Inhibition of platelets and tumor cell adhesion by the disintegrin domain of human ADAM9 to collagen I under dynamic flow conditions

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1. Introduction

Metastasis is a key characteristic of malignant cells, which use the bloodstream to colonize distant target organs. Tumor cell arrest within the vasculature is a prerequisite for metastasis and it can be supported by tumor cell interaction with activated platelets [1]. Once in the circulation, tumor cells and platelets are exposed to shear stress generated by the dynamic flow conditions in the bloodstream [2,3]. Under these conditions, the adhesive properties of cells are pronouncedly different from those that mediate tumor cell attachment under static or in vitro conditions [4].

To form metastasis, circulating tumor cells must attach to vascular endothelial cells or components of the vessel wall and invade through them until reach the target organs. This is mediated by specific adhesive functions of tumor cell receptors, including the integrins. Integrins are a family of cell-surface α/β heterodimer receptors, which mediate adhesive interactions with multiple protein ligands, mostly extracellular matrix (ECM) proteins [5].

On the other hand, the ADAM (A Disintegrin And Metallopeptidase) family of proteins comprises a group of multifunctional proteins that play important roles in many biological processes, such as cell fusion, cell adhesion, proteolysis, and in some diseases as well, including cancer [6]. These type I transmembrane proteins are characterized by the presence of a prodomain, an N-terminal metallopeptidase domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane region, and a cytoplasmic tail with signaling properties [6–11]. The peptidase, disintegrin and cysteine-rich domains of the ADAMs are homologous to the snake venom metallopeptidase (SVMP) family of proteins [12].

Although various studies have focused on the proteolytic activities of some ADAM members, the importance of the disintegrin domain in cell adhesion and tumor progression has only been recently demonstrated [6,13]. The disintegrin domain of many ADAMs has the ability to interact with integrins on the surface of different cell types, including normal and cancer cells [13,14]. The interaction of ADAM15, or human metargidin, is provided by its disintegrin domain. Other ADAM family of proteins contains the RGD sequence in the disintegrin domain. Other ADAM members bind to cells via ECD or
DCD motifs [7,13,15]. In the literature, the adhesive properties of the different ADAMs’ domains are still a matter of controversy. Whereas some works attribute adhesive properties to the disintegrin domain [16–18], others indicate the cysteine-rich domain as having adhesive functions [19]. The same controversy is also observed for ADAM homologues, the SVMP proteins [20].

Among the 30 described members of the ADAM family, ADAM9 or MELTRIN-γ, is a widely expressed, non-RGD-containing protein that has been shown to bind to the α6β1 integrin on fibroblasts [16], αVβ5 on myeloma cells [21] as well as osteoblasts [17]. Furthermore, the disintegrin domain of ADAM9 was demonstrated to bind directly to α6β4 and α2β1 integrins on the surface of colon carcinoma cells [18]. After binding to these integrins, the disintegrin domain of ADAM9 was able to promote different cell signaling responses, such as an increase in IL-6 production via the p38MAPK and cPLA2 pathways [17].

In the present work we demonstrated for the first time, the adhesive properties of the disintegrin domain of ADAM9 under flow conditions, used to simulate the shear conditions found in vivo in the bloodstream. ADAM9D inhibited the adhesion of both tumor cells and platelets to type I collagen, a major component of the endothelium. In addition, we demonstrated that αVβ3 integrin is a new interacting partner for ADAM9D. Moreover, ADAM9D inhibited the invasion of tumor cells through matrigel, strongly suggesting a role for the ADAM9 disintegrin domain on the adhesive properties of this protein.

2. Materials and methods

2.1. Antibodies

For competition assays the monoclonal antibodies directed against human α2 (MAB1233), αβ (MAB13501), β1 (MAB17781) and αVβ5 (MAB2528) integrins were obtained from R&D Systems (Minneapolis, MN, USA). Antibodies against αVβ3 (MAB1976) and α3 (AB1920) integrins were from Chemicon (Billerica, MA, USA), and antibodies to the α4 integrin subunit (I6528) were from Sigma–Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-labeled anti-αVβ3, anti-α2β1, anti-α2 and anti-αVβ3β integrin antibodies and FITC-labeled rat IgG2a isotype control antibodies for flow cytometry analysis were from Becton–Dickinson Biosciences (Franklin Lakes, NJ, USA). Purified control IgG was purchased from Dako (Hamburg, Germany).

