RESEARCH REPORT

Recombinant disintegrin (r-Cam-dis) from *Crotalus adamanteus* inhibits adhesion of human pancreatic cancer cell lines to laminin-1 and vitronectin

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ABSTRACT

Pancreatic cancer is a malignant cancer common worldwide having poor prognosis, even when diagnosed at its early stage. Cell adhesion plays a critical role in cancer invasion and metastasis. Integrins are major mediators of cell adhesion and play an important role in invasion and metastatic growth of human pancreatic cancer cells. Snake disintegrins are the most potent ligands of several integrins and have potential therapeutic applications for cancers. We have previously cloned and expressed a new recombinant RGD-disintegrin from *Crotalus adamanteus* (r-Cam-dis). This recently published r-Cam-dis has an extra nine amino acids derived from the vector (SPGARGSEF) at the N-terminus end and has strong anti-platelet activity. However, this r-Cam-dis contains the contamination of the cleavage of the N-terminal end of the pET-43.1a cloning vector. In this study, we have cloned r-Cam-dis in a different cloning vector (pGEX-4T-1) showing five different amino acids (GSPEF) at the N-terminal part. This new r-Cam-dis was expressed and tested for inhibition of platelet aggregation, specific binding activity with seven different integrins, and inhibition of adhesion of three different pancreatic cancer cell lines on laminin-1 and vitronectin. The r-Cam-dis showed potent binding to $\alpha_v\beta_3$ integrin, but was moderate to weak with $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_2\beta_1$, and $\alpha_6\beta_1$. Interestingly, the inhibition of r-Cam-dis on pancreatic cancer cell lines adhesion to laminin-1 was more effective than that to vitronectin. Based on our binding results to integrin receptors and previous adhesion studies using function-blocking monoclonal antibodies, it is suggested that r-Cam-dis could be inhibiting adhesion of pancreatic cancer cell lines through integrins $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$.

KEYWORDS: Binding activity, *Crotalus adamanteus*, cell adhesion, disintegrins, integrins, pancreatic cancer

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States (Siegel et al, 2014). The overall 5-year survival rate (2003–2009) is 6% (Siegel et al, 2014) and median survival period is 3–6 months (Spinelli et al, 2006). The prognosis of pancreatic cancer patients remains poor due to difficulties in early detection, rapid tumor growth, extensive invasion and metastasis, and a high resistance to treatment (Stathis and Moore, 2010; Wolfgang et al, 2013). Therefore, identification and development of new therapeutic agents against this malignancy is needed.

Cell adhesion is critical for many biological processes such as hemostasis, wound healing, angiogenesis, and also cancer progression and metastasis (Zigler et al, 2010; Eke and Cordes, 2014). Cell adhesion is mediated by the specific interactions of cell surface receptors, integrins, with

extracellular matrix molecules such as collagens, fibronectin, laminins, and fibrinogen. Integrins are the main cell adhesion receptor molecules that modulate a variety of cellular functions such as tumor metastasis (Desgrosellier and Cheresh, 2010).

Integrins are heterodimers (α- and β-subunits) on the surface of cells that mediate cell-cell and cell-extracellular matrix interactions, as well as signal transduction. There are 18 α and 8 β known subunits, which generate at least 24 distinct integrin heterodimers (Hynes, 2002). There are at least 11 different integrins containing the β_1 subunit and mediates the interaction of most extracellular matrix proteins with tumor cells (Felding-Habermann, 2003). Specific integrins preferentially bind to distinct extracellular matrix proteins including fibrinogen, collagens, fibronectin, laminins, vitronectin, and cellular receptor through short peptide sequences such as Arg-Gly-Asp (RGD), Glu-Ile-Leu-Asp-Val (EILDV), or Arg-Glu-Asp-Val (REDV) (Plow et al, 2000; Hynes, 2002). Pancreatic cancer cell lines express several integrins that allow these cells to bind extracellular matrix proteins such as collagen, vitronectin, laminins, and fibronectin and promote the invasive phenotype of pancreatic cancer. However, it has been shown that laminin-1, a major extracellular matrix protein in the basement membrane, is involved in the proliferation, differentiation, and survival of pancreatic precursor cells (Jiang et al, 1999; Jiang et al, 2001). In pancreatic cancer cells, the expression of an integrin profile is modulated on cancer cells in accordance with the extracellular matrix modification. Integrin subunits α_2, α_3, α_5, α_6, α_v, and β_1 are expressed on most pancreatic cancer cell lines including BxPC-3, AsPC-1, and Panc-1 (Löhr et al, 1996; Grzesiak and Bouvet, 2006; Lee et al, 2011; Zhu et al, 2011); whereas the expression of integrins $\alpha_6\beta_1$ and $\alpha_v\beta_3$ in pancreactic cancer cell lines and tissues are associated with invasion (Vogelmann et al, 1999; Hosotani et al, 2002). Integrin $\alpha_2\beta_1$ has also been described to mediate malignant phenotypes by increasing adhesion, proliferation, and migration in pancreatic cancer cells (Grzesiak and Bouvet, 2006). Although integrin $\alpha_2\beta_1$ is known to be a primarily collagen receptor, it has been shown that the $\alpha_2\beta_1$ integrin can interact with different ligands including type I collagen, type IV collagen, and mouse laminin (laminin-1) on fast-growing Colo-357 (FG-RFP) pancreatic cancer cells (Grzesiak et al, 2011). Integrin $\alpha_6\beta_1$ and $\alpha_3\beta_1$ have been reported to be overexpressed and functionally active in metastatic formation through binding to laminin-1 (Vogelmann et al, 1999; Sawai et al, 2003; Binkley et al, 2004; Grzesiak et al, 2007). In addition, integrin $\alpha_5\beta_1$, a fibronectin receptor, plays key roles in invasion by irradiated pancreatic cancer cell lines including Panc-1, BxPC-3, and MiaPaCa-2 (Yao et al, 2011). Moreover, Zhou et al (2013) demonstrated that abnormal expression of integrin β_1 subunit is related to the poor differentiation, rapid progress, easy metastasis, and poor prognosis of pancreatic cancer suggesting that the expression of integrin β_1 mRNA and protein expression in blood may serve as a biomarker for the development and metastasis of pancreatic cancer and as a prognosis indicator for pancreatic cancer.

Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have also been reported to play an important role in tumor cell adhesion and migration and is functionally involved in metastasis and angiogenesis of various tumor types (Weis and Cheresh, 2011). Cirulli et al (2000) demonstrated that both integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (major vitronectin receptors) are highly expressed in pancreatic ductal cells and clusters of undifferentiated cells emerging from the ductal epithelium. The integrin $\alpha_v\beta_6$ is an epithelial-specific integrin that is a receptor for fibronectin, vitronectin, and tenascin. Integrin $\alpha_v\beta_6$ has also reported to be expressed in many types of cancers including pancreatic, cervical, lung, and colon cancers (Van Aarsen et al, 2008; Bandyopadhyay and Raghavan, 2009), whereas its expression in corresponding normal tissue is low or undetectable. Pancreatic ductal adenocarcinomas exhibit the highest integrin $\alpha_v\beta_6$ expression among gastroenteropancreatic adenocarcinomas (Sipos et al, 2004). In addition, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_6$ have recently been identified as target biomarkers in the detection of pancreatic cancer in vivo using imaging systems (Liu et al, 2014; Trajkovic-Arsic et al, 2014).

Disintegrins bind to and block many biological functions of integrins on cell surfaces. These proteins are mainly found in snake venoms from the Viperidae and Crotalidae families (Juárez et al, 2008). Disintegrins specificity depend on a tripeptide motif located in a loop that is formed by the pairing of cysteine residues. Most disintegrins contain a tripeptide, the RGD motif, which bind to the integrin $\alpha_{IIb}\beta_3$ on the platelet surface and inhibit platelets (Calvete, 2013). Some of RGD-disintegrins are able to bind to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the cell surface of some tumor cells and inhibit cell migration and metastasis in various tumor cell types such as lung, breast, and bone cancers (Yang et al, 2005; Oliva et al, 2007; Swenson et al, 2007; Calvete, 2013). Recombinant disintegrins that bind to $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ receptors have also been reported to have anti-angiogenic properties (Ramos et al, 2008; Montenegro et al, 2012; Lucena et al, 2012; Lucena et al, 2014). However, studies on the specific interaction of recombinant disintegrins to several other integrins have been rare.

We have previously reported that r-Cam-dis, recombinant RGD-disintegrin derived from *Crotalus adamanteus*, showed strong anti-platelet effects (Suntravat et al, 2013). However, the r-Cam-dis was partially purified and contained the N-terminal part of the cleaved Nus tag (~14kDa) as a contaminant (Suntravat et al, 2013). In the present study, we cloned a P-II class snake venom metalloproteinase (CamVMPII)-derived RGD-disintegrin using a different cloning vector (pGEX-4T-1 vector) to improve the purity of r-Cam-dis. (CamVMPII, Genebank accession no. JX457344). We show that this new r-Cam-dis inhibits platelet aggregation, binds to soluble integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, and demonstrates the inhibition of adhesion on three different human pancreatic cancer cell lines (AsPC-1, Panc-1, and BxPC-3) to laminin-1 and vitronectin.

MATERIALS AND METHODS

PCR amplification and cDNA cloning of r-Cam-dis

A full-length cDNA encoding a *Crotalus adamanteus* venom metalloproteinase II was used (GenBank accession no. JX457344) as a PCR template to subclone its disintegrin domain. PCR was used to generate double stranded cDNA, with the following disintegrin-specific primers (a forward primer 5′-CGCGAATTCGAGGTGGGAGAAGATTGTGACTG-3′ and a reverse primer 5′-GACTCGAGTTAGCCATA-

GAGGCCATTTCTGGGA-3′, two restriction enzyme sites (underlined): *Eco*RI in forward primer and *Xho*I in reverse primer) as previously described (Suntravat et al, 2013). PCR amplification consisted of a cycle of 94°C (3min), 40 cycles of 94°C (30sec), 60°C (30sec), and 72°C (1min). A final extension step was performed for 10min, at 72°C. The PCR product was digested with *Eco*RI and *Xho*I and gel purified. The PCR product was ligated into *Eco*RI and *Xho*I sites of pGEX-4T-1 expression vector (GE Healthcare Lifesciences, Uppsala, Sweden), which was a different vector as previously described in Suntravat et al (2013). The ligated plasmid was transformed into *E. coli* Top10 competent cells (Invitrogen, CA, USA). Plasmid was extracted using the GenElute plasmid miniprep kit (Sigma-Aldrich, MO, USA). Plasmids containing inserts of the predicted size for Cam-dis were performed by PCR and further confirmed by sequencing for construction of in-frame.

