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Junctional adhesion molecule C (JAM-C) dimerization aids cancer cell migration and metastasis

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ABSTRACT

Most cancer deaths result from metastasis, which is the dissemination of cells from a primary tumor to distant organs. Metastasis involves changes to molecules that are essential for tumor cell adhesion to the extracellular matrix and to endothelial cells. Junctional Adhesion Molecule C (JAM-C) localizes at intercellular junctions as homodimers or more affine heterodimers with JAM-B. We previously showed that the homodimerization site (E66) in JAM-C is also involved in JAM-B binding. Here we show that neoexpression of JAM-C in a JAM-C-negative carcinoma cell line induced loss of adhesive property and pro-metastatic capacities. We also identify two critical structural sites (E66 and K68) for JAM-C/JAM-B interaction by directed mutagenesis of JAM-C and studied their implication on tumor cell behavior. JAM-C mutants did not bind to JAM-B or localize correctly to junctions. Moreover, mutated JAM-C proteins increased adhesion and reduced proliferation and migration of lung carcinoma cell lines. Carcinoma cells expressing mutant JAM-C grew slower than with JAM-C WT and were not able to establish metastatic lung nodules in mice. Overall these data demonstrate that the dimerization sites E66-K68 of JAM-C affected cell adhesion, polarization and migration and are essential for tumor cell metastasis.

1. Introduction

Metastasis is a process by which tumor cells escape from the primary tumor site, into the blood or lymphatic vascular network, to reach a distant organ and form secondary tumors [1,2]. Tumor cells of different origins metastasize to specific preferential sites. For example breast tumors frequently go on to colonize lungs, bones and brain, and lung tumors often metastasize to brain [3]. The majority of tumors originate from highly adhesive transformed epithelial cells, called carcinoma cells. Epithelial cells normally form strong cell-cell contacts to maintain tissue integrity so that epithelial tumor cells have to undergo changes in their adhesive properties in order to migrate out of their original tissue and form metastases. These changes are called epithelial-to-mesenchymal transitions (EMT) because carcinoma cells start to express proteins that are normally expressed by mesenchymal cells, which have lower levels of epithelial proteins and lower cell-cell contact adhesiveness. The classical molecular changes in EMT are the loss of E-Cadherin and up-regulation of N-Cadherin and the transcription repressors SNAILs, the ZEBs, and the TWISTs [4].

Metastatic tumor cells escape from the primary tumor by migrating through the connective tissue and by intravasation into blood or lymphatic vessels. Once in the blood circulation, tumor cells adhere to the endothelial barrier in distant tissues and extravasate. Many of these steps include adhesion molecules which makes it important to study the

Abbreviations: ECM, extracellular matrix; EGF, epithelial growth factor; EMT, epithelial to mesenchymal transition; JAM, junctional adhesion molecule; LEF-1, lymphoid enhancer-binding factor 1; MDCK-II, Madin Darby canine kidney II; MMP, matrix metalloproteinase; PDZ, post-synaptic density protein (PSD95), drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (ZO-1) domain; TNT, tris sodium tween buffer; ZEB1, zinc finger E-box-binding homeobox 1

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behavior of these molecules. While the first migration steps of cancer cells within the tissue of origin followed by intravasation are not well understood, the second steps from blood to distal tissue resemble migration of leukocytes during inflammation. They use cell adhesion molecules such as integrins and Ig-like proteins [5,6].

There are several families of cell adhesion molecules including Cadherins, Integrins, Selectins, and the Immunoglobulin superfamily. In physiological condition their expression and localization are tightly regulated, but during tumor development they are frequently deregulated or mutated, leading to a disruption of cell-cell adhesion [7]. Junctional Adhesion Molecules (JAMs) are a subset of the immunoglobulin superfamily, which have two extracellular immunoglobulin-like domains, a cytoplasmic tail and a PDZ-domain-binding motif [8]. JAM-A, B and C are paralogous and highly similar. They are expressed early during development, especially in brain and testis [9]. They can form homodimers at the surface of one cell and between two neighboring cells and heterodimers can also occur between JAM-B and JAM-C. JAM proteins also interact with human leukocyte integrins. For example, platelet JAM-C binds neutrophil integrin $\alpha_M\beta_2$ (Mac-1/CD11b/CD18) to help neutrophils to transmigrate through endothelium [10]. Human JAM-B also binds to leukocyte integrin $\alpha_4\beta_1$ in a JAM-C-dependent way [11].

We and others have demonstrated that JAM-B and JAM-C are localized at the plasma membrane, close to tight junctions, in epithelial and endothelial cells and that these proteins help leukocytes to cross endothelial barriers [12–15]. In adult mice, JAM-B is mainly located on non-circulating cells, whereas JAM-C is found on hematopoietic stem cells as well [16]. JAM-C is also present on human lymphocytes and platelets [10,17].

