Recombinant disintegrin domain of human ADAM9 inhibits migration and invasion of DU145 prostate tumor cells

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Introduction

Prostate cancer is the second leading cause of death by cancer in men. It has become the most common cancer in men in the Western world, with approximately 40,000 new cases yearly in the UK. In Europe and North America, more than 500,000 cases are diagnosed per year. The disease incidence varies worldwide, being more common in Western countries compared to East Asian and developing countries. The disease incidence has increased in the past 2 decades, probably due to the improvements in early detection and/or treatment, and also an increased public awareness.11 Because of the high prostate cancer incidence, the development of new therapeutic tools should be created to combat this disease.

The ADAM (A Disintegrin And Metalloproteinase) 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.2,3 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.4

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 αvβ1 integrin on fibroblasts,5 αvβ5 on myeloma cells6 as well as osteoblasts.7 Furthermore, the disintegrin domain of ADAM9 was demonstrated to bind directly to αvβ4 and αvβ1 integrins on the surface of colon carcinoma cells.8 After binding to these integrins, the disintegrin domain of ADAM9 is able to promote different cell signaling responses, such as an increase in IL-6 production via the p38MAPK and cPLA2 pathways.7 Recombinant disintegrin domain of ADAM9 (rADAM9D) was demonstrated to bind to MDA-MB-231 tumor breast cells through α1, α3, αvβ3, αvβ5 and α2 integrins and also to inhibit the adhesion of this cell line and platelets to collagen type I under dynamic flow conditions.9

The adhesive properties of the different ADAMs domains are still a matter of controversy. Whereas some works attribute adhesive properties to the disintegrin domain,5,7,9 others indicate the cysteine-rich domain as having adhesive functions.10, 11 Recent data from different model systems suggest that ADAM9 is involved in tumor formation/progression. In many types of human cancers ADAM9, as well as other specific ADAMs, are up-regulated.12–18

In the present work we demonstrated the pro- and anti-adhesive properties of the rADAM9D on DU145 prostate cancer cells. rADAM9D was able to promote adhesion of DU145 cells, therefore acting as an adhesion molecule, similarly to other
molecules such as collagens, laminin and fibronectin. Furthermore, rADAM9D inhibited the adhesion of DU145 cells to laminin-coated wells, but not to collagen type I. The adhesion of rADAM9D to DU145 cells can occur through β1, α6, αvβ5 and αvβ3, since the incubation with anti-integrin blocker antibodies directed against these integrins prevented cell adhesion to rADAM9D. rADAM9D was also able to inhibit DU145 cell migration and invasion through matrigel. Overall, this study may contribute to the development of new therapeutic strategies for prostate cancer.

Results

rADAM9D promotes DU145 cell adhesion
rADAM9D was able to promote adhesion of DU145 cell line, similarly to different extracellular matrix proteins (collagen type I and IV, laminin and fibronectin), therefore supporting cell adhesion as extracellular matrix protein, probably by interacting with DU145 integrins. There was no difference among quantities (5, 10 or 50 μg) of rADAM9D plated on the wells to promote the adhesion of DU145 cells (Fig. 1).

ADAM9D interacts with DU145 prostate tumor cells through β1, α6, αvβ5 and αvβ3
Since rADAM9D supported DU145 cell adhesion we verified which integrins in this prostate cancer cell line would be specific for this disintegrin. For that, we incubated DU145 cells with different anti-integrin blocking antibodies before plate on an rADAM9D (10 μg) coating. The antibodies against β1, α6, αvβ5 and αvβ3, inhibited DU145 cell adhesion to rADAM9D (Fig. 2A), confirming that this recombinant protein could bind to these integrins on the surface of DU145 cells. On the other hand, antibodies against α2 and α4 integrins chains did not inhibit the adhesion of DU145 cells to rADAM9D (Fig. 2A). To confirm this result, rADAM9D (1 μM) was previously incubated with DU145 cells and the mixture was then incubated with anti-integrin antibodies. Subsequently, cells were analyzed by flow cytometry (Fig. 2B) and the results obtained confirmed that rADAM9D inhibits the binding of anti-β1, anti-α6, anti-αvβ5 and anti-αvβ3 antibodies to DU145 cells, probably because rADAM9D binds to these integrins on these cells, preventing previous binding of cited antibodies. The inhibition of anti-α6 antibody promoted by rADAM9D was lower compared to the other antibodies using cytometry analysis (Fig. 2B). rADAM9D did not promote inhibition of the binding of anti-α2 and α-4 antibodies to DU145 cells. Integrin profile in DU145 cell line was measured by flow cytometry, using the antibodies mentioned above. This cell line presented higher levels of α2 and β1, moderate levels of α6, αvβ3 and αvβ5, and low levels of α4 integrin subunit (Fig. 2C).

