Parasitology International 65 (2016) 665–667

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Short communication

Entamoeba histolytica: Overexpression of the *gal/galnac* lectin, *ehcp2* and *ehcp5* genes in an *in vivo* model of amebiasis

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ARTICLE INFO

Article history:
Received 18 April 2016
Received in revised form 18 July 2016
Accepted 28 August 2016
Available online 5 September 2016

Keywords:
Virulence
Entamoeba histolytica
ehcp2
ehcp5
crd

ABSTRACT

The parasite *Entamoeba histolytica* causes intestinal amebiasis and amebic liver abscess as its main extraintestinal manifestation. To study the *in vivo* events related to inflammation and the interactions between hosts and parasites during amebiasis, we designed a novel model of host-parasite interactions using cellulose membrane dialysis bags containing *E. histolytica* trophozoites. A bag is placed into the hamster peritoneal cavity, as has been reported in previous studies of programmed cell death (PCD) in *E. histolytica* trophozoites. To determine if virulence factors such as cysteine proteinases (EhCP2 and EhCP5) and Gal/GalNAc lectin could be involved in the host-parasite interaction using this model, we examined the relative expression of the *ehcp2* and *ehcp5* genes and the carbohydrate recognition domain (*crd*) of Gal/GalNAc lectin using real-time quantitative PCR (qRT-PCR). All analyzed genes were over-expressed 0.5 h after the initiation of the host-parasite interaction and were then progressively down-regulated. However, Gal/GalNAc lectin had the greatest increase in gene expression 1.5 h after host-parasite interaction; Gal/GalNAc lectin had a 250-fold increase with respect to the axenically grown trophozoites, which over-express Gal/GalNAc lectin in *in vivo* models. These results support the important role of these molecules in the initiation of cell damage by *E. histolytica*.

The protozoan parasite *Entamoeba histolytica* causes bowel inflammation, intestinal ulcer, and amebic liver abscess (ALA), which is its main extraintestinal manifestation.

To study the *in vivo* events related to inflammation and host-parasite interactions during amebiasis, we utilized a previously designed host-pathogen interaction model in which cellulose membrane dialysis bags containing *E. histolytica* trophozoites are placed into the peritoneal cavity of hamsters. This model was developed to determine if cellular immunity contributes to the damage of *E. histolytica* trophozoites by releasing molecules that induce amebic-programmed cell death (PCD). We found a 70% change in protein synthesis patterns in *E. histolytica* trophozoites from 3 h post-interaction to 6 h post-interaction [1].

The virulence of *E. histolytica* has been attributed to the capacity of the parasite to provoke tissue damage through the expression and/or secretion of diverse molecules. In turn, components of the cellular host response influence the behavior of amebas, altering the expression of amebic virulence mechanisms [2].

Cysteine proteinases (CPs) and Gal/GalNAc lectin have been proposed as important virulence factors involved in the invasion and destruction of host tissues by amebas [2].

E. histolytica contains at least 50 cysteine proteases, and the most studied proteases are EhCP1, EhCP2 and EhCP5, which have been shown to be responsible for cell damage in some animal models, particularly cysteine proteinase 5 (EhCP5); for this reason, we decided to study EhCP5 and other cysteine proteinases [3–6]. Another important virulence factor is Gal/GalNAc lectin, an amebic surface molecule that mediates parasite adherence *via* a carbohydrate recognition domain (CRD) [7–10]. Very few studies have evaluated the role of the proposed virulence factors EhCP1, EhCP2, EhCP5, and Gal/GalNAc lectin in the susceptibility to amebiasis of animal models. However, these studies have demonstrated the important role of Gal/GalNAc lectin in the pathogenesis of ALA [7–11]. Our group demonstrated that amebic Gal/GalNAc lectin diffuses and binds to human and hamster cultured hepatocytes, and we also evaluated the role of lectin in the evolution of ALA using the hamster model [10]. Moreover, recent studies have demonstrated the dependence of endothelium crossing on Gal/GalNAc lectin [11–12].

In the present study, we determine if the virulence factors cysteine proteinases 2 and 5 (EhCP2 and EhCP5, respectively) and Gal/GalNAc lectin could be involved in the early stages of the host-parasite interaction in a previously described *in vivo* model [1].

For this purpose, we used two groups of 12 male golden hamsters (*Mesocricetus auratus*); the hamsters were 2 months old with a mean weight of 100 g. To stimulate and expand peritoneal inflammatory

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http://dx.doi.org/10.1016/j.parint.2016.08.009
1383-5769/

exudates, one group of animals was intraperitoneally injected with 1 ml of mineral oil (Sigma-Aldrich) one week before the dialysis bag was placed into the peritoneal cavity. The second group, which only received the dialysis bag but not mineral oil, was the non-stimulated animal group; the dialysis bags from both groups contained *E. histolytica* trophozoites.

