Using the optical microscopy and scanning microscopy confocal laser in the evaluation of morphological and structural aspects of human pancreatic tumor cells treated with Jararhagin metalloproteinase.

L.M.C. Gaziola, J.A.L.C. Moreira and D.A. Maria
Laboratory of Biochemistry and Biophysics, Butantan Institute, 1500, Av. Vital Brasil, Butantan, Zip Code 05503-900 São Paulo, Brazil

The human pancreatic adenocarcinoma is an aggressive disease that develops in a relatively symptom-free manner and when diagnosed, usually is in an advanced stage. When studied in three-dimensional culture system of pancreatic tumor the cells migrate through the solid matrix constituted of spheroids growing in multicellular aggregates. Epithelial cells organize themselves into cyst-like structures that contain a spherical monolayer of cells that enclose a central lumen recreating the tumor microenvironment in vivo. The use of optical microscopy and scanning microscopy confocal laser to evaluate and certify the efficiency of Jararhagin metalloproteinase in human pancreatic tumors is extremely important because through it you can see the morphofunctional distortion caused by the compound compared with the control group, making infeasible the entire cellular group that received treatment.

Keywords: optical microscopy, scanning microscopy confocal laser, morphology, pancreatic tumor

1. Introduction

Pancreatic cancer usually develops imperceptibly, without causing symptoms, making early detection virtually impossible. Its evolution is fast making it one of the most lethal types. In most cases it is clinically recognized in very advanced stages. The incidence gradually increases with advancing age. The majority of pancreatic tumors develop in head and neck of the pancreas [1].

The cause specific mortality rates of pancreatic cancer, is quite high. The natural history of pancreatic cancer is evolving with lymph node metastases in most cases, in addition to involving the liver, peritoneum, pleura and the lungs and adrenals. When pancreatic cancer is diagnosed, many patients have already liver metastasis. Adenocarcinomas appear most frequently in head of the pancreas, causing obstruction of common bile duct, causing bile flow back to the liver and then into the bloodstream, thereby causing jaundice. When tumors are advanced pain becomes the most important symptom. It may be of low intensity, however, is typically strong and is located in the back in region of the lower ribs [2].

Approximately 60% of pancreatic cancers arise in head of the gland, 15% in body, tail and 5%; in 20% of cases involve the entire gland neoplasm. Normally are gray masses ill-defined, hard, stellate or whitish. Most are ductal adenocarcinomas that resemble, to some degree, normal ductal epithelium to form glands and secrete mucin. Microscopically, there is no difference between carcinomas of the head, body or tail of the pancreas. The appearance is slightly to moderately differentiated adenocarcinoma, forming abortive tubular structures or clusters of cells and shows a pattern of aggressive and deeply invasive growth.

Genetic analysis of pancreatic cancer revealed frequent loss of heterozygosity in several specific chromosomal regions, including 1p36, 6q21-q24, 9p21, 12q21-q23.1, 17p13, and 18q21. These regions harbor tumor suppressor genes involved in development and progression of pancreatic cancer. Frequently inactivated mutations have been reported: CDKN2A/INK4/p16 on 9p21, 17p13 in TP53/p53 and SMAD4/MADH4/DPC4 in 18q21. Other well-known genes are involved in inherited predisposition to pancreatic cancer, such as BRCA2 (13q12-q13, breast cancer) and LKB1 (19p13, associated with Peutz-Jeghers syndrome). KRAS (chromosome 12p) is the most frequently altered oncogene: 80-90%, is activated by point mutations.

