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Human bronchial epithelial cells injury and cytokine production induced by *Tityus serrulatus* scorpion venom: An *in vitro* study

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ABSTRACT

Tityus serrulatus is the scorpion specie responsible for the majority of scorpion sting accidents in Brazil. Symptoms of envenomation by *Tityus serrulatus* range from local pain to severe systemic reactions such as cardiac dysfunction and pulmonary edema. Thus, this study has evaluated the participation of bronchial epithelial cells in the pulmonary effects of *Tityus serrulatus* scorpion venom (Tsv). Human bronchial epithelial cell line BEAS-2B were utilized as a model target and were incubated with Tsv (10 or 50 μg/mL) for 1, 3, 6 and 24 h. Effects on cellular response of venom-induce cytotoxicity were examined including cell viability, cell integrity, cell morphology, apoptosis/necrosis as well as cell activation through the release of pro-inflammatory cytokines IL-1β, IL-6 and IL-8. Tsv caused a decrease in cell viability at 10 and 50 μg/mL, which was confirmed by lactate dehydrogenase (LDH) measurement. Flow cytometry analyses revealed necrosis as the main cell death pathway caused by Tsv. Furthermore, Tsv induced the release of IL-1β, IL-6 and IL-8. Altogether, these results demonstrate that Tsv induces cytotoxic effects on bronchial epithelial cells, involving necrosis and release of pro-inflammatory cytokines, suggesting that bronchial epithelial cells may play a role in the pulmonary injury caused by Tsv.

1. Introduction

Scorpion stings are considered a global medical-sanitary problem in many tropical and subtropical countries (Chippaux and Goyffon, 2008). In Brazil, the genus *Tityus* is the main genus responsible for the scorpion accidents in humans with the specie *Tityus serrulatus* causing the majority of severe cases and mortality in southeast and northeast regions (Reckziegel and Pinto Jr, 2014). Pulmonary edema is a frequent finding in patients stung by this scorpion, especially among children, in whom the number of severe cases and the lethality rate are increased (Reckziegel and Pinto Jr, 2014; Bucaretchi et al., 1995; Deshpande and Akella, 2012; Pucca et al., 2015; Amaral et al., 1993). Two distinct mechanisms have been suggested to explain the development of pulmonary edema: acute left ventricular failure resulting from massive catecholamine and inflammatory mediators release (Benvenuti et al., 2002; Freire-Maia et al., 1978), which leads to an increase in vascular permeability, and consequently extravasation of blood plasma in the alveolar space (Deshpande and Akella, 2012). Such inflammatory effect is an important event and a group of researches proposed the use of the term scorpion venom respiratory distress syndrome instead of pulmonary edema induced by scorpionism (D'Suze et al., 1999).

In addition to the pulmonary edema, literature shows that scorpion envenomation induces a systemic inflammatory response characterized by a massive and fast release of cytokines (Magalhães et al., 1999; Fukuhara et al., 2003; Pessini et al., 2003; Fialho et al., 2011). Different cytokines are released following scorpion envenomation both in patients as well as in mice exposed to various species of scorpion venom (Fukuhara et al., 2003; Petricevich, 2010). Indeed, an increase of interleukins (IL)-1α, IL-1β, IL-6, IL-8,

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IL-10, Interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α, were observed in the serum of patients who were stung by *T. serrulatus* (Magalhães et al., 1999; Fukuhara et al., 2003). In addition, it has been demonstrated that the increased levels of IL- 1β, IL-6, IL-8, TNF-α and IL-10 in patients envenomed by *Tityus serrulatus* venom (Tsv) were positively correlated with the severity of envenomation (Fukuhara et al., 2003). Moreover, a study developed by Meki et al. (2003) evaluating scorpion accidents from Egypt, also described a positive correlation between IL-8 release and the severity of envenomation.

Bronchial epithelial cells constitute the main layer of the lung airways and play a wide variety of critical roles in the defense of the lungs against the entry of noxious substances (Rennard et al., 1994). These cells, not only form a passive barrier, but also play an active role in the pulmonary immune response, being able to produce a variety of mediators initiating and perpetuating inflammatory responses inducing the recruitment and activation of lung structural cells and leukocytes (Van der Velden et al., 1999). A number of studies have shown the involvement of immune cells on envenoming caused by Tsv. Petrichevich (2002); Petrichevich et al. (2007; 2008) showed that incubation of these cells with Tsv increased the production of the inflammatory mediators IL-1α, IL-1β, TNF-α, IL-6 and IFN-γ. In addition, an increase in the production of IL-6 and TNF-α by J774.1 macrophages was observed after stimulation with Tsv or its toxins (Ts1, Ts2 and Ts6) (Zoccal et al., 2011). Despite the demonstrated effects of the scorpion venom on the airways and the involvement of cytokines and immune cells in the envenoming caused by Tsv, there is a lack of knowledge regarding the participation of other cells, such as bronchial epithelial cells, in scorpion venom inducing pulmonary injury.

