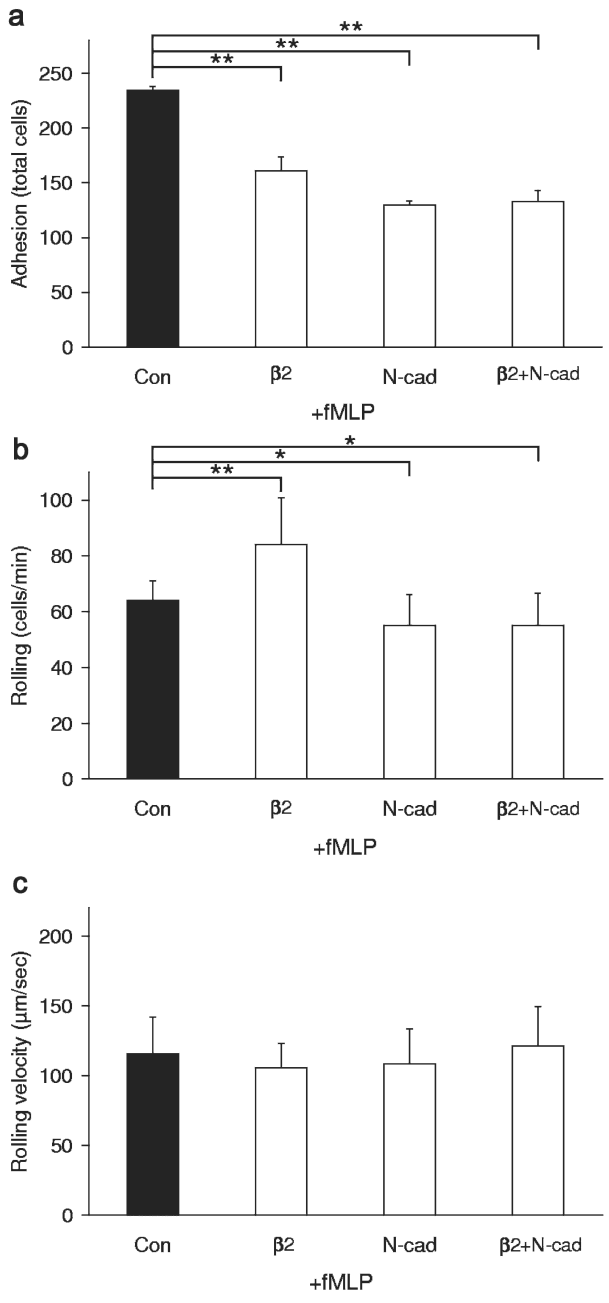

Figure 3. The role of β2-integrin and N-cadherin in the rolling and adhesion of neutrophil granulocytes. (*a*) Total adherent cells during the 20-min observation period. (*b*) Number of rolling cells analyzed in 1-min intervals. (*c*) Velocity of the rolling cells. All graphs show mean values and SD of three independent experiments with neutrophil granulocytes from different donors. Con, control; β2, blockade of β2-integrin; N-cad, blockade of N-cadherin. fMLP was added at a concentration of 10 nM. Statistically different changes: * $p < 0.05$, ** $p \leq 0.001$.

or not (Fig. 4a). In contrast, the surface expression of N-cadherin was increased by fMLP treatment, but not by norepinephrine (Fig. 4c, d). However, both fMLP and norepinephrine had an effect on the intracellular signal transduction of the cells, as seen by changes of the intracellular calcium concentration (Fig. 4e). fMLP led to a strong significant initial increase of the intracellular calcium by 16.7±2.5%, which then decreased to slightly elevated concentrations, which were still significant ($p \leq 0.011$ over the entire measurement period). Norepinephrine reduced the initial increase. After 70 s control concentrations were reached, representing a significant ($p \leq 0.040$) reduction of the fMLP-induced increase (Fig. 4e).

