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Entamoeba histolytica: Lipid rafts are involved in adhesion of trophozoites to host extracellular matrix components

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Index Descriptors and Abbreviations:
Adhesion
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Extracellular matrix
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Lipid raft
DIC, differential interference contrast
$DiIC_{16}$, 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanineperchlorate
ECM, extracellular matrix
FAK, focal adhesion kinase
Gal/GalNAc lectin, galactose/*N*-acetylgalactosamine-inhibitable lectin
IC_{50}, mean inhibitory dose
LCC, lipoprotein–cholesterol concentrate
MβCD, methyl-β-cyclodextrin
PBS, phosphate buffered saline
SD, standard deviation

ABSTRACT

Adhesion is an important virulence function for *Entamoeba histolytica*, the causative agent of amoebic dysentery. Lipid rafts, cholesterol-rich domains, function in compartmentalization of cellular processes. In *E. histolytica*, rafts participate in parasite–host cell interactions; however, their role in parasite–host extracellular matrix (ECM) interactions has not been explored. Disruption of rafts with a cholesterol extracting agent, methyl-β-cyclodextrin (MβCD), resulted in inhibition of adhesion to collagen, and to a lesser extent, to fibronectin. Replenishment of cholesterol in MβCD-treated cells, using a lipoprotein–cholesterol concentrate, restored adhesion to collagen. Confocal microscopy revealed enrichment of rafts at parasite–ECM interfaces. A raft-resident adhesin, the galactose/*N*-acetylgalactosamine-inhibitable lectin, mediates interaction to host cells by binding to galactose or *N*-acetylgalactosamine moieties on host glycoproteins. In this study, galactose inhibited adhesion to collagen, but not to fibronectin. Together these data suggest that rafts participate in *E. histolytica*–ECM interactions and that raft-associated Gal/GalNAc lectin may serve as a collagen receptor.

1. Introduction

Entamoeba histolytica is a human intestinal pathogen that affects more than 50 million people worldwide, leading to 100,000 deaths annually (reviewed in Laughlin and Temesvari, 2005). Transmitted by the fecal–oral route, ingestion of the infective cyst form occurs via contaminated food and water. In the pre-invasive form of the disease, motile trophozoites, which excyst in the small intestine or colon, interact with the mucin layer. In the invasive stage of amoebiasis, *E. histolytica* trophozoites breach the mucus-secreting epithelium of the human colon and encounter the submucosa, which is comprised of loose connective tissue, blood vessels, and ECM components, including collagen and fibronectin. Destruction of epithelium and the ECM that surrounds the epithelial cells produces flask shaped ulcers (Laughlin and Temesvari, 2005). The resultant manifestations, including diarrhea and dysentery, are major public health concerns in developing and underdeveloped countries. In some cases, colonic invasion can result in dissemination of trophozoites to extra-intestinal sites such as the liver, lungs, and brain through the portal vascular system (Laughlin and Temesvari, 2005). Thus, adhesion to ECM components and their subsequent degradation facilitates invasion, and is a critical step in the pathogenesis of amoebiasis.

Several lines of evidence suggest that adhesion of *E. histolytica* to ECM may be likened to focal adhesions of higher eukaryotes (Talamas-Rohana and Meza, 1988; de Lourdes et al., 2001). This interaction is also believed to activate parasite signal transduction pathways and virulence (Talamas-Rohana and Meza, 1988, 2000; de Lourdes et al., 2001; Cruz-Vera et al., 2003; Debnath et al., 2004; Perez et al., 1996, 1998; Flores-Robles et al., 2003; Franco et al., 2002; Talamas-Rohana and Rios, 2000). For

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example, exposure of *E. histolytica* to collagen induces both actin reorganization within the trophozoites and phosphorylation of tyrosine residues on the *E. histolytica* homolog of $pp125^{FAK}$ (de Lourdes et al., 2001; Perez et al., 1996). $pp125^{FAK}$ is a cytosolic FAK that localizes to adhesion plaques (Perez et al., 1996). Exposure of *E. histolytica* to collagen also stimulates $pp125^{FAK}$ association with paxillin and Src (Perez et al., 1998) and phosphorylation of $p42^{MAPK}$ (Perez et al., 1996), a map kinase which may propagate a collagen-based signal from the plasma membrane to the nucleus. Encountering collagen also increases DNA binding of three *E. histolytica* transcription factors, AP-1 (Perez et al., 1998), STAT1, and STAT3 (Cruz-Vera et al., 2003), which, in turn, may regulate changes in gene expression. In support of this, collagen exposure results in increased expression of an amoebapore and a cysteine protease (Debnath et al., 2004), two secreted proteins which play a role in host tissue destruction. Binding of *E. histolytica* trophozoite to a second ECM component, fibronectin, also induces actin reorganization (Talamas-Rohana and Meza, 1988; Talamas-Rohana and Rios, 2000), phosphorylation of $pp125^{FAK}$ (Flores-Robles et al., 2003), $pp125^{FAK}$ association with paxillin and vinculin (Flores-Robles et al., 2003), and activation of protein kinase A (Franco et al., 2002), an enzyme involved in G-protein coupled receptor signaling. Since exposure to ECM components appears to upregulate signaling events that modulate virulence, a better understanding of adhesion to ECM may provide insight into pathogenic mechanisms.