2.2. Cell line and culture

MDA-MB-231 human breast tumor cell line, obtained from ATCC, was maintained at 37 °C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM, Cultilab – Campinas; SP, Brazil) containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and l-glutamine (2 mM). Cell cultures and experiments were conducted in a humidified environment with 5% CO₂ at 37 °C. ADAM9 disintegrin domain cloning

Total RNA from a VMM12 human melanoma cell line was kindly donated by Dr. Jay W. Fox from the University of Virginia Health Systems (Charlottesville, VA, USA). After reverse transcription using the Superscript One-Step RT-PCR kit (Invitrogen – Carlsbad, CA, USA), the resulting cDNA was used for amplification of the disintegrin domain of human ADAM9 (ADAM9D) (GenBank accession no. NM_0033816) with the following oligonucleotides: sense 5’-CAT GGATCCGCCCTCCCCCTGTGCTAATAAGTTG-3’ and antisense 5’-CT GCCTCAGG TTAATACCTTTGGA AAAAAACATCTG-3’. The PCR product for ADAM9D corresponds to the nucleotides 1318–1588 and generates an amplicon of 270 pb which results in a protein with two ECD domains (base pairs 1357–1365 and 1513–1521). To facilitate the DNA cloning, Bam HI and Xho I restriction sites were added to the primers (underlined regions). For ADAM9D expression it was used the pGEX-4T-1 vector which is classically used to produce GST fusion proteins. The PCR product and pGEX-4T-1 vector (GE Healthcare, Little Chalfont, UK) were digested with the same restriction enzymes, purified from 1% agarose gels, and ligated using T4 DNA ligase (Invitrogen). After the transformation of Escherichia coli DH5α cells, the ampicillin-resistant recombinant plasmids were selected for restriction analysis and the positive clones were automatically sequenced in an ABI Prism 377 DNA Sequencer (Perkin Elmer – Foster City, CA, USA). The confirmed recombinant plasmids (pADAM9D) were used to transform the E. coli AD494(DE3) expression strain.

2.4. Protein expression, purification and characterization

Cultures of E. coli AD494(DE3)pADAM9D were induced for expression by addition of 0.5 mM isopropyl β-D-granduror- anoside (IPTG). Four hours after induction, the cells were harvested by centrifugation (7000 rpm, 15 min, 4 °C) in a Sorvall refrigerated centrifuge. Cell pellet was suspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3) and lysed by sonication (5 times, 4 °C, 1 min interval). ADAM9D was released from the fusion protein (GST) by thrombin (GE Healthcare) cleavage. Thrombin was eliminated from samples containing ADAM9D by purification in a Benzamidine-Sepharose 4B column (GE Healthcare). Fractions were analyzed by SDS-PAGE with Coomassie brilliant blue staining and ADAM9D concentration was determined by BCA protein assay (Pierce, Rockford, IL, USA). The ADAM9D expression yield is approximately 2.5 mg/l of culture.

2.5. Platelet aggregation

Platelet aggregation assays were performed in human platelet-rich plasma (PRP). Human blood was obtained from healthy donors and an 8% sodium citrate solution was added into the blood at the proportion of 1/9 (v/v). The mixture was centrifuged at 500 × g for 10 min and PRP was transferred into a clean tube. Different amounts of ADAM9D were added to PRP and allowed to incubate for 2 min, followed by the addition of collagen type I (final concentration of 10 μg/ml) or ADP (10 μM) to initiate aggregation. Platelet aggregation was measured in a Chronolog Aggregometer at 37 °C with stirring (900 rpm). The maximum aggregation response obtained from addition of collagen or ADP and in the absence of ADAM9D was given a value of 100% aggregation.

