The Homophilic Binding of Junctional Adhesion Molecule-C Mediates Tumor Cell-Endothelial Cell Interactions*

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The junctional adhesion molecule C (JAM-C) was recently shown to undergo a heterophilic interaction with the leukocyte β2 integrin Mac-1, thereby mediating interactions between vascular cells in inflammatory cell recruitment. Here, the homophilic interaction of JAM-C is presented and functionally characterized to mediate tumor cell-endothelial cell interactions. Recombinant soluble JAM-C in fluid phase bound to immobilized JAM-C as assessed in a purified system; moreover, JAM-C-transfected Chinese hamster ovary (CHO) cells adhered to immobilized JAM-C. The homophilic interaction of JAM-C was mediated by the isolated amino-terminal Ig domain (D1), but not the carboxyl-terminal Ig domain (D2), of the molecule. Dimerization of JAM-A is dependent on the sequence RVE in the amino-terminal Ig domain. This motif is conserved in JAM-C (Arg64-Ile65-Glu66), and a single amino acid mutation in this motif (E66R) abolished the homophilic interaction of JAM-C. The lung carcinoma cell line NCi-H522 was found to express JAM-C. NCi-H522 cells adhered to immobilized JAM-C, as well as to JAM-C-transfected CHO cells, but not to mock-transfected CHO cells or to CHO cells transfected with the JAM-C mutant (E66R). Adhesion of NCi-H522 cells to JAM-C protein or JAM-C-transfected CHO cells was abolished in the presence of soluble JAM-C or the isolated D1. Furthermore, the adhesion of NCi-H522 cells to endothelial cells was significantly blocked by soluble JAM-C or the isolated D1. Thus, JAM-C undergoes a homophilic interaction via the Arg64-Ile65-Glu66 motif on the membrane-distal Ig domain of the molecule. The homophilic interaction of JAM-C can mediate tumor cell-endothelial cell interactions and may thereby be involved in the process of tumor cell metastasis.

Junctional adhesion molecules (JAMs)2 belong to the Ig superfamily, consisting of two Ig-like domains: a membrane-distal V-type Ig domain and a membrane-proximal C2-type Ig domain, followed by a transmembrane and a cytoplasmic region (1–3). At their final carboxyl terminus all three molecules have a class-II PDZ domain binding motif, which predisposes them to interact with PDZ domain-containing molecules, such as the ones found in tight junctions (1, 2). A dual function has been suggested for JAMs that appear to regulate both leukocyte/platelet/endothelial cell interactions in the immune system and tight junction formation in epithelial and endothelial cells (2, 3). JAM-A, also referred to as JAM-1 or F11R (4, 5), is found on different circulating blood cells including platelets, monocytes, lymphocytes, and erythrocytes as well as on endothelial and epithelial cells. The expression pattern of JAM-B (also called VE-JAM, human JAM-2, mouse JAM-3) is more restricted, as it localizes on vascular and lymphatic endothelium and especially at high endothelial venules (6). JAM-C (also referred to as human JAM-3 and mouse JAM-2) is expressed on epithelial and endothelial cells and on platelets (7–10).

Recently, JAM-A, JAM-B, and JAM-C have been implicated in leukocyte-endothelial and leukocyte-platelet interactions through heterophilic binding in trans with the leukocyte integrins LFA-1, VLA-4, and Mac-1, respectively (9, 11, 12). JAM-A and JAM-B have been shown to undergo a homophilic binding mediating homotypic cell adhesion (13, 14), and especially for JAM-A the homophilic interaction has also been shown biochemically (15). The homophilic interaction of JAM-A is regulated by the dimerization of the molecule, which requires the RVE residues at positions 58–60 of the V₁₂ fold (16). In contrast, it is not clear yet whether a homophilic binding exists for JAM-C, although this is indicated by the subcellular localization of the molecule (7, 17). Recently, we have observed that recombinantly produced JAM-C tends to form multimers (18). However, direct evidence for cell-cell adhesion events mediated through a homophilic interaction of JAM-C has not been reported so far.

