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Bothrops jararaca venom gland secretory cells in culture: Effects of noradrenaline on toxin production and secretion

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ABSTRACT

Primary culture of snake venom gland secretory cells could be a good model to study the mechanism(s) of toxin(s) production. These cells can produce and secrete venom to the medium with a hemorrhagic activity comparable to that induced by venom collected from snakes. Production of new venom is triggered by the sympathetic outflow, through the release of noradrenaline, but the importance of this neurotransmitter on toxin synthesis has not been addressed. This work led to the identification and comparison of the toxin panel produced by cultured secretory cells, during a 12-day time-course analysis, as well as to the effects of noradrenaline on the process. The results showed that in our culture model the synthesis of new toxins is asynchronous, mimicking data previously published from proteomic analyses of venom glands harvested from animal experimentation. Furthermore, noradrenaline did regulate the synthesis and/or secretion of venom toxins over the analyzed period. Finally, we demonstrated that snake venom metalloproteinases present in these cultured cells secretome were mostly in their zymogen forms; consequently, processing occurs after secretion to the gland lumen. Overall, the data support the use of venom gland secretory cells as a reliable model to investigate the mechanism(s) of toxin(s) synthesis and secretion.

1. Introduction

An important characteristic of the venom gland of a Viperidae snake is the presence of a basal-central lumen, where most of the venom produced is stored. The activation of this venom gland occurs when the amount of venom inside the lumen decreases. After activation, the secretory epithelium undergoes morphological and biochemical changes. The secretory cells change their shape from cuboid to columnar, the cisternae of the rough endoplasmic reticulum expands, and the Golgi apparatus becomes well developed (Ben-Shaul et al., 1971; Rotenberg et al., 1971; Oron and Bdolah, 1973). When manual venom extraction is performed, the maximal synthetic activity of the secretory cells and the highest mRNA concentration occur between 4 and 8 days after venom extraction (Kochva, 1987; De Lucca et al., 1974; Carneiro et al., 1991). After that, the secretory cells activity decreases and the venom gradually accumulates in the lumen of the gland, while the epithelium returns to a quiescent stage. During this period, the exocytosis of new toxins synthetized by secretory cells is continuous. Venom production cycle is long, lasting around 30–50 days (Ben-Shaul et al., 1971; De Lucca et al., 1974; Kochva, 1960, 1987; Rotenberg et al., 1971; Oron and Bdolah, 1973, Carneiro et al., 1991; Mackessy, 1991; Yamanouye et al., 1997). Therefore, the secretory cells have two different phases: one is the quiescent stage (when the lumen is full of venom), while the other is the activated stage, when the venom toxins are produced (Kochva, 1960, 1987; Mackessy, 1991).

Oron et al., 1978, using [^{3}H] leucine, showed that the synthesis of new toxins in *Vipera palestinae* is asynchronous, since the relative radioactivity of several toxins from 2 days after venom extraction was significantly different from 4 days after extraction. More

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recently, we showed that the composition of toxins in the venom gland is different at the various stages of the venom production cycle, suggesting that synthesis and secretion of toxins in *Bothrops jararaca* snake is also asynchronous (Luna et al., 2013).

The mechanisms that regulate synthesis and secretion of snake venom toxins are not well understood. However, the noradrenenergic innervation of the venom gland is essential for the venom production cycle, since the depletion of catecholamines (by administration of reserpine) blocks the production/secretion of venom by secretory cells in activated stage (Yamanouye et al., 1997). Both α- and β-adrenoceptors are stimulated in this process, and are involved in venom production cycle triggering (Yamanouye et al., 1997, 2000; Kerchove et al., 2004, 2008; Luna et al., 2009). Nevertheless, the importance of the sympathetic outflow in toxins production has not been addressed yet.

It is well-established that hemorrhage plays an important role in *Bothrops jararaca* envenomation and that snake venom metalloproteinases (SVMPs) are key players in the hemorrhagic syndrome (Moura-da-Silva and Baldo, 2012). We have previously shown that *Bothrops jararaca*'s venom gland secretory cells in primary culture are functional, being able to produce and secrete venom with hemorrhagic activity (Carneiro et al., 2006; Yamanouye et al., 2007). Thus, secretory cell culture could be a good *in vitro* model for investigating the mechanism(s) of production of toxin(s).

Therefore, the aim of this study was to identify and compare the toxin panel produced by secretory cells of *Bothrops jararaca* venom gland along the primary culture, and the effects of noradrenaline on toxin production and secretion.

