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Initiation of inflammation and cell death during liver abscess formation by *Entamoeba histolytica* depends on activity of the galactose/*N*-acetyl-D-galactosamine lectin

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Abstract

The parasite *Entamoeba histolytica* colonizes the human intestine causing amoebic colitis and disseminates through the vascular route to form liver abscesses. The Gal/GalNAc lectin is an adhesion protein complex which sustains tissue invasion by *E. histolytica*. Disruption of the Gal/GalNAc lectin function in engineered parasites (HGL-2 trophozoites) changed the pathophysiology of hamster liver abscess formation. HGL-2 trophozoites produced numerous small inflammatory foci located in the vicinity of blood vessels. The low penetration of HGL-2 trophozoites into hepatic tissue was shown to be associated with weak attraction of neutrophils and macrophages to the infiltrated areas and absence of pro-inflammatory tumour necrosis factor, in contrast to wild type or control vector infections. The low host inflammatory response in HGL-2 infections correlated with a delay in apoptosis of hepatic cells, whereas apoptosis of endothelial cells was not detected. Triggering of apoptosis in both host cell types most likely has a central role in modulating inflammation, a major landmark in hepatic amoebiasis. These data highlight the key role of the Gal/GalNAc lectin in initiation of *E. histolytica* hepatic infection.
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Keywords: Amoebiasis; Apoptosis; Inflammation; Gal/GalNAc lectin

1. Introduction

Entamoeba histolytica is a protozoan parasite that causes dysentery and liver abscesses in humans. The parasite's life cycle comprises cysts (which are present in fecally contaminated water or food) and trophozoites (which are formed by excystation in the intestinal lumen of infected human hosts). In symptomatic infections, *E. histolytica* trophozoites penetrate the colonic mucosa and produce extensive tissue damage at the invasion site. The trophozoites first adhere to colonic mucins and target cells. Parasite–cell interaction triggers the release of amoebic proteolytic enzymes that disrupt cell junctions and degrade the intestinal mucus layer, thus facilitating tissue penetration. Interaction of *E. histolytica* with human cells may lead to the secretion of amoebopores – amoebic protein complexes with membrane pore-forming activity – that may contribute to the lysis of host cells (Leippe et al., 1991). Invasion of the intestinal epithelium facilitates the haematogenous dissemination of trophozoites to systemic sites, in particular to the liver via the portal route.

Amoebic liver abscesses have a characteristic structure in humans and in hamster models, with a necrotic, central region containing inflammatory cells, and lysed hepatocytes. Motile trophozoites migrate to the abscess periphery and form a cell layer surrounded by inflammatory cells, thus delimiting the abscess within the hepatic tissue (Tsutsumi et al., 1984; Rigothier et al., 2002). During the early

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stages of hepatic invasion, trophozoites penetrate the sinusoids. This phenomenon is associated with an influx of host immune cells in general and polymorphonuclear (PMN) leucocytes, in particular. The host inflammatory response has amoebicidal activity but is also believed to contribute to liver tissue damage facilitating invasion by amoebae. The trophozoites that escape lysis in the first hours of invasion cause the formation of numerous infection foci after 24 h of intraportal inoculation (Rigothier et al., 2002).

In addition to cell death directly caused by the immune response, a hallmark of the tissue invasion process is the induction of cell death by apoptosis of epithelial cells (Huston et al., 2003; Seydel and Stanley, 1998). Apoptosis (a form of programmed cell death) is an evolutionarily conserved form of cell suicide based on the activity of a family of cysteine proteases, known as caspases. Contact-dependent cell killing by *E. histolytica* requires activation of host cell caspase-3 (Huston et al., 2000) and genes involved in death receptor pathways are up-regulated when *E. histolytica* is injected into the liver of mice (Pelosof et al., 2006).

