Inhibition of pancreatic tumoral cells by snake venom disintegrins

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ABSTRACT

Pancreatic cancer often has a poor prognosis, even when diagnosed early. Pancreatic cancer typically spreads rapidly and is rarely detected in its early stages, which is a major reason it is a leading cause of cancer death. Signs and symptoms may not appear until pancreatic cancer is quite advanced, and complete surgical removal is not possible. Furthermore, pancreatic cancer responds poorly to most chemotherapeutic agents. The importance of integrins in several cell types that affect tumor progression has made them an appealing target for cancer therapy. Some of the proteins found in the snake venom present a great potential as anti-tumor agents. In this study, we summarize the activity of two integrins antagonist, recombinant disintegrins mojastin 1 and viridistatin 2, on human pancreatic carcinoma cell line (BXPC-3). Both recombinant disintegrins inhibited some essential aspects of the metastasis process such as proliferation, adhesion, migration, and survival through apoptosis, making these proteins prominent candidates for the development of drugs for the treatment of pancreatic cancer.

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1. Introduction

The pancreas is an organ that has endocrine and exocrine functions. As an endocrine gland, it produces important hormones, including insulin, glucagon, somatostatin, and pancreatic polypeptide. These hormones play an imperative role in glucose metabolism and regulation of blood glucose concentration. As an exocrine gland, the pancreas secretes pancreatic juice containing digestive enzymes that assist the absorption of nutrients and the digestion in the small intestine. These enzymes help to further break down carbohydrates, proteins, and lipids (Hegyi and Petersen, 2013; Mastracci and Sussel, 2012). Pancreatic cancers can arise from the exocrine and endocrine parts of the pancreas; approximately 95% of them develop from the exocrine portion. In general, there are three basic types: ductal adenocarcinoma (more than 90% of pancreatic cancers), neuroendocrine tumors, and cystic neoplasm (Saif, 2011).

Ductal adenocarcinoma of the pancreas (PDA), commonly referred to as pancreatic cancer, is a frequent and lethal disease ranking the fourth cause in cancer-related death in western countries. PDA is a genetic disease caused by the successive accumulation of mutations in key oncogenes and tumor suppressor genes that once established is a quite complex, heterogenous and genetically unstable disease (Hidalgo, 2012). The incidence of and number of deaths caused by pancreatic cancer have been gradually rising. Despite developments in detection and management of pancreatic cancer, only about 4% of patients will live 5 years after diagnosis. The only potentially curative therapy for pancreatic cancer is surgical resection. Unfortunately, 80–85% of patients present with advanced unresectable disease. Furthermore, pancreatic cancer responds poorly to most chemotherapy agents (Vincent et al., 2011). The developments of novel and more effective chemotherapeutic agents for patients with advance disease are needed to improve the prognosis of this disease.

Disintegrins represent a family of low molecular weight (40–100 amino acids), cystein-rich polypeptides released in viper venoms by proteolytic processing of PII snake venom metalloprotease (SVMP) precursors. Disintegrins bind specifically to integrins expressed on platelets and other cells, including vascular endothelial cells and some tumor cells, leading to inhibition of platelet aggregation, inhibition of cell adhesion, migration and

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angiogenesis (Calvete, 2013; Marcinkiewicz, 2013). Since integrins are intimately involved in cancer cell motility, invasion, and other processes critical to cancer progression and metastasis, disintegrins hold significant potential for cancer therapy (Calvete, 2013). For this reason, the objective of this study was to determine the effect of two recombinant disintegrins, r-mojastin 1 and r-viridistatin 2, derived from *Crotalus scutulatus scutulatus* and *Crotalus viridis viridis*, respectively on human pancreatic adenocarcinoma cancer cells (BXPC-3).

2. Materials and methods

2.1. Preparation of recombinant disintegrins

r-Mojastin 1 and r-viridistatin 2 were expressed in *Escherichia coli* and further purified by two-step chromatography, using the method of Sánchez et al. (2010) and Lucena et al. (2012), respectively. Briefly, *E. coli* BL21 cells were grown, induced by 0.5 mM of isopropyl β-D thiogalactoside (IPTG) and centrifuged. After bacterial cell disruption with a Branson Sonifier 450 (Danbury, CT), the cell debris was removed by centrifugation and the crude lysate was incubated with glutathione Sepharose 4B (GS4B) (Amersham Biosciences). Recombinant disintegrins peptides were cleaved and eluted from glutathione S-transferase (GST) bound to GS4B by thrombin (80 U/mL, GE Healthcare Life Sciences, USA). Thrombin was removed from r-mojastin 1 and r-viridistatin 2 using a 5 mL HiTrap™ Benzamidine Sepharose 4 Fast Flow column (Amersham Biosciences). Purity of recombinant disintegrins was determined by using a 10–20% Tricine gel (Schägger and von Jagow, 1987) in an XCell SureLock Mini-Cell (Invitrogen Life Technologies, USA).

2.2. Cells lines and culture conditions

The human pancreatic adenocarcinoma (BXPC-3) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The BXPC-3 cells were maintained with RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) and 50 U/mL penicillin, 50 μg/mL streptomycin. The cells were maintained in a humidified 5% CO_2 air incubator at 37 °C.

