Effects of *Lonomia obliqua* caterpillar venom upon the proliferation and viability of cell lines

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Abstract Many active principles produced by animals, plants and microorganisms have been employed in the development of new drugs for the treatment of human diseases. Among animals known to produce pharmacologically active molecules that interfere in human cell physiology, the caterpillar *Lonomia obliqua* has become the focus of toxicological studies due to recent findings about its venom constituents. The objective of this study was to investigate the effects of *L. obliqua* venom upon the viability and the proliferation of different cell lineages and to propose mechanisms for the herein observed induction of cell proliferation in glioma cell lines. MTT analyses indicate that *L. obliqua* venom increases the viability of tumor cell lines U138-MG and HT-29; on the other hand, it inhibits the viability of V-79 nontumor cells.

Cell count based on the *trypan blue* exclusion method suggests a proliferating activity of the venom upon U138-MG cells. Exposure of U138-MG to crude venom extract led to a decrease in the production of nitric oxide, and activation of the cAMP signaling pathway inhibited the effects of the venom, indicating that these mechanisms may influence cell proliferation triggered by the venom. Despite the proliferative effects of crude venom on U138-MG and HT-29 cell cultures, a protein purified from *L. obliqua* hemolymph previously shown to have cytoprotective activity had no effect on U138-MG and HT-29; however, this same protein increased the viability of V-79 cells that had previously been exposed to the cytotoxic activity of the crude venom extract. This study indicates that the venom and the antiapoptotic protein act differently and have different effects on

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cell cultures, depending on the cell line analyzed. Biomolecules displaying either mitogenic or cytotoxic activities are of great biotechnological interest. Further studies encompassing the purification of active principles from *L. obliqua* venom are necessary to further elucidate its effects on different cell types.

Keywords *Lonomia obliqua* · Caterpillar · Venom · Cell proliferation · Cell viability · Glioma

Introduction

Caterpillars of the species *Lonomia obliqua* (Lepidoptera, Saturniidae) (Fig. 1) are responsible for a severe hemorrhagic syndrome in humans who come into contact with their urticating spines; the envenomation can be fatal due to kidney complications and brain hemorrhage (Duarte et al. 1996; Kelen et al. 1995). The number of accidents increased in the past 15 years, driving scientists to investigate the molecular mechanisms that lead to such a bleeding disorder, as well as searching for new biomolecules of pharmacological and biotechnological interest.

Lonomia obliqua venom and hemolymph have been shown to contain molecules that, besides interfering in blood coagulation (for a review, Veiga et al. 2009), can also disturb cell physiology, due to the activities of well-described molecules such as fibrinogenases, phospholipase A2, hyaluronidases, factor X activator, prothrombin activator and an antiapoptotic protein, whose activities have been studied and

described in the literature (Chudzinski-Tavassi et al. 2005; Fritzen et al. 2005; Pinto et al. 2006; Seibert et al. 2006; Souza et al. 2005; Veiga et al. 2003). Among the substances isolated from *L. obliqua* venom and hemolymph, a few have been used in cell culture: a phospholipase A2 (PLA2) with hemolytic activity on human erythrocytes (Seibert et al. 2006), a factor X activator (Losac) (Alvarez Flores et al. 2006) and a prothrombin activator (Lopap) (Fritzen et al. 2005) that increased the viability of human endothelial cells line (HUVEC), and a purified protein from the hemolymph that showed cytoprotective activity on *Spodoptera frugiperda* cells (Sf-9) (Souza et al. 2005).

Most studies about the effects of *L. obliqua* venom on cell lines employed fractions isolated from the bristle extract or from the hemolymph. In one study, by Pinto et al. (2008), crude bristle extract was applied in cultures of human fibroblast of the cell line HS68, for 2 hours at a venom concentration of 50 µg/ml, to assess cell gene expression by cDNA microarray.

Until now, no study has shown the possible activities of *L. obliqua* crude venom extract upon the viability and proliferation of different cell lines over long periods of treatment capable of generating cellular responses. From what has been demonstrated, Losac and Lopap, which were extracted from the caterpillar's bristle extract, as well as an antiapoptotic protein extracted from the hemolymph, displayed proliferative activity and increased the viability of treated cells. Nevertheless, one cannot disregard that the joint action of some active principles of the venom, such as the hemolytic activity of PLA2 and the hyaluronidase activity, may have inhibitory effect on proliferation and cell viability. In addition, *L. obliqua* crude bristle extract contains several other toxins, many of which have not been isolated, identified or characterized yet and which might as well display anti-proliferative and pro-apoptotic activities and present biotechnological potential.

