The Junctional Adhesion Molecule-C Promotes Neutrophil Transendothelial Migration *in Vitro* and *in Vivo*

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The third member of the family of junctional adhesion molecules (JAMs), JAM-3, also called JAM-C, was recently shown to be a novel counter-receptor on platelets for the leukocyte β2-integrin Mac-1 (αMβ2, CD11b/CD18). Here, new functional aspects of the role of endothelial cell JAM-C were investigated. Endothelial cells express JAM-C, which is predominately localized within junctions at interendothelial contacts, since it codistributes with a tight junction component, zonula occludens-1. Whereas JAM-C does not participate in neutrophil adhesion to endothelial cells, it mediates neutrophil transmigration in a Mac-1-dependent manner. In particular, inhibition of JAM-C significantly reduced neutrophil transendothelial migration, and the combination of JAM-C and platelet/endothelial cell adhesion molecule-1 blockade almost completely abolished neutrophil transendothelial migration *in vitro*. In *vivo*, inhibition of JAM-C with soluble mouse JAM-C resulted in a 50% reduction of neutrophil emigration in the mouse model of acute thioglycollate-induced peritonitis. Thus, JAM-C participates in neutrophil transmigration and thereby provides a novel molecular target for antagonizing interactions between vascular cells that promote inflammatory vascular pathologies.

The recruitment of leukocytes is an integral part of inflammatory processes or vascular remodeling as well as during the immediate response toward bacterial infection or injury. Leukocyte recruitment requires multistep adhesive and signaling events including selectin-mediated rolling, leukocyte activation, and integrin-mediated firm adhesion and diapedesis (1). During firm adhesion of leukocytes to the endothelium, members of the β2-integrin family, LFA-1 (αLβ2, CD11a/CD18), Mac-1 (αMβ2, CD11b/CD18), and p150,95 (αXβ2, CD11c/CD18), as well as β2-integrins on the leukocyte surface interact with endothelial counterligands such as ICAM-1, surface-associated fibrinogen, or vascular cell adhesion molecule-1 (2–4). In contrast to the first three steps (i.e. rolling, activation, and adhesion of leukocytes), the molecular mechanisms of leukocyte trans-endothelial migration are far from being understood. Although leukocytes can traverse the endothelial cell layer by transcytosis (5), the major route for a leukocyte to traverse the endothelial barrier is through the cleft between two or three adjacent cells. Several molecular interactions involved in diapedesis have been described. (i) Platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD31), which is expressed diffusely on the surfaces of most leukocytes and at the borders of endothelial cells contacts (6, 7), can undergo homophilic interactions that are important in leukocyte diapedesis (8–10). (ii) CD99, which is expressed on all leukocytes as well as on the lateral interendothelial contacts (11), controls the final step in leukocyte transmigration, which is distal to the one controlled by PECAM-1 (12). Recently, much attention has been attributed to the role of junctional adhesion molecules (JAM) in leukocyte transmigration (13, 14).

JAMs belong to the Ig superfamily and have two extracellular Ig domains followed by a transmembrane and a cytoplasmic region, the latter involved in cytoskeletal or signal transduction interactions (14). The first member of this family, JAM-1 or JAM-A, was shown to participate in monocyte transmigration across endothelial cells, both by undergoing homophilic binding interactions (15) and with a heterophilic interaction with the β2-integrin LFA-1 (16). JAM-A is also proposed to modulate paracellular permeability (17), since it associates with the tight junction components cingulin, occludin, ZO-1, and AF-6 via their PDZ domains (14, 18, 19). JAM-2 or JAM-B interacts with the β1-integrin VLA-4 in T-cells (20) and probably participates in lymphocyte homing (21). We and others have recently identified JAM-3 or JAM-C, expressed in endothelial cells, platelets, T cells, and natural killer cells (22–24). Platelet JAM-C was shown to be a counter-receptor for neutrophil Mac-1, mediating neutrophil-platelet interaction under low shear (22). JAM-C was recently shown to participate in transendothelial migration of lymphocytes; however, this phenomenon could be ascribed to a homophilic interaction of JAM-C, because lymphocytes do not usually engage Mac-1 binding (25). Thus, it is yet unclear whether JAM-C mediates Mac-1-dependent transmigration of neutrophils. These diverse observations have prompted us to investigate the exact role of JAM-C in neutrophil transendothelial migration.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were provided from these sources: blocking monoclonal antibody (mAb) against human CD18, IB4 (Alexis, Grünberg, Germany); mAb L15 against LFA-1 (Dr. Y. van Kooyk, Amsterdam, The Netherlands); mAb LP519c against Mac-1 and mAb against ICAM-1 (DAKO, Hamburg, Germany); mAb 6S6 against β2-chain (CD29) (Chemicon, Hofheim, Germany); and rabbit anti-ZO-1 from Zymed Laboratories Inc. (Berlin, Germany). mAb Gi18 specific for...
PECAM-1 IgG domains 1 and 2 was characterized previously and inhibited homophilic interactions of PECAM-1 (26); mAb Gi11 against human JAM-C was described previously (22); respective isotype-
matched antibodies as well as preimmune IgG (Sigma, St. Louis, MO) were used as controls. DNA reaction for DAKO (Hamburg, Germany); mAb against αβ,-integrin as well as mouse fibronectin were from Chemicon. The blocking mAb against mouse Mac-1 (M1/70, mouse LFA-1 (M17/4), and mouse ICAM-1 (3E2), as well as respective isotype-matched control antibodies, which were used for in vivo inhibition studies, were from Pharmingen (Hamburg, Germany). MnCl2, human fibronectin, and monocyte chemotactant protein (MCP-1) were purchased from Sigma (St. Louis, MO).

