Published in final edited form as:

Toxicon. 2010 September 1; 56(3): 391–401. doi:10.1016/j.toxicon.2010.04.002.

The mojastin mutant Moj-DM induces apoptosis of the human melanoma SK-Mel-28, but not the mutant Moj-NN nor the non-mutated recombinant Moj-WN

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Abstract

In this study, three recombinant mojastin peptides (Moj-WN, Moj-NN, and Moj-DM) were produced and compared functionally. Recombinant Moj peptides were purified as GST-fusions. GST-Moj-WN and GST-Moj-NN inhibited ADP-induced platelet aggregation in platelet rich plasma. The GST-Moj-WN had an IC₅₀ of 160 nM, while the GST-Moj-NN had an IC₅₀ of 493 nM. The GST-Moj-DM did not inhibit platelet aggregation. All three GST-Moj peptides inhibited SK-Mel-28 cell adhesion to fibronectin. The GST-Moj-WN inhibited the binding of SK-Mel-28 cells to fibronectin with an IC₅₀ of 11 nM, followed by the GST-Moj-NN (IC₅₀ of 28 nM), and the GST-Moj-DM (IC₅₀ of 46 nM). The GST-Moj peptides' ability to induce apoptosis on SK-Mel-28 cells was determined using Annexin-V-FITC and nuclear fragmentation assays. Cells were incubated with 5 μM GST-Moj peptides for 24 h. At 5 μM GST-Moj-DM peptide, 13.56% ± 2.08 of treated SK-Mel-28 cells were in early apoptosis. The GST-Moj-DM peptide also caused nuclear fragmentation as determined by fluorescent microscopy and Hoechst staining. The GST-Moj-WN and GST-Moj-NN peptides failed to induce apoptosis. We characterized the SK-Mel-28 integrin expression, as the first step in determining r-Moj binding specificity. Our results indicate that SK-Mel-28 cells express αvβ3, αv, α6, β1, and β3 integrin receptors.

Keywords

anoikis; melanoma cell line; recombinant disintegrin; binding-loop mutants; cell adhesion inhibition; mojastin

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Conflict of interest statement: The authors declare that there are no conflicts of interest.

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1. Introduction

Cells in multicellular organisms use extracellular and intracellular signals that convey cell boundaries and are the basis for the formation of tissues. Cells interact with the extracellular matrix (ECM) by binding to adhesion molecules using cell surface receptors, such as integrins (Huveneers et al., 2007; Stupack, 2007). Integrins are heterodimeric transmembrane receptors composed of α and β subunits (Arnaout, 2002). Depending upon the combination of these subunits, an integrin can transfer a signal that induces the cell to migrate, proliferate, or undergo apoptosis (Lu et al., 2008; Mousa, 2008).

Integrins can relay intracellular signals when mechanical changes arise from cell-ECM detachment (Chiarugi and Giannoni, 2008). When the cell detaches from the ECM, a signal transduction pathway leads to the translocation of pro-apoptotic BCL-2 family members Bad, Bim, and Bax to the outer mitochondrial membrane, initiating the intrinsic apoptosis-signaling pathway (Grossmann, 2002; Bouchard et al., 2008). This type of apoptosis resulting from ECM detachment is termed anoikis (Morozovich et al., 2006; Chiarugi and Giannoni, 2008). The understanding of the regulation of integrin signaling resulting in anoikis has potential in the development of new therapies, as it is clear that the inhibition of anoikis in malignant cells allows them to invade other tissues during metastasis (Chiarugi and Giannoni, 2008; Simpson et al., 2008; Geiger and Peeper, 2009; Su et al., 2009). Antagonists, such as disintegrins, are being studied as these molecules can bind and alter integrin signaling (Barja-Fidalgo et al., 2005; Huveneers et al., 2007). Furthermore, studies have shown the induction of apoptotic activity of endothelial cells after exposure to the disintegrins accutin (Yeh et al., 1998), agkistins (Ren et al., 2006), echistatin (Brassard et al., 1999; Alimenti et al., 2004), rhodostomin (Wu et al., 2003), salmosin (Hong et al., 2003), and the disintegrin-like protein VAP1 (Araki et al., 2002).

Disintegrins are low molecular weight snake venom proteins that are released as part of a larger molecule, a snake venom metalloprotease (SVMP). After proteolytic cleavage, some disintegrins become monomers and others dimerize (Calvete et al., 2003). Disintegrins are non-enzymatic molecules, with a cysteine-rich portion, and an integrin binding-loop (McLane et al., 2004). Disintegrin binding motifs include RGD, KGD, MVD, MLD, VGD, ECD, and MDG (McLane et al., 2004; Calvete et al., 2005).

Disintegrins are classified according to their size and their number of disulfide bonds (Calvete et al., 2003). There are five groups of disintegrin peptides: short (41-51 amino acids; four disulfide bonds), medium-sized (~70 amino acids; six disulfide bonds), long (~84 amino acids; seven disulfide bonds), disintegrin-like domains of PIII-SVMP (100 amino acids; eight disulfide bonds) (Calvete et al., 2000a), and dimeric (67 amino acid subunits; two inter-chain and four intra-chain disulfide bonds) (Calvete et al., 2000b; Calvete et al., 2005).

