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In vivo and *in vitro Leishmania amazonensis* infection induces autophagy in macrophages

Larissa Tavares Cyrino[a], Alexandra Paiva Araújo[a], Paulo Pinto Joazeiro[b], Cristina Pontes Vicente[c], Selma Giorgio[a,*]

[a] Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Caixa Postal 6109, Cep 13.083-970, Campinas, São Paulo, Brazil
[b] Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil
[c] Departamento de Biologia Estrutural e Funcional, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil

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ABSTRACT

Autophagy is the primary mechanism of degradation of cellular proteins and at least two functions can be attributed to this biological phenomenon: increased nutrient supply *via* recycling of the products of autophagy under nutrient starvation; and antimicrobial response involved in the innate immune system. Many microorganisms induce host cell autophagy and it has been proposed as a pathway by which parasites compete with the host cell for limited resources. In this report we provide evidence that the intracellular parasite *Leishmania amazonensis* induces autophagy in macrophages. Using western blotting, the LC3II protein, a marker of autophagosomes, was detected in cell cultures with a high infection index. Macrophages infected with *L. amazonensis* were examined by transmission electronic microscopy, which revealed enlarged myelin-like structures typical late autophagosome and autolysosome. Other evidence indicating autophagy was Lysotracker red dye uptake by the macrophages. Autophagy also occurs in the leishmaniasis skin lesions of BALB/c mice, detected by immunohistochemistry with anti-LC3II antibody. In this study, autophagy inhibitor 3-methyladenine (3MA) reduced the infection index, while autophagy inductors, such as rapamycin or starvation, did not alter the infection index in cultivated macrophages, suggesting that one aspect of the role of autophagy could be the provision of nutritive support to the parasite.

1. Introduction

Autophagy is the primary mechanism of degradation of cellular proteins and organelles, and involves the sequestration of regions of the cytosol within bound double-membrane that matures and degrades cytoplasmic constituents (Levine, 2007; Levine et al., 2011). At least two functions can be attributed to autophagy: increased nutrient supply *via* recycling of the products of autophagy under nutrient starvation, which is an important mechanism in maintaining cellular homeostasis (Levine et al., 2011; Mizushima, 2007); and autophagy could have an antimicrobial function and is involved in the innate immune response (Kirkegaard et al., 2004; Fabri et al., 2011), for example, in the removal of invading bacteria, including group A *Streptococcus* (Nakagawa et al., 2004), *Shigella* (Ogawa et al., 2005), and *Listeria* (Rich et al., 2003). However the relation between pathogens and autophagy appears to be complex. One example is the induction of host cell autophagy by the intracellular apicomplexa parasite *Toxoplasma gondii* (Wang et al., 2009a). It has also been suggested as a pathway by which parasite can compete with the host cell for limited resources (Wang et al., 2009b). *Chlamydia*, an obligate intracellular bacterium, induces autophagy that plays a defensive role in cells infected with chlamydial species not adapted to the host species. On the other hand, autophagy supports bacterial growth in permissive cells (Yasir et al., 2011). For *Leishmania*, an intracellular protozoan parasite that lives and proliferates within parasitophorous vacuoles (PV) of mononuclear phagocytes and causes a spectrum of human diseases (Kima, 2007; Nylén and Gautam, 2010; Mougneau et al., 2011), few reports exists of an association between the parasite and autophagy. One of the earliest reports suggested that *Leishmania mexicana* acquired macromolecules from the host cell *via* the mechanism of autophagy (Schaible et al., 1999). Pinheiro et al. (2009) showed that autophagy induction by starvation or cytokines alters parasite load, depending on the strain of mice used, while Mitroulis et al. (2009) detected autophagy in bone marrow taken from a patient with visceral leishmaniasis.

In this report, our group presents data demonstrating that *Leishmania amazonensis* infection induces autophagy in cultivated macrophages using three different methods; blot detection of

* Corresponding author. Tel.: +55 19 35216287; fax: +55 19 35216287.
E-mail address: sgiorgio@unicamp.br (S. Giorgio).

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LC3II, a marker of the autophagy pathway that is predominantly associated with autophagic organelles, transmission electron microscopy, and Lysotracker red staining (Klionsky et al., 2007). We also investigated whether autophagy modulation alters *in vitro L. amazonensis* infection. In addition, the occurrence of autophagy was examined in the cutaneous lesions of infected mice.

2. Materials and methods

2.1. Animals, parasite, and cell cultures

Female BALB/c and C57Bl/6 mice, aged 6–8 weeks, were obtained from Animal Center of the Campinas State University (Unicamp), Campinas, SP, Brazil. *L. amazonensis* (MHOM/BR/73/M2269) amastigotes were isolated from footpad lesions of BALB/c mice (Centro Multidisciplinar para Investigação Biológica, Unicamp) as described previously (Barbieri et al., 1993), and used immediately following isolation. Promastigotes were cultured as described by Arrais-Silva et al. (2005). The murine macrophage cell line RAW264.7 was maintained in RPMI 1640 medium supplemented with gentamicin with 25 μg/ml gentamicin (Sigma, St. Louis, MO, USA) and 10% fetal calf serum (FCS) (Nutricel, Campinas, SP, Brazil) at 37 °C in 5% CO_2, 5% O_2, and balanced N_2 (Linares et al., 2008). Bone marrow-derived macrophages (BMDM) were obtained from normal BALB/c or C57Bl/6 mice total bone marrow by flushing femurs and tibia with Dulbecco's modified Eagle's (DME) medium; the bone marrow mononuclear cells were propagated by culturing in DME medium supplemented with 10% FCS and 20% L929-conditioned medium for seven days (Swanson et al., 2009). The murine fibrosarcoma cell line L929 were cultured as described previously to generate L929-conditioned medium (Swanson et al., 2009). All the experimental protocols were approved by the Ethics Committee for Animal Research of the Institute of Biology, Unicamp.