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were cultured as previously described (27). Human neutrophils were isolated from peripheral blood as previously described (22).

Flow Cytometry Analysis—HUVEC were analyzed with mAb Gi11 by flow cytometry on FACSCalibur™ (Becton Dickinson) as previously described (22).

Western Blot Analysis—Western blot analysis for the detection of JAM-C was performed as previously described (22). HUVEC were lysed by adding sample buffer. After a 5-min incubation at 95 °C, 20 µl of the lysates were loaded onto SDS-PAGE, and subsequently proteins were transferred onto nitrocellulose membrane and incubated with mAb Gi11 (2.5 µg/ml). Bound IgG was detected using peroxidase-labeled rabbit anti mouse IgG (dilution 1:10,000; Dianova, Hamburg, Germany) and chemiluminescence substrate (Amersham Biosciences).

Nondenaturing Electrophoresis—For the analysis of JAM-C under nondenaturing conditions, JAM-C was loaded onto 10% gels, and polyacrylamide gel electrophoresis was performed in the absence of SDS and mercaptoethanol in all reagents. Proteins were identified by silver staining or by Western blot analysis as described above.

Immunofluorescence—HUVEC (20,000 cells/well) were plated onto 8-well chamber slides (Pernonax; Nunc, Roskilde, Denmark) that were precleaned with 0.2% gelatin and were allowed to adhere for 16 h at 37 °C. After extensive washing, the slides were fixed with ice-cold methanol acetone (1:1, v/v), blocked with 3% (w/v) bovine serum albumin, and incubated with mAb Gi11 (10 µg/ml) and rabbit polyclonal antibody against ZO-1 (5 µg/ml) for 2 h at 22 °C. Cells were washed, and then fluorescein-coupled anti-mouse IgG (Dianova, Hamburg, Germany) or rhodamine-coupled anti-rabbit IgG (Sigma). HUVEC were seeded on Transwell filters 2 days prior to the assay and grown without medium in the lower compartment for 48 h in a humidified atmosphere (37 °C, 5% CO2). For the beginning of the transmigration assay, 600 µl of the migration assay medium (serum-free RPMI in the absence or presence of 50 ng/ml MCP-1) was added to the lower compartment of the Transwell system. An aliquot of 100 µl of neutrophils (4 × 105) was added to the upper compartment on top of the endothelial monolayer. After incubation for 4 h at 37 °C, the number of transmigrated cells in the lower compartment was estimated with a cell counter (CASY-Counter; Scharfe-System, Reutlingen, Germany).

In Vivo Peritonitis Model—Thioglycollate-induced peritonitis in mice was performed as previously described (30, 33). For inhibition studies, the following compounds (total volume 300 µl) were administered intraperitoneally 30–45 min or intravenously 30 min prior to the injection of thioglycollate: various concentrations of soluble recombinant mouse JAM-C; 100 µg of mAb against mouse Mac-1, mouse LFA-1, or mouse ICAM-1 in PBS; 100 µg of mouse fibronectin in PBS; or combinations of the inhibitors. Control mice were treated with the same volume of PBS or isotype-matched control antibodies (100 µg). In order to evaluate neutrophil recruitment, mice were sacrificed at 4 h following injection. Peritoneal lavage fluid, the peritoneal lavage was collected, and the number of emigrated neutrophils was quantitated as previously described (30, 33). In some experiments, thioglycollate-elicted leukocytes were used for adhesion assays.