2.6. Inhibition of cell adhesion

Cell adhesion assays were performed as described earlier [22]. Briefly, 80% confluent monolayer cultures of MDA-MB-231 breast tumor cells were detached from tissue flasks by a short period incubation with 0.25% Trypsin–0.1% EDTA solution. Collagen type I from calf skin (Sigma–Aldrich) in acetic acid (0.1%) was immobilized in 96 well plates (10 μg/well) by incubation at 4 °C overnight. Wells were blocked with 1% BSA in adhesion buffer (20 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgSO4 and 1 mM MnCl2, pH 7.4) (200 μl/well) at room temperature for 2 h. MDA-MB-231 cells (5 × 10⁵ cells/ml) were labeled by incubation with 12.5 μM CMFDA (5-chloromethylfluorescein diacetate) in adhesion buffer at 37 °C for 30 min. Unbound label was removed by washing with adhesion buffer. Labeled cells (1 × 10⁵ cells/well) were incubated with ADAM9D at several concentrations before being transferred to the plate and incubated at 37 °C for 30 min. After washing to remove...
unbound cells, the remaining cells were lysed by the addition of 0.5% Triton X-100. In parallel, a standard curve was prepared in the same plate using known quantities of labeled cells. The plates were read using a Spectra-Max Gemini XS fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA) with a 485-nm excitation and 530-nm emission filters.

2.7. Promotion of cell adhesion and antibody competition assays

For the promotion of cell adhesion assay, collagen type I (10 μg/well) (Sigma–Aldrich) or ADAM9D (1–100 μg/well) dissolved in 0.1% acetic acid or adhesion buffer respectively, were immobilized on a 96-well microtiter plate overnight at 4 °C. Labeled cells (1 × 10^5 cells/well) alone or previously incubated with anti-αvβ3, anti-αvβ5, anti-α2, anti-α3, anti-α4, and anti-β1 blocking antibodies were added to the wells for 30 min at 37 °C. After washing with adhesion buffer to remove unbound cells, the remaining cells were lysed and the plate was read as described above.

2.8. Flow cytometry analysis

The integrin content on MDA-MB-231 breast tumor cells and platelets was determined by flow cytometry analysis using specific anti-integrin antibodies. Harvested MDA-MB-231 breast tumor cells were washed in PBS containing 1% BSA and 5 × 10^5 cells were incubated for 30 min at 4 °C with 10 μl FITC-anti-αvβ3, anti-α2, anti-α3, anti-α4, or anti-β1 isotype antibodies. Cells were immediately analyzed with a FACscalibur flow cytometer (Becton–Dickinson Biosciences).

2.9. Matrigel invasion assay

To measure cell invasion BD BioCoat Matrigel Invasion Chambers (Becton–Dickinson Biosciences) were used. Eighty percent confluent monolayer cultures of MDA-MB-231 breast tumor cells were detached as described above and 5 × 10^6 cells/ml, previously mixed or not (controls) with ADAM9D (1 μM) were placed in the upper well of rehydrate inserts. For the controls, the lower well was filled with 750 μl medium with (+C) or without (–C) 10% FBS. Cells were allowed to invade for 22 h. Then the non-invading cells were scraped from the upper surface of the membrane and the cells on the lower side were fixed with 100% methanol and stained with 1% Toluidine Blue in 1% Borax. Inserts were rinsed with water and membranes were allowed to air dry. Twenty images covering the membrane were taken under microscope and the recovered surface of insert membranes was calculated using the software Image J.

2.10. Flow adhesion assay

The effects of ADAM9D on the adhesion of tumor cells and platelets in vitro under flow conditions were analyzed using a model proposed by Gomes [23]. MDA-MB-231 cells (10^6 cells/ml) labeled with cell tracker red CMTPX (Invitrogen) were incubated (20 min) with ADAM9D (5 and 10 μM, final concentrations) or PBS (control) at 37 °C. Blood obtained from consenting healthy human donors was anticoagulated with standard heparin (13 IU/ml) and centrifuged at 200 × g for 20 min to obtain PRP which was then labeled with 5 μg/ml cell trace calcein green AM (Invitrogen) for 30 min at 37 °C. PRP was mixed with the remaining red blood cells (1:1.5, v/v) and further mixed with ADAM9D-treated MDA-MB-231 labeled cells (1:10, v/v). The mixture was perfused (1500 s^-1) in a perfusion chamber for 10 min covered by a bovine collagen type I-coated coverslip. After washing with PBS for 5 min, twenty different fields were captured using the software Scion Visiocyte Image Acquisition Application and an epifluorescent microscopy (Nikon Eclipse TE 300) coupled to a photo machine. Recovered surface of adhered platelets and cells in each field was differentially counted using the software Image J.