Adhesion of tumor cells to endothelium. Compared to neutrophil granulocytes, MDA-MB-468 human breast carcinoma cells show less adhesion to the endothelial cells (20 ± 9 cells) even after stimulation with norepinephrine (19 ± 11 cells; Fig. 5a). One reason for this weak interaction might be the lack of the β2-integrin expression (which is with some exceptions blood cell specific) of MDA-MB-468 cells (Fig. 5b). In contrast, more tumor cells than neutrophil granulocytes rolled on the endothelium (215 ± 30 cells, and 202 ± 41 cells after norepinephrine treatment; Fig. 5c). Accordingly, the MDA-MB-468 cells have a high surface expression of N-cadherin (Fig. 5d). Despite the higher N-cadherin expression, the rolling velocity of the MDA-MB-468 cells was higher (183 ± 43 mm/s; Fig. 5e) than that of the neutrophil granulocytes (113 ± 34 mm/s; Fig. 2c), which might be due to the higher flow resistance of the tumor cells due to their size. In addition, both endothelial cells used here expressed the fMLP receptor (Fig. 6a, b), and the expression of N-cadherin was selectively and very rapidly (within 10 min) increased after fMLP treatment (Fig. 6c, d). We did not observed such an up-regulation by IL-1β or TNF-α, or an up-regulation of ICAM-1, VCAM-1 or selectins by fMLP (data not shown). Although MDA-MB-468 cells express the fMLP receptor (Fig. 6e), fMLP treatment had no influence on the migratory activity of these cells, as analyzed in our three-dimensional collagen-based migration assay, or on the proliferation of these cells (data not shown). No further increase of the already high N-cadherin expression (Fig. 5d) was observed in MDA-MB-468 cells after fMLP treatment (Fig. 6f). The adhesion of the MDA-MB-468 cells to the endothelium was significantly ($p = 0.001$) increased by 124% from 25 ± 5 cells to 56 ± 4 cells in the presence of fMLP (Fig. 7a). This effect is caused by the up-regulation of N-cadherin on the surface of the endothelial cells, since this effect was significantly ($p = 0.003$) reduced to 40 ± 1 cells by an N-cadherin blocking antibody (Fig. 7a).

Interaction of tumor cells and neutrophils during adhesion to endothelium. Because leukocytes and tumor cells are together in the blood stream *in vivo*, we

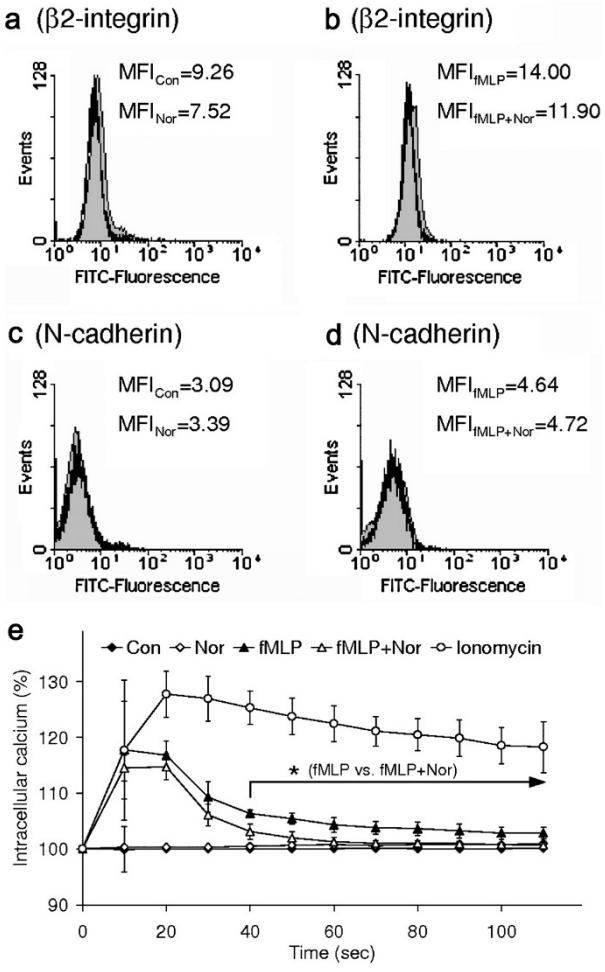

Figure 4. Receptor expression and signal transduction of neutrophil granulocytes in response to fMLP. (*a*, *b*) Expression of β2-integrin without (*a*) and with (*b*) 10 nM fMLP. Treatment of the cells with fMLP resulted in an up-regulation of β2-integrin expression by $40.39 \pm 11.95\,\%$ ($p = 0.004$). Application of norepinephrine alone or in combination with fMLP led to a slight down-regulation of β2-integrin by $3.43 \pm 1.75\,\%$ (*a*) and by $11.72 \pm 4\,\%$ (*b*). (*c*, *d*) Expression of N-cadherin without (*c*) and with (*d*) 10 nM fMLP. Stimulation of the cells with fMLP up-regulated the surface expression of N-cadherin by $41.81 \pm 7.5\,\%$ ($p = 0.03$). Additional treatment with norepinephrine did not have any significant effect on the N-cadherin expression. Gray fields, control; black line, treatment with 10 μM norepinephrine; MFI, mean fluorescence intensity. Graphs hold true for three independent experiments with neutrophil granulocytes from different donors. Additional application (*e*): Changes of the cytosolic calcium concentration after treatment with 10 μM norepinephrine and 10 nM fMLP. The graph shows mean values and SD of three independent experiments with neutrophil granulocytes from different donors (statistically different changes: * $p < 0.05$). The calcium ionophore ionomycin (500 ng/ml) served as positive control.

