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Short communication

Jararhagin, a snake venom metalloprotease-disintegrin, activates the Rac1 GTPase and stimulates neurite outgrowth in neuroblastoma cells

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ABSTRACT

It has been shown previously that the snake venom metalloprotease-disintegrin jararhagin stimulates cell migration and cytoskeletal rearrangement, independently of its effects on cellular adhesion but possibly associated with the activation of small GTP-binding proteins from the Rho family [Costa, E.P., Santos, M.F., 2004. Toxicon 44(8), 861–870.] Here we show that jararhagin stimulates spreading, actin dynamics and neurite outgrowth in neuroblastoma cells, and that this effect is accompanied by the translocation of the Rac1 small GTPase to the membrane fraction, suggesting its activation. Stimulation of neurite outgrowth was observed within minutes and was dependent on the proteolytic activity of the toxin. These results suggest that jararhagin may stimulate neuronal differentiation, being a potential tool for neuronal regeneration studies.

1. Introduction

Jararhagin, a toxin isolated from the venom of *Bothrops jararaca* (Paine et al., 1992), shows structural homology with other proteins such as the cell surface ADAMs, RGD disintegrins from venoms and several matrix metalloproteases (MMPs) (Bode et al., 1993; Bjarnason and Fox, 1994; Gomis-Ruth et al., 1993; Niewiarowski et al., 1994; Usami et al., 1994). In platelets and fibroblasts jararhagin binds the $\alpha 2\beta 1$ integrin. Although platelet aggregation is inhibited (Moura-da-Silva et al., 2001), in fibroblasts jararhagin functions as a collagen-mimetic substrate that activates integrins (Zigrino et al., 2002).

Previous studies have shown that jararhagin stimulates the influx of inflammatory cells in a dorsal air-pouch without having direct chemotactic activity (Costa et al., 2002). This toxin also stimulated the migration of epithelial cells in vitro and the phosphorylation of Focal Adhesion Kinase, associated with integrin signaling, while cellular adhesion to the substrate was inhibited (Costa and Santos, 2004). These effects were accompanied by the rearrangement of the actin cytoskeleton, with increased actin polymerization and formation of motility-associated cell processes, such as filopodia and lamellipodia.

Most actin-dependent processes important for migration, adhesion, morphogenesis, and axon guidance are dependent on the activity of small Rho GTPases (reviewed by Etienne-Manneville and Hall, 2002). These highly conserved proteins shuttle between the inactive GDP-bound and active GTP-bound forms. Upon activation, Rho GTPases translocate to the membrane, where they interact with specific effector molecules (reviewed by Bustelo et al., 2007).

In neuronal cells, Rho GTPases have emerged as key integrators of environmental cues to regulate various aspects of neuronal development, including neuronal migration,

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neurite/axon formation and outgrowth, as well as dendrite and dendritic spine formation and maintenance (reviewed by Van Aelst and Cline, 2004 and Govek et al., 2005). During the nervous system ontogenesis, neurite outgrowth is essential for pathfinding of elongating axons on their way to their target regions and formation of the dendritic tree.

Protease activity seems to be associated with neuronal differentiation, either by cleaving the extracellular matrix or by processing cell surface molecules. Several ADAMs, for example, capable of mediating signaling through integrins and ectodomain shedding, have important functions in neurogenesis, neuronal migration, axonal outgrowth and plasticity (reviewed by Yang et al., 2006). It has also been shown that some environmental cues enhance the expression of MMPs in dorsal root ganglion neurons, PC12 and neuroblastoma cells (Machida et al., 1989; Chambaut-Guerin et al., 2000).

Considering that jararhagin has great similarity with ADAMs and MMPs, and that in other cell types it is capable of regulating the actin cytoskeleton and cell migration, the aim of this study was to characterize the effects of jararhagin on neurite outgrowth, using a well-established in vitro model for neuronal differentiation.