During blood-borne tumor cell metastasis the circulating tumor cells that have detached from the primary tumor interact with the vascular endothelium. This interaction is a crucial step preceding the invasion of the target organ by the tumor cells (19). Integrins, and especially β1 integrins, as well as other cell adhesion receptors play an important role in mediating interactions of several tumor cells, including non-small lung carcinoma and melanoma cells, with endothelial cells (20, 21). The process of tumor cell metastasis is thought to share many similarities with the multi-step process of leukocyte extravasation (22). Whether JAM-C or any other JAM family member may be expressed on tumor cells (especially of epithelial origin) and whether JAM-C may participate in tumor cell-endothelial cell interactions has not been studied so far.

These diverse observations have prompted us to investigate the homophilic interaction of JAM-C, to characterize this interaction biochemically, and to study its functional consequences in tumor cell-endothelial cell interactions. We identified JAM-C-expressing tumor cells and have demonstrated that the homophilic interaction of JAM-C mediates cell adhesive events between JAM-C-expressing tumor cells and the endothelium.

EXPERIMENTAL PROCEDURES

Materials—Recombinant soluble human JAM-C (sJAM-C) was produced as previously described (18). Fc-JAM-A, Fc-JAM-B, antibodies against human JAM-B, and tumor necrosis factor-α were from R & D...
Systems (Minneapolis, MN). Purified I domain of Mac-1 was provided by Dr. D. Tuckwell (School of Biological Sciences, Manchester, UK). Monoclonal antibody (mAb) Gi11 against human JAM-C and biotinylated Gi11 were described previously (9); respective isotype-matched control antibodies, as well as secondary antibodies, were from DAKO (Hamburg, Germany). Blocking mAb against CD18 was from Alexis (Gruenberg, Germany), and blocking mAb against CD29 was from Chemicon (Temecula, CA). All other reagents were from Sigma.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were from Cambrex (East Rutherford, NJ) and were cultivated as previously described (23). The lung adenocarcinoma cell lines (NCl-H522 and NCl-H322M) that both have mutant p53 function were provided from the Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health (Frederick, MD) and were cultivated in RPMI medium supplemented with 10% fetal calf serum (Invitrogen) (24).

Production of Soluble Recombinant JAM-C IgG Fusion Proteins—Fc fusion proteins of soluble recombinant JAM-C (Fc-JAM-C), the amino-terminal first Ig domain of JAM-C (Fc-D1), and the carboxy-terminal second Ig domain of JAM-C (Fc-D2) were produced in High-Five insect cells as previously described (25). The cDNAs of JAM-C encoding for the soluble JAM-C (nucleotides 128–766; Val32-Gly243, numbering according to NCBI/AF448478), D1 (nucleotides 128–457; residues Val32-Thr141), and D2 (nucleotides 428–766; residues Glu132-Glu243) were amplified from the full-length JAM-C in the mammalian expression vector pcDNA4 by PCR using Turbo Pfu polymerase (Stratagene). PCR products were inserted by blunt-end ligation into the EcoRV site of plgplus vector (R & D Systems) and were subcloned into the DH5α high efficiency competent Escherichia coli (Invitrogen). Plasmids from positive clones were then shuttled into pIB/V5-His Topo expression vector (TA expression kit; Invitrogen) by PCR of JAM-C plgplus constructs using TaqGold polymerase (Applied Biosystem) and plgplus-specific primers. All three JAM-C plB constructs were subcloned as described above. Plasmids from selective clones were purified using QiAprep (Qiagen) and verified by nucleotide sequencing on an ABI Prism Genetic Analyzer 3100 (Applied Biosystems) prior to transfection. Aliquots of 2 · 10⁶ High Five insect cells in 1 ml of Express Five SFM medium were transfected with 1 µg (or 5 µg) of plasmids using Cellfectin as recommended by the manufacturer (Invitrogen). After 3 days, stably transfected cells were selected in culture medium containing blasticidin (80 µg/ml; Invitrogen). Culture supernatants derived from stable cell lines were then analyzed by immunoblotting using mAb against V5 peptide (dilution 1:1000), horseradish peroxidase-labeled rabbit anti-mouse IgG (dilution 1:100,000), and chemiluminescence system (ECL; Amersham Biosciences). JAM-C fusion proteins from ~2 liters of culture supernatants were purified using Protein G column (Amersham Biosciences) and analyzed by silver staining. Purified JAM-C fusion proteins from the Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health (Frederick, MD) and were cultivated in RPMI medium supplemented with 10% fetal calf serum (Invitrogen) (24).