In pancreatic tumors there is a pattern of progression of non-neoplastic epithelium, non-invasive, well-differentiated lesions to invasive carcinoma in the ducts. These precursors are called pancreatic intraepithelial neoplasia (PanIN). The genetic alterations in the cells of PanIN are similar to those occurring in invasive carcinomas. The epithelial cells of PanIN show telomere shortening. A critical shortening of telomere length may predispose these lesions to accumulate progressive chromosomal abnormalities and to develop invasive carcinoma. The histological analysis showed in pancreatic tumors development characteristics from:

- PanIN 1A- tall columnar cells, basally located nucleus, abundant apical mucin.
- PanIN 1B- papillary lesions present architecture, micro papillary or pseudo - stratified, the remainder is equal to 1 nickel.
- PanIN 2- exhibit lesions that may be flat, but tend to be more papillary. These lesions showed nuclear abnormalities, but smaller than those seen in the nickel 3.
• PanIN 3- severe cellular atypical, the lesions resemble carcinoma, but without breaking the basement membrane (Fig. 1).

The cell lines pancreatic tumor BXPC-3 was cultured from a 61-year-old woman’s adenocarcinoma of the body of the pancreas. Tumors grown in nude mice resemble the primary tumor of the patient and produced carcinoembryonic antigen, human pancreas cancer-associated antigen, human pancreas-specific antigen, and traces of mucin. The tumor cells pancreatic human Mia Paca-2 was derived from the pancreas adenocarcinoma of a 65-year-old man who presented with abdominal pain for 6 months and a palpable upper abdominal mass. The tumor did not express measurable amounts of carcinoembryonic antigen and an alkaline phosphatase stain was negative.

The snake venoms have been widely investigated for years in search of the isolation of its active components involved in biological processes such as adhesion, migration, proliferation, angiogenesis, platelet aggregation, among others [3][4]. Proteinases are present in the venoms of many snakes and are structurally classified into trypsin-like serine proteinases (SVSPs) and metalloproteinases (SVMPs). SVMPs cause hemorrhage by disturbing the interactions between endothelial cells and the basement membrane through the degradation of endothelial cell membrane proteins (e.g., integrin, cadherin) and basement membrane components (e.g., fibronectin, laminin, nidogen, type IV collagen)[5].

Structurally, the organization of its domains, the SVMPs are classified into three classes: P1 enzymes containing only the metalloproteinase domain (M), P-II consists of M domain and disintegrin domain, and P-III containing the M domain, disintegrin like (D) and rich in cysteine (C) [6]. The PIII class is further subdivided into subclasses based on different post-translational modifications such as homodimerization (P-IIIc) or proteolysis between M and D (P-IIIb) domain, the presence of an additional domain -c lectin -like (P-IIIld) derived from a post-translational modification of the standard class P-III (P-IIIa) of SVMPs of these are linked to SNACLECs include both C-type lectins and similar to C-type lectin proteins (CLPs) that form homodimers linked by disulfide bonds or homo-oligomers and are distinguished by their ability to bind calcium and carbohydrates such as galactose [7][8] (Fig. 2).

The jararhagin toxin, from Bothrops jararaca snake venom, acts upon several biological processes, as inflammation, pain, platelet aggregation and tumor inhibition growth [9][10][11]. Snake venom zinc metalloproteinase-disintegrin-like jararhagin: causes hemorrhage [12][13][14]. This is the result of the degradation of sub-endothelial matrix proteins leading to the disruption of the blood vessel endothelium, with accompanying disturbances in platelet function [15][16]. It is able to degrade von Willebrand factor (vWF) and it hydrolyzes the alpha-chain of fibrinogen (FGA) while leaving the beta and gamma chains unaffected. It inhibits collagen-induced platelet aggregation through the binding to alpha-2/beta-1 integrin (ITGA2/ITGB1) (collagen receptor), and it cleaves the beta-1 subunit of the same integrin, inhibiting platelet interaction and ultimately causing impairment of signal transduction [17][18][19][20]. It has inability to be affected by the plasma inhibitor alpha(2)-macroglobulin. In fibroblasts, it functions as a collagen-mimetic substrate and, in endothelial cells, it causes apoptosis and indirectly inhibits cell proliferation by release of angiostatin-like compounds. It induces a strong pro-inflammatory response characterized by intense leukocyte accumulation and release of cytokines at the site of the injection [21][22][23]. Although hemorrhage and edema are a response to the direct effect of this toxin, the jararhagin toxin induced inflammation and necrosis are dependent on macrophages and key pro-inflammatory cytokines or their receptors. It also possesses anti-tumorigenic properties [24][25][26][27].
2. Materials and methods

