Gap-junctional coupling between neutrophils and endothelial cells: a novel modulator of transendothelial migration

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Abstract: Communication between leukocytes and endothelial cells is crucial for inflammatory reactions. Paracrine cross-talk and outside-in signaling (via adhesion molecules) have been characterized as communication pathways to date. As leukocytes and endothelial cells express connexins, we considered intercellular communication via gap junctions an intriguing additional concept. We found that gap-junctional coupling between neutrophils and endothelium occurred in a time-dependent, bidirectional manner and was facilitated by adhesion. After blockade of connexins, transmigration of neutrophils through the endothelial layer was enhanced, and the barrier function of cell monolayers was reduced during transmigration. Tumor necrosis factor α decreased coupling. In the presence of connexins, transmigration of neutrophils did not alter permeability. Thus, neutrophils couple to endothelium via gap junctions, functionally modulating transmigration and leakiness. Gap-junctional coupling may be a novel way of leukocyte-endothelial communication. *J. Leukoc. Biol.* 73: 118–126; 2003.

Key Words: *connexin · PMN · leukocyte · HUVEC · emigration · diapedesis*

INTRODUCTION

Communication between leukocytes and vascular endothelial cells is mandatory for an appropriate inflammatory response. The present paradigm of inflammatory reactions postulates three steps: leukocyte rolling via adhesion molecules of the selectin class, activation of the rolling leukocyte, and its firm adhesion via β_2 integrins. Subsequently, transmigration through the endothelial lining takes place [1]. This interaction of endothelial cells and leukocytes is controlled by a paracrine way (via chemokines), as well as by outside-in signaling via adhesion molecules [1].

Most types of cells in the mammalian body communicate with each other via gap junctions, thereby coordinating their actions. Communication occurs through electrical coupling and transfer of small molecules (<1 kD). Gap junctions consist of subunits, the connexins (Cx). Endothelial cells reportedly express Cx37, Cx40, and Cx43 [2, 3]. As Cx43 has also been found on stimulated leukocytes [4], it was speculated that endothelial-leukocyte coupling and thus direct intercellular communication might occur [4]. The idea of such an information exchange was supported by ultrastructural studies, describing "gap-junction-like" connections between leukocytes and endothelium [4]. Furthermore, the Cx on endothelial cells are mainly located along the cell-cell contacts [3], i.e., where leukocytes preferentially adhere and migrate [1]. As expression and distribution of Cx on leukocytes and on endothelial cells are changed during pathophysiological conditions, such as inflammation [4–6], disturbed flow [4], or atherosclerosis [7], the concept of leukocyte-endothelial communication via gap junctions becomes even more attractive.

Thus, it is tempting to speculate that leukocyte activation by or transmigration through endothelium might be influenced by low molecular signals passing through gap junctions (e.g., cyclic adenosine monophosphate; cyclic guanosine 5′-monophosphate; Ca^{2+}; inositol trisphosphate) between endothelial cells and leukocytes. Until now, however, there are no quantitative and functional data on a putative gap-junctional communication between leukocytes and endothelial cells and its regulation. Therefore, we addressed the hypothesis that human endothelial cells (HUVECs) and human polymorphonuclear neutrophils (PMN) couple via gap junctions with special emphasis on the time course, the directionality, and the specificity of coupling. We further tested whether specific functions of PMN in the inflammatory process, such as adhesion, activation, and transmigration, were modulated by PMN-endothelial coupling via gap junctions.

MATERIALS AND METHODS

Cell culture

HUVECs were isolated from fresh umbilical cords according to the method of Jaffe et al. [8]. Briefly, the umbilical vein was cannulated, rinsed with phosphate buffer, and then filled with collagenase A (1 mg/ml) for 5 min at 37°C. Subsequently, the endothelial cells were eluted with medium 199, centrifuged, washed, pelleted again, and then seeded with endothelial cell growth medium (Promocell, Heidelberg, Germany) into T 25 culture flasks. Cells were kept under air plus 5% CO_2. After reaching confluence, the primary cultures were detached from the flask with trypsin and transferred into 24-well culture dishes coated with collagen A (Biochrom, Berlin, Germany). Experiments were performed 2 days after the cells had reattained confluence.

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Human cervical carcinoma HeLa cells (ECACC No. 96112022) were cultivated in Dulbecco's modified Eagle's medium with 10% fetal calf serum under air plus 10% CO_2. These cells were wild type (WT) or transfected with Cx37, -40, or -43, as described previously [9]. The medium for the transfected cells contained 1 μg/ml puromycin (Sigma Chemical Co., Deisenhofen, Germany). HeLa cells were a kind gift from Prof. Klaus Willecke (Dept. of Genetics, University of Bonn, Germany).

Isolation of neutrophils

Human PMN were isolated from fresh peripheral venous blood as described previously [10]. Briefly, blood was centrifuged at 350 *g* for 15 min, platelet-rich plasma was removed, and the buffy coat was harvested. After incubation with a magnetically tagged anti-CD15 antibody (Miltenyi, Bergisch Gladbach, Germany), the buffy coat was passed through a magnetized column. After washing, only PMN remained in the column and were eluted after removal of the column from the magnetic field. After isolation, the cells were resuspended in phosphate-buffered saline (PBS) and were used immediately after counting with a Coulter Counter (Coulter Electronics, Krefeld, Germany).

Cell-cell coupling

Coupling between HUVECs and PMN was quantified by a flow cytometry-based dye-transfer method, as described previously [11–13]. In brief, HUVECs or HeLa cells were incubated with 0.1 μM calcein-AM (Molecular Probes, Eugene, OR) for 15 min. This fluorescent dye ester is taken up by cells and is subsequently chemically modified intracellularly by esterases. Thus, the dye becomes membrane-impermeant and cannot leave intact cells on short-term except via gap junctions. Extracellular calcein-AM was removed by three wash steps. Unlabeled PMN (100,000/well) were added to the confluent cultures of labeled HUVECs or HeLa cells and sedimented in a centrifuge at 15 *g* for 2 min to allow for a defined onset of interaction. After coincubation for different periods of time, loosely attached and/or firmly adherent PMN (after detachment with EDTA and trypsin) were collected and examined in a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany). Forward- and sideward-scatter served as discrimination criteria between PMN and HUVECs or HeLa cells. Those PMN showing a higher fluorescence after coincubation than untreated, control PMN at an excitation wavelength of 488 nm and an emission of 515 nm were defined as calcein-positive and thus, had coupled with HUVECs or HeLa cells (**Fig. 1a**). Furthermore, the dye transfer from HUVECs to PMN was visualized by generating sections in the z-plane of the preparations with a confocal laser-scanning microscope (LSM 410 Invert, Zeiss, Jena, Germany), as exemplarily shown in Figure 1b.

The same experiments were performed in the reverse order; i.e., PMN were labeled with calcein, and thereafter, dye transfer into HUVECs or HeLa cells was determined.

The specificity of this model was tested by using the peptides SRPTEK-TVFTV, SRPTEKTIFII, and SRPTEKNVFIV (each 300 μM; Biosource, Solingen, Germany), which have been shown to reduce gap-junctional coupling previously [14, 15] or the gap-junction blocker α-glycyrrhetinic acid (50 μM and 500 μM; Sigma Chemical Co.). HUVECs were preincubated with both substances for 15 min before the addition of the PMN.

