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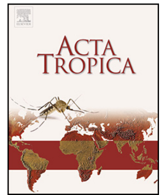

Regulation of intrinsic apoptosis in cycloheximide-treated macrophages by the Sichuan human strain of Chinese *Leishmania* isolates

Jin Zeng[a,1], Qi-Wei Chen[a,1], Ze-Ying Yu[a,2], Jun-Rong Zhang[a,2], Da-Li Chen[a], Chen Song[b], Jie Luo[b], Chen Zhang[b], Shun-Li Wang[b], Jian-Ping Chen[a,c,*]

[a] *Department of Parasitology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan, PR China*
[b] *West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan, PR China*
[c] *Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, Sichuan University, Chengdu, Sichuan, PR China*

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ABSTRACT

Leishmania spp. are able to survive and proliferate inside mammals' mononuclear phagocytes, causing Leishmaniasis. Previous studies have noted that the regulation of apoptosis in host cells by these parasites may contribute to their ability to evade the immune system. However, current results remain unclear about whether the parasites can promote or delay the apoptotic process in host cells, because the regulatory effect of *Leishmania* was assumed to be strain-, species- and even infection time-dependent. The aim of this study was to investigate whether the Sichuan isolates of Chinese *Leishmania* (SC10H2) can alter the process of intrinsic apoptosis induced by cycloheximide in different types of macrophage cell lines and to determine in which steps of the signaling pathway the parasites were involved. Human THP-1 and mouse RAW264.7 macrophages were infected by SC10H2 promastigotes followed by cycloheximide stimulation to assess the alteration of intrinsic apoptosis in these cells. The results indicated that SC10H2 infection of human THP-1 macrophages could promote the initiation of intrinsic apoptosis, but completely opposite results were found in mouse RAW264.7 macrophages. Nevertheless, the expression of Bcl-2 and the DNA fragmentation rates were not altered by SC10H2 infection in the cell lines used in the experiments. This study suggests that SC10H2 promastigote infection is able to promote and delay the transduction of early apoptotic signals induced by cycloheximide in THP-1 and RAW264.7 macrophages, revealing that the regulation of intrinsic apoptosis in host cells by SC10H2 *in vitro* occurs in a host cell-dependent manner. The data from this study might play a significant role in further understanding the relationship between *Leishmania* and different host cells.

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1. Introduction

Protozoan parasites of the genus *Leishmania*, obligate intracellular pathogens, primarily invade macrophages as well as monocytes in mammals and cause zoonotic leishmaniasis. Depending on the different organs where the pathogen is found, human leishmaniasis can be categorized into three main forms: visceral, cutaneous and mucocutaneous leishmaniasis. Substantial human infection can be caused by at least 21 of 30 species of the genus *Leishmania* in different regions (Chandra and Naik, 2008; Olivier et al., 2005). In the relationship between *Leishmania* and humans, the sand fly plays an important role as a vector that carries and transmits the parasite to human beings. The two forms of *Leishmania*, promastigotes and amastigotes, represent the different stages in sand flies and humans, respectively. When transmitted by sand flies, promastigotes develop into amastigotes in macrophages and continue to proliferate inside of the flies. An intriguing component of *Leishmania* pathogenesis is the ability to evade the immune system, which promotes successful survival and further proliferation by protecting the parasites from the killing effect of macrophages.

To avoid immune elimination and guarantee their survival within hosts, *Leishmania* can evade and suppress the immune defense system of hosts by developing multiple tactics, such as modifying the complement system and phagocytosis process, interfering with signaling pathways in macrophages, modulating

* Corresponding author at: Department of Parasitology, West China School of Preclinical and Forensic Medicine, Sichuan University, No. 24, 1st Section of First Ring Road South, Chengdu, Sichuan 610041, PR China.
E-mail address: jpchen007@163.com (J.-P. Chen).
[1] These authors contributed equally to this work.
[2] These authors also contributed equally to this work.

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cytokine and chemokine production, and altering Toll-like receptor and T cell response pathways, *etc.* (Gupta et al., 2013; Mougneau et al., 2011; Bogdan and Rollinghoff, 1998). A prominent mechanism of the immune evasion of *Leishmania* is apoptosis, a process of programmed cell death. Traditionally, the activation of apoptosis in response to various stimuli has been known to terminate the life of cells in a "silent" way without initiating inflammatory processes or causing further injury to the adjacent tissue or cells. However, *Leishmania* parasites have been found to regulate the process of apoptosis by interfering with the transduction of apoptotic signals in infected host cells (Getti et al., 2008; Wanderley et al., 2005). Therefore, the parasites may benefit from this regulation to avoid the killing by internal inflammatory factors produced by host cells in the early stage and thus may continue proliferating inside host cells.