2.1 Culture monolayer cells pancreatic tumor cells Mia Paca-2 and BXPC-3

Were used adenocarcinoma cell lines and pancreatic carcinoma human cells: BXPC-3 from ATCC (CRL-1687) and Mia Paca-2 from ATCC (CRL-1420), belonging to the cell bank of Prof. Dr. Durvanei Augusto Maria from Biochemistry and Biophysics Laboratory of Butantan Institute. After defrosting, the cells were transferred into cell culture bottle (25 cm²) containing DMEM culture medium (Cultilab, Campinas, SP) supplemented with 10% fetal bovine serum (FBS), 200 mM sodium bicarbonate, pH 7.4 5% CO₂ at 37°C. The tumor cell line was incubated at 37°C for 24 hours with different concentrations of Jararhagin diluted in DMEM without fetal calf serum. The cells arranged in monolayer were subjected to enzymatic dissociation solution with 0.2% trypsin + 0.02% EDTA for cell detachment. The enzymatic neutralization was carried out using the same culture medium DMEM containing 10% FBS. After neutralizing the cells in suspension were counted in a Neubauer chamber and the concentration adjusted to 10⁵ cells/mL. Cell viability was determined by exclusion test trypan blue (Fig. 3).
2.2 Culture of pancreatic tumor cells in 3D – Matrigel System

The standardized density for use was 80 cells/µL of medium, were cultured in 3D Matrigel System. The Petri plaques were previously washed with PBS to remove possible fragments of polystyrene. The Matrigel (BD Biosciencies, Belfort, USA) was diluted respectively in serum-free medium culture and medium culture containing 10% FBS and superposed in Petri plaque, the solidification process was complete in 30 minutes at 37ºC. After gel formation, the lineage of pancreatic adenocarcinoma was sown on Matrigel added of DMEM culture medium supplemented with 10% FBS and treated with Jararhagin toxin, from the obtained concentration IC50%. The culture medium utilized for diluting the Jararhagin toxin was used as control (Fig. 4 and 5)

![Scheme 3D cell culture.](image1)

Fig. 4  Scheme 3D cell culture.

![Appearance of pancreatic tumor growth in 3D cell culture over time. Featuring a sturdy peripheral structure the diffusion of the drug.](image2)

Fig. 5  Appearance of pancreatic tumor growth in 3D cell culture over time. Featuring a sturdy peripheral structure the diffusion of the drug.

2.3 Morphological analysis with confocal laser microscopy

Aliquot of cell culture Mia Paca-2 and BXPC-3 in monolayer and 3D matrigel was respectively cultivated in Petri plaques with DMEM culture medium and 10 % FCS maintained in kiln at 5% CO2 in 37°C. The cell samples of groups treated with Jararhagin and controls went through a process to removing the DMEM culture medium. Done this was added 1 ml of 2X PBS for washing and added too 300uL of bovine serum albumin (BSA) for 10 minutes and then a new wash was performed with PBS. In a 0.6mL tube was added 1.2uL of Hoescht fluorophore 33342 (2ug/mL, Invitrogen) and 596uL of PBS 1X, after this process three washes were performed with PBS 1X. Then was added 300uL of formaldehyde at 3.7% by 15 minutes and washed three times with PBS. 03µL of mounting medium was placed in a plaque for fluorescence protection (Fluoroshield, Sigma-Aldrich). This plaque was wrapped in aluminum foil to protect the fluorescent and stored at 4°C until viewing.