To further test the specificity of dye transfer, dye-microinjection experiments were performed (Femto-Jet, Eppendorf, Hamburg, Germany): When calcein was injected into an adherent PMN, the adjacent endothelial cell was also stained within seconds; when an endothelial cell carrying an adherent PMN was injected, the dye was passed on to the PMN (data not shown). This leaves only the conclusion of a direct, cytoplasmatic coupling.

The influence of leukocyte adhesion to HUVECs on coupling was determined. For this purpose, an adhesion-blocking antibody against the leukocyte-adhesion molecule CD18 (clone YFC 118.3, Serotec, Kidlington, UK) was used. The effects of inflammatory stimuli on the coupling process were investigated by incubating HUVECs with tumor necrosis factor α (TNF-α; 2.5 ng/ml), interferon-γ (IFN-γ; 250 ng/ml; both from Endogen, Woburn, MA), lipopolysaccharide (LPS; 100 ng/ml), or thrombin (1 U/ml; both from Sigma Chemical Co.) for 4 h before coincubation of endothelial cells and PMN.

Adhesion of PMN to HUVECs or HeLa cells

The adhesion of PMN to HUVECs or HeLa cells was determined as described previously [16]. Briefly, after stimulation of the HUVEC or HeLa monolayer for 4 h with TNF-α (2.5 ng/ml) and subsequent washing or after stimulation of PMN with phorbol 12-myristate 13-acetate (PMA; 1 μM) for 5 min, 100,000 PMN were added per well of a 24-well culture dish. Accordingly, only HUVECs and HeLa cells were confronted with TNF-α, but PMA was present for some minutes on PMN and the respective monolayer. The dish was centrifuged for 2 min at 15 *g* and then incubated 37°C for 30 min. Afterwards, nonadherent PMN were removed by gentle washing, and the remaining adherent PMN were lysed with 200 μl 1% hexadecyl-trimethyl-ammoniumbromide (Sigma Chemical Co.). The lysate (100 μl) was transferred to microtiter plates and assayed for myeloperoxidase as described previously [16]. The kinetics of the development of the orange-colored adduct was monitored with an enzyme-linked immunosorbent assay reader (MR 7000, Dynatech, Guernsey, UK) at 450 nm in samples and standards of varying PMN numbers. The detection limit of this method was 500 PMN/well; i.e., 0.25% of the added cells.

Transmigration of PMN through monolayers of HUVECs or HeLa cells

The transmigration of PMN through monolayers of HUVECs or HeLa cells was quantified according to the modified method of Boyden [17]. In brief, cells were cultured on filter inserts with a pore diameter of 3 μm (Becton Dickinson) until they were tightly confluent. formyl-Met-Leu-Phe (fMLP; 0.1 μM; Sigma Chemical Co.) was added to the lower compartment as a chemoattractant, and at the same time, 100,000 PMN were added to the upper compartment. After 4 h of coincubation, the insert was removed, and the number of transmigrated PMN in the lower compartment was quantified using a flow cytometer. About 20,000 PMN migrated through filters without endothelium under the conditions described above.

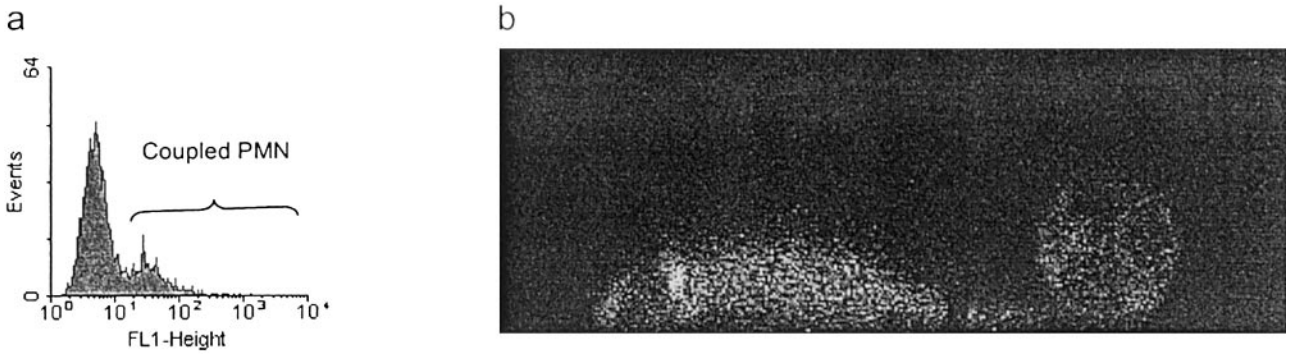

Fig. 1. Dye transfer is detected by flow cytometry and by confocal microscopy. (a) After coincubation of PMN with calcein-loaded HUVECs, a subpopulation of PMN with higher fluorescence emerges, which have taken up dye via gap junctions. (b) In a confocal scan (z-plane), dye distribution in the cytosol of a loaded HUVEC (left) and dye uptake into an adherent PMN (right) are visualized.

Measurement of cell monolayer permeability

To assess changes of cell monolayer integrity, HUVECs or HeLa cells were cultured on cell-culture inserts, as described above. Rhodamin B-labeled dextran (10 μg/ml, 10 kD; Molecular Probes, Eugene, OR) was added as an indicator to the upper compartment. In aliquots of medium in the lower compartment, fluorescence intensity was measured (excitation wavelength, 556 nm; emission, 578 nm) at different time points using a plate reader (SpectraFluor, Tecan, Crailsheim, Germany).

Measurement of leukocyte activation

HUVECs were loaded with calcein and coincubated with PMN for 30 min as described above. Subsequently, the PMN were stimulated in situ for 15 min with the chemotactic peptide fMLP (0.1 and 1μM). As a measure of activation, the surface molecule and early activation marker CD11b [18] were quantified by flow cytometry. To this end, PMN were fixed, washed, and incubated with a phycoerythrin-labeled antibody against CD11b (clone ICRF 44, Serotec). This dye has an emission wavelength different from calcein (575 nm). Thus, we were able to differentiate CD11b expression, i.e., activation of coupled (calcein-positive) and uncoupled PMN (calcein-negative).

Immunofluorescence

To determine the presence and subcellular localization of Cx37, Cx40, and Cx43 in PMN, cells were fixed with buffered formaldehyde (3%), centrifuged onto a microscope slide, air-dried, and subsequently permeabilized by submersion in 0.2% Triton X-100 in PBS for 2 min. The samples were rinsed three times with PBS, blocked with 0.2% bovine serum albumin in PBS for 15 min, and then incubated with the respective affinity-purified, primary antiserum (rabbit anti-mouse Cx37 A11 or rabbit anti-mouse Cx40, Biotrend, Cologne, Germany, or anti-Cx43, Transduction Laboratories, Lexington, KY) for 45 min. After washing four times, the secondary antibody (anti-rabbit immunoglobulin G, labeled with ALEXA 488, Molecular Probes, Leiden, NL) was added, and samples were incubated for 30 min at room temperature. After four final washing steps, the cells were covered with mounting medium (Sigma Chemical Co.) and a coverslip and then surveyed by means of a confocal microscope. Unspecific fluorescence was detected by using an irrelevant, isotype-matched primary antibody. The specificity of the antisera was tested by Western blotting or immunocytochemistry in HeLa cells expressing only one specific type of Cx or no Cx at all. The antibodies bound only in the cell type expressing the respective Cx. Unspecific binding in the Cx-free WT HeLa cells was negligible.