Therefore, further studies involving cell culture to investigate the effects of *L. obliqua* venom are needed to better characterize the activities of the venom and its pharmacological and biotechnological potential. This work aimed to study the effects of *L. obliqua* venom upon the proliferation and viability of different cell lines and propose mechanisms for the observed induction of cell proliferation in a human glioma cell line.

Fig. 1 *Lonomia obliqua* (Lepidoptera, Saturniidae); 6th instar larvae

Materials and methods

Lonomia obliqua venom

Lonomia obliqua caterpillars were kindly provided to the Laboratory of Molecular Biology at UFCSPA by the Fire Department of Erechim (RS) and from the Municipal Sanitary Surveillance Secretary of Videira (SC). Venom samples were prepared from bristles as described in Veiga et al. (2005). Briefly, the caterpillars were placed in Petri dishes on ice. Each *scolus* was cut at the base to obtain the spicules and hemolymph, which were collected and macerated in milli-Q water (1 ml of water for spicules collected from 5 caterpillars), using a glass potter homogenizer (Potter–Elvehjem Tissue Homogenizer-Style). The material was centrifuged at 9,600g for 10 min and the supernatant containing the venom was transferred to a new tube. Protein concentration was determined based on the BCA Assay (Pierce Biotechnology, Rockford, IL, USA), in a SpectraMax (Molecular Devices) microplate reader, according to the manufacturer's instructions; bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) was used for the standard curve. Finally, venom was prepared in 1 ml aliquots at a final protein concentration of 1 mg/ml, and stored at -80°C .

The antiapoptotic protein from the hemolymph of *L. obliqua* that was used in this study was purified and kindly provided by Dr. Ronaldo Zucatelli Mendonça, from the Butantan Institute, University of São Paulo, Brazil (Souza et al. 2005). Studies using this protein showed that it displays a cytoprotective activity upon cell culture (Mendonça et al. 2008; Souza et al. 2005). The lyophilized protein was diluted in milli-Q water to achieve a final concentration of 1 mg/ml and stored at -80°C .

Cell culture

Human glioma cells (U138-MG) obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used. Cells were grown and maintained in 50 ml flasks, each containing 5 ml of culture medium Dulbecco Modified Eagle's Medium (DMEM-High glucose, Gibco, Grand Island, NY, USA) with addition of 15 % (v/v) fetal bovine serum (FBS), 2.5 mg/ml fungizone and 100 U/l gentamicin (Morrone et al. 2003). Exponentially growing cells were detached with trypsin 0.25 % EDTA, transferred

to culture dishes and treated accordingly as follows: in one condition cells were grown in medium with 15 % FBS (v/v), while in another condition cells were grown in decreasing concentrations of FBS to induce quiescence, as described previously (Jacques-Silva et al. 2004; Morrone et al. 2003).

To check if the same effects obtained in the U138-MG cell lineage would be observed in other cells, two other cell types were also used, both obtained from ATCC: Chinese hamster fibroblasts (V-79) and human colorectal cancer cell line (HT-29). V-79 cells were cultured in 50 ml vials, each containing 5 ml of culture medium DMEM (DMEM-High glucose, Gibco) with addition of 5 % (v/v) fetal calf serum, 2.5 mg/ml fungizone and 100 U/l gentamicin. HT-29 were grown and kept in 50 ml sterile flasks with 5 ml of RPMI 1640 culture medium (Invitrogen, Sao Paulo, SP, Brazil) with the addition of 10 % (v/v) FBS, 2.5 mg/ml fungizone and 100 U/l gentamycin. Cells were kept in an incubator at 37°C , with 95 % of minimum relative humidity and a 5 % CO_2 atmosphere.

Exposure of cells to *L. obliqua* venom

Lonomia obliqua crude bristle extract was added to the culture medium of cells cultured in the presence or absence of FBS (cells in proliferation or quiescence, respectively). The following concentrations of venom were tested: 5, 50, 150, 300 and 450 $\mu\text{g/ml}$, for different times of treatment: 24 and 48 h.

