UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular

CYNTIA DE FREITAS MONTENEGRO

EFEITOS DE UMA DESINTEGRINA RGD RECOMBINANTE NA MIGRAÇÃO DE UMA LINHAGEM CELULAR DE CARCINOMA DE CÉLULAS ESCAMOSAS DE ORIGEM ORAL

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Tese apresentada ao programa de Pós-Graduação em Génetica Evolutiva e Biologia Molecular, do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para a obtenção de título de Doutor em Genética e Biologia Molecular.

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RESUMO

O câncer representa um conjunto de doenças relacionadas ao crescimento anormal de células e sua capacidade de invadir tecidos. Essas habilidades resultam no desenvolvimento de metástases, que levam muitos dos pacientes acometidos por essa doença, à morte. Durante o processo de invasão as células encontram um tecido conectivo rico em fibronectina e colágeno, o qual terão que atravessar a fim de chegar aos vasos sanguíneos e posteriormente ao sítio metastático. Receptores de adesão a fibronectica como a αvβ3 e α5β1 são essenciais para que as células sejam capazes de se movimentar sobre as fibras dessa protéína. A integrinas se ligam a fibronectina através do motivo RGD presente, fazendo desses receptores, potenciais alvos no estudo da migração celular durante o câncer. Assim, o efeito da Dis*Ba*-01, uma desintegrina com motivo RGD, foi investigado em duas células especializadas em migração; fibroblastos (BJ) e uma linhagem de carcinoma de células escamosas oral (SCC25). A Dis*Ba*-01 (1 e 2 μM) foi capaz de diminuir a velocidade de migração e a direcionalidade das células de câncer, mas não dos fibroblastos, em ensaios de *time-lapse* realizados em placas recobertas por fibronectina. Com o objetivo de entender se o efeito da Dis*Ba*-01 está relacionado com alterações na adesões celulares, as células foram plaqueadas sobre fibronectina e marcadas com anti-paxilina. Nas imagens adquiridas através de microscopia confocal é possível visualizar mudanças nas adesões, que se tornaram maiores e mais numerosas. Dados que foram confirmados em ensaios de *time-lapse* utilizando a microscopia de fluorescência de reflecção interna total (TIRF – *Total Internal Reflection Fluorescence*) em células transfectadas com paxilina. A expressão dos principais receptores de fibronectina, α5, αv e β3 está presente de forma similar nas duas linhagens, enquanto a subunidade β1 é expressa em quantidades menores nas células OSCC. Para investigar a provavel relação da inibição da migração com a expressão da integrina β3, a mesma foi superexpressa em nas células de OSCC e todos os parâmetros analizados foram normalizados. Em conjunto, esses resultados demonstram a capacidade da Dis*Ba*-01 de afetar a migração celular dependendo do nível de expressão de integrinas e através da modulação da dinâmica das adesões.

ABSTRACT

Cancer is a group of diseases related to abnormal cell growth and ability to invade tissues. These skills result in the development of metastases, leading to death in many of the patients. During the process of invasion, the cells find a connective tissue rich in fibronectin and collagen in which they will have to go through in order to reach the blood vessels and then the metastatic site. Fibronectin adhesion receptors such as αvβ3 and α5β1 integrins are essential for cells to be able to move through the fibronectin fibers. Integrins bind to the extracellular matrix (ECM) proteins via the RGD motif present in fibronectin, making these cell types migration receptors a potential target for the study of cancer. Thus, the effect of Dis*Ba*-01, an RGD containing motif disintegrin, was investigated in two cell specialized on migration; fibroblasts (BJ) and oral squamous cell carcinoma (SCC25). Dis*Ba*-01 (1 and 2 µM) was able to reduce cancer cells migration speed and directionality, but not fibroblasts, on time-lapse experiments performed on fibronectin coated plates. In order to understand if the effect of Dis*Ba*-01 is associated with alterations in cellular adhesion, cells were plated on fibronectin and stained with anti-paxillin. Images acquired by confocal microscopy show changes in focal adhesions, which have become larger and more numerous. These data were confirmed, by time-lapse assays using Total Internal Reflection Fluorescence (TIRF) in cells transfected with paxillin. The expression of key fibronectin receptors, α5, β3 and αv is present similarly in both strains, while the β1 subunit is expressed in lower amounts in OSCC cells. To investigate the probable relationship of migration inhibition with integrin β3 level, this subunit was overexpressed in the OSCC cells and all parameters analyzed were restored. Taken together, these results demonstrate the ability of Dis*Ba*-01 to affect cell migration depending on integrin expression level and by modulating the dynamics of cell adhesions.

MOTIVOS ADESIVOS DAS DESINTEGRINAS

ECD	Ácido glutâmico - Cisteína - Ácido aspártico
DCD	Ácido aspártico - Cisteína - Ácido aspártico
KTS	Lisina - Treonina – Serina
KGD	Lisina - Glicina - Ácido aspártico
MGD	Metionina - Glicina - Ácido aspártico
MLD	Metionina - Leucina - Ácido aspártico
MVD	Metionina - Valina - Ácido aspártico
RGD	Arginina - Glicina - Ácido aspártico
RTS	Arginina - Treonina – Serina
VGD	Valina - Glicina - Ácido aspártico
WGD	Triptofano - Glicina - Ácido aspártico

LISTA DE ABREVIATURAS

ADAMs - A Disintegrin And Metalloproteinase

BCAR1 - Breast Cancer Anti-estrogen Resistance protein 1

CRISP - Proteína secretora rica em cisteína (*Cistein-Rich Secretory Protein*)

DNA – Ácido desoxiribonucéico

EGF – Fator de Crescimento Epidermal (*Epidermal Growth Factor*)

EMT – Transição epitélio-mesenquimal (*Epitelial-Mesenquimal Transition*)

FAK – Quinase de adesão focal (*Focal Adhesion Kinase*)

FGF – Fator de crescimento de fibroblasto (*Fibroblast Growth Factor*)

HCV – Vírus da Hepatite C (*Hepatitis C Virus*)

HIF – Hipoxia Inducible Factors

HPV – Human Papiloma Virus

LAOs - L-aminoácido oxidases

MAT – Transição mesenquimal-amebóide (*Mesenquimal-Ameboid Transition*)

MMPs – Metalopeptidases de Matriz (*Matrix Metalloproteinases*)

MPO – Mieloperoxidase **(***Myeloperoxidase)*

MEC – Matriz Extracelular

OMS – Organização Mundial da Saúde

OSCC – Carcinoma de células escamosas oral (*Oral Squamous Cells Carcinoma*)

PLA_2 – Fosfolipase A_2 (*Phospholipases A_2*)

PDGF – Fator de Crescimento derivados de plaquetas (*Platelets Derived Growth Factor*)

PPB – Peptídeos Potenciadores de Bradicinina

SVMP – Metaloprotease de veneno de serpente (*Snake venom Metalloproteinase*)

TIRF – *Total Internal Reflection Fluorescence*

TNF-α – Fator de Necrose Tumoral-α (Tumor Necrosis Factor-α)

VEGF – Fator de crescimento endotelial vascular (*Vascular Endothelial Growth Factor*)

VEGFR – Receptor do fator de crescimento endotelial vascular (*Vascular Endotelial Growth Factor Receptor*)

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1. INTRODUÇÃO

1.1 CÂNCER E O MICROAMBIENTE TUMORAL

De acordo com a Organização Mundial da Saúde (OMS), em 2030 ocorrerão 27 milhões de novos casos de câncer em todo o mundo, contabilizando 17 milhões de mortes e 75 milhões de pessoas vivendo com a doença. No Brasil, para o ano de 2016, estima-se o registro de 596 mil novos casos de câncer, sendo 205 mil em mulheres e 214 mil em homens, excluindo-se os de pele não melanoma. Em homens, os tipos mais comuns são: próstata, traquéia, brônquios e pulmão, cólon e reto, estômago e cavidade oral. Em mulheres são: mama, cólon e reto, colo de útero, traqueia, brônquios e pulmão e estômago. Devido a esses números alarmantes, o câncer é considerado um problema de saúde pública não apenas no Brasil, mas no mundo ("Estimativa 2014", 2014, "INCA - Dia Nacional de Combate ao Câncer, 2015"). Apesar disso, o conhecimento sobre a biologia do câncer tem levado ao desenvolvimento de novos fármacos, que melhoraram a qualidade de vida e sobrevivência dos pacientes, além de conhecimento sobre novos alvos para os quais novas drogas poderão ser desenvolvidas (MA; ADJEI, 2009).

Por definição, o câncer é uma síndrome de mais de 100 doenças relacionadas com o crescimento anormal de células, capazes de invadir os tecidos vizinhos e afetar, praticamente, qualquer parte do corpo ("Estimativa 2014", 2014, "What Is Cancer? - National Cancer Institute" [s.d.]).

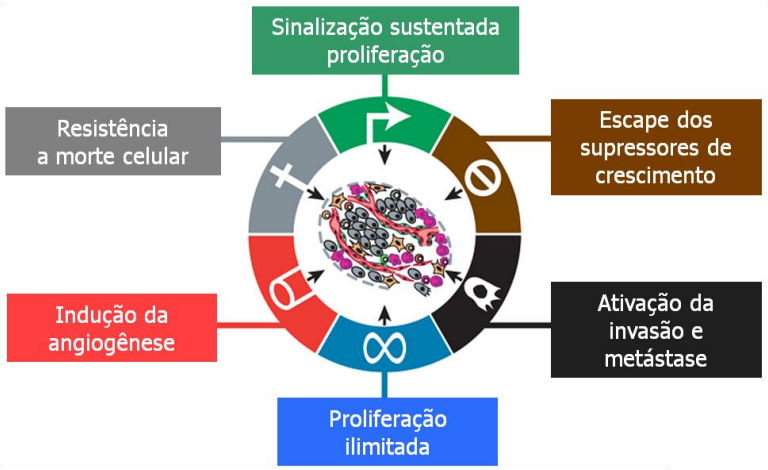
O tumor maligno é proveniente de uma série de mutações no DNA das células, que podem ser causadas por diferentes agentes mutagênicos como os componentes inalados do cigarro, radiação ultravioleta, inflamação crônica ou adquirida de forma hereditária, entre outros, que conferem as células uma grande capacidade de proliferação e adaptação ao microambiente (MBEUNKUI; JOHANN, 2009; STRATTON; CAMPBELL; FUTREAL, 2009).

Durante a evolução do tumor, alterações genéticas e/ou epigenéticas adquiridas pelas células, acumulam-se ao longo dos processos de divisão celular e fazem com que elas se tornem capazes de evadir do sistema imune, sobreviver aos tratamentos e formar metástases em diferentes órgãos, podendo levar o paciente à morte (ALTROK; LIU; MICHOR, 2015).

Segundo Hanahan e Weinberg, as células de câncer possuem seis características cruciais, que permitem o desenvolvimento do tumor e a evolução do câncer, como

demonstrado na figura 1. São elas: estímulo contínuo à proliferação celular, evasão dos inibidores de crescimento, estímulo à invasão e metástase, potencial replicativo ilimitado, indução da angiogênese e resistência à morte celular (HANAHAN; WEINBERG, 2011).

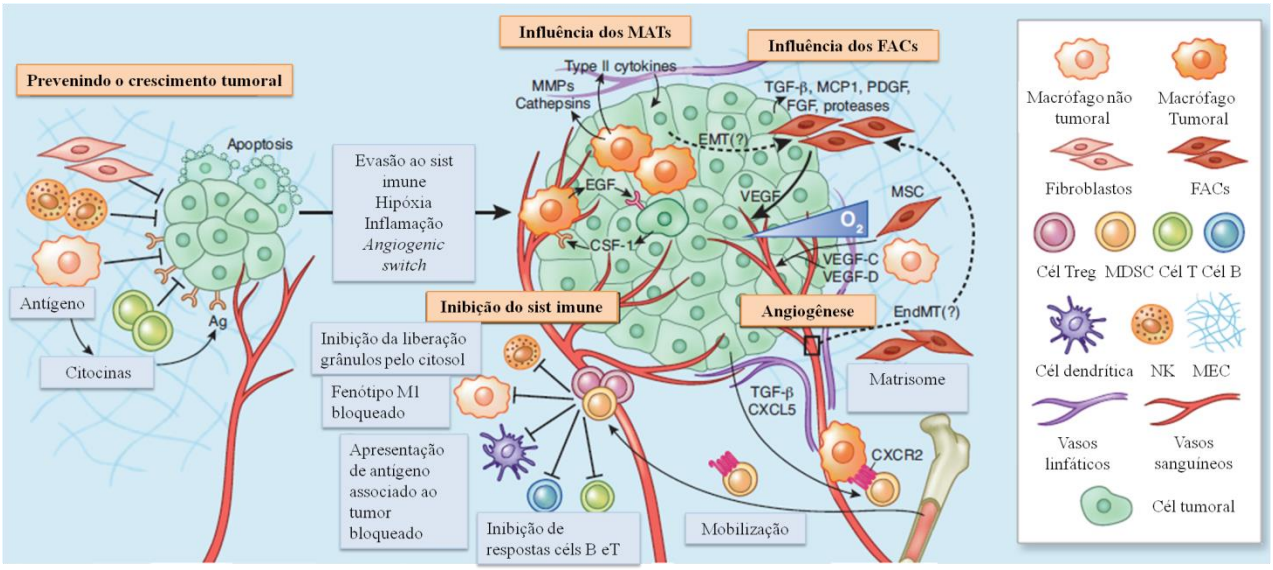
Figura 1. Alterações celulares necessárias para o desenvolvimento do câncer.



Fonte: Modificado de Hanahan e Weinberg, 2011.

Apesar dos estímulos, as células cancerígenas não agem de forma independente, elas são influenciadas pelo meio ambiente que as cercam, podendo adaptar seu fenótipo a medida que este meio é modificado (BISSELL; HINES, 2011; FRIEDL; ALEXANDER, 2011). No microambiente onde o tumor se desenvolve (Figura 2) estão presentes diferentes elementos incluindo desde a matriz extracelular (MEC), vasos sanguíneos e linfáticos, diversos tipos celulares e fatores de crescimento (BHOWMICK; NEILSON; MOSES, 2004; MBEUNKUI; JOHANN, 2009). Esse conjunto de estruturas, células e moléculas que compõe o microambiente tumoral é considerado crítico, na medida que pode influenciar a transformação de células normais para células cancerígenas, assim como a progressão do câncer (PICKUP; MOUW; WEAVER, 2014).

Figura 2. Microambiente tumoral; à esquerda a figura mostra o início do crescimento do tumor, onde células do sistema imune tentam suprimir o seu crescimento enquanto à direita, as mesmas células, em conjunto com outros tipos celulares se tornam parte to tumor.



Fonte: modificado de Quail e Joyce, 2013.

A presença de células inflamatórias nesse ambiente foi vista, inicialmente, como tentativas do organismo para eliminar o tumor. No entanto, uma resposta inflamatória crônica pode agir de forma a criar condições favoráveis para o crescimento do tumor e estimular a proliferação, inibir a morte celular, assim como levar ao aumento da angiogênese e metástases (CHAI et al., 2015; HANAHAN; WEINBERG, 2011). Além disso, doenças que levam a inflamação crônica, como a obesidade, Doença de Crohn e infecções virais como a do HPV (*Human Papiloma Virus*), *Helicobacter pylori* e o vírus da Hepatite C (HCV), geram instabilidade nas moléculas de DNA predispondo as células ao surgimento de mutações e, consequentemente, ao desenvolvimento de alguns tipos de câncer (CHAI et al., 2015; GRIVENNIKOV; KARIN, 2010).

A medida que o tumor cresce, as células inflamatórias presentes no seu microambiente começam a receber estímulos provenientes das células tumorais. Fibroblastos, macrófagos, células endotelias, entre outros tipos celulares respondem a esses estímulos produzindo fatores de crescimento, citocinas, quimiocinas e metaloproteases de forma a favorecer o crescimento das células tumorais ao invés de combatê-las (QUAIL; JOYCE, 2013).

A MEC é composta por proteínas, glicosaminoglicanos, proteloglicanos e fatores de crescimento produzidos principalmente por fibroblastos e células endoteliais

(HARISI; JENEY, 2015; HYNES, 2011; WORTHLEY; GIRAUD; WANG, 2010). A MEC tumoral não funciona apenas como uma estrutura tridimensional, necessária para sustentação e homeostase dos tecidos**.** Como mencionado anteriormente, ela influencia o comportamento do tumor, agindo de forma a favorecer sua sobrevivência e crescimento (NABA et al., 2014).

A proporção das moléculas presentes na MEC é única nos diferentes tipos de tumores, assim proteínas como a fibronectina, tenascin-C e colágeno podem estar presentes em maior ou menor quantidade nos diferentes tumores (GOETZ, 2012; HARISI; JENEY, 2015; PICKUP; MOUW; WEAVER, 2014). Um exemplo interessante é o da fibronectina, que está ausente na mucosa oral de adultos, mas em carcinomas de células escamosas orais bem diferenciadas ela se encontra em alguns pontos do estroma e na frente de invasão. Em cânceres de células escamosas orais menos diferenciados, e portanto mais agressivos, a fibronectina se distribui de forma difusa, também no estroma e nas frentes de invasão. Ou seja, a medida que o grau de diferenciação desse câncer diminui e este se torna mais agressivo, o teor de fibronectina aumenta (KOSMEHL et al., 1999).

1.2 ANGIOGÊNESE

Todo o estímulo da MEC e das células inflamatórias para o tumor seria em vão, caso as células endotelias não estivessem presentes. A formação de novos vasos sanguíneos é necessária, para que ocorra a manutenção da sobrevivência celular em áreas onde a oferta de nutrientes e oxigênio é restrita. Na ausência dos novos vasos, a massa tumoral não cresce além de 1-4 mm^3 (HILLEN; GRIFFIOEN, 2007; SAMPLES; WILLIS; KLAUBER-DEMORE, 2013).

No tumor, existem diferentes mecanismos para a indução da formação de novos vasos sanguíneos e entre os mais comuns estão a angiogênese; formação de novos vasos a partir outros já existentes e a vasculogênese que é formação de vasos a partir de células endoteliais precursoras (SAMPLES; WILLIS; KLAUBER-DEMORE, 2013).

A angiogênese é o principal mecanismo de formação de novos vasos dentro do tumor. Ela ocorre a partir de uma mudança no equilíbrio entre fatores pré e anti-angiogênicos. A medida que o tumor cresce, a oxigenação de algumas áreas não ocorre. Assim, vai haver o acúmulo de HIF (*Hypoxia Inducing Factor*), que levará a produção do VEGF-A (*Vascular Endothelial Growth Factor*) , FGF (*Fibroblast Growth Factor*) e PDGF (*Platelet Derived Growth Factor*). Esses fatores pró-angiogênicos se ligam às

células endoteliais e estimulam sua proliferação e migração celular, e a produção de metaloproteases (MMPs). Assim, a membrana basal e a matriz extracelular que as cercam serão degradadas, para que as células endoteliais migrem e formem o novo vaso necessário para levar nutrientes e oxigênio para as o interior do tumor (HANAHAN; WEINBERG, 2011; SAMPLES; WILLIS; KLAUBER-DEMORE, 2013; TONINI; ROSSI; CLAUDIO, 2003).

O VEGF tem um papel central na regulação da angiogênese, portanto é uma molécula fundamental para o crescimento tumoral (LANGENKAMP et al., 2012). A ligação do VEGF-A, membro da família VEGF relacionado a angiogênese, ao seu receptor tirosina quinase VEGFR2 é responsável pelos efeitos clássicos do VEGF, como aumento da proliferação e migração de células endoteliais, formação de tubo, assim como aumento da permeabilidade vascular. Já a interação com o VEGFR1, resulta em hematopoiese, formação de vasos embrionários e migração de macrófagos. No câncer, o VEGFR1 estimula a angiogênese em carcinomas e glioblastomas e a metástase tumoral (SAMPLES; WILLIS; KLAUBER-DEMORE, 2013; SHIBUYA, 2014).

1.3 METÁSTASE

Com o surgimento de novos vasos, o tumor primário será capaz de expandir, levando a um aumento na quantidade de células que entram na circulação e consequentemente as chances de ocorrer metástases (HANAHAN; WEINBERG, 2011).

A metástase é responsável por 90% das mortes entre os pacientes com câncer e é composta por uma cascata de eventos celulares e moleculares, ainda não totalmente elucidados, que culminam no crescimento de um ou mais tumores secundários em localizações distintas ao tumor primário (ALIZADEH; SHIRI; FARSINEJAD, 2014).

A cascata de eventos tem início com a desadesão de células do tumor primário, que migram através de tecidos vizinhos, atingindo os vasos sanguíneos ou linfáticos**.** Nessa etapa, apenas 0.01% das células transformadas que intravasam conseguem sobreviver na corrente sanguínea para formar metástases. Elas formam agregados com as plaquetas, que as protegem contra o ataque de células do sistema imune e facilitam sua adesão à parede do endotélio no sítio metastático, para em seguida extravasar e colonizar o sítio secundário (QUAIL; JOYCE, 2013). Entretando, elas ainda podem sofrer apoptose, por não se adaptar ao novo ambiente, permanecer dormentes ou

proliferar estimulando a angiogênese e recrutando células do estroma, promovendo o desenvolvimento do novo tumor (CHAFFER; WEINBERG, 2011).

Os novos sítios de metástases são formados em locais pré-condicionados para receber as células tumorais, sendo denominados de nichos pré-metastáticos. Fatores de crescimento secretados pelo tumor primário estimulam a produção de fibronectina, proteínas pró-inflamatórias e recrutam macrófagos, neutrófilos e mastócitos que preparam o local para ser colonizado por células tumorais (SEMENZA, 2015).

De acordo com Alizadeh et al., 2014 a cascata metástatica pode ser dividida em 5 etapas: dissociação, invasão, intravasamento, extravasamento e dormência. Em todas as etapas, os processos e invasão e adesão das células tumorais são essenciais. Elas precisam ser capazes de se aderir aos componentes do microambiente, de modo que possam migrar através da matriz e, consequentemente metastatizar de forma eficiente (ALIZADEH; SHIRI; FARSINEJAD, 2014; YILMAZ; CHRISTOFORI, 2010).

O tumor é composto por diferentes subpopulações de células que possuem diferentes comportamentos, variando na taxa de proliferação, imunogenicidade, resposta aos fármacos e na habilidade para formar metástases. Esses diferentes comportamentos podem ser resultado de diferentes aportes de nutrientes e oxigênio nas diferentes partes do tumor (TABASSUM; POLYAK, 2015).

1.4 TRANSIÇÃO EPITÉLIO-MESENQUIMAL

Células tumorais localizadas nas margens do tumor dão início à metástase passando por um processo denominado de Transição Epitélio-Mesenquimal (EMT: *Epithelial-Mesenquimal Transition*). Em tumores epiteliais as células perdem seu fenótipo, através da perda da polaridade ápico-basal, redução da expressão de E-caderina, alteração das junções celulares e aumento da expressação de N-caderina, vimentina, fibronectina, Twist1, proteínas *zinc fingers,* fibras de estresse e metaloproteases (MMPs), características de células mesenquimais, que resultam no aumento da mobilidade celular (CATALANO et al., 2013). A EMT capacita as células tumorais a sobreviverem em microambientes hostis e tem papel fundamental na promoção de metástases, resistência às drogas e na recorrência do câncer (WONG et al., 2014).

Células que passam pela EMT se tornam indiferenciadas, com um comportamento similar às células mesenquimais e adquirem as características necessárias para se desprender do tumor primário e iniciar o processo de invasão dos

tecidos em direção a corrente sanguínea, um passo crucial da cascata metastática. O nível de diferenciação das células do tumor pode ter relação com seu prognóstico, quanto menos diferenciada for a célula, maior será sua capacidade migratória (ALIZADEH; SHIRI; FARSINEJAD, 2014).

Uma molécula que pode ser usada para indicar o nível de diferenciação celular é a E-caderina. Ela pertence uma família de glicoproteínas transmembrana, composta de mais de 20 membros responsáveis pela a adesão intercelular. São expressas em células diferenciadas, como as epiteliais e são as principais moléculas responsáveis pela adesão célula-célula. A disfunção ou a ausência de E-caderinas está relacionada a carcinomas invasivos. Um outro membro da mesma família, as N-caderinas, parecem possuir uma função inversa a das E-caderinas estando presente em células mesenquimais e endoteliais. Sua expressão está diretamente relacionada com a diminuição de E-caderinas e com o aumento da motilidade de células tumorais, podendo inclusive estimular fenótipos mesenquimais em cânceres de células escamosas (ALIZADEH; SHIRI; FARSINEJAD, 2014). Portanto, a diminuição da expressão de E-caderinas e o aumento das N-caderinas pode ser capaz de promover a desadesão da célula transformada do tumor primário e o início do processo de migração celular.

1.5 MIGRAÇÃO CELULAR

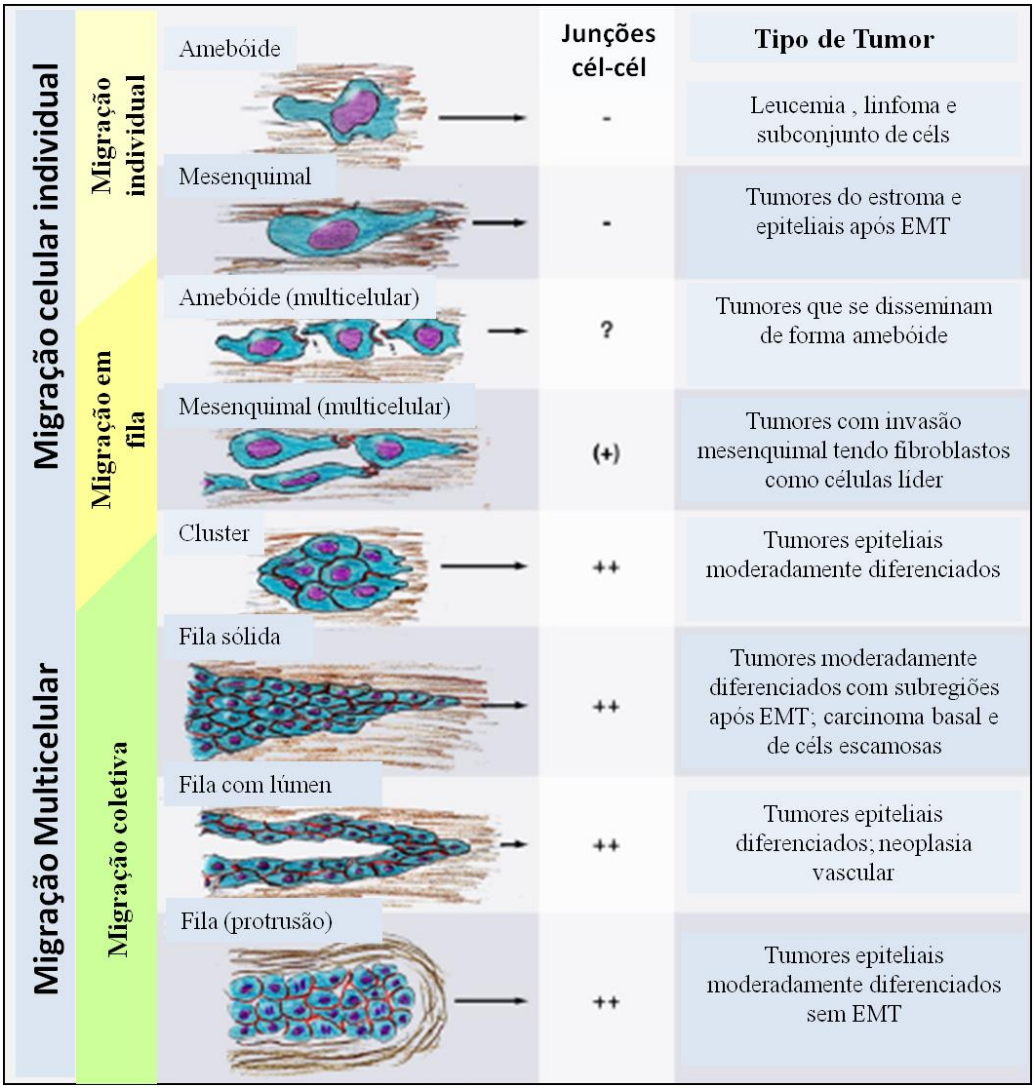
É um processo complexo que envolve uma sequência de eventos bem orquestrados e com a participação de várias moléculas diferentes. A migração celular é crucial para o desenvolvimento normal dos tecidos, cicatrização de feridas e resposta imune. Contudo, em doenças como o câncer, ela ocorre de forma desregulada e disseminada se tornando um mecanismo chave para a progressão tumoral (CHARRAS; SAHAI, 2014; VEISEH et al., 2011).

Em ambas situações, fisiológica ou patológica, células possuem diferentes modos de migração (individual ou coletiva) que diferem na mofologia celular, geração de força contrátil, organização do citoesqueleto e interação célula-substrato (WELCH, 2015). Células do sistema imune como os leucócitos migram de forma individual, respondendo rápidamente a estímulos, enquanto células epiteliais migram de forma coletiva como durante o fechamento de feridas (RØRTH, 2011). Células tumorais podem migrar de diferentes formas, dependendo do tipo de tumor e do ambiente em que se encontram (KURNIAWAN; CHAUDHURI; LIM, 2015).

A migração individual pode ser classificada em amebóide ou mesenquimal, (Figura 3). As células que passam pela EMT e se desaderem do tumor e passam a migrar no modo mesenquimal. Elas adquirem forma de fuso, possuem protrusões proeminentes, adesões que interagem fortemente como substrato (dependentes de integrinas), liberam proteases capazes de clivar a MEC, são ricas em fibras de estresse e em actina na frente de migração. O fibroblasto é um exemplo clássico de célula que migra desse modo. Ele possui uma morfologia alongada, liberando MMPs que vão falcilitar sua passagem por entre as fibras da MEC e é um tipo de migração mais lento devido a forte interação da célula com as proteínas da matriz (KURNIAWAN; CHAUDHURI; LIM, 2015; WELCH, 2015). Células de carcinoma e sarcoma, mais comumente, apresentam esse tipo de migração.