Western blotting

Western blots for identification of Cx were performed as follows: Isolated and purified PMN were lysed in cell lysis buffer containing 20 mM KH_2PO_4, pH 7.0, 1 mM EDTA, 1 mM Pefa block®, 10 μg/ml aprotinin, 0.5 mg/ml leupeptin, 0.7 μg/ml pepstatin, 50 mM NaF, 40 mM Na-pyrophosphate, and 1 mM Na_3VO_3. After keeping the lysates overnight at 4°C, Triton X-100 was added to a final concentration of 1%, and the samples were kept on ice for 30 min. Afterwards, one-half of the cell lysate was centrifuged for 2.5 h (10,000 *g*, 4°C), and the other half was removed. The supernatant of the centrifuged lysate (triton-soluble fraction) was separated from the pellet (triton-insoluble fraction), which was redissolved in lysis buffer supplemented with sodium dodecyl sulfate (SDS) and was disrupted mechanically. Separation on a 12% SDS-polyacrylamide gel was performed using standard procedures.

After electrophoresis, the gels were transferred to nylon membranes using the semi-dry transfer technique. For detection of Cx, the membranes were probed with antibodies specific for Cx37, Cx40, or Cx43 (see Immunofluorescence above) and were visualized on an X-ray film using a secondary antibody labeled with horseradish peroxidase and the SuperSignal system (Pierce, Bonn, Germany). For documentation, the films were scanned with a video camera-based system (GelDoc, BioRad, Munich, Germany).

Statistical procedures

All data shown are mean ± SEM. The number of observations varies between the experimental conditions and is specified in the respective figure legends. Data were compared by ANOVA. Subsequently, the Student-Newman-Keul's procedure was chosen as a post-hoc test. Values were considered significantly different if the error probability P was <0.05.

RESULTS

Dye-transfer experiments reveal gap-junctional coupling between PMN and HUVECs

Gap-junctional coupling between PMN and HUVECs was quantified as transfer of the small fluorescent dye calcein from one cell type to the other, using a well-established flow cytometric method [11–13].

The dye transfer from HUVECs to PMN was time-dependent and required leukocyte adhesion to HUVECs: In loosely adherent PMN (detached by washing), calcein-positive cells amounted to 2.8 ± 0.1% after 10 min and 5.2 ± 0.8% after 60 min of coincubation with calcein-labeled HUVECs. In adherent PMN, the percentages were 28.6 ± 4.1 and 40.6 ± 3.2, respectively (**Fig. 2a**). To test whether the dye transfer was in fact dependent on the presence of functional gap junctions, several control experiments were performed. The peptide SRPTEKTVFTV, which broadly inhibits gap junctions [14], reduced dye transfer to ~50% of control. In contrast, the peptides SRPTEKNVFIV and SRPTEKTIFII, which selectively only block Cx40 or Cx43, respectively [15], had only marginal, inhibitory effects (Fig. 2b). The blocking agent glycyrrhetinic acid concentration dependently lowered the percentage of calcein-positive cells to ~69% and 35% of control (Fig. 2b).

When PMN were loaded with calcein, and dye transfer toward HUVECs was studied, 16 ± 1% of the endothelial cells were calcein-positive after 60 min of coincubation. The transfer of calcein in this direction was also dependent on previous adhesion, as partial blockade of PMN adhesion with an anti-CD18 antibody reduced the number of coupled cells to 8 ± 0.5%. Furthermore, the percentage of dye-positive HUVECs increased proportionally with the number of added PMN (data not shown).

As a further control of the specificity of dye transfer for detecting gap-junctional coupling, calcein transfer was also measured between PMN and HeLa cells, which do not express Cx in their WT status. After 60 min of coincubation, hardly any transfer from WT HeLa to PMN was observed (2.3±0.1%). All three HeLa transfectants when expressing selectively one single Cx showed significantly higher transfer rates (19.4±2.2%, 8.6±0.5%, and 8.3±0.6% for Cx43, Cx40, and Cx37 cells, respectively; **Fig. 3a**). These experiments show that the dye transfer observed is indeed dependent on the presence of Cx. When transfer from PMN to HeLa cells was investigated, similar results were obtained (2.2±0.9% for WT, 31.7±3.7%, 12.9±0.4%, and 10.8±0.8% for Cx43, Cx40, and Cx37 cells, respectively; Fig. 3b). To exclude clonal differences between the single transfectants, dye transfer was successfully blocked by the respective, specific inhibitory peptide (data not shown).

The cytokine TNF-α reduces gap-junctional coupling

To learn more about the potential, biological significance of communication via gap junctions, we studied whether dye

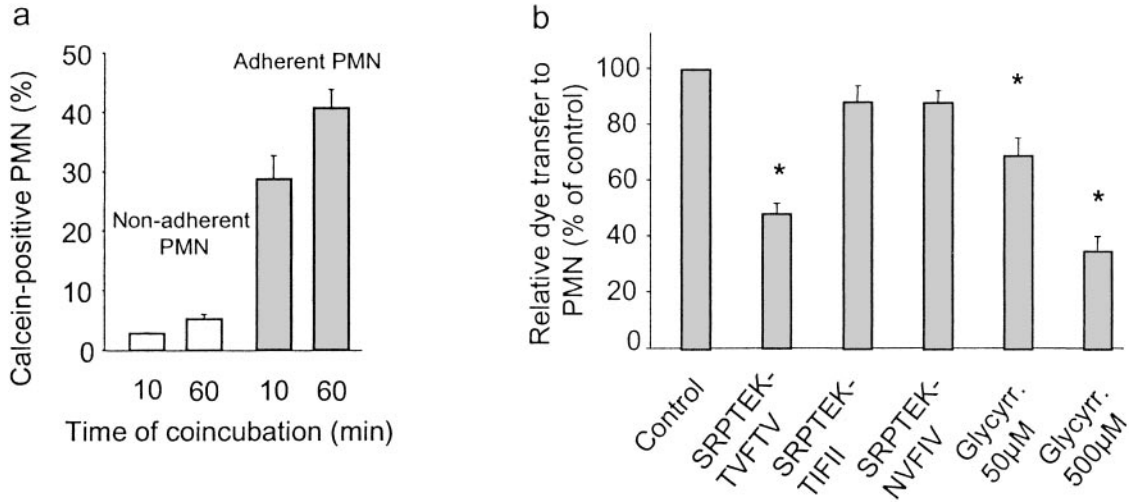

Fig. 2. Calcein is transferred from HUVECs to PMN via gap junctions. (a) The number of calcein-positive PMN increases with time and is higher in adherent cells (shaded bars, n=18) as compared with nonadherent PMN (open bars, n=12). Note that the biggest part of the effect is already reached after 10 min. (b) Dye transfer is reduced by the global gap-junction inhibitory peptide SRPTEKTVFTV and by different concentrations of glycyrrhetinic acid (Glycyrr.); n = 6 in all cases; *, $P \leq 0.05$ versus control. The inhibitory peptides selectively blocking only one type of Cx (SRPTEKTIFII and SRPTEKNVFIV) had only marginal effects.

coupling is influenced by different inflammatory mediators or leukocyte-stimulating agents. Dye transfer from HUVECs to PMN was not significantly altered by IFN-γ, LPS, thrombin, fMLP, or PMA (**Fig. 4a**), and TNF-α led to a dramatic decrease (16% of control; Fig. 4a). Dye transfer from PMN to HUVECs was also hardly influenced by LPS, IFN-γ, thrombin, fMLP, or PMA. However, TNF-α caused a reduction of dye-positive HUVECs to 60% of control (Fig. 4b). This regulation of coupling by an important cytokine suggests a role of this communication pathway during inflammation.