Expression and purification of r-Cam-dis
Once the sequence was obtained, in-frame r-Cam-dis-pGEX-4T-1 plasmid containing an extra five amino acids from this cloning vector was transformed into *E. coli* BL21 (DE3) star cells (Invitrogen). BL21 cells harboring recombinant plasmid DNA was first cultured in 100ml fresh Luria-Bertani (LB) medium overnight at 37°C with shaking at 225rpm on an Innova® 43 incubator shaker (New Brunswick Scientific, CT, USA). After inoculation of the overnight culture into 2l of fresh LB medium, the culture cells were grown at 37°C with shaking at 225rpm on an Innova® 43 incubator shaker (New Brunswick Scientific) until the absorbance at 600nm (OD_{600}) reached 0.6. The culture was induced with a final concentration of 0.1mM isopropyl β-D-thiogalactopyranoside (IPTG) for 5hr to induce expression of recombinant proteins. Bacterial cells were collected by centrifugation at 10000xg for 10min and resuspended in 1x BugBuster Protein Extraction reagent (Novagen CA, USA) by gentle vortexing, using 5ml reagent per gram of wet cell paste. Cells were resuspended and incubated on a shaking platform for 20min at room temperature. The lysate was centrifuged at 16000xg for 20min at 4°C. The soluble supernatant was purified using a glutathione S-transferase (GST)-binding resin (Novagen) in Econo-Column chromatography column (BIO-RAD, CA, USA), which was previously equilibrated with 1x phosphate buffer saline (PBS), pH 7.4. r-Cam-dis proteins were cleaved and eluted from GST bound to GST-binding resin by thrombin cleavage. Thrombin was removed from r-Cam-dis using a 1ml HiTrap™ Benzamidine FF (high sub) column (Amersham Biosciences, NJ, USA) according to the manufacturer's instruction. The column was equilibrated with 5 column volumes of binding buffer (20mM sodium phosphate, 0.15M NaCl, pH 7.5). One milliliter of the sample was loaded into the column and r-Cam-dis protein was obtained by washing the column with a high salt buffer (20mM sodium phosphate, 1M NaCl, pH 7.5). The column was finally washed with 10 column volumes of elution buffer (10mM HCl, 0.5M NaCl, pH 2.0) to remove the thrombin bound to the column. r-Cam-dis was dialyzed in 1x PBS and concentrated using a 3kDa Amicon Ultra-15 centrifugal filter (Millipore, Carrigtwohill, Ireland), electrophoresed on SDS-PAGE under non-reducing condition. Protein concentration was estimated from the absorbance at 280nm.

N-terminal sequencing
r-Cam-dis (4μg) was transferred from an SDS-PAGE onto an Immobilon®-P Membrane, polyvinylidene fluoride (PVDF) (Millipore Corporation, MA, USA) using a Semi-Dry Transblot Cell (BIO-RAD) at 125mA for 1hr. The membrane was stained with Coomassie blue R-250 for 5min and distained with 50% (v/v) methanol for 5min. The sample membrane was sent out for N-terminal amino acid sequencing at the Protein Facility, Office of Biotechnology, Iowa State University, Iowa.

Inhibition of platelet aggregation
The inhibition of adenosine diphosphate (ADP)-induced platelet aggregation by r-Cam-dis was determined by measuring the impedance of human whole blood in a Chrono-Log Whole Blood Aggregometer (Chrono-Log, PA, USA) as previously described (Suntravat et al, 2013). The percent inhibition of platelet aggregation was calculated using the following equation: [(C-E/C)]x100, where C is the units of platelet aggregation (ohms) for the control, and E is the unit of platelet aggregation (ohms) for the experimental fraction. The extent of the inhibition of platelet aggregation was assessed by comparison with the maximal aggregation induced by the control dose of agonist (ADP). The median inhibitory concentration (IC_{50}) was calculated from a dose-dependent curve using Microsoft Excel 2011.

Binding of soluble integrins to immobilized r-Cam-dis
The interaction of r-Cam-dis with soluble recombinant human integrins was performed as described previously (Lucena et al, 2014). All recombinant human integrins were purchased from R&D Systems (MN, USA) including integrin $\alpha_v\beta_3$ (3050-AV), $\alpha_v\beta_5$ (2528-AV), $\alpha_v\beta_6$ (3817-AV), $\alpha_2\beta_1$ (5698-A2), $\alpha_3\beta_1$ (2840-A3), $\alpha_5\beta_1$ (3230-A5), and $\alpha_6\beta_1$ (7809-A6). Mouse anti-integrins monoclonal antibodies $\alpha_v\beta_3$ (23C6 clone), $\alpha_v\beta_5$ (P5H9 clone), α_3 (IA3 clone), α_5 (612557 clone), α_6 (MP4F10 clone), and β_6 (437216 clone) were from R&D Systems. Mouse anti-$\alpha_2\beta_1$ (BHA2.1 clone) monoclonal antibody was from Millipore (CA, USA). Briefly, the microtiter plates (96-well) were coated with 100μl of r-Cam-dis at various concentrations (0.005μM–1.2μM for $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_2\beta_1$ integrins, 0.05μM-16μM for $\alpha_6\beta_1$, and 0.01μM-5μM for $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins) in PBS, pH 7.4 at 4°C for 18hr. After washing three times with washing buffer (PBS buffer, pH 7.4 containing 0.05%, v/v Tween-20), the remaining sites on the wells were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS containing 0.05% (v/v) Tween (PBS-T) for 1hr at room temperature. The plates were then washed with washing buffer and followed by addition of 100μL of soluble integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ (20μg/ml) or $\alpha_v\beta_6$ (5μg/ml), in 0.5% (w/v) BSA in PBS-T and separately incubated with each integrin at room temperature for 2hr, with the exception of the plate with integrin $\alpha_6\beta_1$, which was incubated at 4°C for 24hr. After incubation and washing step, mouse anti-integrins monoclonal antibodies $\alpha_v\beta_3$, $\alpha_v\beta_5$, α_3, α_5, α_6, and α_2 (10μg/ml), and β_6 (5μg/ml) were added and incubated for 1 hr at room temperature. After the washing step, 100μl/well of 1:1500 diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (KPL, MD, USA was added and incubated for 1hr. A final wash was performed and 100μl/well of TMB substrate solution (0.2g/l 3,3',5,5'-tetramethylbenzidine and 0.01% (v/v) H_2O_2 in citric acid buffer; KPL) was

added. The reaction was stopped with 100μl/well of TMB stop solution (KPL), and the absorbance was measured in a microplate reader (Beckman Coulter model AD 340) at 450nm. Commercial echistatin (Sigma-Aldrich), a disintegrin that binds with a high affinity to integrin $\alpha_v\beta_3$, was used as a positive disintegrin control. Wells were coated only with 1mg/ml BSA (no disintegrin) to detect non-specific binding. Data on the graph was net specific binding, which was obtained by subtracting optical density values of the total binding from wells coated only with BSA. The error bars represent the standard deviations.