Exposure of cells to *L. obliqua* antiapoptotic protein

The antiapoptotic protein was purified according to Souza et al. (2005) and the lyophilized protein was then diluted in milli-Q water in aliquots at a concentration of 1 mg/ml and stored at -80°C . U138-MG, HT-29 and V-79 cells were treated with doses of 0.625, 1.25, 2.5, 5, 10 and 20 $\mu\text{g/ml}$ of the isolated antiapoptotic protein alone for 48 h for analysis of cell viability by MTT.

Cell counting

After venom treatment of the U138-MG cell line, medium was removed, cells were washed in PBS and detached with trypsin 0.25 % EDTA; cell counting was performed under an optical microscope using a

hemocytometer, as described in previous studies (Jacques-Silva et al. 2004; Morrone et al. 2003).

Cell viability

Cell viability was analyzed using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Sap Paulo, SP, Brazil), which measures mitochondrial activity. U138-MG and V-79 cell lines were grown in 96-well plates at a concentration of 4×10^3 cells per well in culture medium DMEM supplemented with 15 % FBS and 5 % FBS, respectively, for 24 h. After this period, the medium was discarded and 200 μ l DMEM supplemented with 15 % FBS or 5 % FBS were added in positive controls in U138-MG or V-79 cells, respectively. The doses of venom were diluted in their respective concentrations in culture medium also supplemented with FBS. HT-29 cells were cultured for 24 h in RPMI supplemented with 10 % FBS at a concentration of 7×10^3 cells per well. The control was treated with 10 % FBS RPMI and the different doses were diluted in the same medium for the treatments.

In quiescence treatments, for which the U138-MG strain was used, the medium added to negative control quiescent cells contained only 0.5 % FBS and venom doses were also diluted in 0.5 % FBS medium. In the positive control, quiescent cells were treated with 15 % FBS medium. After 24 or 48 h of treatment, the culture medium was removed from the 96-well plates and cells were washed with HBSS (100 μ l/well). Then, 100 μ l of a 5 mg/ml MTT solution was added to each well. The plate was incubated for 4 h at 37 °C. Then, the medium was carefully removed from the wells, and the plate was set at room temperature until becoming completely dry. Dimethyl sulfoxide (DMSO) was added and the absorbance at 492 nm was measured in a multiplate reader.

Griess reaction

U138-MG cells were plated at a density of 4×10^3 cells/well in a 96-well plate. On the next day, quiescence was induced in the negative control and in cells that had been treated with venom at doses of 50, 150, 300 and 450 μ g/ml diluted in 0.5 % FBS medium. The positive control was always treated with 15 % FBS medium. To measure the amount of nitric oxide (NO) after 24 h of treatment, 50 μ l of the

supernatant were collected and incubated with 50 μ l of Griess solution (1 % sulfanilamide solution and ethyl α -naphthyl diamine solution 0.1 %) for 10 min. The absorbance was monitored at 550 nm.

MTT analysis of cells treated with *L. obliqua* venom in combination with agents that enhance cAMP signaling

In order to investigate whether the observed effects of the venom on cell viability and proliferation are due to a direct activity or an indirect activity that might involve cell signaling through the cAMP pathway, experiments of cell viability and proliferation using the venom in combination with agents such as forskolin, which increases cAMP levels in the cell, and 8-Br-cAMP, a c-AMP analogue, were performed. U138-MG cells were plated in 96-well plates at a density of 4×10^3 cells/well. After 24 h, the following treatment conditions were carried out in separate: control, with 15 % FBS culture medium; treatment only with forskolin (10 mM) or 8-Br-cAMP (10 mM); treatment with forskolin (10 mM) or 8-Br-cAMP (10 mM) for 30 min followed by addition of venom at the dose of 450 μ g/ml; treatment with 450 μ g/ml venom alone, diluted in culture medium. After 48 h of treatment, the MTT technique was performed as previously described.

Statistical analysis

All experiments were performed at least three times. Results were expressed as mean $\pm$ standard deviation of the mean (SEM). Comparisons among different experimental groups were performed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test when ANOVA revealed significant differences between groups. P values less or equal to 0.05 indicated significant differences.