Leukocyte adhesion to HeLa cells, but not to HUVECs, is altered by coupling

To detect functional consequences of intercellular coupling, leukocyte adhesion to HUVECs and to HeLa cells was studied. As shown in **Figure 5a**, partial blockade of Cx with SRPTEKTVFTV did not alter adhesion of PMN to HUVECs under control conditions (110±10% vs. 100% control) or after stimulation with TNF-α (211±11% without vs. 211±11 with SRPTEKTVFTV) or PMA (226±32% without vs. 288±66% with SRPTEKTVFTV).

In contrast, the adhesion of PMN to HeLa cells after stimulation with PMA was different in the absence or presence of Cx (Fig. 5b). There was no response to TNF-α or PMA in WT cells (111% and 131%, respectively). In cells expressing Cx37, Cx40, or Cx43, there was also no significant response to TNF-α (142%, 141%, and 168%, respectively). In contrast, PMA elicited pronounced adhesion of PMN to Cx37, Cx40, and Cx43 HeLa cells (200%, 298%, and 319%, respectively). These results indicate that Cx do not play a role as adhesion ligand in cells, where classical adhesion molecules are present abundantly, but they may serve as accessory "adhesion molecules" in other cells.

Leukocyte transmigration is enhanced by low gap-junctional coupling

As a further, functionally important step of inflammation, transmigration of PMN through cell monolayers was measured in a transwell assay. Under control conditions, ~2800 PMN transmigrated through a HUVEC monolayer into the lower compartment of the chemotaxis chamber during the period of observation. Reduction of PMN/HUVEC coupling enhanced transmi-

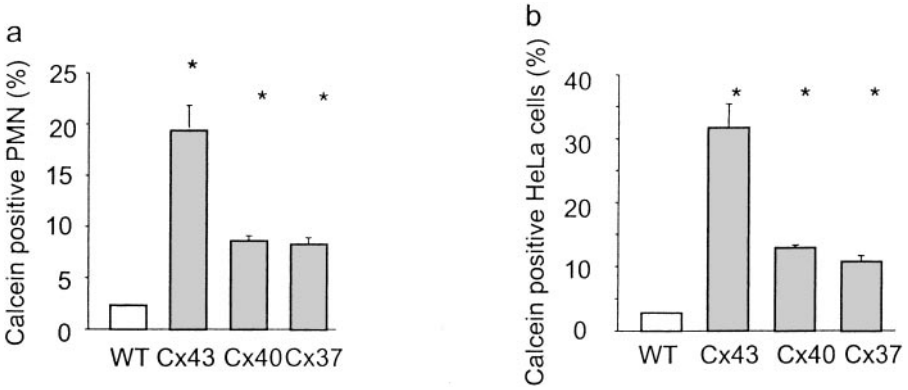

Fig. 3. Cx are necessary for dye transfer. Calcein is transferred from HeLa cells to PMN (a) and from PMN to HeLa cells (b) only in the presence of Cx. WT HeLa cells (no Cx) were compared with HeLa cells selectively expressing Cx43, Cx40, or Cx37; n = 12–24; *, $P \leq 0.05$ versus WT cells.

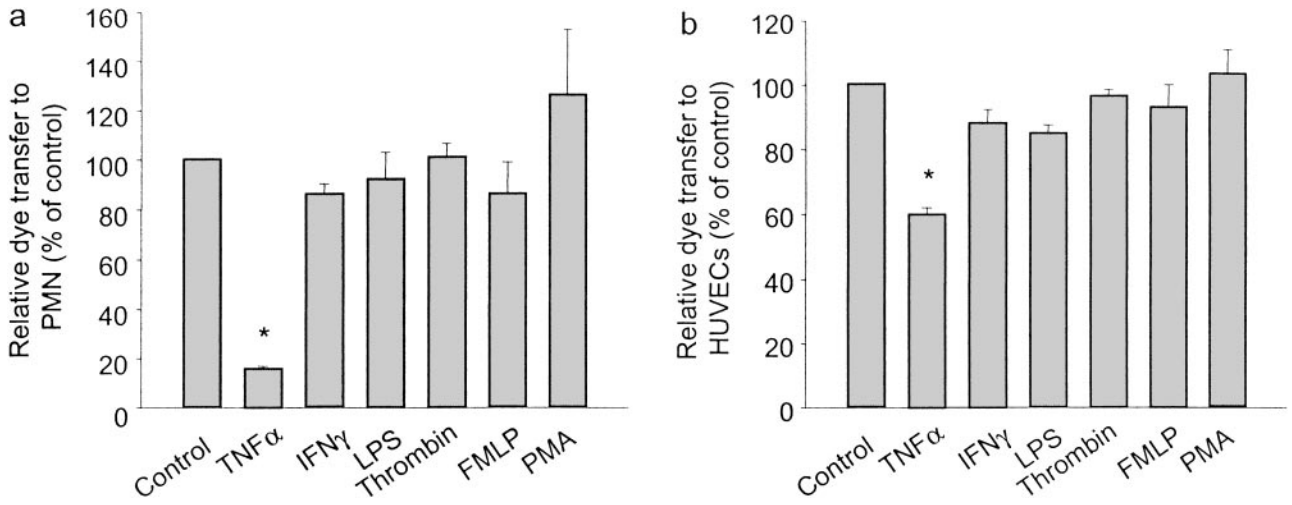

Fig. 4. TNF-α reduces dye transfer in both directions. Dye transfer toward PMN (a) is not altered by the endothelium activators IFN-γ, LPS, or thrombin, and TNF-α markedly reduces coupling. The PMN-activating agents fMLP and PMA have no effect. Dye transfer toward HUVECs (b) behaves in an analog way. *, $P < 0.05$ vs. control.

gration: First, when cellular coupling was partially blocked with SRPTEKTVFTV, the number of cells that had transmigrated increased to 3500, i.e., by ~21% (**Fig. 6**). Second, when no Cx were present in the monolayer (HeLa WT cells), transmigration was increased by 45% (2689±125 cells/well) as compared with the presence of Cx40 on HeLa cells (1492±113 cells/well; Fig. 6).

Cell monolayer permeability during transmigration increases in the absence of gap junctions

To test whether the increase in transmigration after SRPTEKTVFTV might be a result of disruption of interendothelial gap junctions and thus, an infringed endothelial barrier function, permeability of HUVEC monolayers was measured in the absence and presence of SRPTEKTVFTV. Thrombin, a permeability-increasing mediator, was used as positive control. Although thrombin readily elevated permeability, no differences were seen between controls and cells treated with SRPTEKTVFTV (**Fig. 7a**).

To investigate functional alterations in the monolayer as a result of the presence or absence of Cx, changes in permeability during transmigration were measured. In the absence of Cx (HeLa WT cells), permeability was time-dependently increased during transmigration of PMN (Fig. 7b). In contrast, in the presence of Cx40, permeability during transmigration was unchanged as compared with time-matched controls without PMN (Fig. 7b).