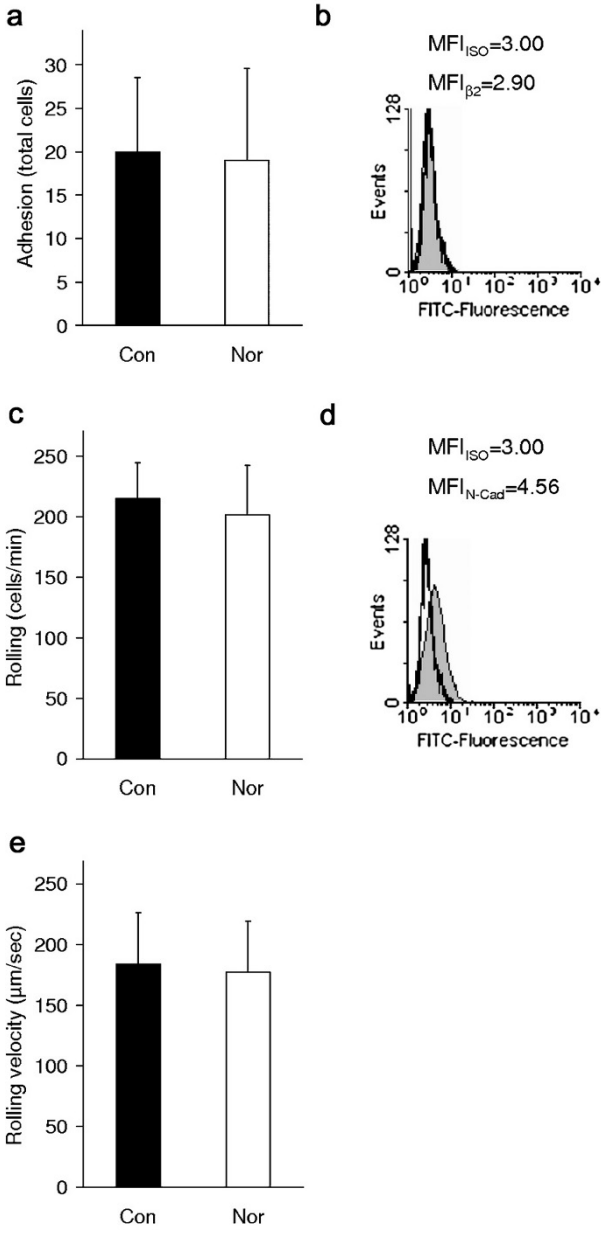

Figure 5. Interaction of MDA-MB-468 cells with endothelial cells under the influence of norepinephrine. (*a*) Total adherent cells during the 20-min observation period. (*b*) Expression of β2-integrin. Gray field, anti-β2-integrin antibody; black line, isotypic control antibody. (*c*) Number of rolling cells analyzed in 1-min intervals. (*d*) Expression of N-cadherin. Gray field, anti-N-cadherin antibody; black line, isotypic control antibody. (*e*) Velocity of the rolling cells. The graphs in *a*, *c*, and *e* show mean values and SD of three independent experiments. Con, control; Nor, norepinephrine. The graphs in *b* and *d* hold true for three independent experiments.

investigated whether these two cell types might interact during extravasation and whether fMLP modulates this interaction. Unfortunately, an analysis of rolling was for technical reasons not feasible in those experiments in which neutrophil granulocytes and tumor cells were applied simultaneously, thus we evaluated only adhesion. The presence of neutrophil granulocytes increased the adhesion of the MDA-MB-468 cells to the endothelium (Fig. 7b). Such an interaction of tumor cells with endothelium *via* neutrophil granulocytes under flow conditions is demonstrated in Figures 8a and b. This association