Cell line and culture conditions

The human pancreatic tumor cell lines (BxPC-3, AsPC-1, and Panc-1) were purchased from American Type Culture Collection (ATCC, VA, USA). BxPC-3 and AsPC-1 were maintained in Roswell Park Memorial Institute medium (RPMI)-1640 with L-glutamine and Phenol Red (ATCC) containing 10% (v/v) fetal bovine serum (Gibco, NY, USA) and antibiotics (50units/ml penicillin and 50μg/ml streptomycin) (ATCC). Panc-1 was maintained in Minimum Essential Medium (MEM) containing 10% (v/v) fetal bovine serum and antibiotics (50units/ml penicillin and 50μg/ml streptomycin). The cells were cultured in a humidified 5% (v/v) CO_2 air incubator at 37°C. All pancreatic cancer cells used in the adhesion assay were from passages 2–6.

Adhesion assay

Since laminin-1 and vitronectin are involved in the malignant phenotype of pancreatic cancer cells as described above and r-Cam-dis was able to bind to integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ (vitronectin receptors), $\alpha_6\beta_1$ (laminin receptor), $\alpha_2\beta_1$ (collagen and laminin receptor), and $\alpha_v\beta_6$ (fibronectin and vitronectin receptor), we decided to use vitronectin and laminin-1 to determine the specificity of adhesion and the inhibition of adhesion of three different pancreatic cancer cell lines on these extracellular matrix proteins by r-Cam-dis. r-Cam-dis was used to inhibit the binding of human pancreatic tumor cells to extracellular matrix proteins including vitronectin and laminin-1 coated plates using a modified method as described by Lucena et al (2012). Duplicate wells in a 96-well plate (Falcon® Tissue Culture Plate) were coated with 0.1ml of vitronectin or laminin-1 (isolated from mouse Engelbreth-Holm-Swarm tumor, Sigma-Aldrich) at 10μg/ml in 0.01M PBS, pH 7.4, and incubated overnight at 4°C. The plate was blocked with 0.2ml of 5% (w/v) BSA in PBS and incubated at 37°C for 1hr. Cells were harvested, counted, and resuspended in minimum essential medium (MEM) containing 1% BSA at 5×10^5 cells/ml (BxPC-3) or 7.5×10^5 cells/ml (AsPC-1 and Panc-1). The r-Cam-dis (0.05ml) was added to the cell suspension (0.45ml) at various concentrations and allowed to incubate at 37°C for 1hr. The blocking solution was aspirated, and the cell/disintegrin suspensions (0.2ml) were added to the wells coated with matrix protein and incubated at 37°C for 1hr for BxPC-3 or 2hr for AsPC-1 and Panc-1. The solution was aspirated and washed three times with PBS-5% (w/v) BSA by filling and aspirating. A total of 0.2ml of MEM medium in 1% (w/v) BSA containing 2.5mg/ml of 3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenltetrazolium bromide (MTT) (5:1, v/v) was added to the wells containing cells and incubated at 37°C for 2hr. The MTT was aspirated and 0.1ml of dimethyl sul-

foxide (DMSO) was added to the wells to lyse the cells. The absorbance was read at 570nm using a Beckman Coulter model AD 340 reader. Untreated cells adhere to the matrix was considered as a negative control. The percent inhibition was calculated by the following formula: [(absorbance of negative control - absorbance of cell/r-Cam-dis)/absorbance of negative control]x100.

Statistical analyses

The results were expressed as the mean±standard deviation (SD). Their significance was analyzed by the student's t-test. The level of significance was at $P<0.05$.

RESULTS

Recombinant production of r-Cam-dis

It was previously demonstrated that r-Cam-dis inhibits platelet activities (Suntravat et al, 2013). In the present study, r-Cam-dis was cloned into the pGEX-4T-1 expression vector containing the Glutathione S-transferase (GST) tag for affinity purification, which has been previously reported to express, in high levels, soluble and active recombinant disintegrins in *E. coli* (Sánchez et al, 2010; Lucena et al, 2012). In addition, this vector allows mild elution conditions for release of fusion proteins from the affinity matrix, thus minimizing effects on functional activity.
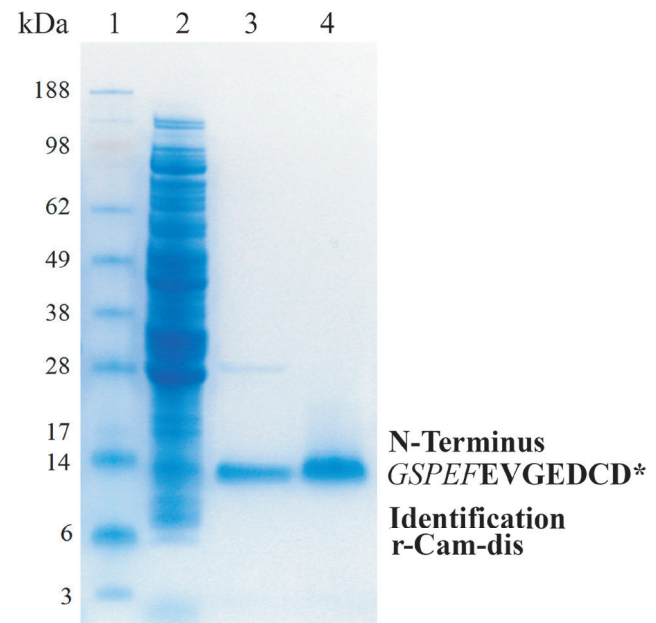

Figure 1. Expression and purification of r-Cam-dis analysis by 4–12% SDS-PAGE gel under non-reducing condition. Samples were run on 4–12% (w/v) Bis-Tris Gel using an Xcell SureLock Mini-Cell at 200V for 30min. The gel was stained with Rapid-Stain. Lane 1: SeeBlue Plus2 Markers; lane 2: soluble fraction of lysates of expressed *E. coli* BL21 cells by BugBuster reagent (150μg); lane 3: cleaved r-Cam-dis after wash with binding buffer (3μg); lane 4: purified r-Cam-dis after wash with high salt buffer (3 μg). An asterisk (*) represents the N-terminal amino acid sequence of purified r-Cam-dis containing the five amino acids from the vector (italicized) before the disintegrin sequence, which are shown in bold letters.