rADAM9D inhibits the adhesion of DU145 cells to laminin but not to collagen type I
Since rADAM9D seemed to bind specifically α6β1 integrin on DU145 prostate cancer cell line, we further investigate whether this rADAM9 domain would inhibit tumor cell adhesion to laminin, a known α6β1 integrin ligand. Therefore, we tested the capacity of rADAM9D to inhibit the adhesion of DU145 cells to laminin and collagen type I, which has no specificity for α6β1 integrin. For that, DU145 cells were previously incubated with different rADAM9D concentrations and then plated on laminin or collagen type I-coated wells. Results indicate that incubation of rADAM9D with DU145 cells inhibited their adhesion to laminin (Fig. 3A) but not to collagen type I-coated wells (Fig. 3B). In other words, the blocking of receptors on DU145 cells promoted by rADAM9D was specific to inhibit the adhesion of this cell line to laminin but not to collagen type I. rADAM9D at concentrations of 2000nM were also tested for the inhibition of DU145 cells to collagen type I, but there was no inhibition (data not shown).

rADAM9D inhibits invasion and migration of DU145 cells
rADAM9D was tested to its ability to inhibit DU145 cell invasion and migration. rADAM9D, at concentrations ranging from 100 to 1000 nM, was able to significantly inhibit DU145 cell invasion (Fig. 4). In a wound healing migration assay, rADAM9D significantly inhibited DU145 cell migration at 100, 500, 1000 and 2000nM (Fig. 5A and B). The effects were more striking after 24 hours of wound repopulation.
Discussion

The progression of malignant tumors results from invasion of the primary tumor to a secondary site, causing metastasis in a multi-step process. These steps can be summarized as follows: cell detachment from the primary tumor, migration and invasion into the ECM, intravasation into a blood or lymphatic vessel, survival within the vasculature, adherence of these tumor cells in the endothelium, extravasation, and formation of secondary tumors.19-21 Therefore, metastasis necessitates disruption of cellular interactions with the tumor microenvironment, increased migratory and invasion capacity and the ability to overcome the pro-apoptotic signals provided by diminished intercellular and cell-ECM interactions mainly through integrin receptors.22

Several members of the integrin family, including α1β1, α2β1, α3β1, α6β1, α7β1 and α6β4 heterodimers serve as laminin receptors on a variety of normal and tumor cell types and DU145 prostate cell line was reported to have abundant content of integrin β1, along with α1, α2, α3, α5 and α6 integrin subunits.24,25

In this work, we have demonstrated that DU145 tumor cell line contains high amounts of α2β1, moderate amounts of αvβ5 and α6 and low quantities of αvβ3 and α4 integrins. Additionally, we showed that the recombinant disintegrin domain of ADAM9 (rADAM9D) was able to inhibit the adhesion of DU145 prostate cancer cells to laminin, mainly by binding to α6β1 integrin receptor, therefore rADAM9D may act at the beginning of metastatic cascade, disrupting the binding of prostate cells to the ECM laminin component. Furthermore, rADAM9D was able to inhibit DU145 cell invasion and migration of this tumor cell line.