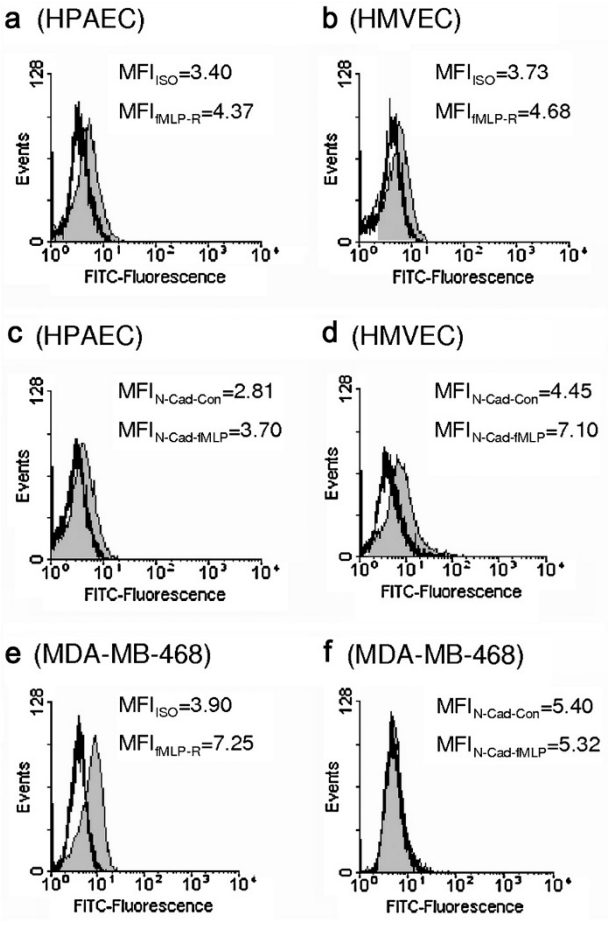

Figure 6. Expression of the fMLP receptor and of N-cadherin in response to fMLP. The expression of the fMLP receptor (fMLP-R) on human pulmonary artery endothelial cells (HPAEC, *a*) and pulmonary microvasculature endothelial cells (HMVEC, *b*) as well as on the MDA-MB-468 human breast carcinoma cells (*e*) was analyzed by flow cytometry. Black line, isotypic control antibody (ISO); gray field, fMLP-R-specific antibody. (*c*, *d*) Expression of N-cadherin on the endothelial cells in response to fMLP. Treatment of the cells with fMLP resulted in an up-regulation of N-cadherin expression by $20.76 \pm 9.75\,\%$ on HPAEC ($p = 0.03$) and by $56.6 \pm 2.52\,\%$ on HMVEC cells ($p < 0.02$). (*f*) Expression of N-cadherin on MDA-MB-468 cells in response to fMLP. Application of fMLP led to a down-regulation of N-cadherin on the surface of MDA cells by $7 \pm 5\,\%$ ($p < 0.03$). Black line, control; gray field, fMLP-treatment (10 nM). All graphs hold true for three independent measurements.

may occur in two ways: first, the neutrophil granulocytes are already in contact with the endothelium and then capture the rolling MDA cells; second, the neutrophil granulocytes interact with the tumor cells and then mediate their adhesion to the endothelium during the rolling process. The adhesion increased from 25 ± 4 cells to 35 ± 8 cells without fMLP and from 55 ± 8 cells to 94 ± 24 cells with fMLP; both effects with fMLP were significant compared with control ($p = 0.005$ and $p = 0.008$, respectively). Both endothelial cells and MDA-MB-468 cells are negative for β2-integrins (Figs. 5b and 7c, d), but express the responding ligand ICAM-1 (Fig. 7e and [29]). It is possible that neutrophil granulocytes, which are β2-integrin positive (Fig. 4a, b), function as a linking cell between these two cells and thereby facilitate the adhesion of tumor cells. Therefore, we down-regulated ICAM-1 expression on the tumor cells using siRNA (Fig. 7e). After 24 h, the siRNA inhibited ICAM-1 expression, which started to be restored after 72 h. Therefore, the tumor cells were used in this time frame (36–48 h) for the flow-through adhesion experiments (Fig. 7f). As already shown in Figures 7a and b, the adhesion of the tumor cells significantly ($p = 0.016$) increased after fMLP treatment from 27 ± 7 cells to 49 ± 1 cells. In both cases, without or with fMLP, the presence of neutrophil granulocytes led to a strong, although not significant, increase of the adhesion of the tumor cells (40 ± 10 cells, $p = 0.14$, and 86 ± 29 cells $p = 0.095$, respectively). In tumor cells, in which ICAM-1 expression was inhibited, this effect of the neutrophil granulocytes was strongly diminished (without fMLP 32 ± 10 cells; with fMLP 61 ± 16 cells; Fig. 7f).