Mojastin 1, a disintegrin found in the *Crotalus scutulatus scutulatus* venom, has 70 amino acids with a molecular weight of 7 kDa (Sanchez et al., 2006). Mojastin inhibited platelet aggregation in whole human blood but was ineffective at inhibiting T24 cells binding to fibronectin. The objectives of the present study were to produce binding-motif mutations on the partial *Mojastin* cDNA sequence (Soto et al., 2007), compare the function of three mojastin recombinant peptides (GST-Moj-WN, GST-Moj-NN, and GST-Moj-DM), and find a mutant capable of inducing apoptosis in the human melanoma cell line SK-Mel-28. Developing more effective chemotherapies against melanomas is imperative as this is the deadliest skin cancer (Petermann et al., 2007). Melanomas are difficult to treat, as radiation is ineffective and the response rate to chemotherapies is in the order of 10% (Sekulic et al., 2008). Recombinant GST-Moj peptides were produced and tested for their ability to inhibit platelet aggregation, SK-Mel-28 cells binding to fibronectin, and apoptosis. The findings of this study form the basis

for further molecular alterations of the *Mojastin* DNA sequence in order to manipulate biological function of the mojastin disintegrin.

2. Materials and methods

2.1. Production of binding-loop mutants of a partial Mojastin cDNA

A partial *Mojastin* cDNA (Genbank accession number **DQ677629.1**) was previously obtained (Soto et al., 2007). The *Mojastin* cDNA was cloned into the *EcoR I-Hind III* sites of the pGEX-KG expression vector, transformed into *E. coli* NEBα cells, and extracted with the Wizard *Plus* plasmid DNA isolation kit. Stratagene's QuikChange Multi Site-Directed Mutagenesis kit was used to result in a single or double amino acid substitution(s) C-terminal to mojastin's RGD.

The Moj39N 5'-CAGCAAGGGGTGATAACAATGACGATACCTGCA-3' primer was used to achieve the single (W to N) mutation. The Moj39D-40M 5'-CAGCAAGGGGTGATGATATGGACGATACCTGCA primer was used to achieve the double (WN to DM) mutation. The multi site-directed reaction consisted of 2.5 μL 10× QuikChange multi reaction buffer, 18.5 μL sterile nuclease-free water, 1 μL (50 ng) of *Mojastin* cDNA-pGEX KG recombinant construct, 1 μL (50 ng) of mutant primer, 1 μL dNTP mix, and 1 μL QuikChange enzyme blend. The PCR amplification consisted of one cycle of 95°C (1 min), followed by 30 cycles of 95°C (1 min), 55°C (1 min), and 65°C (8 min). Following PCR, the reactions were incubated with 1 μL *DPN I* (10 U/mL) for 3 hr at 37°C, followed by transformation in XL-10 gold ultracompetent cells. DNA was extracted and sequenced (Tocore, LLC) to determine if mutants were in-frame. The mutant mojastin proteins were designated mojastin DM and mojastin NN. The designation corresponds to the two amino acids following the RGD tripeptide. Non-mutated recombinant mojastin protein was termed mojastin WN.

2.2. Expression and purification of GST-Moj fusion peptides

To prepare GST-Moj fusion peptides, samples were transformed into *E. coli* BL21 cells. Cultures for the wild type and each mutant were grown to an A_{600} of 0.6-0.8 in 2xYTA broth. Synthesis of GST-Moj was induced by incubating bacteria with 1mM IPTG for 2 h. The culture was then centrifuged at 700× G for 10 min, and the pellet suspended in 1X PBS. Cell lysate was obtained using lysozyme (0.1 μg/μL), with ten freeze/thaw cycles. The supernatant was retained after centrifugation for 10 min at 700× G and applied to a 1 mL GStap column at 0.2-1 mL/min. The column was washed with 5 mL of 1X PBS and eluted with 5 mL of reduced glutathione. Protein concentrations were determined using the Bradford assay. Protein samples were mixed with 1X Laemmli Sample Buffer and boiled at 95°C for 5 min. Samples (40 μL) were loaded in a NuPAGE 4-12%, Bis-Tris gel (Invitrogen) and separated at 120 mA for 40 min in MES Buffer (Invitrogen).

2.3. Structural protein modeling of r-Moj peptides

Three-dimensional structural models of r-Moj peptides were produced by homology. A high resolution X-ray structure of trimestatin (PDB ID 1J2L), a medium-sized disintegrin with a high sequence identity to mojastin, was used as a template. Preliminary models were built for each protein by MODELLER bioinformatics program. The models were analyzed with PROCHECK and the best quality models were chosen after evaluating the Ramachandran plots. Molecular images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

2.4. Inhibition of platelet aggregation using platelet rich plasma (PRP)

Platelet aggregation was estimated by turbidimetry using a dual-channel Chronolog Aggregometer model 560 CA. PRP was prepared by mixing fresh blood sample with trisodium citrate solution (3.2%, w/v) in a volume ratio of 9:1, followed by centrifugation at $190 \times g$, 25°C for 20 min to sediment leukocytes and erythrocytes. The platelet count was adjusted to 3.0×10^8 platelets/mL with platelet-poor plasma. Four hundred and ninety microliters of citrated PRP were pre-incubated at 37°C with a stir bar in a silicone-treated glass cuvette. Then, 10 μL of GST-Moj in PBS buffer or PBS buffer alone were added 4 min before addition of a platelet aggregation inducer. Aggregation was induced by adding 5 μL of ADP (final concentration 10 μM), and the changes in light transmittance were continuously recorded for 6 min. The percentage of inhibition was calculated by comparing light transmittance of GST-Moj samples and control. The IC_{50} value was calculated from a dose-dependence curve that was achieved from at least five different inhibitor concentrations.