2.4 Evaluation of cytotoxicity and proliferative activity in vitro by MTT colorimetric assay

MTT is a colorimetric assay that measures quantitatively the proliferation, cell viability and toxicity. MTT is a yellow tetrazolium salt MTT and redox leads to the formation of insoluble formazan crystal violet color in water. This reaction occurs only in viable cells, by the mitochondrial enzyme succinate dehydrogenase. The tumor cells were incubated in 96 well plates at concentration 2x10^7 cells/ml for 24 hours and treated with Jararhagin toxin at various concentrations,
as control culture medium, vehicle was used to dilute stock solution of Jararhagin toxin. After 24 hours of treatment, the supernatant was collected and added to another plate in 100 mL of MTT (Calbiochem - Darmstadt, Germany) at a concentration of 5 mg/mL, the cells were incubated for 3 hours in incubator containing 5% CO₂ at 37°C. After this period, the contents were removed and added to 100 mL of methanol to dissolve the formazan crystals formed and precipitated. The absorbance measurement was performed in an ELISA reader at wavelength of 540 nm. The concentration that induces toxicity in 50% of the cells (IC50) was determined 24 hours after treatment with different concentrations to evaluate the dose-response effect. The cytotoxicity of each treatment was expressed as the percentage of cell death, calculated relative to the negative control (Fig. 6).

![Fig. 6 Scheme colorimetric MTT.](image)

2.5 Analysis of expression of markers of cell death by flow cytometry
Samples of Mia Paca-2 cells BXPC-3 and stored in 2.5% paraformaldehyde solution were centrifuged, resuspended in buffer Fac's flow and adjusted to a concentration of 10⁶ cells/ml. In samples cytoplasmic markers death cellular caspase-3 (sc-7272), caspase-8 (ab-119892), p53 (ab-26) (Abcam, Cambridge, USA), Bcl-2 (sc-7382), p21 (sc-6246), p27 (sc-1641 AF488), and Bax (sc-7480) (Santa Cruz Biotechnology, California, USA) were evaluated after permeabilization from 0,1% Triton-X 100 (Sigma Company). Then the primary antibody was added and allowed to stand for 1 hour and added the secondary antibody, for 24 hours. The acquisition of marker expression was performed in the flow cytometer Facs Calibur (BD) for fluorescence FL2 channel to the red fluorochrome PE (Phycoerythrin) or to FL1 green fluorochrome FITC (fluorescein isothiocyanate).

2.6 Evaluation of Apoptotic Activity- Annexin V/PI
The tumor cells after 24 hours of treatment with Jararhagin and controls were washed 2 times in PBS at 4°C, resuspended in 100 mL of PBS and incubated for 30 minutes with 1mg of Annexin V- FITC diluted in buffer and 18 mg /ml solution propidium iodide. The reading of the amount of expression of Annexin V (ab14196) (apoptosis) and PI (ab14083) (necrosis) (Abcam, Cambridge, USA) was performed in the flow cytometer - FACScalibur in fluorescence intensity FL-1/FL-2 according to each antibody. The results were analyzed by WinMDI 2.8 software.

3. Results

3.1 Determination of cytotoxicity by MTT method
After 24 hours of adhesion in plate 96 wells, the cells were incubated with different concentrations of Jararhagin diluted in culture medium (DMEM with 10% Fetal Bovine Serum and 1% antibiotic) at concentrations of 1µM at 1.10⁻⁹µM.

The treatments of Mia Paca-2 and BXPC-3 cells were observed morphology formation and lysis of cell debri from the concentration of 1µM (Fig. 7). The other concentrations showed cytotoxic effects, with percentage increase in cell death, loss of cell adhesion, the cytoplasmic membrane fragmentation and loss of progression of cytoplasmic processes.
Fig. 7 Aspect of cellular changes caused after treatment of Mia Paca-2 and-3 with BXPC Jararhagin cells; (A) Control group; (B) Group treated with 1μM concentration, there is significant change in the morphology and cell adhesion; (C) Group treated with 0.13nM concentration, there is a moderate loss of cell adhesion and modification of morphology with consequent cell death; (D) Group treated with a concentration of 0.001nM, there was a slight loss of cell adhesion and modification of cell morphology; (E) Control group; (F) Group treated with 1μM concentration, there is significant change in the morphology and cell adhesion; (G) Group treated with 11.9 nM concentration, there is a moderate loss of cell adhesion and modification of morphology with consequent cell death; (H) Group treated with a concentration of 0.001nM, there was a slight loss of cell adhesion and modification of cell morphology.