No movimento tipo amebóide as células adquirem um formato arredondado ou elipsóide que se deforma rapidamente, permitindo que essas células se comprimam para passarem por entre as fibras da matriz. Elas migram rapidamente, possuem interações fracas com os substratos adesivos (são pouco dependentes de receptores de adesão), formam pequenas protrusões e não são capazes de liberar enzimas proteolíticas (WELCH, 2015; WOLF et al., 2013). O fato de serem pouco dependentes de receptores de adesão faz com que essas células consigam se movimentar mais facilmente por entre as diferentes fibras da MEC. Existem duas subcategorias para esse modo migração: na primeira, as células usam propulsões de *blebs*, utilizados por células do sistema imune e na segunda, projeções de filopódia, como apresentadas na Figura 3, ricas em actina, tais como as utilizadas por células germinativas de peixe-zebra. A diferença entre os modos de migração amebóide está na frente de migração que, no primeiro modo, não possui actina, enquanto o segundo possui filopódias ricas em actina. Leucócitos, células dendrídicas, células tronco hematopoiéticas e células transformadas de leucemias e linfomas migram dessa forma (KURNIAWAN; CHAUDHURI; LIM, 2015; WELCH, 2015).

Figura 3. Modos de migração celular no câncer. Em cada tumor predomina um modo de migração, entretanto mais de um modo pode ocorrer dependendo do nível de diferenciação das células. Além disso, esses modos podem ser convertidos entre si dependendo das interações célula-célula, contratilidade do citoesqueleto e reciclagem das adesões.



Fonte: modificado de Frield e Alexander, 2011.

Na migração coletiva as células se locomovem em conjunto, mantendo a sinalização e o contato intercelular. Um grupo de células se desadere do tumor mantendo as junções célula-célula, através de E-caderinas, e uma célula líder abre caminho por entre as fibras da matriz. As células líder passam pela EMT, adquirindo características mesenquimais, mas ainda aderidas ao *cluster* de células, com alta mobilidade e liberando metaloproteases que irão clivar as fibras da MEC e facilitar a passagem do grupo. O *cluster* de células tem seu movimento coordenado, através da força de adesão entre as células, inibição do movimento celular por contato, força da

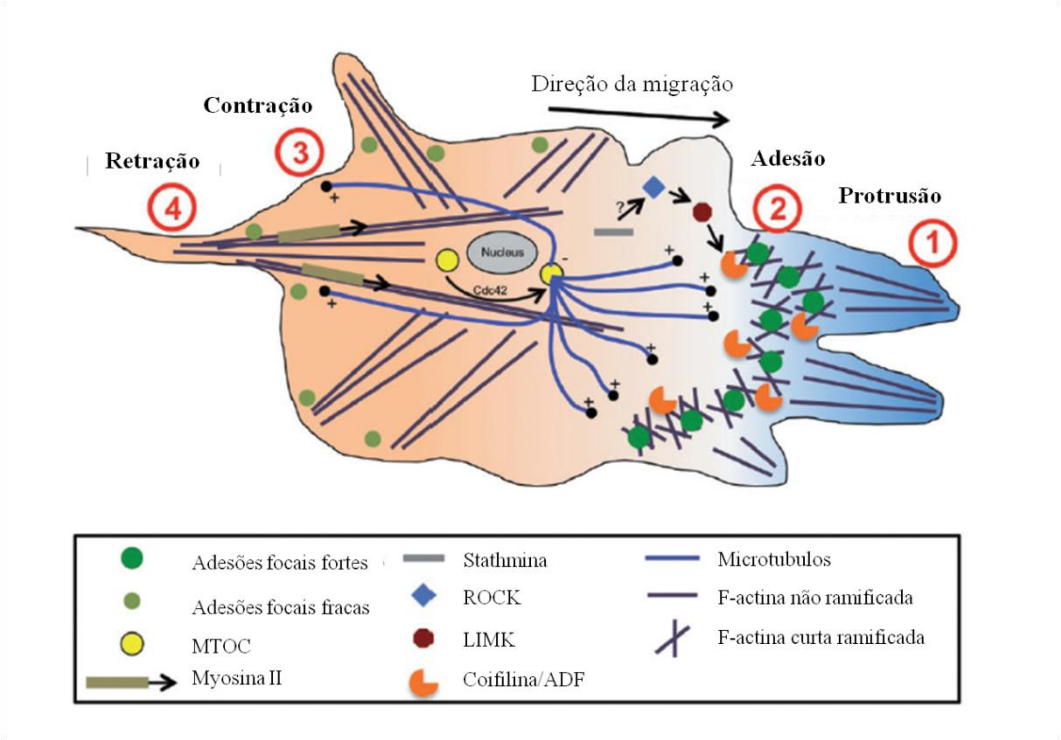
tração exercida no substrato**,** limitações espaciais e densidade celular. Células de melanoma e cânceres de origem epitelial como carcinomas migram dessa forma (HAEGER et al., 2014; KURNIAWAN; CHAUDHURI; LIM, 2015).

As células tumorais são plásticas, portanto possuem a habilidade de alterar seu fenótipo e transitar entre diferentes modos de migração dependendo das condições do meio extracelular e de mecanismos de sinalização extra e intracelulares (CHARRAS; SAHAI, 2014; KURNIAWAN; CHAUDHURI; LIM, 2015; WELCH, 2015). Algumas das características do microambiente que influenciam alterações nos modos de migração são: o confinamento celular, tamanho dos poros e geometria da MEC, dureza e flexibilidade, densidade e os tipos de *crosslink* dos componentes da matriz (HAEGER et al., 2014). Matrizes com poros largos favorecem o arredondamento celular, enquanto poros menores, que restringem o movimento celular, levam ao alongamento celular e a utilização de proteases. Entretanto, em condições de baixa adesividade no plano 2D e alta densidade da matriz no plano 3D, fibroblastos alteram sua forma de migrar de mesenquimal para amebóide, um modo de migração mais rápido, passando pela transição mesenquimal-ameboide (MAT – *Mesenquimal-Ameboid Transition*) (LIU et al., 2015; WELCH, 2015).

1.6 MECANISMOS MOLECULARES DA MIGRAÇÃO

Mudanças físicas e químicas (proteínas presentes na MEC e quimioatraentes, respectivamente) no microambiente irão estimular a migração celular, levando todo o citoesqueleto a se reorganizar para que a célula possa efetuar o movimento (FIFE; MCCARROLL; KAVALLARIS, 2014; MOGILNER; KINNERET, 2009). Assim, a migração tem início com a protrusão da membrana na frente de migração, causada pela polarização dos filamentos de actina (Figura 4). A protrusão se conecta às fibras da MEC através de *clusters* formados por receptores de adesão (integrinas), que estão conectados ao citoesqueleto de actina na sua porção intracelular. Imediatamente à frente de migração ocorre a degradação da matriz por metalopeptidases (MMPs), enquanto o citoesqueleto contrai o corpo e cauda celular causando a translocação do corpo celular. Desssa forma, as adesões presentes na cauda da célula começam a se desfazer, a cauda da célula retrai e a membrana presente na frente de migração dá início a uma nova protrusão e a reciclagem gradual das adesões ocorre (PARSONS; HORWITZ; SCHWARTZ, 2010a; WOLF et al., 2013).

Figura 4. Migração celular em etapas. 1.Protrusão, 2. Adesão, 3. Contração, 4. Retração.



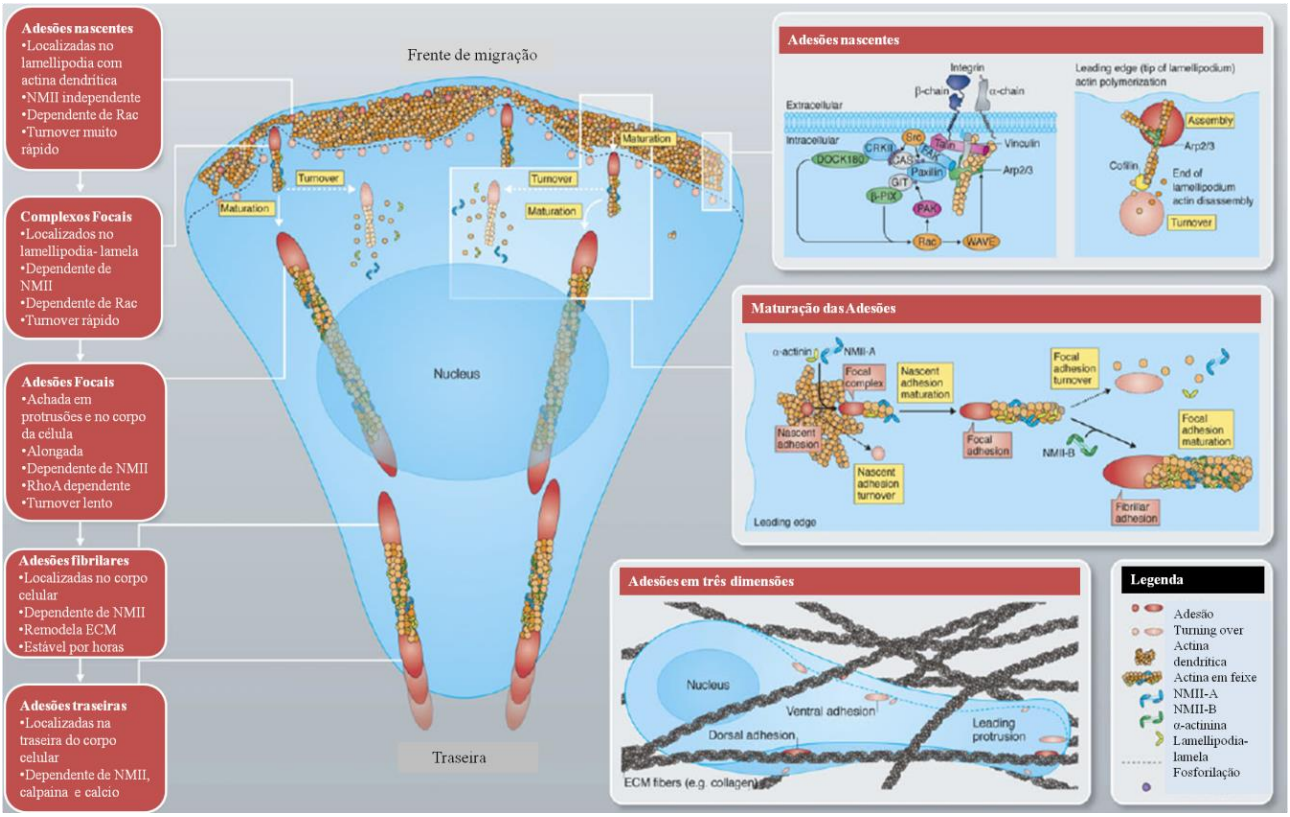
Fonte: modificado de Fife, McCarroll e Kavallaris, 2014.

A protusão da membrana celular pode ser, principalmente, em forma de lamelipódia, como um lençol que se estende na frente de migração de celular, de pseudopódia, protrusões na forma de espinhos ou ainda em ambos. As protrusões, na medida que se estendem, são estabilizadas pela adesões focais, estruturas macromoleculares que conectam as proteínas da MEC ao citoesqueleto de actina e agem como âncoras, fixando a célula a matriz enquanto o corpo celular se contrai e novas adesões se formam a frente (KURNIAWAN; CHAUDHURI; LIM, 2015; PARSONS; HORWITZ; SCHWARTZ, 2010b).

Cerca de 160 moléculas fazem parte da estrutura das adesões focais e, entre elas, reguladores do citoesqueleto de actina, proteínas adaptadoras, moléculas sinalizadoras e seus moduladores (GEIGER; SPATZ; BERSHADSKY, 2009). Como indicado na figura 5, as adesões começam a se formar bem próximas à membrana celular em protrusão (lamelipódia) e são denominadas de adesões nascentes. A medida que a membrana protrui, algumas dessas adesões se desfazem e outras continuam a crescer, aumentando sua interação com os componentes da matriz, passando a ser chamadas de complexos focais. Com a continuação da protrusão, alguns dos complexos focais continuam a amadurecer formando adesões focais. O contínuo movimento celular faz com que as adesões se localizem de forma cada vez mais posterior e sempre

aumentando de tamanho, sendo denominada de adesões fibrilares e finalmente adesões traseiras, quando atingem a "cauda" da célula e se desfazem permitindo a retração da membrana (DI CIO; GAUTROT, 2016; VICENTE-MANZANARES; HORWITZ, 2011).

Figura 5. Maturação das adesões celulares. A figura exemplica de maneira completa o mecanismo da maturação das adesões.



Fonte: modificado de Vicente-Manzanares e Horwitz, 2011.

Integrinas são as moléculas que dão início às adesões, elas são ativadas e formam os *clusters,* que inicialmente são compostos de 3 ou 4 moléculas (adesões nascentes) (GEIGER; SPATZ; BERSHADSKY, 2009). Moléculas adaptadoras como talina e vinculina fazem a ligação entre as integrinas e os filamentos de actina, participando da adesão desde sua formação. Imediatamente após, moléculas como a kindlina, paxilina, α-actinina e tensina se reunem à adesão reforçando a ligação aos filamentos do citoesqueleto de actina, além de proteínas sinalizadoras como a FAK (*Focal Adhesion Kinase*) e a BCAR1 (*Breast Cancer Anti-estrogen Resistence 1*). Nesse primeiro momento, as adesões não dependem das forças contráteis da miosina II, mas já possuem o conjunto de moléculas sinalizadoras e estruturais necessárias para a formação das adesões focais. Moléculas como a FAK, vinculina, paxilina e BCAR1 são

fosforiladas criando sítios de ligação para outras proteínas e promovendo a polimerização da actina. Portanto, a medida que a protrusão avança, as adesões amadurecem, o número de moléculas aumenta, consequentemente, aumentando o tamanho das adesões, assim como sua força contrátil e o seu *turnover* (DI CIO; GAUTROT, 2016; GEIGER; SPATZ; BERSHADSKY, 2009; VICENTE-MANZANARES; HORWITZ, 2011; WEHRLE-HALLER, 2012).

A paxilina, molécula citada acima, está presente desde o momento da formação das adesões, onde ela é fosforilada em diferentes sítios de tirosina, serina e treonina, regulando a interação de diversas proteínas com seus ligantes, até o momento em que as adesões se desfazem, se tornando um importante marcador (BROWN; TURNER, 2004; DEAKIN; TURNER, 2008). Ela é uma molécula adaptadora que facilita a transdução de sinais através do recrutamento de intermediários de vias de sinalização específicas, agindo também como plataforma para o *crosstalk* entre diferentes vias através do recrutamento de intermediários em comum. Essa integração permite qua as células respondam as mudanças no ambiente externo em várias frentes incluindo modulação da organização do citoesqueleto, assim como mudanças na expressão genética (BROWN; TURNER, 2004).

Todos esses passos da migração são coordenados principalmente pela família Rho GTPases. As integrinas transmitem sinais recebidos do meio extracelular, que ativam membros da família Rho GTPases para regular cascatas de sinalização relacionadas à dinâmica do citoesqueleto , direcionalidade da célula, adesão e desadesão das junções célula-célula (FIFE; MCCARROLL; KAVALLARIS, 2014). As principais moléculas efetoras da família, ligadas à motilidade celular, são a Rho, Rac e Cdc42. Estas moléculas agem na formação de fibras de stress e contração do citoesqueleto de actomiosina, promovem a formação de lamelipódia e organização do citoesqueleto de microtubulos, respectivamente (SANZ-MORENO et al., 2008).

A evolução da tecnologia permitiu um avanço significativo das pesquisas sobre migração celular. Diferentes técnicas tem sido utilizadas para investigar o comportamento e o mecanismo das células em movimento. Em estudos morfológicos, pode-se observar o fenótipo celular, para identificar as características migratórias dos diferentes tipos celulares e suas respostas aos diferentes estímulos, além de características morfológicas. Essas observações podem ser realizadas com experimentos utilizando microscopia de campo claro e filmes de *time-lapse,* que são ferramentas amplamente utilizadas para esse tipo de estudo (JUSTUS et al., 2014; MASUZZO et al.,

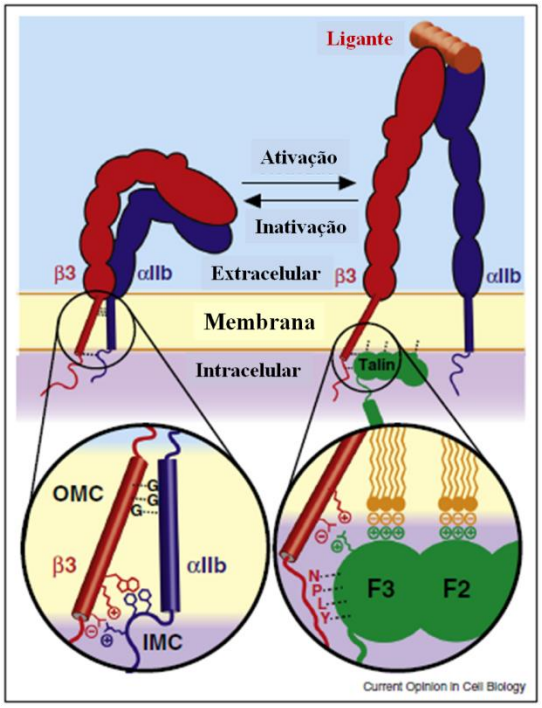
2015). Em estudos relacionados aos mecanismos da migração celular a microscopia de fluorescência de reflexão interna total (TIRF- Total Internal Reflextion Fluorescence), um dos tipos de técnicas microscópias utilizadas nesse estudo, tem ganhado muito espaço. Ela permite a visualização de eventos que ocorrem na membrana basal das células ou imediatamente acima dela assim, processos de endo e exocitose, adesão celular e dinâmica da moléculas do citoesqueleto podem ser observados em alta resolução (VERVEER, 2015).

1.7 INTEGRINAS

Como citado acima, as integrinas são moléculas chave na migração celular. Atualmente, sabe-se da existência de 18 subunidades α e 8 subunidades β formando 24 heterodímeros diferentes (GINSBERG, 2014; IWAMOTO; CALDERWOOD, 2015). Cada subunidade possui uma porção extracelular longa, um domínio transmembrana e uma curta cauda citoplasmática. As integrinas se ligam às proteínas da MEC, no meio extracelular, enquanto que a cauda citoplasmática se liga à quinases e diversas proteínas acessórias, que regulam processos além da migração como proliferação, sobrevivência celular e apoptose. Além da sua função mecânica de ancoramento, as integrinas possuem um papel de receptor, que transduz sinais para o meio intracelular ativando cascatas de sinalização, que regulam a expressão de genes e mudanças no fenótipo das células (DESGROSELLIER; CHERESH, 2010; GINSBERG, 2014).

As duas extremidades das integrinas, uma no exterior e outra no interior da célula podem transmitir os sinais de forma bidirecional. Quando o heterodímero interage com uma proteína da MEC o sinal transmitido de fora para dentro é denominado de sinalização *outside-in*, enquanto que, quando moléculas do meio intracelular interagem com determinada integrina, essa sinalização é denominada de *inside-out* (de dentro para fora). Essa sinalização, no entanto é regulada por estados de ativação ou inativação, nos quais a conformação da integrina é alterada, para que ela possua uma maior ou menor afinidade pelos seus ligantes, como é mostrado na figura 6. Interações, seja pelo meio intra ou extracelular, podem fazer com que a integrina passe de uma conformação inativa, na qual ela se encontra dobrada e com as duas subunidades unidas na porção transmembrana, para uma conformação ativa, na qual as subunidades interagem com proteínas do meio intracelular e desfazem a ligação entre os monômeros, resultando em interações de maior afinidade da integrina com seu ligante (DESGROSELLIER; CHERESH, 2010; IWAMOTO; CALDERWOOD, 2015).

Figura 6. Integrina em seu estado ativo e inativo. À esquerda a integrina se encontra em uma conformação dobrada com seus motivos transmembrana unidos, enquanto à direita a integrina se encontra sob a forma ativa, com os motivos transmembrana desconectados e ligada a um alvo.

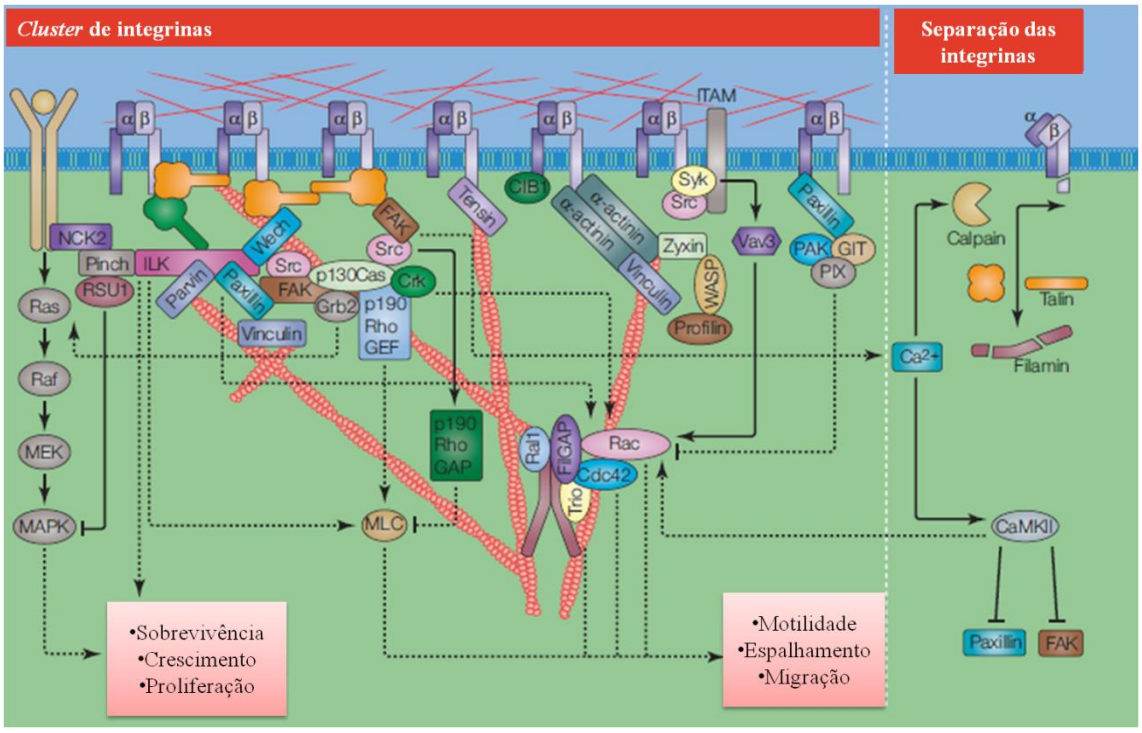


Fonte: modificado de Iwamoto e Calderwood, 2015.

A conformação ativa leva as integrinas a se agruparem formando estruturas como os *clusters* (figura 7), que no processo de migração dão origem as adesões nascentes. Esse agrupamento aumenta ainda mais a afinidade, a avidez da ligação das integrinas por seus alvos e ativam as vias de sinalização intracelulares (DI CIO; GAUTROT, 2016; MAINALI; SMITH, 2013).

Os heterodímeros possuem afinidade por diferentes sequências adesivas presentes tanto em pequenos peptídeos, como em proteínas da MEC (REEKS; FRY; ALEWOOD, 2015). Uma integrina pode ter afinidade por diferentes alvos e diferentes integrinas podem se ligar a um mesmo alvo, mas mediar funções específicas. Um exemplo são as integrinas $\alpha_5\beta_1$ e $\alpha_v\beta_3$, receptores clássicos que se ligam à sequência RGD presente na fibronectina, que estão envolvidas em funções diferentes relacionadas a motilidade celular sobre esta proteína. Enquanto a $\alpha_5\beta_1$ se faz necessária na adesão das células à fibronectina, a $\alpha_v\beta_3$ age principalmente na motilidade celular (DI CIO; GAUTROT, 2016; MISSIRLIS et al., 2016).

Figura 7. Cluster de integrinas. A esquerda pode-se observar a clusterização de integrinas e a rede de moléculas que estão presentes no meio intracelular participando da sinalização.



Fonte: modificado de Harburger e Calderwood, 2009.

Células expressam diferentes integrinas e dependendo do tipo celular e do microambiente, o perfil dessas moléculas pode ser alterado. No câncer, a expressão de determinadas integrinas pode ser relacionadas a uma maior ou menor chance de sobrevivência dos pacientes (DESGROSELLIER; CHERESH, 2010; LAWSON; BURRIDGE, 2014). A integrina $\alpha_v\beta_3$ é mais expressa em células cancerígenas de mama do que em células normais e as subunidades α_3, α_5, α_6, α_v, β_1 e β_3 são mais expressas em células metastáticas sendo, portanto indicadores de metástase (RATHINAM; ALAHARI, 2010).

Além do papel das integrinas de forma isolada, elas podem interagir de forma direta ou indireta com outros receptores de membrana tanto em células normais como tumorais, afetando a expressão, afinidade e sinalização desses receptores (DESGROSELLIER; CHERESH, 2010). Um exemplo é o *crosstalk* entre o receptor 2 do VEGF e a integrina $\alpha_v\beta_3$, que age regulando a sinalização do VEGF durante o processo de vascularização (SOMANATH; MALININ; BYZOVA, 2009). A mesma

integrina também pode ser relacionada com as MMPs. A integrina $\alpha_v\beta_3$ age estimulando a expressão e atividade das MMPs assim como, pode ser encontrada em co-localização com a MMP-2 na frente de migração celular, facilitando a passagem das células por entre as fibras da matriz (VARTAK; LEE; GEMEINHART, 2010). Inclusive, a inbição da integrina $\alpha_v\beta_3$ foi capaz de inibir a atividade da MMP2 em fibroblastos (MONTENEGRO et al., 2012).

Na angiogênese, a integrina $\alpha_v\beta_3$ possui papel crucial não apenas por agir em cooperação com as moléculas dotadas de diferentes funções biológicas como as metaloproteases, fatores de crescimento e seus receptores, mas também por interagir com diversas proteínas da MEC (vitronectina, fibronectina, fibrinogênio, trombina, trombospondina e fator von Willebrand) (BELVISI et al., 2005).

Devido à sua participação em eventos cruciais no câncer e por ser expressa de uma maneira seletiva, a integrina $\alpha_v\beta_3$ passou a ser amplamente estudada, sendo considerada um alvo terapêutico interessante para o tratamento dessa doença (BELVISI et al., 2005; SCARINGI et al., 2013).

1.8 METALOPROTEASES

As metaloproteases de matriz (MMPs) são enzimas expressas na forma de precursores inativos, dependentes de zinco. As MMPs, juntamente com as ADAMs (*A Disintegrin-like And Metalloproteinase*) pertencem a superfamília das metzincinas, caracterizadas pela presença de três histidinas e um zinco no motivo catalítico, seguidos de um resíduo metionina conservada (APTE; PARKS, 2015; KESSENBROCK; PLAKS; WERB, 2010). Todas as MMPs são formadas por um peptídeo sinal, um pró-peptídeo e o domínio catalítico, além do domínio tipo-hemopexina que está presente em quase todas as MMPs, presente no C-terminal, mas que não está presente nas MMPs 7 e 26. Sua ativação pode acontecer dentro ou fora das células e ocorre no momento da dissociação entre um resíduo de cisteína, presente no pró-domínio, e o zinco do domínio catalítico (KESSENBROCK; PLAKS; WERB, 2010). Essas enzimas estão intimamente relacionadas com o processo de invasão, no qual a célula necessita das metaloproteases para se mover através da MEC. Durante o processo de clivagem dos componentes da matriz, as MMPs acabam liberando fatores de crescimento presentes nessa matriz, que agem sobre as células estimulando a produção de outras MMPs (KESSENBROCK; PLAKS; WERB, 2010).

Estudos recentes vêm dando uma maior importância ao papel das MMPs como enzimas de processamento, que agem controlando desde a clivagem de citocinas, quimiocinas, peptídeos antimicrobianos e até proteínas de superfície celular, que resultam em alterações em funções regulatórias relacionadas a imunidade, reparação tecidual, diferenciação e na transformação celular (APTE; PARKS, 2015). No câncer, tanto suas funções catalíticas como não catalíticas podem promover a invasão celular e, normalmente, sua presença em alguns tipos de tumores malignos tem sido associada a presença de um pior prognóstico (BROWN; MURRAY, 2015). A superexpressão da MMP-3 induz a transição epitélio-mesenquimal, resultando no desenvolvimento de tumores de mamas mais invasivos. A MMP-9 eleva os níveis de VEGF disponíveis enquanto cliva as proteínas da matriz em um evento que antecede a vascularização tumoral (KESSENBROCK; PLAKS; WERB, 2010; MOSS; JENSEN-TAUBMAN; STETLER-STEVENSON, 2012). Entretanto, alguns tipos de câncer se comportam de forma diferente. A superexpressão da MMP-9 é associada a um prognóstico favorável em cânceres de mama sem presença de metástase para os gânglios linfáticos e também está relacionada a um menor risco de metástase hepática no câncer colorectal. Isto ocorre, provavelmente, devido a sua capacidade de gerar inibidores angiogênicos como a angiostatina e a tumestatina, a partir da clivagem dos mesmos das proteínas presentes na MEC (LÓPEZ-OTÍN; MATRISIAN, 2007).

MMPs e integrinas podem estar intimamente conectadas, como a integrina αvβ3 que está co-localizada com a MMP-2 na frente migratória das células, realizando uma degradação restrita da MEC para a facilitar a migração celular. Isto pode ocorrer devido a forma ativa da MMP estar ligada a integrina na membrana de células endoteliais (Vartak, Lee et al. 2009).

1.9 VENENO COMO FONTE DE FÁRMACOS

Apesar das políticas de prevenção e os novos meios de detecção ou metodologias para detecção precoce e avanços nos tratamentos, o número de mortes causados pelo câncer ainda são muito elevados. Assim, há uma enorme necessidade de encontrar novos agentes terapêuticos capazes de impedir o avanço dessa doença, principalmente nos casos de metástase, para os quais ainda não existe tratamento efetivo (BISSELL; HINES, 2011).