Discussion

The bacterial peptide fMLP is a strong inducer for the migratory activity of neutrophil granulocytes [14, 26]. The chemotactic movement of these cells toward a site of bacterial contamination is essential for the immune response. It is thus not surprising that fMLP is also a substance that provokes an enhanced recruitment of neutrophil granulocytes from their main source – the blood stream – to the tissue interstitium, as shown here. However, similar to the migration itself [19], the adhesion of neutrophil granulocytes to the endothelium is significantly reduced by norepinephrine. This adds molecular findings to the epidemiology-based theory that chronic stress suppresses the immune system [30]. But what are the molecular mediators for the effects of fMLP and norepinephrine? Most interestingly, both endothelial cell types used here express the fMLP receptor, and N-cadherin is specifically up-regulated after fMLP treatment. Blockade of N-cadherin binding inhibits both the rolling of neutrophil granulocytes on endothelium and their adhesion to the endothelial cells. In contrast, blockade of β2-integrins reduces adhesion but increases rolling. These results can be explained by the distinct involvement of the adhesion molecules in the different stages of extravasation process. On the one hand, N-cadherin takes part in the rolling process. If rolling is inhibited, adhesion is also affected since the deceleration of the cells in the first step of the extravasation process is

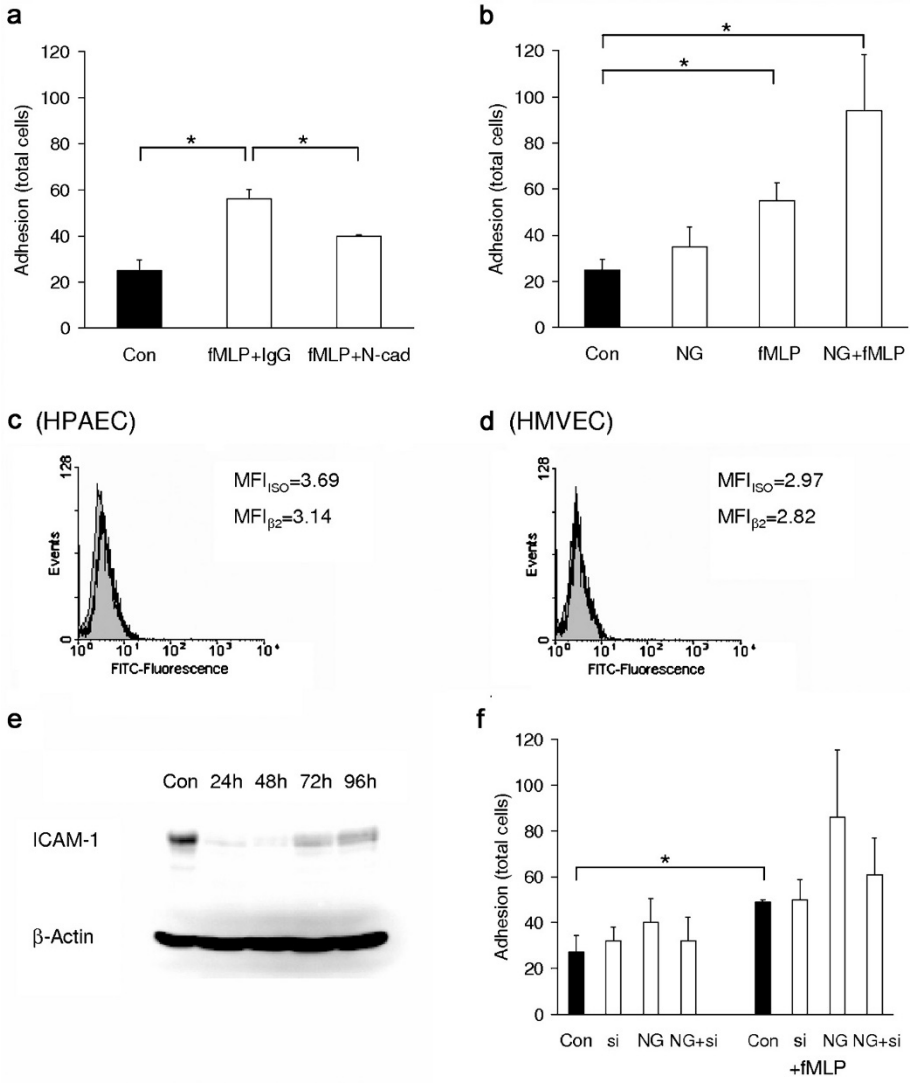

Figure 7. Effect of fMLP on the adhesion of MDA-MB-468 cells and their interaction with neutrophil granulocytes. (*a*) Con, control; fMLP + IgG, treatment with 10 nM fMLP and an isotypic control antibody; fMLP + N-cad, treatment with 10 nM fMLP and an N-cadherin blocking antibody. (*b*) Con, control; fMLP, treatment with 10 nM fMLP; NG, presence of neutrophil granulocytes at the same amount that was used for the experiments with neutrophil granulocytes alone. (*c*, *d*) Expression of β2-integrin on HPAEC and HMVEC. Gray field, anti-β2-integrin antibody; black line, isotypic control antibody. (*e*) Down-regulation of ICAM-1 expression using siRNA. β-Actin was used to control the total amount of applied protein. Flow-though adhesion experiments were performed after 36–48 h of siRNA treatment. (*f*) Con, control (cells treated with control siRNA); si, siRNA specific for ICAM-1; NG, presence of neutrophil granulocytes. fMLP was added at a concentration of 10 nM where indicated (right). The graphs in *a*, *b* and *f* show the total adherent cells during the 20-min observation period. These graphs show mean values and SD of three independent experiments in *b* and *f* with neutrophil granulocytes from different donors. * Statistically different changes, $p < 0.05$.

needed for a strong adhesion. On the other hand, when β2-integrins are inhibited, less cells can tightly adhere, which leads to an increase or a prolongation of the rolling process. Both of these adhesion molecules are increased on the surface of neutrophil granulocytes after fMLP treatment, but only β2-integrin is down-regulated by norepinephrine. Both of these substances generate characteristic changes of the intracellular calcium concentration (Fig. 4e). Thus, the changes of the cell surface receptor expression are reflected in intracellular signaling events. Nevertheless, it has been