Fig. 8 (A) The graph shows the 50% inhibitory concentration, obtained by the straight line equation, 0.13nM. (B) The graph shows the 50% inhibitory concentration, obtained by the straight line equation, 11.9nM. n = 8. * Statistical differences obtained by ANOVA test of variance, followed by multiple Tukey-Kramer.
3.2 Morphological Analysis with Confocal Laser

Confocal laser microscopy was used to visualize morphological changes of Mia Paca-2 and BXPC-3 tumor cells and the formation of spheroids 3D, after 24 hours of treatment with Jararhagin concentrations 0.13 nM and 11.9 nM respectively (Fig. 9).

Fig. 9  Photomicrograph of 3D culture of BXPC-3 and Mia Paca-2 cells acquired Confocal laser microscopy. (A) control group, the cells are glandular conformation, acinar formation; (B and C) treated group jararhagin concentration 11.9 nM, acinar cells lose this conformation, there is increased cell volume, detachment and loss of cell adhesion, as in (D) Group treated with 1nM. (E) control group, the cells have glandular conformation, acinar formation; (F and G) Group treated with jararhagin concentration 0.13 nM, the acinar cells lose this conformation, there is increased cellular volume, detachment and loss of cell adhesion, as in (H) Group treated with 1nM.
3.3 Analysis of markers of cell death by flow cytometry

After treatment with Jararhagin toxin, tumor cells Mia Paca-2 and 3-BXPC markers were analyzed by flow cytometry and percentage of expression of the compared to control and Jararhagin groups. Data were acquired on FACS Calibur flow cytometer and analyzed using WinMDI software version 2.9, and are shown in the bar graph means ± standard deviation. The mean ± SD of percentage of tumor cell expression of markers Mia Paca-2 and BXPC-3 after treatment with jararhagin toxin is shown in figure 10.

Fig. 10  Graph of the percentage of expression of markers of cell death in (A) Mia Paca-2 (B) BXPC-3 cells after treatment with Jararhagin. * Statistical differences obtained by ANOVA test of variance, followed by multiple Tukey-Kramer.

3.4 Evaluation activity apoptotic- annexin V/PI flow cytometric

The purpose of this analysis is to determine quantitatively the percentage of cells undergoing apoptosis by virtue of the ability to bind annexin V and exclude propidium iodide (PI). Annexin V is a phospholipid with vascular anticoagulant activity which is largely found in the cytosolic side of the cell membranes. The use of Annexin V in flow cytometry applications is derived from their selective affinity for negatively charged phospholipids. The Mia Paca-2 and BXPC-3 cells were treated with jararhagin toxin at concentrations of 0.13 and 11.9 nM respectively (Figs. 11 and 12).
4. Discussion

Research in cell culture systems and animal models have reported coming venoms of several species of snakes and effects in various tumor types, in order to find substances with possible therapeutic potential, acting as a single agent or as an adjuvant [29].

The Jararhagin toxin, isolated from the venom of Bothrops jararaca, induces changes in morphology and cell viability of human melanoma (SK-MEL-28), and reduces the number of in vivo metastasis when pretreated and injected in mice, according to data obtained by our research group [30]. Furthermore, our group showed that treatment with jararhagin toxin also promotes morphological changes such as cytoplasmic shrinkage, loss of adhesion and formation of large multicellular aggregates; these changes were accompanied by decreased expression of CDK2 and CDK4 genes.