Stable Expression of JAM-C Wild-type (Glu66) and Mutant Isoforms (Arg66) and JAM-A in CHO Cells—Full-length mutant JAM-C in pcDNA4 encoding for Arg66 JAM-C isoform was produced from wild-type JAM-C (Glu66) pcDNA4 vector by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) as previously described (9). For the construction of primer pair encompassing nucleotides 212–250, two mismatches (GA -> AG) at positions 230 and 231, respectively, were introduced. After subcloning, constructs were validated by nucleotide sequencing analysis for subsequent transfection studies (see above). JAM-C expression vector was then transfected into the CHO cells by the use of Effectene (Invitrogen) as recommended by the manufacturer. Stable cell lines expressing wild-type and mutant JAM-C were selected with Zeocin (200 µg/ml; Invitrogen) and were analyzed by flow cytometry using mAb Gi11.

CHO cells were transfected with full-length JAM-A construct as previously described (9). Stably transfected cells were selected with Zeocin and subcloned for homogeneous JAM-A expression. Surface expression was measured by flow cytometry using mAb F11 specific for JAM-A (4) as described below.

Flow Cytometry—Surface expression of JAM-C on CHO cells was analyzed either with JAM-C or the mutant JAM-C isoform (Arg66), as well as on the non-small lung cancer cell lines NCI-H522 and NCI-H322M, which was analyzed with mAb Gi11 and fluorescein-labeled secondary antibody as previously described (9, 18). For the analysis of Fc-JAM-C binding to CHO cells, 40 µl cell suspensions (5 · 10⁶/ml) were incubated with 40 µl of JAM-C IgG fusion proteins (80 µg/ml) at room temperature for 1 h in U-bottom microtiter wells. After centrifugation at 1200 rpm for 90 s, cells were washed with 200 µl of phosphate-buffered saline supplemented with 10% fetal calf serum (washing buffer; Invitrogen) and stained with 100 µl of fluorescein-conjugated rabbit anti-human IgG at 4 °C for 30 min. Labeled cells were resuspended in 300 µl of washing buffer and analyzed by flow cytometry on a FACSCalibur™ (BD Biosciences).

In Vitro Binding Experiments—Binding of Fc-JAM-C to immobilized sJAM-C was performed according to a previously described protocol (15, 23). Microtiter wells were coated with 50 µl of sJAM-C (20 µg/ml; 1 µg/well) or bovine serum albumin (BSA) (50 µg/ml; Molecular Probes) in TBS at room temperature for 1 h and then blocked with 2% BSA in TBS. After washing twice with TBS, increasing concentrations of Fc-JAM-C were added to the wells in blocking buffer. After incubation for 60 min, peroxidase-conjugated anti-human IgG (1:3000; Dianova) was added and incubated for an additional 60 min, followed by addition of the substrate 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS)). Binding was quantified at 405 nm with a microplate reader (BIO-TEK, Winooski, VT). In other experiments Fc-JAM-C, Fc-D1, or Fc-D2 (each at 2 µg/ml) were added and allowed to react for 60 min. Microtiter wells then were washed three times. Bound fusion proteins were detected as described above, except that bound antibodies were measured using ortho-phenyldiamine (Dako Cytomation, Hamburg, Germany) as substrate in a microplate reader at 492 nm (TECAN). In all experiments, nonspecific binding to BSA-coated wells was used as blank and was subtracted to calculate specific binding.

BLACore Surface Plasmon Resonance Analysis—Real-time protein-protein interaction was examined using surface plasmon resonance on a BLACore 2000 (Biacore AB). Purified sJAM-C or the I domain of Mac-1 (each 50 µg/ml) was immobilized on a research-grade CM5 sensor chip via the amine coupling method as recommended by the manufacturer (Biacore) to the level of 560 and 1079 resonance units, respectively. A flow cell without sJAM-C or I domain was used as reference, the background signal from the reference being subtracted from every surface plasmon resonance data set. Various concentrations (10–100 µg/ml) of 30 µl of Fc-JAM-C, Fc-D1, Fc-D2, or Fc alone in surface plasmon resonance running buffer (20 mM Tris, 145 mM NaCl, pH 7.4) were injected serially using BLACore control software (KINJECT) at 25 °C with a flow rate of 20 µl/min. Measurements (n = 3) were performed in two steps: association, which corresponds to the sample injection, followed by a dissociation step, which corresponds to buffer injection. Between two consecutive measurements, chip surfaces were regenerated with SDS 0.5%. Data were analyzed using the BLAevaluation software version 3.2. The KD constants of both the homophilic and heterophilic interactions were calculated using the 1:1 Langmuir interaction model.
JAM-C in Tumor Cell-Endothelium Interactions