Molecules on the surface of *E. histolytica* are responsible for parasite adhesion to host cells. The major parasite adhesive protein complex is the Gal/GalNAc-specific lectin (Mann et al., 1991; Petri et al., 1987). It is a 260 kDa heterodimer complex composed of two subunits bound by disulfide bonds: a light (Lgl) 31/35 kDa subunit and a heavy (Hgl) 170 kDa subunit. The complex is associated with an intermediate 120 kDa subunit (Igl) (Cheng et al., 2001). Blockage of out-in signalling through the heavy chain was observed in parasites transfected with a plasmid expressing the carboxyl terminal end of Hgl (HGL-2 trophozoites (Tavares et al., 2005) and HGL-3 trophozoites (Vines et al., 1998)). Impairing the Gal/GalNAc function in HGL-2 parasites significantly reduces amoebic adhesion to both epithelial cells and lymphocytes. Following intraportal inoculation of HGL-2 parasites in hamsters used as a model for pathophysiological studies of liver abscesses, a large number of small, structurally disorganized abscesses have been observed (Tavares et al., 2005). These abscesses contained a high number of parasites, but their low adhesion to host cells significantly reduced their penetration in the liver tissue (Coudrier et al., 2005).

In this work, we compared the pathophysiological parameters of abscesses formed by the virulent *E. histolytica* HM1:IMSS wild type (WT) strain and trophozoites transfected with the control vector pExEhNeo (NEO) with those observed for the derived construct $Hgl2_{TM\text{-}COO}$- (HGL-2 trophozoites) (Tavares et al., 2005). We focused our analysis on the onset of host inflammatory response and cell destruction by apoptosis. Four types of target cells were examined: endothelial cells, PMNs, macrophages, and hepatocytes. Our results demonstrate that the in vivo triggering of the host immune response depends on Gal/GalNAc lectin activity. When the adhesion-deficient HGL-2 trophozoites were inoculated by the portal route into hepatic tissue, fewer PMNs and macrophages were attracted towards the trophozoites and no TNF secretion was detected when compared with WT and NEO trophozoites. The induction of cell apoptosis following contact between HGL-2 trophozoites and hepatic tissue was delayed when compared with the apoptotic process triggered by WT trophozoites.

2. Materials and methods

2.1. *Entamoeba histolytica culture*

The pathogenic WT strain was cultured axenically in TYI-S-33 medium (Diamond et al., 1978) for 48 h at 37 °C. Prior to each experiment, the engineered trophozoites were maintained for 48 h in the presence of 30 μg/ml geneticin (Gibco-BRL). Parasites were transfected with NEO or with HGL-2. The recombinant plasmid's expression status was checked by Western blots prior to each inoculation of HGL-2 parasites in the test animals and 5 days p.i. (Tavares et al., 2005).

2.2. *Animal model experiments and tissue processing*

Animal handling and experimentation were conducted in accordance with European Union and Pasteur Institute approved protocols.

Male Syrian golden hamsters (*Mesocricetus auratus*) were infected intraportally with WT, NEO or HGL-2 parasites (8×10^5 trophozoites per animal) (Rigothier et al., 2002). Livers were excised and fixed immediately after necropsy at 1 or 6 h or 1, 2, or 3 days post-inoculation. Samples were fixed in 10% buffered formalin (pH 7.4) and embedded in paraffin. Analysis was performed on three 5 μm sections (separated from each other by 15 μm) from two hepatic lobes of three hamsters for each inoculated parasite (WT, NEO, and HGL-2). The parasites were immunolabelled with a human anti-*E. histolytica* antibody and host cells were counterstained with hematoxylin.

Macrophages were identified using Perl's Prussian Blue staining method (Churukian, 2002). After dewaxing and rehydration, sections were immersed in 2% acid ferrocyanide reagent for 1 h; the nuclei were counterstained for 5 min with 0.5% aqueous safranine, dehydrated, cleared in xylene and mounted in resinous medium. Macrophages are stained blue by a precipitate formed by the Fe^{3+} released from ferritin.

TNF reactivity was determined using a purified polyclonal goat serum raised against mouse TNF (AF-410-NA, R & D Systems). We made a comparison using the BESTFIT algorithm (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA) between the amino acid sequence of TNF from mice (Accession No. **NP**_038721) and that from hamsters (Accession No. AAC40100) and found that these two peptides have an identity of 87.5% with a similarity of 91.7%. An ELISA was performed to verify that hamster TNF was recognized by anti-mouse TNF antibody in blood samples of LPS i.p. treated animals (1 μg of LPS for 1 h 30 min). The anti-mouse TNF antibody could recognize hamster TNF at 940 pg/ml of hamster

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