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Hemiscorpius lepturus venom induces expression and production of interluckin-12 in human monocytes

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ABSTRACT

The objective of this study was to evaluate the capacity of the venom from *Hemiscorpius lepturus* to induce expression and production of interleukin-12 (IL-12) on isolated human monocytes. For this purpose, isolated human monocytes (250,000–300,000 cells/ml) were exposed to different concentrations of the venom (0.625, 1.25, 2.5, 5, 10 and 20 μg/ml) in 96-well plates for varying incubation periods (6, 12, and 24 h). The end point of assessment included LDH cytotoxicity assay, measurement of expression of IL-12,p40 mRNA by real-time PCR, and quantification of IL-12 release using sandwich ELISA technique. The results showed that this venom produced concentration- and time of incubation-dependent cytotoxicity. The level of enhancement of expression and production of IL-12 were found significantly higher with lowest concentration and after 6 h of incubation. The findings demonstrated that the venom from this scorpion contains active constituents which can direct the immune system to produce IL-12.

1. Introduction

Scorpions are venomous arthropods with capacity for producing different types of venoms. The venom is composed of about 50–100 different toxic polypeptides and until now almost 250 bioactive proteins and peptides have been characterized (Possani et al., 2000). Induction of inflammatory responses among human subjects following envenomation with *Hemiscorpius lepturus* (*H. lepturus*) has been previously demonstrated (Jalali et al., 2011). Moreover, after scorpion envenomation, several reports have demonstrated an increase in production of pro- and anti-inflammatory cytokines as well as other mediators, such as prostaglandins, leukotrienes, bradykinins and platelet activating factor, which orchestrate the inflammatory processes and pain response (Abdel-Haleem et al., 2006; Jalali et al., 2011; Petricevich, 2010).

H. lepturus is one the most dangerous and medically important scorpion in Iran (Mohseni et al., 2013; Pipelzadeh et al., 2007). This scorpion belongs to Hemiscorpiidae family and its venom is very cytotoxic (Pipelzadeh et al., 2006). The results of immunoassay experiments showed that *H. lepturus* venom activated the host immune responses in accidentally envenomed patients towards production of specific antibodies against this scorpion (Khanbashi et al., 2014).

Studies on natural toxins prepared from venomous animal species from different areas of the world reported different immunological and toxicological manifestations. These findings proposed the notion that these venoms can to be employed as starting points for designing new strategies for treatment of various diseases (Gomes et al., 2010; Joseph and George, 2012; Kapoor, 2010). Previously studies have reported significant anti-tumor activity of scorpion venom (Jain and Kumar, 2012; Ortiz et al., 2015; Liu et al., 2003). A previous *in vitro* study reported that the toxic components from *Tityus serrulatus* scorpion (Ts1, Ts2 and Ts6) induce different types of immune response following macrophage activation (Zoccal et al., 2011, 2013). While Ts1 and Ts6 stimulated the production of NO, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, Ts2 stimulated the production of the anti-inflammatory cytokine IL-10.

The heterodimeric IL-12 cytokine has multiple biological

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functions bridging innate and adaptive immunity (Airoldi et al., 2002; Del Vecchio et al., 2007) and is an important immunoregulatory cytokine that is produced mainly by antigen-presenting cells. This cytokine induces differentiation of naive CD4+ T cells towards Th1 cells type, activates NK cells and induces interferon-γ (IFN-γ) production (Bonecchi et al., 1998). Empowering immune system with IL-12 immunotherapy could be importance in the treatment of diseases where a Th1 response is desirable, such as viral, bacterial infections and cancers (van Wanrooij et al., 2012; Del Vecchio et al., 2007).

A previous clinical study on accidentally envenomed patients has demonstrated that *H. lepturus* scorpion induces TNF-α pro-inflammatory cytokine production (Jalali et al., 2011). In addition, a recent study showed that the venom has unique immune stimulating properties, leading to production of specific antibodies (Khanbashi et al., 2014). However, the exact mechanism that mediates this activity has not been studied previously. No previous study has specifically assessed the potential of this venom to induce the expression and production of IL-12. The aim of the present *in vitro* study was, therefore, to evaluate mRNA expression and production of IL-12 in human monocytes following exposure to *H. lepturus* venom on human monocytes under cell culture condition.

2. Materials and methods

2.1. Venom preparation

H. lepturus adult scorpions were collected near Baghmalek and Ramhormoz cities in Khuzestan province, Southwest of Iran. The crude venom was collected monthly by mild electrical simulation (20 V, 500 mA) and dissolved in sterile double distilled water. In order to remove the mucous, this solution was centrifuged at 13,000 g for 15 min at 4 °C, the supernatant was immediately freeze-dried and stored at −70 °C until used (Khanbashi et al., 2014; Zargan et al., 2011). The crude venom protein content was determined by Bradford method (Bradford, 1976).