2.5. Cell line and culture conditions

The human melanoma SK-Mel-28 cell line was obtained from ATCC. The SK-Mel-28 cell line was maintained with Eagle's minimum essential medium, supplemented with 10% fetal calf serum in a humidified 5% CO_2 air incubator at 37°C .

2.6. Cellular adhesion inhibition assay

Inhibition of SK-Mel-28 cell binding to fibronectin induced by GST-Moj peptides was measured as described previously (Wierzbicka-Patynowski et al., 1999). Triplicate wells of a 96-well plate were coated with fibronectin at $10\mu\text{g/mL}$, in 0.01 M PBS pH 7.4, and incubated overnight at 4°C . The plate was blocked with 0.2 mL of 5% bovine serum albumin (BSA) in PBS and incubated at 37°C , for 1 h. Cells were harvested, counted, and resuspended in medium containing 1% BSA, at 5×10^5 cells/mL. GST-Moj peptides were added to the cell suspension at various concentrations and incubated at 37°C for 1 h. The blocking solution was aspirated, and the cell/ peptide fraction suspensions (0.2 mL) were added to the wells coated with fibronectin, and incubated at 37°C for 1 h. Synthetic echistatin (SIGMA, Lot. 023K12301) was used as a positive control. In these wells, SK-Mel-28 cells failed to bind to fibronectin. The negative control consisted of SK-Mel-28 cells incubated with PBS. In these wells, the cells bound to fibronectin. Wells were washed three times with PBS-5% BSA. Two hundred microliters of medium in 1% BSA containing MTT (5:1 vol/vol) were added to the wells containing cells and incubated at 37°C , for 2 h. MTT was aspirated and 100 μL of DMSO were added to lyse the cells. The plate was gently shaken, and the absorbance read at 570 nm using a Beckman Coulter model AD 340 reader. The percent inhibition was calculated by the formula: $[(\text{absorbance of negative control} - \text{absorbance of cell/venom sample}) \div \text{absorbance of negative control}] \times 100$.

2.7. Apoptosis Detection

SK-Mel-28 cells were cultured in a 24-well plate. Five hundred thousand cells were added to 1 mL of media for each well and grown for 24 h. Three wells in every experiment were dedicated for each different treatment. After initial incubation, wells were treated with either 5 μM GST, GST-Moj-WN, GST-NN, or GST-Moj-DM peptides, and incubated for 24 h. An untreated control was also performed. Cells were then detached from the plate surface using 0.05% trypsin EDTA. Trypsin was neutralized with culture media. Cells were centrifuged, washed twice with cold PBS, and resuspended in 250 μL of 1X binding buffer. Then, 100 μL were removed and exposed to 5 μL each of Annexin-V-FITC and Propidium Iodide (BD Biosciences). The reaction was incubated at room temperature, in the dark for 15 min. Four hundred microliters of 1X binding buffer were added and 10,000 events per sample were analyzed using a Becton Dickinson FACScan flow cytometer and FACSCalibur software.

Statistical analysis of the percent apoptotic cell population, using ANOVA, was performed using Systat.

2.8. Nuclear Fragmentation

SK-Mel 28 cells were grown on chambered slides. One and a half million cells were grown in 2 mL of media for 24 h. After initial incubation, cells were treated with either 5 μ M GST, GST-Moj-WN, GST-Moj-NN, or GST-Moj-DM peptides, for 24 h. An untreated control was also performed. The media was aspirated and the cells washed twice with 1X PBS. Cells were fixed on the slide with 3% formaldehyde for 1 h, followed by two washes with 1X PBS. Ten micrograms/mL of Hoechst stain was added to cells and incubated in the dark for 15 min. Excess Hoechst stain was removed by two 1X PBS washes. Slides were mounted with 30% glycerol and viewed at 100 $\times$, oil immersion, with a Zeiss AXIO fluorescent microscope.

2.9. Characterization of integrins expression using RT-PCR

The Invitrogen FastTrack 2.0 mRNA Isolation Kit was used to obtain mRNA from 1×10^8 SK-Mel-28 cells. RT-PCR was used to generate double stranded cDNA (Promega Access RT-PCR kit). Primers used to amplify the corresponding binding sites of integrin subunits $\alpha 1$, $\alpha 1b$, $\alpha 3$, $\alpha 6$, αv , $\beta 3$, $\beta 4$, $\beta 5$, and $\beta 6$ were the following.