2.2. Animal infection

Six-week-old female BALB/c mice were subcutaneously infected in the right hind footpad with 10^5 amastigotes. The course of the infection was monitored by measuring the increase in footpad thickness with a dial caliper compared with the contralateral uninfected footpad (Arrais-Silva et al., 2006).

2.3. Macrophage treatment and infection

For autophagy induction through starvation, cells were washed three times with PBS and incubated with Hanks balanced salt solution (Sigma) at 37 °C in 5% CO_2, 5% O_2, and balanced N_2; alternatively autophagy was induced by treatment with rapamycin 50 μg/ml in DME medium with 10% FCS (Singh et al., 2006). The specific inhibitor of autophagy used was 3-methyladenine (3MA) (Sigma) (Wang et al., 2009a). RAW and BMDM cells were infected with *L. amazonensis* amastigotes at different parasite–cell ratios for 1–24 h, as previously described (Colhone et al., 2004). Briefly, following a period of interaction, the cultures were washed to remove extracellular parasites and fresh medium was added to the cell cultures. To determine the percentage of infected macrophages and number of amastigotes per macrophage, cells on coverslips were stained with Giemsa and examined microscopically at 1000× magnification. At least 200 cells were counted per triplicate coverslip.

2.4. LC3II protein detection

Immunohistochemistry of LC3II in murine cutaneous lesions were performed using rabbit polyclonal anti-LC3 (Novus Biologicals, Littleton, CO, USA) developed with goat polyclonal anti-rabbit antibody conjugated with peroxidase (Sigma). Serial 5 μm thick paraffin sections of footpad were treated as follows: deparaffination, rehydration, endogenous quenching, and citrate buffer microwave retrieval and visualized with a peroxidase substrate solution containing 3,3′-diaminobenzidine and hydrogen peroxide, as previously described (Arrais-Silva et al., 2005; Araújo et al., 2010). The images were recovered with a digital imaging system, a light microscope (Eclipse E800, Nikon, Tokyo, Japan), a Cool Snap-Pro Color camera (Media Cybernetics, Bethesda, MD, USA) and capture software Image-Pro plus (Media Cybernetics). The LC3II were detected by immunoblotting in cultured macrophages. For immunochemical analyses of LC3II in macrophages cultured *in vitro*, cells were scraped from culture flasks, checked for viability, and then rinsed twice with PBS. Lysis buffer (62.5 mM Tris–HCl, pH 6.8, 69 mM SDS, 10% glycerol, 2% 2-mercaptoethanol, 34 mM ethylenediaminetetraacetic acid, 2 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride; Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added to the cell pellets. Proteins were denatured at 40 °C for 1 min, electrophoresed on tricine-SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). After blotting, membranes were blocked with 5% (w/v) nonfat dry milk for 1 h and incubated with rabbit polyclonal anti-LC3 (Novus Biologicals) or mouse anti-β actin (Sigma) overnight at 4 °C. The secondary antibodies consisted of peroxidase-conjugated anti-rabbit and mouse IgGs (Sigma Aldrich) and were developed with 3,3-diaminobenzidine (Degrossoli et al., 2004). Immunoreaction images were scanned and the densitometric value of each band was determined using Image Master Total Lab version 1 software (Amersham Pharmacia Biotech).

2.5. Ultrastructural analyses

The macrophages (2×10^5) were seeded in eight-well chamber slides (Permanox Lab Tek, Nunc, Naperville, IL, USA) and a fixative consisting of 2.5% glutaraldehyde (Electron Microscope Science, Hatfield, PA) in 0.1 M sodium cacodylate (Electron Microscope Science) buffer at pH 7.4 was added to the medium of adherent cells for 15 min. Then, the fixative and medium mixture was replaced by pure fixative for 45 min. Next, the cells were rinsed (3–10 min) in 0.1 M sodium cacodylate buffer, pH 7.4 and then post-fixed in 1% OsO_4 (Electron Microscope Science) solution for 1 h. Following dehydration in an ethanol gradient, adherent cells were embedded in Epon 812 resin (Electron Microscope Science). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a LEO 906 (Leica, Wetzlar, Germany) transmission electron microscope operated at 60 kV (Degrossoli et al., 2011).

2.6. Lysotracker red staining

Lysotracker (Lysotracker red, Lonza, Walkersville, MD, USA) was used in accordance with the manufacturer's instructions. Briefly, cells were stained with 100 nM Lysotracker red at 37 °C for 1 h. After washing three times with PBS, cells were immediately analyzed under phase-contrast and fluorescence using fluorescence inverted microscopy (Nikon-Eclipse TS100).

2.7. Statistical evaluation

All experiments were repeated at least three times and the results are expressed as the mean ± SD. Data obtained under different conditions were analyzed statistically by the Student *t* test for independent samples ($P \leq 0.01$).