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Cytotoxicity of the coagulant *Moringa oleifera* lectin (cMoL) to B16-F10 melanoma cells

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ABSTRACT

Moringa oleifera seeds are used in alternative medicine to treat inflammation, tumors and bacterial and protozoan infections, for example. The seeds contain lectins, which are carbohydrate-binding proteins with several biological properties including cytotoxicity to cancer cells. In this work, we examined the cytotoxicity of the coagulant *M. oleifera* lectin (cMoL) on B16-F10 murine melanoma cells. cMoL cytotoxic effects were evaluated through trypan blue assay and flow cytometry analysis. Mitochondrial superoxide levels and activation of caspases 3, 8 and 9 were measured. cMoL (1.5–16 μM) reduced viability and caused cell death of B16-F10 cells with an IC_{50} of 9.72 μM. Flow cytometry analysis indicated induction of necrosis and suggested the presence of cells in late apoptosis. Specificity for tumor cells was observed since death of normal human fibroblasts (GN) was not higher than 20% in treatments with cMoL from 1.5 to 16 μM. Microscopy images revealed rounded shape and reduction of volume in B16-F10 cells treated with cMoL. cMoL increased mitochondrial ROS production and promoted caspases 3, 8 and 9 activation in B16-F10 cells, indicating the activation of apoptosis-related pathway. In conclusion, this study demonstrates that cMoL is cytotoxic to B16-F10 cells, which stimulates more investigation on the anticancer potential of this lectin.

1. Introduction

Moringa oleifera Lam. (Moringaceae) is a medium-sized tree autochthonous from northeastern India and widely distributed worldwide throughout the tropics and subtropics (Teixeira et al., 2012). In developing countries, it is a vegetable and medicinal plant, and is source of oil with uses in industry and culinary (Santos et al., 2015). It has been reported the pharmacological potential of *M. oleifera* as source of anxiolytic, antiepileptic, anticancer, antiulcer, anti-obesity and anti-inflammatory agents (Cheenpracha et al., 2010; Araújo et al., 2013; Choudhary et al., 2013; Jung, 2014; Al-Asmari et al., 2015; Ingale and Gandhi, 2016; Metwally et al., 2017).

M. oleifera seeds are broadly used in water treatment for human consumption due to their coagulant properties (Santos et al., 2015). Two lectins deemed cMoL (coagulant *M. oleifera* lectin) and WSMoL (water-soluble *M. oleifera* lectin) are among the coagulant proteins present in the seeds (Santos et al., 2009; Ferreira et al., 2011). Lectins are proteins of non-immune origin that interact reversibly and specifically with sugars; this carbohydrate-binding effect endows diverse biological properties to lectins, including the ability to alter the functioning of cells (Procópio et al., 2016). The cMoL is a heat-stable and basic lectin (theoretical pI of 11.67) with a native molecular mass of 30 kDa, composed by subunits with 101 amino acids, and belonging to the α/β tertiary structure class. It is able to recognize both monosaccharides and oligosaccharide moieties of glycoproteins, with highest affinity for glucose, galactose, asialofetuin and azocasein (Santos et al., 2009; Luz et al., 2013). This lectin demonstrated anticoagulant properties on hemostatic parameters of human blood coagulation (Luz et al., 2013) as well as insecticidal activity against *Anagasta kuehniella* (Oliveira et al., 2011). However, no study on the anticancer properties of cMoL was performed until now.

Mitochondria play an important role in cell death process since they regulate energy production and execution of cell death (Fulda and Kroemer, 2011). Mitochondria are also important in generating reactive oxygen and nitrogen species and are involved in pathways linked to cell proliferation, intracellular death signaling and disease pathogenesis (Figueira et al., 2013). Lectins have been able to interfere with mitochondria function causing cell death. The *Cratylia mollis* seed lectin

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induced death of *Trypanosoma cruzi* epimastigote by promoting mitochondrial Ca^{2+} overload and stimulation of reactive oxygen species (ROS) production followed by necrosis-like cell death (Fernandes et al., 2010). The *Canavalia virosa* seed lectin was toxic to rat C6 glioma cells by disrupting the mitochondrial membrane potential (Osterne et al., 2017). The treatment of HT29 human colon adenocarcinoma cells with a lectin from *Bothrops jararacussu* venom lead to mitochondrial respiration decrease and increase of cytochrome *c* release (Damasio et al., 2014).

In the present work, we evaluated the *in vitro* cytotoxic effects of cMoL to B16-F10 murine melanoma tumor cells and to normal human fibroblasts (GN) as well as mechanisms involved in B16-F10 cell death. In the context of studies searching for positive effects against cancer cells, this cell line was selected since it is very aggressive and presents high capacity for metastatic dissemination (Zang et al., 2015). Some of the experiments were also conducted with normal fibroblasts obtained from gingival biopsy, since they correspond to untransformed primary cells, which brings them very close to a physiological situation. In addition, these cells were chosen due to their different origin being appropriate to test the specificity of the lectin cytotoxicity.

2. Materials and methods

2.1. Plant material

The seeds of *M. oleifera* were collected in Recife City, State of Pernambuco, northeastern Brazil and stored at − 20 °C. A sample of the collected material is archived as voucher specimen (number 73,345) at the herbarium Dárdano de Andrade Lima from the *Instituto Agronômico de Pernambuco* (Recife, Brazil). The authors have authorization from the *Instituto Chico Mendes de Conservação da Biodiversidade* from Brazilian Ministry of the Environment for plant collection (number 38690).