Results

Previous studies have shown the diversity of active principles present in *L. obliqua* venom (Veiga et al. 2005). Considering that many arthropod venoms may act upon the proliferation and viability of cancer cells (Heinen and Veiga 2012), this study analyzed the effects of *L. obliqua* venom upon different tumor cell

lines. *L. obliqua* crude bristle extract was prepared as previously described (Veiga et al. 2003) and used in different treatment conditions.

An antiapoptotic protein purified from the venom was also used in some analyses.

Viability of human glioma cell line treated with *L. obliqua* crude venom extract

Cell viability was measured by MTT. MTT is a salt that measures the activity of mitochondrial enzymes; once reduced by these enzymes, MTT forms formazan crystals, which can be quantified in a colorimetric assay. This reaction occurs only when the mitochondrial reductase enzymes are active in living cells; thus, the conversion of MTT to formazan crystals is used to determine viable cells.

For the evaluation of results, the treatments were calculated in relation to the positive control, which was considered 100 %. The plates of each cell type and different treatment times were prepared at least three times and treatment results represent the mean values compared to control.

Human glioma cells (U138-MG) were treated with different doses of crude venom extract (10, 50, 150, 300 and 450 µg/ml) for 24 and 48 h (Fig. 2a). The control was treated with DMEM with 15 % FBS and the different doses were diluted in the same medium for the treatment of the cells. After 24 h of treatment, a significant increase in cell viability with the dose of 300 µg/ml was observed when compared to control ($P = 0.033$). There was also a significant increase in cell viability after 48 h with the dose of 450 µg/ml compared to control ($P = 0.021$).

To verify if the venom induced an increase in the number of viable cells even in the absence of growth factors and other components present in serum, cells were first grown in quiescent conditions and then treated with 0.5 % FBS, 15 % FBS or venom diluted in 0.5 % FBS (Fig. 2b). After 48 h of treatment, we found a significant increase in cell viability with venom at 450 µg/ml compared to control (C) in which cells had been treated with 15 % FBS medium ($P = 0.05$). There was also a significant increase in cell viability with doses of 300 and 450 µg/ml compared to the negative control, which was supplemented with 0.5 % FBS medium ($P = 0.013$ and $P = 0.001$, respectively). A significant increase in cell viability was also observed in cells treated with

450 µg/ml venom compared to the positive control ($P = 0.04$), which was first supplemented with 0.5 % FBS to induce quiescence and then supplemented with 15 % FBS medium at the time of treatment.

Viability of human colorectal cancer cells and Chinese hamster fibroblasts treated with *L. obliqua* crude venom extract

In order to verify if the increase in cell viability induced by *L. obliqua* venom observed in U138-MG cells would be similar in other cell types, colorectal cancer cells (HT-29) and Chinese hamster fibroblasts (V-79) were also treated with venom at the doses that had the greatest effects on glioma cells: 150, 300 and 450 µg/ml. After 48 h of treatment (Fig. 3), there was a significant increase in cell viability with the doses of 150 and 300 µg/ml compared to control in HT-29 ($P = 0.028$). However, there was a significant decrease in cell viability of V-79 cells at doses of 300 and 450 µg/ml compared to control ($P = 0.048$ and 0.002, respectively).

Cell counting

Considering the results obtained in cell viability analysis, we performed experiments of cell counting to verify if the increase in the number of U138-MG viable cells was due to an increase in cell proliferation. Using venom doses of 150, 300 and 450 µg/ml we found that, after 48 h of treatment, there was a significant increase in cell number with treatments of 150 and 450 µg/ml compared to control ($P = 0.039$ and 0.014, respectively) (Fig. 4a). Although there was no significant result in cell counts within 24 h of treatment, there is a trend towards an increase in the number of treated cells. The increase in cell number could be clearly observed under light microscopy (Fig. 4b).