After r-Cam-dis was cleaved from the GST by thrombin treatment, a yield of 1mg of protein per liter of culture was obtained. Purified r-Cam-dis was identified by N-terminal sequence analysis. The r-Cam-dis contained an additional five amino acids from the vector at the N-terminus end (GSPEF), for a total calculated molecular weight of r-Cam-dis with GSPEF of about ~8.4kDa with a pI 4.36 by Protein Identification and Analysis Tools on the Expasy Server (Figure 1).

Inhibition of platelet aggregation
r-Cam-dis was initially tested for the inhibition of ADP-induced platelet aggregation activity. The r-Cam-dis inhibited ADP-induced platelet aggregation in a dose-dependent manner with the IC_{50} value of 8.88nM (Figure 2).

Binding of r-Cam-dis to integrins
To confirm that r-Cam-dis is capable of direct integrin binding, we employed indirect ELISA assay. As shown in Figure 3, r-Cam-dis was able to bind to integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_2\beta_1$, and $\alpha_6\beta_1$ (Figure 3A-E) but not to $\alpha_3\beta_1$ and $\alpha_5\beta_1$ (Figure 3F and 3G). The binding activity was most potent in the presence of integrin $\alpha_v\beta_3$. Echistatin, a well-known RGD-disintegrin that bind preferentially to the integrin $\alpha_v\beta_3$, showed binding specificity to only integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and was considerably less effective when compared with r-Cam-dis.

Inhibition of cell adhesion to vitronectin and laminin-1
The r-Cam-dis inhibited BxPC-3 adhesion to vitronectin and laminin-1, in a concentration-dependent manner with IC_{50} values of 10.73μM and 6.85μM, respectively (Figure 4A). Adhesion to vitronectin in Panc-1 was dose-dependently inhibited up to 28.9±5.2% (Figure 4B). The inhibition of Panc-1 binding to laminin-1 was not investigated because untreated Panc-1 control cells did not adhere to laminin-1. The r-Cam-dis also inhibited AsPC-1 adhesion to vitronectin and laminin-1 by 19.3±1% and 45±1%, respectively (Figure 4C).

DISCUSSION

Pancreatic cancer is a leading cause of cancer death throughout the world due to its rapid metastasis rendering late detection. Metastasis and the invasion of tumor cells to both nearby and distant organs are the most critical aspects of cancer (Spano et al, 2012; Alizadeh et al, 2014). Cell adhesion is a critical process for tumor growth as well as tumor metastasis, which is regulated by integrin adhesion molecules. Therefore, integrins have become a target for future anti-cancer drugs (Cayrol et al, 2011; Chen et al, 2013; Shi et al, 2014; van der Horst et al, 2014). Integrin antagonists are currently in clinical trials for cancer therapy including monoclonal antibodies such as etaracizumab, abegrin (McNeel et al, 2005; Hersey et al, 2010), RGD-based
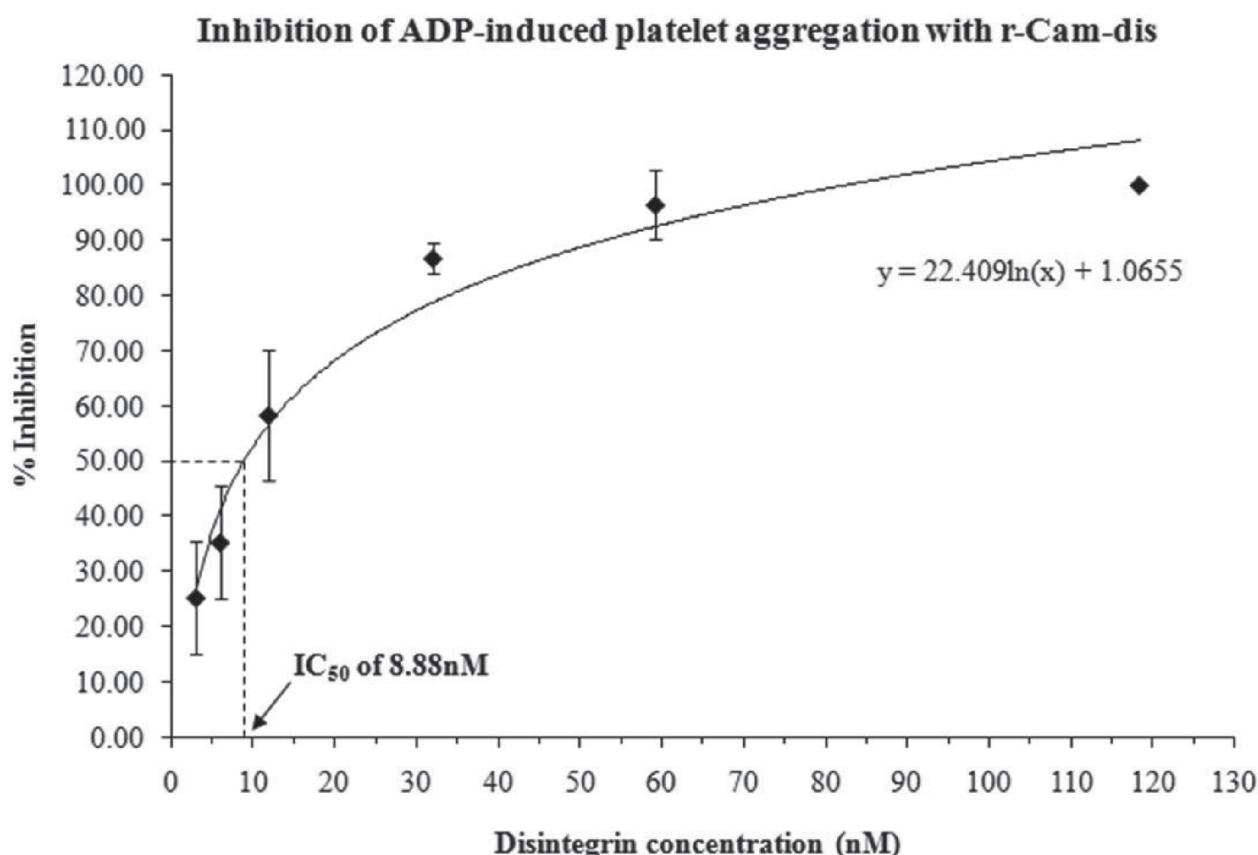