CHO Cell Adhesion Assay—Microtiter wells were coated with 50 μl of purified JAM-C (1 μg/well) for 2 h at 37 °C and blocked with 3% BSA in HBS buffer (119 mM NaCl, 4 mM KCl, 11 mM glucose, 20 mM Hepes, pH 7.4) for 1 h at room temperature. After washing with HBS buffer, 50 μl of CHO cell suspensions (4 × 10^6 cells/ml) were allowed to adhere for 1 h at 37 °C. Microtiter wells were then washed twice with 150 μl of HBS. Adherent cells were fixed with methanol/acetone (1:1) at 4 °C for 30 min, stained with crystal violet, and quantified by measuring absorbance at 590 nm on a microplate reader.

Fluorescence Adhesion Assay—Adhesion of the non-small lung cancer cell lines NCI-H522 and NCI-H322M, or different CHO cells to immobilized proteins (sJAM-C, Fc-JAM-C, Fc-JAM-A, Fc, or BSA), to surface-adherent mock-transfected CHO cells (transfected with plasmid pcDNA3) or to CHO cells transfected with either JAM-C or the mutant JAM-C isoform (Arg^66) or to tumor necrosis factor α-prestimulated (6 h, 10 ng/ml) surface-adherent confluent HUVEC was tested according to a previously described protocol (9, 18, 23). Briefly, wells were coated with different proteins (each 1 μg/well) in phosphate-buffered saline and blocked with 3% BSA in HBS. In cell-cell adhesion experiments, mock-transfected CHO cells, CHO cells transfected with either JAM-C or the mutant JAM-C isoform (Arg^66), or HUVEC cells were grown to confluence onto 96-well plates. Fluorescence-labeled cells (NCI-H522 and NCI-H322M) or fluorescence-labeled CHO cells (5 × 10^3/well) in 96-well plates were washed twice and added to immobilized proteins or to surface-adherent cells for 60 min at 37 °C in the absence or presence of effectors. After washings, adhesion of cells was quantified as the percentage of total cells added using a fluorescence microplate reader (BioTek, Winooski, VT).

RESULTS

Homophilic Interaction of JAM-C—We have previously shown that JAM-C undergoes a heterophilic interaction with the leukocyte integrin Mac-1, although it has not been clarified yet whether JAM-C may also interact in a homophilic manner. Therefore, we investigated whether JAM-C is capable of interacting homophilically. In particular, we tested whether Fc-JAM-C that was used as fluid phase ligand bound to immobilized sJAM-C (solid phase ligand). A dose-dependent and saturable binding of Fc-JAM-C to immobilized JAM-C was detected (Fig. 1A). Binding was not affected at all in the presence of EDTA, indicating that the homophilic interaction of JAM-C takes place in a cation-independent manner (not shown). In contrast, no binding of Fc or Fc platelet endothelial cell adhesion molecule-1 (PECAM-1) fusion protein to immobilized JAM-C (Fig. 1B) was observed (data not shown). Additionally, excess sJAM-C in the fluid phase blocked the binding of Fc-JAM-C to immobilized JAM-C (Fig. 1B). Furthermore, the homophilic interaction of JAM-C was compared with the heterophilic binding of JAM-C to the immobilized I domain of Mac-1. As shown in Fig. 1B, the IC_{50} of sJAM-C for inhibiting the homophilic interaction of JAM-C was higher than the IC_{50} of sJAM-C for inhibiting the interaction between Fc-JAM-C and the I domain of Mac-1, indicating that the homophilic binding of JAM-C may be stronger than the heterophilic interaction with Mac-1. Taken together, JAM-C can undergo a homophilic interaction.