Functional influences of coupling on the leukocytes were examined by determining the sensitivity of coupled and uncoupled PMN for fMLP: The responses of the PMN were concentration-dependent and did not differ between leukocytes that had coupled to HUVECs and those that had remained uncoupled (data not shown). This would suggest that the

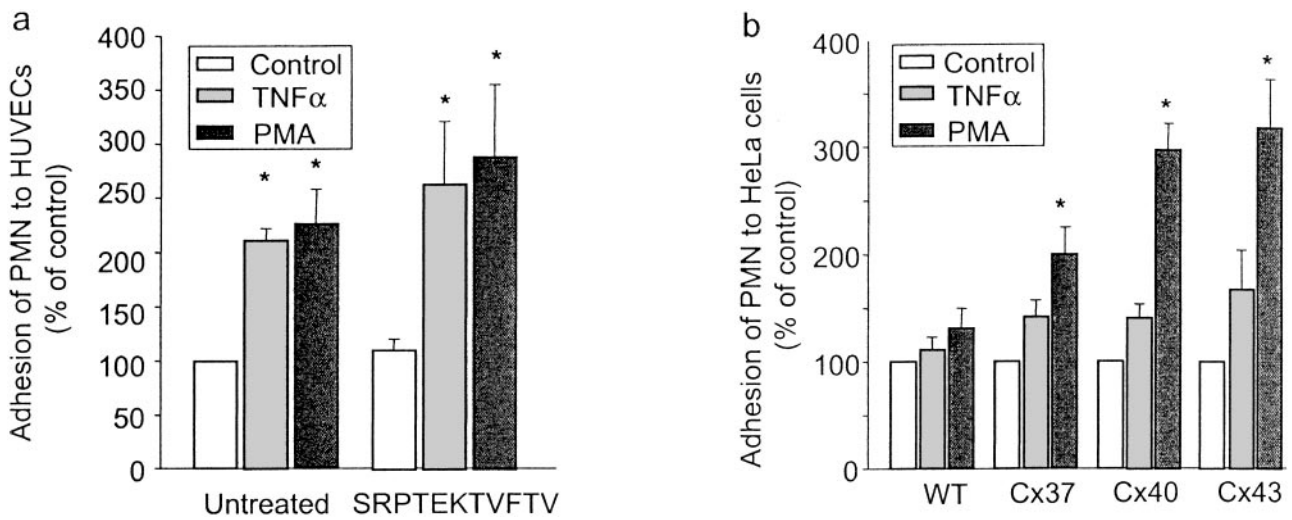

Fig. 5. Adhesion of PMN to HUVECs is independent of Cx. (a) TNF-α and PMA increased adhesion of PMN in the absence and presence of the gap-junction inhibitory peptide SRPTEKTVFTV; n = 12; *, $P \leq 0.05$ versus the respective control. (b) Adhesion of PMN to HeLa cells is increased by TNF-α and PMA. The response to PMA is higher in HeLa cells expressing any of the three Cx, Cx37, Cx40, or Cx43, as compared with WT cells; n = 12; *, $P \leq 0.05$ versus the respective value in WT cells.

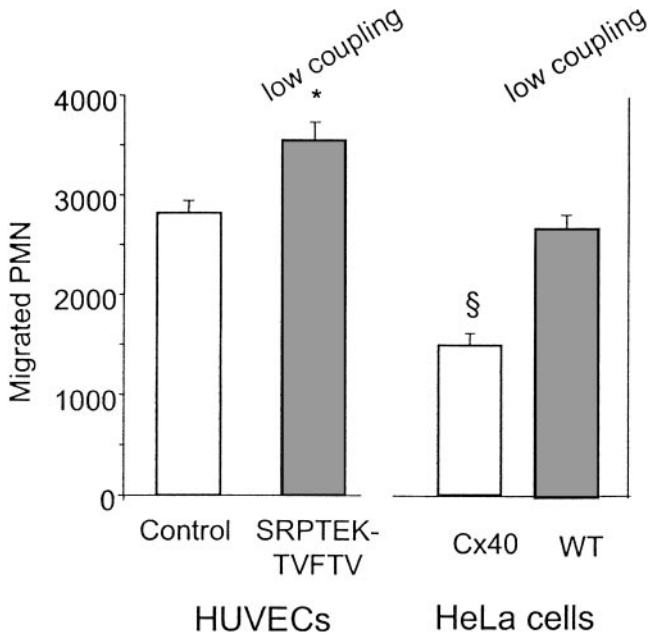

Fig. 6. Migration of PMN is modulated by the presence of functional Cx. Migration of PMN through monolayers of HUVECs along a chemotactic gradient of fMLP increases after inhibition of gap-junctional coupling with SRPTEKTVFTV. Migration through a monolayer of HeLa cells is higher in the absence of Cx (WT cells); n = 6; *, $P \leq 0.05$ versus control; §, $P \leq 0.05$ versus WT cells.

changes observed during transmigration would be mainly a result of an altered behavior of the cell monolayer.

Immunofluorescence and Western blotting detect the presence of various Cx on PMN

To investigate whether resting PMN express Cx on their surface, we stained PMN with antibodies against Cx37, Cx40, and Cx43. Cx37 was mainly localized in pseudopodia of PMN, and Cx40 and Cx43 have shown a more granular organization (**Fig. 8a**). The specificity of our staining was tested by using the same antibodies for Western blotting. All three antibodies recognized proteins of the appropriate molecular weight in whole-cell lysates of PMN (Fig. 8b). Western blots of membrane preparations of PMN revealed that a part of the cellular content of any Cx analyzed was present in cell membranes (Fig. 8b) of resting cells.

DISCUSSION

The close apposition of adherent leukocytes and the endothelium [4] and the discovery of Cx on endothelial cells [2, 3] and on leukocytes [4, 19–23] raise the possibility that in addition to paracrine and outside-in signaling, gap-junctional communication might play a role during inflammatory events. We provide evidence for the hypothesis that PMN and endothelial cells can directly communicate via gap junctions and that this coupling has functional consequences.

Cell-cell coupling

As an experimental human model, cultured HUVECs and freshly isolated human PMN were used. HUVECs have been demonstrated to possess the same Cx that are expressed on endothelial cells in vivo [2, 3]. PMN have been studied, as they play an important role in a number of inflammatory disorders, such as reperfusion injury [24] or rheumatoid arthritis [25]. A flow cytometry-based dye-transfer technique was used as a quantitative measure for cell-cell coupling. The validity and specificity of this approach have been demonstrated before in a number of different cell models [11–13]. As PMN have a considerable phagocytic potential, the critical question of whether detection of dye in the PMN after coincubation with calcein-loaded HUVECs could really be attributed to transfer via gap junctions was addressed: Even after a prolonged period