described by other groups that neutrophil granulocytes also use β2-integrin-independent strategies for the fMLP-induced adhesion to lung endothelium, since blockade of the β2-integrin did not completely prevent adhesion [2, 31], which is in accordance with our results. We suggest that N-cadherin might be a candidate for this replacement. In comparison to the neutrophil granulocytes, less MDA-MB-468 cells adhere to the endothelium but more cells roll on it. The small number of adherent cells can be explained by the lack of β2-integrin expression. Thus, similar to the blockade of β2-integrins on neutrophil granulocytes, more cells roll. In addition, the MDA-MB-468 cells display a high expression of N-cadherin on their surface. Cadherins are well-known receptors for intercellular adhesion, especially E-cadherin. However, N-cadherin is the most abundant cadherin on MDA-MB-468 cells [32]. A role of N-cadherin in extravasation has, so far, only been described for the diapedesis, where it guides the tumor cells through the endothelial junction [33]. During normal vascular morphogenesis N-cadherin is known to mediate adhesion, recognition and signaling between pericytes and endothelial cells [34, 35]. We show that fMLP increases the rolling of the MDA-MB-468 cells, which is caused by an increased N-

Figure 8. Interaction of MDA-MB-468 cells with HMVEC cells *via* neutrophil granulocytes as linker cells. (*a*, *b*) Two different examples of this direct linking process. The pictures demonstrate how an already adherent neutrophil granulocyte increases the adhesion of tumor cells to the endothelium. All these steps happen within 2 s under flow-through conditions. The arrow indicates the neutrophil granulocyte that is already in contact with the endothelium and then captures the rolling MDA cells. The corresponding movie can be visited at http://www.uni-wh.de/NeutrophilsMDA468Flow. EC, HMVEC; NG, neutrophil granulocyte; MDA, MDA-MB-468 breast carcinoma cells.

cadherin expression of the endothelial cells and results finally in an increased number of adherent tumor cells. In contrast to fMLP, norepinephrine does not influence any of the investigated parameters of the extravasation process of MDA-MB-468 cells (Fig. 5), although norepinephrine is one of the most potent known physiological inducers of migration of these cells [15].

Since leukocytes and tumor cells are together in the blood stream, we investigated the interaction of these cells. Liang and colleagues [36] have previously published results on the interaction of melanoma cells and neutrophil granulocytes *via* ICAM-1 and β2-integrins, and they ascribed IL-8, produced by the melanoma cells, a mediating role for this interaction [37]. We show here that the MDA-MB-468 cells constitutively interact with the neutrophil granulocytes during extravasation, and that this interaction is mediated by ICAM-1/β2-integrin interactions. Neutrophil granulocytes function as a linker between the tumor cells and the endothelial cells, since both of those cells are β2-integrin negative but ICAM-1 positive, and neutrophil granulocytes express β2-integrin. However, this interaction is intensified by the presence of fMLP for the above-discussed effects on β2-integrin, but also because of N-cadherin up-regulation. Here, fMLP is an indirect promoter for tumor cell adhesion by its influence on neutrophil granulocytes and endothelial cells.

