Microbial Pathogenesis 123 (2018) 285–295

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

O-deGlcNAcylation is required for *Entamoeba histolytica*-induced HepG2 cell death

Young Ah Lee, Arim Min, Myeong Heon Shin*

Department of Environmental Medical Biology and Institute of Tropical Medicine, Yonsei University College of Medicine, Seoul, 03722, South Korea

ARTICLE INFO

Keywords:
Entamoeba histolytica
O-GlcNAc
Cell death
OGT
OGA

ABSTRACT

Entamoeba histolytica is an enteric tissue-invading protozoan parasite that causes amoebic colitis and occasionally liver abscess in humans. *E. histolytica* can induce host-cell apoptosis by initiating various intracellular signaling mechanisms closely associated with tissue pathogenesis and parasitic immune evasion. O-GlcNAcylation, similar to phosphorylation, is involved in various cell-signaling processes, including apoptosis and proliferation, with O-GlcNAc addition and removal regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively. However, whether O-GlcNAc alterations in host cells affect *E. histolytica*-induced cell death and which signal molecules participate in *E. histolytica*-induced deglycosylation remain unknown. In this study, co-incubation of HepG2 cells with *E. histolytica* increased DNA fragmentation and LDH release as compared with control cells. Additionally, Gal-lectin-mediated amoebic adherence of live trophozoites to HepG2 cells decreased O-GlcNAcylated protein levels within 5 min. We also observed a rapid decrease in cellular OGT protein level, but not OGA, in HepG2 cells in a contact-dependent manner. Furthermore, HepG2 pretreatment with OGA inhibitors or OGA siRNA prevented *E. histolytica*-induced O-deGlcNAcylation, DNA fragmentation, and LDH release. Our results suggested that *E. histolytica*-induced O-deGlcNAcylation in HepG2 cells was an important process required for hepatocyte cell death induced by *E. histolytica* adherence.

1. Introduction

Entamoeba histolytica is an enteric protozoan, tissue-invasive parasite that causes amoebic colitis and occasionally liver abscess in humans. In developing countries, *E. histolytica* causes disease in 50 million people and kills from 40,000 to 100,000 individuals annually [1,2]. *E. histolytica* has virulence factors such as Gal/GalNAc lectin, amoebapore and cysteine protease. *E. histolytica* is capable of inducing apoptotic cell death in many host cells, including immune [9,38,39] and non-immune cells [3,8], thereby allowing the parasite to minimize host-tissue damage and maintain infectivity and virulence *in vivo*. *E. histolytica* can induce host cell death in contact dependent [7,8] and independent manner [36,41]. Specially, when amoebae adhere to host cells through an amoebic Gal/GalNAc lectin on their surface, amoebae induce rapidly host cell death with interaction between host cell and amoeba resulting in rapid elevation of intracellular Ca^{2+} in host cells and leading to cell death [4]. *E. histolytica* triggers the activation of calpain, a calcium-dependent cysteine protease (CP), in Jurkat T cells. This activation is closely related to cleavage of various host proteins associated with cell signaling and regulation, such as tyrosine phosphatases (PTPs), including PTP1B [5], SHP-1, and SHP-2 [6], and caspase-3 [7] and calpastatin [8], which might cause dephosphorylation and DNA fragmentation in Jurkat T cells [6]. In particular, intracellular caspase-3 activation in Jurkat T cells [7] and reactive oxygen species in human neutrophils [9] are required for *E. histolytica*-triggered host-cell apoptosis. In addition, *E. histolytica* also induced DNA fragmentation and LDH release in colon epithelial cells such as CaCo2, HT29 [29,43]. Previous studies revealed that *E. histolytica* induces host-cell death in amoebic liver abscess via a non-Fas-dependent, non-TNF-α-dependent pathway of apoptosis [10]. Additionally, the initiation of inflammation and cell death during liver abscess formation by *E. histolytica* [38,39] depends upon the activity of the Gal/GalNAc lectin [11,37,40]. However, cell-death-signaling molecules associated with *E. histolytica*-induced HepG2 cell death remain unknown.