Cell viability after treatment with *L. obliqua* antiapoptotic protein

In view of the results showing an increase in cell viability of U138-MG and HT-29 cells, we tested different doses of a protein that has been shown to display antiapoptotic activity on Sf-9 cells (Souza et al. 2005). The antiapoptotic protein alone, at doses of 1.25, 2.5, 5, 10 and 20 µg/ml, was added to cultures

Fig. 2 **a** Effect of *L. obliqua* venom on the viability of growing U138-MG cells. After 24 and 48 h of treatment, cells were analyzed by the MTT method. There was a significant increase in cell viability at venom doses of 300 µg/ml at 24 h and 450 µg/ml at 48 h compared to control (*, $P = 0.033$ and *, $P = 0.021$, respectively); $n = 3$. **b** Effect of *L. obliqua* venom on the viability of U138-MG quiescent cells. After 48 h of treatment, cells were analyzed by the MTT method. * indicates a significant increase in cell viability at a venom dose of 450 µg/ml

compared to the control treated with 15 % FBS containing medium (C) ($P = 0.05$); ^ indicates a significant increase in cell viability with doses of 300 and 450 µg/ml compared to the control supplemented with 0.5 % FBS containing medium (C-) ($P = 0.013$ and $P = 0.001$, respectively); # indicates a significant increase in cell viability at a dose of 450 µg/ml compared to the control supplemented first with 0.5 % FBS containing medium and then with 15 % FBS containing medium at the time of treatment (C+) ($P = 0.04$); $n = 3$

Fig. 3 Effect of *L. obliqua* venom on the viability of HT-29 and V-79 growing cells. After 48 h of treatment, cells were analyzed by the MTT method. There was a significant increase in cell viability with venom doses of 150 and 300 µg/ml compared to control (*: $P = 0.028$) in HT-29 cells. There was a significant decrease in cell viability with doses of 300 and 450 µg/ml compared to control ($P = 0.048$ and $P = 0.002$, respectively) in V-79 cells; $n = 3$

of U138-MG cells in quiescence and of HT-29 cells in proliferation; after 48 h of treatment, no effect on cell viability was observed (Fig. 5a, b). Similar treatments with U138-MG cells in proliferation and HT-29 cells in quiescence also showed no significant differences (data not shown). However, when V-79 cells were treated with the same doses and the dose of 0.625 µg/ml, we observed a significant increase in cell viability with the doses of 0.625 µg/ml ($P = 0.036$), 1.25 µg/ml ($P = 0.003$), 2.5 µg/ml ($P = 0.002$), 5 µg/ml ($P = 0.004$), and 10 µg/ml ($P = 0.006$) when compared to control (Fig. 5c).

Nitric oxide production

Previous studies showed that Lopap, a protein purified from the venom of *L. obliqua* caterpillar, induces an

Fig. 4 **a** Effect of *L. obliqua* venom on U138-MG cell numbers. After 24 and 48 h of treatment, cells were counted in a Neubauer chamber. Statistical analysis revealed no significant difference in cell count after 24 h of exposure to the venom, despite showing a tendency to cell proliferation. After 48 h of treatment, there was a significant increase in cell number with venom doses of 150 and 450 µg/ml compared to control ($P = 0.039$ and $P = 0.014$, respectively); $n = 3$.

b Optical microscope photograph of U138-MG cells after 24 h of treatment with *L. obliqua* venom. **a** Negative control treated with DMEM containing 0.5 % FBS. **b** Positive control treated with DMEM containing 15 % FBS. **c** Treatment with venom at 150 µg/ml diluted in DMEM containing 0.5 % FBS. **d** Treatment with venom at 300 µg/ml diluted in DMEM containing 0.5 % FBS. **e** Treatment with venom at 450 µg/ml diluted in DMEM containing 0.5 % FBS

increase in cell viability in human endothelial cells (HUVECs) and rLopap increases viability in primary cultures of neutrophils and endothelial cells of rats by increasing nitric oxide (NO) production (Fritzen et al. 2005; Waismam et al. 2009). It is known that NO has an important role in the progression of gliomas (Himlin et al. 2006). To verify the production of NO, quiescent U138-MG cells were treated with doses of 50, 150, 300 and 450 µg/ml of venom (Fig. 6). The negative control was treated with 0.5 % FBS medium and the positive control with 15 % FBS medium. After 24 h of treatment, the culture medium was collected and incubated with Griess reagent for subsequent reading at 550 nm on a plate reader. The results showed a decrease on NO production in the positive control ($P < 0.001$), as well as in treatments with

doses of 150 µg/ml ($P = 0.003$), 300 µg/ml ($P = 0.002$) and 450 µg/ml ($P < 0.001$), when compared to the negative control (Fig. 6). The dose of 50 µg/ml, which had no significant effect on cell viability, also showed no effect in NO production compared to negative control.