5'-TACCCATGGGACAGTGTTACAGC-3' ($\alpha 1$ forward),
 5'-TCCGTGGATAGACTGGCCAAAAATTCAG-3' ($\alpha 1$ reverse),
 5'-TGCCCTGGAGGGCCGAGGGCGG-3' ($\alpha 1b$ forward),
 5'-GGGGAAGGGGCTGTCCAGGACCTG-3' ($\alpha 1b$ reverse),
 5'-CTCCATCGGCAGACAGAGCGGCAGCAGCGC-3' ($\alpha 3$ forward),
 5'-CAGCTTCTCTCCATGGATTACCTGCTGGGG-3' ($\alpha 3$ reverse),
 5'-ATCGAGTTTGATAACGATGCTGACCCACG-3' ($\alpha 6$ forward),
 5'-AGGTGATATACCCCTTGAGAACCTGTGTTGG-3' ($\alpha 6$ reverse),
 5'-TACCATTGGAGAACTGAGATGGAGCGAGAG-3' (αv forward),
 5'-AGCAGCCCACTGCCCTTAAAGGATTTGAGATGG-3' (αv reverse),
 5'-CTCCGGCTCCGGCCAGATGATTCGAAG-3' ($\beta 3$ forward),
 5'-TTTCTGGGATAGCTTCTCAGTCATCAGCCCCAAAG-3' ($\beta 3$ reverse),
 5'-GTCCGTCTGCGGCCCGGTGAGGAGCGG-3' ($\beta 4$ forward),
 5'-GTGCTTGGCGAGCAGGCGCACCAGGGTGGG-3' ($\beta 4$ reverse),
 5'-GTAACCTCCGGCCCGGTGACAAGACCAC-3' ($\beta 5$ forward),
 5'-CGGTTCTCTGCCAATTTCTCTCCAAGCAAGGC-3' ($\beta 5$ reverse),
 5'-CTTAAGTTGAGACCAGGTGGTGCGCAGACTCTG-3' ($\beta 6$ forward),
 5'-GTTTTGTACCAGTTTATCAATGAGTTGTCCAATTG-3' ($\beta 6$ reverse).

Baneres et al., (2000) primer sequences for $\alpha 5$ and $\beta 1$ were used. The RT-PCR reaction was set-up following the manufacturer's users manual. The reverse transcription reaction was performed at 45°C, for 45min. PCR amplification consisted of 40 cycles of 94°C (30 s), 60°C (1 min), and 68°C (2 min). A final extension step was performed for 7 min, at 68°C. DNA fragments were isolated using Invitrogen's E Gel system and sequenced using integrin-specific

primers (Sequetech, Co.). Sequences were compared using BLAST and aligned using BioEdit Sequence Alignment Editor version 7.0.4.1.

2.10. Characterization of integrins expression using flow cytometry

The following antibodies were used to determine integrin expression on SK-Mel-28 cells. Antibodies against $\alpha\text{v}\beta 3$ ($\alpha\text{v}\beta 3$ sc-7312 FITC), $\alpha 6$ ($\alpha 6$ sc-19622-FITC), αv (αv -53360-FITC), $\beta 1$ ($\beta 1$ sc-9970-FITC), $\beta 3$ ($\beta 3$ sc-51738 FITC), and respective isotypes were purchased from Santa Cruz Biotechnology. Cells were cultured to 90-95% confluency in a 75 cm² tissue culture flask and detached from the flask surface using 0.05% trypsin EDTA. Trypsin was neutralized with complete culture media. Cells were resuspended in 1X PBS to a final concentration of 10⁶ cells/mL, and blocked with 1 $\mu\text{L}/\text{mL}$ normal rabbit serum, for 25 min at room temperature. After blocking, cells were washed with 1mL 1X PBS, centrifuged, and resuspended in FCM Wash Buffer (Santa Cruz Biotechnology) to 10⁶ cells/mL. One hundred microliters of cells were distributed in three, 12 $\times$ 75 mm round bottom glass test tubes and fixed in 1% formaldehyde for 1 h. After fixation, cells were washed three times in 1X PBS. The first tube received 5-20 μL of a cocktail containing fluorochrome-conjugated monoclonal antibody. The second tube received the same volume of matched isotype control antibody. The third tube of cells received an equivalent volume of 1X PBS to serve as an autofluorescence control to establish appropriate flow cytometer settings. Cells were incubated with conjugated antibodies or control for 1 h, at room temperature, in the dark. Then three washes with 1X PBS were performed to remove unbound antibodies. Four hundred microliters of 1X binding buffer were added and the samples analyzed using a Becton Dickinson FACScan flow cytometer and FACSCalibur software.

2.11. Phylogenetic analysis

DNA sequences of RGDDM, -RGDDL, or KDGDG-containing disintegrins were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and included in the phylogenetic analysis. The following medium-sized disintegrins were included: bothrostatin (Genbank accession number [AF345931](#)), halystatin (Genbank accession number [E05552](#)), halystatin 2 (Genbank accession number [D28871.1](#)), jarastatin (Genbank accession number [DQ375441](#)), jerdonitin (Genbank accession number [AY364231.1](#)), MD2 (Genbank accession number [AF117637.1](#)), salmosin (Genbank accession number [AF054626.1](#)), saxatilin (Genbank accession number [AY005480.1](#)), and trigramin (Genbank accession number [X51530.1](#)). The following dimeric disintegrins were included: acostatin beta (Genbank accession number [ABO78906](#)), contortrostatin (Genbank accession number [AF212305](#)), and piscivostatin beta (Genbank accession number [ABO78906](#)). All sequences were aligned on the program BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall, 1999). DNA clustalW alignments were generated from 208-233 nucleotides over a 261-position span. Neighbor-joining phylogenetic trees were constructed with 1000 bootstrap replicates using MEGA version 3.1 (Kumar et al., 2004).

3. Results

3.1. Production of r-Moj mutants

Three mojastin mutants were produced and tested. All lacked ten amino acids at the amino terminus when compared to mojastin 2, and 12 amino acids when compared with mojastin 1 (Fig. 1A). The r-Moj-WN contained the original binding motif (RGDWN), while r-Moj-NN contained a single amino acid substitution C-terminal to the RGD motif. The r-Moj-DM contained two amino acid substitutions.