Testes utilizando moléculas que incluem miRNA, anticorpos monoclonais e inibidores de tirosina quinases vem sendo realizados visando atingir processos

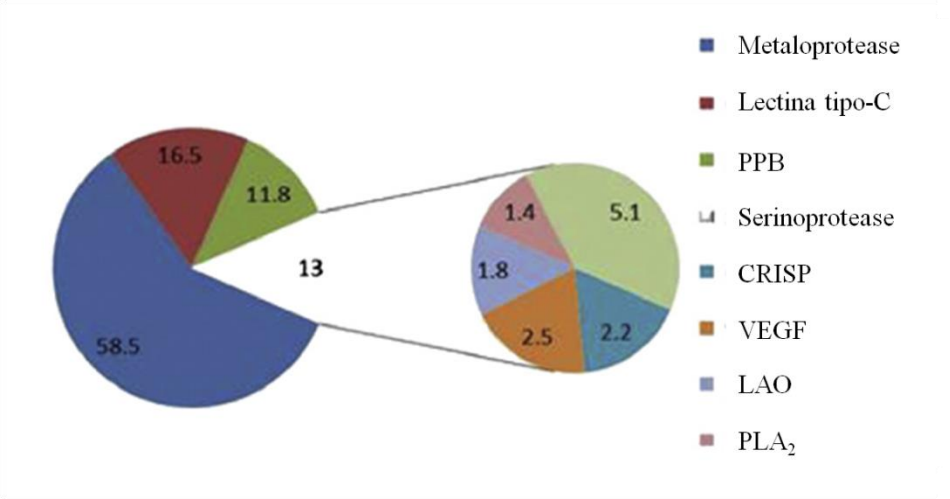
relacionados à metástase e ao microambiente tumoral, como a EMT, *anoikis*, motilidade celular e angiogênese, os principais alvos terapêuticos envolvidos no processo metastático (GUAN, 2015; HOJJAT-FARSANGI, 2014; WELLS et al., 2014).

Os venenos de serpentes desde a antiguidade fascinaram pesquisadores por sua complexa composição de moléculas biologicamente ativas. Mesmo em baixas concentrações essas moléculas afetam sistemas fisiológicos vitais como os sistemas neurológico e cardiovascular e são altamente seletivas e específicas para seus alvos (MARCINKIEWICZ, 2013; ZAMBELLI et al., 2016). Essas características fazem os venenos tóxicos, porém quando as moléculas são isoladas podem se tornar fontes para desenvolvimento de novos fármacos (EBLE, 2010).

Atualmente, estão no mercado alguns fármacos derivadas de venenos de serpentes. O primeiro a ser descoberto e o exemplo mais clássico é o Captopril®, medicamento anti-hipertensivo que inibe a enzima conversora de angiotensina (ECA), que foi desenvolvido a partir de um peptídeo isolado do veneno da *Bothrops jararaca*. Podem ser citados também a Haemocoagulase, um pró-coagulante descoberto a partir do veneno da *Bothrops atrox,* além do Aggrastat e Eptifibatide® (Integrilin), que são utilizados como antiagregantes e foram produzidos a partir das desintegrinas echistatina e barbourina (KOH; KINI, 2011; ZAMBELLI et al., 2016).

Venenos são compostos por uma variedade de substâncias desde sais inorgânicos até grandes moléculas como enzimas e proteínas. A composição de cada veneno varia de acordo com cada gênero e espécie, idade e sexo do animal. Neste caso, a espécie envolvida foi a *Bothrops alternatus,* popularmente conhecida como urutu e amplamente encontrada no sul do Brasil, Argentina, Bolívia e Paraguai. As principais classes de moléculas presentes nesse veneno são: fosfolipases A_2 (PLA_2), serinoproteases, lectinas tipo C, peptídeos potenciadores de bradicinina (PPB) e, presente em maior quantidade, como mostra a figura 8, as metaloproteases ou *Snake Venom Metaloproteinases* (SVMPs) (CARDOSO et al., 2010; DE PAULA et al., 2014; GONZA; LANARI; ROSSET, 2010; ZAMBELLI et al., 2016).

Figura 8. Composição do veneno da *Bothrops alternatus*, em porcentagem. Diferentes moléculas estão presentes no veneno, sendo as enzimas denominadas de SVMPs as mais expressas, compondo 58,5% das toxinas do veneno e em menor quantidade, serinoproteases, CRISP (proteína secretora rica em cisteína), VEGF, LAO (L-aminoácido oxidases) e PLA_2 (fosfolipase A2).



Fonte: modificado de Paula et al, 2014.

1.10 METALOPROTEASES DE VENENO DE SERPENTE

As metaloproteases de veneno de serpente são enzimas compostas por múltiplos domínios e dependem de Zn^{2+} para exercer sua atividade catalítica. Possuem importante papel na patologia do envenenamento degradando não apenas a MEC, mas uma variedade de outros substratos, causando hemorragias locais e sistêmicas, edema, inflamação e necrose nas vítimas (DE PAULA et al., 2014; FOX; SERRANO, 2009).

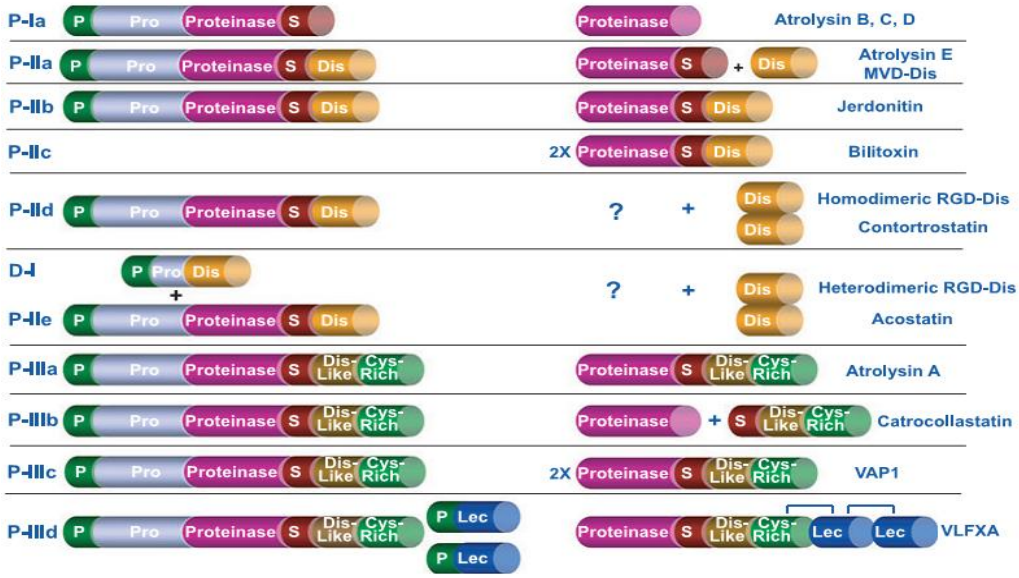
As metaloproteases foram classificadas em três grupos, de acordo com as diferentes massas moleculares e estruturas de seus domínios. São denominadas P-I, as menores SVMPs, as enzimas que possuem apenas o pró-domínio e um domínio de metaloprotease. A classe P-II é composta pelas SVMPs de tamanho médio (30-60kDa) e são acrescidas de um domínio desintegrina. O domínio desintegrina desse grupo geralmente contém os motivos adesivos RGD, VGD ou KGD. As P-III são acrescidas de um domínio tipo desintegrina, normalmente contendo os motivos E/DCD, e de um outro domínio rico em cisteína. Poderia ainda haver uma classe P-IV, que possui todos os domínios da P-III, além de dois domínios tipo-lectinas ligados ao domínio rico em cisteína por pontes de sulfeto, entretanto a sequência nucleotídica dessa classe nunca foi

encontrada e ela foi incorporada à classe das P-IIId (FOX; SERRANO, 2008; MARKLAND; SWENSON, 2013).

Todas as SVMPs são sintetizadas na forma de um precursor inativo e posteriormente sofrem processamento proteolítico, liberando seus domínios proteinase, desintegrinas, tipo desintegrinas e rico em cisteínas ou tipo lectinas, dependendo da classe a que pertencem, como indicado na Figura 9 (FOX; SERRANO, 2008).

Os domínios desintegrinas são processados a partir da classe P-II, não são moléculas tóxicas e são desprovidas de ação enzimática, contendo uma sequência adesiva composta por um tripeptídeo, como citado acima, geralmente RGD, KGD ou VGD, mas também podem possuir outros motivos como o RTS, KTS, MGD, MLD, MVD e WGD (CALVETE, 2013; FOX; SERRANO, 2008).

Figura 9. Classes de SVMPs. Metaloproteases antes e depois do processamento. Os pontos de interrogação representam compostos ainda não identificados no veneno e o S situado entre os domínios significa *spacer*.



Fonte: Fox e Serrano, 2008.

1.11 DESINTEGRINAS

No final da década de 80 foi descoberta a primeira desintegrina, trigramina, o que levou à descoberta de uma nova classe de proteínas. Stefan Niewiarowski e Tur-Fu Huang isolaram do veneno da *Trimeresurus gramineus*, serpente encontrada no sul da Índia, essa pequena proteína capaz de inibir a agregação plaquetária de forma específica e eficiente. A inibição ocorreu devido à competição da trigramina com o fibrinogênio pelo seu receptor, a integrina $\alpha_{IIb}\beta_3$, quando as plaquetas foram estimuladas por ADP

(CALVETE, 2013; MARCINKIEWICZ, 2013). Portanto, a trigramina se ligou à integrina impedindo a interação do fibrinogênio com o receptor e bloqueando a agregação plaquetária.

A partir da descoberta das desintegrinas, cientistas iniciaram uma série de pesquisas para investigar as possíveis interações e efeitos dessas proteínas em outros tipos integrinas. Atualmente, existem potenciais agentes terapêuticos derivados de desintegrinas para o tratamento de trombose, metástase tumoral, inflamação e outras doenças relacionadas as atividades das integrinas (CALVETE, 2013).

As desintegrinas são polipeptídeos ricos em cisteínas, que contém de 40-100 aminoácidos e podem ser subdivididas de acordo com sua estrutura ou função. De acordo com a estrutura, elas podem ser monoméricas ou diméricas, sendo homodiméricas ou heterodiméricas. As desintegrinas monoméricas podem ser ainda subclassificadas de acordo com o tamanho da sua cadeia polipeptídica e o número de pontes dissulfeto. As classificadas como pequenas possuem de 41 a 51 aminoácidos e 4 pontes dissulfeto, as médias possuem aproximadamente 70 aminoácidos e 6 pontes e as grandes possuem cerca de 84 aminoácidos e 7 pontes dissulfeto. A subclassificação relacionada à função se refere à sequência do tripeptídeo presente no *loop* das desintegrinas pondendo ser RGD, KTS ou MLD (CALVETE et al., 2005; MARCINKIEWICZ, 2013).

A sequência adesiva dessas proteínas se encontra no ápice de uma alça (*loop*) móvel e saliente do centro da proteína. A sequência de aminoácidos presentes na alça e adjacentes a ela, assim como a formação correta das pontes de dissulfeto, são críticos para a atividade biológica das desintegrinas (MCLANE et al., 1998; REEKS; FRY; ALEWOOD, 2015).

Portanto, as sequências encontradas no *loop* de cada desintegrina resultam em interações com diferentes integrinas, com especificidades e afinidades distintas, e consequentemente, atividades biológicas diferentes. Alguns exemplos de desintegrinas com motivo KGD como a bauborina se ligam de forma seletiva à integrina $\alpha_{IIb}\beta_3$, enquanto a obstutatina, uma desintegrina KTS se liga à integrina $\alpha_1\beta_1$ e desintegrinas diméricas como a EC3, que possui uma motivo MLD e outro VGD, interagem com as integrinas $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_9\beta_1$ e $\alpha_5\beta_1$ (CALVETE, 2013; REEKS; FRY; ALEWOOD, 2015).

Dada a relevância das integrinas nos processos de metástase, as desintegrinas poderiam ser potenciais inibidoras nos processos de adesão, proliferação e a migração

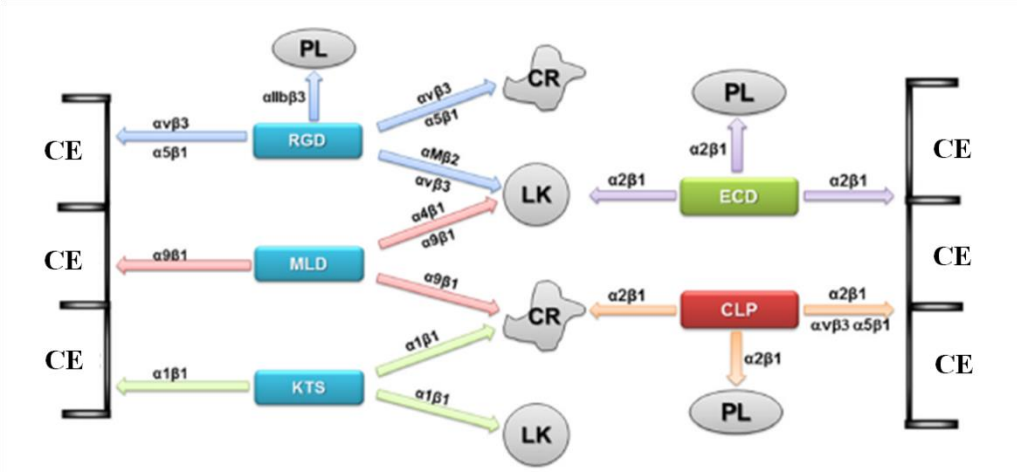
de células cancerígenas (MARCINKIEWICZ, 2013; REEKS; FRY; ALEWOOD, 2015). Diversas desintegrinas RGD como a triflavina, ussuristatina 1, rodostomina, colombistatina e viridistatina demonstraram ser eficazes na inibição da adesão de células tumoras a proteínas da MEC. A eristostatina, uma desintegrina RGD, foi capaz de inibir a colonização do pulmão por células de melanoma em camundongos, bem como a migração celular em ensaio de *wound healing* analisado por *time-lapse* de diferentes linhagens de células de melanoma, utilizando placas recobertas por fibronectina (TIAN et al., 2007). Já a contortrostatina, outra desintegrina com motivo RGD e ligante da integrina $\alpha_v\beta_3$, inibiu o crescimento e metástase tumoral em células de câncer de mama (MDA-MB-435) em modelo de camundongo, assim como o crescimento de gliomas em cérebros de camundongo (MARCINKIEWICZ, 2013).

Os vasos sanguíneos são o primeiro ponto do organismo de interação com o veneno sendo assim, a atividade das desintegrinas na angiogênese vem sendo intensamente investigada por pesquisadores. Entretanto, existem duas finalidades para essas pesquisas e elas são opostas. No caso da angiogênese relacionada a doenças cardiovasculars, as pesquisas são voltadas para a estimulação desse processo, enquanto que no câncer, para onde foram direcionado a maioria dos estudos, as pesquisas são voltados para a inibição da angiogênese (MARCINKIEWICZ, 2013). Diversas desintegrinas possuindo motivos RGD, KTS e RTS se mostraram capazes de inibir a angiogênese, Figura 10 (REEKS; FRY; ALEWOOD, 2015). Desintegrinas RGD como acustina e rodostomina inibiram a angiogênese *in vitro*, em ensaios de formação de tubo em matrigel e *in vivo* em ensaio com membrana cório-alantoide de ovo de galinhas. A salmosina, desintegrina que age bloqueando a integrina $\alpha_v\beta_3$, foi capaz de inibir a proliferação de células microvasculares de boi através da inibição da ativação da FAK e diminuindo a expressão de paxilina e da $p130^{cas}$, além de bloquear a angiogênese *in vitro* e *in vivo* (MARCINKIEWICZ, 2013). Jerdostatina, uma desintegrina RTS que se liga a integrina $\alpha_1\beta_1$, receptor de colágeno, é capaz de inibir a formação de tubo dependente desse receptor em células HUVEC (BOLÁS et al., 2014). A lebestatina, uma desintegrina KTS, também ligante da integrina $\alpha_1\beta_1$, inibiu a migração de células HMEC-01 (*Human Microvascular Endothelial Cells*) na concentração de 1μM, assim como a angiogênesis *in vivo* (OLFA et al., 2005).

Apesar da maioria das desintegrinas terem seus receptores desvendados, alguns efeitos ainda não foram compreendidos por completo como no caso da eristostanina que apesar de ter o motivo adesivo RGD, não interage com a integrina $\alpha_v\beta_3$. Entretanto, um

estudo sugere que seu receptor seria a integrina $\alpha_4\beta_1$, por estar presente em todas as células utilizadas nos estudos anteriores nos quais na eristostatina foi testada (MARCINKIEWICZ, 2013).

Figura 10. Integrinas alvo das sequências adesivas presentes em desintegrinas e os tipos células em que elas atuam. Desintegrinas da classe PII são representadas em caixas azuis, PIII na caixa verde e Lectinas tipo-C estão na caixa vermelha - CLP (C-type Lectin Protein), enquanto os tipo de células estão abreviados: plaquetas – PL; leucócitos – LK; células endoteliais – CE; células de câncer – CR.



Fonte: modificado de Marcinkiewicz, 2013.

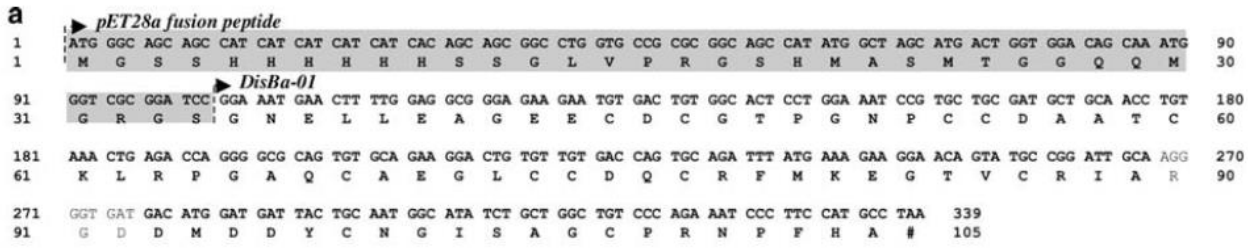
O motivo RGD foi o primeiro ser descoberto e está presente na maior parte das desintegrinas monoméricas (CALVETE et al., 2003). Esse tripeptídeo possui diferentes afinidades e especificidades pelas integrinas $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_8\beta_1$ e $\alpha_v\beta_1$, mas principalmente pelas integrinas $\alpha_v\beta_3$, $\alpha_5\beta_1$ e $\alpha_{IIb}\beta_3$ que são os receptores de vitronectina, fibronectina e fibrinogênio respectivamente. A ligação dessas moléculas as integrinas, usualmente, é potente, sendo necessárias baixas concentrações, na escala de nanomoles para a visualização dos efeitos biológicos *in vitro* e micromolares para testes *in vivo* (SELISTRE-DE-ARAUJO et al., 2010).

1.12 Dis*Ba*-01

A Dis*Ba*-01, desintegrina de *Bothrops alternatus*, é uma desintegrina recombinante RGD (figura 11), monomérica, média, sendo composta por 78 aminoácidos e 6 ligações dissulfeto (12 cisteínas). Ela foi produzida a partir de uma biblioteca de cDNA construída com o mRNA da glândula venenífera de uma única serpente do gênero *Bothrops alternatus*, popularmente conhecida como urutu. Sua massa molecular é de 11,780 Da, entretanto, nela está inclusa a cauda de histidina,

composta por 6 histidinas, adicionada a sequência com o objetivo de facilitar o processo de purificação da proteína. O primeiro trabalho publicado sobre a Dis*Ba*-01 contém uma modelagem molecular, seguida de um ensaio de ressonância plasmônica, realizado com a subunidade β_3 da integrina $\alpha_v\beta_3$, comprovando sua afinidade pela subunidade β_3 (K_D: $1{,}6.10^{-7}$ M) das integrinas (RAMOS et al., 2008).

Figura 11. Senquência nucleotídica da Dis*Ba*-01. Na sequência protéica deduzida a cauda de histidina está presente no N-terminal da proteína e o motivo RGD (indicado por letras mais claras) próximo ao C-terminal.



Fonte: Ramos et al., 2009.

Outros artigos demonstram os efeitos da Dis*Ba*-01 sobre plaquetas, células de câncer e endoteliais, *in vitro* e *in vivo*. Em 2008, Kauskot e colaboradores investigaram o efeito dessa desintegrina sobre plaquetas de camundongos, coelhos e humanos constatando que ela, além inibir fortemente a agregação plaquetária e prolongar o tempo de sangramento na cauda de camundongos, ela foi capaz de inibir a fosforilação da FAK em plaquetas humanas (KAUSKOT et al., 2008). Ramos e colaboradores, também em 2008, realizaram experimentos *in vitro* e *in vivo*, utilizando linhagens de células endoteliais e tumorais. Dis*Ba*-01 foi capaz de inibir fortemente a adesão e a proliferação de células de melanoma murino B16F10 e HMEC-1 (IC_{50} = 225 e 555nM, respectivamente) plaqueadas sobre vitronectina, proteína presente MEC, alvo da integrina $\alpha_v\beta_3$. Experimentos *in vivo*, realizados em camundongos demonstraram uma diminuição significativa da formação de novos vasos induzida por bFGF (*basic Fibroblast Growth Factor*). Também *in vivo,* em modelo animal de formação de metástase pulmonar, células de melanoma foram aplicadas em camundongos, para avaliar a capacidade da Dis*Ba*-01 de prevenir metástases no pulmão. Os animais tratados com a desintegrina tiveram uma redução de 69,8 ± 11,7% na colonização dos pulmões pelas células de melanoma (RAMOS et al., 2008).

A Dis*Ba*-01 demonstrou ser capaz de diminuir a expressão do VEGF e seus receptores VEGFR1 e VEGFR2 em células HMEC-1 e a expressão do VEGF em fibroblastos e MDA-MB-231 nos tempos de 24 e 48 horas, respectivamente (MONTENEGRO et al., 2012). Além de inibir a migração em células de câncer de mama, próstata e fibroblastos, em ensaio de migração *transwell* e diminuir a atividade da MMP-2 (SELISTRE-DE-ARAUJO et al., 2010; MONTENEGRO et al., 2012).

O mais recente artigo publicado sobre a Dis*Ba*-01 envolve ensaios *in vivo* em modelo de inflamação demonstrando a capacidade da desintegrina de diminuir os níveis de inflamação, neovascularização e deposição de colágeno através da diminuição dos níveis de atividade de MPO, hemoglobina, VEGF, bFGF, TNF-α, CXCL1 e CCL2. Demonstrando o potencial terapêutico da Dis*Ba*-01 no controle da angiogênese e doenças fibroproliferativas (CASSINI-VIEIRA et al., 2014).

2. JUSTIFICATIVA

O Brasil, assim como o Paquistão, Indonésia e França são países com os mais altos índices de câncer de cavidade oral do mundo. Esse tipo de câncer é o responsável por 3% do total de casos de câncer no mundo e 90% desses são de carcinoma de células escamosas. No Brasil, 80% dos casos são diagnósticados em estadios avançados e apenas 20% de forma precoce, em adição a isso, esses tumores apresentam altas taxas de reincidencia local, contribuindo para uma baixa taxa de sobrevivência na qual apenas 40-50% dos pacientes acometidos sobreviverão por 5 anos (GLEBER-NETTO et al., 2015; SILVA et al., 2011; TANIS et al., 2014). Esse câncer pode ocorrer em diferentes sítios anatômicos da cavidade oral como língua, orofaringe, lábios, gengiva, palato duro e mucosa oral e possuem uma baixa resposividade a quimioterapia, sendo cirurgia e radioterapia a primeira escolha de tratamento, exceto em casos inoperáveis (SILVA et al., 2011)**.**

Apesar dos avanços no diagnóstico, técnicas cirúrgicas e tratamentos, na maior parte dos casos, não existe tratamento para pacientes com câncer metastático. As drogas disponíveis no mercado hoje são utilizadas apenas para fins paliativos, sendo a metástase, responsável pelos altos índices de morte causados pela doença (BISSELL; HINES, 2011).

O processo de migração é uma das etapas do câncer considerada como um alvo susceptível para tratamento por sua capacidade de limitar os processos de invasão e metástase (WELLS et al., 2014). Assim, tendo em vista o número de desintegrinas que já se tornaram medicamentos e, portanto, o enorme potencial terapêutico dessa classe de peptídeos, ligantes específicos de integrinas e capazes de bloquear a adesão celular, uma etapa crucial na migração celular, se faz necessário aprofundar o conhecimento sobre os mecanismos moleculares da ação dessa desintegrina no processo migratório.

3. OBJETIVOS

Objetivo geral

Verificar os efeitos e o mecanismo de ação da Dis*Ba*-01 sobre a migração de células normais e tumorais.

Hipótese inicial: a Dis*Ba*-01 por ter afinidade pela integrina αvβ3 poderia ter um efeito antimetastático em células tumorais durante a invasão celular que ocorre através de um tecido rico em fibronectina, como o tecido conectivo.

Objetivos Específicos

- Comparar os efeitos da Dis*Ba*-01 entre as linhagens de fibroblastos e de carcinoma de células escamosas orais em um ambiente rico em fibronectina;

- Utilizar técnicas de *time-laspse* para verificar o efeito da Dis*Ba*-01 sobre a migração celular;

- Determinar a dinâmica das adesões nas células afetadas pela Dis*Ba*-01 através de diferentes técnicas de microscopia;

- Identificar o receptor ao qual ela interage através de ensaios envolvendo transfecção de subunidades de integrinas.

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5. MANUSCRITO - EM FASE DE REDAÇÃO

A recombinant RGD disintegrin inhibits the migration of Oral Squamous Cell Carcinoma due to modulation on cell adhesion

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Abstract

The connective tissue is an environment formed by extracellular matrix (ECM) rich in fibronectin and collagen that cancer cells have to overpass to reach blood vessels and then a metastatic site. Cell adhesion to fibronectin is mediated by αvβ3 and α5β1 integrins through an RGD motif present in this ECM protein, thus making these receptors a key target for cell migration studies. In this context, we investigated the effect of an RGD disintegrin, Dis*Ba*-01, on the migration of two highly migratory cells: human fibroblasts, BJ, and oral squamous cancer cells (OSCC), SCC25, on a fibronectin rich environment. Initially, time-lapse images were acquired on fibronectin-coated glass-bottomed dishes and migration speed and directionality analysis indicated that OSCC cells, but not fibroblasts, showed significant decrease in both parameters when plated in the presence of Dis*Ba*-01 (1μM and 2μM). Integrin expression levels of the α5, αv and β3 subunits were similar in both cell lines, while β1 subunit is present in lower levels on the cancer cells. Next, we determined whether the effects of Dis*Ba*-01 are related to changes in adhesion properties by using paxillin immunostainning and total internal reflection fluorescence (TIRF) microscopy. OSCCs in the presence Dis*Ba*-01 showed increased adhesion sizes and number of maturing adhesion when compared to controls as well as changes in the morphology. To further analyze if these findings are related to the integrin molecule, images of β3-GFP overexpressing cells were acquired using optical and TIRF microscopy. The same parameters were analyzed and showed that β3 overexpression restored cell migration velocity and the number of maturing adhesion that were increased by Dis*Ba*-01. These results indicate that Dis*Ba*-01 affects tumor cell migration according to the integrin expression level and that this selective effect is related to the modulation of cell adhesive properties.

Introduction

Oral squamous cell carcinoma (OSCC) is responsible for 90% of all oral cancers and it might occur at different sites of the oral mucosa (1,2). The exposition to chemical, physical or biological agents with mutagenic and carcinogenic properties, leads mutated keratinocytes to proliferate and invade the connective tissue. OSCCs responsiveness to chemotherapy is low probably due to the heterogeneity of tumor cell population, and high local recurrences rates of this disease result in a 5 year survival

rate of 40-50% (2,3).

Tumor cell migration is the initial step to promote invasion and metastases. Changes on cell phenotype allow their detachment from the primary tumor to invade the surrounding tissues reaching out the blood and lymph vessels (4,5). Throughout this process, epithelial-derived tumor cells need to undergo adaptations in the migration process, since there is a transition from a laminin-enriched basal membrane to a connective tissue rich in fibronectin and collagen. Previous studies showed that the more aggressive is the OSCC, the higher is the presence of fibronectin throughout the tissue and specially at the invasion fronts (6,7), which might be associated to an induction of fast single tumor cell migration (8).

Cell migration is a complex and cyclic process involving actin polymerization, engagement of adhesions and actomyosin-mediated contractility, which enables cells to move forward (9,10). Adhesion molecules such as the integrin receptors are essential in the migration process since the interaction with extracellular matrix (ECM) components results in traction forces necessary for cell migration. Integrins initiate intracellular signaling cascades that not only influence actin cytoskeleton dynamics, but also affect cell proliferation, differentiation and survival (10–12). They are composed of two different subunits, α and β which together may assemble into 24 differently combined heterodimers with individual specificities to ECM proteins. Although some integrins share affinity to the same ligands, they may trigger different signaling pathways (13–15) such as the integrins α5β1 and αvβ3 that bind to fibronectin through a RGD (arginine-glycine-aspartic acid) motif but differentially regulates adhesion maturation through distinct mechanisms of activating RhoA (16,17). This way, the use of peptides containing integrin-binding motifs could be considered an alternative strategy to impair cell motility and decrease the possibility of metastasis.

The blockage of integrin activity affects several adhesion dependent processes. For instance, disintegrins, a family of low molecular weight cystein rich peptides are released by snake venoms causing platelet aggregation inhibition, by binding to the fibrinogen receptor (integrin αIIbβ3) which leads, along with other components of the venom, to local and systemic hemorrhage (18,19). There is a variety of adhesive sequences in the disintegrin structure (KTS, WGD, KGD, MLD, VGD), which are able to interfere with cell migration, proliferation, apoptosis and other processes related to integrin receptors (20–24). Dis*Ba*-01 (disintegrin of *Bothrops alternatus*) is a recombinant RGD containing disintegrin that inhibits cell migration (25) *in vivo*

angiogenesis (26), pulmonary metastasis (27) and is able to decrease the expression of VEGF receptors in endothelial cells (28).