MTT analysis of cell viability treated with the venom in combination with agents that enhance cAMP signaling

To determine whether the cAMP cell-signaling pathway was involved in cell proliferation induced by the venom, we conducted a joint approach using venom at the dose of 450 µg/ml in combination with forskolin (an adenylyl cyclase activator that activates cAMP)

Fig. 5 **a** Effect of the antiapoptotic protein on the viability of quiescent U138-MG cells. After 48 h of treatment, cells were analyzed by the MTT method. The doses as well as the control (C) were diluted in DMEM containing 0.5 % FBS. There was no difference between treatments and control; $n = 6$. **b** Effect of the antiapoptotic protein on the viability of HT-29 growing cells. After 48 h of treatment, cells were analyzed by the MTT method. The doses were diluted in RPMI containing 10 % FBS and the control

was treated with RPMI containing 10 % FBS. There was no significant difference in cell viability between treatments and control; $n = 3$. **c** Effect of the antiapoptotic protein on the viability of growing V-79 cells. After 48 h of treatment, cells were analyzed by the MTT method. There was a significant increase in cell viability with venom at the doses of 0.625 μg/ml ($P = 0.036$), 1.25 μg/ml ($P = 0.003$), 2.5 μg/ml ($P = 0.002$), 5 μg/ml ($P = 0.004$) and 10 μg/ml ($P = 0.006$) compared to control; $n = 3$

Fig. 6 Effect of *L. obliqua* venom on the production of NO in quiescent U138-MG cells. After 24 h of treatment, the cell medium was analyzed by the Griess method. Results show a decrease in production of NO in the positive control ($P < 0.000$), as well as with venom at doses of 150 μg/ml ($P = 0.003$), 300 μg/ml ($P = 0.002$) and 450 μg/ml ($P < 0.000$) compared to negative control; $n = 7$

(Fig. 7). The treatment with forskolin alone, and with forskolin combined with venom at a dose of 450 μg/ml, showed no differences compared to the control treated

with culture medium alone. However, venom alone at the dose of 450 μg/ml showed a significant increase in cell viability compared to control ($P = 0.001$), forskolin alone ($P = 0.011$) and forskolin plus venom ($P = 0.018$). To confirm these results, a similar experiment was performed using 8-Br-cAMP (a cAMP analogue). The results were similar, showing a significant increase in cell viability with the dose of 450 μg/ml compared to control ($P < 0.001$), to 8-Br-cAMP ($P < 0.001$) and to 8-Br-cAMP plus venom ($P = 0.042$).

Discussion

Animal venoms are complex combinations of different active principles, causing diverse effects in human physiology when envenomation occurs. Therefore, they are a rich source of pharmacologically active molecules that may be used in the pharmaceutical or biotechnological industries.

Fig. 7 Effect of *L. obliqua* venom on U138-MG cells in the presence of agents that increase cAMP. After 48 h of treatment, cells were analyzed by the MTT method. **a** There was a significant increase in cell viability with venom at the dose of 450 µg/ml compared to control (*, $P = 0.001$), forskolin alone (#, $P = 0.011$), as well as compared to the adjuvant treatment

with venom at 450 µg/ml + forskolin (^, $P = 0.018$). **b** There was a significant increase in cell viability with venom at a dose of 450 µg/ml compared to control (*, $P < 0.000$), 8-Br-cAMP alone (#, $P < 0.000$) as well as compared to the adjuvant treatment with venom at 450 µg/ml + 8-Br-cAMP (^, $P = 0.042$); $n = 4$

Caterpillars of the species *L. obliqua* are well known for producing venomous compounds that interfere in blood coagulation and fibrinolysis (Veiga et al. 2009). These caterpillars do not have a venom gland, and the toxic active principles are produced and stored in many parts of the animal. One of the main sources of these molecules is the hemolymph that circulates inside the animal, bathing the tegumental structures and the cells underneath them, including the caterpillar's spicules (Veiga et al. 2001). Even though some active principles seem to be concentrated in the spicules, there is no morphological barrier separating these structures from the hemolymph, therefore different venomous preparations may have similar activities (Pinto et al. 2006; Veiga et al. 2001). On the other hand, active principles that circulate in the hemolymph and therefore are present in the spicules may be inhibited by molecules produced in only a specific cell type or tissue, leading to the wrong assumption that such an activity is not present in that specific structure. Thus, it is important to consider as "venom" all sample preparations from *L. obliqua* caterpillars. While some researchers use only crude bristle extract as the main source of venom (Chudzinski-Tavassi et al. 2005; Fritzen et al. 2005), others use the hemolymph (p.e. Carmo et al. 2012; Mendonça et al. 2008; Souza et al. 2005).