2.2. Preparation of human monocytes

Venous blood samples from healthy volunteers were drawn into heparinized syringes, diluted 1:2 in PBS (phosphate-buffered saline), layered onto Ficoll–Paque (GE Hearelthcare, Buckinghamshire, UK), and centrifuged for 30 min at 900 g. Using a sterile pipette, the upper layer that contained the plasma and most of the platelets was removed. Using another pipette, the mononuclear cell layer was transferred to another centrifuge tube. The cells were washed by adding excess Hank's balanced salt solution (HBSS) (Gibco, Waltham, MA, USA) (3 times the volume of the mononuclear cell layer) and centrifuged at 18–20 °C for 10 min at 400 g, The supernatant cells were removed and resuspended in HBSS, and washed once more to remove the remaining platelets (Fuss et al., 2009). Peripheral blood mononuclear cells were suspended in supplemented serum-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltham, MA, USA) at 2×10^6 cells/ml. Ten ml of the cell suspension was added to each 75-cm^2 flask and incubated for 1 h in a humidified 37 °C, 5% CO_2 incubator. The medium which contained non-adherent cells, was decanted, washed twice with 10 ml supplemented serum-free DMEM to remove any residual non-adherent cells, and replaced with 10 ml fresh DMEM. The adherent monocytes were removed by gentle scraping with a plastic cell scraper. The monocytes were resuspended in supplemented serum-free DMEM and monocytes were counted (Wahl and Smith, 1991). At least 95% of the adherent cells were monocytes as determined by both Wright–Giemsa staining.

2.3. Cell treatment

Adherence-purified monocytes were cultured at a final concentration of 250,000–300,000 cells/ml 96-well plates. The cells were incubated overnight without stimulation in 1.5 ml polypropylene tubes at 37 °C in 5% CO_2 atmosphere. Depending on the experimental protocol, the monocytes were stimulated with varying of concentrations of the venom and tested after different incubation periods (Zargan et al., 2011; Siegemund et al., 2007). All samples were cultured in duplicate and the cell culture supernatants of each duplicate were harvested after the indicated time points.

2.4. Cytotoxicity assay

The effect of scorpion venom on cell cytotoxicity was determined by lactate dehydrogenase (LDH) assay. Release of LDH was used as a marker for evaluation of cell membrane integrity for determination cell viability after exposure to the venom. Cells were seeded in 96-well plate at a density of 200,000–300,000 cells/well in culture medium. After an overnight incubation period, the medium was replaced and cells were exposed to varying concentrations of venom (0.625, 1.25, 2.5, 5, 10 and 20 μg/ml). Cells were incubated for 2, 4, 6, 12 and 24 h and LDH activity was measured in the supernatants using *in vitro* cytotoxicity assay kit (Takara, Shiga, Japan) in accordance with the manufacturer's instructions. The absorbance was determined at 490 nm using plate reader. All samples were cultured in triplicate. Two independent experiments were performed. Cells in the positive control (high control) were treated with Triton X-100 solution (Sigma–Aldrich, Germany), and negative control (low control) in which cells were incubated in culture media alone. Blank contained the corresponding extract concentrations or Triton X-100 solution or media without cells. Percentage of cytotoxicity was calculated using the following relationship:

$$\%\ cytotoxicity = \frac{experimental\ value - low\ control}{high\ control - low\ control} \times 100$$

2.5. Measurement of expression of IL-12 mRNA in human monocytes by real-time PCR (qRT-PCR)

The monocytes were pelleted (8000 g for 5 min) and the medium was removed. Total RNA was isolated using high pure RNA isolation kit (Jene BioScience, Jena, Germany) according to the manufacturer's recommendations. The concentration and quality of extracted mRNA were determined spectrophotometrically at 260 nm wavelength and 260/280 nm wavelength ratio, respectively. Two μg of extracted RNA, diluted to a final volume of 20 μl, was reverse transcribed into cDNA using g RevertAid™ First Strand cDNA Synthesis Kit according to the manufacturer's recommendations (Thermo Scientific, Waltham, MA, USA). Expression of IL-12 mRNA in human monocytes was investigated using Applied Biosystems StepOne™ (Life technologies, Waltham, MA, USA) Real-time PCR system. Data were normalized based on GAPDH expression as the housekeeping gene. Real-time PCR was performed using the following specific primers derived from previously studies (Omata et al., 2001) Human IL-12,p40 (sense, 5′-TGGAGTGCCAGGAGGACAGT-3′, and anti-sense, 5′-GTTGATGTCCCTGATGAAGAAGC-3′); GAPDH (sense, 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, and anti-sense, 5′-TAGAAGCATTTGCGGTGGACGATGGAGGG-3′). PCR reaction mixtures contained: 2 μl of cDNA, 10 μl SYBR Premix ExTaq™ II (2×) (Takara, Shiga, Japan) 1 μl