Recent evidence suggests that highly-ordered cholesterol- and sphingolipid-rich microdomains, termed lipid rafts, are present in the plasma membrane of *E. histolytica* (Laughlin et al., 2004). In higher eukaryotes, rafts are thought to harbor specialized transmembrane, GPI-linked, and dually acylated proteins (reviewed in Maxfield, 2002; Simons and Toomre, 2000). They are also thought to serve as signaling platforms in which adhesion/signaling molecules, such as integrins, accumulate in a signal-dependent fashion (Maxfield, 2002; Simons and Toomre, 2000). In *E. histolytica*, rafts have been shown to play a role in endocytosis and adhesion to host cells (Laughlin et al., 2004). In addition, an important adherence protein of *E. histolytica*, the Gal/GalNAc lectin, is localized to these membrane microdomains (Laughlin et al., 2004). The Gal/GalNAc lectin is a heterotrimer comprised of light, intermediate, and heavy subunits, and binds to galactose and *N*-acetylgalactosamine residues of host glycoconjugates on mucin, epithelial cells, and erythrocytes (Adler et al., 1995; Ravdin and Guerrant, 1981; Petri et al., 2002). Given that rafts participate in cell–matrix interactions in other systems (Ramprasad et al., 2007; Gopaladrishan et al., 2000), it is conceivable that lipid rafts may also play a role in the interaction of *E. histolytica* with host ECM. Moreover, lipid raft-resident adhesion molecules, such as the Gal/GalNAc lectin, may mediate this function of rafts. In the present study, we have examined the role of lipid rafts in adhesion of *E. histolytica* trophozoites to elements of host ECM. Here, we demonstrate that disruption of rafts inhibits adhesion to host ECM, and that rafts accumulate at parasite–ECM interfaces. Moreover, we demonstrate that the Gal/GalNAc lectin may mediate interactions to collagen but not to fibronectin. Together these data suggest a role for rafts and their resident proteins in *E. histolytica* interaction with host ECM.

2. Materials and methods

2.1. Strains and culture conditions

Entamoeba histolytica trophozoites, strain HM-1:IMSS, were cultured axenically in TYI-S-33 medium in screw-cap glass tubes at 37 °C (Diamond et al., 1978). Log phase-harvested trophozoites were used for all experiments.

2.2. Measurement of E. histolytica adhesion

To assess parasite–ECM interactions, we developed a fluorescence-adapted assay based on a similar test used to assess the binding of *E. histolytica* trophozoites to host epithelial cells (Powell et al., 2006). *Entamoeba histolytica* trophozoites were labeled with 5 μg/ml calcein AM (Invitrogen, Carlsbad, CA), a green fluorescent vital stain, at 37 °C, for 60 min in serum-free medium (TYI-33). These cells were then seed onto collagen type I- or fibronectin-coated 96-well plates (BD Cellware, Bedford, MA). Following incubation at 37 °C, the wells were gently washed twice with warm PBS to remove non-adherent cells. The relative fluorescence (a measure of adhesivity) was assessed using a fluorescence plate reader (Model FLX800, BioTek Instruments, Winooski, VT). Alternatively, the number of adherent cells was determined by counting five fields per well at a magnification of 400× on an Olympus CK2 inverted light microscope. The number of cells to be seeded into the wells for subsequent experiments, as well as the incubation time, was determined empirically by examining a range of cell concentrations and a range of incubation times.

To test the role of lipid rafts, adhesion assays were performed with trophozoites that had been treated with a range of concentrations of the cholesterol depleting agent, MβCD (Sigma–Aldrich, St. Louis, MO), during the last 30 min of calcein AM staining. Alternatively, adhesion assays were performed with untreated or MβCD-treated cells that were exposed to a cholesterol source, lipoprotein–cholesterol concentrate (LCC) (57 mg of cholesterol/dL) (MP Biomedicals, Solon, OH), for 15 min at 37 °C. Analysis by the vendor of the lot used throughout the study indicated that LCC consisted of cholesterol (1548 mg/dL), triglycerides (7%), protein (4.5 mg/dL), chloride (50 mequiv units/L), ammonia (56 μM), calcium (5 mg/dL), and sodium (98 mequiv units/L). If applicable, adhesion data were corrected for slight losses in fluorescence due to MβCD treatment.

To test if the Gal/GalNAc lectin was involved in adhesion to ECM, adhesion assays were performed in the presence of a range of concentrations of D(+) galactose (Sigma–Aldrich) or 100 mM mannose (control) (Sigma–Aldrich) (Adler et al., 1995; Ravdin and Guerrant, 1981). In all cases, adhesion data were reported as a percent of control, which was arbitrarily set to 100%.

2.3. Confocal microscopy

Trophozoites were allowed to adhere to collagen type I- or fibronectin-coated cover slips (BD Biosciences) in serum-free medium. Following incubation at 37 °C for 15 min, the medium was aspirated and the non-adherent cells were removed by washing twice with warm PBS. The cells were fixed by treatment with 4% (vol/vol) paraformaldehyde for 10 min at room temperature and then incubated with the fluorescent lipid raft stain, $DiIC_{16}$, according to the protocol of Nguyen and Hildreth (2000) (4.5 mM; Invitrogen), for 10 min. The cover slips were then washed twice with PBS, mounted in PBS, and observed using a LSM 510 confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). As a control, trophozoites were allowed to adhere to uncoated glass cover slips.

2.4. Statistical analyses

All values are represented as the mean of the results from at least three trials (±standard deviations [SD]). Statistical analyses were performed using GraphPad Instat (version 3.05; IBM) with ANOVA. *P*-values less than 0.05 were considered statistically significant (*). *P*-values less than 0.01 were considered highly statistically significant (**). The IC_{50} values were calculated using the line of best fit generated by TableCurve2D version 5.01 (Systat Software Inc., San Jose, CA).