Hamsters were intraperitoneally anaesthetized with sodium pentobarbital (Anestesal; Smith Kline) at a dose of 4.72 mg per 100 g body weight. After a mid-longitudinal incision of the abdominal wall, the dialysis bag containing 4×10^6 amebas/ml was placed into the peritoneal cavity. The dialysis cellulose membranes were 50 mm long and 7.5 mm in diameter, were permeable to molecules up to 25 kDa, and were allowed to interact for 0.5, 1.5, 3, and 6 h in the peritoneum of mineral oil-stimulated and non-stimulated hamsters (three animals by each time point from each group). Thereafter, the animals were sacrificed, the dialysis bags were extracted, and the amebas were collected under sterile conditions. The animal management protocols were approved by the institutional committee (IACUC; number 423-08). Our Institution fulfills the technical specifications for the production, care and use of laboratory animals and is certified by national law (NOM-062-Z00-1999). All hamsters were killed by overdose of sodium pentobarbital and were handled according to the guidelines of the 2000 AVMA Panel of Euthanasia.

Axenic *E. histolytica* trophozoites (4×10^6 amebas/ml) [13] from cultures were harvested during the logarithmic phase of growth and used as a control.

Trophozoites were collected from three dialysis bags at each time point of incubation. Total RNA was isolated, quantified, and DNase treated. qRT-PCR was performed using the corresponding cDNA from each sample. qRT-PCR assays were performed in triplicate. Primer sequences for the analyzed genes and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) are summarized in Table 1.

RNA was reverse transcribed using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. qRT-PCR was performed using a ABI PRISM 7000 Sequence Detection System (Applied Biosystems), and fluorescence in real time was detected using the SYBR Green PCR Master Mix (Applied Biosystems). Immediately after amplification, melt curve analysis was performed to ensure that self-complementary primer-dimers and other non-specific products were minimized or eliminated.

Relative expression was calculated using the CT method, which uses the arithmetic formula $2^{-\Delta\Delta C_T}$ [14]. To validate the $2^{-\Delta C_T}$ method, we verified that the amplification efficiency of target genes and the internal control *gapdh* were nearly equal. Statistically significant differences in gene expression between trophozoites grown axenically (control) and trophozoites collected from dialysis bags at different time points were analyzed using Kruskal-Wallis one-way analysis of variance on ranks, and multiple comparisons were analyzed using Tukey's test with SPSS Sigma Stat statistical software ver.2.0.

Changes in gene expression for *ehcp2*, *ehcp5* and *crd* were evaluated by qRT-PCR after the specified time periods of incubation. PCR efficiencies were nearly equal among each of the transcripts investigated and the endogenous control *gapdh*. Single peaks were identified in the melt curve for each screened gene, indicating that unique PCR products were produced (data not shown).

The relative expression levels of transcripts in *E. histolytica* trophozoites grown axenically were determined, and the parasites that were incubated for different lengths of time (0.5, 1.5, 3, and 6 h) from the stimulated and non-stimulated groups are shown in Fig. 1.

We found that gene expression appears to be up-regulated followed by a progressive gene down-regulation over time and in comparison to the trophozoites grown axenically. However, Gal/GalNAc lectin up-regulation was greater, and its down-regulation occurred later (6 h) compared to that of EHCP (3 h).

Others have demonstrated that the CRD of Gal/GalNAc lectin has a similar sequence to the receptor-binding domain of hepatocyte growth factor (HGF) and competes with HGF for binding to the c-Met HGF receptor [15]. For this reason, our results could be explained as follows: in the dialysis bag model, adherence does not occur, and the lack of specific binding of Gal/GalNAc lectin (*via* its CRD) to c-Met on hepatocytes may cause the ameba to increase Gal/GalNAc lectin expression.

These results were similar to other *in vivo* studies that showed a significant up-regulation of *gal/galnac* lectin and *ehcp* genes [3–11]. However, the expression of Gal/GalNAc lectin reported here is the greatest level of over-expression reported in an *in vivo* model.

Most likely, the lack of interaction between target cells and trophozoites could be a key event in this model; in addition, molecules released by *E. histolytica* trophozoites are not diffusible, forming a microenvironment that modulates ameba virulence. Some researchers have demonstrated that the very early stages of infection and the environment are critical for trophozoite survival. For example, amebas that were purified from ALA can be adapted to *in vitro* culture conditions; in this process of continuous passage in culture conditions, the ameba may change in their ability to form liver abscesses. In addition, differences in the early response to *E. histolytica*, specifically in tissue invasion, have been observed between female and male mice. Females recruited a greater number of Natural Killer T cells to the site of infection and produced more IFN-γ, which is a key regulator of early inflammation that activates macrophages *via* TNF-α production and in turn promotes nitric oxide (NO) synthesis by inflammatory cells [12,16]. These results could explain the physiological differences between females

Table 1
Oligonucleotides used in real-time quantitative PCR (qRT-PCR).