Since ECM components are able to modify the adhesion properties of tumor cells and elicit a migratory behavior (8), we hypothesized that the selective blockage of integrin receptors by disintegrins might impair the migratory process of OSCC cells on a fibronectin rich environment. In this study, we show that Dis*Ba*-01 decreased migration speed, directionality and changed the migration mode of a highly invasive OSCC cell but had no effect on fibroblasts. This selective effect on epithelial-derived tumor cells was related to the modulation on adhesion dynamics and was reverted by the overexpression of β3 integrin. Together, these data show that Dis*Ba*-01 affects tumor cell migration according to the integrin expression level, altering the OSCC migration mode to a less aggressive phenotype and that this selective effect is related to modulation of cell adhesive properties.

Material and Methods

Dis*Ba*-01 expression and purification

Dis*Ba*-01 is a recombinant disintegrin produced from a cDNA venom gland library of *B. alternatus*, since the native disintegrin could not be purified from the venom due to low yields in proteins preparations (27). The coding region corresponds to an RGD containing motif disintegrin of 78 amino acids residues (GenBank accession AY259516). Expression and purification of the recombinant His-tag protein were performed as previously described (27). Molecular modeling and adhesion assays suggested that the fusion His-tag peptide was not involved on integrin binding and therefore its proteolytic removal would not be needed (27, 28).

Cell Culture and Transfection

SCC25 cells (ATCC1 CRL-1628™) were cultivated in DMEM/F12 with 15mM HEPES and 0.5mM sodium pyruvate (Gibco), FBS 10% and hydrocortisone (400ng/ml, Sigma), while BJ cells (ATCC® CRL2522™) in DMEM Glutamax with 1% non-essential amino acids (NEAA) and 10% fetal bovine serum. Cal27 (ATCC1 CRL-2095™) were cultivated in DMEM high glucose (Gibco) supplemented with 10% Fetal Bovine Serum (FBS). All cells were maintained in incubator at 37°C and 5% CO2. SCC25 cell were considered being a highly aggressive oral squamous cell carcinoma (OSCC) cell line

due to its E-cadherin low content when compared to Cal27 (also an OSCC) cell line with high levels of E-cadherin. For Total Internal Reflectance Fluorescence (TIRF) microscopy, SCC25 were transfected using TransIT-2020 (Mirus) 24hs before the experiment with 0.5µg Paxillin-GFP plasmid (29) and for β3 subunit overexpression 0.5µg Paxillin-mko and 0.5µg β3-GFP.

Migration and Adhesions Dynamics Assays

For the phase microscopy time-lapse, two cell lines were used, BJ cell lines (human fibroblasts) and SCC25 (OSCC). Images were acquired during 8 hours with a 10 minute interval, at 37°C using a Nikon TE300 microscope (10x 0.25 NA CFI Achro DL106 Nikon objective), with a charge coupled device camera (Orca II, Hamamatsu Photonics, Iwata-City, Japan) using Metamorph software (Molecular Devices), as previously described (30). For time-lapse movies on TIRF microscopy, only SCC25 cells were used. Images were captured every 3 seconds during 20 minutes using an Olympus IX70 inverted microscope (63x 1.45 NA oil Olympus PlanAplo 660 TIRFM objective), fitted with a Ludl modular automation controller (Ludl Electronic Products) with a charge-couple device camera (Retiga Exi, Qimaging) and images were captured using Metamorph software. GFP was excited using the 488nm laser line of an Argon laser (Melles Griot), a dichroic mirror (HQ485/30) and an emission filter (HQ525/50). Image J software plug in manual tracking was installed to analyze all data regarding migration parameters. Migration speed was determined by the ratio between the total distance and duration of cell migration while directionality was determined by X and Y coordinates normalized to a "zero" starting point, all collected from the manual tracking plug in. For the rate of maturing adhesions, were considered nascent adhesions the adhesions formed at the border of the cell membrane during protrusion and mature adhesions were considered the ones that persisted through membrane protrusion and gave place to newly formed adhesions.

On both assays, were used 3cm glass-bottomed dishes containing fibronectin (2µg/mL) and Dis*Ba*-01 (1 and 2µM) as a substrate. Cells were plated with CCM1 media and left at the incubator at 37ºC for 1 hour (phase microscopy) and 20 min (TIRF microscopy) before being placed at the microscope. At least, 4 different experiments were realized for each group.

Western blottings

Cells were trypsinized, washed and lysed in RIPA Buffer (25mMTris-HCL pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease and phosphates cocktails. The lysates were separated by 4-20% precast polyacrylamide gel (BioRad) and proteins transferred to PVDF membranes (Biorad), blocked for 1 hour with 5% deffated milk in a PBS/0.5% Tween 20 at room temperature. Samples were immunoassayed for α_v, β_3, α_5, β_1 and α-Tubulin (Cell Signaling, USA) using Pierce ECL Western Blotting Substrate (Thermo Scientific).

Immunofluorescence

Cells were incubated with Dis*Ba*-01 for 3 hours on fibronectin (2µg/ml) covered glass coverslips on CCM1 media. After that period they were washed with PBS, fixed with formaldehyde 4%, and permeabilized using TritonX-100 (0.3%). The blocking was realized with normal goat serum and incubated overnight with anti-paxilin. Secondary antibodies contained Alexa488 dye (Molecular Probes, Oregon, USA) and the actin filaments were marked with phalloidin conjugated to rhodamine (Molecular Probes, Oregon, USA). Images were obtained on a confocal microscope (Olympus FluoView 1000, Tokyo, Japan) with the 63x objective (UPlanSApo x63, 1.20 NA, oil immersion objective). Alexa488 was excited with the 488nm laser line of an Argon ion laser (Melles Griot, Albuquerque, NM), and rhodamine with the 543nm laser line of a Helium-Neon laser (Melles Griot,Albuquerque, NM). Images were acquired using FluoView software (Olympus, Tokyo, Japan). The Z-stacks were aquired from cells (0.1µm step size) and slices 3 slices obtained, corresponds to an equivalent 0.3µm of the cell, and were merged using ImageJ software tool the "Z-stack/maximum projection".

Statistical Analysis

One way analysis of variance (ANOVA) was used followed by Tukey´s post test and student t-test for the TIRF microscopy assays.

Results

Dis*Ba*-01 selectively affects OSCC migration

Disintegrins containing the RGD adhesive motif sequence act as β3 and β1 integrins antagonists. As shown in previous work, Dis*Ba*-01 was able to inhibit cancer

cell and fibroblasts on a transwell migration assay (25). Therefore, to investigate its potential effect on cell migration in a fibronectin rich environment we used two human cell lines that exhibit fast-single cell migration: fibroblasts (BJ) and a highly invasive/low differentiated oral squamous carcinoma cell line (SCC25). Time-lapse movies (8h) were performed on both cell lines plated on glass-bottomed dishes coated with fibronectin (2µg/ml) in the presence/absence of Dis*Ba*-01 (1 or 2µM) in the media or in the substrate. Individual migratory cells were tracked and migration speed and directionality (total net translocation from the origin point) analysis indicated that fibroblasts (control = 40 cells; Dis*Ba*-01 media = 27 cells; Dis*Ba*-01 substrate = 40 cells) were not affected by Dis*Ba*-01, even at a high concentration (2µM) when compared with control cells (Figure 1A, Supplementary Movie 1). However, the OSCC migration speed was decreased by ~40% (control = 37 cells; 1µM media = 35 cells; 1µM substrate = 39 cells; 2µM substrate = 34 cells and DisBa-01 2µM substrate = 48 cells, $p<0.0001$) when plated in the presence of Dis*Ba*-01 and this effect was more pronounced for migration directionality (Figure 1B, Supplementary Movie 2). While OSCC plated only in fibronectin exhibited a fast, individual and directionally persistent migration behavior, cells treated with 1µM of Dis*Ba*-01 in the media or in the substrate lost their directionality and moved in circles with a tendency to migrate in pairs (collectively). This selective effect of Dis*Ba*-01 in the migration of tumor cells suggests a differential effect on cell adhesive properties.

Dis*Ba*-01 modulates cells adhesive properties of OSCC

Fast migratory cells, such as leucocytes, are characterized by small and rapidly turning over adhesions while slow adherent migratory cell possesses persistent adhesions that mature into larger and elongated focal complexes (1,2). Due to the changes on OSCCs migration behavior, we decided to study whether Dis*Ba*-01 effects were related to changes in adhesion properties by staining OSCCs and fibroblasts for paxillin, a marker for nascent adhesions (32). Fibroblasts have small adhesions at the cell border, which agrees with fast migratory cells phenotype, and Dis*Ba*-01 showed no alterations on paxillin distribution for these cells (figure 2A). In contrast, significant changes were observed on the pattern of adhesions in OSCCs such as an increase in adhesion sizes and quantities in the presence of Dis*Ba*-01 in the media or in the substrate when compared to cells plated only on fibronectin (Figure 2B).

To further investigate the adhesion dynamics seen at the paxillin immunostaining, SCC25 cells were transfected with paxillin-GFP and 24h later plated on fibronectin coated dishes (2µg/mL) in the presence/absence of Dis*Ba*-01 in the media or in the substrate. Then, adhesion dynamics was imaged on TIRF microscopy every 3 seconds during 20 minutes. Dis*Ba*-01 increased the rate of maturing adhesions on OSCCs which was accompanied by an increase on the adhesion area (Figure 2, supplementary movies 3 and 4). During the migration process, cells begin extending their membrane to form the lamellipodia and then attach the membrane to the extracellular matrix. The area of these protruding membranes was also measured and it was seen that Dis*Ba*-01 makes the cell protrusion area significantly larger than control cells (n= 3 cells, p>0,0005). Taken together, these data indicate that Dis*Ba*-01 shows a differential effect on cell adhesion properties according to cell type.

Fibronectin is a glycoprotein found on the ECM that interacts mainly with integrins αvβ3 and α5β1 through the RGD motifs in its structure (16). A possible mechanism for the differential effects of Dis*Ba*-01 on adhesion properties is that the cell lines show different levels of integrin receptors for fibronectin. To test this hypothesis, we analyzed the integrin expression level on fibroblasts (BJ), a highly invasive/low differentiated (SCC25) and a low invasive/highly differentiated (Cal27) OSCC cell line (Figure 3). It was observed that the low invasive/high differentiated OSCC cell line (Cal27) presents only low levels of αv subunit, which might explain the poor migratory performance observed on fibronectin (8). On the other hand, fibroblasts showed high levels of all fibronectin-related integrin subunits, which corroborates to the fast single cell migration phenotype. When we analyzed the integrin levels of the highly invasive OSCC cell line, besides the high level of integrin alpha-subunits, these cells show low levels of β1 and high levels of β3 subunits, which indicates that the migration process of this epithelial-derived tumor cell line on fibronectin relies mostly on β3 activity. Since Dis*Ba*-01 is an RGD disintegrin, this result suggests that Dis*Ba*-01 could be selectively acting on αvβ3 integrin of OSCC cells.

β3 overexpression recovered Dis*Ba*-01 effects

Dis*Ba*-01 has the potential to interact with αvβ3 integrins (27), which suggests that the interaction between Dis*Ba*-01 with integrin receptors impairs the migratory activity of OSCC cells. To investigate this hypothesis OSCCs were transfected with β_3

subunit, plated on fibronectin-covered dishes in the presence/absence of Dis*Ba*-01, and migration properties were assessed. We used two plasmid concentrations for β_3 subunit, in order to reach similar levels of expression observed in BJ (Figure 4A, supplementary movies S5 and S6). It was observed that the overexpression of β_3 subunit restored the migration speed and directionality of cells plated in the presence of Dis*Ba*-01. In order to analyze adhesion properties, cells overexpressing β_3 integrin were immunostained for paxillin or co-transfected with paxillin-mko and analyzed in confocal or TIRF microscopy, respectively. It was observed that overexpression of β_3 integrin recovered cells from the phenotype induced by Dis*Ba*-01 (2μM), which corroborates with the hypothesis that the inhibition of β_3 integrin by Dis*Ba*-01 is the probable mechanism by which Dis*Ba*-01 impairs the migration properties of OSCC cells.

Discussion

Tumor metastasis is the main lead of clinical failure in cancer treatment and might be influenced by extrinsic factors, such as the components of the environment, as well as by intrinsic migratory properties. Also, tumor cell plasticity is a response to microenvironmental changes and often influences tumor progression, invasion and resistance to therapy (5). Alterations in matrix density, expression or function of adhesion receptors such as integrins or cadherins and gene expression of cytoskeletal adaptor proteins can lead to interconvertible changes on migration mode depending on each cell intrinsic capacities (8,33), which makes the development of strategies to block the interaction of tumor cells with the extracellular matrix an interesting complementary tool for cancer treatment.

Here, we demonstrated that Dis*Ba*-01, an RGD disintegrin, present on media or substrate, was able to significantly decrease cell migration and cell directionality of highly invasive OSCC, on fibronectin coated dishes but it did not affect fibroblasts. Since soluble and immobilized disintegrin forms triggered opposite results in the past (34), both forms were tested. Migration initiates with biochemical or mechanical cues from the extracellular environment (35). As demonstrated by previous studies, fibronectin induces migration of different cells lines including low and highly differentiated OSCC cells (8,31,36). One interesting finding was that OSCC cells in the presence of Dis*Ba*-01 migrated in a similar fashion of low invasive OSCC cells, Cal27, also plated over fibronectin. Cells moved in circles and in a collective way, indicating a

change on the migration mode that leads toward a less aggressive cell migration phenotype (8). Another small RGD containing motif disintegrin, eristostatin, was reported to decrease migration speed in a wound healing assay using fibronectin coated plates and melanoma cells. Although it is not mentioned on the report, it is possible to notice impairments on cells directionality and changes on cells morphology especially on the highly invasive melanoma cell c8161 time-lapse videos (21). According to Mirssilis et al (2016) the inhibition of αvβ3 or α5β1 integrins, both on fibronectin coated surfaces caused cells to lose their directionality, but with increased migration speed (36).

A possible mechanism of action for the impairment of cell migration by Dis*Ba*-01 is the modulation of the adhesion properties. In order to better understand our results on the effects of Dis*Ba*-01 on OSCC migration, we looked deeper into cell adhesions dynamics and morphology. Highly invasive OSCC cells have small and dynamic adhesions when plated on fibronectin, as was already demonstrated (10). However, when Dis*Ba*-01 was added to the cells, adhesions became larger, with a higher rate of maturation and some instability could be noticed on membrane protrusions. Blocking of α5β1 but not αvβ3 by a specific ligand increased focal adhesion sizes (36). So adhesions function as anchors that respond to different stimuli and forces from the ECM and/or cytoskeleton altering its shape, size and dynamics according to the environment during the migration process (37).

Variation on integrin expression could lead to different migratory phenotypes and how cells are affected by Dis*Ba*-01. Our results on the migration speed inhibition could be due to Dis*Ba*-01 interaction with αvβ3 integrin that leaves cells with no proper adhesion and traction forces to migrate, since β1 subunit is present in very limited amount and the overexpression of β3 subunit on OSCC rescued the migratory phenotype. Moreover, β1 subunit has been characterized for promoting random cell migration on fibronectin coated surfaces, α5β1 mediated cell adhesion causes lost of polarity and directionality (38) such as the motile behavior seen on highly aggressive OSCC cell line in the presence of Dis*Ba*-01. This data indicate that, during invasion, epithelial-derived tumor cells switch the expression of integrins, favoring those related to connective tissue, and that the specific blockage of these receptors, might impair the invasive process.

In an attempt to find new therapies for oral cancers, here we showed that an RGD disintegrin capable of changing the migration behavior of an aggressive cancer

cell by decreasing its migration speed, directionality and changing adhesion phenotype possibly by αvβ3 integrin binding. This interesting phenomenon could lead one to think about how highlighting cell plasticity and turning it to our own benefit could be an achievable way to fight against metastasis, instead of completely blocking cell processes involved on the process.

Figure 1. Dis*Ba*-01 inhibits the migration of OSCC cell line. (A) Dis*Ba*-01 significantly inhibited the migration speed of SCC25 cells but not BJ on both conditions tested (Media = DisBa-01 in the media; Subs = DisBa-01 in the substrate). Results were calculated as % of control. Statistical analysis was performed using ANOVA (one-way) followed by Tukey's post-test, $p<0.0001$. (B) Migration tracks of SCC25 cells treated with Dis*Ba*-01 in the concentrations of 2µM indicates a loss of directionality when compared to the control tracks. Each individual line represents a cell path translated to a common origin.

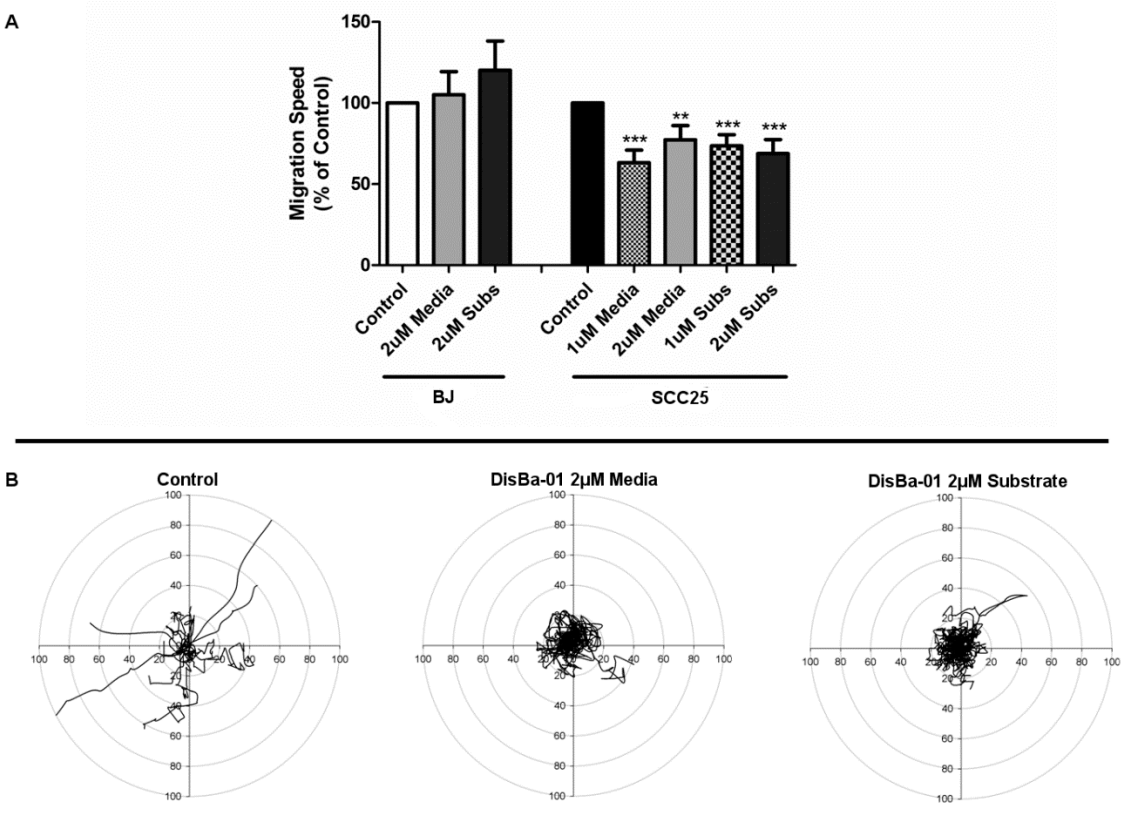


Figure 2. OSCCs treated with Dis*Ba*-01 show an increase in adhesions maturation and area and protrusion area. Cells were allowed to spread for 3 hours on fibronectin (2µg/ml) coated dishes, subsequently fixed, stained for paxillin and analyzed by confocal microscopy. **(**A) Fibroblasts show a large number of small adhesions with no differences between cells with or without Dis*Ba*-01 treatment, while SSC25 treated cells show changes in adhesions and morphology, appearing to be more rounded. Paxillin transfected cells in the presence of Dis*Ba*-01 (2µM)**.** (B) Percentage of maturing adhesions on TIRF time-lapse movies in the absence or presence of Dis*Ba*-01, (C) in the adhesion area (D) and in the protrusions area ($p<0,05$).

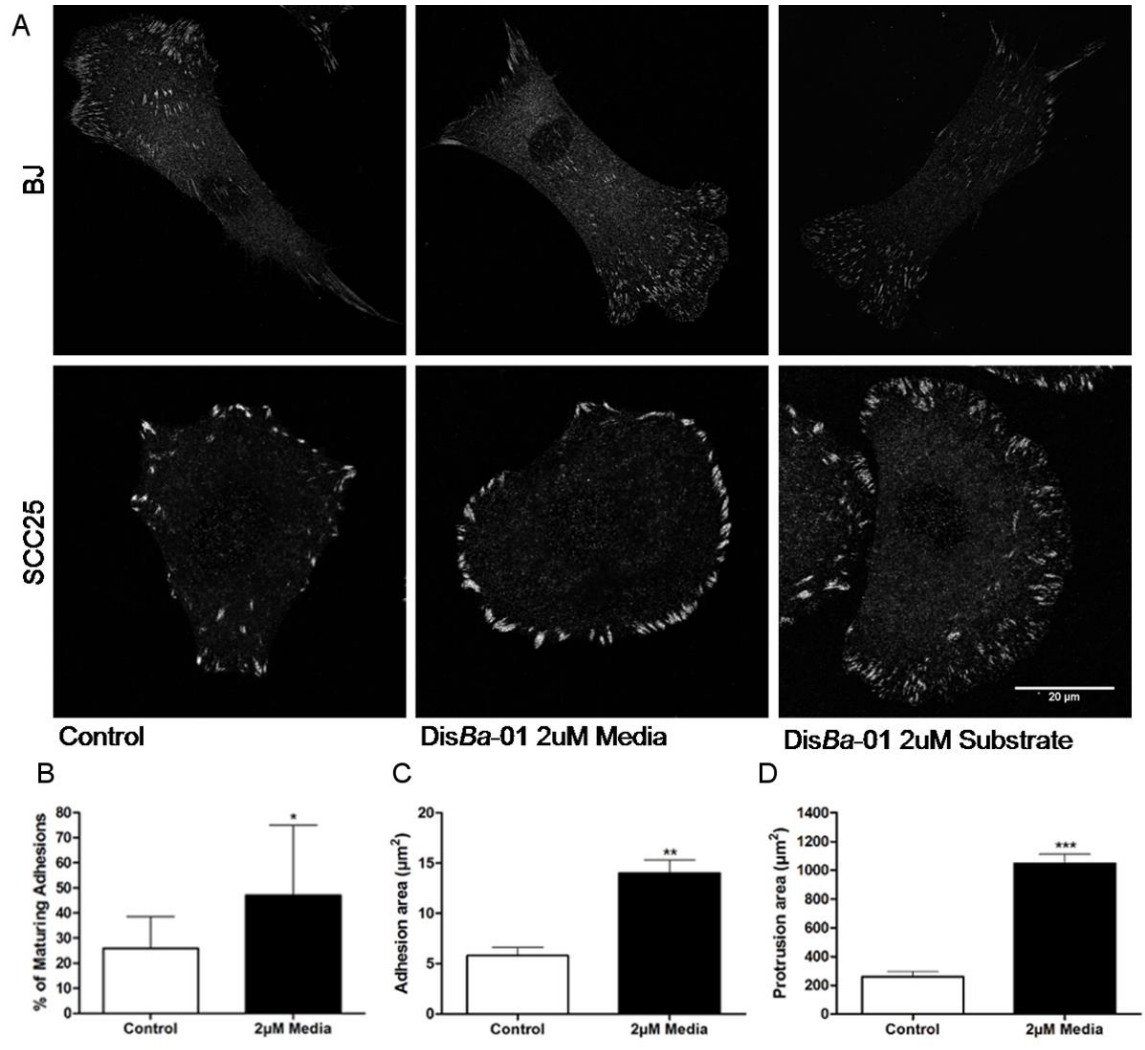


Figure 3. Fibronectin-related integrin receptors show differential expression according to the differentiation level of the tumor cell. Cells lysates were submitted to western blot. BJ and SCC25 contain α_v, β_3 and α_5 integrin subunits in similar amounts while β_1 is present in smaller amounts on SCC25 cells. E-cadherin and N-cadherin are differentiation markers.

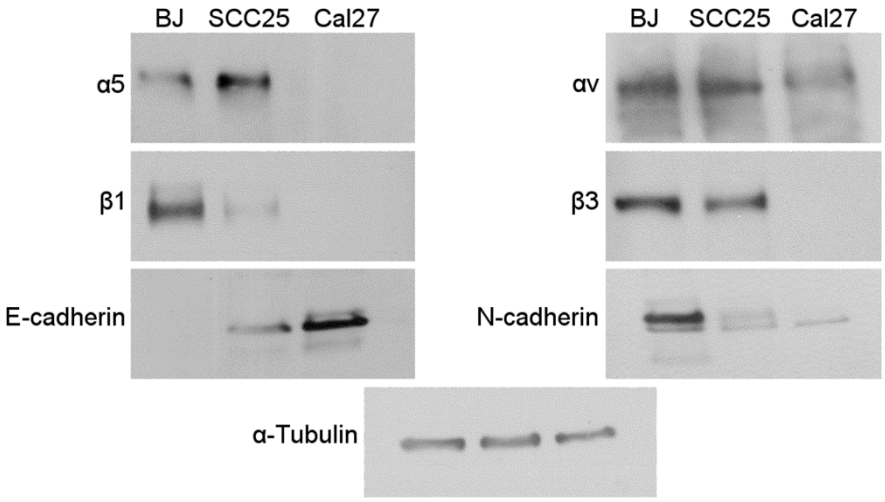
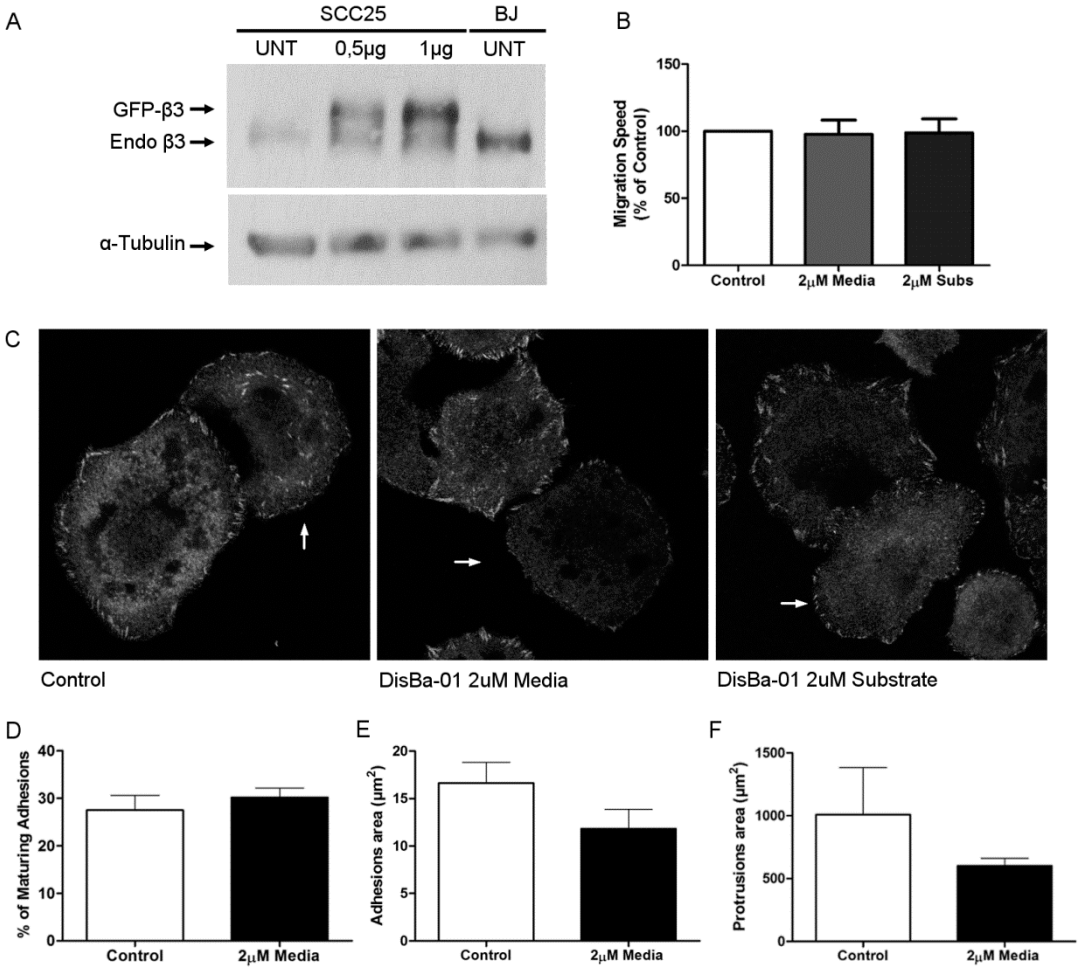


Figure 4. Overexpression of β3 subunit recovered DisBa-01 effect on migration speed and adhesion dynamics of OSCC cells. (A) SCC25 cells were transfected with GFP-β_3 0,5 and 1µg of plasmid. Protein bands show the levels of GFP-β_3 and endogenous β_3 expressed by SCC25 cells. (B) Migrations speed analysis shows no differences between control and Dis*Ba*-01 treated cells. (C) Representative images of β_3 overexpressing cells (indicated with arrows) stained for paxillin. (D) β_3 overexpressing cells with Dis*Ba*-01 reduced the number of adhesions maturation, similar to the controls, when compared with the previous TIRF experiment**.** (E) DisBa-01 effect over the area of adhesions and (F) area of protrusion was also was reverted on β_3 overexpressing cells treated with Dis*Ba*-01.



Supporting information (Recorded on the CD attached)

S1 movie. Fibroblasts migration in presence the absence/presence of Dis*Ba*-01 (2µM).

S2 movie. Highly invasive cells (OSCC) migration in presence the absence/presence of Dis*Ba*-01 (2µM).

S3 movie. Adhesion dynamics of highly invasive cells (OSCC) transfected with paxillin-GFP plated in fibronectin.

S4 movie. Adhesion dynamics of highly invasive cells (OSCC) transfected with paxillin-GFP plated in fibronectin and in the presence of Dis*Ba*-01 in the media.