Statistical Analysis—Results are expressed as mean ± S.D. Comparisons between groups were analyzed by analysis of variance with Bonferroni adjustment (SPSS 11.5 software). p values of <0.05 were considered statistically significant.

RESULTS

JAM-C Is Expressed by Endothelial Cells and Localizes at Intercellular Junctions—We have previously shown that among various peripheral blood cells, JAM-C is expressed only on platelets (22). Here, the expression of JAM-C was tested on endothelial cells. Surface expression of JAM-C on HUVEC was analyzed by flow cytometry (Fig. 1A) and was confirmed by Western blot (Fig. 1B) and by analysis of JAM-C mRNA using RT-PCR (not shown).

In order to investigate the localization of JAM-C on endothelial cells, we performed immunostaining analysis. JAM-C was found to localize at cell-cell contacts similar to proteins that are associated with tight junctions, such as ZO-1. Double staining indicated that JAM-C and ZO-1 colocalize at interendothelial contacts (Fig. 2). Together, endothelial cells express JAM-C that localizes at interendothelial junctions.

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nor soluble JAM-C inhibited the migration of neutrophils through the bare filter (Fig. 4B). From these data, we conclude that soluble JAM-C interferes with Mac-1 at the stage of neutrophil transmigration across the endothelial cells but not at a later stage of neutrophil migration through the transwell filter. Moreover, the concentration of soluble JAM-C effective in inhibition of transendothelial migration of neutrophils was relatively low provided that JAM-C is present as a monovalent soluble adhesion molecule. We therefore tested whether our preparations of soluble JAM-C contained aggregated forms of the molecule. When soluble JAM-C was analyzed by nondenaturing gel electrophoresis, the majority of the protein migrated as multimers at a position between 80 and 90 kDa (Fig. 4C). From these data, we conclude that soluble JAM-C has the propensity to form multimers that provide the basis for the profound inhibitory effect at relatively low concentrations, comparable with the used monoclonal antibodies.

In order to test whether soluble JAM-C blocks neutrophil transendothelial migration due to interference with the interaction between Mac-1 on neutrophils and endothelial JAM-C, we tested adhesion of neutrophils to plate-immobilized JAM-C. Neutrophil adhesion to JAM-C was blocked by blocking mAb against Mac-1 but not by mAb against LFA-1. This is in accordance with our previous findings that Mac-1 but not LFA-1 interacts with JAM-C (22). Moreover, binding of neutrophils to plate-immobilized JAM-C was blocked by soluble JAM-C (Fig. 5). Taken together, these data suggest that the heterophilic interaction between Mac-1 and JAM-C may be involved in neutrophil transendothelial migration.

Participation of JAM-C in Neutrophil Recruitment in Acute Peritonitis in Mice—To test whether JAM-C participates in neutrophil recruitment in vivo, we studied its role in a mouse model of acute inflammation. Peritonitis was induced by thioglycollate injection, and after 4 h, there was the expected increase in the total leukocyte count in the peritoneum, mostly attributable to emigrated neutrophils. The portion of neutrophils among all leukocytes after 4 h was 50–60% as compared with 3–5% 1 h following stimulation (30, 33). Blocking of LFA-1, Mac-1, or ICAM-1 30 min prior to the induction of peritonitis with specific antibodies resulted in a significant 60–85% inhibition of neutrophil extravasation into the inflamed peritoneum at 4 h following thioglycollate injection (Fig. 6A). Interestingly, pretreatment with recombinant soluble mouse JAM-C also resulted in a significant inhibition of neutrophil extravasation (Fig. 6A), and this inhibitory effect was dose-dependent (Fig. 6B). Neutrophil emigration into the inflamed peritoneum was also inhibited by mAb to Mac-1 or to ICAM-1 as well as by soluble JAM-C when these reagents were administered intravenously 30 min prior to the induction of peritonitis (Fig. 6C). Interestingly, the combination of mAb against ICAM-1 together with recombinant soluble mouse JAM-C, administered either intraperitoneally or intravenously, resulted in a more profound blockade of neutrophil recruitment comparable with the inhibition obtained with mAb against Mac-1 (Fig. 6, A and C). A soluble protein control, mouse fibronectin, did not affect neutrophil emigration. Moreover, since murine soluble JAM-C contains a six-histidine tag, we have tested an irrelevant control protein containing a six-histidine tag that did not interfere with neutrophil emigration in the thioglycollate-induced peritonitis model, thereby underlining the specificity of the inhibitory effect of mouse soluble JAM-C (data not shown). Taken together, JAM-C mediates neutrophil recruitment in the acute inflammatory model of thioglycollate-induced peritonitis in vivo.