2.3. Proliferation inhibition studies

Anti-proliferation activity on BXPC-3 cells of recombinant disintegrins, r-mojastin 1 and r-viridistatin 2 were performed by measuring cell proliferation using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). Two hundred microliters of cells were cultured on 96-well flat-bottom microtiter plates at 10^5 cells/well, in triplicate, and incubated at 37 °C in 5% CO_2 for 24 h.

Twenty microliters of each recombinant disintegrin (r-mojastin 1 and r-viridistatin 2) at various concentrations were added to the BXPC-3 cell suspension at 37 °C for 48 h. Then, 10 μL of MTT (5 mg/mL) was added to each well. After incubation for 4 h at 37 °C, MTT was aspirated and 100 μL of DMSO was added to lyse the cells. The absorbance at 570 nm was read using a Beckman Coulter™ model AD 340 reader. Doxorubicin, paclitaxel, gemcitabine, and 5-fluorouracil, anticancer drugs used in the treatment of prancreatic cancer and known to induce apoptosis, were used as positive controls (Neesse et al., 2014; Kratz et al., 2013; Saif, 2013). The negative control was cells treated with PBS buffer, pH 7.4. The percentage of cell proliferation was calculated relative to the negative control, which was defined as 100%. The 50% cytotoxic concentration (CC_{50}) of sample is defined as the protein concentration, which reduced 50% of proliferation. The values of the percentages of cell proliferation inhibition were plotted against disintegrins concentrations, and the CC_{50} was determined. Experiments were performed in triplicate.

2.4. Cellular adhesion inhibition assay

r-Viridistatin 2 and r-mojastin 1 were used to inhibit the binding of BXPC-3 cells on two extracellular matrix proteins (laminin 1 and vitronectin at 10 μg/mL) coated plates (Sánchez et al., 2009). The negative control consisted of BPXC-3 cells incubated with PBS. The negative controls allowed binding of cells to extracellular matrix proteins. The percent inhibition was calculated by the following formula: [(absorbance of negative control − absorbance of cell/r-disintegrin sample) ÷ absorbance of negative control] × 100.

2.5. Cellular adhesion inhibition using anti-integrin antibodies assay

To determine which integrins on the BXPC-3 cell surface are the targets for r-mojastin 1 and r-viridistatin 2, adhesion inhibition assays were performed using α_5 (VC5 clone), α_2 (AK-7 clone), α_6 (GoH3 clone), α_3 (C3 II.1 clone), β_4 (439-9B clone), $\alpha_v\beta_3$ (23C6 clone), and β_1 (MAR4 clone) monoclonal anti-integrin antibodies (BD Biosciences), and β_6 (437,211 clone) monoclonal anti-integrin antibody (R&D System). Duplicate wells of a 96-well plate (Falcon® Tissue Culture Plate) were coated with 0.1 mL of r-mojastin 1 and r-viridistatin 2at a concentration of 40 μg/mL, in 0.01 M Phosphate buffer saline (PBS), pH 7.4, and incubated overnight at 4 °C. Cells were harvested, counted and resuspended in medium containing 1% BSA at 5×10^5 cells/mL. Anti-integrins antibodies (α_5, α_2, α_6, α_3, β_4, β_6, $\alpha_v\beta_3$ and β_1) were added to the cell suspension at 10 μg/mL and allowed to incubate at 37 °C for 1 h. The positive control of adhesion consisted of BXPC-3 cells incubated with PBS (No IgG). Also, a control with murine IgG was used (mIgG). Negative control of adhesion was performed with the plate coated with BSA at 40 μg/mL.

In order to provide proof of the specific interaction between integrin $\alpha_3\beta_1$ with r -mojastin 1 and r-viridisitatin 2, a binding assay of BXPC-3 cells to immobilized monoclonal anti-integrins α_3 and β_1 was performed. In this experiment, duplicate wells of a 96-well plate (Falcon® Tissue Culture Plate) were coated with 0.1 mL of monoclonal anti-integrins α_3 and β_1 at a concentration of 40 μg/mL, in 0.01 M Phosphate buffer saline (PBS), pH 7.4, and incubated overnight at 4 °C. Cells were harvested, counted and resuspended in medium containing 1% BSA at 5×10^5 cells/mL. Recombinant disintegrins were added to the cell suspension at 5 μM and allowed to incubate at 37 °C for 1 h. The positive control of adhesion consisted of BXPC-3 cells incubated with PBS (no disintegrin). Negative control of adhesion was performed with the plate coated with murine IgG (mIgG) at 40 μg/mL.

2.6. Cellular migration inhibition assay

BXPC-3 cell migration was measured after scraping cells from the bottom of the well as described by Galán et al., 2008. The negative control consisted of BXPC-3 cells incubated with PBS, which allowed cell migration to occur. Cells were then incubated in a CO_2 chamber and were only removed from the incubator for microscopy images at times 0, 3, 6, 12, and 24 h after recombinant disintegrins incubation. The concentration of r-disintegrins used were 5, 2.5 and 1.25 μM. Percent of closure was calculated by the following equation: % Closure: $[(C-E)/C] \times 100$, where C is the units of distance of cell edge (mm) at zero time for the control, and E is the distance from the cell edge (mm) at the final incubation time for the disintegrin.