O-linked β-N-acetylglucosamine (O-GlcNAc) is the major glycosylation type found within the cytosolic and nuclear compartments of eukaryotic cells [12]. Additionally, numerous studies in different cell types showed that O-GlcNAcylation occurs as an inducible, cytoprotective stress response [13,14]. GlcNAcylation, similar to phosphorylation, is the post-translational cycling of a single O-GlcNAc on the hydroxyl groups of Ser and/or Thr residues of target proteins [15]. A large number of GlcNAcylated proteins are involved in regulating

* Corresponding author.
E-mail address: myeong@yuhs.ac (M.H. Shin).

https://doi.org/10.1016/j.micpath.2018.07.012
Received 31 January 2018; Received in revised form 12 July 2018; Accepted 12 July 2018

intracellular signaling, including pathways involved in apoptosis, proliferation, transcription regulation, cytoskeletal networks, stress responses, and the ubiquitin-proteasome system [16,17]. Currently, only two enzymes are known to regulate GlcNAcylation in mammals: O-GlcNAc transferase (OGT), which catalyzes the addition of O-GlcNAc, and β-N-acetylglucosaminidase (OGA), a neutral hexosaminidase responsible for O-GlcNAc removal [15]. Several studies reported that GlcNAcylation protects against cellular stress and cell death. For examples, increases in O-GlcNAc levels due to glucosamine treatment protect cardiac myocytes following ischemia/reperfusion injury by increasing levels of the anti-apoptotic protein Bcl2 [18], which improved cardiac function in a rat model of trauma-hemorrhage during resuscitation [19] and reduced circulating inflammatory cytokines by inhibiting the NF-κB pathway. Conversely, decreased O-GlcNAc levels due to OGA expression play a role in triggering the programmed cell death observed in diseases [20]. However, the ability of *E. histolytica* to induce increases or decreases in O-GlcNAc modifications in host cells during *E. histolytica*-induced host-cell death remains unknown.

In this study, we investigated whether HepG2 cell-death-signaling molecules induced by *E. histolytica* are associated with cellular O-GlcNAc levels and evaluated the effect of amoeba-induced intracellular fluctuations of O-GlcNAc modifications through the use of various pharmacological inhibitors.

2. Materials and methods

2.1. Reagents and antibodies

O-(2-acetamido-2-deoxy-D-glucopyranosyldene-amino-N-phenylcarbamate) (PUGNAc) was purchased from Toronto Research Chemicals (North York, Canada). Thimet G was obtained from the laboratory of Dr. Cho (Yonsei University, Korea). A rabbit monoclonal Ab against OGT and mouse monoclonal Abs against O-GlcNAc and OGA were purchased from Abcam (Cambridge, UK). Lipofectamine was purchased from Invitrogen (Carlsbad, CA, USA). All other products were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. E. histolytica culture conditions and E. histolytica-derived secretory products (EhSPs)

Trophozoites of the pathogenic *E. histolytica* HM-1, non-Pathogenic *E. histolytica* Rahman, and ICP1$^+$ (endogenous Inhibitor of Cysteine Protease inhibitor 1) which the ICP1 gene was overexpressed [21] were grown at 37 °C in TYI-S-33 medium. Trophozoites were harvested after 48–72 h during the logarithmic phase by chilling the culture tubes on ice for 10 min. After centrifugation at 200*g* and 4 °C for 5 min, the amoebae were washed with PBS twice and suspened in MEM media containing 10% (v/v) heat-inactivated FBS. The viability of amoebae as judged by trypan blue exclusion assay was consistently shown to be > 99%. To collect EhSPs from *E.histolytica* HM-1, trophozoites were incubated in Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY, USA) for 2 h at 37 °C. The viability of *E. histolytica* trophozoites following incubation was 98% as determined by a trypan blue exclusion assay. Protein concentration was measured using the BCA method with bovine serum albumin as a standard.