3.2. Molecular modeling of r-Moj WN, r-Moj-NN, and r-Moj-DM

Trimestatin, a medium-sized disintegrin with an RGD sequence in which the Arg and Asp side chains point in opposite directions, was used as a model to construct predicted mojastin three-dimensional models (Fujii et al., 2003). Of all the crystallized medium-sized disintegrins, mojastin had the highest sequence identity (87.3%) to trimestatin (Fig. 1A). MODELLER aligned r-Moj-WN, r-Moj-NN, and r-Moj-DM with the trimestatin template and produced five preliminary models for each peptide. PROCHECK was used to analyze each one and the best quality models were chosen after examining the Ramachandran plots. Ramachandran plots are invaluable when assessing protein models, as they are one of the best indicators of stereochemistry quality (Kleywegt and Jones, 1996). Each of the models chosen possessed over 91% of its residues in favorable regions, suggesting that the structures produced by MODELLER were stereochemically accurate.

Three-dimensional models for r-Moj-WN, r-Moj-NN, and r-Moj-DM peptides were visualized using CHIMERA (Fig. 1B). Models of the three r-Moj peptides showed predicted, altered orientations in their RGD motifs and C-termini. Thus, we hypothesized that r-Moj mutants may possess different pharmacological functions as a result of possible variations in the stereochemistry of the RGD motif and the C-terminal domain.

3.3. Production and purification of r-Moj peptides

GST-Moj fusion peptides for the r-Moj-WN, r-Moj-NN, and r-Moj-DM mutants were produced and purified (Fig. 2A). SDS-PAGE results showed that the pGEX-KG vector, without the mojastin insert, produced a band at 29 kDa (the predicted size of the GST protein), whereas pGEX-KG with the mojastin insert produced a band at the predicted size of 34 kDa.

3.4. Platelet aggregation activity of GST-Moj-WN and GST-Moj-NN

GST-Moj-WN and GST-Moj-NN inhibited ADP-induced platelet aggregation in platelet rich plasma (Fig. 2B). The GST-Moj-WN had an IC_{50} of 160 nM, while the GST-Moj-NN had an IC_{50} of 493 nM. This difference was not significant ($p = 0.0567$). The GST-only protein (used as a negative control) and the mutant GST-Moj-DM did not inhibit platelet aggregation.

3.5. The GST-Moj peptides inhibited SK-Mel-28 cell adhesion to fibronectin

The GST-Moj-WN inhibited the binding of SK-Mel-28 cells to fibronectin with an IC_{50} of 11 nM (Fig. 3). The GST-Moj-NN had an IC_{50} of 28 nM, while the GST-Moj-DM had an IC_{50} of 46 nM. The potency difference between the GST-Moj mutants was not significant ($p = 0.0977$).

3.6. GST-Moj-DM peptide induced apoptosis of SK-Mel-28 cells

The GST-Moj peptide's ability to induce apoptosis on SK-Mel-28 cells was determined using Annexin V-FITC and nuclear fragmentation assays. Cells were incubated with 5 μ M GST-Moj peptides for 24 h (Fig. 4). At 5 μ M GST-Moj-DM peptide, 13.56% $\pm$ 2.08 of treated SK-Mel-28 cells were in early apoptosis, while the GST-Moj-WN, GST-Moj-NN, and negative controls (GST-only protein and untreated cells) failed to induce apoptosis at significant levels. The apoptotic activity of the GST-Moj-DM peptide was statistically significant ($p < 0.005$).

The GST-Moj-DM peptide was also able to induce nuclear fragmentation as determined by fluorescent microscopy and Hoechst staining (Fig. 5A-B). The morphology of SK-Mel-28 cells was also affected by the treatment with GST-Moj-DM peptide, as these cells had a loose arrangement on the slide (Fig. 5B). Nuclear fragmentation and morphology alterations were not detected in untreated cells (Fig. 5C-D), cells treated with GST-only protein, GST-Moj-WN, nor GST-Moj-NN (data not shown).

3.6. Characterization of integrin expression on SK-Mel-28 cells

RT-PCR and flow cytometry were used to characterize integrin expression on SK-MEL-28 cells, as the first step in determining which integrins bind to r-Moj-WN, r-Moj-NN, and r-Moj-DM. RT-PCR results showed that SK-Mel-28 cells contained mRNA for $\alpha 6$, αv , $\beta 1$ and $\beta 3$ integrin subunits, but not for $\alpha 1$, $\alpha 3$, $\alpha 5$, αIIb , $\beta 4$, $\beta 5$, or $\beta 6$ (Fig. 6A). Flow cytometry and FITC-labeled antibodies against $\alpha v\beta 3$, and integrin subunits $\alpha 6$, αv , $\beta 1$, and $\beta 3$ were used to corroborate results obtained by the RT-PCR experiments. Our results indicate that SK-Mel-28 cells express $\alpha v\beta 3$, $\alpha 6$, αv , $\beta 1$, and $\beta 3$ integrin receptors (Fig. 6B).

4. Discussion

4.1. Recombinant disintegrin research

Recombinant versions of disintegrins provide investigators with ways of increasing potencies and altering activities. Studies using recombinant disintegrins are aimed at increasing binding specificity to integrins and improving their bioavailability, with the goal of potentially developing therapeutic agents (Eble and Haier, 2006).