It was demonstrated in the literature that ADAM9 transcripts are alternatively spliced to express a transmembrane protein (ADAM9-L) and a secreted variant (ADAM9-S) with opposite functions in breast cancer cells and that the balance between these isoforms is an important determinant in manifestation of aggressive migratory phenotypes associated with breast cancer progression. According to the authors, ADAM9-S is an enhancer, whereas ADAM9-L is a suppressor of cell migration.26 Mazzocca and colleagues8 have demonstrated the presence of these alternatively spliced variants of ADAM9. In hepatic stellate cells, it was demonstrated that ADAM9-S induced a highly invasive phenotype and promotes tumor cell invasion. Moreover, they demonstrated that ADAM9-S binds directly to α6β4 and α2β1 integrins on the surface of colon carcinoma cells through the disintegrin domain.8 Our results are not in accordance with these data since we have demonstrated that rADAM9D is an inhibitor of invasion and migration in prostate tumor cells. ADAM9-S is a construct composed by a signal sequence, a prodomain, a metalloproteinase domain and by disintegrin and cysteine-rich domains. In our work, however, only the disintegrin domain of ADAM9 was recombinantly produced in a bacterial system and this difference in protein structure may explain the differential biological activities of both molecules. Additionally, it is important to point out that the adhesion properties of the isolated rADAM9D domain are not sufficient to explain the molecular interactions and functional significance of the entire protein in a cellular and in vivo system. Moreover, the native disintegrin domain of ADAM9 has a N-glycosylation site,27 which
undoubtedly influences its adhesion properties. In this work, however, rADAM9D does not have the presence of this N-glycosylation, since bacterial systems to produce recombinant proteins are not able to perform such types of post-translational modifications. Furthermore, Takeda and co-workers, resolving the crystal structure of VAP-1, a homolog of mammalian ADAMs, demonstrated that the binding area of the disintegrin domain in a C-type conformation is not accessible for protein binding and a Hyper-Variable Region (HVR) of the cysteine-rich domain may instead be involved in substrate interaction. Therefore, alone the disintegrin domain may only contribute, when in native conformation, but not mediate cell adhesion in physiological conditions.

On the other hand, Zigrino and coworkers prepared a construct only with disintegrin and cysteine-rich domains of ADAM9 and reported that this protein functions as a cell adhesion molecule. These authors also demonstrated that the recombinant ADAM9 disintegrin-cysteine-rich domains specifically interact with the β1 integrin subunit on keratinocytes. However, engagement of integrin receptors by the ADAM9 disintegrin-cysteine-rich domains resulted in ERK phosphorylation and increased MMP9 synthesis. Additionally, keratinocytes adhering to the immobilized disintegrin and cysteine-rich domains showed increased motility, which was partially due to the induction of MMP9 secretion in this cell line. In our work, instead, rADAM9D inhibited DU145 cell migration and invasion, again demonstrating the influence of different protein constructs on its functional activity.

This is the first time that the effects of a recombinant domain of an human ADAM are demonstrated on adhesion, migration and invasion of prostate tumor cells. Taken together, our results demonstrate that recombinant ADAM9D has specific migratory properties when compared with different constructs having additional domains, specially those of metalloproteinase and cysteine-rich domains. Furthermore, we showed that rADAM9D was able to inhibit cell adhesion, migration and invasion mainly through interacting with α6β1 in DU145 tumor cell line.

**Conclusion**

The potential of the rADAM9D as an anti-adhesive molecule can be explored as tool to combat metastasis and cancer progression and for the design of selective inhibitors against the adhesion and extravasation of cancer cells. Other studies, using animal models, should be conducted to confirm this hypothesis.
were used to transform the analysis. The confirmed recombinant plasmids (pADAM9D) lin-resistant recombinant plasmids were selected for restriction

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induced for expression by addition of 0.5 mM isopropyl thio-

strained. Cultures of

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Expression and purification of the recombinant disintegrin domain of ADAM9 (ADAM9D) (GenBank accession no. NM003816). For ADAM9D expression it was used the pGEX-4T-1 vector as described.9 Briefly, total RNA from a VMM12 human melanoma cell line was reverse transcribed and resulting cDNA was used for amplification of the disintegrin domain of human ADAM9 (ADAM9D) (GenBank accession no. NM003816). For ADAM9D expression it was used the pGEX-4T-1 vector which is classically used to produce GST fusion proteins. After the transformation of Escherichia coli DH5-α cells, the ampicillin-resistant recombinant plasmids were selected for restriction analysis. The confirmed recombinant plasmids (pADAM9D) were used to transform the E. coli AD494(DE3) expression strain. Cultures of E. coli AD494(DE3)pADAM9D were induced for expression by addition of 0.5 mM isopropyl thio-

β-D-galactopyranoside (IPTG). Four hours after induction, cells were harvested by centrifugation (7000 rpm, 15 min, 4°C) and 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 cleavage. Thrombin was eliminated from samples containing ADAM9D by purification in a Benzamidine-

thrombin cleavage. Thrombin was eliminated from samples

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rADAM9D concentration was determined by BCA protein assay (Pierce).

Antibodies
For flow cytometry and competition assays the monoclonal blockers antibodies used against human α2 (MAB 1233), α6 (MAB13501), β1 (MAB17781) and αvβ5 (MAB2528) integrins, were obtained from R&D Systems (R&D Systems Inc., Minneapolis, MN, USA). Antibodies against αvβ3 integrin (MAB1976) were from Chemicon, and antibodies to the α4 integrin subunit (I6528) were from Sigma-Aldrich. Control IgG was from Dako. Secondary antibody goat anti-mouse IgG-FITC (Fluorescein isothiocyanate, sc-2010), used for flow cytometry, were purchased from Santa Cruz Biotechnology.