2.11. Statistical analysis

Each experiment was repeated three times in triplicate and a standard error mean was calculated. The results were compared statistically with a one-way analysis of variance (ANOVA). Since the ANOVA tests showed significant differences (acceptable p level < 0.05) Dunnett’s significant difference post hoc analysis was performed to determine differences between simple main-effect means.

3. Results

3.1. ADAM9D cloning, expression, purification and characterization

A band of 270 bp was amplified from total RNA extracted from human VMM12 cells using specific primers for the disintegrin domain of human ADAM9 (GenBank accession no. NM003816) (Fig. 1A). The PCR product and the vector pGEX-4T-1 were purified and ligated. The success of the cloning process was confirmed by restriction analysis and sequencing (not shown). E. coli AD494(DE3) cells were transformed by pADAM9D and the ADAM9D was produced after IPTG induction (Fig. 1B, lanes 1 and 2). Most of the recombinant protein (60%) was found in the soluble extract (Fig. 1B, lane 3), yielding approximately 2.5 mg of ADAM9D per liter of bacterial culture. However, a great quantity of protein was produced as inclusion bodies and was found in the pellet fraction (Fig. 1B, lane 4). The ADAM9D was released from the pGEX-4T-1 fusion protein (GST) using thrombin cleavage. Fig. 1C represents the thrombin cleavage at 0 h at room temperature (lane 1) or 4 °C (lane 2) and after 2, 4, 6, 8, 10 and 12 h, under room temperature, respectively (Fig. 1C, lanes 3–8). Thrombin was eliminated from samples containing ADAM9D by purification in a Benzamidine-Sepharose 4B column and the fractions containing ADAM9D were concentrated (Fig. 1D) to be used in the biological assays. ADAM9D was submitted to N-terminal sequencing of its first 8 residues (Gly-Ser-Ala-Pre-Ser-Cys-Gly-Asn). The amino acid sequence had 100% identity with the predicted sequence of the disintegrin domain of ADAM9 (bold residues after the thrombin cleavage site).

3.2. Platelet aggregation

Recombinant ADAM9D was tested on platelet aggregation using human PRP and collagen type I or ADP as agonists for aggregation. Recombinant ADAM9D, at concentrations as high as 10 μM, was unable to inhibit platelet aggregation induced by both agonists, collagen type I or ADP (results not shown).

3.3. Effects of ADAM9D on the adhesion of MDA-MB-231 cells under static conditions

In a static model, ADAM9D inhibited the adhesion of MDA-MB-231 breast cancer cells to the collagen type I. This inhibition was partially proportional to the concentration of ADAM9D used. Concentrations of 1 nM of ADAM9D inhibited 22.96 ± 6.8%, whereas concentrations of 10, 100 and 1000 nM inhibited 32.75 ± 4.8%, 36.56 ± 0.05% and 37.43 ± 1.4%, respectively, the adhesion of MDA-MB-231 cancer cells compared to the positive control, collagen type I, to which the adhesion was considered as 100% (Fig. 2).
To analyze whether the recombinant ADAM9D could support the adhesion of breast tumor cancer cells, the protein was immobilized on the wells of a 96-well plate. When coated on wells, ADAM9D significantly induced concentration-dependent adhesion of breast tumor MDA-MB-231 cells, working as an adhesion molecule for this cell type (Fig. 3A). ADAM9D at quantities of 1, 10 and 100 \( \mu \text{g} \) supported the adhesion of tumor cells by 67.7/6.2%, 99.8/4.5% and 110/10.2%, respectively, when compared to the adhesion of collagen type I (Fig. 3A). MDA-MB-231 cells adhered very poorly on BSA (11.5/1.8%), used as a negative control (Fig. 3A). The morphology of cells plated on different substrates was also analyzed (Fig. 3B). After 6 h of incubation the majority of cells plated on BSA appeared rounded, contrasting with cells plated on collagen type I, which spread out through the coating forming cell projections. A similar pattern was observed when cells were plated on ADAM9D coating (10 \( \mu \text{g} \), 6 h and 24 h) (Fig. 3B) suggesting that this protein can serve as a substrate for supporting cell adhesion.