In our study, we expressed and functionally tested three recombinant mojastin peptides produced as glutathione-S-transferase (GST) fusions. Previous research demonstrated that recombinant disintegrins produced as GST fusion proteins in *E. coli* have biological activity. For instance, recombinant GST-elegantin (Rahman et al., 1998) and GST-rhodostomin (Chang et al., 1993) retained their structural morphology and biological activity. Furthermore, a recombinant GST fusion peptide containing the disintegrin domain of ADAM 9 was used to determine the integrin-binding specificity of ADAM 9 (Mahimkar et al., 2005). The GST-ADAM 9 disintegrin peptide failed to bind to the human renal cancer cell line, HEK-293, in the presence of $\alpha 1$, $\alpha 3$, $\alpha 6$, αv , and $\beta 1$ antibodies.

GST-elegantin had the same inhibitory potency (IC_{50} 0.3 mM) as the purified toxin in ADP-induced platelet aggregation of platelet rich plasma (Rahman et al., 1998). Binding of human hepatoma cells to GST-rhodostomin coated plates was 10 to 20 fold higher than those culture plates containing GST alone (Chang et al., 1993). Furthermore, two synthetic peptides were produced to test whether the binding of human hepatoma cells was specific to the RGD-loop of GST-rhodostomin. The first was GRGDSP, which retained the RGD sequence and the second was GRGESP with an Asp amino acid substituted with a Glu amino acid. Increasing amounts of GRGDSP decreased the binding of human hepatoma cells to GST-rhodostomin. However, increasing amounts of GRGESP did not affect binding of human hepatoma cells, indicating that the cells were specifically binding to GST-rhodostomin. The biological functionality of recombinant rhodostomin in these assays led the investigators to conclude that the rhodostomin molecule bound to GST was properly folded (Chang et al., 1993).

4.2. Changing the RGD motif of r-Moj results in loss and gain of function

We produced truncated mojastin peptides that lack the first ten amino acids found in the native peptide. The truncations were produced in order to diminish or abolish the platelet aggregation activity, and to potentially alter other biological activities of recombinant mojastin peptides. The truncation was not sufficient to abolish the platelet aggregation activity of the GST-Moj-WN and GST-Moj-NN peptides, but potentially it could have resulted in the elimination of this activity of the GST-Moj-DM peptide.

In our study, we compared the function of three mojastin recombinant peptides, two of which had mutations in the RGD motif and one with the wild type RGD motif. Our hypothesis was supported as the data showed that altering mojastin's RGD motif caused a potential change of binding specificities. A single amino acid substitution (RGDWN to RGDNN) was not

sufficient to render the GST-Moj-NN significantly less potent at inhibiting platelet aggregation than the GST-Moj-WN peptide. However, two amino acid substitutions carboxyl to the RGD motif (RGD~~WN~~ to RGD~~DDM~~) resulted in the loss of platelet aggregation inhibition activity, and gain of apoptosis-inducing activity for the GST-Moj-DM mutant.

The GST-Moj-WN had an IC₅₀ of 160 nM, while the IC₅₀ of the column purified mojastin peptide, with the same RGD motif, from the *Crotalus scutulatus scutulatus* venom was 13.8 nM (Sanchez et al., 2006). It is important to note that the platelet aggregation assays were not performed in the same manner in both studies. It is possible that the difference in IC₅₀ data between the recombinant and native mojastin peptides is attributed to the different assays. Sanchez et al. (2006) used whole blood, while PRP was used in the present study. Differences in IC₅₀ data between experiments performed in whole blood and PRP have been previously observed. For instance, Gonzales et al. (2007) studied the influence of acetylsalicylic acid and salicylic acid on whole blood and PRP platelet aggregation. They found that for PRP, the concentration needed to reach IC₅₀ was 281 $\pm$ 16 μ M, whereas in whole blood it was 24.9 $\pm$ 1.2 μ M. They attributed this difference to the presence of neutrophils found in whole blood.

Differences in biological activity have been obtained in recombinant disintegrins with mutations in the binding motif. For instance, diminished potencies in platelet aggregation and wound closure activities were found in RGD motif mutants of r-eristostatin (Tian et al., 2007).

The binding specificity and affinity of disintegrins to integrin receptors are attributed to the amino acids flanking the RGD motif of disintegrins (Kini and Evans, 1995a; Kini and Evans, 1995b; Rahman et al., 1998). Kini and Evans found that the antiplatelet activity of a small peptide increased 13-fold when two amino acids were replaced with Pro (Kini and Evans, 1995a). One replaced amino acid was located N-terminal and the other C-terminal to the RGD motif. The mutated sequence was altered from IARGDMN to IPRGDMP. Furthermore, Rahman et al. (1998) were able to change the potency of elegantin by replacing the N-terminal and C-terminal amino acids flanking the RGD motif.

The disintegrin-binding loop is critical for receptor recognition, while the C-terminal domain is critical for the induction of conformational changes of the receptors (Wierzbicka-Patynowski et al., 1999). Wierzbicka-Patynowski et al., (1999) substituted the C-terminal sequence of eristostatin (WNG) with that of echistatin (HKGPAT). It was found that the hybrid inhibited VNRC3 cell adhesion to fibronectin eight times stronger than the wild type (Wierzbicka-Patynowski et al., 1999).