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6. CONSIDERAÇÕES FINAIS

A Dis*Ba*-01 é uma desintegrina capaz de desencadear efeitos interessantes relacionados principalmente com a migração celular e angiogênese. Apesar de não ser conhecido o mecanismo pelo qual a Dis*Ba*-01 age, estudos *in silico*, assim como dados da literatura foram capazes de sugerir o possível alvo desta desintegrina. Neste trabalho, foi possível observar com maiores detalhes os efeitos causados pela Dis*Ba*-01 em células de câncer oral durante o processo de migração, contribuindo para a elucidação do seu mecanismo de ação, assim como a identificação de seu alvo. Além disso, é um estudo que pode gerar novas perguntas e resultar no desenvolvimento de novos projetos.

7. ANEXO – ARTIGOS PUBLICADOS

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Research paper

Blocking αvβ3 integrin by a recombinant RGD disintegrin impairs VEGF signaling in endothelial cells

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ABSTRACT

Vascular endothelial growth factor (VEGF) and αvβ3 integrin are key molecules that actively participate in tumor angiogenesis and metastasis. Some integrin-blocking molecules are currently under clinical trials for cancer and metastasis treatment. However, the mechanism of action of such inhibitors is not completely understood. We have previously demonstrated the anti-angiogenic and anti-metastatic properties of Dis*Ba*-01, a recombinant His-tag RGD-disintegrin from *Bothrops alternatus* snake venom in some experimental models. Dis*Ba*-01 blocks αvβ3 integrin binding to vitronectin and inhibits integrin-mediated downstream signaling cascades and cell migration. Here we add some new information on the mechanism of action of Dis*Ba*-01 in the tumor microenvironment. Dis*Ba*-01 supports the adhesion of fibroblasts and MDA-MB-231 breast cancer cells but it inhibits the adhesion of these cells to type I collagen under flow in high shear conditions, as a simulation of the blood stream. Dis*Ba*-01 does not affect the release of VEGF by fibroblasts or breast cancer cells but it strongly decreases the expression of VEGF mRNA and of its receptors, vascular endothelial growth factor receptors 1 and 2 (VEGFR1 and VEGFR2) in endothelial cells. Dis*Ba*-01 at nanomolar concentrations also modulates metalloprotease 2 (MMP-2) and 9 (MMP-9) activity, the latter being decreased in fibroblasts and increased in MDA-MB-231 cells. In conclusion, these results demonstrate that αvβ3 integrin inhibitors may induce distinct effects in the cells of the tumor microenvironment, resulting in blockade of angiogenesis by impairing of VEGF signaling and in inhibition of tumor cell motility.

1. Introduction

Metastasis is the primary cause of mortality for most cancer patients. In order to metastasize, cancer cells must achieve new abilities such as an invasive phenotype. Complex interactions between cancer cells and the tumormicroenvironment mediate the main steps of the metastatic cascade that involves migration at the primary site, intravasation into lymphatic vessels or the blood stream, where they must survive from the immune system attack, arrest and extravasation to a new site. Either at the primary tumor or at the distant site, cancer cells interact with the components of the tumor microenvironment such as stromal cells, the endothelium and extracellular matrix components. It is well established that these interactions may facilitate metastatic growth and tissue selectivity. Most metastatic lesions do not respond to conventional cancer treatments for primary tumors and the development of metastatic lesions signals for a poor diagnosis. Therefore, a better understanding of the metastatic process may lead to improved therapies for cancer patients [1].

The contribution of stromal fibroblasts for the development of metastasis has been previously demonstrated [2–4]. Fibroblast stimulation by breast tumor cells results in a proinvasive and prometastatic phenotype with altered expression of matrix metalloprotease (MMP) activity via upregulation of key cytokine expression [2–4]. It has been recently reported that metastatic prostate cancer cells irreversibly activated normal fibroblasts to tumor-associated fibroblasts (TAFs) with subsequent reduced expression of ECM components, increased expression of MT1-MMP and enhanced motility [5].

The presence of hypoxia pockets in the tumor mass has been described as one of the major factors for the development of metastasis. In hypoxic conditions, tumor cells respond not only to paracrine signaling but also autocrine signaling to angiogenic

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factors such as the vascular endothelial growth factor (VEGF) [6,7]. VEGF autocrine signaling in tumor cells induced by hypoxia leads to the activation of the PI3-kinase signaling pathway resulting in decreased apoptosis and stimulation of breast cancer cell migration toward a chemo attractant gradient [6–8]. VEGF binds to and activates three types of tyrosine kinase receptors, VEGFR1 (Flt-1) [8], VEGFR2 (KDR/Flk-1) and VEGFR3 [6,8–11], and several co-receptors, triggering intracellular signaling pathways that result in cell migration, survival, and proliferation of endothelial cells. Negative regulation is mostly due to a soluble VEGFR1 variant that clears VEGF therefore preventing its binding to VEGFR2 [12]. VEGF receptors have also been described in tumor cells [6,9]. Thus, VEGF-targeted therapy has become an important alternative for cancer treatment and a number of molecules have been submitted to clinical trials including anti-VEGF antibodies [13]. However, its effectiveness has a very short-life and many patients do not respond to VEGF-targeted therapy, while others develop drug resistance mechanisms and in some cases with increased number of metastatic foci [14,15]. Therefore, a better understanding of the tumor microenvironment and search for new targets are relevant issues.

In most steps of the metastatic cascade, changes in tumor cell adhesion to stromal cells and to the extracellular matrix (ECM) as well as proteolytic degradation of ECM by MMPs are needed for cell invasion. Tumor cell adhesion to ECM is mediated by integrins, heterodimeric receptors of ECM components [16]. Integrins are composed by 1 of 18 α and 1 of 8 β chains of transmembrane proteins that bind to specific proteins in the ECM. Ligand binding to integrins switches the receptor from an inactive form to an active state, which triggers specific intracellular signaling cascades [17]. There is accumulated evidence that integrins play a key role in cancer cell proliferation, invasion and apoptosis [18,19]. The α6β4 integrin, a key laminin receptor in adhesion structures such as hemidesmosomes, induces tumor cell migration by activating PI3k/Akt pathway [9]. The vitronectin receptor, αvβ3 integrin, is highly expressed in endothelial cells of angiogenic vessels [20]. The αvβ3 integrin is also very important in the process of intravasation and extravasation of tumor cells [21], and is strongly involved in the malignant spread of several tumor cell types such as breast and prostate carcinoma [22]. Blocking αvβ3 integrin is therefore expected to have some impact in cancer therapy. A few integrin blockers have entered in clinical trials with encouraging results [16]. Most ligands for αvβ3 and αIIbβ3 integrins bind to the receptors by the adhesive motif arginine-glycine-aspartic acid (RGD) [23]. For instance, the dual blocking of αvβ3 and αvβ5 integrins for inhibition of both angiogenesis and invasion was the basis of cilengitide design [24]. Cilengitide was demonstrated to induce endothelial and glioma cell apoptosis by inhibition of the focal adhesion kinase (FAK) pathway [25].

Previous studies have demonstrated that metastasis is facilitated by platelet adhesion to tumor cells, forming coaggregates and contributing to the arrest at the vasculature [26]. Convincing evidence for the relevance of platelets in the metastatic process is the reduced number of metastasis in thrombocytopenic mice relative to normal animals [26]. Previous studies demonstrated that tumor cells use the αvβ3 integrin to bind platelet αIIbβ3 integrin via cross-linking plasma protein ligands [27]. However, antagonists of both platelet integrin receptors only partially block tumor cell adhesion [26]. These results demonstrated that the search for new molecules with more specific anti-integrin activity is necessary.

Disintegrins comprise a family of nonenzymatic, low-molecular-weight, cystein-rich peptides from snake venom that specifically and potently inhibit integrin functions. Several disintegrins have demonstrated their anti-cancer properties [28,29]. Contortrostatin, salmosin, accutin or rhodostomin were described with anti-angiogenic and anti-metastatic activities [30–32]. Many disintegrins contain the RGD or the KGD adhesive motif sequence which acts as a potent antagonist of β1 and β3 integrins, in particular, α5β1, αIIbβ3 and αvβ3 integrins [33]. However, KTS/RTS-disintegrins that bind specifically to α1β1 integrin were recently demonstrated to have anti-angiogenic properties [34–36].

We have previously reported that Dis*Ba*-01, a recombinant His-tag fusion RGD-disintegrin from *Bothrops alternatus* snake venom, inhibits both angiogenesis and lung metastasis in nude mice [21]. Dis*Ba*-01 is a high-affinity αvβ3 and αIIbβ3-integrin binding protein with strong anti-platelet and anti-thrombotic effects [37]. Recently, it has been demonstrated that Dis*Ba*-01 also inhibits tumor cell migration *in vitro* [38]. Here we show that Dis*Ba*-01 also decreased the expression of VEGF and its receptors in endothelial cells, but not in fibroblasts or tumor cells. In addition, this disintegrin modulates MMP-2 and -9 activities. As far as we know, this is the first report of such activities for this type of integrin blockers. These results add some new light on the understanding of the role of αvβ3 integrin antagonism on the tumor microenvironment.

2. Experimental procedures

2.1. DisBa-01 expression, purification and characterization

Since the native Dis*Ba*-01 disintegrin could not be purified from the venom due to very low yields in protein preparations, **r**ecombinant Dis*Ba*-01 was produced from a cDNA venom gland library of *B. alternatus* [21]. The coding region corresponds to a medium disintegrin (78 amino acid residues) with an RGD adhesive motif (GenBank accession AY259516). Expression and purification of the recombinant His-tag protein were performed as previously described [21]. N-terminal sequencing confirmed the expected sequence of the 12 kDa fusion protein which was then used in all biological assays. Molecular modeling suggested that the fusion His-tag peptide was not involved in integrin binding and therefore its proteolytic removal could not be needed [21].

2.2. Cell lines and culture

Human Fibroblasts were purchased from the Cell Bank of Rio de Janeiro (Brazil) where they were certified by short tandem repeat (STR) analysis. Human breast tumor cell line (MDA-MB-231) was from ATCC. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Vitrocell, Campinas; SP; Brazil) supplemented with 10% (v/v) fetal bovine serum (FBS). Microvascular endothelial HMEC-1 was cultured in MCDB-131 (GIBCO) with 10% FBS. All cell lines were cultured in the presence of penicillin (100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM), in a humidified environment with 5% CO_2 at 37 °C. For all cell assays, the cells were harvested with 0.25% trypsin-0.1% EDTA solution.

2.3. Flow cytometry analysis

The integrin content of the cells was determined using specific anti-integrin antibody by flow cytometry. Brief, 1×10^6 cells were incubated with 1 μg of anti-integrin at 4 °C for 30 min. After, the cells were washed with PBS and centrifuged at 4 °C, for 10 min at 150 × *g*. Then, 0.5 μg of FITC-IgG was added to each sample and incubated for 30 min at 4 °C in the dark. In the end of this time, the cells were washed again with PBS, centrifuged and the cells were immediately analysed with FACsCalibur flow cytometer (BD Bioscience).

2.4. Adhesion assays

For the **s**tatic adhesion assay, a 96-well microplate was coated overnight with collagen type I, III or IV (1 μg/well) in acetic acid (0.1%), fibronectin (1 μg/well) or the disintegrin (0.1; 1; 10 μg/well) and then blocked with 1% BSA. Cells (5×10^4 cells/well) in serum-free DMEM medium were added to the wells and allowed to adhere to the substrate during 30 min at 37 °C. After, the unbound cells were gently removed and the remaining cells were fixed and stained with 0.5% crystal violet for 30 min. Cells were then washed and lysed by 1% SDS for 30 min and the absorbance was read at 540 nm. Untreated cells adhered to the collagen type I and to BSA were considered as positive (100%) and negative control, respectively. A synthetic His-tag peptide (Sigma, USA) was also used as a control. The adhesion assays under flow were made as previously described [39,40]. Briefly, tumor cells (10^7cells/ml) were labeled with cell tracker red CPMTX (Invitrogen) for 30 min and incubated with different concentrations of Dis*Ba*-01 at 37 °C. Whole blood obtained from consenting healthy human donors was anticoagulated with heparin (13 IU/ml) and centrifuged for 20 min at 200 × g to obtain platelet-rich plasma (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 disintegrin-treated tumor labeled cells (1:10, v/v). The cover slips coated with collagen I (2.5 mg/ml) were placed into the chamber and blood containing labeled tumor cells was perfused through the chamber for 10 min at 1500 s^{-1} at a constant blood flow rate of 10 mL/min using a peristaltic pump. After, the cover slips were washed in PBS for 5 min and fifteen different fields were captured using the software Scion Visiocapture Image Acquisition Application on an epifluorescent microscope (Nikon Eclipse TE 300) coupled to a photo machine. Fluorescent adhered platelets and cells in each field were differentially counted using the software Image J. Results are expressed as the percentage of adhered cells compared to the control without the disintegrin.

2.5. Isolation of total RNA and synthesis of cDNA

Cells in culture medium (DMEM or MCDB-131) plus 10% FBS were incubated in 6 cm dishes (Corning) coated with collagen type I (1 mg/ml) in medium for 90 min at 37 °C and 5% CO_2. The cells were then incubated with 0, 1, 10 or 1000 nM Dis*Ba*-01. After time incubation of 4, 24, 48 and 72 h, culture medium was removed and stored at −80 °C for immunological assays. Cells were lysed with cold TRIzol Reagent (Invitrogen) according to the manufacture's protocol for total RNA isolation. RNA concentrations were determined by spectrophotometry at 260 nm and RNA integrity was verified by ethidium bromide staining on a 1% agarose-formaldehyde gel. Total RNA was reverse transcribed into cDNA using the M-MLV Reverse Transcriptase (Promega). cDNA was stored at −20 °C until use.

2.6. Real-time PCR

VEGF, VEGFR1 and VEGFR2 mRNA levels were quantified by real-time using the ABI SYBR® Green PCR Master Mix (Applied Biosystems, Fosters City, CA). For VEGFR1 and VEGFR2, the PCR conditions were the same as previously described (29). The primer sequences for the analysis of VEGF expression were: forward 5′-CACATTGTTGGAAGAAGCAGCCCA; reverse 5′- ACTCACACACACA-CAACCAGGTCT, and the PCR conditions were: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 20 s.

2.7. VEGF enzyme-linked immunoassay

The conditioned medium of treated and untreated (control) cells was used for analysis of VEGF expression by ELISA. The assays were performed using the kit Quantikine immunoassay for human VEGF (R&D Systems, Minneaopolis, MN) according to the manufacturer's instructions.

2.8. Gelatin zymography

MMP activity was analyzed by the gelatin zymography on a wound healing assay. Cells (2×10^5) were seeded in 12 well-plates, grown at 37 °C until confluency and scratched with a 200 μl plastic tip. Confluent monolayers were washed once with the appropriate medium to remove cell debris, and medium without serum and with different Dis*Ba*-01 concentrations was added to the wells. After the treatment, culture medium was collected, centrifuged at 10,000 × *g* for 5 min at 4 °C and incubated with sample buffer without β-mercaptoethanol. The samples were resolved at 4 °C in a 10% polyacrylamide gel containing 0.1% gelatin. The gel was washed 2 times with 2.5% Triton X-100 and incubated at 37 °C during 16–22 h in 50 mM Tris buffer pH 8.0, 5 mM $CaCl_2$, 0.02% NaN_3 and 10 mM $ZnCl_2$. After staining with Commassie Blue R-250 and destaining with acetic acid:methanol:water (1:4:5), the clear bands were quantified by densitometry using the Gene Tool version 3.06 software (Syngene, Cambridge, UK).

2.9. Statistical analysis

Each experiment was repeated three times and mean and standard error were calculated. The results were compared statistically using one-way analysis of variance (ANOVA) and Dunnett's statistical test was used when p level were $^*p < 0.05$.

3. Results

3.1. Integrin expression by human fibroblasts and MDA-MB-231 cells

To study the role of Dis*Ba*-01 in interfering on αvβ3 integrin-mediated cell adhesion, we have first analyzed by flow cytometry the content of some major integrins in the cell types studied. Human fibroblasts express αvβ3 and αvβ5 as well as α6, αv and β1 but not α2. Similar results were observed for MDA-MB-231 cells but these cells express high levels of α2 (Supplementary material 1). The human microvascular endothelial cell line-1 (HMEC-1) was reported to express high levels of αvβ3 [38]. Therefore all the cell lines were considered good models to investigate the role of a αvβ3 integrin inhibitor of tumor cell adhesion to ECM.

3.2. DisBa-01 acts like a matrix protein for fibroblasts and tumor cells

The cells are able to adhere to different ECM proteins by recognizing adhesive sequences through cellular receptors, such as integrins. We demonstrate here that Dis*Ba*-01 was able to support the adhesion of fibroblasts and MDA-MB-231 cells similar to collagen and fibronectin (Fig. 1A–B, respectively). Dis*Ba*-01 was effective in supporting close to maximum fibroblast adhesion with the lowest amount (0.1 μg/well). A control synthetic His-tag peptide was ineffective in supporting cell adhesion.

3.3. DisBa-01 affects tumor cell adhesion under flow conditions

We have previously demonstrated that Dis*Ba*-01 dose-dependently inhibited the static adhesion of B16F10 melanoma cells and of HMEC-1 to vitronectin, with IC_{50} of 225 nM and 555 nM, respectively [21]. Here we have tested the effect of this disintegrin in inhibiting the adhesion of MDA-MB-231 cell line

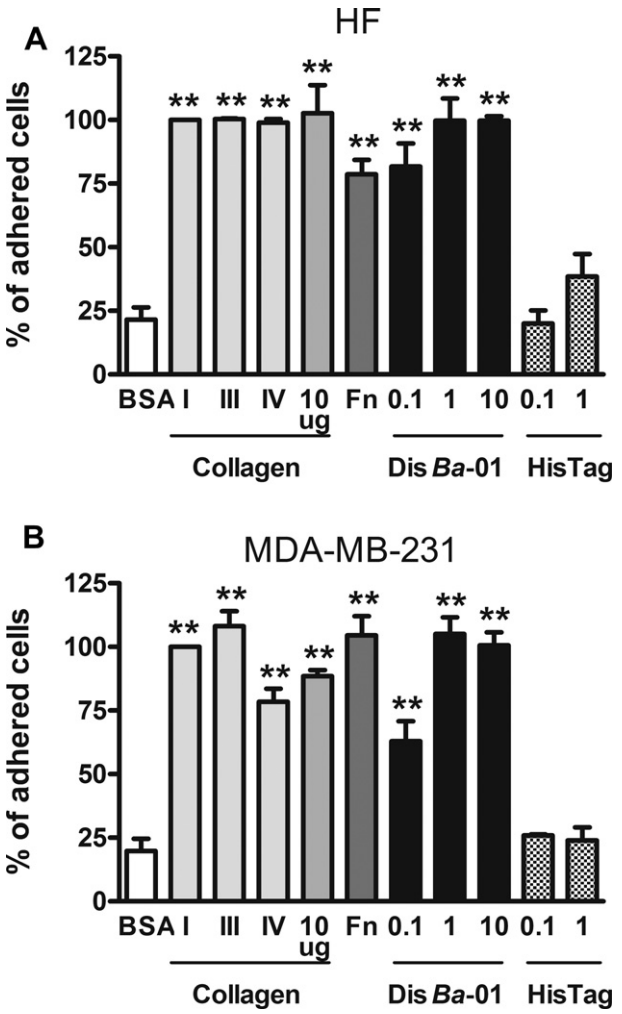

Fig. 1. Dis*Ba*-01 acts like a matrix protein for fibroblasts and tumor cells. Dis*Ba*-01 supports the adhesion of human fibroblasts (A) and MDA-MB-231 breast tumor cells (B). Ninety six-well plates were coated with denatured collagen I, III, IV (1 μg/well), fibronectin (Fn, 1 μg/well), Dis*Ba*-01 (0.1; 1; 10 g/well) and His-tag peptide (0.1 and 1.0 μg/well) overnight at 4 °C. After blocking with 1% BSA, cells (1×10^5cells/well) were seeded in the wells and allowed to adhere for 30 min. Cells were then fixed, staining with crystal violet and the plate was read at 540 nm. Results are expressed as percentage of control (considered as 100% attachment) of three independent experiments ($^{**}p < 0.01$). BSA was used as negative control.

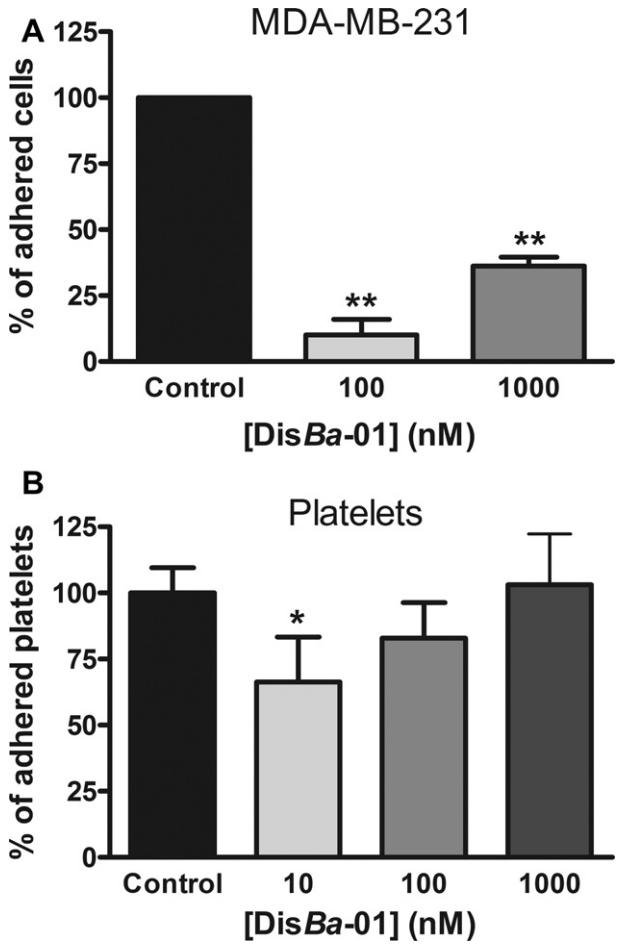

Fig. 2. Inhibition of MDA-MB-231 tumor cell and platelet adhesion to collagen type I by Dis*Ba*-01 under flow conditions. CPMTX-labeled cells (10^7 cells/ml) were incubated 30 min with Dis*Ba*-01 (10, 100, 1000 nM) or PBS (control) and added to whole blood, prepared in 1:5 dilution with calcein labeled-PRP. The mixture of MDA-MB-231 cells and blood was perfused (1500 sec^{-1}) for 10 min on collagen type I-coated cover slips, in a perfusion chamber. Tumor cell (A) and platelet (B) adhesion were measured by the analysis of images taken from fifteen random fields on each cover slip using the software Image J. Results are the mean ± standard error of three independent experiments. Statistical analysis was done using Dunnett's test ($^{*}p < 0.05$ and $^{**}p < 0.01$).

under flow conditions at shear rate of 1500 s^{-1}, a condition considered to simulate the human arterial shear rate [29]. We observed that Dis*Ba*-01 strongly inhibited the adhesion of MDA-MB-231 cells to collagen type I at 100 and 1000 nM concentrations (90% and 64%, respectively) compared with the control (Fig. 2A). In addition, the adhesion of activated platelets to collagen I was inhibited only by 10 nM Dis*Ba*-01 (33.75% ± 3.93%) confirming its specificity for αv integrins (Fig. 2B).

3.4. DisBa-01 decreases VEGF expression in HMEC but not in fibroblasts or MDA-MB-231 cells

Since it was previously demonstrated that ALT-C, an ECD-disintegrin, Cys-rich protein from *B. alternatus* venom induced VEGF expression in fibroblasts [41], we have checked if Dis*Ba*-01, an RGD disintegrin could also induce the same effect. We observed here that VEGF gene expression levels in human fibroblasts were not significantly altered independently of the incubation time (4–72 h) and concentration of Dis*Ba*-01 (1, 10, 100 and 1000 nM) (Fig. 3A). A significant increasing effect was observed only after 24 h of 1000 nM Dis*Ba*-01 treatment. However, Dis*Ba*-01 strongly decreased VEGF mRNA expression in HMEC in all times and tested concentrations (Fig. 3B). Interestingly, MDA-MB-231 cells also expressed significant levels of VEGF mRNA that were not affected by the disintegrin with the only exception of 1000 nM of Dis*Ba*-01 treatment after 72 h (Fig. 3C).

Surprisingly, VEGF protein levels were significantly increased after 48 and 72 h of 10 and 1000 nM Dis*Ba*-01 treatment in the conditioned medium of fibroblast cultures as detected by ELISA (Fig. 4A). Since the culture medium was not replaced by fresh medium during the incubation period, these results could indicate VEGF accumulation in the conditioned medium. In that case, the same observation should also be made in the controls. However, increase in VEGF levels in the conditioned medium of Dis*Ba*-01-treated cells were above the controls thus reflecting an effect induced by the disintegrin.

In agreement with the gene expression results, VEGF protein levels were not significantly modified in disintegrin-treated MDA-MB-231 cells (Fig. 4B). It was also observed that these cells express

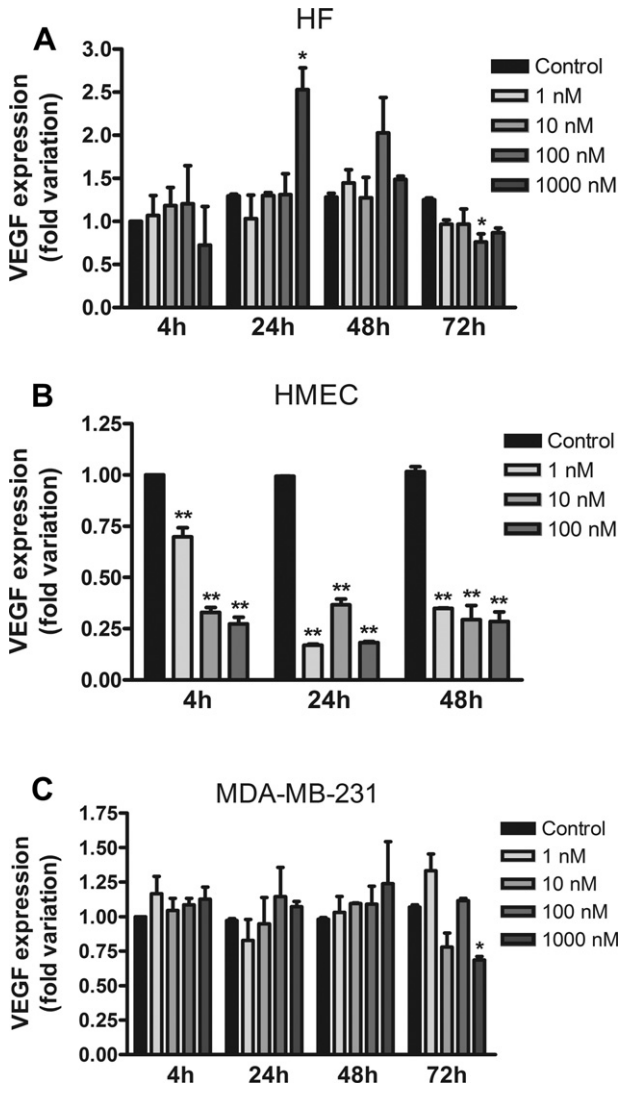

Fig. 3. Dis*Ba*-01 modulates VEGF mRNA expression. The levels of VEGF mRNA of Dis*Ba*-01-treated human fibroblasts (A), HMEC (B) and MDA-MB-231 cells (C) were analyzed by qPCR. Cells were plated on collagen I-coated wells, incubated with the indicated concentrations of Dis*Ba*-01 at several times and then lysed for RNA extraction. The relative levels of VEGF mRNA were normalized to those of β-actin mRNA compared with the non-treated control. Results are mean ± SEM of three independent experiments. The *p* value was determined using the using Dunnett's test ($^{*}p < 0.05$; $^{**}p < 0.01$).

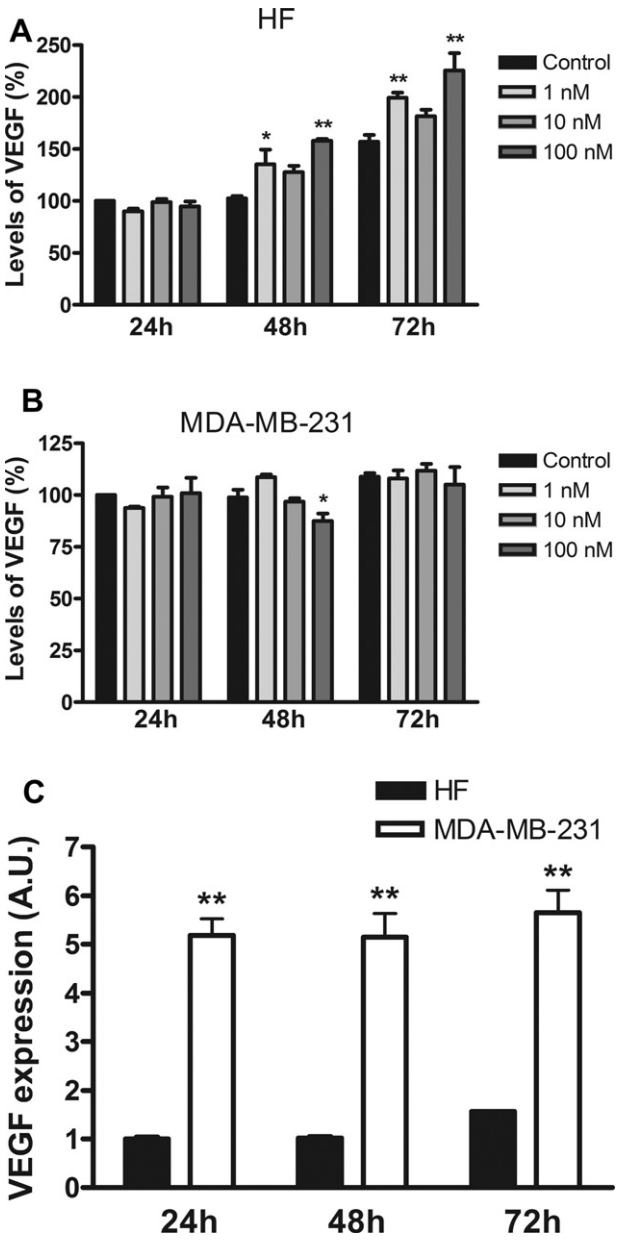

Fig. 4. Dis*Ba*-01 increases VEGF protein expression in fibroblasts. ELISA assays for VEGF detection were performed using the Quantikine immunoassay kit for human VEGF in the conditioned medium of (A) Human fibroblasts (HF) or (B) MDA-MB-231 cells incubated with the indicated disintegrin concentrations. Results are represented as the percentage from the control (100%) as the mean ± SEM of three independent experiments. In (C), the absolute VEGF levels produced by the two types of cells are compared. The *p* value was determined using the using Dunnett's test ($^{*}p < 0.05$; $^{**}p < 0.01$).

higher amounts (about 5 times) of VEGF than fibroblasts (Fig. 4C). Dis*Ba*-01 did not affect the number of the viable fibroblasts and MDA-MB-231 cells at the tested concentrations up to 72 h (not shown). However, the viability of HMEC cells was reported to be decreased by Dis*Ba*-01 at about 42% by concentrations ranging from 5 to 100 nM [21].