Finally, in order to demonstrate that mouse Mac-1 indeed interacts with mouse JAM-C, we tested the adhesion of thio-
glycollate-elicited leukocytes from mice to immobilized mouse JAM-C. Adhesion of mouse leukocytes to immobilized mouse JAM-C was blocked by the mAb against Mac-1 but not by the antibody against LFA-1. Moreover, soluble mouse JAM-C blocked the adhesion of mouse leukocytes to immobilized mouse JAM-C (Fig. 7). Thus, in mice a direct interaction between Mac-1 and JAM-C occurs.

DISCUSSION

Leukocyte activation and adhesion to the endothelium and the subsequent transendothelial migration are pivotal steps in the recruitment of cells to the inflamed tissue. This highly coordinated multistep process requires tight regulation of adhesive events, including the regulation of expression of adhesion receptors as well as the modification of the affinity and avidity of these adhesion receptors (1–4). In contrast to rolling and adhesion of leukocytes, the molecular mechanisms of leukocyte trans-endothelial migration remain incompletely understood. The recent observation that JAM-A, JAM-B, or JAM-C can independently function as counter-receptors for the leukocyte integrins LFA-1, VLA-4, or Mac-1, respectively, led us to test the hypothesis of whether endothelial cell surface adherent leukocytes interact via their \( \beta_2 \)- and \( \beta_2 \)-integrins with JAM family members at the most apical regions of the interendothelial junctions in order to pass through this cellular barrier (13).

The present work provides in vitro and for the first time in vivo evidence that the interaction between Mac-1 and JAM-C may indeed be involved in the leukocyte transendothelial migration process.

We have previously shown that JAM-C on platelets undergoes a heterophilic interaction with the leukocyte integrin Mac-1, thereby mediating leukocyte-platelet interactions under low shear stress (22). Here we characterized the participation of this binding in leukocyte-endothelial interactions. In particular, the following findings are consistent with a role of JAM-C in neutrophil-endothelial interactions. (i) In accordance with a previous report (25), JAM-C protein was found to be expressed on endothelial cells. Moreover, endothelial JAM-C was localized at cell-cell contacts, showing a similar pattern as proteins that are associated with tight junctions, such as ZO-1. This is in accordance with a recent report showing an association of JAM-C with ZO-1 in transfected cells (34). (ii) This junction-associated JAM-C cannot mediate adhesion of neutrophils to endothelial cells, probably because JAM-C is found in the interendothelial contacts and under quiescent conditions is not available for Mac-1 on the adhering neutrophil. In this case, ICAM-1 as a counter-receptor of both LFA-1 and Mac-1 predominately mediates neutrophil adhesion to the endothelium. (iii) In contrast, transendothelial migration was inhibited in the presence of soluble JAM-C that interfered with the
interaction between neutrophil Mac-1 and endothelial JAM-C. Moreover, the simultaneous blockade of PECAM-1 and JAM-C almost completely abolished transmigration, and the same phenomenon was also observed when PECAM-1 and Mac-1 were simultaneously blocked. Thus, an additive inhibitory effect of mAb against PECAM-1 with either soluble JAM-C or mAb against Mac-1 was observed. The fact that relatively low concentrations of soluble JAM-C had a profound inhibitory effect is most likely due to multimerization of soluble JAM-C, as evidenced by nondenaturing gel electrophoresis. (iv) In acute thioglycollate-induced peritonitis, recombinant soluble mouse JAM-C resulted in a significant inhibition of neutrophil extravasation. Strikingly, a combined inhibition of neutrophil adhesion and transmigration by using mAb against ICAM-1 and recombinant soluble mouse JAM-C, respectively, resulted in a more dramatic blockade of neutrophil recruitment comparable with the inhibition obtained with mAb against Mac-1.