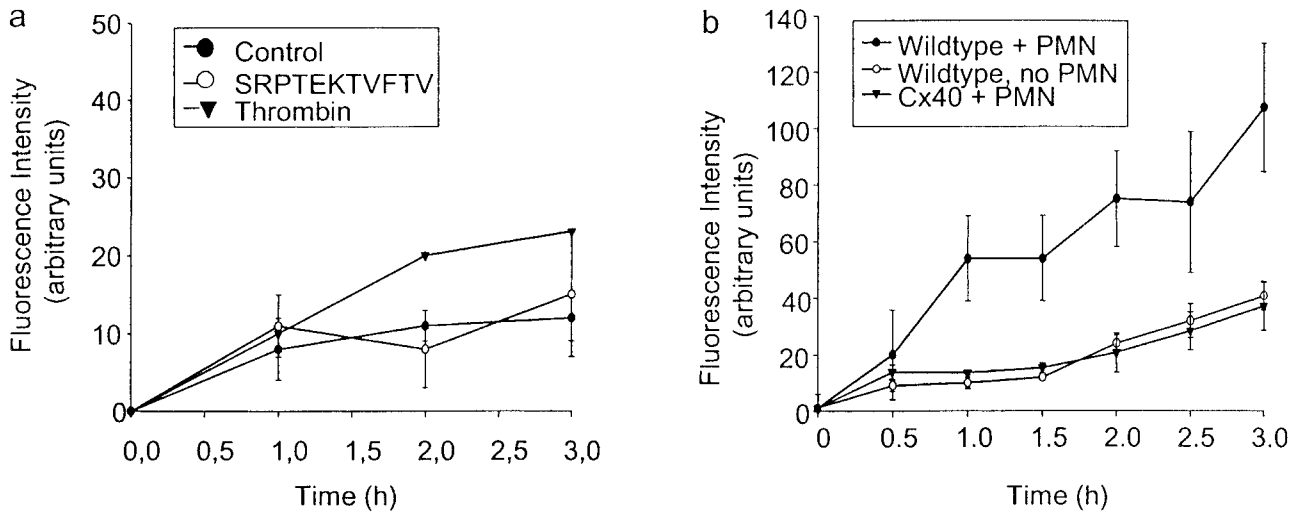

Fig. 7. PMN increase permeability during migration in the absence of Cx. (a) Permeability of HUVEC monolayers increases after application of thrombin, a well-known inducer of vascular permeability. Inhibition of gap junctions with SRPTEKTVFTV has no such effect; n = 6 in all cases. (b) Permeability of HeLa cell monolayers increases during transmigration of PMN in the absence of Cx (WT). In the presence of Cx40, this effect is much less pronounced. Data for Cx40 cells in the absence of PMN are identical to that with PMN and are thus omitted for graphical reasons; n = 6 in all cases.

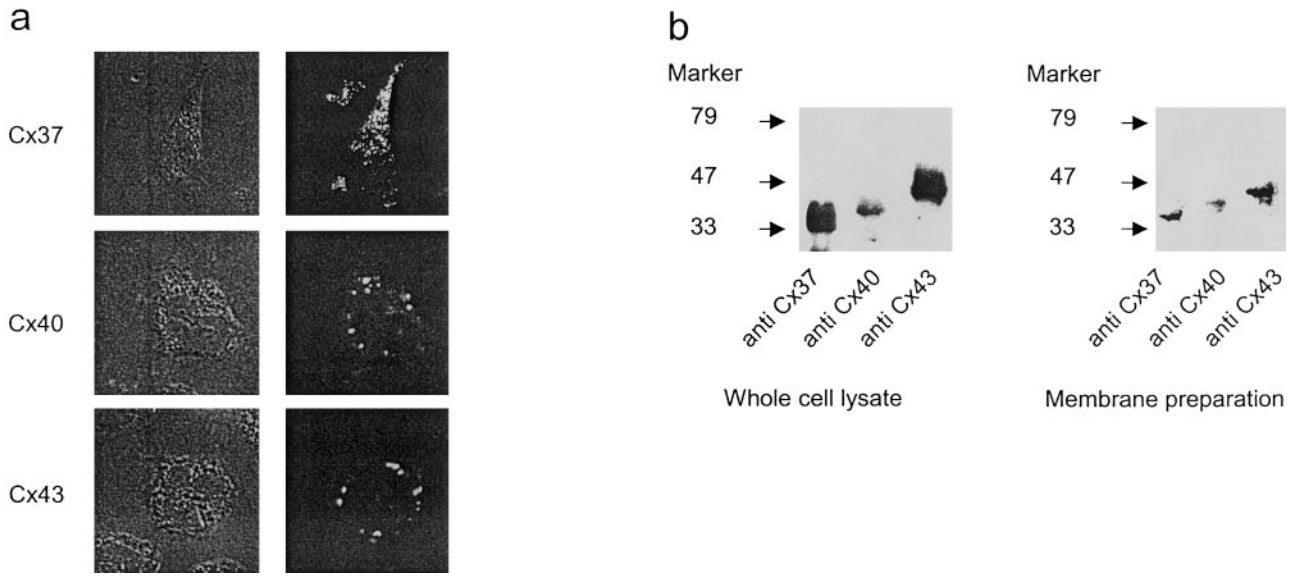

Fig. 8. Immunofluorescence staining and Western blotting of PMN for different Cx. (a) From top to bottom: staining with an anti-Cx37 antibody, staining with an anti-Cx40 antibody, and staining with anti-Cx43 antibody. Left column, phase contrast; right column, fluorescence. Brightness and contrast of the fluorescence images were adjusted in a way that the negative controls showed a black image. (b) On the left panel, Western blots of whole-cell lysates of PMN are shown for Cx37, Cx40, and Cx43. On the right panel, the same is depicted for membrane preparations of PMN.

of time, the transferred calcein was diffusely distributed in the PMN, which argues against phagocytotic uptake (data not shown). In addition, the de-esterified form of calcein (which would be released by dead or leaky HUVECs) was not taken up by PMN even when exposed for 2 h to 10 times higher concentrations of the dye than during the usual loading procedure (data not shown). Thus, it is highly unlikely that calcein was taken up by PMN in ways other than via gap junctions. Furthermore, direct injection of calcein into adherent PMN led to dye transfer to the respective endothelial cell, and injection into HUVECS caused dye transfer into adherent PMN (data not shown). The inhibitory peptide SRPTEKTVFTV [14] as well as the junction blocker glycyrrhitinic acid [15] reduced the dye transfer from HUVECs to PMN (Fig. 2b). To test the specificity of the inhibitory peptide, two other peptides, which only block one single type of Cx, were applied: SRPTEKNVFIV for Cx40 and SRPTEKTIFII for Cx43 [15]. Indeed, both peptides caused only weak inhibition of dye transfer (Fig. 2b), probably because the dye was able to pass through the respective, nonblocked Cx. Both inhibitory principles (peptides and glycyrrhitinic acid) typically do not afford complete inhibition of coupling in nontoxic concentrations [14, 15]. Other agents that are inhibitory (e.g., carbenoxolone, octanol, heptanol) are also not very selective and have to be used in high (millimolar) concentrations. As halothane, a gap junction inhibiting volatile anesthetic [26], also inhibits several functions of PMN [27], it was also inappropriate as a tool in the present study. Therefore, to circumvent these problems, HeLa cells were used that expressed no (WT) or only one single type of Cx. Indeed, virtually no dye transfer was detected between PMN and WT cells (Fig. 3a), and in all HeLa cells transfected with one of the Cx that occurs in endothelial cells (Cx37, Cx40, or Cx43), respectively, dye was transferred into PMN or vice versa (Fig. 3b). Thus, dye transfer in our model is indeed dependent on the presence of functionally intact Cx.