Name	Sequence(5′-3′)
ehcp2	F: TGGACCATTTGCTGCTATGA
	R: TAACATGATCCGCATTGTGC
ehcp5	F: CGCTGCTATTGATGCTTCTG
	R: CTCCCCAAATAGTTCCCCAT
crd	F: GCATATTGTACATACGAAATAACAACA
	R: TTTTCCTTCAATGAATGCTTTACG
Gapdh	F: CCGTCCACAGACAATTCGAA
	R: TTGAGCTGGATCTCTTTCAGCTT

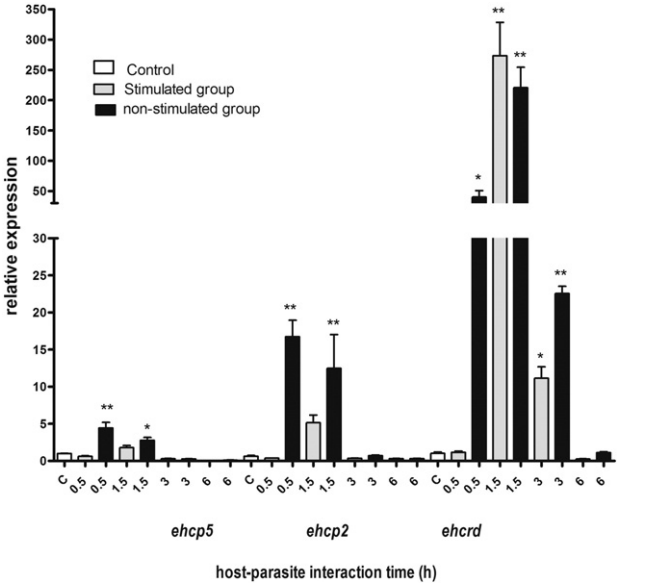

Fig. 1. Relative expression of *ehcp5*, *ehcp2*, and *crd* during the course of host-parasite interaction. The graph shows the relative expression of *ehcp5*, *ehcp2*, and *crd* of trophozoites recovered from dialysis bags after 0.5, 1.5, 3 and 6 h post-interaction in the hamster peritoneum. $^*p < 0.05$ and $^{**}p < 0.001$ indicate trophozoites that showed significant differences in relative expression with respect to the trophozoites grown axenically.

and males in their susceptibility to infection; consequently, the environmental physiological conditions could be important in the behavior of the virulence of *E. histolytica* trophozoites.

Regarding the down-regulation of genes from *E. histolytica* trophozoites, we considered that trophozoites could have adapted as a result of the molecules derived from both the host and the parasite. The main molecule that could be involved is nitric oxide (NO), which has been reported as a major cytotoxic molecule that kills *E. histolytica* trophozoites and also inhibits cysteine proteinases and regulates the Gal/GalNAc lectin of parasites [17–19]. In addition, nitrites and nitrates, which are the stable end products of NO, were detected inside the dialysis bag, in the serum and in the peritoneal fluid at 3 and 6 h of incubation in the hamster. In the same work, we demonstrated that PCD occurs in *E. histolytica* [1]. Other molecules could be involved in the adaptation of a new environment, including reactive oxygen species (ROS) released by the peritoneal exudate cells, arginase, an enzyme involved in the protection of the parasite against oxidative stress, and an excess of 150 oxidized proteins that were recently shown by proteomic analysis to be involved in *E. histolytica* resistance to oxidative stress [20].

When expression levels were evaluated, *crd* expression increased the most in this model. The maximum values of crd expression in trophozoites from stimulated and non-stimulated animals were very similar, approximately 250-fold more with respect to crd expression in the trophozoites that were grown axenically (control group). In contrast to the over-expression of *crd,* the levels of *ehcp2 and ehcp5* were lower, which was similar to the results from other models [3–6]. The maximum fold increase in *ehcp* expression in trophozoites collected from stimulated and non-stimulated animals was approximately 4- and 14-fold for *ehcp5* and *ehcp2,* respectively, compared with amebas grown axenically.

The increase in the expression of CPs and Gal/GalNAc lectin in the dialysis bag model supports the important role of these molecules in the initiation of parasite invasion as described in other works [3–11].

Moreover, we observed that the response of trophozoites was slower in the stimulated group (1.5 h of interaction) compared to the non-stimulated group (0.5 h of interaction). This suggests that the increase in peritoneal cell exudate from the stimulated group is able to kill *E. histolytica* because the exudate contains neutrophils (70%), macrophages (30%), and nitric oxide. In particular, NO has been reported to be the major cytotoxic molecule that kills *E. histolytica* trophozoites, and it has also been reported to inhibit cysteine proteinases from the parasite. In addition, some researchers have demonstrated that NO regulates Gal/GalNAc lectin [17–19]. However, it is important to consider that other molecules <25 kDa, such as TNF-α, INF-γ, IL-1 β, IL-8 and IL-10, could be involved in the regulation of proteases and Gal/GalNAc lectin. In regard to the dialysis bag model, we believe that it is a good model to understand the relationship between the interfaces in both cases: the virulence molecules from *E. histolytica* and the derived molecules from the immune response that interact with the amebas.

Currently, studies are being conducted to allow us to understand the role of the peritoneal exudate products and also to quantify molecules (ROS and cytokines) inside the dialysis bag and in the peritoneal liquid and serum; these molecules may modulate ameba virulence factors and thus participate in the early events of tissue damage.

Acknowledgments

We would like to thank Karla Gil-Becerril, Angélica Silva-Olivares and Silvia Galindo-Gómez for their valuable technical assistance.

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