Therefore, the aim of the present work was to study the biological effects of Tsv on human bronchial epithelial cell focusing on cell cytotoxicity, cell death, as well as the ability of the venom to induce cytokines release.

2. Material and methods

2.1. Venom

The lyophilized crude *T. serrulatus* scorpion venom (Tsv) was obtained from the Butantan Institute. The venom was stored at −20 °C until the time of use, when it was diluted in sterile phosphate-buffered saline (PBS).

2.2. Cell culture

The human bronchial epithelial cell line (BEAS-2B) provided by Dr. Roger Chammas (School of Medicine of University of São Paulo, Brazil) was used as the venom target. BEAS-2B cells were maintained at subconfluent levels in growth medium consisting of bronchial epithelial basal medium (BEBM) (Lonza) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic and incubated at 37 °C in a humidified atmosphere containing 5% CO2. The cells were maintained at subconfluence densities and passage every two days. BEAS-2B were plated in 96 well plates (1×10^4 cell/well) and incubated for 48 h for cell adherence. After this period, the cells were incubated with Tsv at concentrations of 10 or 50 μg/ for 1, 3, 6 and 24 h.

2.3. Cell viability assay

Mitochondrial activity was measured to assess bronchial epithelial cell viability, as described previously (Franco et al., 2016). This analysis was based on cell mitochondrial measured by the 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) based cytotoxicity assay (Sigma Aldrich, St. Louis, MO, USA). After incubation with venom for 1, 3, 6 and 24 h, the cells were washed with 100 μL of PBS and MTT was added to cell culture to a final concentration of 0.5 μg/mL and the cells were incubated for 3 h, at 37 °C. After the incubation time, 100 μL of isopropanol was added to each well to dissolve the formazan crystals. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 620 nm. Each sample was assayed in triplicate wells, in at least three independent experiments.

2.4. Cell integrity

To confirm the cytotoxicity caused by the venom, the enzymatic activity of Lactate dehydrogenase (LDH) present in the supernatant of cultures was determined as the parameter of cell integrity, as previously described (Villalobos et al., 2007). The effect of Tsv on cell permeability of bronchial epithelial cell wall was evaluated by the release of LDH at 1, 3, 6 and 24 h, in the supernatant of endothelial cell culture. For this purpose, the BEAS-2B cells were incubated with Tsv or BEBM medium only (control), and LDH activity was determined in 20 μL of cell supernatant by the addition of 200 μL of substrate containing 200 mm NaCl, 0.2 mM pyruvate, 1.6 mM NADH, by using a commercial kit (Labtest, Minas Gerais, Brazil). The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 340 nm. The results were expressed by the decrease of the optical density values, resulting from the oxidation of NADH in the presence of pyruvate. Each sample was assayed in triplicate wells, in at least three independent experiments.

2.5. Light microscopy

BEAS-2B cells were photographed before and after 24 h incubation of Tsv (50 μg/mL) for morphology analysis. An TS-100 light microscope (Nikon, objective 40× and 100×) coupled to a video camera (CCD DS-Fi 1, Nikon) and connected to a microcomputer was used for capturing images. BEAS-2B cell counting was performed automatically by the software ImageJ.

2.6. Analysis of apoptosis/necrosis by flow cytometry

The assessment of the apoptotic and necrotic effects induced by Tsv on BEAS-2B was determined by flow cytometry, using a kit containing annexin V-FITC and 7-amino-actinomicyn D (7-AAD) in a FACS Accuri C6 (BD Biosciences, CA, USA). The population of necrotic cells were marked with 7-AAD (7-AAD+), while the cells in apoptosis are marked with annexin-V FITC (AN+) and the population of both necrotic and apoptotic cells is marked as 7-AAD+/AN+ (Nogueira-Pedro et al., 2013).

BEAS-2B cells were plated into 24-well plates (1×10^6 cells/well), followed by incubation for 24 h at 37 °C in a humidified incubator containing 5% CO_2. After this period, the cells were treated with 50 μL of PBS (negative control) or 50 μL of Tsv samples at concentration of 50 μg/mL. After 24 h of treatment, cells were transferred to flow cytometry tubes, centrifuged at 900 *g* for 5 min, the supernatant was discarded and cellular pellet ressuspended in 1 mL of PBS. The same process was repeated, this time with addition of 50 μL of annexin buffer, 3 μL of annexin V-FITC (Becton Dickinson - USA) and 5 μg/mL of 7-amino-actinomicyn D (7-AAD) (Invitrogen - USA) for 20 min protected from light. After incubation, cells were washed and then ressuspended with 200 μL of annexin buffer for data acquisition on flow cytometer. A total of 50.000 events were acquired on FACs Accuri C6 (Becton Dickinson – USA). Cell Quest Pro Analysis (version 5.1 - Becton Dickinson – USA) and