4.3. The mojastin mutant GST-Moj-DM induced apoptosis of SK-Mel-28

Integrins are the transmembrane proteins that mainly interact with the ECM (Moschos et al., 2007). Besides attaching cells to the ECM, integrins also interact with the cytoskeleton intracellularly where signal transduction molecules can be activated upon ECM-integrin alterations (Chiarugi and Giannoni, 2008). In normal cells, growth factors activate ERK and PI3K pathways resulting in survival. Cell-ECM detachment disrupts these survival signals and activates anoikis (Chiarugi and Giannoni, 2008). In our study, the GST-Moj mutant peptides tested inhibited SK-Mel-28 cell adhesion to fibronectin but only GST-Moj-DM induced apoptosis of SK-Mel-28 cells. The GST-Moj-DM peptide induced early apoptosis in 13.56% $\pm$ 2.08 of SK-Mel-28 cells. This level of apoptotic-inducing activity is comparable to the results obtained with the recombinant disintegrin domain of metagirdin (Trochon-Joseph et al., 2004). r-metagirdin induced early apoptosis in 12.8% $\pm$ 2.5 of human vein endothelial cells (HUVEC). In addition, venom-purified rhodostomin induced apoptosis in 12.91% of HUVECs, as determined by flow cytometric measurements of DNA content (Wu et al., 2003).

It is possible that GST-Moj-WN, GST-Moj-NN, and GST-Moj-DM bind and potentially activate different integrin receptors. A potential distinctive binding of GST-Moj-DM to integrin receptors on SK-Mel-28 cells may result in the activation of signal transduction pathways resulting in apoptosis.

The GST-Moj-DM mutant radically altered the morphology of SK-Mel-28 cells upon treatment. Drastic morphological changes were also observed in HUVECs upon treatment with VAP1 disintegrin-like protein (Araki et al., 2002). Furthermore, Oliva et al. (2007) showed that treating the human melanoma cell line B16F10 with jarastatin, flavoviridin, or kristin induced morphological changes. Cells' morphology is provided by the action of actin-associated proteins and upstream signaling molecules controlling the formation of the actin cytoskeleton (Moseley and Goode, 2006). Since this a dynamic process, cells have the ability to change their cytoskeleton in response to signals originating from the ECM. Research has shown that the cytoskeleton is reorganized as a result of the activation of anoikis and non-ECM-dependent apoptotic pathways (Su et al., 2009).

4.4. Integrin receptors on SK-Mel-28 cells

Disintegrins are natural antagonists of integrin binding with cell matrix proteins. Recombinant proteins have been produced to test the binding affinity and specificity of disintegrins to integrins receptors (Chang et al., 2001). We found that the SK-Mel-28 melanoma cell line expresses $\alpha\text{v}\beta 3$, $\alpha 6$, αv , $\beta 1$, and $\beta 3$ integrin receptors. Integrins $\alpha\text{v}\beta 3$ and $\alpha 6\beta 1$ are important for cell survival (Chiarugi and Giannoni, 2008). Furthermore, some melanoma cells express $\alpha\text{v}\beta 3$ receptors before they metastasize (Sekulic et al., 2008). It is possible that GST-Moj-DM may bind to the $\alpha\text{v}\beta 3$ receptor, as this receptor has been shown to be involved in apoptosis-induction by disintegrins (Yeh et al., 1998; Alimenti et al., 2004). Phylogenetic, integrin specificity, and functional data support this possibility. Research has shown that despite disintegrins' differences in amino acid length, number of cysteines, or structure (monomeric or dimeric) between groups, disintegrins bind to the same receptors if the RGD-motif is the same (Fig. 7). For instance, the short disintegrin echistatin and the medium-sized disintegrins jarastatin and saxatilin have an RGDDM motif and bind to $\alpha\text{v}\beta 3$ receptors (Oliva et al., 2007; Kim et al., 2008). In addition, the medium-sized disintegrin salmosin and the dimeric disintegrin contortrostatin have a RGDDL motif in their binding loop and bind to $\alpha\text{v}\beta 3$ receptors as well (Chung et al., 2003; Hong et al., 2003; Swenson et al., 2005).

Integrin specificity for some of the apoptosis-inducing disintegrin or disintegrin-like peptides was determined. For instance, accutin binds to $\alpha\text{v}\beta 3$ (Yeh et al., 1998). Echistatin binds to $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ integrin receptors (Wierzbicka-Patynowski et al., 1999), phosphorylates FAK, and induces caspase-3 activation of apoptosis (Alimenti et al., 2004). In addition, it has been suggested that echistatin binds with higher affinity to $\alpha 5\beta 1$ than $\alpha\text{v}\beta 3$ (Alimenti et al., 2004). Salmosin recognizes $\alpha\text{v}\beta 3$ receptors and induces apoptosis of the human melanoma SK-Mel-2 cell line (Chung et al., 2003), and bovine capillary endothelial (BCE) cells (Hong et al., 2003). VAP1 exerts its apoptotic-inducing activity by binding to $\alpha 3$, $\alpha 6$, and $\beta 1$ integrins (Araki et al., 2002).

4.5. Conclusion

The restricted availability of toxins from natural sources has urged production and mutations of recombinant toxins with therapeutic potentials. Our data suggest that changing one-to-two amino acids C-terminal to the RGD changed the conformation of their RGD motifs and C-termini and resulted in altered binding specificity of GST-Moj peptides. We found that changing one amino acid did not alter the potency of platelet aggregation inhibition, and changing two amino acids (WN to DM) in the RGD motif altered the function of the GST-Moj peptide.