Figure 2. Inhibition of platelet aggregation using whole blood by r-Cam-dis. A Chronolog aggregometer was used to measure ADP-induced platelet aggregation by impedance. A total of 10μl of r-Cam-dis at varying concentrations was added to whole blood and incubated 1min at 37°C prior to adding 10μM of ADP. The error bars represent the standard deviation from three independent experiments with n=3.
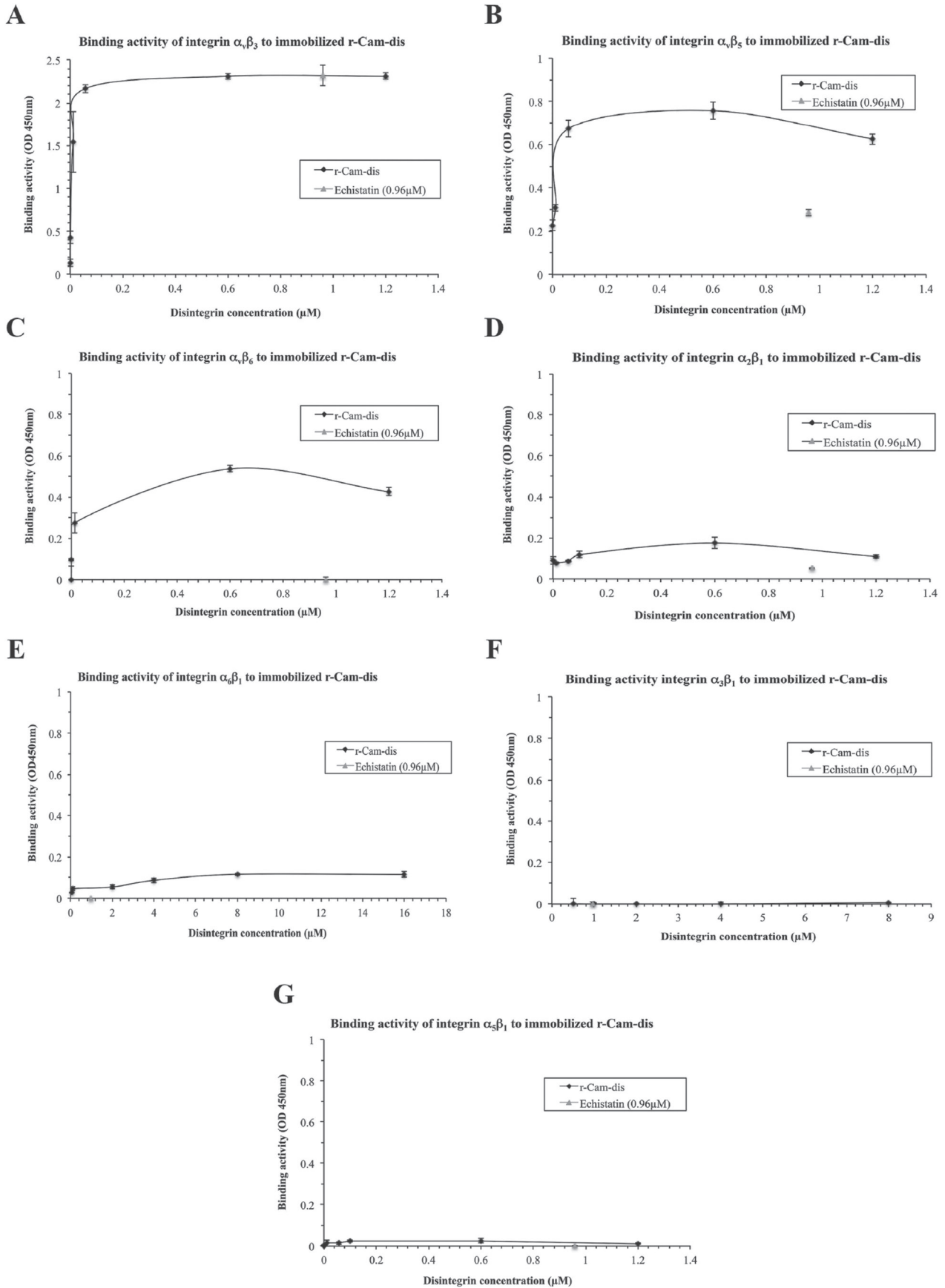
Figure 3. Interaction of immobilized r-Cam-dis with integrins A) $\alpha_v\beta_3$, B) $\alpha_v\beta_5$, C) $\alpha_v\beta_6$, D) $\alpha_2\beta_1$, E) $\alpha_6\beta_1$, F) $\alpha_3\beta_1$, and G) $\alpha_5\beta_1$. Integrin binding was measured by indirect ELISA assay as described in Materials and Methods. Absorbance at 450 nm of the individual well was measured to determine the binding activity. The error bars represent the standard deviation from two independent experiments with n=2.
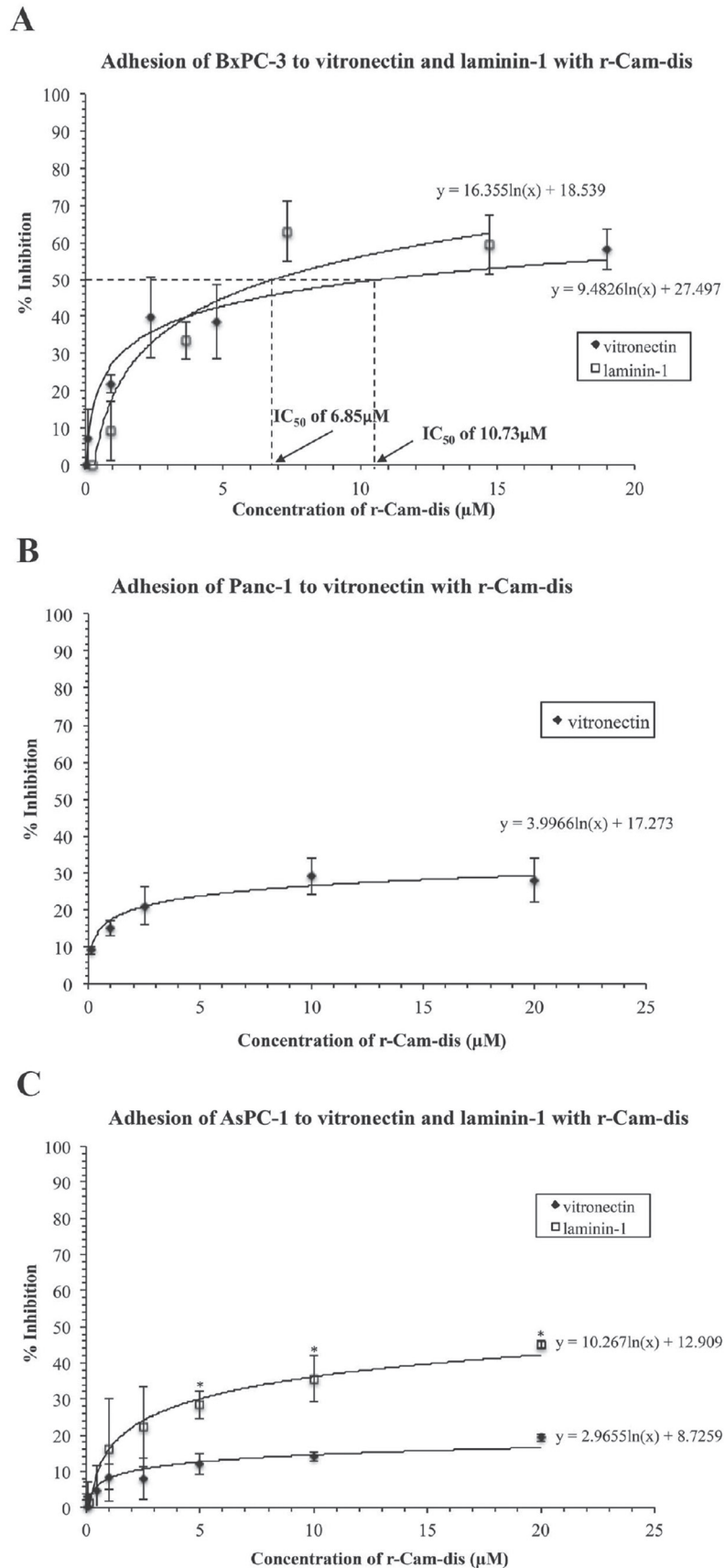
A