2.2. Lectin preparation

cMoL was obtained in accordance with the protocol described by Luz et al. (2013). Seed flour proteins were extracted with 0.15 M NaCl at 25 °C for 6 h. The extract was treated with ammonium sulphate (0–60% saturation) at 25 °C for 4 h and a precipitated fraction was obtained, dialyzed and applied (10 mg of protein) on a guar gel column (10 cm × 1.0 cm) previously equilibrated (20 mL/h flow rate) with 0.1 M NaCl. After washing step with 0.15 M NaCl, cMoL was eluted with 0.3 M NaCl. UV absorbance was used to monitor protein elution. cMoL concentration was evaluated according to Lowry et al. (1951) using bovine serum albumin (31.25–500 μg/mL) as standard. Carbohydrate-binding ability was monitored by hemagglutinating activity assay performed according to Silva et al. (2016).

2.3. Cell culture

B16-F10 cells were obtained from American Type Culture Collection (Virginia, USA) and cultured in 75-cm^2 plastic bottles (TPP, Trasadingen, Switzerland) in RPMI 1640 (Vitrocell, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL gentamicin, 100 IU/mL penicillin and 100 μg/mL streptomycin (Vitrocell, São Paulo, Brazil). Primary GN fibroblasts were obtained from normal gingival biopsy and kindly provided by Dr. Ricardo Della Coletta from the *Universidade Estadual de Campinas*. GN cells were grown in DMEM with high glucose supplemented with 10% FBS (Vitrocell, São Paulo, Brazil), 2 mM glutamine, 100 μg/mL gentamicin, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.4. cMoL effects on B16-F10 cell viability

The effect of cMoL on cell viability was evaluated as previously described (Rossato et al., 2014). Briefly, B16-F10 cells were plated in 6-well culture plates (3.5×10^4 cells per well) and after 24 h, the medium was replaced and cells were treated with the lectin (1.5–16 μM). The concentrations were calculated considering a native molecular mass of 30 kDa for cMoL (Santos et al., 2009). After further 48 h, cells were washed in PBS, treated with a trypsin-EDTA solution (Vitrocell, São Paulo, Brazil), centrifuged at 1500 *g* for 4 min and resuspended in 300 μL of medium containing FBS. Cell viability was assessed using trypan blue dye (0.1%, w/v) added to aliquots of cell suspensions, and the percentages of stained (unviable) cells were determined microscopically. Cell viability in lectin treatments was expressed as the percentage of the number of viable cells in control. The assay was considered valid only when cell viability in control was above 95%.

2.5. Flow cytometry analysis of cell death

Samples were analyzed in a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with an argon laser and Cell-Quest software (version 4.1). For the cell death analysis, B16-F10 cells (10^6) from control or treated with cMoL (1.5–16 μM; 48 h) were incubated in labeling buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing annexin V (Anx V) conjugated to FITC (1:500) and propidium iodide (PI; 20 μg/mL) at room temperature for 20 min in the dark (Rossato et al., 2014). Ten thousand events were acquired for each sample and apoptosis was quantified as the percentage of Anx V^{pos}/PI^{neg} while necrosis was quantified as the number of Anx V^{neg}/PI^{pos} cells. GN cells treated or not with cMoL (1.5–16 μM; 48 h) were also evaluated for cell death.

2.6. Microscopy

B16-F10 melanoma cells (3.5×10^4 cells/mL) were treated with cMoL at 8 or 16 μM for 48 h. Then, the cells were photographed under a microscope Leica DFC360 FX with an increase of 20 ×, using LAS AF software (Leica Microsystems, Germany).

2.7. Determination of mitochondrial superoxide generation

B16-F10 cells (10^6) were treated with cMoL (8 μM) for 6 h, harvested, and stained with 5 μM MitoSOX (Molecular Probes, USA) for 10 min at 37 °C in order to detect mitochondrial superoxide levels. MitoSOX fluorescence intensity was analyzed by flow cytometry (FACSCalibur, BD, San Jose, USA).

2.8. Detection of caspases 3, 8 and 9 activation

To determine caspases 3, 8 and 9 activation, B16-F10 cells were treated with cMoL at 8 μM and, after 48 h, 10^6 cells were stained with the fluorescent markers FITC-DEVD-FMK, FITC-IETD-FMK or FITC-LEHD-FMK (1:300, Calbiochem, USA) in serum-free medium for 40 min at 37 °C in a humidified atmosphere with 5% CO_2. Then, cells were washed, resuspended in the same medium, and analyzed by flow cytometry according to the manufacturer's instructions. B16-F10 cells treated with 1.25 μg/mL cycloheximide (Sigma-Aldrich, USA) and 10 nM tumor necrosis factor α (TNFα; Peprotech, USA) for 24 h were used as a positive controls.

2.9. Statistical analysis

Data were expressed as the mean ± the standard error of the mean (S.E.M.) of at least five independent experiments. Differences between means values were analyzed using one-way ANOVA followed by Tukey's multi-comparison test. A p-value < 0.05 was considered significant. The concentration of cMoL that causes 50% death of melanoma cells (IC_{50}) was calculated by probit analysis using the software

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