In a recent report, Johnson-Le´ger et al. (25) demonstrated that JAM-C participates in lymphocyte transendothelial migration. However, this system is probably distinct from JAM-C-dependent neutrophil transendothelial migration described here. Several current observations lead to this conclusion. (i) Human lymphocytes express JAM-C, whereas neutrophils do not (22, 25). Thus, a homophilic interaction between leukocyte and endothelial JAM-C can only be relevant for the former cell type. (ii) Mac-1 plays a minor role in lymphocyte transmigration, as opposed to neutrophils. (iii) JAM-C has not been detected on any mouse leukocytes (25). Thus, our in vivo findings cannot be attributed to a homophilic interaction of JAM-C. Therefore, we propose a dual function for JAM-C in leukocyte transendothelial migration. The homotypic interaction between JAM-C on lymphocytes and endothelial cells, as has been suggested for PECAM-1 (11), may be important in the constitutive transmigration of recirculating lymphocytes across the high endothelial venules within lymph nodes or in high endothelial venule-like vessels, as the ones formed in nonlymphoid tissues during chronic inflammatory processes.
endothelium (11, 35). These hypotheses need further experimental proof in vivo. In particular, the precise expression and localization of JAM-C of different vascular endothelial beds as well as its regulation have to be determined, and studies engaging JAM-C-deficient mice will be instructive. In both scenarios, further potential processes, such as the homotypic interaction between JAM-C on apposing endothelial cells as well as the potential regulation of interendothelial permeability by JAM-C have to be considered. The former interaction might modify the structure of junctions, whereas JAM-C was previously reported to enhance paracellular permeability (36), and both processes may facilitate leukocyte transendothelial migration under pathophysiological conditions.

JAM-C was not found to mediate neutrophil adhesion to nonstimulated endothelial cells, which can probably be attributed to the fact that JAM-C localizes at interendothelial contacts of nonstimulated endothelial cells and is therefore not available for the adhering leukocyte. However, it cannot be excluded that upon endothelial stimulation (e.g. with proinflammatory cytokines), a redistribution of JAM-C might take place, thereby enabling the molecule to act as a leukocyte adhesion receptor due to its property of interacting with Mac-1. In fact, such a situation has been previously demonstrated for JAM-A. Upon treatment of endothelial cells with tumor necrosis factor-α and interferon-γ, JAM-A dissociates from the junctions and redistributes to the apical surface, making the molecule available for leukocyte LFA-1 and conferring upon the molecule a regulatory function for junction integrity (37).

In a recent paper, Schenkel et al. (38) identified a novel step in monocyte extravasation, the locomotion of monocytes to the endothelial junction following cell-cell adhesion and prior to transendothelial migration. According to this study, monocyte locomotion depends mainly on the β₂-integrins, whereas these integrins appear to play only a minor role during the diapedesis step. It is therefore intriguing to test whether the locomotion step is also relevant for neutrophils that engage a slightly different integrin repertoire than monocytes during their extravasation. If the locomotion step is indeed existent in neutrophils, then the function of JAM-C in neutrophil extravasation could be attributed to a role of the molecule during locomotion and/or during transmigration. As our present experimental system does not distinguish between these two processes, the role of JAM-C in locomotion versus transmigration needs to be addressed in future studies.

Our data suggest a further interendothelial molecule to be a
crucial player in leukocyte transmigration. Previously, JAM-A was shown to participate in this process both by undergoing a homophilic interaction and by binding to LFA-1 (15, 16). Moreover, PECAM-1 that is expressed diffusely on the surfaces of most leukocytes and is concentrated at interendothelial borders (39) has been implicated as a critical mediator of transendothelial migration (8, 11). Recently, PECAM-1 was found to recycle along interendothelial borders into segments of the junction, across which leukocytes are actively migrating (10). Whether a similar mechanism can also be envisioned for JAM family members needs to be investigated. In addition, CD99, which is found on all leukocytes as well as on the lateral interendothelial contacts (11), was shown to mediate the final step in leukocyte transmigration (12). Whether a cross-talk between these different steps of leukocyte transendothelial migration exists remains to be investigated. At least our present data, showing an additive effect of JAM-C and PECAM-1 blockade, indicate that these two mechanisms are distinct and independent of each other.

Finally, uncontrolled activation of leukocytes and/or endothelial cells is a feature of pathological chronic inflammation such as in atherosclerosis, rheumatoid arthritis, and other disorders. The present report demonstrates that JAM-C participates in such inflammatory reactions and in particular in neutrophil transendothelial migration in vitro and in vivo; thus, JAM-C may provide a novel target in inflammatory vascular pathologies. These concepts need to be addressed in future studies.

Note Added in Proof—In a very recent manuscript, Zen et al. (40) demonstrated that the interaction between JAM-C and Mac-1 is also operative in another system: JAM-C promotes Mac-1-dependent trans-epithelial migration in vitro.

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