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Differentiation of *Leishmania donovani* in host-free system: analysis of signal perception and response

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Abstract

Leishmania donovani are the causative agents of kala-azar in humans. They undergo a developmental program following changes in the environment, resulting in the reversible transformation between the extracellular promastigote form in the sand fly vector and the obligatory intracellular amastigote form in phagolysosomes of macrophages. A host-free differentiation system for *L. donovani* was used to investigate the initial process of promastigote to amastigote differentiation. Within an hour after exposing promastigotes to differentiation signal (concomitant exposure to 37 °C and pH 5.5), they expressed the amastigote-specific A2 protein family. At 5 h they started to transform to amastigote-shaped cells, a process that was completed 7 h later. This morphological transformation occurred synchronously, while cells arrested at G1. By sequential exposure to elevated temperature (for 24 h) and then acidic pH, we found that heat was responsible for the growth arrest and acidic pH to its release and subsequent route to differentiation into amastigotes. Lastly, ethanol and Azetidine 2 carboxylic acid (a synthetic proline analog) that induced heat shock response in promastigotes were capable of replacing heat in the differentiation signal.

Keywords: *Leishmania donovani*; Differentiation; Cell cycle; Heat shock response; Signaling

1. Introduction

During their lifetime, parasitic protozoa cycle between vectors (that transmit the parasites) and hosts (to whom they are pathogenic), and as a result encounter extreme environmental changes. Parasitic protozoa respond to these changes by differentiating to highly adapted forms for each of these new environments, which enables them to invade and proliferate inside their hosts. Our knowledge of the molecular mechanisms that regulate these processes is limited. We aim to reveal these mechanisms using *Leishmania donovani* as a model parasite.

L. donovani, the causative agents of visceral leishmaniasis, are intracellular parasitic protozoa that cycle between phagolysosomes of mammalian macrophages and alimentary tract of sand flies [1,2]. In the vector, they grow as extracellular flagellated promastigotes, in the host they proliferate as intracellular aflagellated amastigotes. Normally, in the insect mid-gut, the actively dividing, immature, procyclic promastigotes differentiate into non-dividing metacyclic forms, which migrate to the thoracic mid-gut and proboscis. These latter forms have been shown to be the infective stage of the parasite [3]. They are introduced into the host during a vector's blood meal and are subsequently phagocytosed by macrophages, where they differentiate to amastigotes [2].

Studies of the last decade indicated that shifting promastigotes to an intralysosomal-like environment (e.g. 37 °C and pH 5.5 in a 5–7% CO_2 environment for visceral species) induced differentiation into amastigotes in host-free culture [4–8]. Such an experimental system has been developed in

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our laboratory and has already been used to investigate various stage-regulated functions in *L. donovani* [9–14]. These studies revealed that concomitant exposure to acidic pH and high temperature signal for differentiation. Regardless of the method used for differentiation, all axenic amastigotes express the known stage-specific proteins including A2, amastin, specific proteases, nucleotidases and phosphatases, and down-regulate the expression of surface coat lipophosphoglycan. Axenic amastigotes are virulent as they infect hamsters and macrophage cell lines. Moreover, differentiation in host-free culture resumes virulence of long-term attenuated promastigotes [4,6]. Hence, it is established that these cultures are excellent representatives of animal-derived amastigotes.

Whereas we know what trigger promastigotes to differentiate, there is no data available on the course of events that occur between the signal and completion of differentiation. The objective of this work was to determine these activities. We found that the differentiation signal induced cell cycle arrest at G1 and that morphological transformation into amastigotes initiated during this time. The role of each parameter of the differentiation signal was assessed. Heat induced growth arrest in promastigotes, acidic pH released the arrest and route the heat adapted promastigote to differentiate into amastigotes. Furthermore, modulators of heat shock can replace heat in the differentiation signal.

2. Materials and methods

2.1. Materials

[^{3}H]thymidine was obtained from New England Nuclear; propidium iodide and hydroxyurea from Sigma; medium 199 and fetal calf serum from Biological Industries, Inc.; flavopiridol was donated by Dr. Jeremy C. Mottram from Glasgow University. All other reagents were of analytical grade.

2.2. Cell culture

A cloned line of *L. donovani* 1SR was used in all experiments [6]. This cell line was maintained as a clone by inoculating single colonies of promastigotes from medium 199 agar plates. Promastigotes were grown in medium 199 and supplemented with 10% fetal calf serum at 26 °C.

Promastigote to amastigote differentiation in a host-free culture and the maintenance of axenic amastigotes were performed either as described by Saar et al. [6], or by inoculating late log phase promastigotes in medium 199 at pH 5.5 containing 25% fetal calf serum. The axenic amastigotes remained stable in culture for a long time. They were recycled routinely every 10 weeks by differentiating them back to promastigotes, in parallel initiating a new line of amastigotes.

2.3. Cloning the green fluorescence protein gene (GFP) into promastigotes chromosome

A 0.75 Kbp of *GFP* coding region was cut from pEGFP (NcoI/Not1, Clontech Inc) and subsequently cloned into pF4X1.HYG (Jena Bioscience, Germany; pF4X.HYG-GFP, S. Goyard, unpublished). In this construct 18s rRNA flanked the HYG-GFP genes. Following linearization with SwaI, HYG.GFP was integrated into 18s rRNA locus in *L. donovani* promastigotes chromosome (Ld1S-GFP).

2.4. Flow cytometry

Flow cytometry of promastigotes, amastigotes and differentiating promastigotes was performed as follows: For each assay, 5 ml of cell culture (1×10^6–1×10^7 cells/ml) was aliquoted, washed twice with a phosphate buffered saline and then suspended in 90% ice-cold methanol for fixation. These cells were kept at −20 °C for further use. Prior to analysis, the cells were treated with 20 mg/ml RNase for one hour at 37 °C. Subsequently, DNA was stained using propidium iodide and analyzed for DNA content using FACSCalibur (Becto Dickinson, San Jose, CA.). In each assay, 20,000 cells were counted. The distribution of G1, S and G2/M phases in each experiment was calculated from each histogram using the Phoenix algorithm (Phoenix Flow Systems Inc., San Diego, CA).

2.5. Thymidine incorporation

Thymidine incorporation into cell nuclei was determined according to Noll et al. method [15] as follows:

To 1 ml of cells (0.5–1×10^7 cell/ml), 1–2 μCi/ml of [^{3}H] thymidine was added in 24-well ELISA plates. After 0.5 h of incubation, the cells were washed in 10 ml cold phosphate buffered saline (PBS), then suspended in 2 ml lysis buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 8.6 and 0.5% NP-40) and supplemented with a 4 mM Vanadyl ribonucleoside complex. The suspension was vortexed for 10 s and then centrifuged for three minutes ($6000 \times g$ at 4 °C). The nuclei-containing pellet was suspended in 10 ml ice-cold 10% trichloroacetic acid (TCA), kept at 4 °C for at least 20 min and subsequently filtered through GF/C filters (Whatman International Ltd.). The filters were washed with 10 ml 10% TCA and then with 10 ml 95% ethanol. The filters were then subjected to scintillation counting.

2.6. Western blot analyses

Cellular proteins from the indicated time points were separated on 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane [16] reacted with anti-A2 monoclonal antibody [17], and developed using Super-Signal® kit for peroxidase-conjugated antibody detection (Pierce Inc., Rockford).