The in vitro treatment with Jararhagin toxin was effective in their ability to inhibit the growth of tumor cells of human pancreas treated with the toxin. The Jararhagin toxin has shown cytotoxic effects on cells BXPC-3 pancreatic adenocarcinoma and pancreatic carcinoma Mia Paca-2. Cell viability was evaluated after 24 showed a reduction in cell viability/time dependent concentration. At the concentration of 0.13nM Jararhagin toxin showed a significant decrease
in cell viability and marked morphological changes such as loss of cell adhesion in pancreatic carcinoma Mia Paca-2. The same occurred with pancreatic adenocarcinoma cells BXPC-3 at a concentration of 11.9nM Jararhagin toxin.

The MTT is a quantitative indicator of mitochondrial metabolic activity method, with a linear relationship between absorbance and cell activity. The loss of membrane integrity often happens after completing apoptosis in most cellular systems. Thus, treatment with Jararhagin toxin induced decrease in mitochondrial activity at a concentration of 0.13nM Mia Paca-2 cells and concentration of 11.9nM BXPC-3 cells.

Tumor cells Mia Paca-2 and BXPC-3 grown in 3D culture system treated with Jararhagin toxin showed changes as aggregate of cells with disorganized nuclei and destruction lumen formed in the control culture which received no treatment. The spheroids had strong cell junctions and dead cells in the cortical and medullary portions after treatment with Jararhagin. On the origin of the lumen in cell culture process, the selection of resistant cells to anoikis process, with the formation of cell groups and formation of spheroids occurs [30][31]. The growth of spheroids is a result of aggregation, proliferation, polarization, modulation of adhesion molecules and death of cells in the lumen.

In tumor cells Mia Paca-2, a significant expression of p53 and BAX was observed. However, a suppression of the production of Bcl-2 and p27 was observed. The Caspase-3, p21 and Caspase-8 expression markers remained equal to control, with no significant differences. A significant expression of active caspase-3 was observed in tumor cells BXPC-3, suggesting cell death by apoptosis dependent intrinsic pathway of caspase-3 as a significant expression of p27 and BAX. However, a suppression of the production of Bcl-2 was observed. The p53, p21 and Caspase-8 expression markers remained equal to control, with no significant differences.

The difference between the increased apoptosis or necrosis was assessed by marking method with Annexin V/PI flow cytometry using treatment with Jararhagin toxin. At the concentration of 0.13nM in Mia Paca-2 cells in Jararhagin toxin was able to induce significant increase in the percentage of dead cells in early and late apoptosis. While at a concentration of 11.9nM in cells BXPC-3 induced toxin also induced a significant increase in the proportion of early apoptosis, late apoptosis and necrosis killed by a lesser number, these data confirm the data obtained with the expression of Caspase-3 activity. However, Tanjoni (2005) demonstrated the induction of apoptosis with loss of cell adhesion, anoikis in endothelial cells treated with Jararhagin toxin, which indicates special attention to possible side effects on vascular system [32].

There are several antiproliferative mechanisms among which we highlight apoptosis and senescence, as described above. Although senescence, apoptosis similarly, it is also an extremely complex process, some forms of senescence are defining more precisely. Senescence by replication that occurs in primary cells that are cultured in vitro is dependent on telomere length and is usually reversed by expression of telomerase. In recent years, however, it became evident that there is an induced by oncogenes (OIS- oncogene-induced senescence) senescence, which is triggered by the activation of oncogenes such as Ras and Raf and that requires p53, acting via inhibitors of CDKs, and p21CIP p16INK. In these two cases is common to irreversible growth arrest and expression of β-galactosidase acids in cellular compartments which allows the measurement of senescence. In various normal tissues senescent premalignant lesions were found and it is believed that these do not progress to malignancy due to senescence, indicating the importance of senescence as an antitumor process. This information added to the results obtained with respect to expression of p53, p21 and p27 showed the participation of mechanisms that induce senescence and autophagy -dependent concentration of Jararhagin studied in pancreatic cancer cell lines.

The images of confocal fluorescence laser microscopy indicated that at both concentrations were observed decrease in the number of morphologically viable cells, cell detachment mangling tumor parenchyma and formation of agglomerates. While, in control intensive proliferation with formation of ductal structure and luminal was observed.

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References


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