According to the literature, not every type of Cx can couple to any other. For example, Cx43 has been demonstrated to be hardly able to interact with Cx40 [9], as we also have observed. Thus, as PMN were able to couple to HeLa cells expressing selectively Cx40, it is highly probable that PMN at least express one more type of Cx. Taking into consideration that significant levels of Cx43 were demonstrated on PMN only after hours of stimulation [4], and in the present work, coupling occurred within minutes, it lay close at hand that resting PMN express Cx other than Cx43. Indeed, we were able to show for the first time the presence of Cx37 and Cx40 in unstimulated PMN (Fig. 8, a and b). Furthermore, analysis of membrane preparations of PMN by Western blotting revealed that part of the Cx expressed by PMN is incorporated in the membrane, even under resting conditions. Thus, even unstimulated PMN are able to couple to endothelial cells as soon as they have contact of sufficient intensity and duration. Consequently, it has to be expected that preformed Cx as whole, functional hemi-channels are present in the membrane of resting PMN, as had recently been demonstrated for a different cell type [28]. The functionality and the biological role of these hemi-channels in suspended, freely flowing PMN can only be speculated about at the moment. A further point that is unclear is the place where and the mode how PMN form gap junctions with endothelial cells. Although speculative, it is most probable that coupling occurs at the interendothelial contacts, which are a predilective site of leukocyte adhesion. This hypothesis is supported by confocal microscopy, where coupled PMN are found far distal from the nuclear region of the endothelial cell (Fig. 1b).

To investigate basal coupling between PMN and endothelial cells, the initial experiments were performed without further stimuli, which might be an analogy to the physiologic margination of resting PMN in vessels. However, if the coupling between PMN and endothelial cells serves a biological function

during inflammatory processes, it has to be postulated that this action is modulated by inflammatory stimuli. Others have already shown that expression of Cx is tightly regulated by inflammatory mediators [6]. Especially TNF-α has been demonstrated to reduce gap-junctional communication between endothelial cells [3]. In our study, the inflammatory mediators IFN-γ, or thrombin had no effect on dye transfer in both directions (from PMN to HUVECs and vice versa). In contrast, TNF-α efficiently reduced calcein transfer from PMN to HUVECs and to an even greater extent, from HUVECs to PMN (Fig. 4). Thus, coupling between PMN and HUVECs is selectively modulated during an inflammatory reaction, suggesting that this process is of physiological significance.

Leukocyte adhesion

It is interesting that dye transfer was bidirectional, which implies that small, molecular messengers might also pass in both directions. Dye transfer in both directions was facilitated by PMN adhesion: Only loosely adherent PMN were coupled to a lesser degree than firmly adherent ones (Fig. 2a), and coupling was reduced by an adhesion-blocking antibody (anti-CD18). Given that we worked in a static model, where adhesion is no prerequisite for cell-cell contact (as opposed to the in vivo situation), this implies that Cx and adhesion molecules in PMN and/or HUVECs might be functionally connected as has been shown for epidermal cells [29]. Further evidence for this hypothesis is the colocalization of Cx [3] and adhesion molecules [1] along the endothelial cell-cell contacts.

In our study, adhesion of PMN to HUVECs was increased by activation of endothelial cells (via TNF-α) as well as by simultaneous activation of PMN and HUVECs (via PMA). However, Cx seem to play no role for adhesion between these cells that are equipped with abundant adhesion molecules, as reduction of coupling with SRPTEKTVFTV did not influence basal or stimulated adhesion (Fig. 5a). In comparison to HUVECs, HeLa cells showed low adhesivity for PMN under basal conditions (about one order of magnitude less). In these HeLa cells where adhesion molecules seem to be of lower importance than in HUVECs, adhesion of PMN increased in the presence of Cx. Although TNF-α had no effect, adhesion after stimulation of PMN with PMA was increased with Cx37, Cx40, and Cx43 cells as compared with WT HeLa cells (Fig. 5b). Thus, in cells lacking efficient adhesion molecules, Cx might afford a certain potential for leukocyte recruitment. A similar role of Cx has recently been shown with respect to adhesion of malignant glioma cells [30].

Transmigration of PMN and permeability

Whenever coupling of PMN was reduced, the rate of transmigration increased (Fig. 6). This conclusion can be drawn from two different sets of experiments: When coupling of PMN to HUVECs was decreased with SRPTEKTVFTV, the number of transmigrated PMN rose. Conversely, an increase in coupling in Cx40 HeLa cells as compared with WT HeLa cells (low coupling) resulted in a reduction of transmigration by 50%. The lower level of PMN migration through HeLa cells than through HUVECs might be attributable to a lack in HeLa cells of adhesion molecules that are needed for outside-in signaling during migration. It seems unlikely that an inhibitory signal is passed from the HUVECs to the PMN, as reactivity upon activation of PMN that had coupled to HUVECs was identical to that of PMN that had not coupled (data not shown).

The permeability for a fluorescence-labeled dextran did not differ between untreated HUVEC monolayers and endothelial cells, which had been incubated with the inhibitory peptide SRPTEKTVFTV (Fig. 7a). The basal permeability also was identical in WT and Cx40 cells (data not shown). This is not surprising, as the barrier function depends on tight junctions and not on gap junctions. Thus, differences in the degree of coupling between the cells in the monolayer are no likely cause for changes of PMN transmigration. However, during transmigration of PMN, permeability considerably increased in WT cells, and in Cx40 cells, there was no change as compared with baseline permeability (Fig. 7b), although some degree of transmigration occurred. Thus, the speculation lies close at hand that HeLa cells that are able to couple to PMN show a lower formation of intercellular clefts than WT HeLa cells during transmigration of PMN. Transmigration and barrier function are not related on a linear basis.

From the data presented here, we conclude that PMN communicate with endothelial cells via gap junctions following adhesion. Adhesion of PMN does not automatically lead to transmigration, which is thus likely to be controlled separately. According to our results, the degree of coupling between PMN and endothelial cells could be involved in this control process: PMN, well-coupled to unstimulated endothelium, migrate to a lesser extent than uncoupled PMN. Furthermore, the migration process seems to be qualitatively different: Although transmigration of coupled PMN hardly causes any leak formation, uncoupled PMN significantly increase permeability during transmigration. The reduction of the expression of Cx and of their conductivity [3, 6] by inflammatory parameters would then represent a signal that facilitates transmigration. Thus, on the one hand, TNF-α would increase adhesion, and conversely, it would decrease leukocyte-endothelial coupling, thereby facilitating and controlling emigration of PMN into the desired tissue in a dual way. The concept that inflammatory events are modulated by gap-junctional coupling between leukocytes and the endothelium is also appealing with respect to some pathophysiological states of the vasculature. For example, it has been shown that interendothelial communication via Cx43 is impaired under conditions of disturbed flow [31]. This could render the affected endothelium more susceptive to leukocyte infiltration. The differences between the behavior of "coupled" and "uncoupled" PMN could be one of the features that distinguishes "physiological" extravasation (without increased permeability) from the "pathological" transmigration during acute inflammation, which is accompanied by formation of edema. Gap-junctional coupling between leukocytes and endothelial cells might be a broadly used and excitingly novel way of intercellular communication, as several recent papers have demonstrated coupling of lymphocytes to endothelium [22] and some functional consequences, e.g., for angiogenesis [23].

In conclusion, we have demonstrated for the first time that resting, human PMN express Cx37 and Cx40 on their surface and couple to endothelial cells via gap junctions in a rapid,

bi-directional, and adhesion-dependent manner. A central finding of our study is that a lack of gap-junctional coupling of PMN increased transmigration. This interaction modulates inflammatory reactions in addition to previously known ways of communication such as chemokine or integrin signaling. Future studies have to elucidate the active principle that is passed between PMN and endothelial cells via gap junctions and that coordinates the actions of these two cell types.