2.3. Cultivation of human cell lines

The HepG2 human hepatoma cell line (American Type Culture Collection, Manassas, VA, USA) was grown in MEM media containing 10% (v/v) heat-inactivated FBS at 37 °C in a humidified 5% CO_2 atmosphere. The viability of HepG2 cells as judged by trypan blue exclusion assay was consistently shown to be 99%.

2.4. Pretreatment of host cells with various chemical inhibitors

HepG2 cells were pretreated with pharmacological inhibitors, including PUGNAc, streptozotocin and Thimet G, for 4 h at 37 °C in a humidified CO_2 incubator (5% CO_2 and 95% air atmosphere). After preincubation with inhibitors for the indicated time, host cells were washed twice with PBS before treatment with *E. histolytica* or EhSPs. We observed no inhibitor-induced cytotoxicity at the concentrations used. The inhibitor-induced cytotoxicity were evaluated by trypan blue exclusion assay.

2.5. OGT-overexpressing cells

The pFLAG-CMV-5.1 expression vector (Sigma-Aldrich) with *ogt* was used for this study. For mock transfections, all reagents were used, except for the vector. Transfection of the vector containing *ogt* or vector control was performed using Lipofectamine reagent (Invitrogen) according to manufacturer instructions. Cell viability was monitored throughout the experiments. The cells viable throughout the course of all experiments according to trypan blue exclusion assays (data not shown) were used. At 72 h post-transfection, the efficiency of OGT overexpression was confirmed by western blot using the OGT Ab, with a β-actin-specific Ab used as the loading control. At 72 h post-transfection, the transfected HepG2 cells were washed, resuspended in fresh cell-culture medium, and co-incubated with *E. histolytica* for the experiment.

2.6. OGA knockdown by siRNA in HepG2 cells

ON-target plus SMARTpool OGA siRNA (L-012805-00-0005) and scrambled siRNA (D-001210-02-20) were purchased from Dhamarcon (Lafayette, CO, USA). For mock transfections, all reagents were used, except for siRNA, and siRNA cellular transfections were performed using Lipofectamine reagent (Invitrogen) according to manufacturer instructions. To optimize the conditions of the RNAi treatments, different siRNA concentrations (50 or 100 nM) and varying incubation times (24–72 h) were examined. Cell viability was monitored throughout the experiments, with cells viable throughout the course of all experiments according to trypan blue exclusion assays (data not shown). At 72-h post-transfection, the efficiency of siRNA knockdown of OGA was confirmed by western blot using the OGA Ab, with a β-actin-specific Ab used as the loading control. At 72-h post-transfection, the transfected HepG2 cells were washed, resuspended in fresh cell-culture medium, and co-incubated with *E. histolytica* for the experiment.

2.7. Measurement of E. histolytica-induced cell death by DNA-fragmentation and LDH-release assays

HepG2 cells (4×10^6 cells/sample) were incubated with various *E. histolytica* trophozoites (4×10^5 cells/sample) at a ratio of 5:1 or 10:1 (HepG2 cells to *E. histolytica*) for 60 min at 37 °C in a humidified CO_2 incubator. After incubation, cells were harvested by centrifugation and washed with cold PBS. DNA was extracted using a TaKaRa kit (MK600; Shiga, Japan) according to manufacturer protocol. DNA samples were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide to assess DNA fragmentation. LDH release was determined by evaluating the amounts of LDH in culture supernatants using the CytoTox 96 cytotoxicity assay system (Promega, Madison, WI, USA) according to manufacturer protocol. Briefly, HepG2 cells (2×10^5 cells/sample) were seeded onto 24-well plates 1 day prior to the addition of *E. histolytica*. Culture supernatants were collected after incubation with *E. histolytica* for 1 h at a 5:1 or 10:1 ratio (HepG2 cells to *E. histolytica*) and centrifuged at 250 g for 4 min. The amount of LDH in the supernatants was measured using a 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA) according to manufacturer protocol. The background (spontaneous LDH release) value was