The integrin content on tumor MDA-MB-231 cell and platelets were measured by flow cytometry using specific antibodies against different integrins (Fig. 4). Cytometry analysis of MDA-MB-231 cells showed that they express high levels of \( \alpha V \beta 3 \), \( \alpha 1 \), \( \alpha 2 \) and \( \alpha 3 \) integrins, however they do not express the platelet \( \alpha 2 \beta 1 \) integrin on their surface (Fig. 4). For the surface of human platelets, the integrin profile is already well documented [24]. They contain at least five members of integrin family proteins, including a collagen receptor, the \( \alpha 2 \beta 1 \) integrin, which is the most widely distributed collagen-binding integrin on platelets [24].

Fig. 1. Cloning, expression and purification of recombinant disintegrin domain of human ADAM9 (ADAM9D). (A) cDNA amplification of ADAM9D (270 bp) starting from total RNA of VMM12 cell line (1% agarose gel). (B) Recombinant ADAM9D protein was expressed by E. coli AD494(DE3). Bacterial culture sample before IPTG induction (lane 1) or 4 h after IPTG induction (lane 2) are represented. The protein content of the cell extract representing the soluble (lane 3) and insoluble (lane 4) fractions are also showed (SDS-PAGE in a Coomassie brilliant blue-stained 15% gel). (C) Digestion of ADAM9D/GST recombinant protein after expression and purification on a Glutathione-Sepharose 4B resin, following 0 h of thrombin incubation at room temperature (lane 1) or 4 \( \mu \text{g} \) (lane 2) and 2, 4, 6, 8, 10 and 12 h (lanes 3–8) of thrombin incubation at room temperature, respectively (15% SDS-PAGE). Samples containing ADAM9D were fractionated by application on a Benzamidine-Sepharose 4B column for thrombin elimination. (D) Concentrated fraction of ADAM9D (15% SDS-PAGE). M: molecular mass marker.

Fig. 2. ADAM9D inhibits the adhesion of MDA-MB-231 breast tumor cells to collagen type I. Ninety six well plates were coated with collagen I (10 \( \mu \text{g/well} \)) overnight at 4 °C. After blocking with 1% BSA, CMFDA-labeled MDA-MB-231 cells (1 \( \times 10^5\) cells/well) previously incubated for 30 min at 37 °C with different concentrations of ADAM9D (1–1000 nM) were seeded in the wells. After washing, remaining cells were lysed and the plate was read for the release of fluorescence. Results are expressed as mean ± SEM of three independent experiments. The results were normalized by the collagen values which were considered as 100% adhesion. The \( p \) value was determined using the using Dunnett’s test comparing ADAM9D bars with the positive control bar (collagen type I) (* \( p < 0.05 \); ** \( p < 0.01 \).
Other integrin receptors in platelets include a fibrinogen receptor ($\alpha_{IIb}\beta_3$), a vitronectin receptor ($\alpha_V\beta_3$), a fibronectin receptor ($\alpha_5\beta_1$), and a laminin receptor ($\alpha_6\beta_1$) [24].

3.4. ADAM9D binds to MDA-MB-231 tumor breast cells

by $\beta_1$, $\alpha_3$, $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_2$ integrins

To verify which integrin subunits of tumor cells were specific for the binding of ADAM9D, we performed competition assays using blocking anti-integrin antibodies (Fig. 5). MDA-MB-231 cells were incubated with different inhibitory anti-integrin antibodies and then applied on ADAM9D-coated wells. Antibodies directed against the $\beta_1$ integrin subunit efficiently inhibited MDA-MB-231 cell adhesion to ADAM9D (88.13 $\pm$ 3.4%) compared to the control, where cells were incubated only with PBS and plated on ADAM9D (Fig. 5). Antibodies against $\alpha_3$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_2$ integrins also inhibited MDA-MB-231 cell adhesion to ADAM9D by 74 $\pm$ 17.1%, 55.1 $\pm$ 1.8%, 50.66 $\pm$ 3.7% and 39.63 $\pm$ 7.6%, respectively. This result demonstrates for the first time that the disintegrin domain of ADAM9 interacts with $\alpha_V\beta_3$ integrin. On the other hand, antibodies raised against $\alpha_6$ and $\alpha_4$ integrin subunits did not have a significant effect on the inhibition of MDA-MB-231 cell adhesion to ADAM9D-coated wells (Fig. 5).