2. Material and methods

2.1. Animals and venom glands

Bothrops jararaca (Wied, 1824) female adults (n = 7), weighing 200–350 g, were classified by the Special Laboratory of Zoological Collection from Instituto Butantan; animals were treated and kept as described by Breno et al. (1990). These snakes had no access to food for 40 days to make sure that the cells were in quiescent stage. Snakes were anesthetized with sodium pentobarbital (30 mg/kg, s.c.), and the blood was collected by the aorta to reduce the interference of red cells in the primary culture of secretory cells (Yamanouye et al., 2007). After that, snakes were decapitated and the venom glands removed and dissected as described previously by Yamanouye et al. (2007). Animal care and procedures used were in accordance with guidelines of the Animal Ethics Committee of the Instituto Butantan (891/12) and the Brazilian Institute for Environment and Renewable Natural Resources (IBAMA, License 01/2009).

2.2. Secretory cells culture

The primary cultures of cells were treated with noradrenaline (0.1 mM) (n = 3) or not (control, n = 4). Preparation of dispersed cells was based on the protocol described by Yamanouye et al. (2007). Viable cells were counted in the presence of 0.4% Trypan Blue. The viability of the cells was 3.30 ± 0.98 × 10^6 cells per preparation. One milliliter of culture medium [DMEM supplemented with 5% fetal bovine serum, 10 μg/mL insulin, 5 μg/mL transferrin, 0.07 μg/mL sodium selenite, 10 μg/mL dexamethasone, 80 ng/mL epidermal growth factor, 0.1 μM retinoic acetate, and antibiotics (100 U/mL of penicillin and 100 μg/mL streptomycin)] was added to each well of a 24-well plate; 2 × 10^6 cells were then added per well. The culture was maintained at 30 °C in a humidified incubator (5% CO_2) until the twelfth day. The culture medium was collected every three days. Thus, the culture media collected at different times were identified as follows: from 0 to 3 days of culture (3D), from 3 to 6 days of culture (6D), from 6 to 9 days of culture (9D), and from 9 to 12 days of culture (12D). These media were stored at −20 °C until processing.

2.3. Albumin removal

The culture medium contained 5% fetal bovine serum and the albumin concentration present therein could interfere with the detection, by our proteomic approach, of toxins eventually secreted by the cells. Thus, all collected media were subjected to albumin removal procedure using Affi-Gel Blue (Bio-Rad-Hercules, CA, USA) following the manufacturer's instructions. After this procedure, only a few toxins were lost (data not shown). Protein concentration was determined by Bradford assay (Bradford, 1976) using bovine serum albumin as a standard. The following media protein concentrations (mean ± SEM, n = 4) were determined: DMEM – 3256.09 ± 802.21 μg/mL; 3D – 3491.21 ± 612.52 μg/mL; 6D – 3697.5 ± 802.57 μg/mL; 9D – 3715.29 ± 829.45 μg/mL. After albumin removal (n = 5) the protein concentration was DMEM – 570.38 ± 66.01 μg/mL; 3D – 586.86 ± 103.30 μg/mL; 6D – 578.15 ± 72.92 μg/mL; 9D – 561.26 ± 58.63 μg/mL; 12D – 619.09 ± 52.24 μg/mL.

2.4. 2D-PAGE

Protein samples (80 μg) were first precipitated with acetone (Jiang et al., 2004). The pellet was suspended in 125 μL of DeStreak rehydration solution (GE Healthcare, Uppsala, Sweden) and 0.5% immobilized pH gradient (IPG) buffer pH 3–10 (GE Healthcare). IPG strips of 7 cm, pH range 3–10, were passively rehydrated overnight at room temperature. Isoelectric focusing (IEF) was performed on an Ettan IPGphor II (GE Healthcare) using a program for a total of 5952 Vh, according to manufacturer's instructions, including an initial step at low voltage of 100 V for 2 h (to allow salt to migrate out of the strip). After IEF, the strips were initially incubated with equilibration buffer (75 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing 1% dithiothreitol for 15 min. Next, solution was discarded and the strips were incubated in equilibration buffer containing 2.5% iodoacetamide for 15 min at room temperature. After equilibration, the strips were applied to the second dimension to separate proteins on a 12.5% SDS-PAGE using a Mini Protean Tetra Cell electrophoresis system (Bio-Rad). Experiments were performed using the media from three cultures (n = 3) and in technical triplicate.

2.5. 1D-SDS-PAGE

Protein content (30 μg) for each culture medium (containing the cell secretome) of the different time frames assayed was submitted to denaturation and disulphide bond reduction in sample buffer, at 100 °C for 5 min (Laemmli, 1970). The proteins were separated by SDS-PAGE (12%) with the buffer system described by Laemmli (1970), stained overnight with Coomassie Brilliant Blue-G, and processed for mass spectrometric protein identification as previously described (Luna et al., 2013). All experiments were performed using the media from three cultures and in technical triplicate. When gels were to be used for further immunoblotting analysis for SVMP prodomain detection, 200 μg of protein were applied per lane.

2.6. Immunoblotting analyses

The detection of toxins produced by secretory cells in culture