The Amino-terminal Ig Domain of JAM-C Mediates the Homophilic Interaction—We then sought to determine which domain of JAM-C mediates the homophilic binding. To do so, domains 1 and 2 (amino- and carboxyl-terminal Ig domains of JAM-C, respectively) were produced as Fc fusion proteins (Fig. 2A). The binding of Fc-D1 and Fc-D2 to immobilized sJAM-C was compared with the binding of Fc-JAM-C. As shown in Fig. 2B, Fc-D1 was capable of interacting with sJAM-C, whereas no binding of Fc-D2 to sJAM-C was noticed. Thus, the amino-terminal Ig domain D1 of JAM-C is responsible for the homophilic interaction.

To further determine the specificity of the homophilic binding of JAM-C, real-time interaction between purified JAM-C proteins was analyzed by surface plasmon resonance analysis (Fig. 3). sJAM-C was attached to the sensor surface, and the binding of various JAM-C fusion proteins was tested at a concentration of 30 μg/ml. Sensograms were plotted and aligned (overlay mode). Although specific interaction...
The Arg64-Ile65-Glu66 Motif in the Amino-terminal Ig Domain of JAM-C Is Important for the Homophilic Interaction—The sequence RVE in the amino-terminal Ig domain of JAM-A has been reported to mediate dimerization of the molecule due to the formation of salt bridges between Arg58 and Glu60, and vice versa, on two separate JAM-A molecules (16). As this tripeptide is conserved in JAM-C (Arg64-Ile65-Glu66), we next investigated whether this sequence is responsible for the homophilic interaction of JAM-C. For this purpose a mutant isoform of JAM-C in which Glu66 was mutated to Arg66, thus preventing a putative salt bridge, was expressed in CHO cells. As shown in Fig. 4A, CHO cells bearing this mutant JAM-C isoform were not capable of binding Fc-JAM-C. In contrast, CHO cells bearing wild-type JAM-C strongly bound Fc-JAM-C. In the control experiment, both transfected cells expressed JAM-C protein on the surface, which is detectable with mAb Gi11 (Fig. 4A). Furthermore, the importance of the Arg64-Ile65-Glu66 motif in D1 of JAM-C for the homophilic interaction of JAM-C could be confirmed when cell adhesion to immobilized sJAM-C was studied. JAM-C-transfected CHO cells significantly adhered to immobilized sJAM-C, and adhesion was inhibited by excess JAM-C in the fluid phase. In contrast, CHO cells bearing the Arg66 mutant isoform of JAM-C and the CHO cells transfected with plasmid pcDNA3 (mock) showed background adhesion to immobilized sJAM-C, and this background adhesion was not affected by excess sJAM-C (Fig. 4B). Moreover, we found that JAM-C-transfected CHO cells adhered more readily than mock or mutant (Arg66) JAM-C-transfected cells to surface-adherent JAM-C-transfected cells (Fig. 4C). Together, these data indicate that the homophilic interaction of JAM-C mediates cell adhesion and is dependent on the Arg64-Ile65-Glu66 motif located in D1 of the molecule.

As the tripeptide sequence responsible for the homophilic interaction is conserved in JAM-A (RVE) and JAM-C (RIE), we then tested whether an interaction between JAM-C and JAM-A may exist. To test this possibility, we studied the adhesion of JAM-C- and JAM-A-transfected CHO cells to immobilized JAM-C or JAM-A. As demonstrated in Fig. 4D, specific binding of JAM-C-transfectants to immobilized JAM-A and of JAM-A transfectants to immobilized JAM-C was detected. Binding of JAM-C-expressing cells to JAM-A, and vice versa, was weaker than the homophilic interactions of JAM-C or JAM-A. Furthermore, the mutant (Arg66) JAM-C-transfected or the mock-transfected CHO cells did not adhere to JAM-A. Thus, JAM-C may interact with JAM-A, and this interaction is mediated at least in part by the R[V,I]E motif.