Many cell surface proteins have dimerization capacities between each other once in the membrane. For example, it has been demonstrated that recombinant soluble JAM-A is 90% dimeric and 10% tetrameric [18]. These intermolecular contacts between JAM-A molecules have been extensively studied by crystallography. One contact region has two salt bridges, formed by Arg 58 on one monomer and Glu 60 on the other. Mutation of the Glu60 residue to Arg in mouse JAM-A inhibited dimerization with wild-type JAM-A. Analysis showed that soluble JAM-A can form oligomers and that binding is mediated by the tri-peptide sequence $R^{58}V^{59}E^{60}$ in the membrane distal domain of the molecule. Mutation of this peptide sequence leads to significantly impaired JAM-A/JAM-A homophilic interaction in vitro [18,19]. In the paralogs JAM-B and JAM-C, the tri-peptide Arg58-Val59-Glu60 dimerization motif in the N-terminal Ig-domain of JAM-A is conserved, as RLE and RIE respectively. Correct localization and stabilization of JAM-C at intercellular junctions requires trans-interactions as homophilic (JAM-C/JAM-C) or heterophilic (JAM-C/JAM-B) complexes [14,18,20,21]. By analogy to JAM-A, mutation of the putative dimerization site of JAM-C (E66R) impedes homophilic interaction [19,20]. The heterophilic interaction between JAM-C and JAM-B stabilizes JAM-C in junctions and facilitates binding of leukocyte integrin $\alpha_4\beta_1$ [22]. The JAM-B/JAM-C interaction has a higher affinity than, and can displace, the homophilic JAM-C/JAM-C interaction [19]. The site of heterophilic interaction between JAM-C and JAM-B turned out to be the V domain of JAM-C as assessed by reduced binding of JAM-B to truncated or mutated, soluble JAM-C [20].

Since the JAM-C/JAM-B interaction is important for JAM-C stabilization at cell/cell junctions we identified the critical residues of JAM-C necessary for this interaction by directed mutagenesis of the V-domain of JAM-C. We tested JAM-C and mutant JAM-Cs overexpression for adhesion to JAM-B and studied their localization to cell-cell contacts as well as their effect on tumor cell behavior.

2. Materials and methods

2.1. Animal procedures

NOD-SCID immune-deficient (NOD.CB17-Prkdc^{scid}/NCrCrl) mice and NSG immune-deficient (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice were purchased from Janvier laboratory (France) and then inbred in house. NSG-*Jam-b*$^{-/-}$ immune-deficient mice were generated by crossing male NSG mice with *Jam-b*$^{-/-}$ mice. Mice were backcrossed for more than six generations on a BALB/cByJ background. F1 male animals with the *Il2rg*^{tm1Wjl} and *Jam-b*$^{-/-}$ mutation were backcrossed with NSG females to produce the N1 generation, which was characterized by microsatellites and *Prkdc* locus sequencing. Accelerated backcrossing, using microsatellites, was then performed for six generations before intercrossing, which produced the NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*Jam2* $^{Tm1.1Maal}$/SzJ mice used in this study. All animal procedures were performed in accordance with the Institutional Ethical Committee of Animal Care in Geneva and the Swiss Cantonal Veterinary Office (Authorization number GE/158/14).

2.2. Site directed mutagenesis and transfection of mutants

Directed mutagenesis was performed by PCR (PfuTurbo®, STRATAGENE®, La Jolla, CA) using murine JAM-C in the pcDNA3.1 vector as a template and the following oligonucleotides: 5′-CAG TAC TAC TGC ATT GCT AGC AAT GAC GCA GC-3′, 5′-GCT GCA CCT GCG TCA TTG CTA GCA ATG CAG TAG TAC TG-3′ for the JAM-C mutant E66R, 5′-CCT AGG ATT GAA TGG GAG AAA ATC CAA GAT GGC CAA ACC-3′, 5′-GGT TTG GCC ATC TTG GAT TTT CTC CCA TTC AAT CCT AGG-3′ for JAM-C K68E mutant, and 5′-GAC CCT AGG ATT AGA TGG GAG AAA ATC CAA GAT GGC CAA ACC-3′, 5′-GGT TTG GCC ATC TTG GAT TTT CTC CCA TCT AAT CCT AGG GTC-3′ for the JAM-C E66R-K68E mutant. DNA was digested with *Dpn* I and precipitated with ethanol. Electro-competent bacteria (E.coli XL-1 blue) were transformed with pcDNA3.1 containing mutated JAM-C genes. Plasmid DNA was mini-prepped from four colonies and the presence of mutated insert was assessed by digestion with Hind III and *Xba* I (Invitrogen®, Thermo Fisher Scientific, Waltham, MA U.S.A.) and by DNA sequencing on both strands.

pcDNA3.1-containing JAM-C mutants were transfected into MDCK-II and KLN205 cells (previously tested for the absence of JAM-C expression) using Fugene Transfection reagents according to manufacturer's instruction (Roche Diagnostics, Rotkreuz, Switzerland). Briefly, cells were grown to 60% confluency on 100 mm dishes. 5 μg of plasmid DNA were incubated with Fugene HD for 15 min in serum-free Opti-MEM medium, (Invitrogen®, Thermo Fisher Scientific, Waltham, MA U.S.A.). This solution was added to cells and incubated for 72 h. Cells were kept under selection with G418 (400 μg/ml) for 2 weeks. Cells were sorted two times for JAM-C expression by flow cytometry and kept under G418 selection (400 μg/ml).

2.3. In vivo metastasis assay

Mouse lung squamous carcinoma KLN205 cells expressing JAM-C mutants were injected subcutaneously into 7 weeks-old female NOD-SCID mice (250,000 cells/mouse) [23,24]. After 20 days, animals were anesthetized to remove primary tumor, closed with surgical clips and treated with buprenorphine as painkiller (Temgesic 50 μg/kg). 4 weeks after tumor resection, animals were anesthetized with a mix of Ketamine and Xylasine (80 mg/kg, 10 mg/kg respectively) before intratracheal instillation of 10% Formalin to fix lungs. After paraffin embedding, samples were cut into sections at five different levels and stained with hematoxilin/eosin. Slides were scanned using an automatic slide scanner, Axioscan (Zeiss, Plan-Apochromat 20x/0.8) and lung metastasis were quantified and measured using Image J software.