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REFERENCES

1. Ebnet, K., Vestweber, D. (1999) Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines. *Histochem. Cell Biol.* **112,** 1–23.
2. van Kempen, M. J., Jongsma, H. J. (1999) Distribution of connexin37, connexin40 and connexin43 in the aorta and coronary artery of several mammals. *Histochem. Cell Biol.* **112,** 479–486.
3. van Rijen, H. V., van Kempen, M. J., Postma, S., Jongsma, H. J. (1998) Tumour necrosis factor alpha alters the expression of connexin43, connexin40, and connexin37 in human umbilical vein endothelial cells. *Cytokine* **10,** 258–264.
4. Jara, P. I., Boric, M. P., Saez, J. C. (1995) Leukocytes express connexin 43 after activation with lipopolysaccharide and appear to form gap junctions with endothelial cells after ischemia-reperfusion. *Proc. Natl. Acad. Sci. USA* **92,** 7011–7015.
5. Hillis, G. S., Duthie, L. A., Brown, P. A., Simpson, J. G., MacLeod, A. M., Haites, N. E. (1997) Upregulation and co-localization of connexin43 and cellular adhesion molecules in inflammatory renal disease. *J. Pathol.* **182,** 373–379.
6. Hu, J., Cotgreave, I. A. (1997) Differential regulation of gap junctions by proinflammatory mediators in vitro. *J. Clin. Invest.* **99,** 2312–2316.
7. Polacek, D., Lal, R., Volin, M. V., Davies, P. F. (1993) Gap junctional communication between vascular cells. Induction of connexin43 messenger RNA in macrophage foam cells of atherosclerotic lesions. *Am. J. Pathol.* **142,** 593–606.
8. Jaffe, E. A., Nachman, R. L., Becker, C. G., Minick, C. R. (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52,** 2745–2756.
9. Elfgang, C., Eckert, R., Lichtenberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hulser, D. F., Willecke, K. (1995) Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J. Cell Biol.* **129,** 805–817.
10. Zahler, S., Kowalski, C., Brosig, A., Kupatt, C., Becker, B. F., Gerlach, E. (1997) The function of neutrophils isolated by a magnetic antibody cell separation technique is not altered in comparison to a density gradient centrifugation method. *J. Immunol. Methods* **200,** 173–179.
11. Guinan, E. C., Smith, B. R., Davies, P. F., Pober, J. S. (1988) Cytoplasmic transfer between endothelium and lymphocytes: quantitation by flow cytometry. *Am. J. Pathol.* **132,** 406–409.
12. Kiang, D. T., Kollander, R., Lin, H. H., LaVilla, S., Atkinson, M. M. (1994) Measurement of gap junctional communication by fluorescence activated cell sorting. *In Vitro Cell. Dev. Biol. Anim.* **30A,** 796–802.
13. Czyz, J., Irmer, U., Schulz, G., Mindermann, A., Hulser, D. F. (2000) Gap-junctional coupling measured by flow cytometry. *Exp. Cell Res.* **255,** 40–46.
14. Warner, A., Clements, D. K., Parikh, S., Evans, W. H., DeHaan, R. L. (1995) Specific motifs in the external loops of connexin proteins can determine gap junction formation between chick heart myocytes. *J. Physiol.* **488,** 721–728.
15. Chaytor, A. T., Martin, P. E., Evans, W. H., Randall, M. D., Griffith, T. M. (1999) The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication. *J. Physiol.* **520,** 539–550.
16. Zahler, S., Kupatt, C., Mobert, J., Becker, B. F., Gerlach, E. (1997) Effects of ACE-inhibition on redox status and expression of P-selectin of endothelial cells subjected to oxidative stress. *J. Mol. Cell. Cardiol.* **29,** 2953–2960.
17. Keller, H. U., Borel, J. F., Wilkinson, P. C., Hess, M. W., Cottier, H. (1972) Re-assessment of Boyden's technique for measuring chemotaxis. *J. Immunol. Methods* **1,** 165–168.
18. Kuijpers, T. W., Tool, A. T., van der Schoot, C. E., Ginsel, L. A., Onderwater, J. J., Roos, D., Verhoeven, A. J. (1991) Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* **78,** 1105–1111.
19. Vliagoftis, H., Hutson, A. M., Mahmudi-Azer, S., Kim, H., Rumsaeng, V., Oh, C. K., Moqbel, R., Metcalfe, D. D. (1999) Mast cells express connexins on their cytoplasmic membrane. *J. Allergy Clin. Immunol.* **103,** 656–662.
20. Martin, C. A., el Sabban, M. E., Zhao, L., Burakoff, R., Homaidan, F. R. (1998) Adhesion and cytosolic dye transfer between macrophages and intestinal epithelial cells. *Cell Adhes. Commun.* **5,** 83–95.
21. Oviedo-Orta, E., Hoy, T., Evans, W. H. (2000) Intercellular communication in the immune system: differential expression of connexin40 and 43, and perturbation of gap junction channel functions in peripheral blood and tonsil human lymphocyte subpopulations. *Immunology* **99,** 578–590.
22. Oviedo-Orta, E., Errington, R. J., Evans, W. H. (2002) Gap junction intercellular communication during lymphocyte transendothelial migration. *Cell Biol. Int.* **26,** 253–263.
23. el Sabban, M. E., Merhi, R. A., Haidar, H. A., Arnulf, B., Khoury, H., Basbous, J., Nijmeh, J., de The, H., Hermine, O., Bazarbachi, A. (2002) Human T-cell lymphotropic virus type 1-transformed cells induce angiogenesis and establish functional gap junctions with endothelial cells. *Blood* **99,** 3383–3389.
24. Jordan, J. E., Zhao, Z. Q., Vinten-Johansen, J. (1999) The role of neutrophils in myocardial ischemia-reperfusion injury. *Cardiovasc. Res.* **43,** 860–878.
25. Pillinger, M. H., Abramson, S. B. (1995) The neutrophil in rheumatoid arthritis. *Rheum. Dis. Clin. North Am.* **21,** 691–714.
26. He, D. S., Burt, J. M. (2000) Mechanism and selectivity of the effects of halothane on gap junction channel function. *Circ. Res.* **86,** E104–E109.
27. Mobert, J., Zahler, S., Becker, B. F., Conzen, P. F. (1999) Inhibition of neutrophil activation by volatile anesthetics decreases adhesion to cultured human endothelial cells. *Anesthesiology* **90,** 1372–1381.
28. Bruzzone, S., Guida, L., Zocchi, E., Franco, L., De Flora, A. (2001) Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells. *FASEB J.* **15,** 10–12.
29. Jongen, W. M., Fitzgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi, M., Yamasaki, H. (1991) Regulation of connexin 43-mediated gap junctional intercellular communication by Ca2+ in mouse epidermal cells is controlled by E- cadherin. *J. Cell Biol.* **114,** 545–555.
30. Lin, J. H., Takano, T., Cotrina, M. L., Arcuino, G., Kang, J., Liu, S., Gao, Q., Jiang, L., Li, F., Lichtenberg-Frate, H., Haubrich, S., Willecke, K., Goldman, S. A., Nedergaard, M. (2002) Connexin 43 enhances the adhesivity and mediates the invasion of malignant glioma cells. *J. Neurosci.* **22,** 4302–4311.
31. DePaola, N., Davies, P. F., Pritchard, W. F., Florez, L., Harbeck, N., Polacek, D. C. (1999) Spatial and temporal regulation of gap junction connexin43 in vascular endothelial cells exposed to controlled disturbed flows in vitro. *Proc. Natl. Acad. Sci. USA* **96,** 3154–3159.