The Homophilic Interaction of JAM-C Mediates Tumor Cell Adhesion to Endothelial Cells—We then investigated whether the homophilic interaction of JAM-C is capable of mediating cell-cell adhesion. JAM-C is expressed on epithelial cells (1, 2, 27), and numerous tumor cells are of epithelial origin. We therefore investigated whether JAM-C-expressing tumor cells might engage JAM-C to adhere to endothelial cells. First, we identified that the non-small lung cancer cell line NCI-H522 showed a strong expression of JAM-C. Another non-small lung cancer cell line, NCI-H322M, which was negative for JAM-C expression, was used as a control cell line (Fig. 5A). As expected, the JAM-C-expressing NCI-H522 cells adhered strongly to immobilized sJAM-C, whereas the NCI-H322M cells did not adhere to sJAM-C. In addition, the adhesion of the NCI-H522 cells to immobilized sJAM-C was blocked by Fc-JAM-C or Fc-D1 but not by Fc-D2, indicating that JAM-C on the NCI-H522 cells interacted homophilically with the immobilized sJAM-C to mediate cell adhesion (Fig. 5B). JAM-C has been reported to interact with JAM-B (12). We therefore tested the expression of JAM-B on the tumor cell lines NCI-H522 and NCI-H322M, as JAM-B on these cells might mediate the adhesion to immobilized JAM-C. Expression of JAM-B was found neither on NCI-H522 cells nor on NCI-H322M cells as assessed.
by flow cytometry and Western blot analysis (not shown). Thus, the possibility that NCI-H522 cells adhere to JAM-C via JAM-B was excluded.

In the next step, the adhesion of fluorescence-labeled NCI-H522 or NCI-H322M cells to confluent surface-adherent CHO cells was studied. The NCI-H322M cells showed only weak background adhesion to mock-transfected CHO cells, JAM-C-transfected CHO cells, or CHO cells bearing the Arg<sup>66</sup> mutant isoform of JAM-C. This weak adhesion of the NCI-H322M cells was not affected in the presence of sJAM-C (Fig. 6A). In contrast, the NCI-H522 cells strongly adhered to the JAM-C-transfected CHO cells, and this adhesion was prevented in the presence of Fc-JAM-C. Only weak background adhesion to mock-transfected CHO cells or the CHO cells bearing the Arg<sup>66</sup> mutant isoform of JAM-C was observed, indicating that the homophilic interaction of JAM-C was operative in mediating the adhesion of the NCI-H522 cells to the JAM-C-transfected CHO cells (Fig. 6B). Furthermore, the adhesion of NCI-H522 cells to surface-adherent JAM-C-transfected CHO cells was blocked by the Fc-D1, but not the Fc-D2 (Fig. 6C).

We then sought to determine whether the homophilic interaction of JAM-C could be involved in the adhesion of the NCI-H522 cells to confluent surface-adherent endothelial cells. As opposed to the NCI-H322M cells the adhesion of NCI-H522 cells to surface-adherent HUVEC was partially blocked (~40% inhibition) by Fc-JAM-C and Fc-D1 but not Fc-D2 (Fig. 7, A and B). The adhesion of both lung carcinoma cell lines to HUVEC was partially inhibited by functional blocking mAb against β1 integrin (CD29) (Fig. 7, A and B), which is known as a receptor responsible for tumor cell-endothelial cell interactions. Both non-small lung cancer cells showed comparable expression of CD29 (data not shown). Interestingly, the combined blockade of CD29 and JAM-C provided an additive inhibitory effect and almost completely abolished the adhesion of NCI-H522 cells to HUVEC (Fig. 7B). Moreover, the possibility that adhesion of the NCI-H522 cells to HUVEC was mediated by the interaction between JAM-C on the NCI-H522 cells and JAM-B on HUVEC was also tested. As shown in Fig. 7B, no inhibition of adhesion was obtained with Fc-JAM-B. In addition, adhesion of the lung carcinoma cells to HUVEC was not affected in the presence of Fc-JAM-A (not shown). Thus, JAM-B or JAM-A are not involved in the adhesion of NCI-H522 cells to HUVEC. Together, these data demonstrate that the homophilic interaction of JAM-C is involved in tumor cell-endothelial cell interactions.

**DISCUSSION**

In the present work the homophilic binding of JAM-C is established and its functional relevance for tumor cell-endothelial cell interactions is demonstrated. We have previously shown that JAM-C undergoes a heterophilic interaction with the leukocyte integrin Mac-1 (9). Here, we described the homophilic interaction of JAM-C biochemically and provided novel evidence for its potential (patho)physiological function in adhesive interactions between tumor cells and endothelial cells.