All venom preparations have been shown to contain diverse active principles, including molecules with activities upon blood coagulation, fibrinolysis and hemolysis (review in Veiga et al. 2009). Such biomolecules present a high potential in the development of new therapeutic drugs; further, the venom is a

rich source of molecules of biotechnological interest, such as antiapoptotic and antibacterial activities (Carmo et al. 2012; Mendonça et al. 2008; Souza et al. 2005). In this study, *L. obliqua* crude venom extract was shown to increase the viability and proliferation of U138-MG and HT-29 cell lines, but decreased dramatically the viability of the V-79 cell line. These results suggest that the bristle extract contains both pro- and anti-proliferative activities that act differently depending on the cell type. Also, these molecules might display their pharmacological activities at different incubation times, so that the observed effects depend not only on venom concentration, but also treatment times.

Cells treated with venom at doses that significantly increased the proliferation of U138-MG showed a decrease in nitric oxide production compared to the negative control. These data suggest that decreasing the concentration of nitric oxide by treating cells with the venom leads to an increase in cell proliferation in gliomas. Interestingly, previous studies have shown that a protein from *L. obliqua* venom known as Lopap induces an increase in cell viability in human endothelial cell line (HUVECs) and rLopap increases viability in primary cultures of neutrophils and endothelial cells of rats through an increase in the production of NO (Fritzen et al. 2005; Waismam et al. 2009). It is known that, in gliomas, nitric oxide displays dual pro- and antitumor action, depending on the site and concentration of the molecule. Higher concentrations of NO tend to be cytostatic and cytotoxic to tumor cells, while lower concentrations tend to increase tumor growth (Jadeski et al. 2002),

which may explain the results shown in the present study, where the concentrations of NO in cells treated with *L. obliqua* venom at the doses of 150, 300 and 450 µg/ml were similar to the positive control, with cell proliferation (Fig. 6). In fact, this did not occur in the negative control cells that showed a higher concentration of NO along with an arrest in cell progression induced by quiescence. Several other studies in the literature report that a higher concentration of NO in glioma is related to cytotoxic and cytostatic activities (Kurimoto et al. 1999; Viani et al. 2003; Weyerbrock et al. 2009).

The decrease in NO production induced by the venom does not completely explain the proliferative effect of the venom, because, as shown in Fig. 6, the amount of NO observed in the positive control was not different from those found with doses of 150, 300 and 450 µg/ml, suggesting that the venom also acts through other pathways to induce cell proliferation.

To verify if the venom interacts in some cell signaling pathways, we combined venom treatments with agents that increase cAMP. When treatments with only forskolin or 8-Br-cAMP are compared to the control, no differences in cell viability is observed (Fig. 7), as expected based on the literature (Farias et al. 2008). *L. obliqua* venom at a dose of 450 µg/ml diluted in culture medium increased cell viability compared to control; however, when this same dose is used with forskolin or 8-Br-cAMP, differences are not observed, suggesting that the venom interacts with the cAMP pathway, decreasing cell viability. Other studies have shown that forskolin and 8-Br53-cAMP can interact with other substances, influencing the viability of U138-MG cells (Farias et al. 2008). Considering that the crude extract is composed of numerous active compounds, other signaling pathways may be activated or be able to interact with the venom. More studies encompassing new cell pathways, like PI3 K or MAPK pathways, are needed to better explain such an action of the venom.

Quite interestingly, we found that the antiapoptotic protein isolated from *L. obliqua* hemolymph by Souza et al. (2005) had a strong cytoprotective effect on V-79 cell line, which was not observed when U138-MG and HT-29 cell lines were exposed to this same protein, highlighting the fact about the different interactions of the caterpillar's secretions with the cells, which may vary in dependence of the cell lineage. On the other hand, crude venom extract displayed a strong

cytotoxic effect on the V-79 lineage, inhibiting the viability of these cells.