Several previous studies have demonstrated that *Leishmania* undermine the apoptotic process in infected host cells. The amastigotes of *Leishmania mexicana* have been found to inhibit apoptosis of infected monocyte-derived dendritic cells *in vitro* (Gutierrez-Kobeh et al., 2013). Early stage infection with both *Leishmania donovani* and *Leishmania infantum* has also been shown to involve a delay in the induced apoptosis process of primary mouse macrophages (PMM) and RAW264.7 macrophages (Deschacht et al., 2012). Moreover, infection with *Leishmania major* has been shown to delay spontaneous apoptosis and prolong the life span of infected neutrophil granulocytes (Sarkar et al., 2013; Aga et al., 2002). On the other hand, there are also opposite findings arguing that *Leishmania* is able to promote apoptosis in host cells. When incubated with *L. major* and two additional Old World *Leishmania* species for long time periods of time, THP-1 cells showed increased numbers of apoptotic bodies with amastigotes inside (Getti et al., 2008). Although the results regarding the regulation of apoptosis by *Leishmania* remain controversial, most of the investigators are more or less in agreement that the regulatory effects of the parasites on host cell apoptosis are dependent on different strains, different species and different experimental time points (Getti et al., 2008; Deschacht et al., 2012; Donovan et al., 2009). Notably, most of the studies have employed either mouse cells or human cells for their investigation and have obtained consistent results from them. Whether the utilization of cells from both humans and mice as the host cells for one *Leishmania* species can lead to different outcomes regarding the regulation of apoptosis has not yet been investigated.

In this study, MHOM/CN/90/SC10H2 *Leishmania* isolates were isolated from symptomatic patients in Sichuan Province, China. Great efforts have been made to investigate this isolated strain in different aspects in previous studies, including *in vitro* cultivation and critical gene expression (Cao et al., 2012; Guan et al., 2012; Li et al., 2007; Hu et al., 2002; Tian et al., 2004). The aim of this study is to gain insight into whether and how *Leishmania* SC10H2 regulates cycloheximide (CHX)-induced intrinsic apoptosis of macrophages by measuring of several hallmarks of the signaling pathway. Considering the possibility of different outcomes due to the different mammalian cells used, we extended this study by using two cell lines, human THP-1 and mouse RAW264.7. Notably, *Leishmania* SC10H2 was found to regulate cycloheximide-induced apoptosis in human and mouse macrophages, but the two cell types had totally contrasting results. In the case of RAW264.7 cells, the parasites successfully prevented early apoptotic signal transduction by delaying the loss of mitochondrial transmembrane permeabilization, decreasing the activities of caspase-9 and caspase-3 and inducing the expression and synthesis of XIAP. However, surprisingly, the induced apoptosis in THP-1 cells was promoted by *Leishmania* infection under the same experimental conditions. At the same time, the expression of Bcl-2, an important death inhibitor located on mitochondria, was not affected by parasite infection. Finally, the parasites-mediated regulation was found to persist throughout the early stage of apoptosis, with an absence of changes of DNA fragmentation rates. Altogether, our findings may help to clarify the immune evasion of *Leishmania* regarding its role in apoptosis, and this study is the first to compare the regulation of the signaling pathway of intrinsic apoptosis in different mammalian cells by the SC10H2 strain.

2. Materials and methods

2.1. Cultivation of parasites and macrophage cells

Leishmania MHOM/CN/90/SC10H2 was maintained as promastigotes *in vitro* in liquid nitrogen. The promastigotes were cultured at 27 °C in M199 medium (Hyclone), enriched with 10% new-born calf serum (NBCS) (Sijiqing), 50 μg/ml streptomycin and 50 U/ml penicillin. The promastigotes were used at the late logarithmical growth phase.

A murine macrophage-like stable cell line RAW 264.7 was a kind gift from Dr. Xue F., Friendship Hospital of Capital Medical University, China and cultured at 37 °C in a 5% CO_2 −95% air mixture in RPMI-1640 media (Thermo) containing 2.05 mM L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum (HiFBS) (Bioind), 100 μg/ml penicillin, 100 μg/ml streptomycin, with passage every two days. A human monocytic cell line THP-1 was purchased from Shanghai Institutes for Biological Sciences and routinely maintained in the same conditions as RAW264.7 cells, with medium changed three times a week.

2.2. Preparation and treatment of macrophages

Those cells were maintained in cell culture flasks of multi-well plates. To induce differentiation into macrophage-like cells, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) stimulation (0.6 μg/ml) (Sigma) for 1 day. Cells were washed twice with sterile phosphate-buffered saline (PBS) to remove PMA and were incubated for an additional 3 days in fresh RPMI-1640 medium prior to infection.

Both terminally differentiated THP-1 and RAW264.7 cells were subjected to infection with SC10H2 promastigotes (10:1 parasite: cell ratio) for 4 h only or followed by treatment of pro-apoptotic agent cycloheximide (CHX) (5 μg/ml for THP-1, 2.5 μg/ml for RAW264.7 cells) for 16 h. All incubations were performed at 37 °C in a 5% CO_2 −95% air mixture for variable periods of time. After a 4-h post-infection, all medium with non-infecting promastigotes was washed away and replaced by fresh RPMI-1640 medium, then followed by CHX treatment. After 16 h of treatment with CHX, all medium was discarded and cells were harvested for following experiments. Uninfected and untreated macrophages were used as control.

2.3. Optical microscopy and transmission electron microscopy

Terminally differentiated THP-1 cells were infected by *Leishmania* SC10H2 promastigotes at a desired parasite to cell ratio. After the desired time of infection, cells were harvested for preparation of samples. The infected macrophages were methanol fixed, Wright-stained and observed by optical microscopy. Spare sample of the macrophages was collected by centrifugation, fixed in glutaraldehyde, processed and observed by transmission electron microscopy.

2.4. Mitochondrial transmembrane potential

To detect the impact of the parasites infection on the mitochondrial transmembrane potential of the macrophages, a mitocapture™ mitochondrial apoptosis detection fluorometric kit

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