2. Materials and methods

2.1. Materials

Culture ware was purchased from Corning Glass Works (Corning, NY). Medium, fetal bovine serum (FBS) and other cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Other chemicals and biochemicals were obtained from Sigma (St. Louis, MO) and Mallinkrodt (Phillipsburg, NJ). Polyclonal antibody against Rac1 was obtained from Santa Cruz Biotech (Santa Cruz, CA). Rhodamine-phalloidin was obtained from Molecular Probes (Eugene, OR).

2.2. Jararhagin purification

Jararhagin was purified as previously described (Paine et al., 1992) from the venom of *B. jararaca* snakes (kindly donated by Dr. Ana Maria Moura-da-Silva). Briefly, the venom was fractionated by hydrophobic interaction using FPLC Phenyl-Superose columns and, after elution, the purity of the toxin was verified by SDS-PAGE. The protein concentration was estimated by the Bradford method. Inactivation of the toxin was obtained by incubation with 1 mM EDTA for 30 min at 37 °C. For experiments using inactivated jararhagin, control jararhagin was also incubated under the same conditions, without EDTA. EDTA alone was also used as a second control.

2.3. Cell culture and neurite outgrowth

Neuro-2a neuroblastoma cells (Klebe and Ruddle, 1969) were maintained in a humidified atmosphere of 95% air–5% CO_2, in Dulbecco's Modified Eagle's Medium containing 4.5 g/L glucose, 10% fetal bovine serum, antibiotics (penicillin and streptomycin) and L-Glutamine.

For neurite outgrowth experiments, control and jararhagin-treated cells were imaged for up to 1.5 h (3 min intervals) using a 3CCD camera (MTI) coupled to an Aristoplan microscope (Leitz). Image J software (NIH) was used for image capture, and measurements were made using a new software developed in order to permit manually assisted marking of the soma and neurites. The soma was delimitated by a polygon with vertices selected by the user, while the neurites were delimitated by connected line segments and respective branches. After the neurons were marked, the software generated measurements such as area, perimeter and diameter of the soma; the neurites tree length (sum of all line segments approaching the neurites of a neuron), the maximum tree length (sum of lengths of the line segments leading to the longest neurite segment in the tree); the number of branches in a neurite tree, as well as the number of neurites per neuron. Only cellular extensions larger than the cell diameter were considered neurites, and the measurements were made individually in all cells that appeared wholly in the field.

2.4. F-actin staining and confocal microscopy

For staining, cells were cultured on glass coverslips and, after treatment for different periods of time (3–12 h), they were fixed and permeabilized with 2% formaldehyde/0.2% Triton X-100 in PEM buffer (10 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8) for 10 min at room temperature (RT) and post-fixed with 95% ethanol for 5 min at −20 °C. F-actin was detected by rhodamine-phalloidin staining according to the manufacturer's instructions. Samples were imaged using a Nikon PCM 2000 Laser Scanning Confocal microscope.

2.5. Adhesion assays using jararhagin as substrate

Cell culture 35 mm-dishes were coated with 2.5 μg jararhagin (active and inactivated) for 2 h at 37 °C, followed by blocking with 10% bovine serum albumin during 1 h. After washing with HBSS, cells were plated and let to adhere for 1.5 h. After extensive washing with PBS, adherent cells were fixed and counted using an inverted microscope, as previously described by Costa and Santos (2004).

2.6. Measurement of Rac1 translocation to the membrane fraction

For measurement of Rac1 translocation, cells were homogenized on ice in the presence of a protease inhibitor cocktail using a 1 ml-glass Dounce. Cell debris were removed by centrifugation at 500 *g* for 10 min at 4 °C, and the supernatant was centrifuged at 20,000 rpm for 60 min at 4 °C. The pellet (membrane fraction) was resuspended in 200 μl of lysis buffer and used for SDS-PAGE with 15% gels. Protein concentration was determined by the Bradford method. After transferring the proteins to nitrocellulose membranes, non-specific binding sites were blocked with 5% deffated milk in Superblock Blocking Solution (Pierce, Rockford, IL), and incubated overnight at 4 °C with antibody against Rac1. After washing, the membranes were incubated with goat anti-rabbit-peroxidase (ECL™ kit; Amersham) for 2 h at RT and detection of labeled