Acknowledgments

We thank Cleber Ouverney, Takele Teklemariam, and Lucy Arispe for technical assistance. Funding for this project was provided by NSF-REU # DBI 0354149, NIH-NIGMS Minority Access to Research Careers (MARC) # 5T34GM 08253, NIH/SCORE # 2SO6 GM008192, NCRR/Viper # 2P40RR018300, NIH/RIMI #5 5P20MD000216, NIH/SCORE #5 S06 GM0081079, NIH/RISE Grant# 5R25GM071381, and HHMI Science Education Grant# 52006312.

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Fig. 1.

(A) Alignment of amino acid sequences for trimestatin, mojastin 1, mojastin 2, r-Moj-WN, r-Moj-NN, and r-Moj-DM mutants. Underlined amino acids differ from those in trimestatin. The RGD tripeptide is shaded in gray. In bold are shown the two amino acids carboxyl to the RGD motif. Two gaps shown at the amino terminus of trimestatin and mojastin 2 indicate the absence of amino acids E and A, which are present in mojastin 1. The single gap shown at the 6th position from the carboxyl end in mojastin 1, 2, and the recombinant Moj-WN, Moj-NN, and Moj-DM indicate the lack of a W amino acid found in trimestatin. (B) Recombinant Moj-WN, Moj-NN, and Moj-DM peptide models displayed in surface style. The RGD, the two amino acids carboxyl to the RGD, and the C-terminus are colored. The other amino acids are shown in gray. The characteristic RGD motif is shown in red, and the C-terminus is shown in green. For simplicity, the mutants are labeled with the two amino acids carboxyl to the RGD motif. On the WN peptide, the W amino acid is shown in blue. The N amino acids (as in RGD_N) in WN and the NN peptides are shown in yellow. On the NN mutant, the first N amino acid closest to the G (as in RGD_N_) is colored in light brown. On the DM mutant, the D amino acid is shown in purple, while the M amino acid is shown in light orange.

Fig. 2.

(A) Purification of GST-Moj-WN. Peptides were separated on a NuPAGE 4-12 %, Bis-Tris gel. Lane 1: MW; Lane 2: GST uninduced; Lane 3: GST whole-lysate; Lane 4: purified GST (29 KDa); Lane 5: GST-Moj-WN whole-lysate; Lane 6: purified GST-Moj-WN (34 KDa). Similar results were obtained for the GST-Moj-NN and GST-Moj-DM peptides (data not shown). (B) The effects of GST-Moj WN and GST-Moj-NN on ADP-induced platelet aggregation. Various concentrations of disintegrin were preincubated with platelet rich plasma for 2 min at 37°C prior to the addition of ADP. The data are a representation of triplicate experiments.

Inhibition of SK-Mel-28 to Fibronectin with Mojastin Mutants

Fig. 3.

The effects of GST-Moj-WN, NN, and DM on cell adhesion. Various concentrations of disintegrin were preincubated with SK-Mel-28 cells for 1 h at 37°C prior to adding them to fibronectin. The GST-only protein (as a negative control) failed to inhibit cell adhesion. The data are a representation of triplicate experiments.

Fig. 4. GST-Moj-DM induction of apoptosis in SK-Mel-28 cells. Cells were incubated with 5 μ M each of GST-only protein, GST-Moj-WN, GST-Moj-NN, or GST-Moj-DM peptides for 24 h. Untreated cells were used as a negative control. Experiments were done in triplicates. Standard deviation data are included as bars.

Fig. 5. GST-Moj-DM induced nuclear fragmentation and morphological alterations of SK-Mel-28 cells. Cells were incubated with 5 μ M each of GST-only protein, GST-Moj-WN, GST-Moj-NN, or GST-Moj-DM peptides for 24 h. Untreated cells were used as a negative control. (A) Hoechst-stained cells after treatment with 5 μ M GST-Moj-DM for 24 h. (B) Differential Interference Contrast (DIC) microscopy image of cells in (A). Arrows indicate cells with nuclear fragmentation. (C) Untreated cells stained with Hoechst. (D) DIC image of cells in C. Scale bar 10 μ m.

Fig. 6.

Integrin expression of SK-Mel-28 cells. (A) RT-PCR was used to determine integrin expression. A dash indicates no expression of the tested integrin. (B) Flow cytometry was used to corroborate results obtained in (A). Isotype (negative control antibody) profile is indicated as a light gray line. Specific anti-human integrin monoclonal antibody profile is indicated as a gray shaded graph.

Fig. 7.

Phylogenetic analysis of DNA sequences of some disintegrins containing RGDDM, RGDDL, or KGDDL binding motifs and their integrin-binding specificity. DNA sequences were aligned using ClustawL and phylogenetic trees constructed using the Neighbor joining (NJ) analysis. Bootstrap values (1,000 trees) above 50 are shown at the node. The scale bar represents substitutions per site. Integrin-specificity for disintegrins jarastatin (Oliva et al., 2007; Wermelinger et al., 2009), saxatilin (Kim et al., 2008; Sohn et al., 2008), trigramin (Chiang et al., 1995), piscivostatin beta (Okuda and Morita, 2001), contortrostatin (Swenson et al., 2005), and salmosin (Kang et al., 1998; Chung et al., 2003; Hong et al., 2003) are shown.