3.5. ADAM9D inhibits the matrigel invasion of MDA-MB-231 breast tumor cells

ADAM9D (1 $\mu$M) inhibited the invasion of MDA-MB-231 breast tumor cells through the matrigel in an in vitro assay by 60 $\pm$ 11.3% (Fig. 6). Complete medium was used as a chemoattractant and the negative control was medium without serum. Complete medium was used as a chemoattractant and the negative control was medium without serum.

3.6. ADAM9D inhibits the adhesion of MDA-MB-231 breast tumor cells and activated platelets to collagen type I under dynamic flow conditions

The effects of ADAM9D on the adhesion of MDA-MB-231 cells and activated platelets to collagen type I under dynamic flow conditions were tested (Fig. 7). ADAM9D (5 $\mu$M) was able to inhibit the adhesion of platelets and tumor cells by 65.6 $\pm$ 14.2% and 69.5 $\pm$ 13.2% respectively, compared to the control (Fig. 7). Results obtained with 10 $\mu$M ADAM9D were not significantly different from those with 5 $\mu$M (63.4 $\pm$ 29.1% for platelets and 81.8 $\pm$ 17.2% for tumor cells).
4. Discussion

ADAM9D was produced in a bacterial system in an active form, which was demonstrated by several biological assays. Similar results were previously described [17,21] suggesting that no post-translational modifications are needed for its activity. ADAM9D inhibited MDA-MB-231 cell adhesion to collagen I and functioned as an adhesive protein itself. Inhibition of cell adhesion by ADAM9D was only partial probably by the presence of other collagen-binding receptors on MDA-MB-231 cells, such as \( \alpha_1 \beta_1 \) integrin [25–27].

The interaction of ADAM9D with tumor cells was mediated mainly by \( \beta_1 \) integrin subunits in these cells, as demonstrated by antibody competition assays. It has been previously demonstrated that the recombinant disintegrin and cysteine-rich domains from human ADAM9 mediate cellular adhesion through \( \beta_1 \) integrins [19,28]. The disintegrin domain of human ADAM9 was also demonstrated to be a ligand for \( \alpha_3 \beta_5 \) integrin on myeloma cells [21]. Our data are in agreement with these results and moreover indicate, for the first time, an interaction of ADAM9D with \( \alpha_3 \beta_3 \) integrin on MDA-MB-231 cells, such as \( \alpha_1 \beta_1 \) integrin [25–27].

In this work we demonstrated that ADAM9D is able to inhibit the matrigel invasion of MDA-MB-231 breast cancer cells. In a model proposed by Mazzocca et al. [18], a form called ADAM9-S, composed by a prodomain, metallopeptidase, disintegrin and cysteine-rich domains, localized in a proteolytically active form proximal to the cell surface of invasive cells would regulate cell motility, facilitating invasion. On the other hand, Zigrino et al. [19] demonstrated that ADAM9 functions as a cell adhesion molecule via disintegrin and cysteine-rich domains. Therefore, it seems that both disintegrin and the cysteine-rich domains have important and probably cooperative roles in protein interaction and cell adhesion.

In agreement with these results, Mahimkar et al. [28] produced the stable expression of ADAM9 constructs in HEK-293 cells and showed that the disintegrin domain (and not the cysteine-rich domain) is the responsible for the competitive inhibition of these cells to the ECM components, by binding to different \( \beta_1 \) integrins (\( \alpha_1, \alpha_3, \alpha_6 \) and \( \alpha_\gamma \)). On the other hand, Zigrino et al. [19] demonstrated that ADAM9 functions as a cell adhesion molecule via disintegrin and cysteine-rich domains. Therefore, it seems that both disintegrin and the cysteine-rich domains have important and probably cooperative roles in protein interaction and cell adhesion.

In this work we demonstrated that ADAM9D is able to inhibit the matrigel invasion of MDA-MB-231 breast cancer cells. In a model proposed by Mazzocca et al. [18], a form called ADAM9-S, composed by a prodomain, metallopeptidase, disintegrin and cysteine-rich domains, localized in a proteolytically active form proximal to the cell surface of invasive cells would regulate cell motility, facilitating invasion. On the other hand, according to our results, the isolated disintegrin domain is blocking MDA-MB-231 breast tumor cell invasion through matrigel and this result is in agreement with in-flow assays. It is important to mention that ADAM9-S is composed by other domains besides the disintegrin domain [18], and the presence of these additional domains probably also contributes to invasion.