**FIGURE 4.** The role of the Arg<sup>64</sup>-Ile<sup>65</sup>-Glu<sup>66</sup> motif in the homophilic interaction of JAM-C. A, JAM-C-transfected CHO cells or CHO cells transfected with the Arg<sup>66</sup> mutant isoform of JAM-C were incubated with isotype mouse IgG (blue thin line), mAb against JAM-C, Gi11 (blue bold line), Fc (red thin line), or Fc-JAM-C (red bold line). After washings, binding of mouse IgG or Fc was detected by flow cytometry with fluorescein-labeled rabbit anti-mouse or anti-human IgG, respectively. Binding of mAb Gi11 and Fc-JAM-C to transfected cells is also indicated by the arrows. *B*, the adhesion of CHO cells transfected with plasmid pcDNA3 (mock), or JAM-C-transfected CHO cells, or CHO cells transfected with the Arg<sup>66</sup> mutant isoform of JAM-C to immobilized sJAM-C is shown in the absence (filled bars) or presence (open bars) of Fc-JAM-C. Adhesion is shown as absorbance at 590 nm. *C*, the adhesion of CHO cells transfected with plasmid pcDNA3 (mock), or JAM-C-transfected CHO cells, or mutant (Arg<sup>66</sup>) JAM-C-transfected CHO cells to surface-adherent JAM-C-transfected CHO cells is shown. Cell adhesion is represented as percentage of total added cells. *D*, the adhesion of mock-transfected CHO cells (open bars), of JAM-C-transfected CHO cells (filled bars), of CHO cells transfected with the Arg<sup>66</sup> mutant isoform of JAM-C (gray bars), or of JAM-A-transfected CHO cells (hatched bars) to immobilized Fc-JAM-C, Fc-JAM-A, or Fc is presented. Cell adhesion is represented as percentage of total added cells. Data are mean ± S.D. (*n* = 3) of a typical experiment; similar results were observed in three separate experiments.
The following observations were consistent with a specific homophilic interaction of JAM-C: (i) A dose-dependent, saturable binding of Fc-JAM-C to immobilized sJAM-C was observed. The specificity of the homophilic binding of JAM-C was also shown by real-time analysis using surface plasmon resonance technology. The homophilic interaction of JAM-C was corroborated when the adhesion of JAM-C-transfected CHO cells to sJAM-C was tested. (ii) The homophilic interaction of JAM-C could be attributed to the amino-terminal Ig domain D1 of JAM-C, as opposed to the carboxyl-terminal Ig domain D2 that did not participate in the interaction. As assessed by surface plasmon resonance analysis, the isolated Fc-D1 bound to sJAM-C as strongly as the entire Fc-JAM-C. By estimating the $K_D$ of the homophilic interaction of JAM-C and the heterophilic interaction of JAM-C with Mac-1, we demonstrated that both interactions are of high affinity. However, the affinity of the homophilic interaction was found to be slightly higher than the affinity of the heterophilic binding.

Based on the crystallographic data obtained with JAM-A indicating that the dimerization of the molecule is mediated by the tripeptide Arg58-Val59-Gly60 and, in particular, by the formation of salt bridges between Arg58 and Glu60 and vice versa on two separate JAM-A molecules (16), we performed mutation analysis demonstrating that the conserved tripeptide sequence Arg64-Ile65-Glu66 in the amino-terminal Ig domain of JAM-C is responsible for the homophilic binding of JAM-C. Interestingly, we found that this sequence also mediates an interaction between JAM-C and JAM-A, as JAM-C-expressing cells adhered to JAM-A and vice versa, whereas the CHO cells transfected with the Arg66 mutant isoform of JAM-C did not adhere to JAM-A. Binding of JAM-C-expressing cells to JAM-A and vice versa was weaker than the homophilic interactions of JAM-C or JAM-A. Thus, the R(I,V)E motifs in the amino-terminal domain of JAM-C and JAM-A may at least in part mediate an interaction between the two molecules. However, further components in each molecule may enhance or modulate the homophilic interaction, as both molecules bind more strongly to themselves than to each other. As several blood cells, including neutrophils and monocytes, express JAM-A (1, 5), it is possible that JAM-A on these blood cells may interact with endothelial or epithelial JAM-C in different tissues. Nevertheless, detailed biochemical and functional analysis of the interaction between JAM-C and JAM-A has to be performed in order to exactly address these issues.