3.5. DisBa-01 inhibits the expression of VEGFR1 and VEGFR2 in HMEC-1 cells

The expression of VEGF receptors in endothelial cells was not significantly altered by ALT-C, an ECD-disintegrin-like, Cys-rich protein as previously demonstrated by our group [29]. However, we show here that Dis*Ba*-01 (1–100 nM) strongly downregulates VEGFR1 (Fig. 5A) and VEGFR2 (Fig. 5B) gene expression in HMEC cells, and this effect was more pronounced with 10 nM Dis*Ba*-01. Similar results were found for cells treated with 100 nM but not with 1 nM of Dis*Ba*-01.

3.6. DisBa-01 modulates MMP-2 and MMP-9 activity

We tested next if Dis*Ba*-01 could affect MMP activity, which is considered to be relevant for angiogenesis and cell migration. For that, the conditioned medium from Dis*Ba*-01-treated cells after a wound healing assay was analyzed in gelatin zymography. Interestingly, MMP-2 activity was strongly decreased in fibroblast conditioned medium (Fig. 6A) and this effect was concentration-dependent in an inverse way, with the highest inhibition induced by the lowest dose. MMP-9 activity was observed only in the controls suggesting that it was also inhibited by the disintegrin (not

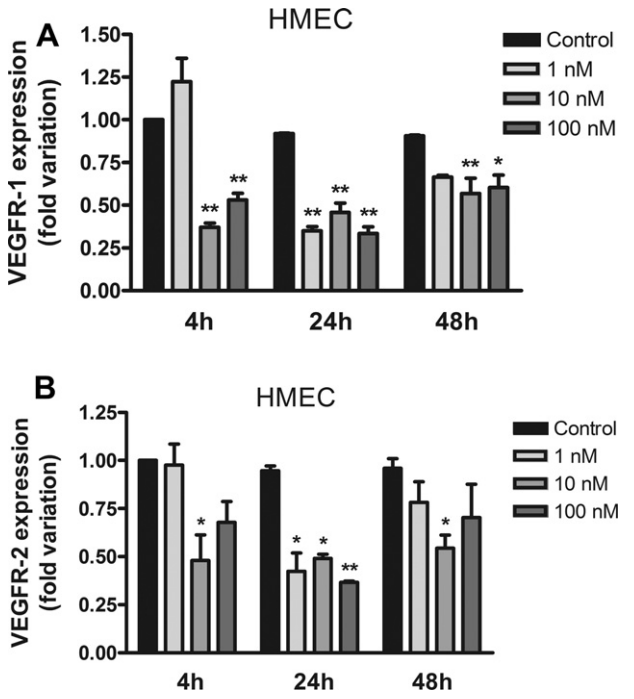

Fig. 5. Dis*Ba*-01 inhibits VEGF receptor expression in HMEC-1. Gene expression of VEGFR1 (A) and VEGFR2 (B) In HMEC-I cells was dose-dependently inhibited by Dis*Ba*-01. Cells were incubated with different concentration of Dis*Ba*-01 for several incubation times (4, 24, 48 and 72 h). RNA was reversed transcribed into cDNA and used in a real-time PCR with VEGFR1 and VEGFR2 primers. Data are mean ± SEM of three independent experiments. The p value was determined using the Dunnett's test. ($^{*}p < 0.05$; $^{**}p < 0.01$).

shown). Interestingly, an opposite situation was seen for MDA-MB-231 cells which expressed increased MMP-9 activity levels in the presence of Dis*Ba*-01 but MMP-2 was not detected (Fig. 6B).

4. Discussion

Metastatic dissemination depends on a cascade of integrin-mediated contacts between the tumor and stromal cells as well as the tumor and the extracellular matrix. Integrin signaling through αvβ3 plays a critical role in endothelial cell proliferation and angiogenesis [42] and in tumor cells can promote migration and metastasis [43]. Therefore, blocking αvβ3 integrin function may be interesting to target several steps of the metastatic cascade and the tumor microenvironment. We have previously demonstrated that Dis*Ba*-01, an RGD-disintegrin that inhibits αvβ3 integrin, has anti-angiogenic and anti-metastatic properties [21]. Here we present new findings regarding the action of this protein that helps to understand its mechanism of action on the tumor microenvironment.

We have chosen three cell types to study Dis*Ba*-01 effects on a tumor microenvironment: Fibroblasts (stromal cells), endothelial cells (HMEC), and a tumor cell line from human breast cancer (MDA-MB-231) in agreement with our previous studies [21]. All studied cell lines express significant levels of αvβ3 integrin that explains the ability of Dis*Ba*-01 to support cell adhesion. However, inhibition of cell adhesion studies showed that this protein does not affect fibroblast and MDA-MB-231 cell binding to collagen I, II, IV, fibronectin, laminin, thrombospondin, and matrigel [21]. These results suggest that Dis*Ba*-01 acts preferably through binding to αvβ3 integrin. Negative adhesion results obtained with a synthetic His-tag peptide confirm our previous molecular modeling data [21] that the fusion peptide does not interfere with the biological activity of Dis*Ba*-01.

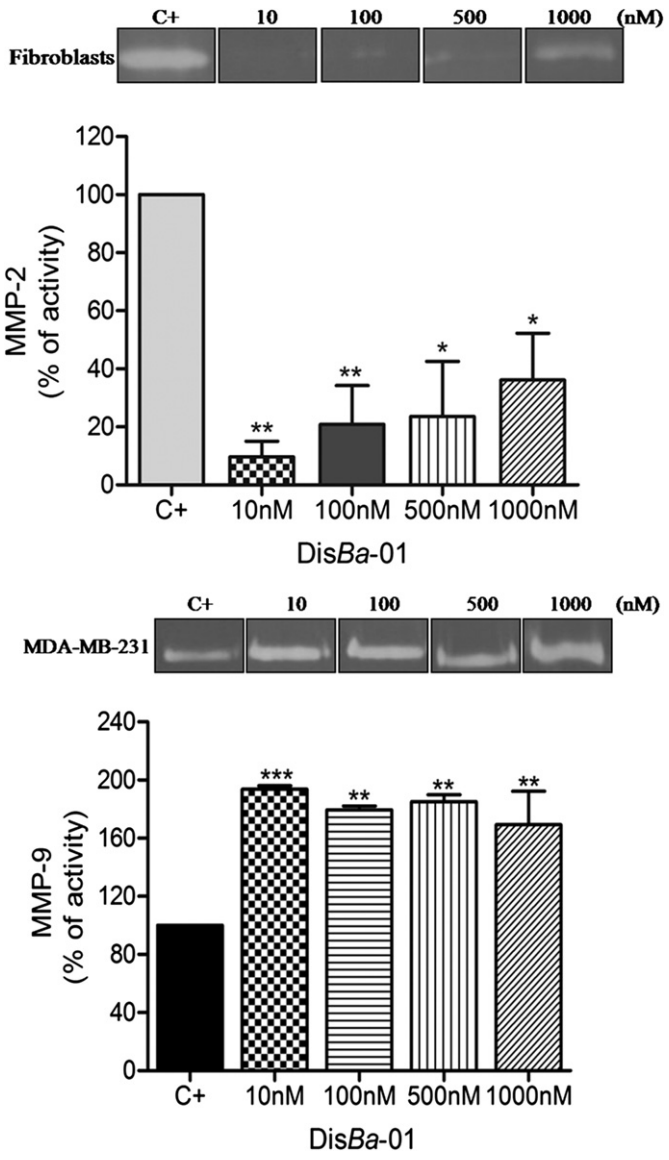

Fig. 6. Effects of Dis*Ba*-01 over MMP-2 and MMP-9 activities in conditioned media from fibroblast and MDA-MB-231 cultures, respectively. Fibroblasts (upper panel) and MDA-MB-231 cells (lower panel) were treated with the indicated concentrations of Dis*Ba*-01 for 24 h in a wound healing assay, and the supernatants subjected to gelatin zymography. Medium without serum from non-treated cells was used as positive control. Zymograms are representative of a single experiment in triplicate. Each bar represents the percentage of MMP activity ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

In the present paper we show that Dis*Ba*-01 also inhibits (90%) the adhesion of MDA-MB-231 cells to collagen I under flow conditions in the presence of blood and platelets. Collagen type I plays a critical role in initiating angiogenesis by binding to endothelial cells after basement membrane degradation [44]. The αvβ3 integrin is a canonical vitronectin receptor but it also binds to collagen I and loss of β1 integrin expression has been reported to activate αvβ3 integrin to become functionally dominant and to induce collagen synthesis [45,46]. Therefore, inhibition of tumor cell binding to collagen I may be helpful in inhibiting not only metastasis but also angiogenesis. Interestingly, the lowest concentration of Dis*Ba*-01 induces the more pronounced effect, a characteristic that is also observed for other assays. RGD-binding integrins are known to produce bell-shaped curves where low doses (nanomolar concentrations) may stimulate angiogenesis and

high (micromolar) concentrations αvβ3/αvβ5—inhibitors inhibited angiogenesis [47]. There are several examples in the literature of compounds that act as pro-angiogenics at low concentrations and anti-angiogenics at higher concentrations such as bortezomib [48], RGD-mimetic integrin inhibitors [49,50], and TGF-β1 [51] but the mechanism underlying these effects is poorly understood. This bell-shaped effect has been suggested as a factor responsible for resistance to angiogenesis inhibitor treatment [47]. Importantly, nanomolar concentrations of αvβ3/αvβ5—integrin inhibitors induced the recycling of both αvβ3—integrin and VEGFR2 suggesting an interplay between these receptors [49].

On the other hand, Dis*Ba*-01 does not inhibit platelet adhesion to collagen I in concentrations that inhibit tumor cell adhesion, with the exception of 10 nM concentration, despite our previous report of anti-platelet and anti-thrombotic activities of this protein [37]. These results are probably due to the fact that Dis*Ba*-01 does not affect collagen binding to platelets which is mediated mostly by α2β1 integrin and GPVI protein [52]. The ways that platelets cooperate in tumor cell dissemination is well described in the literature [53]. Platelets adhere to tumor cells to form coaggregates that protect them from the cytotoxicity of natural killer cells and also help tumor cell extravasation to the metastatic niche. Also, platelets are stores of pro-angiogenic factors that help tumor invasion and growth. Therefore, inhibition of platelet adhesion could help in anti-metastatic therapy. However, Gomes et al., [26], using a similar flow model, demonstrated that the adhesion of MDA-MB-231 cells to collagen I was only partially (45%) inhibited by two different non-peptide antagonists (lamifiban and SB-273005) of platelet αIIbβ3 and αvβ3 integrin, respectively [26]. Our results showed that Dis*Ba*-01 may represent a better option for blocking tumor cell adhesion to collagen since it was more effective, inhibiting approximately 90% of MDA-MB-231 cell adhesion. Dis*Ba*-01 represents a His-tag fusion peptide and despite its significant inhibitory activity, it may produce even stronger effects if cleaved off from the fusion peptide but this possibility remains to be determined.

We have previously demonstrated that Dis*Ba*-01 inhibits bFGF-induced angiogenesis in athymic nude mice using the matrigel plug assay but the mechanism of this inhibition was not known. Here we show that Dis*Ba*-01 strongly inhibits the expression of VEGF and its receptors in endothelial cells and these effects are probably mediated by αvβ3 integrin inhibition. However, the contribution of other RGD-binding integrins such as αvβ5, αvβ6 and αvβ8 has to be considered but remains to be determined. Since Dis*Ba*-01 decreased endothelial cell viability, it is possible that the decrease in VEGF levels could be due to endothelial cell death. However, we measured only the soluble fraction of VEGF. It is possible that a fraction of VEGF remains associated with the cells as it has been demonstrated for cells supporting autocrine VEGF signaling [54].

Contrastingly, VEGF protein levels in the conditioned medium of fibroblasts culture were increased in the presence of Dis*Ba*-01. Apparently, both fibroblasts and MDA-MB-231 cells express VEGF constitutively, although the expression level is much higher for the tumor cells. More importantly, blocking αvβ3 integrin did not interfere with VEGF expression in the tumor cells as it did in endothelial cells thus suggesting that distinct pathways must be involved in growth factor synthesis in the two types of cells. It has been recently demonstrated that endothelial FAK deletion in mice inhibited tumor growth and tumor angiogenesis as well as VEGF-induced angiogenesis [55] confirming the strong correlation between angiogenesis and integrin-mediated pathways. Recently, a cross-talk between endothelial cells and pericytes has been demonstrated [54]. Pericytes recruited by endothelial cells secrete vitronectin which through αv integrin- and NF-κB- mediated signaling pathway results in upregulation of VEGF by endothelial cells. These data are in agreement with the results of the present study since the blockade of αvβ3 integrin by Dis*Ba*-01 inhibits VEGF protein expression in HMEC.

Dis*Ba*-01 also downregulates the expression of VEGFR1 and 2 in endothelial cells, in addition to the decrease in VEGF protein expression. VEGFR2 is considered the key mediator of VEGF-induced angiogenesis [56] and therefore its down regulation decreases the angiogenic response. However, a compensatory angiogenic signaling pathway involving the fibroblast growth factor (FGF) was described as a resistance mechanism to VEGF-targeted therapies for cancer treatment [57,58]. In addition, it was reported that VEGF deletion in myeloid cells accelerates tumorigenesis [59]. Therefore, αvβ3 integrin-targeted therapy may be an alternative of anti-angiogenic treatment; however, the development of resistance remains to be studied. Unexpectedly, Dis*Ba*-01 also affected the expression of VEGF receptors in fibroblast and MDA-MB-231 cells. Recent work has provided strong evidence that VEGFR expression is not restricted to endothelial cells but it can also be detected in other breast cancer cells [60] and in thyroid follicular cells [61].

Tumor cell migration is a hallmark of metastasis spreading. Migration is an integrin-mediated process that involves focal adhesion kinase (FAK) activation and phosphorylation, which, along with the recruitment of a set of accessory proteins, results in the activation of the mitogen-associated protein kinase (MAPK) pathway [62]. Blocking integrin and FAK signaling therefore results in inhibition of cell migration. We have previously demonstrated that Dis*Ba*-01 strongly inhibits FAK phosphorylation and downstream signaling in platelets [37] and also inhibits the migration of human fibroblasts, prostate cancer cells (DU-145) and MDA-MB-231 cells toward conditioned medium in similar concentrations to the ones used in the present study [38].

ECM degradation by MMPs is crucial in order to allow cells to migrate. A close association between MMP-2 and αvβ3 integrin has been suggested since both MMP-2 and MMP-9 localize to the membrane by binding to αvβ3 integrin and CD44, respectively [63]. Here we show that inhibition of αvβ3 (and perhaps α5β1) integrin by Dis*Ba*-01 significantly inhibits MMP-2 activity of fibroblasts which certainly contributes to a decreased migratory ability. However, MMP-2 activity of MDA-MB-231 cells was not affected by Dis*Ba*-01.

Contrastingly, Dis*Ba*-01 stimulates MMP-9 activity in MDA-MB-231 cells which could also contribute to its anti-angiogenic effect as we have previously demonstrated [21]. MMP-9 has been associated to favorable diagnosis in some cases of breast and liver cancer, probably due to its ability to generate endogenous angiogenesis inhibitors such as angiostatin from collagen IV [64]. Interestingly, contortrostatin, a homodimeric RGD disintegrin was reported to inhibit the invasion of glioma cells through matrigel without affecting MMP activity [65]. If these distinct effects are due to the differences in disintegrin structure, cell types or assays remains to be determined.

In summary, we show here that the blockade of αvβ3 integrin by Dis*Ba*-01 strongly affects the tumor microenvironment and that stromal and tumor cells respond differently under disintegrin exposure. These results contribute to the understanding of the molecular mechanism of new anti-metastatic therapies targeting integrin-mediated processes.

[2]Dis*Ba*-01: a recombinant RGD-disintegrin from *Bothrops alternatus* snake venom.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biochi.2012.04.020.

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Research paper

ADAM9 silencing inhibits breast tumor cell invasion *in vitro*

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ABSTRACT

ADAM9 (A Disintegrin And Metalloproteinase 9) is a member of the ADAM protein family which contains a disintegrin domain. This protein family plays key roles in many physiological processes, including fertilization, migration, and cell survival. The ADAM proteins have also been implicated in various diseases, including cancer. Specifically, ADAM9 has been suggested to be involved in metastasis. To address this question, we generated ADAM9 knockdown clones of MDA-MB-231 breast tumor cells using silencing RNAs that were tested for cell adhesion, proliferation, migration and invasion assays. In RNAi-mediated ADAM9 silenced MDA-MB-231 cells, the expression of ADAM9 was lower from the third to the sixth day after silencing and inhibited tumor cell invasion in matrigel by approximately 72% when compared to control cells, without affecting cell adhesion, proliferation or migration. In conclusion, the generation of MDA-MB-231 knockdown clones lacking ADAM9 expression inhibited tumor cell invasion *in vitro*, suggesting that ADAM9 is an important molecule in the processes of invasion and metastasis.

1. Introduction

Attachment of cells to the extracellular matrix (ECM) depends mainly on a family of glycoproteins known as integrins [1], which are expressed on the cell surfaces of many cultured cell types at specialized adhesion sites known as focal contacts [2]. A number of structural and signaling proteins, such as integrins, cytoskeletal proteins, and kinases are concentrated at these sites and are known to initiate signal transduction pathways [3,4]. The aggregation of integrin receptors, ligand occupancy and tyrosine kinase-mediated phosphorylation are the key events that result in different processes, including cell migration, differentiation, tissue remodeling, cell proliferation, angiogenesis, tumor cell invasion and metastasis [1,5].

Members of the ADAM (an acronym for A Disintegrin And Metalloprotease) protein family are involved in several human diseases such as inflammatory disorders, neurological diseases, asthma and cancer metastasis [6,7]. ADAM9 is a transmembrane protein with a number of characteristic domains, including a pro-domain, a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich region, a transmembrane domain, and a short cytoplasmic tail [8]. The ADAM9 disintegrin domain binds to numerous integrins, such as $\alpha_6\beta_1$ integrins in fibroblasts [9], $\alpha_V\beta_5$ in myeloma cells [10] and $\alpha_V\beta_3$ in MDA-MB-231 breast tumor cells [11]. Mahimkar et al. [12] and Zigrino et al. [13] have demonstrated that the recombinant disintegrin and cysteine-rich domains from human ADAM9 mediate cellular adhesion through β_1 integrins. Furthermore, the disintegrin-like and cysteine-rich domains of ADAM9 mediate interactions between melanoma cells and fibroblasts [14].

Over-expression of ADAM9 has been reported in several human carcinomas, including kidney [15], prostate [16], breast [17], liver [18,19], pancreatic [20], gastric [21], cervix [22] and oral [23]. Expression of ADAM9 is elevated in skin melanoma but is restricted to the invading front [24]. Peduto et al. [25] found a correlation between ADAM9 titer and cancerous changes in mouse models of prostate cancer, especially in well-differentiated tumors. Increased expression of ADAM9 led to increased structural abnormalities and growth of early-stage tumors compared to controls.

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The ADAM9 protein also appears to interfere with various cell signaling systems. In prostate cancers, the fibroblast growth factor (FGF) signaling pathway is believed to be particularly important [26], with down-regulation of the fibroblast growth factor receptor 2 isoform IIIb (FGFR2IIIb) being a feature of prostrate tumor progression [27]. Transfection of FGFR2IIIb into malignant tumors is enough to inhibit their growth [28]. Therefore, it is potentially significant that over-expression of ADAM9 increases shedding of FGFR2IIIb from cells, which is expected to disrupt FGFR2IIIb signaling and reduce its function [25]. Additionally, over-expression of ADAM9 leads to increased release of epidermal growth factor (EGF) [25], a factor known to induce prostate cancer growth in rat pups [29].

Although ADAM9 is normally considered a transmembrane protein, a soluble form ADAM9-S has been described [30], which is derived from alternative splicing of the gene [31]. The ADAM9-S protein promotes the invasive phenotype of carcinoma cell lines, and ADAM9 is strongly expressed at the invading front of hepatic metastases, although the authors did not distinguish ADAM9 and ADAM9-S [31]. Taken together, these studies suggest that ADAM9 has a significant role in tumorigenesis and metastasis.

To better understand the role of ADAM9 in breast cancer progression, we generated knockdown clones lacking ADAM9 using RNAi in the MDA-MB-231 human breast tumor cell line. As far as we know, this is the first demonstration that decreased ADAM9 expression impaired the invasiveness of this cell line. In addition, the present work demonstrated, for the first time, ADAM9 silencing in a breast tumor cell line and provided evidence that ADAM9 may play an important role in the metastatic progression of human breast cancer.

2. Material and methods

2.1. Cell culture

MDA-MB-231 breast tumor cells were cultivated in DMEM medium (Invitrogen) containing 10% bovine fetal serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 μg/ml) (Invitrogen) in 5% CO_2 at 37 °C. The anti-ADAM9 antibody was from Abcam (anti-RP2ADAM9) and the anti-β-actin antibody was from Santa Cruz Biotech (sc-1616).

2.2. Design of siRNA primers

Primer set #104056, which targets exon 13 (disintegrin domain) (Silencer® Pre-designed siRNA, Ambion), was selected to ensure that other ADAMs would not be silenced simultaneously. The primer sequences were: sense (5′-rCrCrArGrArGrUrArCrUrGrCrArArUrGrGrUrUrCrUrUrCTC-3′) and antisense (5′-rGrArGrArArGrArArCrCrArUrUrGrCrArGrUrArCrUrCrUrGrGrArA-3′). The negative control (scrambled) used in the assays was *Silencer*® Select Negative Control No. 1 siRNA (Ambion). This sequence does not target any gene product and have no significant sequence similarity to human gene sequences, being essential for determining the effects of siRNA delivery.

2.3. ADAM9 RNA silencing

On the day before transfection, 2×10^5 MDA-MB-231 cells were plated in 5 ml of DMEM medium supplemented with 10% FBS without antibiotics. Ten microliters of lipofectamine were mixed with 490 μl OPTI-MEM serum-free medium (Invitrogen) and incubated at room temperature for 5 min. A total of 10 nM of RNA silencing primer was diluted in OPTI-MEM, added to the lipofectamine/OPTI-MEM mixture and incubated for 20 min at room temperature. This mixture was then added to the cells. The medium was changed 24 h after transfection. Controls comprised of non-treated cells, cells treated with transfection reagent only (lipofectamine), and cells treated with a scrambled primer. Cells were washed in phosphate buffered saline, harvested with Trizol reagent (Invitrogen) according to the manufacturer's protocol, and frozen immediately. For western blotting assays, cells were lysed with Triton X-100 in Hepes buffer [150 mM NaCl, 50 mM Hepes, 1.5 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Sigma), 100 mM NaF and 100 mM Na_3VO_4]. Protein concentrations in the lysed samples were determined by the BCA method (Pierce), and 30 μg of each sample was resolved by SDS-PAGE [32]. Protein bands were transferred to nitrocellulose membranes and probed with anti-RP2ADAM9 and anti-β-actin antibodies. Western blots were scanned on an Image Scanner (GE – General Electric). All the assays using ADAM9 knockdown MDA-MB-231 cells were performed after the third day of siRNA transfections.

2.4. Extraction of RNA and synthesis of cDNA

Total RNA was extracted from cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. All samples were treated with DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen). After quantification, a total of 1 μg of RNA was mixed with 0.5 μl of oligo dT (0.5 μg/μl) (Promega) and nuclease-free water to a volume of 7 μl and incubated at 70 °C for 5 min, followed by 5 min on ice. Next, 0.5 μl of 200 units/μl of Moloney Monkey Leukemia virus (MMLV) reverse transcriptase (Promega), 2.5 μl of 5× MMLV buffer (Promega), and 2.5 μl of 10 mM dNTP mix was added to the reaction. The whole mixture was incubated at 37 °C for 1 h, and was used posteriorly for qPCR.

2.5. Design of qPCR primers

ADAM9 primers targeting the disintegrin domain were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers spanned exon boundaries so that only mRNA sequences would be amplified. The primers were ADAM9DF1 (5′CTT GCT GCG AAG GAA GTA CC); and ADAM9DR1 (5′AAC ATC TGG CTG ACA GAA CTG A). Primers targeting HPRT1F1 (5′TGA CAC TGG CAA AAC AAT GCA), HPRT1R1 (5′GGT CCT TTT CAC CAG CAA GCT), GAPDHF1 (5′GAT GCT GGT GCT GAG TAT GT) and GAPDHR1 (5′GTG GTG CAG GAT GCA TTG CT) were used as endogen controls.

2.6. Gene expression

ADAM9 mRNA expression was measured in a Corbett Rotorgene RG 3000 (Corbett Research) using the following thermocycling conditions: 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s, 55 °C for 5 s and 72 °C for 20 s. The master mix in each well consisted of 12.5 μl ABsoluteTM QPCR SYBR Green mix (6 mM $MgCl_2$, reaction buffer, DNA polymerase and SYBR green dye) (Advanced Biotechnologies), 1.25 μl each of 5 μM forward and reverse primer and 10.5 μl of nuclease-free water in a total volume of 25 μl.

2.7. Proliferation assays

To measure the effect of RNAi-mediated ADAM9 silencing on cell proliferation the transition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used [33]. Cells without any treatment, cells treated only with lipofectamine, and cells transfected with siRNAs were seeded in 96-well plates and left at 37 °C for 24 and 48 h. The cells were then washed with

PBS and incubated in 50 μl of 0.5 mg/ml MTT in culture medium at 37 °C for 4 h. Following the addition of 100 μl of isopropanol, the absorbance was read at 595 nm in an ELISA plate reader. The mean proliferation of cells without any treatment was expressed as 100%.

2.8. Adhesion assays

The effect of RNAi-mediated ADAM9 silencing on the adhesion of MDA-MB-231 cells was analyzed in 96-well plates (Corning). A solution of type I collagen (10 μg) was immobilized on the plates in 0.1% acetic acid. Fibronectin and laminin (10 μg) were dissolved in adhesion buffer (20 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$ and 1 mM $MnCl_2$ pH 7.35), overnight at 4 °C. On the next day, the wells were blocked with a solution of 1% BSA diluted in adhesion buffer for 1 h. Cells were counted and their concentration was adjusted in proportion to 5×10^6/ml. The blocking solution was removed from the wells and they were washed twice with adhesion buffer (100 μl). After this period the cells were incubated for 45 min on the coatings and subsequently wells were washed in order to remove non-adherent cells. A solution of 70% ethanol (100 μl) was added to the wells and the plate was incubated for 10 min at room temperature. Subsequently, the ethanol was removed and 60 μl of crystal violet (0.5%) was added and incubated for 20 min at room temperature. After this time, the solution containing the crystal was removed and the wells were washed with PBS to remove excess. Finally 100 μl of 1% SDS was added and incubated for 30 min at room temperature. The reading of absorbance was performed at a wavelength of 595 nm, and three treatments were compared, including cells without any treatment, cells treated only with lipofectamine, and cells transfected with siRNAs. The adhesion of control cells to each substrate was determined as 100%.

2.9. Wound healing assay

Wound-healing migration assay is based on the repopulation of wounded cultures. The cells were seeded into 24-well culture plates at 1×10^5 cells/well and the cell monolayer were cultured in medium containing 10% FBS until reach 100% of confluence. The monolayers were carefully wounded using a yellow pipette tip, and any cellular debris present was removed by washing twice with DMEM medium. The wounded monolayers were then incubated in DMEM medium containing 10% FBS. Photographs of the exact wound areas taken initially (0 h) were again taken after 16 and 24 h. The images were compared between three treatments, including cells without any treatment, cells treated only with lipofectamine, and cells transfected with siRNAs, and with or without incubation with the ADAM9D protein [11]. Photographs were analyzed using ImageJ software and the formula of % of wound closure [34].

2.10. Cell migration

Cell migration was assessed in 24 well Boyden chambers (BD Biosciences). MDA-MB-231 (5×10^4) cells were seeded on the upper chamber in FBS-free DMEM medium. DMEM containing FBS (10%) was added to the bottom chamber and acted as a chemoattractant. Tumor cells were allowed to migrate for 22 h at 37 °C and 5% CO_2 in a humidified environment. Then, the cells that remained in the upper chamber were removed using a cotton swab. The cells that migrated to the other side of the upper chamber membrane were fixed with methanol and stained with 1% toluidine blue in 1% borax. Cells were counted using the ImageJ software (public domain software) in 5 fields (100× magnification) per well that essentially covered 80% of the well surface. The average number of cells from each of the triplicates represents the average number of cells that migrated in the different groups. Each experiment had triplicate wells for every treatment group and we repeated each experiment three times. The mean of all results from controls was considered as 100%. After that, the images were compared among three treatments, including cells without any treatment, cells treated only with lipofectamine, and cells transfected with siRNAs.

2.11. Matrigel invasion assay

Cellular invasion assays were carried out using BioCoat Matrigel Invasion Chambers (BD Biosciences) with 8-μm pores in 6-well plates. A total of 2.5×10^4 cells were added to each chamber. Complete medium was used as a chemoattractant in the lower chamber. After incubation for 22 h at 37 °C and 5% CO_2, cell invasion was measured in the same way as performed for migration assay (item 2.10). The invasion of cells without any treatment was determined as 100%.