In summary, these results show that tumor cells, which are almost unable to extravasate by themselves, can use neutrophil granulocytes *via* β2-integrin/ICAM-1 as linker for interaction with the endothelium, especially at the second stage of the extravasation process. By this linking function, even those soluble signaling substances such as proinflammatory mediators, to which the tumor cells are insensitive but neutrophil granulocytes are sensitive, have to be considered as modulators for the interaction of tumor cell with endothelium. Furthermore, endothelial cells themselves can also be modulated by these signaling substances. We have identified fMLP as one of these substances that acts on neutrophil granulocytes and on endothelial cells by changing their adhesion receptor profile. In this context we have found that N-cadherin is a key molecule in the rolling and adhesion of neutrophil granulocytes as well as of tumor cells.

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1 van Buul, J. D. and Hordijk, P. L. (2004) Signaling in leukocyte transendothelial migration. Arterioscler. Thromb. Vasc. Biol. 24, 824–833.
2 Wagner, J. G. and Roth, R. A. (2000) Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. Pharmacol. Rev. 52, 349–374.
3 Katayama, Y., Hidalgo, A., Chang, J., Peired, A. and Frenette, P. S. (2005) CD44 is a physiological E-selectin ligand on neutrophils. J. Exp. Med. 201, 1183–1189.
4 Glinsky, V. V., Glinsky, G. V., Rittenhouse-Olson, K., Huflejt, M. E., Glinskii, O. V., Deutscher, S. L. and Quinn, T. P. (2001) The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium. Cancer Res. 61, 4851–4857.
5 Khaldoyanidi, S. K., Glinsky, V. V., Sikora, L., Glinskii, A. B., Mossine, V. V., Quinn, T. P., Glinsky, G. V. and Sriramarao, P.