B

C

Figure 4. Effects of r-Cam-dis on adhesion of BxPC-3, Panc-1, and AsPC-1 pancreatic cancer cell lines on vitronectin and laminin-1. A) BxPC-3, B) Panc-1, and C) AsPC-1 were seeded in 96-well plates, which were pre-coated with vitronectin or laminin-1 in the absence (PBS added), or presence of various concentrations of r-Cam-dis. Cell adhesion was measured by MTT technique and the results were expresses as percent of inhibition. The results are expressed as mean$\pm$SD (n=3). An asterisk (*) indicates the significant difference between the inhibition of vitronectin adhesion and laminin-1 adhesion in each cell type by r-Cam-dis at $P<0.05$.

antagonists such as cilengitide, a cyclic RGD-peptapeptide antagonist of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Beekman et al, 2006; Nabors et al, 2007), and non-RGD-based antagonists such as ATN-161 (Cianfrocca et al, 2006).

We have previously demonstrated that r-Cam-dis (containing extra nine amino acids, SPGARGSEF) is a potent anti-platelet inhibitor. Unexpectedly, the r-Cam-dis was partially purified and contained the N-terminal part of the cleaved Nus tag (~14kDa) as a contaminant (Suntravat et al, 2013). In this study, we improved the purity by cloning r-Cam-dis into a pGEX-4T-1 vector (N-terminus GST tagged vector) (Figure 1). r-Cam-dis, containing five different amino acids (GSPEF), dose-dependently inhibited ADP-induced platelet aggregation with an IC$_{50}$ of 8.88nM (Figure 2), which was about 1.5 times less efficient than our previously reported r-Cam-dis (6nM) (Suntravat et al, 2013). This indicated that the addition of amino acids at N-terminus end is thought to cause conformation changes that alter its biological activity. However, this r-Cam-dis is more efficient than other recombinant disintegrins with IC$_{50}$ values ranging from 34nM to 6μM (Sánchez et al, 2010).