A functional relevance of the homophilic interaction of JAM-C in mediating tumor cell-endothelial cell interactions was demonstrated here for the first time. We identified that the non-small lung cancer cell line NCI-H522 was positive for JAM-C expression. The adhesive properties of this cell line were compared with the JAM-C-negative non-small cancer cell line NCI-H322M. JAM-C on the NCI-H522 cells was capable of interacting homophilically, thereby mediating the adhesion of these cells to immobilized JAM-C. Moreover, the heterotypic adhesion of these cells to endothelial cells was partially mediated by the homophilic binding of JAM-C as it was inhibited by Fc-JAM-C or

![Figure 5. Adhesion of non-small lung cancer cells to immobilized sJAM-C.](http://www.jbc.org/)

A, the expression of JAM-C on the non-small lung cancer cells NCI-H522 and NCI-H322M was analyzed by flow cytometry. Binding is shown of isotype mouse IgG (filled curves) and mAb against JAM-C, G11 (open curves). B, the adhesion of the NCI-H322M cells (filled bars) and the NCI-H522 cells (open bars) to immobilized sJAM-C is shown in the absence (--) or presence of Fc-JAM-C, Fc-D1, or Fc-D2 (each at 20 μg/ml). Cell adhesion is represented as percentage of total added cells. Data are mean ± S.D. (n = 3) of a typical experiment; similar results were observed in three separate experiments.
Fc-D1, but not Fc-D2. In contrast, JAM-C did not affect the adhesion of the JAM-C-negative NCI-H322M cells to endothelial cells.

In a previous report we overlooked the engagement of JAM-C in a homophilic interaction by using a single experimental system (9). That report was focused on the role of JAM-C on platelets as a counter receptor for Mac-1 on neutrophils, thereby mediating neutrophil-platelet interactions. Here, by the use of several experimental systems, we could characterize the homophilic interaction of JAM-C in detail. As presented here, the homophilic interaction of JAM-C may account for tumor cell-endothelial cell interactions. Thus, JAM-C on different cells may engage in different interactions. Differences in the levels of JAM-C expression and in its subcellular localization on different cells, as well as differences in the levels of expression of JAM-C counter receptors and of other adhesion receptors on these cells, may account for the engagement of JAM-C in heterophilic or homophilic interactions (2, 9, 10, 27, 28).

The adhesive interactions between tumor cells and endothelial cells are important in the process of blood-borne metastasis. Our present findings suggest that JAM-C-expressing tumor cells may engage the homophilic binding of JAM-C to adhere to endothelial cells. There is substantial evidence that the β1 integrin system is important for the adhesion and migration of tumor cells during metastasis (20, 21). The homophilic interaction of JAM-C may be operative besides β1 integrin-dependent interactions. In fact, we observed an additive inhibitory effect of mAb against β1 integrin with Fc-JAM-C, and the simultaneous blockade of β1 integrin and JAM-C almost completely abolished the adhesion of the NCI-H522 cells to endothelial cells, indicating that the β1 integrin- and JAM-C-dependent pathways may act synergistically in the process of metastasis. Frequently, tumor cells are larger and less deformable than leukocytes; therefore, because of physical size constraints tumor cells may be forced to stop in small capillaries (29). Thus, it is an intriguing hypothesis that the JAM-C-mediated tumor cell-endothelial cell interactions may promote the entrapment of metastatic cells in small capillaries during metastasis. Furthermore, whether JAM-C is up-regulated in specific tumors and whether JAM-C expression in tumors correlates with their metastatic potential needs to be investigated. Studies addressing the exact role of JAM-C in tumor cell metastasis need to be performed, and this is an ongoing project in our laboratory.

Another conceivable function of the homophilic interaction of JAM-C may be in endothelial and epithelial junction assembly. During the formation of tight junctions, the (homophilic) interaction between transmembrane molecules like occludins, claudins, and JAMs (as shown for JAM-A) on two adjacent cells mediates the contact between these cells and participates in junctional organization (26), as these transmembrane molecules interact with cytoplasmic PDZ domain-containing components of the tight junctions that are also linked to the actin cytoskeleton, such as AF-6, ZO-1, PAR-3, or MUPP1 (2). Thus, the homophilic interaction of JAM-C may participate in the regulation of endothelial and epithelial junction formation and paracellular permeability under some circumstances.
Taken together, the present work describes the homophilic interaction of JAM-C that represents a novel adhesive interaction between tumor cells and endothelial cells, thereby indicating that JAM-C may participate in the process of tumor cell metastasis. Thus, JAM-C may provide a novel molecular target for preventing metastasis, and detailed studies are needed to address this concept.

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