Cell line and culture
DU145 human prostate tumor cell line was obtained from ATCC and maintained at 37°C in 5% CO2 in RPMI culture medium (Cultilab) containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2mM). Cell cultures and experiments were conducted in a humidified environment with 5% CO2 at 37°C.

Cell adhesion and antibody competition
Cell adhesion and inhibition of cell adhesion were performed as described before.9 Briefly, collagen type I, collagen type IV, laminin, fibronectin (10 μg), rADAM9D (5, 10 and 50 μg) or 1% BSA were immobilized overnight at 4°C in 96 well plate. Wells were blocked with 1% BSA in adhesion buffer (HEPES 20mM, pH 7.35; containing NaCl 150 mM, KCl 5 mM, MgSO4 1 mM and MnCl2 1 mM) for 2 h. DU145 cells were labeled with 12.5 μM CMFDA (37°C for 30 min) and then 1 × 105 cells/well were plated and maintained for 30min at 37°C. Wells were washed and the remaining cells were lysed using 0.5% Triton X-100. Fluorescence was read in a fluorimeter Spectra-Max Gemini XS (Molecular Devices, Sunnyvale, CA, USA) with 485nm excitation and 530nm emission filters. For inhibition of adhesion assay, cells were labeled and measured as described above, except that the cells were pre-incubated with rADAM9D in different concentrations (100, 500, 1000 nM) and then plated on 10 μg of collagen type I (Sigma-Aldrich) in acetic acid (0.1%) or on 10 μg of laminin (Sigma-Aldrich) in adhesion buffer. For antibody competition assays CMFDA-labeled cells were incubated with different anti-integrin

Material and Methods
Recombinant ADAM9 disintegrin domain (rADAM9D)

Expression and purification of the recombinant disintegrin domain of ADAM9 (rADAM9D) was performed as previously described.9 Briefly, total RNA from a VMM12 human melanoma cell line was reverse transcribed and resulting cDNA was used for amplification of the disintegrin domain of human ADAM9 (ADAM9D) (GenBank accession no. NM003816). For ADAM9D expression it was used the PGEX-4T-1 vector, which is classically used to produce GST fusion proteins. After the transformation of Escherichia coli DH5-α cells, the ampicillin-resistant recombinant plasmids were selected for restriction analysis. The confirmed recombinant plasmids (pADAM9D) were used to transform the E. coli AD494(DE3) expression strain. Cultures of E. coli AD494(DE3)pADAM9D were induced for expression by addition of 0.5 mM isopropyl thio-

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antibodies (αvβ3, αvβ5, β1, α2, α4, α6 at 10 μg/ml) and IgG control (10 μg/ml) before being plated on rADAM9-coated wells.

**Flow cytometry analysis**

DU145 cells were submitted to flow cytometry analysis to verify their integrin cell profile. Cells (1 × 10^5 cells/ml) were incubated for 40 min at 4°C with specific antibodies against αvβ3, αvβ5, β1, α2, α4, α6 and control IgG. Cells were washed and incubated with secondary antibody labeled with FITC, at same conditions described before, washed and fixed with FACs buffer containing 1% formaldehyde overnight at 4°C. To verify the interaction with integrins, rADAM9D (1 μM) was previously incubated (30 min at room temperature) with DU145 cells, before the addition of antibodies. Cells were analyzed in FACSCanto (BD Biosciences).

**Invasion**

Invasion assays were performed with DU145 prostate cancer cells incubated with rADAM9D using the BioCoat Matrigel Invasion Chambers (BD Biosciences). Briefly, DU145 cells (1.25 × 10^5 cells/ml) were seeded on the inserts (12 well-plate) of the invasion chamber in the presence or absence of rADAM9D (1 μM). Complete medium was used as a chemoattractant at the lower chamber. Plates were incubated for 22 h at 37°C and 5% CO2. Non-invading cells were removed with a cotton swab from the upper surface of the membrane and invading cells were fixed using 100% methanol and stained with 1% toluidine blue in 1%borax. Ten random fields from microscope slides were photographed and cells were counted using Image J software. Positive control was made in the presence of chemoattractant (100% FBS) at the lower chamber and negative control was FBS free medium at the lower chamber.

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