Since snake disintegrins are potent and specific antagonists of several integrins. Grzesiak and Bouvet (2006) reported that pancreatic cancer cells including BxPC-3, Panc-1, and AsPC-1 are expressed in varying degrees of immunoreactivity for the laminin-binding integrins α_2, α_3, α_6, the vitronectin-binding α_v together with the β_1, β_3, β_5 subunits. In this study, we showed that r-Cam-dis bound most potently to $\alpha_v\beta_3$ integrin. While the interaction of r-Cam-dis with integrins $\alpha_v\beta_5$ and $\alpha_v\beta_6$ was moderate and weak with integrins $\alpha_2\beta_1$ and $\alpha_6\beta_1$ (Figure 3). We also provide the preliminary experiments of the inhibition of adhesion of BxPC-3, Panc-1, and AsPC-1 cells on vitronectin and laminin-1 by r-Cam-dis. r-Cam-dis inhibited all three different pancreatic cancer cell lines to vitronectin and laminin-1 having the most potent adhesion inhibition effect on laminin-1, except for Panc-1 cells, which do not attach on laminin-1 (Figure 4). Tani et al (1997) reported that pancreatic carcinoma cell lines including BxPC-3, CFPAC-1, and Panc-1 preferably adhere to laminin-5 compared to laminin-1, however, BxPC-3 and Panc-1 cells adhere to laminin-1 to some extent. BxPC-3 cells showed similar adhesion to vitronectin and laminin-1, while Panc-1 cells preferred vitronectin over laminin-1 and fibronectin. In addition, Grzesiak and Bouvet (2008) using inhibition experiments with function-blocking anti-integrin antibodies including anti-β_1, anti-β_3, anti-β_4, anti-β_5, showed that β_1 integrin plays an important role in promoting adhesion of pancreatic cancer cell lines including AsPC-1, Panc-1, MiaPaCa-2, and BxPC-3 to fibrinogen, laminin-1, and type IV collagen. On type I collagen, cells mediate specifically by the $\alpha_2\beta_1$ integrin (Grzesiak and Bouvet, 2006).

Additionally, in vitro shRNA knockdown studies by Grzesiak et al (2011) demonstrated that knockdown of the β_1 and α_2 integrin subunits significantly inhibits cell adhesion and migration of fast-growing Colo-357 (FG-RFP) pancreatic cancer cells on type I and type IV collagen as well as laminin-1. By contrast, on vitronectin, cells bind predominantly via the integrin $\alpha_v\beta_5$, with involvement from

β_1 integrins as well (Grzesiak and Bouvet, 2006; Grzesiak and Bouvet, 2008). Taken together, it is possible that r-Cam-dis mediates the inhibition of adhesion through the binding of integrins $\alpha_2\beta_1$ and $\alpha_6\beta_1$ and involvement from $\alpha_v\beta_5$ integrin as well. However, r-Cam-dis was also bound to integrin $\alpha_v\beta_6$ (vitronectin receptor) and no studies on function-blocking antibody directed against $\alpha_v\beta_6$ on adhesion of AsPC-1, Panc-1, and BxPC-3 to extracellular matrix proteins have been investigated, therefore, integrin $\alpha_v\beta_6$ should not be excluded to be a possible target of r-Cam-dis. To verify the specific interaction of r-Cam-dis directly against these integrins on the surface of pancreatic cancer cell lines, the inhibition of cell binding to immobilized monoclonal anti-integrin antibodies by r-Cam-dis should be further investigated.

Interestingly, the adhesion of AsPC-1 cells to laminin-1 and vitronectin and Panc-1 cells to laminin-1 were only partially inhibited by r-Cam-dis, which might be due to the different intensity of expressed integrins on different cell types. It has been previously reported that the expression level of α_2 integrin in AsPC-1 is lower than that compared to BxPC-3 cells (Grzesiak and Bouvet, 2006; Ikenaga et al, 2012). Previous studies on $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression during pancreatic islet ontogeny demonstrated that adult islet cells show consistently low levels of both intergrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression as compared with fetal cells (Cirrulli et al, 2000). The molecular mechanisms involved in the inhibitory effect of r-Cam-dis on adhesion in pancreatic cancer cell lines remain to be elucidated.

The roles of platelets in tumor stability, growth, and metastasis have been implicated (Menter et al, 2014). Integrin-mediated inside-out and outside-in signaling and/or crosstalk play an essential role in the biologic responses of platelets. Platelet integrins include primarily $\alpha_{IIb}\beta_3$ (binds fibrinogen or von Willebrand factor), $\alpha_v\beta_3$ (binds vitronectin), $\alpha_2\beta_1$ (binds collagen), $\alpha_5\beta_1$ (binds fibronectin), and $\alpha_6\beta_1$ (binds laminin). Platelet integrins and their adhesive ligands that serve as bridging proteins participate in tumor-induced platelet aggregation (McNicol and Israels, 2008). Once activated, platelets release contents such as alpha granules and microparticles that facilitate tumorigenesis including adhesion, proliferation, and metastasis (Bambace and Holmes, 2011). It has been reported that human pancreatic cancer cell lines including PC-3, PC-44, AsPC-1, BxPC-3, Capan-2, Panc-1 are able to induce platelet aggregation in vitro, suggesting that platelet activation might support metastasis in pancreatic cancer (Heinmöller et al, 1995). Recently, the use of the anti-platelet drug, Clopidogel, decreased the size of the tumors and restored hemostasis in an ectopic model of pancreatic cancer and significantly inhibited the development of metastases in a syngeneic orthotopic mice model of pancreatic cancer (Mezouar et al, 2015). Our results showed that r-Cam-dis is a very potent inhibitor of platelet aggregation and is able to bind to several integrins that are found on both platelets and pancreatic cancer cell lines, however, the in vitro adhesion of Panc-1 and AsPC-1cells to laminin-1 and vitronectin were only partially inhibited. It is possible that r-Cam-dis may exert a stronger inhibitory effect if the pancreatic cancer cell lines were co-cultured with activated platelets and r-Cam-dis, a study which deserves further exploration.

CONCLUSIONS

We provide preliminary data showing that r-Cam-dis recognizes many integrins including, those involved in many pathological processes such as cell adhesion, migration, tumor invasion and metastasis and also inhibits an adhesion effect of three different pancreatic cancer cell lines. However, further studies on functional inhibition using integrins $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ monoclonal antibodies and apoptosis would greatly help in uncovering the exact molecular mechanism of r-Cam-dis in inhibiting adhesion of pancreatic cancer cell lines. r-Cam-dis could have a foundation for the development of targeted therapeutic approaches.

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COMPLETING INTERESTS

None declared.

ABBREVIATIONS

CamVMPII; P-II class snake venom metalloproteinase from *Crotalus adamanteus*
cDNA; complementary deoxyribonucleic acid
SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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