In a previous study, it was shown that low concentrations of *L. obliqua* hemolymph increase the viability of Sf-9 insect cells in culture; however, high concentrations display a deleterious effect (Maranga et al. 2003). These results suggest that the venom components act at different optimal concentrations, therefore the combined effect may vary depending on sample preparation, concentration used and also cell type. Accordingly, our results show that the venom increases the viability of tumor cells and decreases the viability of fibroblasts, which may be due to molecular differences between cell types.

It is known that cancer cells, as glioma and colorectal cancer cells, have many deactivated mechanisms that lead to cell death, like a mutated p53 apoptosis pathway. The first step to verify this difference would be to identify the cellular mechanism activated in fibroblasts that lead these cells to death and try to determine if this same mechanism is activated or not in tumor cells treated with the venom. Probably the mechanism leading to cell death in fibroblasts by exposure to the venom is mutated in cancer cells studied in this work.

Another explanation for these results is that cancer cells often react strongly to external stimuli by activation of the immune system, leading to an increased expression of NF-κB, which will activate different cell proliferation pathways. This mechanism also occurs in normal cells, in which proliferation is well regulated, which does not occur in tumor cells. Tumor cells take advantage of the activation of the immune system to continue proliferating. These mechanisms of increasing or decreasing cellular proliferation are extremely important for the understanding of the venom effects in different cell types and should be a research subject for future studies. Furthermore, new tumor cell lines with different mutations in cell proliferation pathways should be tested as well as the use of primary cultures of normal cells in order to verify the occurrence of the same effects observed in cells previously studied. In view of the effects that *L. obliqua* venom has on mammal angiogenesis (Kelen et al. 1995; Seibert et al. 2003), it would be interesting to verify the effects of the venom upon malignant blood cells, and the influence of angiogenesis in tumor cells treated with the venom.

A molecule with a potent activity in increasing cell proliferation and viability is of great interest in the

biotechnology industry. Culture of mammalian cells represent a major resource for producing high-value bi-therapeutic drugs (Wurm 2004), which includes monoclonal antibodies (Rycyzyn et al. 2008), recombinant proteins (Carmo et al. 2012; Mendonça et al. 2008), viral vaccine production (Chung et al. 2010), gene therapy (Apel et al. 2009), expansion of stem cells (D'Andrea et al. 2008), epithelial cell transplantation to treat burned patients (Wood et al. 2006), among other applications. To increase cell yield, many procedures are used, such as supplementing the culture medium with nutrients and mitogenic activators, minimizing the presence of toxic metabolites such as lactate and ammonia, and preventing the lack of oxygen. The minimization of apoptosis has considerable interest for biotechnological applications. The fact that the venom significantly increases the amount of viable cells, even when diluted in culture medium supplemented with 0.5 % serum compared to control treated with 15 % serum medium, further strengthens the idea that there is some component in the venom that induces the proliferation of the U138-MG cell line more effectively than the actual growth factors present in serum.

The present results suggest that the bristle extract contains both pro- and antiproliferative agents that act differently depending on the characteristics of each cell, such as cellular receptors and signaling pathways. Both results of this study, either an increase or a decrease in cell proliferation, are important. The venom extracted from *L. obliqua* has a great potential for the discovery of new active compounds. An active component which showed the ability to strongly and selectively inhibit the viability of V-79 cells suggests that the same isolated molecule could have a greater effect on these cells and perhaps in other lineages, including even some tumor cells, which would have an application against cancer. Not least, a molecule present in the venom that can be isolated to act as an adjuvant to increase the viability and proliferation of certain cell cultures, developing strategies for improving production of viral systems, protein expression, antibody production, among others, have a strong impact on the biotechnology industry.

Conclusion

The present study shows that *L. obliqua* venom increases the viability of U138-MG human

glioblastoma cells and human colorectal cancer HT-29 cells. However, the venom decreases the viability of V-79 Chinese hamster fibroblasts. These results show that the effects of the venom depend on the cell type tested. The antiapoptotic protein alone had no effect on U138-MG and HT-29, but increased the viability of V-79. Activators of cAMP inhibit the effects of crude venom on U138-MG and the enhancement in cell proliferation could be attributed to a decrease in NO production by glioma cells treated with the venom. But, despite the decrease in NO production, the venom acts inducing proliferation of cells by other cellular mechanisms. Further studies encompassing the purification of active components of venom are needed to further elucidate the effects presented in the cell lines used in this work.

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

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