(2003) MDA-MB-435 human breast carcinoma cell homo- and heterotypic adhesion under flow conditions is mediated in part by Thomsen-Friedenreich antigen-galectin-3 interactions. J. Biol. Chem. 278, 4127–4134.
6 Aigner, S., Sthoeger, Z. M., Fogel, M., Weber, E., Zarn, J., Ruppert, M., Zeller, Y., Vestweber, D., Stahel, R., Sammar, M. and Altevogt, P. (1997) CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood 89, 3385–3395.
7 Friederichs, J., Zeller, Y., Hafezi-Moghadam, A., Grone, H. J., Ley, K. and Altevogt, P. (2000) The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells. Cancer Res. 60, 6714–6722.
8 Christofori, G. (2003) Changing neighbours, changing behaviour: Cell adhesion molecule-mediated signalling during tumour progression. EMBO J. 22, 2318–2323.
9 Hazan, R. B., Phillips, G. R., Qiao, R. F., Norton, L. and Aaronson, S. A. (2000) Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J. Cell Biol. 148, 779–790.
10 Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A. and Springer, T. A. (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J. Exp. Med. 184, 1101–1109.
11 Hughes, B. J., Hollers, J. C., Crockett-Torabi, E. and Smith, C. W. (1992) Recruitment of CD11b/CD18 to the neutrophil surface and adherence-dependent cell locomotion. J. Clin. Invest. 90, 1687–1696.
12 Kalogeris, T. J., Kevil, C. G., Laroux, F. S., Coe, L. L., Phifer, T. J. and Alexander, J. S. (1999) Differential monocyte adhesion and adhesion molecule expression in venous and arterial endothelial cells. Am. J. Physiol. 276, L9-L19.
13 Entschladen, F., Lang, K., Drell, T. L., Joseph, J. and Zaenker, K. S. (2002) Neurotransmitters are regulators for the migration of tumor cells and leukocytes. Cancer Immunol. Immunother. 51, 467–482.
14 Bastian, P., Lang, K., Niggemann, B., Zaenker, K. S. and Entschladen, F. (2005) Myosin regulation in the migration of tumor cells and leukocytes within a three-dimensional collagen matrix. Cell. Mol. Life Sci. 62, 65–76.
15 Drell, T. L., Joseph, J., Lang, K., Niggemann, B., Zaenker, K. S. and Entschladen, F. (2003) Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. Breast Cancer Res. Treat. 80, 63–70.
16 Lang, K., Drell, T. L., Lindecke, A., Niggemann, B., Kaltschmidt, C., Zaenker, K. S. and Entschladen, F. (2004) Induction of a metastatogenic tumor cell type by neurotransmitters and its pharmacological inhibition by established drugs. Int. J. Cancer 112, 231–238.
17 Masur, K., Niggemann, B., Zanker, K. S. and Entschladen, F. (2001) Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. Cancer Res. 61, 2866–2869.
18 Palm, D., Lang, K., Niggemann, B., Drell, T. L., Masur, K., Zaenker, K. S. and Entschladen, F. (2006) The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. Int. J. Cancer. 118, 2744–2749.
19 Bastian, P., Posch, B., Lang, K., Niggemann, B., Zaenker, K. S., Hatt, H. and Entschladen, F. (2006) Phosphatidylinositol 3-kinase in the G protein-coupled receptor-induced chemokinesis and chemotaxis of MDA-MB-468 breast carcinoma cells: A comparison with leukocytes. Mol. Cancer Res. 4, 411–421.
20 Gupta, G. P. and Massague, J. (2004) Platelets and metastasis revisited: A novel fatty link. J. Clin. Invest. 114, 1691–1693.
21 Borsig, L., Wong, R., Feramisco, J., Nadeau, D. R., Varki, N. M. and Varki, A. (2001) Heparin and cancer revisited: Mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc. Natl. Acad. Sci. USA 98, 3352–3357.
22 Neeson, P. J., Thurlow, P. J., Jamieson, G. P. and Bradley, C. (2003) Lymphocyte-facilitated tumour cell adhesion to endothelial cells: The role of high affinity leukocyte integrins. Pathology 35, 50–55.
23 Di Carlo, E., Forni, G., Lollini, P., Colombo, M. P., Modesti, A. and Musiani, P. (2001) The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97, 339–345.
24 Entschladen, F., Drell, T. L., Lang, K., Masur, K., Palm, D., Bastian, P., Niggemann, B. and Zaenker, K. S. (2005) Analysis methods of human cell migration. Exp. Cell Res. 307, 418–426.
25 Palm, D., Lang, K., Brandt, B., Zaenker, K. S. and Entschladen, F. (2005) *In vitro* and *in vivo* imaging of cell migration: Two interdepending methods to unravel metastasis formation. Semin. Cancer Biol. 15, 396–404.
26 Entschladen, F., Gunzer, M., Scheuffele, C. M., Niggemann, B. and Zanker, K. S. (2000) T lymphocytes and neutrophil granulocytes differ in regulatory signaling and migratory dynamics with regard to spontaneous locomotion and chemotaxis. Cell. Immunol. 199, 104–114.
27 Gergely, L., Cook, L. and Agnello, V. (1997) A simplified method for Ca^{2+} flux measurement on isolated human B cells that uses flow cytometry. Clin. Diagn. Lab. Immunol. 4, 70–74.
28 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
29 Carman, C. V. and Springer, T. A. (2004) A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. J. Cell Biol. 167, 377–388.
30 Segerstrom, S. C. and Miller, G. E. (2004) Psychological stress and the human immune system: A meta-analytic study of 30 years of inquiry. Psychol. Bull. 130, 601–630.
31 Mackarel, A. J., Russell, K. J., Brady, C. S., FitzGerald, M. X. and O'Connor, C. M. (2000) Interleukin-8 and leukotriene-B(4), but not formylmethionyl leucylphenylalanine, stimulate CD18-independent migration of neutrophils across human pulmonary endothelial cells *in vitro.* Am. J. Respir. Cell. Mol. Biol. 23, 154–161.
32 Potthoff, S., Entschladen, F., Niggemann, B., Zaenker, K. S. and Lang, K. (2007) N-Cadherin engagement provides a dominant stop signal for the migration of MDA-MB-468 breast carcinoma cells. Breast Cancer Res. Treat. 105, 287–295.
33 Qi, J., Chen, N., Wang, J. and Siu, C. H. (2005) Transendothelial migration of melanoma cells involves N-cadherin-mediated adhesion and activation of the beta-catenin signaling pathway. Mol. Biol. Cell. 16, 4386–4397.
34 Gerhardt, H., Wolburg, H. and Redies, C. (2000) N-Cadherin mediates pericytic-endothelial interaction during brain angiogenesis in the chicken. Dev. Dyn. 218, 472–479.
35 Tillet, E., Vittet, D., Feraud, O., Moore, R., Kemler, R. and Huber, P. (2005) N-Cadherin deficiency impairs pericyte recruitment, and not endothelial differentiation or sprouting, in embryonic stem cell-derived angiogenesis. Exp. Cell Res. 310, 392–400.
36 Liang, S., Sharma, A., Peng, H. H., Robertson, G. and Dong, C. (2007) Targeting mutant (V600E) B-Raf in melanoma interrupts immunoediting of leukocyte functions and melanoma extravasation. Cancer Res. 67, 5814–5820.
37 Peng, H. H., Liang, S., Henderson, A. J. and Dong, C. (2007) Regulation of IL-8 expression in melanoma-stimulated neutrophil inflammatory response. Exp. Cell Res. 313, 551–559.