2.12. Gelatin zymography

The effect of ADAM9 silencing on the proteolytic activity of MDA-MB-231 cells was determined by zymography [35]. MDA-MB-231 cells (2×10^6) in FBS-free DMEM medium were seeded in 6-cm dishes. After incubation for 24 h at 37 °C and 5% CO_2, cells were lysed with a buffer containing Tris–HCl (0.2 M) (pH 7.4) and Triton X-100 (0.2%). The cell lysates were centrifuged (10 min at 13,000× *g* and 4 °C), and the supernatants were separated. The total protein concentration in each sample was measured using the BCA colorimetric detection kit (BCA Protein Assay, Pierce). Protein samples (20 μg) were subjected to electrophoresis under non-reducing conditions in 10% SDS polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis, gels were washed twice in 2.5% of Triton X-100 to remove SDS and incubated in substrate buffer [50 mM Tris–HCl (pH 8.0); 5 mM of $CaCl_2$ and 0.02% NaN_3] at 37 °C for 20 h. To confirm the metalloproteinase activity, EDTA in a final concentration of 15 mM was added to the samples and substrate buffer. Proteins were stained with Coomassie brilliant blue for 1.5 h and destained with an acetic acid, methanol and water mixture (in a 1:4:5 v:v:v ratio). Gels were photographed with a Canon G6 Power Shot 7.1 machine. Gelatinase activity was visualized as clear bands in the stained gels, and the average band intensities was measured using the Gene Tools v3.06 software (Syngene). MMP-2 and MMP-9 activity were quantified as arbitrary units and compared between three treatments, including cells without any treatment, cells treated only with lipofectamine, and cells transfected with siRNAs.

2.13. Statistical analysis

For all assays, each experiment was repeated three times in triplicate (independent experiments), and standard errors of the mean were calculated. The results were compared statistically using a one-way analysis of variance (ANOVA) and Tukey's test was applied for multiple comparisons. All statistical tests used $p \leq 0.05$ as a cut-off for significance. Cases where $p < 0.05$ were marked as follows: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. ADAM9 silencing is detected at the mRNA and protein levels

ADAM9 gene expression was dramatically decreased in MDA-MB-231 cells treated with siRNAs at both the mRNA and protein (Fig. 1A and B, respectively) levels when compared to the controls (MDA-MB-231 cells without treatment and lipofectamine or

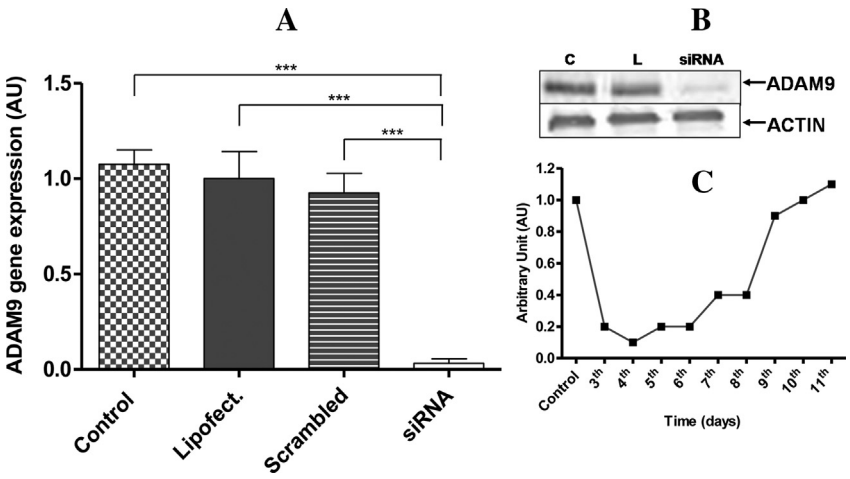

Fig. 1. Silencing of ADAM9. (A) Expression of ADAM9 mRNA in RNAi-mediated ADAM9 silenced MDA-MB-231 cells was analyzed by qPCR. The values are in arbitrary units (AU) and the *p* value was determined using ANOVA with a Tukey's test comparing control, cells treated with lipofectamine, negative control (scrambled) and cells treated with ADAM9 siRNAs (***$p < 0.001$). (B) Western blotting analysis of MDA-MB-231 cell lysates, with three treatments: cells alone (C), lipofectamine alone (L), and ADAM9 siRNAs using anti-ADAM9RP2 primary antibody and goat anti-rabbit IgG as a secondary antibody. β-actin was used as endogen control. (C) The silencing of ADAM9 using siRNAs is transient because it is effective only from the third to the sixth day. The values are in arbitrary units (AU).

scrambled-treated cells). qPCR analysis showed a down regulation of 91.35 ± 6.32% of ADAM9 expression in RNAi-mediated knockdown MDA-MB-231 cells when compared to control cells (Fig. 1A). At the protein level, western blotting analysis using anti-ADAM9 antibody presented similar results (Fig. 1B).

Gene silencing using synthetic duplexes siRNA is transient. As a result, 10 plates (6-cm) containing 2×10^5 MDA-MB-231 cells were silenced. One plate was removed randomly each day to determine the efficiency of ADAM9 silencing over time. RNA knockdown was measured from the third to the eleventh day after transfection and the kinetics of ADAM9 silencing in MDA-MB-231 cells are shown in Fig. 1C. The highest efficiency of ADAM9 silencing was observed from the third to the sixth days, although on the seventh and eighth days, the gene expression still remained low. From the ninth and tenth days onwards, the expression of ADAM9 expression increased exponentially and reached similar levels to those obtained by control cells (Fig. 1C).

3.2. ADAM9 silencing does not affect MDA-MB-231 cell proliferation or adhesion

ADAM9 silencing had no effect on the proliferation of MDA-MB-231 cells on the third (Fig. 2A) or sixth (Fig. 2B) days after silencing in 24 or 48 h of incubation. No significant differences were observed among the groups. ADAM9 silencing also had no effect on the adhesion of MDA-MB-231 cells to different ECM proteins, such as collagen type I, fibronectin or laminin (Fig. 3).

3.3. ADAM9 silencing strongly inhibits MDA-MB-231 cell invasion without affecting cell migration

RNAi-mediated ADAM9 silencing was not able to significantly inhibit the migration of MDA-MB-231 human breast tumor cells when compared with non-transfected cells or with lipofectamine-transfected cells after 16 (Fig. 4A) or 24 h that the wounds were made (Fig. 4B). ADAM9 knockdown cells were incubated with different concentrations of ADAM9D, the disintegrin domain of ADAM9 [11]. ADAM9D in concentrations of 500, 1000 and 2000 nM had no effects in inhibiting ADAM9 knockdown MDA-MB-231 cell migration after 16 (Fig. 4A) or 24 h (Fig. 4B) of incubation. Photographs were taken after 0, 16 or 24 h after wound (Fig. 4C).

To ensure these results we also performed migration assays using Boyden chambers. RNAi-mediated ADAM9 silencing had had no effect on MDA-MB-231 cell migration when compared with control cells or lipofectamine-treated cells after 22 h of incubation (Fig. 5A and B).

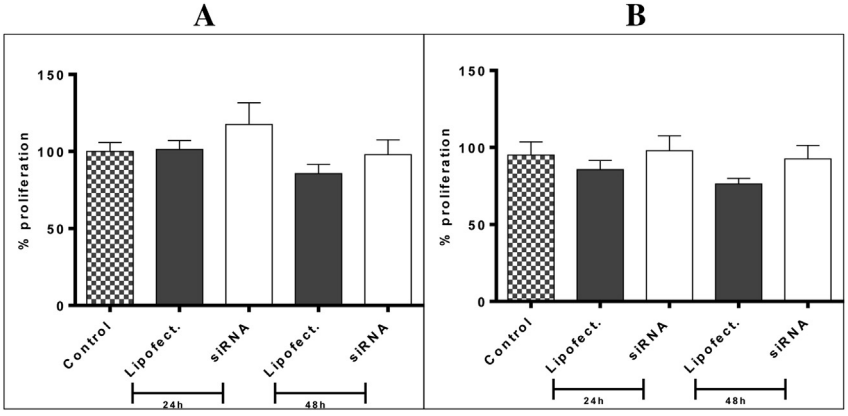

Fig. 2. Proliferation assay. Silencing of ADAM9 had no effect on the proliferation of the MDA-MB-231 cells after three (A) or six days (B) of transfection. After the different times, the cells were incubated with MTT for 24 h or 48 h and compared with control cells or with lipofectamine-treated cells. The absorbance of the samples was measured at 595 nm and the proliferation of control cells was determined as 100%.

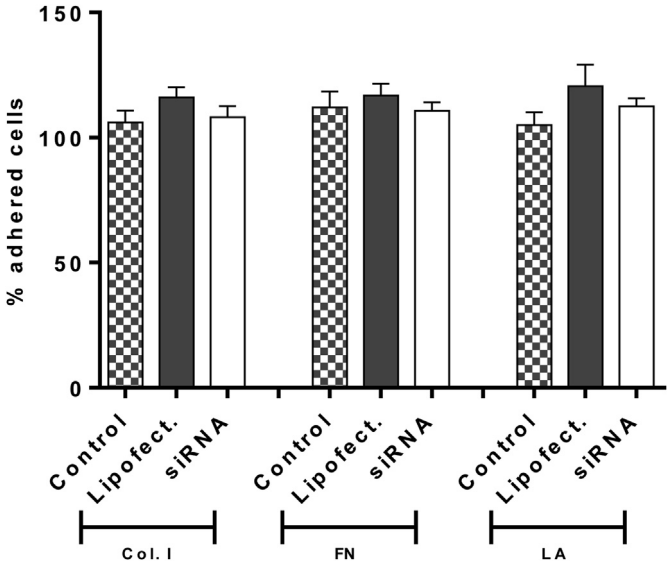

Fig. 3. Adhesion assay. RNAi-mediated ADAM9 silencing had no effect on the adhesion of the MDA-MB-231 under different extracellular matrix proteins, such as collagen I (Col. I), fibronectin (FN) and laminin (LA). The extracellular matrix proteins were coated on the wells of the plate, and on the following day, after the blocking of wells, the cells were allowed to adhere for 45 min. The percentage adhesion was determined as described in the materials and methods. The results were compared using a one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc analysis.

On the other hand, RNAi-mediated ADAM9 knockdown MDA-MB-231 cells strongly inhibited the invasion in an *in vitro* matrigel assay by 71.51 ± 8.02% when compared to control untransfected cells (Fig. 6A). Lipofectamine and negative control-transfected cells (scrambled) remained invasive and no statistically significant differences were observed when compared to untransfected cells (Fig. 6A). Photographs were taken after 22 h of incubation (Fig. 6B).

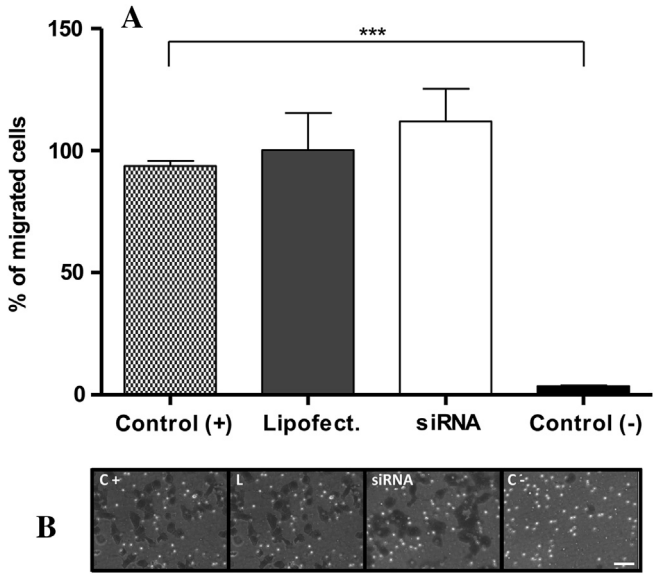

Fig. 5. Effect of RNAi-mediated ADAM9 silencing on migration of MDA-MB-231 cells. (A) A transwell migration assay was used to determine the effect of ADAM9 silencing migration of MDA-MB-231 cells. Control cells, lipofectamine-treated cells or siRNA-ADAM9 cells were allowed to migrate toward medium containing 10% FBS for 22 h. Graphs are representative of three independent experiments. The results were compared using a one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc analysis (***$p < 0.001$). (B) Morphology of cells in the three different treatments: control cells (C+), lipofectamine-treated cells (L), and siRNA-ADAM9 treated cells (siRNA) migrating toward a 10% FBS containing medium. The negative control (C−) was control cells migrating toward a FBS-free medium. Bar represents 10 μm.

3.4. MMP-2 and MMP-9 concentration and activity

In order to investigate the mechanisms involved in the inhibition of the invasion ability of RNAi-mediated ADAM9 silencing of

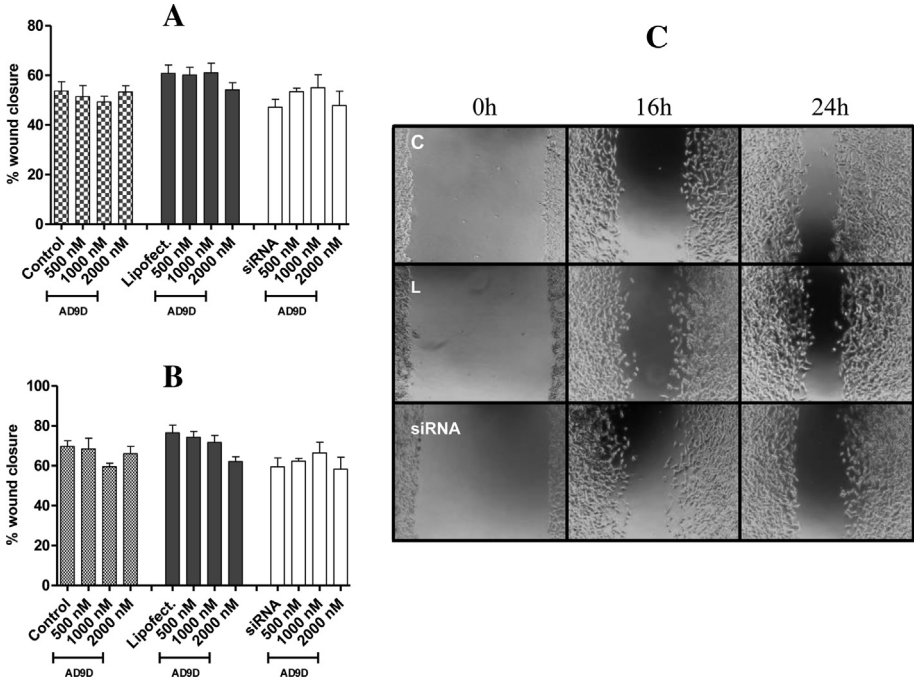

Fig. 4. Migration of MDA-MB-231 cells after wounding. Effects of ADAM9 silencing and ADAM9D recombinant protein on MDA-MB-231 cells migration were plotted as a percentage of wound closure in 16 (A) or 24 h (B) after wounding. Representative photos of wounds were taken at time zero, 16 h, and 24 h after wounding, (C). In these photos we show pictures representing control (C), lipofectamine (L) and siRNA-ADAM9 (siRNA) cells without previous treatment with ADAM9D recombinant protein.

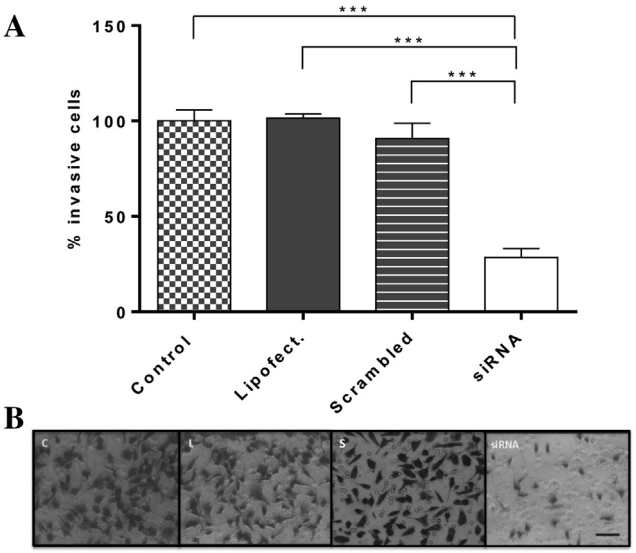

Fig. 6. Effect of RNAi-mediated ADAM9 silencing on the invasion of MDA-MB-231 cells. ADAM9 silencing significantly inhibits the invasion of MDA-MB-231 human breast tumor cells through matrigel compared to the invasion of control cells (A). The cells were plated in wells containing matrigel and FBS was used as a chemoattractant in the lower chamber. The invasive cells were fixed and counted (an average of eight fields from each treatment). The assay was performed in triplicate. The results were compared using a one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc analysis (***$p < 0.001$). (B) Cell morphology in the four different treatments: untreated control (C), lipofectamine-treated cells (L), scrambled siRNA-treated control (S), and siRNA-ADAM9 treated cells (siRNA). Bar represents 10 μm.

MDA-MB-231 cells, we performed zymography assays to evaluate the activity of MMP-2 and MMP-9. There was no variation in the total concentration of MMP-2 or MMP-9 among the three treatment types analyzed in this study, as demonstrated by 1% gelatin-SDS-PAGE (Fig. 7A). The incubation with EDTA resulted in the inhibition of MMP-2 and 9 activities confirming the nature of metalloprotease activity (Fig. 7B). The average activity of MMP-2 and MMP-9 was measured as indicated in Section 2.12 and plotted on a graph (Fig. 7C and D, respectively). This result suggests that RNAi-mediated ADAM9 silencing does not affect the activity of MMP-2 or MMP-9.

4. Discussion

The progression of malignant tumors results from the invasion of the primary tumor to a secondary site, causing metastasis in a multi-step process that requires cell–cell and cell–matrix interactions within the host tissue. These steps can be summarized as follows: cell detachment from the primary tumor, migration 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 [36,37]. These interactions lead to the production, release and activation of a variety of cytokines and growth factors and subsequent generation of signals to directly or indirectly promote tumor growth and survival [24]. Different proteases have been implicated in these processes, such as MMPs, ADAMs and ADAMTSs [7,24,38].

Due to the strong involvement of ADAM9 in the metastatic process, in this study we have generated knockdown clones of MDA-MB-231 human breast tumor cells that lack ADAM9 expression and then tested these clones to their ability to adhere, migrate, proliferate and invade through ECM using *in vitro* assays. The RNAi-mediated silencing in MDA-MB-231 cells was very successful, with more than 90% of ADAM9 knocked down, as estimated by quantitative PCR and western blotting analysis. The expression of ADAM9 was easily silenced using a relatively small (10 nM) concentration of ADAM9 siRNAs. A similar result was obtained by other investigators in highly invasive SCC68 cells, a squamous cell carcinoma cell line, but with tenfold higher concentration of siRNAs (100 nM) [39].

Using a matrigel invasion assay, we showed that the ADAM9 silencing significantly inhibited the invasion capacity of MDA-MB-231 human breast cancer cells, which suggests that this protein plays an important role in cell invasion. However, the silencing of ADAM9 had no effect on MDA-MB-231 cell adhesion, migration, proliferation, or MMP-2 and 9 activities. Our results showed that ADAM9 silencing had no impact on MMP-2 and MMP-9 expression or gelatinase activity indicating that reduced invasion in cells

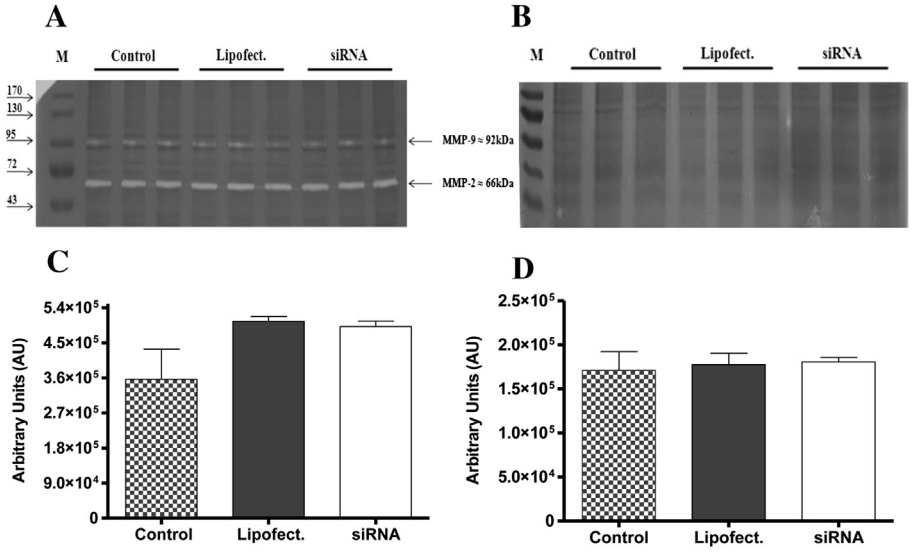

Fig. 7. Analysis of concentration and activity of MMP-2 and MMP-9 in the MDA-MB-231 breast tumor cells. (A) Zymography in 1% gelatin-SDS-PAGE or (B) EDTA-treated samples. Lane 1: molecular mass marker; lanes 2–4: control cells; lanes 5–7: cells treated with lipofectamine; and lanes 8–10: cells treated with ADAM9 siRNAs ($n = 3$; 20 μg of total protein was loaded in each lane). (C) MMP-2 and (D) MMP-9 concentrations were determined by the sum of integrated optical density (IOD) obtained for the intermediate bands. Gels were analyzed by densitometry, and activity was expressed as arbitrary units.

expression ADAM9 siRNA is unlikely to be due to indirect inhibition of MMP-2/9. We propose that ADAM9 proteolytic activity may directly contribute to matrigel invasion by MDA-MB-231 cells since ADAM9 has been reported to cleave laminin [31], a major constituent of matrigel.

Shintani et al. [40] showed that the overexpression of ADAM9 enhances adhesion and cell invasion in lung cancers, via modulation of other adhesion molecules and changes in sensitivity to growth factors. According to this study, ADAM9 may either directly degrade the ECM or induce the activation of other proteases in the ECM, such as matrix MMPs, thereby allowing tumor cell penetration into the brain matrix.

Our results are in agreement with Mazzocca et al. [31] who showed that ADAM9-S, an alternatively spliced variant secreted by activated hepatic stellate cells, induces colon carcinoma cell invasion *in vitro* and that this process requires both protease activity and binding to the $\alpha_6\beta_4$ and $\alpha_2\beta_1$ integrins.

Contradictorily to our results, Fry and Toker [41] demonstrated that the silencing of both soluble (ADAM9-S) and transmembrane (ADAM9-L) isoforms, increased the migration of BT549 breast cancer cells. In this work, they also showed that the overexpression of ADAM9-S is responsible for increasing cell migration in BT549 cells through its metallopeptidase domain. Moreover, they also showed that ADAM9-L is responsible for inhibiting cell migration through its disintegrin domain. Thus, both isoforms have different and opposite responses during cancer progression. Whether MDA-MB-231 cells have ADAM9-S and L isoforms and the effects of isoform silencing in this cell line will be further investigated.

Some ADAMs may induce proliferation by catalyzing the cleavage of growth factors, such as HB-EGF, and its membrane anchored form (proHB-EGF) can act as a negative regulator of proliferation [42,43]. Izumi et al. [44] showed that after induction with TPA (an activator of protein kinase C), ADAM9 interacted with PKCδ and cleaved proHB-EGF; however, we have demonstrated here that ADAM9 is not involved in the proliferation of MDA-MB-231 cells. In another work, RNAi-mediated ADAM9 silencing was responsible by a reduction in adenoid cystic carcinoma metastasis both *in vitro* and *in vivo* [19]. In this work, the authors also demonstrated that ADAM9 is essential for cancer cell proliferation and invasion and that its expression could be used as a prognostic of metastatic risk, since it was elevated in a high metastatic potential cell line (SACC-LM) when compared to a low metastatic potential cell line (SACC-83) [19].

Klessner et al. [39] demonstrated that ADAM9 participates in the shedding of desmoglein 2 (Dsg2), resulting in stronger cell–cell adhesion, which could, in turn, reduce the rate of migration and cell invasion. The ADAMs can also interact with β_1 integrins, and this association facilitates the recognition and location of their substrates for proteolytic shedding [45,46], as reported by Mahimkar et al. [47].

In a recent work, Hamada et al. [48] reported that miR-126 was found to target ADAM9 and that siRNA-based knockdown of ADAM9 in pancreatic cancer cells resulted in reduced cellular migration, invasion, and induction of epithelial marker E-cadherin.

Taken together, the literature results and the data found in the present study suggest that ADAM9 participates in the invasion of tumor cells by either directly degrading the ECM, by inducing activation of other proteases, such as MMPs, by co-localizing with other molecules, such as β_1 integrin, present on the surface of MDA-MB-231 cells (data not shown) or by interacting with other regulators such as miRNAs. A more conclusive demonstration that ADAM9 is a suitable target for metastatic breast cancer will require the use of a stable expression vector *in vivo* and/or inhibitors of this protein alone or in combination with conventional clinical therapies.

5. Conclusions

The results presented in this study reinforce the importance of the ADAM9 role in the invasion of breast tumor cells. Considering the significance of cell invasion in metastatic progression, ADAM9 can be pointed as an interesting target for the design of drugs involved in the treatment or prevention of breast cancers. We conclude that ADAM9 has an essential role in cell invasion and may be involved in metastatic spread. Therefore, it may be an interesting target for anti-metastatic therapy.

Acknowledgments

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DisBa-01 inhibits angiogenesis, inflammation and fibrogenesis of sponge-induced-fibrovascular tissue in mice

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ABSTRACT

Integrins are involved in a number of physio-pathological processes including wound healing, chronic inflammation and neoplasias. Blocking its activity is potentially of therapeutic value in these conditions. We investigated whether DisBa-01, a recombinant His-tag RGD-disintegrin from *Bothrops alternatus* snake venom, could modulate key events (inflammatory cell recruitment/activation, neovascularization and extracellular matrix deposition) of the proliferative fibrovascular tissue induced by polyether polyurethane sponge implants in mice. The hemoglobin content (μg/mg wet tissue), blood flow measurements (laser Doppler perfusion imaging) and number of vessels in the implants, used as indices of vascularization, showed that the disintegrin dose-dependently reduced angiogenesis in the implants relative to the Saline-treated group. DisBa-01 inhibited neutrophil and macrophage content as determined by the myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAG) activities, respectively. Similarly, down regulation of the fibrogenic component studied (collagen deposition) was observed in DisBa-01-treated implants. VEGF, bFGF, TNF-α, CXCL1 and CCL2 levels were also decreased by the disintegrin. The inhibitory effect of this $\alpha_v\beta_3$-blocking disintegrin on the angiogenic, inflammatory, and fibrogenic components of the fibrovascular tissue induced by the synthetic matrix extends the range of DisBa-01 actions and may indicate its therapeutic potential in controlling angiogenesis in fibroproliferative diseases.

1. Introduction

In a number of pathological conditions such as rheumatoid arthritis, psoriasis, atherosclerosis, and tumor growth, inflammation and angiogenesis are key events that

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act simultaneously and synergistically contributing to disease progression (Folkman, 1995; Carmeliet and Jain, 2011). Analyses of both processes have revealed the involvement of integrins on adhesion and proliferation of the cells involved in these biological events (Silva et al., 2008). Particularly, the αvβ3 integrin plays a major role in angiogenesis and tumorigenesis participating in adhesion signaling, activation of matrix metalloproteinases (MMPs), proliferation, migration, invasion and protection against apoptosis (Eliceiri and Cheresh, 2001; Somanath et al., 2009). Although, poorly expressed in most adult tissues, this integrin is highly expressed and active on metastatic tumor cells and on vascular endothelial cells undergoing angiogenesis (Contois et al., 2009; Robinson and Hodivala-Dilke, 2011). Thus, it has been proposed that inhibition of integrins involved in the inflammatory and angiogenic cascades may be of potential therapeutic value in pathological conditions where these processes co-exist. As a result, they have been widely investigated for their therapeutic potential in a number of conditions in which controlled angiogenesis is sought (wound healing, tumor propagation, diabetic retinopathy) (Rapraeger, 2013; Goodman and Picard, 2012). There is also increasing evidence that integrin-inactivating proteins are crucial for appropriate integrin function *in vitro* and *in vivo* and that the regulation of integrin–ligand interactions is a fine-tuned balancing act between inactivation and activation (Bouvard et al., 2013). A number of molecules have been shown to inactivate the interactions between the integrins and target cells (Cox et al., 2010). Among them, disintegrins, a family of nonenzymatic, low molecular weight, cysteine-rich peptides from snake venom, have been shown to specifically and potently inhibit integrin actions, including tumor development, metastasis, and angiogenesis (Selistre-de-Araujo et al., 2010; Calvete, 2013). In fact, blocking of αvβ3 with DisBa-01 (a αvβ3 binding disintegrin) decreased bFGF-induced angiogenesis in a matrigel plug assay in athymic nude mice and inhibited melanoma metastasis (Ramos et al., 2008). These reports on the effects of this peptide on key events of the angiogenic cascade prompted us to hypothesize that it might also modulate inflammatory angiogenesis in the *in vivo* mouse sponge model. In this chronic inflammation model, the acellular and avascular synthetic matrix, implanted subcutaneously in the animals' dorsa, induces the migration, proliferation, and activation of various cell types responsible for the development of a fibrovascular tissue that underlies chronic pathological conditions (Andrade et al., 1987; Pereira et al., 2012). Thus, it has been proven to be instrumental in evaluating a number of potential compounds and molecules bearing anti-angiogenic, inflammatory and/or fibrogenic activities (Barcelos et al., 2009; Araujo et al., 2010). We report here that DisBa-01 was able to dose-dependently attenuate inflammation, neovascularization

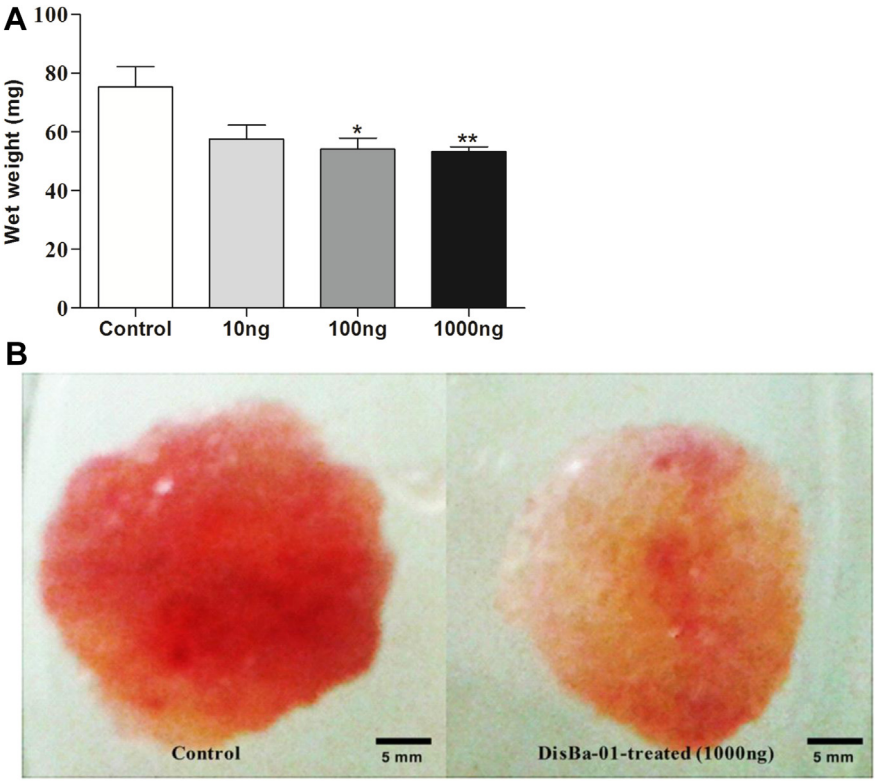

Fig. 1. The sponge tissues were excised, photographed, and weighed. (A) Note that the implant treated with DisBa-01 doses (1000 ng) exhibited reduce weight and intense of staining when compared to the control group. (B) Representative image showing a polyester-polyurethane sponge 9 days post-implantation. Values shown are the means (±SEM) from groups of 8 animals for each group. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. Control group (ANOVA). Bar = 5 mm.

and collagen deposition *in vivo*, a potential therapeutic effect in controlling fibroproliferative diseases.