Fig. 4. MDA-MB-231 cells express \( \alpha_\gamma \beta_3 \), \( \beta_1 \), \( \alpha_2 \), and \( \alpha_3 \), but not \( \alpha_\gamma \beta_3 \) integrin receptors. The presence of integrin receptors on MDA-MB-231 cell surface was determined by flow cytometry with FITC-anti-\( \alpha_\gamma \beta_3 \), anti-\( \alpha_2 \), anti-\( \beta_1 \), anti-\( \alpha_3 \), or anti-\( \alpha_\gamma \beta_3 \) integrin antibodies, as described in Section 2. Shaded curve represents background fluorescence in the presence of isotype-matched antibody.
Metastasis to different organs involves dissemination of tumor cells via the bloodstream [1]. This process depends on tumor cell intravasation, adhesion to the vessel wall, extravasation, infiltration, and proliferation into target tissue. Integrin αvβ3 has been implicated in the pathphysiology of malignant tumors. It plays a role on endothelial cells, where it is required for tumor angiogenesis. In breast cancer, αvβ3 characterizes the metastatic phenotype, as this integrin is up regulated in invasive tumors and distant metastases. In the bloodstream, αvβ3 integrin supports tumor cell interaction with adherent platelets and leukocytes [1]. However, to mediate this interaction, αvβ3 integrin must be activated. In breast cancer cells, the integrin αvβ3 can exist in activated or non-activated forms. Furthermore, expression of activated αvβ3 integrins on the surface of cancer cells is associated with a highly aggressive metastatic phenotype [1]. Thus, expression of αvβ3 on circulating tumor cells in a constitutively activated form, or in a state that allows rapid activation under blood flow dynamic conditions, provides an advantage for successful metastasis [29].

The shear stress generated by the dynamic blood flow conditions in the bloodstream affects the characteristics of cell adhesion receptors [2,3]. Under flow conditions, the adhesive properties of cells are markedly different from those that mediate cell attachment under static conditions. Arrest during blood flow can be supported by tumor cell interaction with attached, activated platelets [4]. Platelets are also implicated in tumor progression by facilitating metastasis formation and enhancing tumor cell adhesion to the subendothelial matrix under blood flow conditions [29]. Furthermore, platelets have been shown to promote tumor cell adhesion to ECM under flow [30]. The mechanisms through platelets and melanoma cells interact are still unknown, but some hypotheses have been created. Some works demonstrate that platelet integrin αIIbβ3 and melanoma cell integrin αvβ3 interact via divalent or multivalent RGD-containing plasma proteins, such as fibrinogen, von Willebrand factor, fibronectin, or thrombospondin [31]. However, the results of aggregation studies using ADP suggest that ADAM9D does not antagonize αIIbβ3 integrin. Since ADAM9D has no effects on collagen-induced platelet aggregation, we hypothesize that this protein, by binding to MDA-MB-231 cells, would inhibit the platelet/cancer cell conjugates recruitment. This hypothesis would explain the discrepant results between in-flow platelet adhesion to collagen inhibited by ADAM9D, and collagen-induced platelet aggregation, not inhibited by ADAM9D.

Regarding the effects on the inhibition of tumor cell invasion and platelet and tumor cell adhesion under dynamic flow conditions, ADAM9D can be a candidate as a model for the design of
selective inhibitors against the adhesion and extravasation of cancer cells. Anti-adhesion therapy is a promising therapeutic concept in oncology [32] and many pharmacological and toxicological studies indicate that antagonists of different integrins, such as disintegrins, RGD peptides, small molecules, and blocking antibodies are well tolerated in different animal species, suggesting that these molecules may be potentially effective and safe for therapeutic purposes [33]. However, the secondary effects of targeting ADAM9D in tumor breast cancer remains to be identified.

In conclusion, we have demonstrated that ADAM9D is able to inhibit tumor and platelet cell adhesion under flow to simulate in vivo conditions. Our results suggest a role for the disintegrin domain of ADAM9 in the metastatic process. In addition, ADAM9D can be used as a tool for investigating the role of ADAMs in metastasis and cancer progression.

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