2. Material and methods

2.1. Animals

Male Swiss mice 7–8 weeks (20–30 g body weight), provided by the Central Animal Facility at the Institute of Biological Sciences, Federal University of Uberlandia-Brazil, were used in these experiments. The animals were housed individually and provided with chow pellets and water *ad libitum*. The light/dark cycle was 12:12 h with lights on at 7:00 a.m. and lights off at 7:00 pm. Efforts were made to avoid all unnecessary distress to the animals. Housing, anesthesia and postoperative care complied with the guidelines established by our local Institutional Animal Welfare Committee (process number: CEUA *n*° 007/11).

2.2. DisBa-01 expression, purification and characterization

Since the native DisBa-01 disintegrin could not be purified from the venom due to very low yields in protein preparations, recombinant DisBa-01 was produced from a cDNA venom gland library of *Bothrops alternatus* (Montenegro et al., 2012). The coding region corresponds to a medium disintegrin (78 amino acid residues) with an RGD adhesive motif (GenBank accession AY259516). Expression and purification of the recombinant His-tag protein were performed as previously described (Ramos et al., 2008). N-terminal sequencing confirmed the expected sequence of the 12 kDa fusion protein which was then used in all biological assays. Molecular modeling suggested that the fusion His-tag peptide was not involved in integrin binding and therefore its proteolytic removal could not be needed (Ramos et al., 2008; Montenegro et al., 2012).

2.3. Sponge discs implantation and DisBa-01 treatment

Polyether-polyurethane sponge (Vitafoam Ltd., Manchester, UK) was used as the implanted material. The implants were in the shape of discs, 5 mm thick × 8 mm diameter. They were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 min before implantation. The animals were anaesthetized with a mixture of ketamine 150 mg/kg and xylazine 10 mg/kg and the dorsal hair shaved and the exposed skin wiped with 70% ethanol. The sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1-cm long dorsal mid-line incision. Post-operatively, the animals were monitored for any signs of infection at the surgical site, discomfort, or distress; any animals showing such signs were promptly euthanized. DisBa-01 at 10, 100 or 1000 ng in 10 μl of saline was injected daily intraimplant for 8 days. The implants were evaluated 9 days post implantation to assess vascularization (blood flow, hemoglobin content, citokines levels and histological analysis), inflammatory markers (Myeloperoxidase (MPO), N-acetyl-β-D-glucosaminidase (NAG) activities and chemokines levels) and fibrogenesis markers (total collagen soluble and TGF-β1) and fibrogenesis markers (total collagen soluble and TGF-β1).

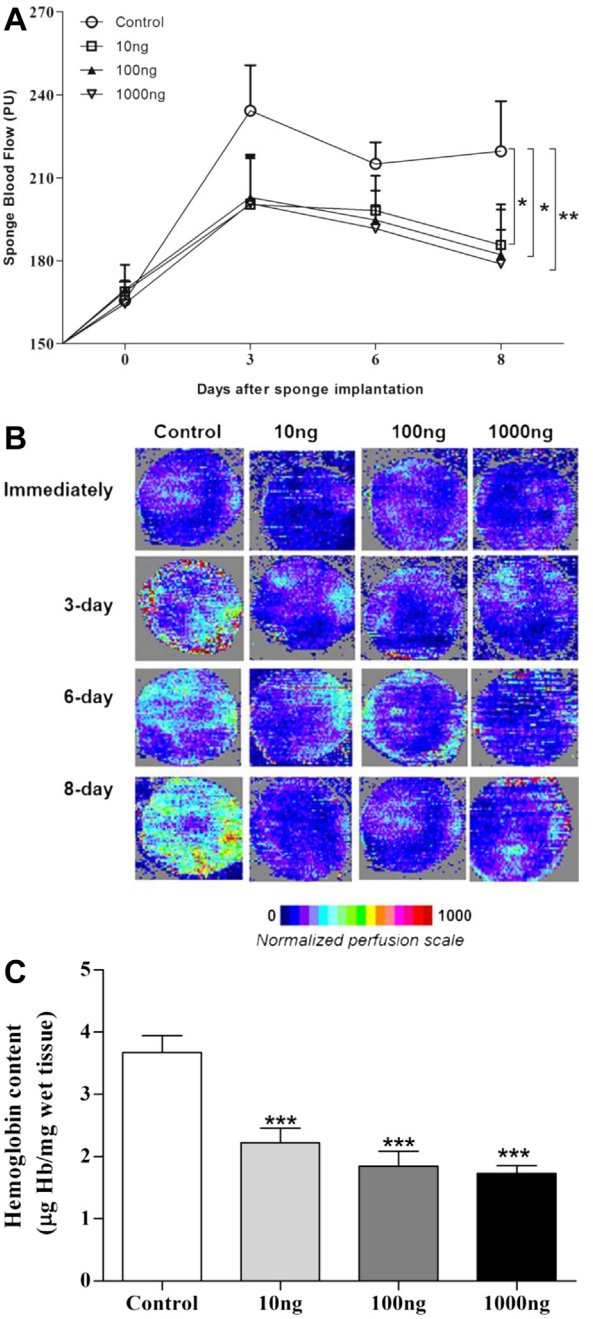

Fig. 2. Effects of various doses of DisBa-01 on blood flow. (A) Implants treated with DisBa-01 showed decrease blood flow monitored by laser Doppler perfusion image (LDPI). A typical image from the laser Doppler perfusion technique is shown (B). The blue bands are the low flow area and the red colors indicate high blood flows within the implant. A significant reduction in hemoglobin content of the treated implants DisBa-01 (C) was also observed. Values shown are the means (±SEM) from groups of 8 animals for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Hemoglobin extraction (Hb)

The extent of vascularization of the sponge implants was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin method (Araujo et al., 2010; Marques et al., 2011). At 9 days post implantation, the animals were euthanized with an excess of anesthetic and the sponge implants were carefully removed, dissected, cleared of any adherent tissue, and weighed. Each implant was homogenized (Tekmar TR-10, OH) in 5 ml of Drabkin reagent (Labtest, São Paulo, Brazil) and centrifuged at 12000 × *g* for 20 min. The supernatants were filtered through a 0.22-μm Millipore filter. The hemoglobin concentration in the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and comparing it against a standard hemoglobin curve. Hemoglobin content in the implant was expressed as μg Hb per mg wet tissue.

2.5. Blood flow measurements

The assessment of blood flow by the laser Doppler perfusion image (LDPI) non-invasive technique. Measurements were carried out in anesthetized mice (ketamine 150 mg/kg and xylazine 10 mg/kg) immediately, 3, 6 and 8 days after implantation. Blood flow in sponge implant was assessed using an LDPI analyzer (Moor Instruments, Devon, U.K). A dark plastic ring was positioned around the sponge implant compartment to direct the light beam and prevent cutaneous blood flow from interfering with the measurements. While capturing perfusion images, the ambient light level was kept at a minimum to impede any influence on the laser light and recorded signals. The animals were kept at a constant temperature of 37 °C for 5 min before and during measurements to control for temperature variations. The mean pixel value of each scanned image was calculated using MoorLDI V5.3 software and the calculated mean flux was expressed as perfusion units (PU), which represent the average blood flow in the implants.

2.6. Measurement of VEGF, TNF-α, bFGF, TGF-β1, CXCL-1 and CCL2 production in the sponge implants

Implants were removed at day 9 post implantation, homogenized in PBS pH 7.4 containing 0.05% Tween, and centrifuged at 10,000 × *g* for 30 min. The levels of the cytokines in the supernatant from each implant were measured in 50 μl of the supernatant using Immunoassay Kits (R and D Systems, USA) and following the manufacturer's protocol. Briefly, dilutions of cell-free supernatants were added in duplicate to ELISA plates coated with a specific murine monoclonal antibody against cytokine,

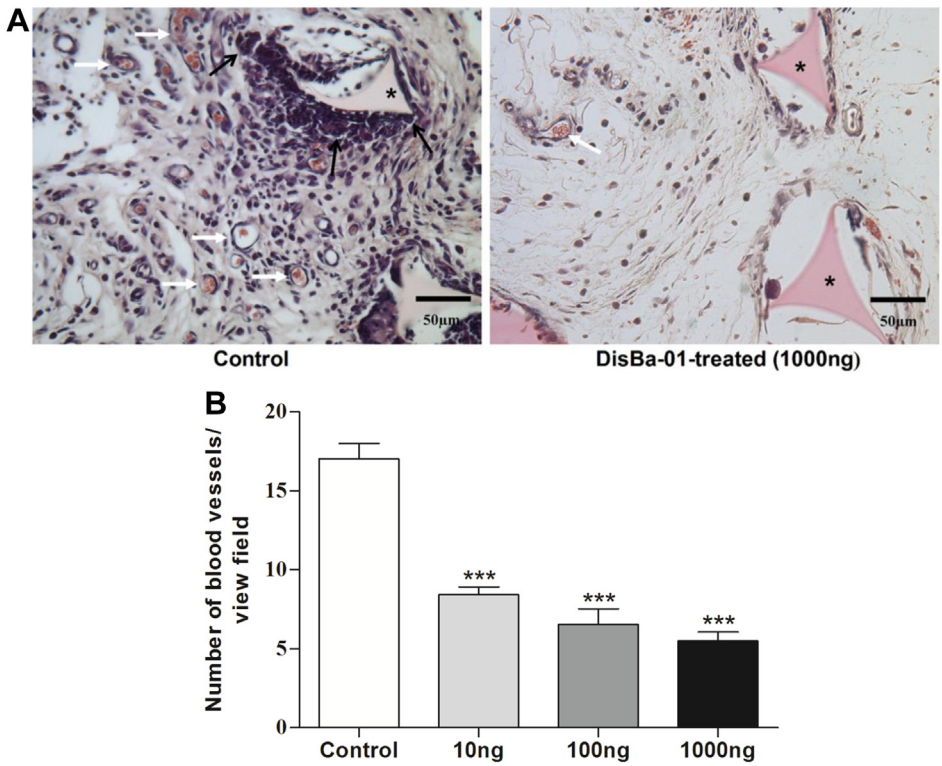

Fig. 3. Representative histological sections (5 μm, stained with H&E) of 9-day old implants treated with eight consecutive doses of DisBa-01 or saline. In A, the control fibrovascular tissue is denser and more vascularized than the DisBa-01-treated implants. The pores of the synthetic matrix (triangular shapes) are filled with inflammatory cells, spindle-shaped fibroblasts and blood vessels (*synthetic matrix; black arrow, fused macrophage; white arrow, blood vessels). There were fewer implant vessels in the DisBa-01–treated group when compared with the control group, as assessed by morphometric analysis in 15 fields from 8 different animals in each group (B). $^{***}p < 0.001$ vs. control group (ANOVA). Bar 50 μm.

followed by the addition of a second horseradish peroxidase-conjugated polyclonal antibody, also against cytokine. After washing to remove any unbound antibody-enzyme reagent, a substrate solution (50 μL of a 1:1 solution of hydrogen peroxide and tetramethylbenzidine 10 mg/ml in DMSO) was added to the wells. Color development was halted after 20 min incubation with 2 N sulfuric acid (50 μL) and the intensity of the color was measured at 540 nm on a spectrophotometer (E max – Molecular Devices). Standards were 0.5-$\log_{10}$ dilutions of recombinant murine cytokines from 7.5 pg ml^{-1} to 1000 pg ml^{-1} (100 μl). The threshold of sensitivity for each chemokine is 15.625 pg/ml. The results were expressed as pg cytokine per mg wet tissue.

2.7. Tissue extraction and determination of myeloperoxidase and N-acetyl-β-D-glucosaminidase activities

The content of neutrophils in implants was measured by assaying myeloperoxidase (MPO) activity as previously described (Araujo et al., 2010). The implants were weighed, homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M $NaPO_4$, 0.015 M NaEDTA), and centrifuged at 12,000 × g for 10 min. The pellets were then re-suspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by adding 50 μl of H_2SO_4 (4 M). Results were expressed as a change in OD/g wet tissue.

Infiltration of mononuclear cells into the implants was quantified by measuring the levels of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) present in high levels in activated macrophages (Barcelos et al., 2005). The implants were homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega) and then centrifuged (3000 × g; 10 min at 4 °C). Samples (100 μl) of the resulting supernatant were incubated for 10 min with 100 μl of p-nitrophenyl-N-acetyl-beta-D-glucosaminide (Sigma) prepared in a citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO4; pH4.5) to yield a final concentration of 2.24 mM. The reaction was terminated by adding 100 μl of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. Results were expressed as nmol/mg wet tissue.

2.8. Soluble collagen measurement

Total soluble collagen was measured in whole tissue homogenates by the Sirius Red reagent based-assay (Campos et al., 2008). The implants were homogenized in 1 ml of PBS and 50 μl of sample were mixed with 50 μl of Sirius Red reagent. Samples were mixed by gentle inversion. The collagen-dye complex was precipitated by centrifugation at 5000 × *g* for 10 min. The supernatants were drained off and discarded and the pellet washed with 500 μl of ethanol (99% pure and methanol free). One milliliter of a 0.5 M NaOH solution was added to the remaining pellet of collagen-bound dye. After solubilization, samples were transferred to a 96-well plate and read at 540 nm. The calibration curve was set up on the basis of a gelatin standard (Merck, USA). The results are expressed as microgram collagen per milligram wet tissue.

2.9. Histological analysis

The sponge implants from a separate group of mice ($n = 8$) were carefully excised, dissected free of adherent tissue, and fixed in formalin (10% w/v in isotonic saline). Sections (5 μm) were stained with hematoxylin and eosin (H&E) and processed for light-microscopic studies. To perform a morphometric analysis, cross section images obtained from 15 sequential fields (8533 μm^2) from each implant were captured with a planapochromatic objective (40×) in light microscopy (final magnification = 400×). The images were digitized through a JVC TK-1270/JCB microcamera and transferred to an analyzer (Kontron Electronics, Carl Zeiss – KS300 version 2). A single observer blinded to the condition and treatment performed the analysis. A countable vessel was defined as a structure with a lumen whether or not it contained red blood cell (Barcelos et al., 2009).

2.10. Statistical analysis

All data were expressed as mean ± SEM. Comparisons between three or more groups were made using one-way analysis of variance (ANOVA) followed by the

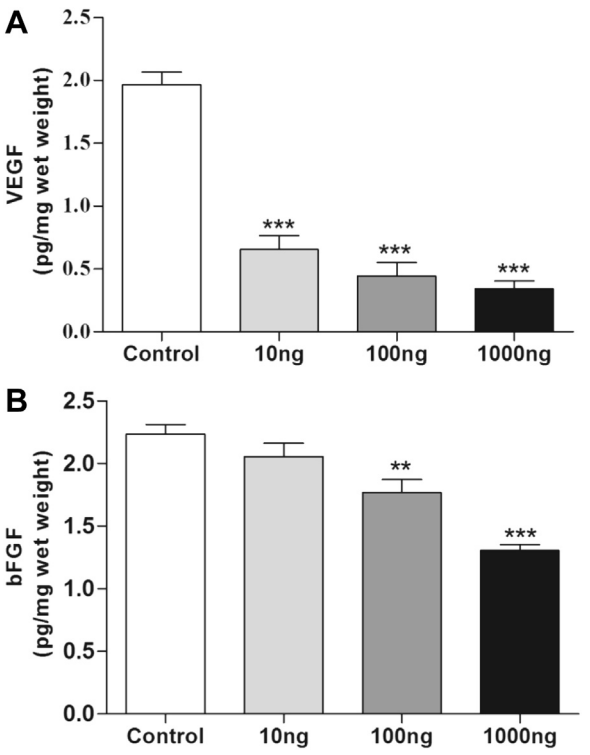

Fig. 4. Effects of DisBa-01 on proangiogenic cytokines. (A) VEGF and (B) bFGF. Values shown are the means (±SEM) from groups of 8 animals for each group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group (ANOVA).

Newman–Keuls correction factor for multiple comparisons as a post-test, except for the Laser Doppler Perfusion Imaging (LDPI) were made using two-way analysis of variance (Two-way ANOVA) followed by the Bonferroni post-test. Differences between means were considered significant when p values were <0.05. The Statistical analysis was performed using the GraphPad Prism program, version 6.0.

3. Results

The sponge matrix was well tolerated by all animals. No signs of infection or rejection were observed in the implant location during the 9-day period of the experiment as the implants became infiltrated by a fibrovascular tissue. In the DisBa-01-treated group, there was reduction in the weights of the implants when compared with the control non-treated group. *In situ* gross examination showed distinct difference in the color intensity between DisBa-01-treated (1000 ng) implants versus saline-treated implants (Fig. 1A and B).

3.1. Angiogenesis in DisBa-01-treated implants

A gradual inhibitory effect of different doses of DisBa-01 (10, 100, 1000 ng/implant) on the development of the angiogenic component of the fibrovascular tissue was observed in 9-day old implants, as assessed by blood flow measurement using a laser Doppler perfusion image (Fig. 2A and B) and hemoglobin content (Fig. 2C). In the control group, the hemoglobin content (μg/mg wet tissue)

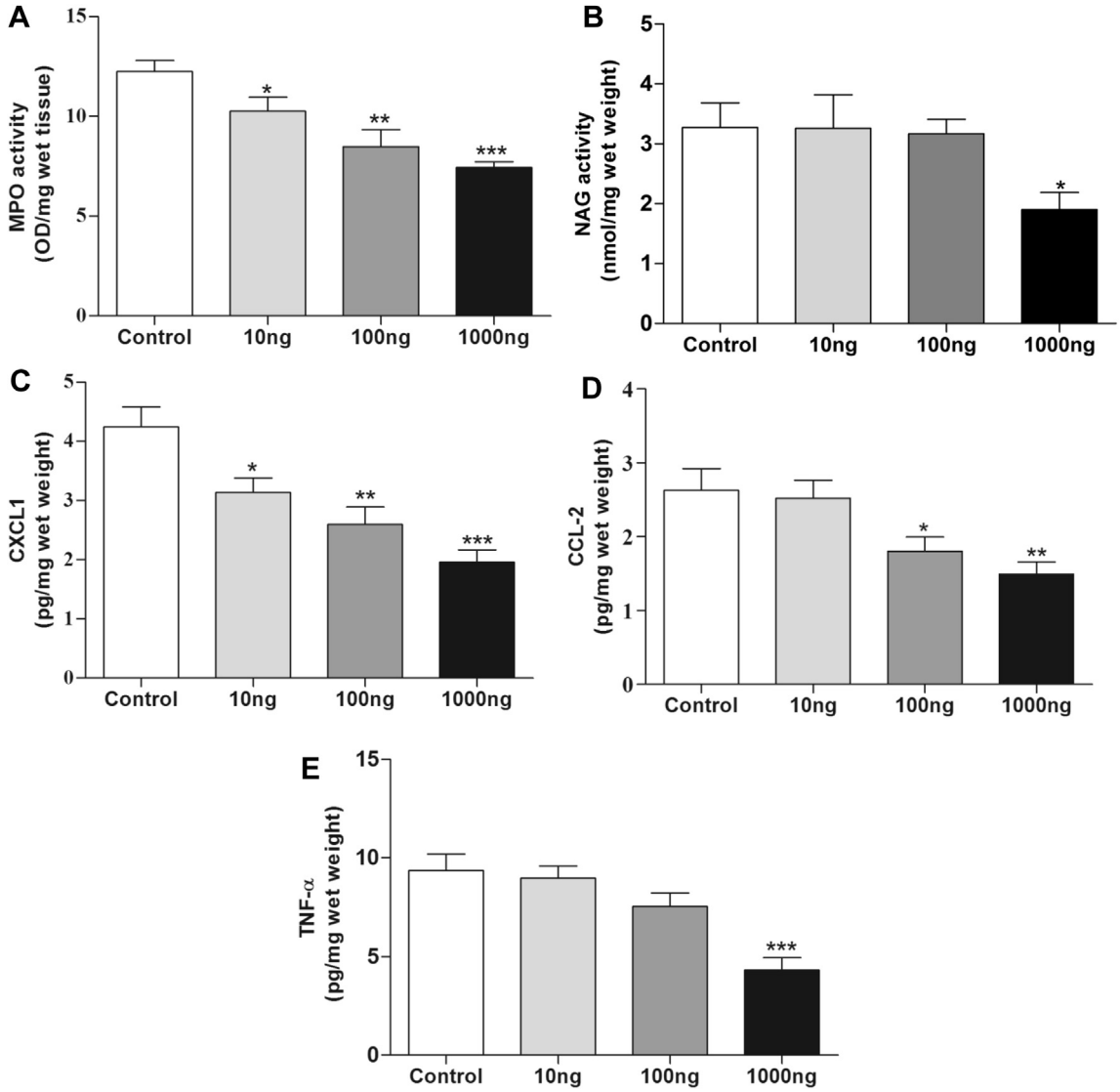

Fig. 5. Effects of various doses of DisBa-01 on inflammatory markers. DisBa-01 treatment caused significant decrease in Myeloperoxidase-MPO-activity (A), N-acetyl-β-D-glucosaminidase -NAG-activity (B), CXCL1 (C), CCL2 (D), and TNF-α (E). Values shown are the means (±SEM) from groups of 8 animals for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group (ANOVA).

was 3.67 ± 0.2, after DisBa-01 (10 ng) it fell to 2.22 ± 0.23. Further decreases were obtained with 100 and 1000 ng of the disintegrin. The functional and biochemical parameters indicative of angiogenesis attenuation by the disintegrin were corroborated by counting the number of histologically stained blood vessels in the implant sections (H&E staining; Fig. 3A and B). The measurement of relevant pro-angiogenic cytokines (VEGF and bFGF) were also decreased dose-dependently by Disba-01 as shown in Fig. 4A and B.

3.2. Inflammation in DisBa-01-treated implants

Several measures were made of the inflammatory state of the implants and consistent with the role of integrins on inflammation, the αvβ3 integrin inhibitor was able to reduce all the evaluated inflammatory markers. Thus, neutrophil content (assayed as MPO activity) and macrophages numbers (assayed as NAG activity) were progressively attenuated by the different doses of DisBa-01. Similarly, the levels of the chemokines CXCL1 and CCL2 and the production of TNF-α decreased after intra-implant application of DisBa-01 at different doses (Fig. 5A–E).

3.3. Fibrogenesis in DisBa-01-treated implants

Collagen deposition, but not the levels of the pro-fibrogenic cytokine (TGF-β1) was reduced after the treatment with DisBa-01 (Fig. 6A and B).

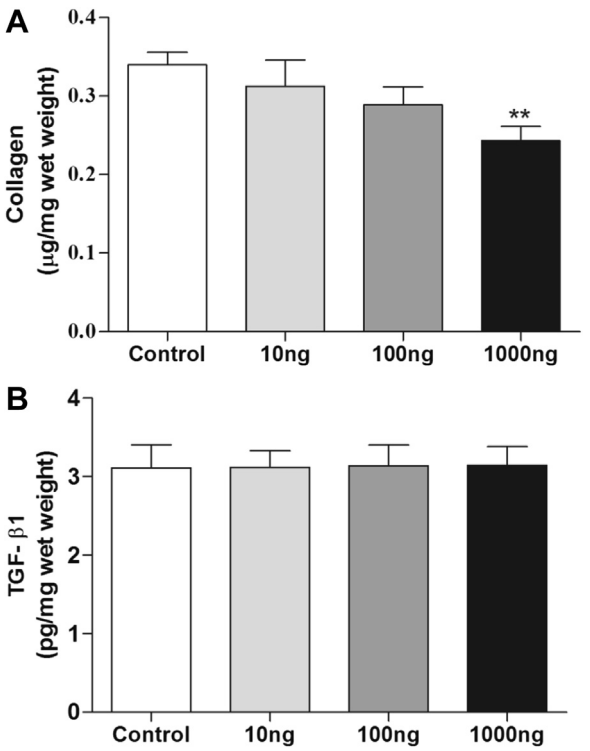

Fig. 6. Effects of DisBa-01 on (A) collagen deposition and TGF-β level (B) in the sponge implant. Values shown are the means (±SEM) from groups of 8 animals for each group. $^{**}p < 0.01$ vs. control group (ANOVA).

4. Discussion

Integrins have been proven to be valuable therapeutic targets in the treatment of several inflammatory and autoimmune diseases, in which they regulate leukocyte adhesion processes. Furthermore, they play an important role in pathological angiogenesis and tumor metastasis, being a promising target for cancer therapy (Contois et al., 2009; Weis and Cheresh, 2011) In this study, we investigated the effects of DisBa-01 (a recombinant His-tag RGD-disintegrin from *B. alternatus* snake venom) on the angiogenic, inflammatory and fibrogenic components of the fibrovascular tissue induced by polyether polyurethane in mice. The implantation technique induces a proliferating fibrovascular tissue similar to that after mechanical and naturally occurring injuries such as balloon angioplasty, atherosclerosis, and surgical wounds. Similar type of tissue is associated with a number of physiological (tissue repair) and pathological processes (chronic inflammation, tumor growth). Our results showed that DisBa-01 dose-dependently inhibited the major components of the newly formed fibrovascular tissue (angiogenesis, inflammation and fibrogenesis) as assessed by a combination of functional, biochemical, and histological approaches. So far, this is the first study to demonstrate such effects concurrently. However, a similarly effect has been reported in DisBa-01 such as inhibition of bFGF-induced angiogenesis using matrigel assay (Ramos et al., 2008) and reduction *in vitro* of pro-angiogenic cytokines (Montenegro et al., 2012). Those data are in agreement with various reports that demonstrated the inhibitory action of other disintegrins on the processes investigated in this study in different *in vitro* and *in vivo* systems. Olfa et al. (2005), for example, reported that Lebestatinn inhibited angiogenesis *in vitro* and *in vivo* (chorioallantoic membrane). The fact that this disintegrin was able to reduce the production of VEGF and bFGF intraimplant is also consistent with the inhibitory action of this and other disintegrins (Lebestatinn, Saxatilin) and other anti-αvβ3 agents (Doxorubicin) (Olfa et al., 2005; Jang et al., 2007; Murphy et al., 2008). It has been demonstrated that anti-αvβ3 agents interfere with the signaling of tyrosine-kinase receptors, which mediate the actions of various growth factors including VEGF. Therefore, it seems reasonable to speculate that our disintegrin may have acted through similar mechanisms (Danhier et al., 2012; Goel and Mercurio, 2013).

We measured five markers of inflammation (MPO, NAG activities, and three cytokines TNF-α, CXCL1 and CCL2) in implants treated with different doses of DisBa-01, Again, this disintegrin was able to dose-dependently attenuate all of them. In a number of studies, the inhibitory effects of various disintegrins (Rhodostatin, Saxatilin, Agretin) on activation, migration, adhesion, and down regulation of pro-inflammatory cytokines (TNF-α, CXCL1, CCL2) have been reported (Yeh et al., 2001; Jang et al., 2007; Hsu et al., 2011). Our results, have not only confirmed the role of the αvβ3 integrin on inflammation but also extended its action to another type of inflammation, namely the chronic inflammatory process induced by subcutaneous implantation of synthetic matrix in mice.

Fibrogenesis in tissue remodeling is regulated by complex interactions of pro- and anti-fibrogenic proteins within the inflammatory tissue. TGF-β1 is a key pro-fibrotic cytokine that induces differentiation of fibroblasts into myofibroblasts that, in turn, synthesize collagen (Wynn, 2007; Duffield et al., 2013). Collectively, our measurements of the effects the disintegrin on the fibrogenic response would indicate that attenuation of collagen deposition is not dependent on production of TGF-β1 levels. Thus, DisBa-01 acted on pro-angiogenic and pro-inflammatory cytokines, but not in the fibrogenic cytokine. This, in turn, would indicate that the signaling pathway of the repair phase is at least partially preserved.

Our present results have identified and quantified the effects of DisBa-01 on some critical features (angiogenesis, inflammation and fibrogenesis) associated with a number of pathological processes, implying that the DisBa-01 may be of therapeutic potential in controlling fibroproliferative diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

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